OPTIMIZATION AND CHARACTERIZATION OF THE GROWTH OF THE PHOTOSYNTHETIC BACTERIUM *BLASTOCHLORIS VIRIDIS* AND A BRIEF SURVEY OF ITS POTENTIAL AS A REMEDIATIVE TOOL

A Dissertation

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by

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Abstract

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The growth of B. viridis was characterized in an undefined rich medium and a well-defined medium, which was later selected for further experimentation to insure repeatability. This medium presented a significant problem in obtaining either multigenerational or vigorous growth because of metabolic limitations; therefore optimization of the medium was undertaken. A primary requirement to obtain good growth was a shift in the pH of the medium from 6.9 to 5.9. Once this shift was made, it was possible to obtain growth in subsequent generations, and the media formulation was optimized. A response curve suggested optimum concentrations of 75 mM carbon, supplemented as sodium malate, 12.5 mM nitrogen, supplemented as ammonium sulfate,
and 12.7 mM phosphate buffer. In addition, the vitamins $p$-Aminobenzoic acid, Thiamine, Biotin, B$_{12}$, and Pantothenate were important to achieving good growth and good pigment formation. Exogenous carbon dioxide, added as 2.5 g sodium bicarbonate per liter media also enhanced growth and reduced the lag time. The optimized medium enhanced the total growth of cells slightly, and increased the culture optical density and pigmentation by a factor of 2 and 3, respectively, bringing the culture performance in the defined medium above the performance in the rich, undefined medium.

A total of 31 chemicals from 17 chemical families were screened at either 10 mM or 2 mM concentration to determine their effect on the growth and pigment formation of \textit{B. viridis}. Most alcohols surveyed caused significant enhancement to growth rate, and the primary carbon in a secondary alcohol was proposed as the site of attack allowing usage of these compounds. Aromatic compounds were not as successfully cultured, and potential mechanisms for cell damage and compound degradation were discussed. \textit{B. viridis} demonstrated the ability to grow unimpaired in the presence of MEK, methyl chloride, mimethyl sulfoxide, dimethyl formamide, chloroethane, acetonitrile, and dioxane.

Several methods for working with bacteria were presented. The Two-Point method, a rapid and reliable method to compare cell growth and health, was demonstrated. A mechanized device for spin plating was also introduced, both significantly reducing operator fatigue and injury, and increasing the consistency of plate count data.
To my parents, Lance and Christine, who instilled in me a love of learning, made it a priority from day one, and moved heaven and earth to let me pursue it.

To my sister, Caitlin, end of discussion.

To my husband, Eric, without whom I would have laughed much less and lost my perspective much faster and more often.
CONTENTS

FIGURES .......................................................................................................................................viii

TABLES ..........................................................................................................................................xvi

ACKNOWLEDGMENTS .................................................................................................................xx

CHAPTER 1: INTRODUCTION, MOTIVATION, AND BACKGROUND ...................1
   1.1 Introduction .......................................................................................................................1
   1.2 The Toxin Problem ...........................................................................................................3
      1.2.1 Hazardous substances .............................................................................................3
      1.2.2 Sources of hazardous substances ...........................................................................4
      1.2.3 Release rates of hazardous substances ....................................................................4
      1.2.4 Determining acceptable levels of hazardous substances ........................................5
   1.3 Current Methods for Toxin Recovery ..............................................................................8
      1.3.1 Overview ...................................................................................................................8
      1.3.2 Non-biological .........................................................................................................9
      1.3.3 Biological ..............................................................................................................12
      1.3.4 Why are new methods needed? ...............................................................................18
   1.4 Types of Bacteria Utilized in Processing Waste ...............................................................20
   1.5 Purple Non-Sulfur Bacteria (PNSB) ..............................................................................24
      1.5.1 Classification ............................................................................................................24
      1.5.2 Known toxins degraded by PNSB ..........................................................................27
      1.5.3 Natural environment of PNSB ..............................................................................30
      1.5.4 Relation of B. viridis in PNSB group .....................................................................31
      1.5.5 Why B. viridis for toxins? .......................................................................................32
   1.6 Brief Overview of This Work ..........................................................................................33
      1.6.1 Measured parameters ..............................................................................................33
      1.6.2 Behavior in RMPABA and Minimal medias ..............................................................34
      1.6.3 Optimizing growth conditions ..................................................................................35
      1.6.4 Examining the effect of target compounds ...............................................................35
      1.6.5 Choice of compounds .............................................................................................36
   1.7 References .........................................................................................................................39
CHAPTER 2: QUANTIFYING CELL GROWTH ..........................................................47
  2.1 Overview ........................................................................................................47
  2.2 Background on Methods Used to Quantify Bacterial Cell Growth ............48
    2.2.1 UV-Vis Spectrophotometry ..............................................................48
    2.2.2 Colony Forming Unit (CFU) count .................................................51
  2.3 Methods Not Adopted for Use in this Project .............................................53
    2.3.1 Particle counters ...........................................................................53
    2.3.2 Cell mass measurements .............................................................54
    2.3.3 Microscope methods ....................................................................54
    2.3.4 TOC ..........................................................................................57
  2.4 Materials and Methods ...............................................................................59
    2.4.1 Media composition .........................................................................59
    2.4.2 Culture preparation .........................................................................60
    2.4.3 UV-Vis spectrophotometry ............................................................61
    2.4.4 Colony Forming Unit (CFU) counting ............................................62
  2.5 Results and Discussion ..............................................................................65
    2.5.1 Determining the culture density range for most accurate UV-Vis
         measurement ....................................................................................65
    2.5.2 A Correlation for the relationship between A660 and CFUs .............69
  2.6 Conclusions ................................................................................................78
  2.7 References ..................................................................................................80

CHAPTER 3: STANDARD GROWTH BEHAVIOR OF B. VIRIDIS .....................84
  3.1 Introduction ................................................................................................84
    3.1.1 Previous work and the next step ...................................................84
    3.1.2 Goals & Justification ....................................................................85
    3.1.3 Background on plotting culture growth .......................................87
  3.2 Materials & Methods ...............................................................................90
    3.2.1 Culture and sample preparation ...................................................91
    3.2.2 Characterization of cell growth and health for all performed
         experiments .........................................................................................92
    3.2.3 Developing and testing the Two Point Method ..............................93
  3.3 Results & Discussion ..............................................................................95
    3.3.1 Growth curve for RMPABA, Minimal, and a 50/50 RMPABA and
         Minimal mix .......................................................................................95
    3.3.2 Testing culture growth variability in Minimal media .....................110
    3.3.3 Validation of the Two Point Method ..........................................117
  3.4 Conclusions ............................................................................................119
  3.5 References ..............................................................................................120

CHAPTER 4: KNOWN GROWTH REQUIREMENTS OF B. VIRIDIS AND OTHER
          PNSB ........................................................................................................124
  4.1 Introduction ...........................................................................................124
  4.2 Known Requirements for Growth .........................................................125
    4.2.1 Inoculation volume, density and mechanical handling ..............125
    4.2.2 Vitamins .....................................................................................127
6.2.6 Buffer type and concentration ........................................................232
6.2.7 Vessel volume and illuminated surface area considerations ..........233
6.2.8 Light intensity ................................................................................234
6.2.9 Validation of Optimized media ......................................................235

6.3 Results and Discussion .................................................................................236
6.3.1 Baseline culture behavior ...............................................................236
6.3.2 Validation of Two-Point Method Modification for cultures in pH 5.6 ........................................................................244
6.3.3 Inoculation concentration ...............................................................245
6.3.4 Vitamin supplementation ...............................................................249
6.3.5 Carbon and nitrogen effects ...........................................................252
6.3.6 Buffer type and concentration ........................................................262
6.3.7 Volume and surface area considerations ........................................265
6.3.8 Light intensity ................................................................................269

6.4 Conclusions: Optimized Media ..............................................................272
6.4.1 Optimized media formulation ........................................................272
6.4.2 Validation of Optimized media ......................................................274

6.5 Conclusions ...................................................................................................280

6.6 References .....................................................................................................283

CHAPTER 7: SURVEY OF THE DEGRADATIVE POTENTIAL AND CHEMICAL TOLERANCE OF B. VIRIDIS ..........................................................285
7.1 Introduction ...................................................................................................285
7.1.1 Capabilities of PNSB .....................................................................286
7.1.2 Anaerobic and aerobic pathways for chemical degradation by PNSB ........................................................................288
7.1.3 Mechanisms of toxicity and solvent tolerance in bacteria ...........293

7.2 Materials and Methods ................................................................................294
7.3 Results and Discussion .................................................................................297
7.3.1 Screen 1: Alcohols .........................................................................298
7.3.2 Screen 2: Aromatic compounds .....................................................303
7.3.3 Screen 3: Aldehydes, ketones, and other common solvents ..........308
7.3.4 Screen 4: N, S, and halogen containing compounds and miscellaneous pollutants ..............................................................311

7.4 Conclusions ...................................................................................................315
7.5 References .....................................................................................................318

CHAPTER 8: FUTURE WORK ..............................................................................324
8.1 Introduction ...................................................................................................324
8.2 Suggestions for Future Work .................................................................325
8.2.1 Optimizing media formulation and culture conditions ...............325
8.2.2 Toxicity ..........................................................................................329
8.2.3 Degradation confirmation and pathways .....................................330
8.2.5 Alternate uses of the bacteria .........................................................334

8.3 References .....................................................................................................336
FIGURES

1.1 Non-Biological and Biological Treatment Methods ..................................................9
1.2 Common Microbial Attacks on Aromatic Ring Substituents .................................29

2.1 General Setup for a Spectrophotometer .................................................................49
2.2 A 10-Fold Serial Dilution Series ..............................................................................52
2.3 A Typical Counting Chamber Grid Layout ...............................................................56
2.4 The Shimadzu TOC-V Series Organic Carbon Analyzer .......................................58
2.5 The Pigment Spectrum of Extracted Membranes from B. viridis .........................62
2.6 The Inherent Measurement Limitation for UV-Vis Measurements .........................67
2.7 The Effect of Dense and Dilute Cultures .................................................................68
2.8 B. viridis Absorption Behavior for Varying Culture Densities ..............................69
2.9 The Relationship Between CFU Count and Calculated A660 ...............................70
2.10 For Minimal Media, the Correlation Between Live Cell Count and Absorbance at
     Early Time and Stationary ..........................................................................................73
2.11 For RMPABA Media, the Correlation for CFU and Absorbance at Early Time .......74
2.12 Comparison of the Correlation between CFU Count and A660 and CFU Count and
     A1020 .........................................................................................................................77

3.1 An Idealized Growth Curve for Microbes ...............................................................87
3.2 Error Calculation for Two-Point Method .................................................................94
3.3 Growth Curves Monitored by A660 for RMPABA, 50/50 Mix, and Minimal Medias .................................................................................................................97
3.4 Growth Monitored by A660 for RMPABA, 50/50 Mix, and Minimal Medias ...............................................................................................................................98
3.5 The pH Behavior of Cultures Grown in RMPABA, 50/50, and Minimal Medias ... 100
3.6 Pigment Increase Measured by A1020 for RMPABA, 50/50 Mix, and Minimal Media ........................................................................................................................................102
3.7 Ratios of Pigment to Cells for RMPABA, 50/50 Mix, and Minimal Media .......... 103
3.8 Comparison of CFU Count between Two Cultures Grown in Minimal Media ...... 111
3.9 Comparison of Growth Rate as Measured by A660 for Two Minimal Cultures ..... 112
3.10 The pH Behavior for Two Minimal Batches ....................................................... 113
3.11 Comparison of Pigment Signature, Represented by A1020, for Two Cultures in Minimal Media ........................................................................................................................................114
3.12 Pigment to Cell Ratios for Two Batches of Minimal Grown B. viridis .......... 115
3.13 Minimal Growth Curve and Rate of Growth Two-Point Method ................. 115

4.1 Glycolysis Converts Glucose to Pyruvate in a Series of Enzymatic Steps ..........129
4.2 The Calvin Cycle .................................................................................................. 130
4.3 The TCA and Reverse TCA Cycles ..................................................................... 132
4.4 The X-ray Crystallography Structure of the Fe-protein and FeMo-protein of Nitrogenase ...............................................................................................................139
4.5 The Flow of Electrons through Ferridoxin, Fe-protein, and MoFe-protein during Nitrogenase Mediated Nitrogen Fixation ...............................................................................140
4.6 The Reduction of N₂ to NH₃ on the MoFe-protein during Nitrogenase Mediated Nitrogen Fixation ..................................................................................................................140
4.7 Generalized Photosynthesis Proton Pump ..........................................................144
5.20 The Final pH and pH Change after 5 Days of Growth in Varying Concentration of Potassium Phosphate Buffer .................................................................207

5.21 The Total Growth, Measured by A660, Observed in RMPABA Media Adjusted to Various pH Values .........................................................................................................................209

5.22 The Final pH for Cultures Based on Their Initial pH ...............................................210

5.23 A Loose Correlation between Growth and pH Change for RMPABA Grown Cells .................................................................................................................................211

5.24 The Change in Culture Density for Cultures Grown in Varied pH Minimal Media ........................................................................................................................................212

5.25 The Growth Rate for Cultures Grown in Minimal Media at Various pH Values ...213

5.26 The Final pH of Cultures Grown in Minimal Media Based on Initial pH .................214

5.27 The Correlation of pH Change to Change in Culture Density, as Measured by A660 .......................................................................................................................................215

5.28 The Behavior of Inorganic Carbon Dissolved in Water at Various pH values ......218

6.1 The Arrangement of Vessels for Varied Light Intensity ............................................235

6.2 The Growth and Culture Density Increase of Cells Grown in pH Adjusted Minimal Media ....................................................................................................................................237

6.3 The Increase in Pigment Measured by A1020 for Cells Grown in pH 5.6 Minimal Media ......................................................................................................................................238

6.4 The Pigment to Cell Ratio of \textit{B. viridis} Cells Grown in pH 5.6 Minimal Media .....240

6.5 The pH Behavior of \textit{B. viridis} Cultured in Minimal Media Initially Adjusted to pH 5.6 .............................................................................................................................................241

6.6 Two-Point Method Modification for pH 5.6 Minimal Media ....................................245

6.7 The Effect of Initial Inoculation on Growth Rate for Cells in Minimal 5.6 Media ....247

6.8 The Effect of Initial Inoculation on Final Culture Density .........................................248

6.9 The Effect on Growth Rate of Modifying the Concentration of Vitamins and Supersalts Present in the Standard Media Formulation .........................................................249
6.10 The Effect of Vitamin Supplementation on the Growth Rate of *B. viridis* in Minimal 5.6 Media .................................................................250

6.11 The Pigment Ratios of Cultures Supplemented with Various Vitamins ..........251

6.12 The Effect of Varying Concentration of Malate on the Growth of *B. viridis* in Minimal 5.6 Media .................................................................253

6.13 The Effect of Bicarbonate Supplementation on Growth of *B. viridis* ..........256

6.14 The pH behavior of Cultures Supplemented with Bicarbonate ..................257

6.15 The Effect of Alternate Carbon Sources on the Growth of *B. viridis* in Minimal 5.6 Media ..............................................................................258

6.16 The Structure of Malate, Fumarate, and Sucrose .................................................................260

6.17 The Growth Rate of *B. viridis* as a Function of Fixed Nitrogen Concentration .....261

6.18 The Effect of Varying Concentrations of Phosphate, Carbonate, and Acetate Buffers on Growth of *B. viridis* ........................................................................................................263

6.19 The Final pH of Cultures Buffered with Phosphate, Carbonate, and Acetate ......263

6.20 The Effect of Phosphate, Carbonate, and Acetate Buffers on Pigment Formation in *B. viridis* ...............................................................................................................264

6.21 The Growth of *B. viridis* in Bottles of Varying Volume ..................................267

6.22 The Effect of Surface Area/Volume on Growth and Pigment/Cell Ratio for *B. viridis* .......................................................................................................................268

6.23 The Effect of Incident Light on the Growth Rate of *B. viridis* .........................270

6.24 The Pigment Behavior of *B. viridis* under Various Light Intensities ...............272

6.25 The Culture Density Behavior of *B. viridis* Grown in Minimal 5.6 Media and Optimized Media .................................................................275

6.26 The Live Cell Behavior of *B. viridis* in Minimal 5.6 and Optimized Medias ..........276

6.27 The Pigment Increase of *B. viridis* for Minimal 5.6 and Optimized Medias ........277

6.28 The Ratio of Pigment to Cells for *B. viridis* in Optimized and Minimal 5.6 Medias .................................................................278
6.29 The pH Behavior of *B. viridis* Grown in Minimal 5.6 and Optimized Medias ......279

7.1 The Enzymatic Pathways of a Wide Variety of Organic Compounds to Benzoyl-CoA .....................................................................................................................................................................................289

7.2 The Anaerobic Degradation Pathways of the BTEX Chemicals ........................................291

7.3 The Growth Rates of *B. viridis* in Minimal and NC Medias when Supplemented with Various Alcohols and Phenol at 10 mM Concentration .................................................................299

7.4 The Final pH Observed for *B. viridis* Cultures Supplemented with Various Alcohols or Phenol ..........................................................................................................................................................................................300

7.5 The Proposed Site of Attack on 2-Propanol (R=H) and t-Butanol (R=CH₃) ..........302

7.6 The Growth Rates of *B. viridis* in Minimal and NC Medias Supplemented with 2 mM of Compound ........................................................................................................................................................................304

7.7 The Pigment Behavior of Cells in the Presence of Aromatic Compounds .................305

7.8 The Effect of Aldehydes and Other Common Solvents on the Growth Rate of *B. viridis* ..............................................................................................................................................................................................309

7.9 The Pigment Ratio for Cells in Minimal 5.6 and NC (Carbon Free) Media, Supplemented with 2 mM of Various Common Solvents ..................................................................................................................310

7.10 The Effect of N, S, and Halogen Containing Compounds and Common Pollutants on the Growth of *B. viridis* ..................................................................................................................................................................................312

7.11 The Effect of N, S, and Halogen Containing Compounds and Common Pollutants on the Final Pigment Ratio of *B. viridis* ..........................................................................................................................313

7.12 The Final pH of Cultures of *B. viridis* Amended with Various Common Pollutants ..................................................................................................................................................................................314

8.1 The Arrangement of Vessels to Achieve 500-2000 lux Light Intensity ...................327

8.2 Proposed Design of Flat Panel Reactor ....................................................................328

8.3 Four Possible Outcomes of Contact with Various Concentrations of the Target Compound ..........................................................................................................................................................................................329

8.4 The Removal of Toluene By Filtering with *B. viridis* Cell Mass .........................335
A.1 The Three Primary Items Used in the Assembly of the Automated Spin Plate Device
..........................................................................................................................................356
A.2 The Simple Design of the Spin Plating Device ..........................................................357
A.3 The Final Design for the Device ..............................................................................357
A.4 Effects of Rotation Speed and Spreading Time .......................................................363
A.5 Examples of Contamination and Smearing ..............................................................364
A.6 Effect of Speed of Rotation and Spreading Time on Contamination ......................366
A.7 Examples of Colony Distribution ............................................................................367
A.8 The Postures Used with the Mechanized Device can Improve its Ergonomic
Friendliness .....................................................................................................................368

C.1 A Typical Wastewater Plant......................................................................................385
C.2 A Continuous Stirring Tank Reactor.........................................................................390
C.3 A Plug Flow Reactor .................................................................................................393
C.4 Model of a Trickle Bed Reactor ..............................................................................394

D.1 The Pigment Spectrum of Extracted Membranes from B. viridis .........................397
D.2 The Arrangement of Antenna LHCs and a RC ........................................................398
D.3 The Reaction Center and Membrane Proteins of a Photosynthetic Bacterium........399

F.1 The Light Box............................................................................................................404

G.1 Anaerobic Toluene Degradation Pathway ..............................................................405

H.1 Anaerobic Ethylbenzene Degradation Pathway......................................................406
I.1 Anaerobic Phenol Degradation Pathway ................................................................. 407

J.1 The Anaerobic Benzoate Degradation Pathway from Benzoate to 3-Hydroxypimelyl-CoA ........................................................................................................ 408

J.2 The Degradation Pathway from 3-Hydroxypimelyl-CoA to Acetyl-CoA .......... 409

K.1 Three Potential Pathways of Aerobic Degradation of Toluene to Acetaldehyde and Pyruvate ................................................................................................. 410

K.2 Two Potential Pathways of Aerobic Degradation of Toluene to Benzoate or 4-Hydroxybenzoate ........................................................................................................ 411

K.3 Aerobic Benzoate Degradation: Continuation of Toluene Degradation from Benzoate through Catechol .............................................................. 412

K.4 The Aerobic Nitrobenzene Degradation Pathway .............................................. 413

K.5 The Aerobic Vanillin Degradation Pathway .......................................................... 414

L.1 The Anaerobic 1,2-Dichloroethane Degradation Pathway ............................. 415

M.1 Methyl Ethyl Ketone Degradation Pathway .................................................... 416

N.1 The Degradation Pathway for Tetrahydrofuran ................................................ 417

O.1 The Dimethyl Sulfoxide and Organosulfide Cycle ............................................ 418
TABLES

1.1 TOTAL RELEASE OF DIOXINS IN 2003 .............................................................. 5
1.2 TOTAL RELEASE RATES FOR BTEX COMPOUNDS 2003 .............................. 6
1.3 TOTAL RELEASE RATES FOR PHENOL CONTAINING COMPOUNDS IN 2003 ....................................................................................................................... 6
1.4 EPA PRIMARY DRINKING WATER STANDARDS (INORGANICS) ............... 7
1.5 EPA PRIMARY DRINKING WATER STANDARDS (CHLORINATED) .......... 7
1.6 SECONDARY TREATMENT STANDARDS ......................................................... 8
1.7 THE DEGRADATION OF ENVIRONMENTALLY RELEVANT COMPOUNDS BY UNDEFINED MIXTURES OF BACTERIA ................................................................. 14
1.8 DEGRADATIONS OF SPECIFIC CHEMICALS BY SPECIFIC SPECIES OF MICROBES ...................................................................................................................... 17
1.9 KEY FEATURES OF FOUR COMMONLY USED MICROBE TYPES .......... 22
1.10 CHEMICAL COMPOUNDS KNOWN TO BE DEGRADED BY PNSB .......... 28

2.1 SIX DIFFERENT DILLUTIONS OF CELL BROTH AND THEIR CELL CONCENTRATION (CFU/ML) AND A660 VALUES .............................................................. 65
2.2 THE DISCREPANCY BETWEEN ACTUAL AND EXPECTED SPECTROPHOTOMETER MEASUREMENTS ................................................................. 66
2.3 THE DIFFERENCE BETWEEN MEASURED VALUES AND VALUES CALCULATED USING THE CORRELATION FOR MINIMAL CELL GROWTH FROM EQUATION 5 ....................................................................................................... 73
2.4 THE ERROR BETWEEN MEASURED VALUES AND VALUES CALCULATED USING THE CORRELATION FOR RMPABA ..............................................................75

3.1 SIX CHARACTERISTICS OF GROWTH USED TO QUANTIFY THE BEHAVIOR OF B. viridis .................................................................92

3.2 CHARACTERISTICS OF GROWTH OBSERVED FOR B. VIRIDIS IN RMPABA, 50/50, AND MINIMAL MEDIAS .................................................................107

3.3 COMPARISON OF CHARACTERISTICS OF GROWTH OF B. VIRIDIS IN RMPABA AND MINIMAL MEDIA .................................................................119

4.1 VITAMINS AND COFACTORS COMMONLY REQUIRED FOR GROWTH OF PNSB ..............................................................................................................128

5.1 EXPERIMENTAL CONDITIONS TO DETERMINE THE INTRINSIC GROWTH OF CELLS IN MINIMAL MEDIA CULTURE INOCULATED WITH BROTH PREPARED IN RMPABA MEDIA .................................................................163

5.2 THE COMPOSITION OF SUPERSALTS INCLUDING TRACE ELEMENTS AND ITS CONCENTRATION IN THE FINAL BROTH ...........................................164

5.3 THE BASE MEDIAS AND ADDITIONS TO INVESTIGATE THE EFFECT OF THIAMINE, P-ABA, AND SUPERSALTS ........................................................................165

5.4 SATURATION EQUIVALENT BICARBONATE ADDITIONS AND THEIR MOLAR CONCENTRATION .................................................................166

5.5 CARBON ADDITIONS TO ACHIEVE A 20 mM CONCENTRATION .................167

5.6 FUMARATE AS A SOLE CARBON SOURCE OR COMBINED WITH MALATE .........................................................................................................................168

5.7 DETERMINING THE OPTIMUM CONCENTRATION OF MALATE FOR THE GROWTH OF B. VIRIDIS .................................................................169

5.8 SPARGED GAS AND MEDIA TYPE TO DETERMINE THE NITROGEN FIXING ABILITY, OXYGEN SENSITIVITY, AND PREFERED NITROGEN SOURCE FOR B. VIRIDIS .................................................................................................................................170
5.9 CONCENTRATION OF AMMONIUM SULFATE TO DETERMINE OPTIMUM CONCENTRATION FOR GROWTH OF B. VIRIDIS ..............................................................170

5.10 POTASSIUM PHOSPHATE BUFFER ADDITIONS TO BUFFER FREE MEDIA ........................................................................................................................171

5.11 SELECTED PH VALUES IN THE RANGE 4.5 TO 9.5 CULTURED TO EXPLORE THE PH DEPENDANT GROWTH BEHAVIOR OF B. VIRIDIS ..........173

6.1 VITAMIN ADDITIONS TO BASE MEDIA TO INVESTIGATE THE EFFECT OF VITAMIN SUPPLEMENTATION ON THE GROWTH OF B. VIRIDIS .................229

6.2 SATURATION EQUIVALENT BICARBONATE ADDITIONS TO MEDIA BROTH AND THE CORRESPONDING MOLAR CONCENTRATIONS OF BICARBONATE ............................................................................................................231

6.3 THE PHYSICAL DIMENSIONS FOR VESSELS USED TO DETERMINE VOLUME AND SURFACE AREA EFFECTS ON THE GROWTH OF B. VIRIDIS ....233

6.4 THE LIGHT INTENSITY ON CULTURES OF B. VIRIDIS ...........................................234

6.5 CHARACTERISTICS OF GROWTH OBSERVED FOR B. VIRIDIS IN MINIMAL MEDIAS AT pH 6.9 AND 5.6 .........................................................................................................................242

6.6 THE IMPORTANT VITAMIN SUPPLEMENTS FOR GROWTH OF B. VIRIDIS IN MINIMAL MEDIA AT pH 5.6 .....................................................................................................................252

6.7 COMPARISON OF THE FORMULATIONS FOR MINIMAL 5.6 AND OPTIMIZED MINIMAL MEDIAS FOR THE GROWTH OF B. VIRIDIS ..............273

6.8 COMPARISON OF THE CULTURE CONDITIONS FOR GROWTH OF B. VIRIDIS ASSOCIATED WITH MINIMAL 5.6 AND OPTIMIZED MINIMAL MEDIAS .........................................................................................................................274

6.9 CHARACTERISTICS OF GROWTH OF B. VIRIDIS A COMPARISON OF PRE-OPTIMIZED MINIMAL 5.6 MEDIA AND OPTIMIZED MEDIA BASED ON THE EXPERIMENTAL RESULTS PRESENTED IN 6.4.2 .................................................................281

7.1 CHEMICAL COMPOUNDS SCREENED FOR THEIR EFFECT ON B. VIRIDIS DIVIDED INTO FOUR EXPERIMENTAL GROUPS BASED ON CHEMICAL FAMILY .........................................................................................................................295
7.2 COMPARISON OF GROWTH RATE AND FINAL pH OF \textit{B. VIRIDIS} CULTURES SUPPLEMENTED WITH ALCOHOLS WHICH CAUSED GROWTH ENHANCEMENT COMPARED TO THE MINIMAL 5.6 CONTROL .................300

B.1 TOTAL RELEASES FOR 2003 IN THE US ..........................................................372

D.1 THE BACTERIACHLOROPHYLLS AND THEIR CHARACTERISTIC WAVELENGTHS AND THE BACTERIA ASSOCIATED WITH EACH ......................397

E.1 THE FORMULATION FOR MEDIAS USED IN THIS WORK .........................402

E.2 THE FORMULATION OF MALATE SOLUTION USED IN MAKING MEDIA ............................................................................................................................403

E.3 THE FORMULATION OF POTASSIUM PHOSPHATE BUFFER USED IN MAKING MEDIA......................................................................................................403

E.4 THE FORMULATION OF SUPERSALTS SOLUTION USED IN MAKING MEDIA ............................................................................................................................403

E.5 THE FORMULATION OF TRACE ELEMENTS SOLUTION USED IN MAKING SUPERSALTS ...............................................................................................403

F.1 THE MEASURED LIGHT INTENSITY (LUX) IN THE LIGHT BOX ASSEMBLED TO INCUBATE CELL CULTURES..............................................................404
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I would like to recognize the invaluable benefit of having a mother who is an English teacher. The responsibility for any grammatically correct sections rests firmly with her, whether direct assistance as “editor” or by virtue growing up in her household. Special thanks for reviewing my citations to insure compliance with Turabian/Chicago formatting rules. My gratitude to my father who tirelessly handed the phone over immediately when I needed consultation, no matter how much he wanted to talk with his firstborn. It was my family and husband who cheered me up, calmed me down, and kept me on track when I was ready to give up or turn back. Thanks to my sister, Caitlin, who went through her Masters thesis process a year ago, and commiserated with me just when I needed it most. Special thanks, love, and devotion goes to my husband, Eric, who
suffered through the worst of my exhaustion, frustration and moods as a fiancé and said “I do” anyway. May I be just as understanding when he writes his thesis.

My deepest gratitude to Sue Myers, who acted as a catalyst for me to remember what it meant to be healthy; she helped me get back to it more times than I can count when I ran my body and mind into the ground in pursuit of science and my degree.

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Without the assistance of Mr. Jim Smith in implementing the design of the spin-plating device, I would certainly have carpel tunnel syndrome by now. It is with gratitude and pain free arms that I extend my appreciation for his expertise.

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completed her thesis in 1988 and recovered extremely well, for offering her perspective and support whenever it seemed I had lost mine.
CHAPTER 1

INTRODUCTION, MOTIVATION, AND BACKGROUND

1.1 Introduction

In the last 25 years, there has been an increase in concern about treatment and disposal of human and industrially-generated waste, as well as an upsurge in regulatory requirements. With this increase in concern, there has been much interest in identifying microbes with inherent abilities to biodegrade compounds of environmental or regulatory infamy. As a result, many organisms have been discovered which biodegrade compounds previously considered untouchable.

One of the most promising microbial families is the Purple Non-Sulfur Bacteria (PNSB). These bacteria are photosynthetic and can therefore harvest light energy from the sun in order to drive reactions that might otherwise be energetically unfavorable. This is in contrast to a wide variety of chemoheterotrophic bacteria which derive all their energy from the breakdown of complex organic chemicals.

Several studies have been done on bacteria in the Purple Non-Sulfur Bacteria family, most specifically *Rhodopseudomonas palustris*, which demonstrate that these
bacteria are quite nutritionally diverse and can break down a wide variety of compounds.\(^5\) *Blastochloris viridis* (formerly *Rhodopseudomonas viridis*), a close relative, has been studied fairly extensively to better understand the electron transport involved in photosynthesis, but almost nothing has been done to determine the ecological usefulness of this bacteria. A strain of *B. viridis* EJB4, containing a readily modified genetic system, was obtained.\(^6\) This genetic system makes EJB4 an ideal subject for biodegradation research, since it would be possible, in the future, to enhance the capabilities or functionality of this organism through the use of genetic engineering.

The goal of this work was initially to characterize the ability of *B. viridis* to break down a wide variety of chemical compounds considered environmentally relevant. First, acceptable methods of measurement and quantification of culture growth and health were defined. (Chapter 2) Because *B. viridis* is a photosynthetic bacterium and contains a pigment, many standard methods of microbiology required adjustment and characterization in order to be useful in this effort. In the effort to develop improved methods, a device for improved live cell counting was developed. (Appendix A) Next, the basic behavior of the bacteria was defined. (Chapter 3) This provided an understanding of how the bacteria behaved in an optimized, rich media environment and what could be expected in terms of growth performance and light absorbing pigment production of normally-growing healthy bacteria. While a very rich and undefined media was sufficient to grow large quantities of this bacterium for the purposes of isolating the photosynthetic apparatus, this media was found to be insufficient for exploration of the innate waste processing abilities of *B. viridis*. The undefined nature of the rich medium made it impossible to distinguish the media component responsible for growth, and
impossible to have truly repeatable growth. Before any tailoring of the media was undertaken, research into the basic nutritional and environmental preferences of the bacteria was performed. (Chapter 4) Subsequently, a minimal, defined media formula was established and optimized to give sustainable and repeatable growth of healthy cells with significant levels of pigment. (Chapter 5 and 6) Finally, a brief exploration of the ability of *B. viridis* to survive in the presence of representative waste compounds was surveyed. (Chapter 7) Since the goal of this exploration was to provide a starting point for future research and identify families of chemicals which *B. viridis* can take an active role in remediating, brief suggestions for future work are provided in Chapter 8.

1.2 The Toxin Problem

1.2.1 Hazardous substances

A “hazardous substance” is defined by the EPA as one which

because of its quantity, concentration, or physical, chemical, or infectious characteristics may cause, or significantly contribute to, an increase in mortality; or cause an increase in serious irreversible or incapacitating reversible illness; or pose a substantial present or potential hazard to human health and the environment when improperly treated, stored, transported, or disposed of, or otherwise managed.  

Hazardous waste can be so defined because it contains a chemical specifically identified as hazardous, or because it meets the general criteria of flammable, corrosive, reactive, or toxic. Flammable wastes are defined by the flash point of the material. Corrosive wastes have pH measurements of less than 2 pH units or greater than 12.5 pH units. Reactive wastes react violently with water, form potentially explosive mixtures, are typically unstable, or may evolve toxic fumes. Toxic wastes are defined by bioconcentration (the likelihood of building up in the environment), LD$_{50}$ and LDC$_{50}$,
(the lethal dose or concentration for 50% of organisms tested), and phototoxicity (the effect on plants). These compounds can also be classified by their chemical family.

1.2.2 Sources of hazardous substances

Each year, approximately one ton of hazardous waste per person is produced in the United States, which makes waste treatment an extremely vital issue. There are two primary classes of waste: domestic and industrial. Domestic sewage is relatively simple and mild in composition. Industrial waste, on the other hand, can contain a variety of more hazardous contaminants. Even industrial waste that is not introduced directly to the sewer can make its way into the environment via accidental spills, as leachates from improperly disposed waste or waste disposed of in buried drums, and by leaks from underground chemical or waste storage tanks.

1.2.3 Release rates of hazardous substances

A startlingly large amount of hazardous wastes make their way into sewage, ground water, and the environment. In 2003, 522 chemical compounds and groups were reported, and a total of 4,443,166,690 pounds of chemical releases were noted for On- and Off-site Disposal or Other Releases. (See Appendix B for complete list) Dioxins, since they are considered particularly hazardous, must also be reported separately, in grams. The total release of dioxins was over 100 kilograms. (See Table 1) Large amounts of chemicals of common concern are also released. Over 114 million pounds of BTEX compounds(benzene, toluene, ethylbenzene, and the xylenes) and 11 million pounds of compounds containing phenol alone were released in 2003. (See
Tables 2, 3) Because a vast amount of chemicals are making their way into the environment, finding new and effective ways to treat these releases is critical.

1.2.4 Determining acceptable levels of hazardous substances

A successful treatment for domestic wastes is focused on modifying the general characteristics of the water, such as suspended solids, dissolved solids, inorganic compounds including heavy metals, organic compounds, BOD (Biological Oxygen Demand), TOC (Total Organic Carbon), COD (Chemical Oxygen Demand), and Total Nitrogen.\textsuperscript{11} The EPA established limits of acceptability for these characteristics in both wastewater and drinking water. Inorganic compounds in drinking water are typically limited to fractions of a milligram.\textsuperscript{12} (See Table 4) Organic chemicals are significantly regulated, both because many can cause severe health problems and because organic chemicals can provide a food source for algal and bacterial blooms in surface water, which can cause significant ecological harm.\textsuperscript{13} (See Table 5) In order to be discharged into surface water, BOD, suspended solids, and pH must meet both a monthly and a weekly standard.\textsuperscript{14} (See Table 6)

<table>
<thead>
<tr>
<th>TABLE 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>TOTAL RELEASE OF DIOXINS IN 2003\textsuperscript{15}</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Total On- and Off-site Disposal or Other Releases (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dioxin and Dioxin Like Compounds</td>
<td>129,571.35</td>
</tr>
</tbody>
</table>
TABLE 2

TOTAL RELEASE RATES FOR BTEX COMPOUNDS 2003\textsuperscript{16}

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Total On- and Off-site Disposal or Other Releases (pounds)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BENZENE</td>
<td>6,746,553</td>
</tr>
<tr>
<td>ETHYLBENZENE</td>
<td>6,978,471</td>
</tr>
<tr>
<td>M-XYLENE</td>
<td>580,739</td>
</tr>
<tr>
<td>O-XYLENE</td>
<td>675,306</td>
</tr>
<tr>
<td>P-XYLENE</td>
<td>1,331,125</td>
</tr>
<tr>
<td>TOLUENE</td>
<td>57,974,725</td>
</tr>
<tr>
<td>XYLENE (MIXED ISOMERS)</td>
<td>40,575,531</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>114,862,450</strong></td>
</tr>
</tbody>
</table>

TABLE 3

TOTAL RELEASE RATES FOR PHENOL CONTAINING COMPOUNDS IN 2003\textsuperscript{17}

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Total On- and Off-site Disposal or Other Releases (pounds)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,4,5-TRICHLOROPHENOL</td>
<td>5,128</td>
</tr>
<tr>
<td>2,4,6-TRICHLOROPHENOL</td>
<td>2,088</td>
</tr>
<tr>
<td>2,4-DICHLOROPHENOL</td>
<td>10,661</td>
</tr>
<tr>
<td>2,4-DIMETHYLPHENOL</td>
<td>159,634</td>
</tr>
<tr>
<td>2,4-DINITROPHENOL</td>
<td>66,735</td>
</tr>
<tr>
<td>2-NITROPHENOL</td>
<td>95</td>
</tr>
<tr>
<td>2-PHENYLPHENOL</td>
<td>6,178</td>
</tr>
<tr>
<td>4,4'-ISOPROPYLDIENEDIPHENOL</td>
<td>1,765,068</td>
</tr>
<tr>
<td>4-NITROPHENOL</td>
<td>1,529</td>
</tr>
<tr>
<td>CHLOROPHENOLS</td>
<td>34,159</td>
</tr>
<tr>
<td>DINITROBUTYL PHENOL</td>
<td>9,410</td>
</tr>
<tr>
<td>PENTACHLOROPHENOL</td>
<td>2,968</td>
</tr>
<tr>
<td>PHENOL</td>
<td>8,499,213</td>
</tr>
<tr>
<td>TETRABROMOBISPHENOL A</td>
<td>643,250</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>11,206,116</strong></td>
</tr>
</tbody>
</table>
### TABLE 4

**EPA PRIMARY DRINKING WATER STANDARDS:**

**MAXIMUM CONTAMINANT LEVELS**

**FOR INORGANIC CHEMICALS**\(^{15}\)

<table>
<thead>
<tr>
<th>Contaminant</th>
<th>Level (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arsenic</td>
<td>0.05</td>
</tr>
<tr>
<td>Barium</td>
<td>1.00</td>
</tr>
<tr>
<td>Cadmium</td>
<td>0.010</td>
</tr>
<tr>
<td>Chromium</td>
<td>0.05</td>
</tr>
<tr>
<td>Lead</td>
<td>0.05</td>
</tr>
<tr>
<td>Mercury</td>
<td>0.002</td>
</tr>
<tr>
<td>Nitrate (as N)</td>
<td>10.00</td>
</tr>
<tr>
<td>Selenium</td>
<td>0.01</td>
</tr>
<tr>
<td>Silver</td>
<td>0.05</td>
</tr>
</tbody>
</table>

### TABLE 5

**EPA PRIMARY DRINKING WATER STANDARDS:**

**MAXIMUM CONTAMINANT LEVELS**

**FOR CHLORINATED HYDROCARBONS AND CHLOROPHENOXY**\(^{16}\)

<table>
<thead>
<tr>
<th>Group</th>
<th>Chemical</th>
<th>Level (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorinated</td>
<td>Endrin (1,2,3,4,10,10-hexachloro-6,7-wpozy-1,4,4a,5,6,7,8,8a-octo-hydro-1,4-end,endo-5,8-dimethanonapthalene)</td>
<td>0.0002</td>
</tr>
<tr>
<td>Hydrocarbons</td>
<td>Lindane (1,2,3,4,5,6-hexachlorocyclohexane, gamma isomer)</td>
<td>0.004</td>
</tr>
<tr>
<td></td>
<td>Methoxychlor (1,1,1-trichloro-2,2-bis {p-methoxy-phenyl}ethane)</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>Toxaphene (C(<em>{10})H(</em>{10})Cl(_t)-technical chlorinated camphene, 67-69% chlorine)</td>
<td>0.005</td>
</tr>
<tr>
<td>Chlorophenoxy</td>
<td>2,4-D (2,4-dichlorophenoxyacetic acid)</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>2,4,5-TP silvex (2,4,5-trichlorophenoxypropionic acid)</td>
<td>0.01</td>
</tr>
</tbody>
</table>
1.3 Current Methods for Toxin Recovery

1.3.1 Overview

There are a wide variety of approaches employed in treating waste. The chosen method is determined based on the characteristics of the waste stream and the regulations affecting its components and eventual release. The methods are typically broken down into non-biological and biological methods, (See Figure 1) however several methods are often combined to achieve more complete remediation. Typically a method will include some manual processing such as removing large objects, some chemical processing, such as precipitation of heavy metals, and some biological processing, such as contacting with activated sludge. As time has gone by, many methods have become outdated or deemed ecologically irresponsible and banned. As previously used methods are deemed unacceptable, new methods with greater ability to degrade the compounds of interest must be devised to replace them.

### TABLE 6

SECONDARY TREATMENT STANDARDS\(^{17}\)

<table>
<thead>
<tr>
<th>Characteristics of Discharge</th>
<th>Unit of Measurement</th>
<th>Average Monthly concentration</th>
<th>Average weekly concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>BOD(_5)</td>
<td>mg/L</td>
<td>30(^*)†</td>
<td>45†</td>
</tr>
<tr>
<td>Suspended solids‡</td>
<td>mg/L</td>
<td>30(^*)†</td>
<td>45†</td>
</tr>
<tr>
<td>Hydrogen-ion concentration</td>
<td>pH units</td>
<td></td>
<td>6.0-9.0§</td>
</tr>
</tbody>
</table>

**NOTE:**

* Or no more than 15% of influent value.
† Arithmetic mean.
‡ Treatment plants with stabilization ponds and flows less than 7570 m\(^3\)/day (2 Mgal/d) are exempt.
§ Continuous, only enforced if caused by industrial wastewater or in-plant treatment.
1.3.2 Non-biological

Historically, dilution was considered an appropriate method of waste treatment.¹⁸ Eventually, this was deemed inadequate to meet the goal of maintaining the environmental health of the streams into which waste was being discharged, especially in the case of the higher volumes of domestic waste produced in urban locations and the particularly hazardous waste produced in industrial settings. For the treatment of domestic waste, the Wastewater Treatment Facility was developed. (See Appendix C)
Industrial generators of waste are often not permitted to release wastewater into the public sewage system until it meets certain concentration and composition criteria. This means that a large amount of hazardous waste treatment happens on sight, and that some waste is transported to offsite treatment facilities.

There are a number of chemical-based treatments for industrial waste, particularly inorganic compounds. If chemicals can be neutralized, a chemical addition is made to render the chemical inert. This can sometimes be accomplished by combining wastes from several onsite processes, but it often requires the purchase of acids or bases.\(^{19}\) Oxidation can also be used to destroy a dangerous compound. Another method is to add agents to cause the hazardous chemicals to precipitate out as recoverable, and hopefully less hazardous, solids.\(^{20}\) Many heavy metals, which pose an environmental threat, can be precipitated out readily when the pH is raised to the pH 8-10 range, and this is generally accomplished by the addition of lime.\(^{21}\)

A wide variety of physical treatments are employed for handling of hazardous waste. Some waste can have a portion of the water removed in order to concentrate it and reduce its total volume.\(^{22}\) This is often accomplished by filtration or pressing.\(^{23}\) One common method is to mix the waste with a cement and lime slurry, and allow it to harden into a concrete block. The hope is that this will keep the material safely contained, however the materials can leach out over time and become environmental contaminants.\(^{24}\) Adsorption can also be used to remove a contaminant from an otherwise disposable waste stream. This concentrates contaminants on the surface of activated charcoal. The contaminant can then be either recovered for reuse or incinerated during regeneration of the charcoal.\(^{25}\) Incineration is a very popular method combined with adsorption or used

10
independently, which can reduce future liability for the chemicals if adequate reduction of waste is achieved. This has several potential problems because complete combustion can never be achieved, leading to sometimes more hazardous incomplete combustion products, discharge of chemicals as hazardous emissions during the process, and ash rich with contaminants.26

There have been many improvements in waste treatment since the “dilution is the solution” days, but a large amount of waste is still disposed of by containment in “secure” landfills, instead of being treated. Recent regulations require that the liquid disposal in dumpsites be minimized, and some liquids such as solvents, dioxins, chlorinated organics, and liquids containing dissolved metals are not allowed at all,27 creating a class of wastes that require improved methods of management. Despite the regulations on liquids, sludges and solids containing hazardous wastes can still make their way into landfill sites. Since there is no truly secure way to store waste, this material will eventually find its way into the soil, water, or air, or need to be dug up and treated.28 This means that an increased focus must be on developing novel and complete ways of dealing with hazardous wastes before they have an opportunity to become environmental remediation sites.

If hazardous waste is allowed to make its way into the environment, there are several methods of treating it. In the most severe cases, where human life is threatened, contaminated soil and materials are removed and transported to a safer disposal area or offsite treatment. This is often extremely cost prohibitive.29 More common is to establish or improve containment by adding non-permeable materials near the contaminated site, but this is still only a temporary solution to the problem.30 Pump and
treat methods are also common. In this method, water is pumped out of the ground, treated in an onsite facility, and then pumped back into the ground. This is generally combined with wells drilled for containment to prevent ground water from leaving the area. Over time, the volume of treated water will be adequate to dilute the concentration of contaminant in the groundwater, and the concentrations reach environmentally acceptable levels.\(^{31}\) Another method is in situ treatment, which involves the addition of chemicals or bacteria into the contaminated site. The chemical additions generally have one of two purposes. The first is to chemically fix the contaminants so that they cannot leach into as yet uncontaminated areas. The second purpose is to enhance the ability of native bacteria to live in the environment and degrade the contaminants.\(^{32}\) The injection of bacteria is a fairly novel technique, and it is dependant on the discovery of novel strains that decompose materials previously thought to be undegradable, as well as the development of methods to enhance their efficiency.\(^{33}\)

1.3.3 Biological

Many compounds known in nature can be readily broken down if the correct microorganisms are employed. One previously common method of land disposal, especially employed by the petroleum industry, was to spread the waste on land and allow the native microorganisms to biodegrade it. It seemed to have a fairly high efficacy, however the EPA disallowed this practice because chemicals are hard to control once they are introduced to the environment.\(^{34}\) It is also highly undefined process with very little guarantee of repeatability. This stresses the importance of developing biological techniques, even for known degradation processes, which are well understood.
and will stand up to the rigors of EPA requirements. The problem becomes more complex with many anthropogenic compounds; they are relatively novel and nature has not had the time to develop strategies for reincorporating them into the natural cycle. It is very likely that there are bacteria or other microorganisms which could be encouraged to consider these compounds food sources, and if so, a pure culture under ideal conditions could be maintained for the purpose of breaking them down. The task then becomes isolation or creation of such a strain, and the identification of the optimum conditions for degradation of a certain compound or family of compounds.

There have been a variety of papers written on the degradation of various compounds by microbes. (See Table 7) In some cases, the microbes used are simply a sampling of various natural environments amended with the compounds of interest. Nales et al. surveyed soil from six different locations and added various electron acceptors such as sulfate, nitrate, and iron (III) to determine if the microbe distribution would degrade benzene under anaerobic conditions. They found that benzene was degraded by the microcosms created from 4 of the 6 sites, but that the presence of other aromatic compounds could inhibit this process. In one of the sites, benzene was initially degraded, however over time the degradation processes failed, and they were unable to determine the reason for this behavior.
Weiner et al. hoped to improve potential anaerobic benzene degradation in methanogenic sediments from aquifers contaminated with petroleum by the addition of sulfate, nitrate, or Fe(III). They found that sulfate was useful in stimulating benzene degradation in this particular aquifer, but noted that this was not consistent with the findings from other aquifers they had studied.\(^{41}\) Just the differing results between the work of Nales et al. and Weiner et al. show the vast uncertainty in trying to understand the behaviors of an undefined microcosm created from a soil sample.

### TABLE 7

**THE DEGRADATION OF ENVIRONMENTALLY RELEVANT COMPOUNDS BY UNDEFINED MIXTURES OF BACTERIA**

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Bacteria</th>
<th>Additives</th>
<th>Result</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzene</td>
<td>soil mix</td>
<td>sulfate, nitrate, iron (III)</td>
<td>Mixed (4/6 samples) Other BTEX inhibited</td>
<td>Nales, et al., 1998(^{36})</td>
</tr>
<tr>
<td>Benzene</td>
<td>methanogenic sediments</td>
<td>sulfate, nitrate, iron (III)</td>
<td>1 sample, required sulfate</td>
<td>Weiner et al., 1998(^{37})</td>
</tr>
<tr>
<td>Trichloroethene (TCE)</td>
<td>methanogens, sulfate reducers, methanotrophs (isolated from soil samples)</td>
<td>none</td>
<td>Some degradation in situ, not observed under laboratory conditions</td>
<td>Etienne et al., 2001(^{38})</td>
</tr>
<tr>
<td>HT, “sulfur mustard”</td>
<td>activated sludge (Black River WW Treatment Plant)</td>
<td>begin with hydrolysis at 90°C</td>
<td>28-44 fold reduction</td>
<td>Harvey et al., 1998(^{39})</td>
</tr>
<tr>
<td>Vinyl chloride</td>
<td>indigenous microorganisms (industrial site)</td>
<td>none</td>
<td>Complete oxidation to CO(_2)</td>
<td>Bradley and Chapelle, 2002(^{40})</td>
</tr>
</tbody>
</table>

**NOTE:** Many studies have been done using an undefined microbial mix isolated from soil or a contaminated site. These studies have mixed success; success followed by unexplained failure is common.
Etienne et al. assayed for co-metabolic trichloroethene (TCE) biodegradation by soil samples in a TCE contaminated plume, which contained primarily methanogens, but also sulfate reducers, and methanotrophs. Although these bacterial groups have been previously associated with degradation of TCE, and some degradation appeared to be happening *in situ*, degradation was not identified in the cultures under laboratory conditions. Several possible reasons for this were proposed, and the authors called for further study.42 These poorly defined species mixtures can have fantastic results or no result at all, and the outcome is unpredictable and can change suddenly with few identifiable corrective measures available. Studies of indigenous undefined mixed populations highlight the need for a system in which the microbial behaviors are defined and repeatable.

In some cases where partial success was achieved, the results with an undefined mixture are more promising and consistent. The vesicant agent HT, also known as the chemical warfare agent “sulfur mustard” has been significantly detoxified by a combination of hydrolysis, achieved by reacting 3.8% wt HT with 90°C water for 2 hours with vigorous agitation, followed by biodegradation. The biodegradation was performed by reactors seeded with activated sludge from the Black River Wastewater Treatment Plant, which though undefined, has been proven by ongoing success treating wastewater. After the optimum treatment, a 28- to 44- fold reduction in total toxicity was obtained.43 Bradley and Chapelle44 determined that vinyl chloride could be reductively dechlorinated under sulfate reducing conditions, and the resultant ethene could be further oxidized to CO₂ by indigenous microorganisms to an industrial site. Despite consistent success, however, the mechanism of degradation and role of these bacteria in *in situ*
biodegradation have not been determined.\textsuperscript{45} Since the process is unknown, if a failure occurs, it is potentially difficult to start up again. In addition, genetic drift can occur in the source of the microbes requiring different handling and conditions over time.

Studies that target specific chemicals with specific microorganisms are also done for a wide variety of compounds, including particularly toxic or recalcitrant chemicals. (See Table 8) DDT, which persists for long periods of time in the environment, has been a target of some of this research. Bacteria such as \textit{Proteus vulgaris}, \textit{E. coli}, several \textit{Pseudomonas} species, including \textit{P. aeruginosa}, various \textit{Bacillus} sp., \textit{Flavobacterium} sp., \textit{Enterobacter cloaceae}, \textit{E. aerogenes}, cyanobacteria, \textit{Ralstonia eutropha}, and fungi such as \textit{Saccharomyces cerevisiae}, \textit{Aspergillus flavus}, \textit{Thanatephorus cucumeris}, and \textit{Pleurotus ostreus} are all indicated in various mechanisms of DDT degradation. By understanding the mechanism of DDT degradation associated with various species, the appropriate conditions for degradation could be provided for pure culture bioreactors and encouraged by species-specific nutrient additions for \textit{in situ} degradations.\textsuperscript{46}

Various explosives, largely trinitrotoluene (TNT) and its derivatives are another chemical family of great interest, as the disposal of obsolete explosives is a problem for the military. Anaerobic biodegradation is reported for the sulfate reducers \textit{Desulfovibrio vulgaris}, \textit{D. gigas}, \textit{D. desulfuricans}, \textit{Desulfovibrio} sp. (B strain), and \textit{Desulfobacterium indolicum}, and for the methanogens \textit{Methanococcus} strain B, \textit{M. deltae}, and \textit{M. thermolithotrophicus}. The optimum microbe was \textit{Desulfovibrio} sp. (B strain), which follows a 5 step metabolic pathway ending in toluene, which, though still considered a hazardous compound, is significantly less hazardous and more biodegradable than TNT.
TABLE 8
DEGRADATIONS OF SPECIFIC CHEMICALS BY SPECIFIC SPECIES OF MICROBES.

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Bacteria</th>
<th>Result</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>DDT</td>
<td><em>Proteus vulgaris</em> E. coli</td>
<td>Works, various mechanisms. Primarily oxygenic.</td>
<td>Foght et. al, 2001 [47]</td>
</tr>
<tr>
<td></td>
<td><em>Pseudomonas</em> species</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>P. aeruginosa</em> Bacillus sp.</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Flavobacterium</em> sp.</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Enterobacter cloaceae</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>E. aerogenes</em> cyanobacteria</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Ralstonia eutropha</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Fungi</em> Saccharomyces cerevisiae</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Aspergillus flavus</em> Thanatephorus</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Pleuratus ostreus</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Desulfovibrio vulgaris</em></td>
<td>Works, anaerobic. Optimum: <em>Desulfovibrio</em> sp. (B strain) pyruvate= e’ donor, sulfate= e` acceptor, TNT= sole N source pathway resulted in toluene.</td>
<td>Boopathy et al, 1998 [48]</td>
</tr>
<tr>
<td>Trinitrotoluene (TNT) and derivatives</td>
<td><em>Desulfovibrio vulgaris</em> D. gigas D. desulfuricans</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Desulfovibrio</em> sp. (B strain)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Desulfo bacterium indolicum</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Methanococcus</em> strain B M. deltae M. thermolithotrophicus</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NOTE: Unless otherwise identified, microbes are bacteria. By identifying specific species and pathways, repeatable results are more easily obtained.

This reduction was achieved when pyruvate was provided as an electron donor, sulfate was provided as the electron acceptor, and TNT was the sole nitrogen source available. [49]

It is this type of in depth understanding of the specific requirements to optimize degradation that must be sought in developing biological solutions to waste and contamination problems. Using this level of quantification, new strategies must be developed to supplement and replace current poorly defined, inefficient, or expensive processes.
1.3.4 Why are new methods needed?

1.3.4.1 Financial and regulatory needs

The reasons for reducing the volume and toxicity of hazardous waste and developing methods for remediation of sites contaminated with hazardous waste are obvious. If the waste can be rendered harmless and environmentally friendly, then special disposal management is not required. The environment is coming under a critical waste contamination load and disposal regulations are becoming ever more stringent. In purely financial terms, it is no longer cost-effective for companies to simply pay for storage-disposal of waste. The cost of environmental and safety regulations has increased from $80 billion per year in 1977 to $258 billion in 1999. This puts a particularly hefty burden on small businesses, averaging around $1,246 per employee each year. Environmentally friendly methods to reduce the volume and toxicity of the waste must replace dumping the waste into temporarily secure dump sites.

1.3.4.2 Inadequacy of non-biological methods

As already discussed, while non-biological methods have evolved much over time, they are still imperfect. The most successful are domestic wastewater treatments for the milder wastes produced by domestic sources. However the more toxic and recalcitrant waste often produced by industrial generators poses a greater problem. Precipitation and concentration may allow a larger portion of the waste stream to be discharged, but both processes leave behind something that must often be stored in a waste storage facility. In addition, these methods require a constant purchase of precipitants and storage is expensive. Containment methods, such as fixing waste into concrete blocks are temporary solutions, because over time these materials can leach out
and become environmental contaminants. Incineration often discharges hazardous emissions during the process, and the residual ash may still be rich with contaminants. Although combining several methods can increase the effectiveness of waste treatment, recent regulations demand many more chemicals, such as solvents, dioxins, chlorinated organics, and liquids containing dissolved metals, must be recovered and may not be discharged in any concentration.

1.3.4.3 Inadequacy of current biological methods

Biological methods of treating the more recalcitrant and problematic chemicals, while promising, are relatively immature. Since microorganism scavengers are a natural part of the ecological system, they work within the natural biogeochemical cycle to break down compounds and recycle them to biologically available forms. The hope is that they are less likely to cause a cascade of problems that must be subsequently solved.

Traditional waste treatment already uses “biomass” or “activated sludge” in their secondary treatment. (See Appendix C) The general action of this biological hodgepodge is known, and the probable organisms present can be guessed, however in many waste treatment texts, the genus species is not even mentioned. These microbes may be referred to as nitrifiers, methane formers, or some other vernacular name, however these descriptive names refer to heterogeneous groups and have no official standing in nomenclature. Given this initially poorly defined mix, genetic drift can occur and is not noticed until the reactor fails for some reason, at which time the solution is to reseed from another, more successful reactor. The undefined nature of the culture means that the biological reactions and their rates can be generalized and approximated, but not understood in a well-defined, repeatable way. Finally, traditional wastewater treatment
that includes bioprocessing generates a sludge, in which toxic and hazardous components not broken down by the biological processes are concentrated, and this sludge is then considered a hazardous waste.62

Wastewater treatment facilities typically employ aerobic species, and aeration is applied to the digesters in use. In many cases, aerobic degradation can be merely superficial modification of the compound, but not complete degradation.63 Oxygen is also frequently absent in native environments because it has been rapidly consumed by oxygenic processes, leaving an anaerobic environment. A large amount of the toxins released into the environment also make their way into anaerobic sediments.64 In bioreactors, the requirement of aeration adds an entire set of equipment to the parts list. The use and quantification of anaerobic or microaerobic organisms offer solutions to many of these difficulties, but these organisms are relatively unexplored.

1.4 Types of Bacteria Utilized in Processing Waste

There are several different groups of bacteria that have been used in waste treatment and waste treatment research. (See Table 9) The most common include activated sludge, methanogens, and sulfur reducers. In recent years, there has been an upsurgence in research on photosynthetic bacteria because of some of their unique properties. Each group has particular abilities, weaknesses, and culture requirements.

Activated sludge is an undefined mixture of aerobes used in nearly every waste treatment plant in the US.65 Activated sludge is an obligately aerobic process, and requires intensive mixing and aeration to succeed, which makes the infrastructure expensive. This method is generally successful for the milder substances encountered in
municipal wastes, however strict regulations prevent the release of industrial wastes into the municipal treatment system until it has been significantly treated to avoid a shock load that could kill the microbes and render the reactor useless. Because this system is ill defined, system failure cannot be predicted; the solution is typically to seed from a successful reactor when failure does occur. Sludge behavior problems are also common, and are typically treated empirically. Although the typical wasted sludge from an activated sludge system is relatively innocuous, repeated land application has been shown to result in a buildup of heavy metals and toxic compounds due to trace amounts remaining in the sludge. In addition, EPA investigation has demonstrated that virus concentrations between $3.8 \times 10^3$ and $11.6 \times 10^3$ PFU/Liter of sludge (Plaque Forming Units) can remain in treated sludge; it takes around 10 days for the virus count in land applied sludge to drop below 1000 PFU/gram.
### TABLE 9

**KEY FEATURES OF FOUR COMMONLY USED MICROBE TYPES**

<table>
<thead>
<tr>
<th>Bacterium Type</th>
<th>PSB</th>
<th>Activated Sludge</th>
<th>Methanogens</th>
<th>Sulfate reducers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Requires O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Strict Anaerobe?</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Requires CO&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Derive energy from</td>
<td>Sunlight and/or chemicals</td>
<td>Chemicals</td>
<td>Chemicals+ H&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Chemicals</td>
</tr>
<tr>
<td>Drive unfavorable reactions</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Carbon source</td>
<td>Organic carbons, CO&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Organic carbons</td>
<td>CO&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Organic carbons</td>
</tr>
<tr>
<td>Requires additives?</td>
<td>Vitamins, CO&lt;sub&gt;2&lt;/sub&gt;</td>
<td>sulfate, nitrate, iron (III)</td>
<td>sulfate</td>
<td></td>
</tr>
<tr>
<td>Common End Product</td>
<td>Biomass + amino acids + pigments, H&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Biomass + low level toxic compounds</td>
<td>Biomass + methane</td>
<td>Biomass + acetate + H&lt;sub&gt;2&lt;/sub&gt;S</td>
</tr>
<tr>
<td>End Products toxic or difficult to degrade</td>
<td>No</td>
<td>Yes</td>
<td>Maybe</td>
<td>Yes</td>
</tr>
<tr>
<td>End product contains pathogens</td>
<td>Some antiviral action noted</td>
<td>Possible</td>
<td>Possible</td>
<td>Not known</td>
</tr>
<tr>
<td>Metabolically Flexible</td>
<td>Yes</td>
<td>Maybe (culture shock can occur)</td>
<td>No</td>
<td>Often</td>
</tr>
<tr>
<td>Repeatable</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Defined</td>
<td>Yes</td>
<td>No: mixed bacteria and protozoa</td>
<td>Sometimes</td>
<td>Sometimes</td>
</tr>
</tbody>
</table>
Another common group used are the methanogens.\textsuperscript{68} These obligate anaerobes do not require mixing or aeration, which reduces the cost of a system, however they do have an extremely long residence time. Methanogens produce methane as they grow, and if this can be recovered, it is useful as an energy source. In an open tank, this product can be released to the environment; if it is collected, it must be handled carefully to avoid explosion or fire. In these types of reactors, accumulation of intermediates such as acetate, hydrogen, and various acids is common. These bacteria are very sensitive to a changing environment.\textsuperscript{69} The accumulation of acids can cause the reactor to fail, and tight pH control is therefore necessary.

Sulfur reducers have been investigated recently for their potential in waste treatment and they are capable of using a variety of substrates such as fatty acids, phenyls, and some aromatic compounds.\textsuperscript{70} These bacteria typically use a low molecular weight compound as an electron donor. They are obligate anaerobes, and the presence of oxygen is toxic to them.\textsuperscript{71} Sulfate reducers are most noted for their end product, hydrogen sulfide, which is toxic to plants and animals. While fixed sulfur is important to life, in areas with large numbers of these bacteria, the sediment will be blackened due to formation of ferrous sulfide, or the aquifer will be acidified. Sulfate reducers also often proceed through a degradation of the compound of interest to acetate. These bacteria are useful in a biogeochemical sense, however using them as the primary method of waste treatment results in several undesirable end products.

A group of studies has shown that members of the family of photosynthetic purple bacteria can also purify wastewater, do not generate sludge, and do not leave hazardous compounds in the resulting biomass.\textsuperscript{72} Further, in these studies, the biomass was used in
land application and significantly enhanced the growth of rice, mandarins, tomatoes, and other agriculture. When used as a supplement to fish and poultry feed, larger animals and more frequent egg laying was observed.\textsuperscript{73,74} Because these pigmented bacteria can derive energy from light, they are able to drive reactions which are energetically unfavorable for bacteria which must derive energy only from chemical compounds. They are known to degrade organic acids, amino acids, fatty acids, alcohols, carbohydrates, C-1 compounds,\textsuperscript{75} and recently a wide variety of aromatic compounds previously considered undegradable.\textsuperscript{76} Typically, the final products are biomass and simple compounds, and problematic traces are not detected. Several phototrophs have also been noted to have antiviral properties, which indicates that they could be expected to produce a pathogen free result. \textit{Rhodopseudomonas capsulata} was able to inactivate poliovirus, Sindbis virus, several fish viruses, and coliphages.\textsuperscript{77} Although these bacteria are considered anaerobic, they are facultative and can survive in moderately oxygenated environments. They are, in fact, quite metabolically flexible and can grow in a wide variety of environments and on a wide variety of substrates. (See 1.5) These factors suggest photosynthetic bacteria as a prime area for further research in waste remediation.

1.5 Purple Non-Sulfur Bacteria (PNSB)

1.5.1 Classification

Purple Non-Sulfur Bacteria (PNSB) belong to the recently defined class \textit{Proteobacteria}. \textit{Proteobacteria} are largely aquatic, and can be found in both fresh and salt-water environments. This class includes the \textit{α-proteobacteria}, purple non-sulfur bacteria and aerobic purple bacteria, \textit{β-proteobacteria}, purple non-sulfur bacteria of
differing fatty acid and quinone composition, and $\gamma$-proteobacteria, purple sulfur bacteria that form sulfur globules inside or outside the cells. *B. viridis* was of the class $\alpha$-proteobacteria and family *Rhodospirillaceae*, however based on more recent 16SRNA classification, it has been determined that species defined as *Rhodospirillaceae* appear in the $\alpha$-proteobacteria as well as the $\beta$-proteobacteria. It is considered a major phylegetic misclassification, and the family name *Rhodospirillaceae* has been abandoned in favor of the popular term Purple Nonsulfur Bacteria (PNSB), which will be used to describe anoxygenic bacteria of the $\alpha$-proteobacteria and $\beta$-proteobacteria until the exact taxonomic criteria for family classification are determined.

Their most striking commonality is that they all contain bacteriochlorophyll and carry out photosynthesis. (See Appendix D) This bacteriochlorophyll can be any one of several types, which lends the distinctive colors of bacteria in the group PNSB, including green, yellow-green, brown-green, brown, brown-red, red, pink, purple, and purple-violet. The light dependent photosynthesis mediated by these bacteriochlorophyll and their complement structures provide an additional energy source that can be used in metabolic processes. All PNSB share some type of internal membranes in which these photosynthetic apparatus, reaction centers, and light harvesting pigment-protein complexes are found, but the specific membrane properties vary within the class.

Photosynthesis in PNSB does differ from that found in plants, algae, and cyanobacteria in several ways. First, photosynthetic pigments are damaged by oxygen. In more complex cells containing chloroplasts, the cell can exist in oxygenic conditions because the chloroplast also function to create a protective micro-environment for the proteins. PNSB are prokaryotes, so the entire contents of the cell are subject to the same
environment. This means the cells must be in a relatively low oxygen tension environment for photosynthesis to occur. A second difference is that the bacteria are unable to use water as an electron donor. This is because they only contain Photosystem II, a light absorbing enzymatic complex, which alone does not generate adequate energy to break water. This prevents the cells from evolving oxygen, as is typical in chloroplast mediated photosynthesis. Instead, it is typical for the cells to use any variety of alternative electron donors, including reduced sulfur compounds, hydrogen, and a myriad of small organic molecules.79

The α-proteobacteria are complex because they contain both the Purple Nonsulfur Bacteria (PNSB), as well as chemotrophic bacteria which are similar enough to be considered their descendants. PNSB are a very diverse group of organisms; the morphology, membrane structures, carotenoids, lipids, quinones, fatty acids, utilizable carbon sources, and electron donors vary widely within the group.80 The cells are gram negative, 0.3-1.8 μm, and can be spherical, ovoid, rods, or spirals. They typically divide by binary fission or budding, and are motile by flagella.81 In general, PNSB prefer to grow by photoorganotrophic metabolism using simple organic substances. They are not limited to this type of growth, however, as they can also grow photolitho-autotrophically with molecular hydrogen as an electron donor, and photoautotrophically fixing carbon via the Calvin Cycle or the Reverse TCA cycle.82 Under dark, microaerobic conditions, the cells can also grow chemoheterotrophically. Most PNSB are known to contain nitrogenase, and can assimilate molecular nitrogen, however they tend to prefer ammonia or an organic nitrogen compound, which causes a halt in nitrogenase production.83 A summary of the main metabolic pathways of B. viridis is given in Chapter 4.
1.5.2 Known toxins degraded by PNSB

PNSB are known to degrade a number of pollutants, including volatile fatty acids, H₂S, and CH₃SH. (See Table 10) *Rhodopseudomonas capsulata* achieved major reduction in acetic, propionic, isobutric, n-butyric, isovaleric, and n-valeric acid concentrations, and reduced each to undetectable levels within 14 days.⁸⁴ Phototrophic bacteria have also been shown to metabolize dimethylnitrosamine, a carcinogenic mutagen. After 28 days of incubation with phototrophic bacteria, the concentration decreases below detection limits.⁸⁵

PNSB have been shown, in recent years, to display remarkable versatility in the degradation of aromatic compounds. In particular, Gibson and Harwood have done much work with the amazingly versatile organism *R. palustris*. In a survey of aromatic compounds using auxanographic plates, *R. palustris* was shown to grow on caffeate, cinnamaldehyde, cinnamate, cyclohexanecaboxylate, cyclohexaneprionate, Δ-1- and Δ-3-cyclohexene-carboxylate, ferulate, hydrocaffeate, hydrocinnamaldehyde, 4-hydroxybenzaldehyde, 4-hydroxybenzoate, 4-hydroxybenzoylformate, 4-hydroxycinnamate, 4-phenylbutyrate, 3-phenylpropionate, and 5-phenylvalerate under aerobic or anaerobic conditions. Another four substances were only utilized under aerobic conditions, and five substances were only utilized under anaerobic conditions. Using the chemical relationships between these substances, the researchers proposed two major routes of anaerobic breakdown of the substance to central metabolites. They suggested that breakdown of aromatic compounds proceeded either through benzoate or through 4-hydroxybenzoate.⁸⁶ In later work, they proposed common microbial attacks on aromatic rings substituents, (See Figure 3) suggesting how a wide variety of aromatic
# TABLE 10

**CHEMICAL COMPOUNDS KNOWN TO BE DEGRADED BY PNSB**

<table>
<thead>
<tr>
<th>PNSB</th>
<th>Chemical</th>
<th>Successful?</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>R. palustris</em></td>
<td>caffeate, cinnamaldehyde, cinnamate, cyclohexanecaboxylate,</td>
<td>Grew aerobically or anaerobically with these as sole carbon sources.</td>
<td>Gibson and Harwood, 1988&lt;sup&gt;87&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>cyclohexaneproionate, Δ1-cyclohexencarboxylate, Δ3-cyclohexencarboxylate,</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>ferulate, hydrocaffeate, hydrocinnamaldehyde, 4-hydroxybenzaldehyde,</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4-hydroxybenzaldehyde, 4-hydroxybenzoate, 4-hydroxybenzoyleformate,</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4-hydroxycinnamate, 4-phenylbutyrate, 3-phenylpropionate, 5-phenylvalerate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>*R. palustris, Rhodobacter</td>
<td>pyridine, nicotinic acid, 4-dimethyl amino pyridine, inidazole, captan,</td>
<td>Degradation was identified for these nitrogen containing chemicals</td>
<td>Rajasekhar et al, 2000&lt;sup&gt;91&lt;/sup&gt;</td>
</tr>
<tr>
<td>sphaeroides, Rhodopseudomonas</td>
<td>pyrazine, quinoline, 8-hydroxy quinoline, guanine, uracil, and carbenxazim</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sp.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>*R. palustris, R.</td>
<td>Indole</td>
<td>Non-lethal below 3 mM</td>
<td>Rajasekhar et al, 1999&lt;sup&gt;92&lt;/sup&gt;</td>
</tr>
<tr>
<td>sphaeroides*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>R. capsulata</em></td>
<td>acetic acid, propionic acid, isobutric acid, n-butyric acid, isovaleric</td>
<td>undetectable within 14 days</td>
<td>Lee and Kobayashi, 1992&lt;sup&gt;85&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>acid, n-valeric acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Rhodomicrobium vannielli</em></td>
<td>Benzoate, benzyl alcohol, 4-hydroxy-3-methoxybenzoate (vanillate), 4-</td>
<td>Excellent</td>
<td>Wright and Madigan, 1991&lt;sup&gt;80&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>hydroxy-3,5-dimethoxybenzoate (syringate)</td>
<td>Moderate</td>
<td></td>
</tr>
<tr>
<td>General phototrophs</td>
<td>Dimethylnitrosamine</td>
<td>Below detection limits after 28 days</td>
<td>Kobayashi et al, 1978&lt;sup&gt;86&lt;/sup&gt;</td>
</tr>
<tr>
<td>General phototrophs</td>
<td>volatile fatty acids, H₂S, CH₃SH</td>
<td>Below detection limits after 14 days</td>
<td>Lee and Kobayashi, 1992&lt;sup&gt;85&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

**NOTE:** A wide variety of compounds, especially aromatic compounds, have been studied and remarkable success has been achieved with bacteria in this classification.
compounds can be transformed to a proposed starting point for known degradation mechanisms, as well as proposing more complete metabolic pathways through both benzoate and 4-hydroxybenzoate. A major metabolic intermediate in almost all of the pathways proposed by this group to date is Benzoyl-CoA, an esterified compound. The coenzyme A appears to assist in the degradation, and from benzoyl-CoA, there is a metabolic pathway ending at Acetyl-CoA, a metabolic intermediate, which can be utilized for cell growth and other metabolic processes. A number of other researchers have also achieved success with aromatic degradations and other photosynthetic bacteria. Anaerobic photocatabolism of aromatic compounds was shown by the PNSB *Rhodomicrobium vannielii*. They were able to grow quite well on benzoate and benzyl alcohol, and they were able to support moderate growth on 4-hydroxy-3-methoxybenzoate (vanillate) and 4-hydroxy-3,5-dimethoxybenzoate (syringate).

Another area of significant interest is the biodegradation nitroaromatics, which have a
tendency to be xenobiotic. Rajasekhar et al. surveyed the ability of *R. palustris*, *Rhodobacter sphaeroides*, and a newly isolated *Rhodopseudomonas* species to degrade a variety of nitrogen containing aromatic compounds, and identified degradation of pyridine, nicotinic acid, 4-dimethyl amino pyridine, imidazole, captan, pyrazine, 2-amino pyrazine, quinoline, 8-hydroxy quinoline, guanine, uracil, and carbenxazim. This group also identified an indole metabolism for *R. palustris* and *R. sphaeroides*, and tentatively proposed a new degradation pathway ending in anthranilate for *R. sphaeroides*. They also showed that there was some inhibitory effect of indole to bacterial growth at 3 mM, but showed that the effect was non-lethal by demonstrating normal growth after the removal of the indole.

1.5.3 Natural environment of PNSB

PNSB were originally thought to be primarily photoorganotrophs, however recent research indicates that they are significantly more metabolically versatile, and can grow as anaerobic photoheterotrophs in the light or microaerophilically in the dark. Their preferred metabolism, however, is photoorganotrophy in the light. PNSB are also acid tolerant, and have also been noted to survive at pH below 5, and are often found in moderately acidic habitats. They typically isolated from bogs, acid lakes, streams, and moist soil. A wet or moist environment is crucial, and they prefer to grow in an aqueous environment. They typically inhabit moderate climates, and are most commonly isolated from shallow aquatic systems and mud environments. PNSB can also be isolated from aerobic waters of lakes, rivers, and streams, as well as sewage and wastewater, but their concentrations in these environments tends to be low.
1.5.4 Relation of \textit{B. viridis} in PNSB group

\textit{Blastochloris viridis} was first classified by Drews and Geisbrecht in 1966 in waters near Freiburg, Germany.\textsuperscript{96} They are rod shaped or ovoid, and generally 0.6-0.9 mm wide and 1.2-2.0 mm long. The young cells are motile by subpolar flagella. \textit{B. viridis} was, until recently, a member of the genus \textit{Rhodopseudomonas}, which previously classified organisms containing several types of bacteriochlorophyll.\textsuperscript{97} The reclassification to \textit{Blastochloris} (\textit{blastos}, bud, shoot; \textit{chloros}, green) included \textit{B. viridis} and \textit{B. sulfoviridis}, which both contain bacteriochlorophyll \textit{b}, which give these species their characteristic green color in dense culture.\textsuperscript{98} Bacteriochlorophyll \textit{b} has characteristic light absorption maxima at 400 nm, 605 nm, 835-850 nm, and 1020-1030 nm.\textsuperscript{99} The major carotenoids are 1,2-dihydroneurosporene and 1,2-dihydrolycopene.\textsuperscript{100} Characteristic peaks at 451 and 483 nm are attributed to these carotenoids.\textsuperscript{101} The primary wavelengths of light used by these bacteria are in the 1020-1030 nm range, which gives them a unique difference from other photosynthetic bacteria, allowing them to use a generally unused portion of the spectrum. Their photosynthetic apparatuses are located in lamellae, which are intracytoplasmic membranes parallel to the cytoplasmic membranes.\textsuperscript{102} Photoheterotrophic growth with small organic molecules is their preferred mode of growth, however they can also grow aerobically in the dark, leading to colorless cultures. When they grow photosynthetically, they gather additional energy, allowing them to drive otherwise energetically unfavorable reactions in order to obtain carbon to generate biomass.
1.5.5 Why *B. viridis* for toxins?

*B. viridis* has been extensively studied to determine the structure of its reaction center by X-ray crystallography, and there is much interest in this organism based on its usefulness in research on photosynthesis.\(^\text{103,104,105,106}\) Almost no literature is available on its ability to perform degradation of hazardous chemicals, and it is neglected in this realm. Its close genetic relationship to the other PNSB indicates that there may be a wide variety of useful degradations possible. *B. viridis* is the type species for *Blastochloris*, and shares 98.7% sequence similarity with *B. sulfoviridis*,\(^\text{107}\) which is known to degrade toluene, trans-cinnamate, and benzylsuccinate, an intermediate in the expected toluene biodegradation pathway.\(^\text{108}\) *B. sulfoviridis* strains which use ethanol, glycerol, propionate, benzoate, crude oil, butyrate, acetate, caprylate, and 4-hydroxybenzoate have also been identified.\(^\text{109}\) *B. viridis* is relatively accessible to lateral gene transfer or transconjugation.\(^\text{110}\) Because it grows in and is isolated from similar environments as the other species in its Family, it is likely that gene sharing has occurred, giving *B. viridis* similar abilities and genetic suites.\(^\text{111}\) Its unique energy utilization because of its bacteriochlorophyll \(b\) pigment gives it potential in areas where other photosynthetic bacteria might be less well adapted, such as low light conditions.\(^\text{112}\) This is certainly a relatively uncharted area in which further research has great potential to discover beneficial processes.

Although it is beyond the scope of this research, the strain of *B. viridis* used in this work with does have an existing system for genetic modifications.\(^\text{113}\) This makes it possible to easily add metabolic pathways via plasmid addition in order to create a new process with this well characterized bacterium.\(^\text{114}\) This would make it possible to add a
single enzyme, facilitating a missing bottleneck reaction, allowing this bacterium to degrade a wide variety of compounds. Because it would be an addition by plasmid, and the addition would be small, the change could be made with relatively little interruption to the native abilities and behavior of the bacteria.

1.6 Brief Overview of This Work

The goals of this project are threefold. First, measured parameters that accurately characterize the growth and health of the bacteria will be defined. Using these, we will characterize the native behavior of the bacteria in nutrient rich conditions and in a defined minimal media. Next, the baseline media will be optimized to support the most vigorous and healthy growth. The optimum is that which offers either the greatest growth or the healthiest cells. Finally, a handful of compounds will be surveyed to establish the native abilities of *B. viridis* to grow in the presence of and degrade typical waste chemical compounds.

1.6.1 Measured parameters

In order to quantitatively understand the behavior of the bacteria, a reliable method of tracking their growth and health must be determined. (Chapter 2) There are a number of methods that are used in bacteriology, each with inherent strengths and weaknesses, and they often provide different types of information. Typical assays for cell growth determine:

- the total density of the culture
- the total number of cells present
the total number of live cells present

the total amount of biomass

Looking at more detailed information such as protein or pigment concentration, the substrate amount or the formation of byproducts can also be instructive. A useful method:

- is informative
- correlates with a real physical characteristic of the cells
- is simple and rapid
- takes into account both the cell growth as well as the pigment behavior

1.6.2 Behavior in RMPABA and Minimal medias

In order to evaluate the effects of imposed conditions on the bacterium, there must be some standard against which it can be compared. (Chapter 3) The ideal media conditions for *B. viridis* are an exceedingly rich, undefined media designated “RMPABA.” (See Appendix E: Media Formulations) Under these conditions, dense, thick growth can be expected. This can be considered the absolute optimum achievable for *B. viridis*. This media, however, is undefined because of the addition of Yeast Extract and Bactopeptone, which are homogenized blends of complex organic material. When these are included in the media, the exact chemical composition cannot be determined. In order to achieve quantifiable, repeatable results, a defined media must be used. This defined media is designated “Minimal,” and contains sodium malate as a carbon source, ammonium sulfate as a nitrogen source, mineral salts, thiamine, and *p*-aminobenzoic acid. Although this solves the problem of undefined components, the growth in this
media is only approximately 1/3 as strong as that in RMPABA. (See Chapter 3 for data)
This calls for optimization of the base condition, allowing for the best possible growth
against which to compare growth in the presence of hazardous compounds.

1.6.3 Optimizing growth conditions

In order to determine an optimum defined growth condition, several factors must
be considered. The first is physical conditions such as light, volume, temperature, and
agitation. If these factors are not optimized, then they must be selected based on previous
work and set to provide reliable repeatability. The second, and more complex goal is
finding the appropriate media conditions and optimizing the Minimal media formulation.
This includes chemical variables such as pH, buffer type and amount, and bubbled
gas/headspace. It also includes biochemical conditions such as carbon source, nitrogen
source, and the addition of minerals, vitamins, and other growth factors. By looking
at each of these myriad growth conditions independently, an optimized minimal, defined
growth condition can be designed. (See Chapters 5 and 6)

1.6.4 Examining the effect of target compounds

Once an optimum baseline is determined, there is a standard against which growth
in the presence of the target compounds can be compared. The effect of a single
concentration of a variety of compounds on B. viridis in the presence and absence of
malate will be determined. (Chapter 7) There are several possible positive effects; the
compound could enhance growth or pigment formation. This would cause an increase in
growth rate, or in the relative UV-Vis absorbance of pigment at key characteristic
wavelengths such as 1020 nm (A1020), and total cell growth after the defined growth period. The compound could also have a neutral effect and be ignored by the cells. This would be indicated by growth and pigment formation identical to that in the base media. Possible negative outcomes are that the compounds will kill the cells outright, prevent them from growing, or prevent them from producing pigment. This would be indicated by a decrease in growth rate, the absence of growth (growth rate equal to zero) or a decrease in the relative pigment density of the culture. Finally, it is also possible that the compound will actively destroy the cells, which would be demonstrated by a decrease in the culture or pigment density over time. Where significant bleaching of the cells occurs, as measured by the primary pigment signature, A1020, compared to cell concentration, the cells are damaged, and can no longer perform photosynthesis to generate energy.

1.6.5 Choice of compounds

We will choose a small selection of compounds to survey. (See Table 11) The goal of this selection was not to be inclusive of all chemicals encountered in an industrial setting, but rather to select compounds from several chemical families and develop a method for understanding how these compounds will interact with this specific biological entity. Research on aromatic compounds in the BTEX family dates back 50 years, but has experienced a recent resurgence due to these compounds presence in a wide variety of industrial wastes.116 Aromatic nitrogen compounds are also a factor for environmental concern due to their persistence and toxicity.117 Chlorinated hydrocarbons are also of environmental concern because they can contaminate drinking water supplies, and because some in that family are considered carcinogens.118 Solvents such as MEK,
alcohols, and chlorinated hydrocarbons are very commonly used, in both chemical processes and as cleaning agents, and they can easily make their way into the environment either by accidental spills or improper disposal. Organic acids and organic nitrogen are common in a mixed sewage blend, and should therefore be considered in order to determine the effectiveness of *B. viridis* for more mundane use. The representative compounds will allow a screen of the relevant chemical families to determine the native abilities of *B. viridis* to survive the presence of and degrade chemicals of interest.
<table>
<thead>
<tr>
<th>Chemical Family</th>
<th>Compounds Included</th>
<th>Typical Sources</th>
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<tbody>
<tr>
<td>Alcohols</td>
<td>Methanol, Ethanol, 1-propanol, 2-propanol (isopropanol), 1-butanol, t-butanol, isoamyl alcohol</td>
<td>VOC emissions, Surface coating, Industrial Solvents, Cleaning compounds, Fuel component</td>
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<td>Phenols</td>
<td>Phenol</td>
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<td>BTEX</td>
<td>Toluene, Xylenes, Benzene</td>
<td>Industrial Solvents, Petrochemical plants, Surface coating, Fuel component</td>
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<td>Aromatic Aldehyde</td>
<td>Benzaldehyde</td>
<td>Solvents, Cleaning compounds</td>
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<tr>
<td>Aromatic Acid</td>
<td>Benzoic Acid</td>
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<td>Tetrahydrofuran</td>
<td>VOC emissions, Industrial Solvent</td>
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<td>Oxygenated Arene</td>
<td>Dioxane</td>
<td>Industrial Solvent, Degreaser</td>
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<td>Multiple Carbon Ring</td>
<td>Potassium Biphthalate, Ninhydrin</td>
<td>VOC emissions, Fuel component</td>
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<td>Organic Nitrogen</td>
<td>Indole</td>
<td>Agricultural waste</td>
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1.7 References


10. TRI On-site and Off-site Reported Disposed of or Otherwise Released (in pounds), for facilities in All Industries, for All Chemicals, U.S., 2003 Data Update as of June 8, 2005. <http://www.epa.gov/triexplorer/>


12. TRI On-site and Off-site.

13. TRI On-site and Off-site.
14. TRI On-site and Off-site.

15. TRI On-site and Off-site.

16. TRI On-site and Off-site.

17. TRI On-site and Off-site.

18. Peavy, 208.


27. Ray, 411.


29. Vesilind, 422-424.


31. Peavy, 397.


33. Vesilind, 422-424.

34. Vesilind, 427.


39. Harvey, 191-203.


41. Weiner, 159-173.

42. Etienne, 27-50.

43. Harvey, 191-203.

44. Bradley, 1-8.


46. Foght, 225-246.

47. Foght, 225-246.

48. Boopathy, 81-89.

49. Boopathy, 81-89.


53. Peavy, 208.

54. Vesilind, 425.
55. Ray, 402.

56. Vesilind, 419-420.

57. Vesilind, 425.


61. Personal Communication: R.L. Irving, Biochemical Engineering Class, Department of Civil Engineering, Spring 2002, University of Notre Dame, Notre Dame, IN.

62. Ray, 408.

63. Boopathy, 81-89.


69. Vesilind, 302.


113. A. E. Ostafin, 53-68.


115. Foght, 225-246.


117. Boopathy, 81-89.

118. Etienne, 27-50.

CHAPTER 2

QUANTIFYING CELL GROWTH

2.1 Overview

One of the main goals of this work is to optimize the conditions necessary for cells to grow and to survive an applied condition, food source, or waste component. To facilitate this task, an accurate and repeatable measurement method for cell growth, which is both suitable for batch screening and also scaleable to larger processes, was established. The most important qualities of measurement are:

- To be informative and information rich
- To correspond to a real physical characteristic of the cell
- To be simple and rapid
- To be independent of the culture vessel
- To provide information about both the cell growth and the pigment health.

Typical assays for cell growth determine the concentration of the culture, the number of cells or live cells present, or the total amount of biomass. Each method has inherent strengths and weaknesses. Because of the particular nature of *B. viridis*, including features like its inherent size, typical culture density, and high degree of
pigmentation, some methods were more easily adapted for use in this project than others. For the special case of photosynthetic bacteria, damage to the pigment robs the cell of its primary energy source, which is used to fix carbon and to produce high-energy molecules such as ATP, NADH, and NADPH.

The measurement of cell culture absorbance, which is generally proportional to the culture density, and a count of the number of colony forming units were identified as methods of significant usefulness for *B. viridis* and relatively easy use. The use of a particle counter for culture density determination, an evaluation of the total cell mass by weight, microscopy techniques, and determination of the Total Organic Carbon (TOC) were determined to have some technical or functional difficulty preventing them from being successfully applied.

2.2 Background on Methods Used to Quantify Bacterial Cell Growth

2.2.1 UV-Vis spectrophotometry

One very common method used in studies of bacteria, including pigmented photosynthetic bacteria, is to determine the density of cells by UV-Vis spectrophotometry, which gives the absorbance and/or light scattering by the culture.\(^1\)\(^2\) This method is, in part, popular because the data can be quickly and easily gathered either in sampled batches or online from an active reactor.\(^3\)^4 The UV-Vis spectrophotometer reports an apparent absorbance, \(A\), at one or several wavelengths of interest. (Figure 1) This apparent absorbance is reported at a single or series of wavelengths, based on the total difference between the incident light produced by the machine and the transmitted light observed by the detector. This includes both light absorbed by molecules in the sample and light scattered from the optical path by the cells in the suspension.
Depending on the mode of operation, this is reported as either the percent transmittance (%T) or the Absorbance (A), which are related to one another. (See Equation 1 and Equation 2)\(^5\) The absorbance is the negative log of the detected light intensity, or power (P) over the initial light intensity (P\(_o\)).

\[
\begin{align*}
%T &= T \times 100 = \frac{P}{P_o} \times 100 \\
A &= -\log \frac{P}{P_o} = -\log T
\end{align*}
\]

\(\text{Equation 1}\)

\(\text{Equation 2}\)

Absorbance is directly proportional to the concentration of the sample, whereas T and %T are inversely proportional. In either form, the measurement can be calibrated to physical properties of the cells, typically the concentration of cells in the culture, via the Beer-Lambert Law, (Equation 3) which describes the relationship of absorbance, a dimensionless quantity, to concentration in an ideal sample, where \(\epsilon\) is the molar extinction coefficient (L mol\(^{-1}\) cm\(^{-1}\)), \(b\) is the path length (cm) or length of sample through which light travels, and \(c\) is the concentration of the material that absorbs the light (mol L\(^{-1}\)).\(^6\)

\[
A = \epsilon bc
\]

\(\text{Equation 3}\)
To obtain the molar extinction coefficient, the absorbance is measured for a series of samples prepared at different concentrations. If the same cuvette and machine configuration is used throughout all measurements, insuring constant path length and identical machine error, the equation can be simplified to a three parameter equation, \( A = Kc \), where \( K \) is a lump parameter describing both the molar extinction coefficient and the path length. A sample concentration can then determined with an absorbance measurement, and is subject to the expression \( c = K^{-1}A \).

For a cell culture, the parameter \( K \) can often be determined to relate the absorbance to the concentration of cells. Sometimes, the absorbance is also affected by cellular components. Then the absorbance is a function of the cell concentration and the concentration of the internal components. Absorbance may also be affected by physical conditions like pH or temperature, which affect the absorbing substance’s molar absorptivity, \( \varepsilon \), and make the measured light intensity disproportionate to cell concentration. Another problem is that the absorbance cannot be directly related to the number of living cells present, since dead and live cells will cause the same behavior of incident light. In order to insure the relevance of a spectrophotometric method, it must be confirmed by and correlated to another method. Colony forming unit (CFU) counting gives the number of live cells present in the media, and is often used in conjunction with a measurement of the optical density of the culture.

In order to get the best quantitative results from absorption methods, the spectrum must be relatively featureless at the wavelength where the measurement is taken so that slight measurement errors do not result in large variations in absorbance. A second requirement is that any independently changing cell components do not affect the light
absorbance. For photosynthetic bacteria, light of wavelength 660 nm, or A660, has been most often used because none of the major photosynthetic pigments of the cell, such as bacteriochlorophyll or carotenoids, absorb light appreciably at this wavelength.9,10,11,12 (See Appendix D: Photosynthesis and Figure 5 located in Section 2.4.3.) A separate measure of the primary pigment, A1020, can be useful in determining changes in the relative pigment concentration and heath.

2.2.2 Colony Forming Unit (CFU) count

CFU count is a popular and commonly used method to determine live cell number in a bacterial culture, in contrast to absorbance which measures the total cell number.13,14,15,16,17 CFU count is a reliable way to determine the total number of cells early in the growth cycle, before cell death begins to occur.18 CFU counting can be used to determine an overall correlation between the absorbance of the culture and the live cell concentration as long as the cell undergoes no significant compositional changes during growth, there is no clumping of cells, and no extensive cell death.

In CFU counting, cells are diluted to between 30 and 3000 cells per milliliter by serial dilution (See Figure 2), to reduce the number of cells to a number per volume that can be reasonably counted. For growth quantification purposes, 10-fold dilutions usually provide a realistically achieved wide spread of dilutions likely to provide a countable plate.19,20 To perform a serial dilution, the one milliliter of the initial broth is diluted into 9 mL of buffer. This 1/10 dilution is well-mixed and then 1 mL of the dilution is added to 9 mL of fresh buffer. This process is repeated until adequate dilution is achieved. Typical live cell counts number around 10^6-10^8 cells/mL, suggesting between
Figure 2: A 10-Fold Serial Dilution Series. Serial Dilution is required to generate a sample that contains a countable number of cells in the assayed volume, typically 100 μL.
four and seven dilution steps. The dilution broth is then spread onto a rich agar gel and incubated until countable colonies form on the agar.

Although CFU counting provides important information about the viability of the culture, this method requires several assumptions to justify that the CFU count reflects the total number of cells in the broth:

- Most of the cells are live. This is a reasonable assumption during the exponential growth and early stationary phase. In the late stationary phase cell death may be occurring. Since dying cells will not create colonies, these cells will not be counted.

- One CFU, or colony-forming unit, is a single bacterium. In a well-mixed dilution of a non-flocculating culture, this is a reasonably safe assumption, but for other types of organisms it may not be true. An anticoagulant is frequently added to the dilution buffer to insure separation of cells.

CFU counting generates an intense workload, especially for multiple specimens, and methods to increase efficiency and reduce plating time are often sought.\(^{21}\)

2.3 Methods Not Adopted for Use in this Project

2.3.1 Particle counters

Particle counters, which provide information about the concentration of particulate in a sample, are sometimes employed to determine the density of cells.\(^{22}\) Particle counting, or nephelometry, uses light scattering information to determine the number of particles present in a solution, based on a standard of similarly sized particles.\(^{23}\) If a standard of size similar to the bacteria in question can be obtained, the number of bacteria in the solution can theoretically be known. The particle counter is
usually equipped with a He-Ne-LASER at 632.8 nm, which scans a portion of the suspension. The particle counter available, a Spectrex Corporation Laser Particle Counter, with a He-Ne-LASER at 632.8 nm, is a static device in which a beam of light scans a portion of the suspension. The device must be calibrated with particles of approximately similar size as the specimen, and the device can then be used to estimate the number of particles in an unknown sample.

To obtain a good measurement, it is important that the cells scatter but not directly absorb visible light. Cells that are pigmented, absorb light, have an irregular shape, or are of a significantly different size than the standard will cause difficulty in obtaining a repeatable measurement. The use of an inappropriate calibration suspension, flocculation of bacterial cells, and surface contamination on the vessel also pose problems for obtaining reliable results. *B. viridis* cells are between 0.6 and 0.9 microns, and a 5-micron calibrating solution was the closest size available. This deviates by almost a factor of 10 from the particles in question. Since *B. viridis* cells are pigmented, and a standard of approximately the correct size was not available, this method was considered inappropriate for use in this project.

2.3.2 Cell mass measurements

Cell mass determinations are popular in wastewater treatment, and can be done by measuring either the wet or dry mass of cells in grams. In this method, a fixed volume of cell broth is passed through a filter and only the cell mass is retained. The initial broth must be solids free in order to count only the cell mass. For wet weight, the filters are measured immediately. For dry weight, the filters are dried to constant weight. The
weight of the cells, either wet or dry, is then recorded and the change in the cell mass per volume can be determined. For highly dilute samples, or samples at early time, where the cell dry weight is less than 0.1 g/L, a very large volume of sample is required for accurate measurement and the error is quite high. For *B. viridis*, a gram of cell mass would require at least 100 mL of broth for cells which had reached stationary and had a high culture density. For broth earlier in the growth phase, even greater amounts would be required. Volumes on such a magnitude are not practical for the laboratory scale work being done. In addition, this would be too large a volume and too many cells to apply to the filter, so a new filter arrangement would be required. Although this method could be considered for a larger scale operation, it was determined to be inappropriate for use in the bench scale stage of this project.

2.3.3 Microscope methods

Microscopic observation is a direct method of counting total cell numbers. The cells can be counted directly by viewing them under magnification. A slide or a well with a grid pattern is often employed, which allows a standard volume to be counted. The grid pattern assists in insuring that each cell is counted only once and speeds data gathering, allowing only a fraction of squares to be counted to determine an overall count. (See Figure 3) For statistical reliability, at least 20 squares must be counted.

Two common gridded devices are the hemacytometer and the Petrof-Hausser cell counter, both available from Hausser Scientific, Horsham, PA. A hemacytometer is designed to help in counting blood cells, with cell size typically in the range 5.0-8.5
μm,\textsuperscript{31,32} and platelets, typically 2-3 μm in diameter.\textsuperscript{33} A Petrof-Hausser cell counter has a defined volume well, and is 3 mm by 3 mm total area. The gridded device is injected with a known volume of cell suspension, being careful not to overflow the well, and then the number of cells in each square is counted for at least 20 squares. The average for the squares is multiplied by 20 million to give the total number of cells per milliliter. For the Petrof-Hausser grid, a concentration of at least 10 million cells per milliliter is required.\textsuperscript{34}

Figure 3: A Typical Counting Chamber Grid Layout. The chamber insures a standard volume and the grid allows several squares to be counted and averaged to obtain a cell count. Around 20 squares should be counted to obtain a statistically reliable count. From Hausser Scientific. The chamber is 3 mm X 3 mm.
Microscopic visualization is required because the cell size too small to be observed by the naked eye. The microscope available for this work consisted of a Model LTCM-II base (John Chatillion & Sons, New York, NY), a 20X NA 50X objective (Spencer, USA), and a Panasonic Digital Industrial Color Camera Model GP-KR222 capable of digital magnification up to 4 times. Digital magnification makes the image larger but does not increase the resolution. This allowed for the cells to be viewed at up to 80X magnification, but only about 40X magnification with good resolution. The *B. viridis* cells, which are generally 0.6 – 0.9 microns, are able to be viewed as objects about 0.24-0.72 millimeters. This was still extremely small, therefore distinction between cells was difficult. In addition, not all cultures provided adequate concentrations for statistically meaningful results, so this method was not used in this study.

2.3.4 TOC

The TOC analyzer determines the Total Organic Carbon (TOC) present in a sample of filtered, particle free media. (See Figure 4) In a TOC analyzer, a diluted sample is injected into a 600°C to 950°C oven to oxidize all organic carbon to CO₂. An infrared (IR) detector then measures the CO₂. Some TOC analyzers, especially those using Wet-Oxidation, will also allow the carbon content of a solid, such as filter concentrated cell mass, to be determined. In that case, the carbon content of cells from a known volume of culture can be measured. TOC is a useful method in that it is independent of the oxidation state of the organic matter, and measures only organic carbon and not other organically bound elements. Wastewater and drinking water
treatment applications commonly employ a TOC analyzer, which determines the total concentration of organic carbon in the aqueous sample.\textsuperscript{37,38,39}

In a culture where a known amount of organic substrate is offered and subsequently consumed, determination of the incorporated carbon in the cell mass (C_{cellmass}) is made by subtracting the carbon content of a media sample during the growth (TOC_{media}) curve from the carbon content of the initial media sample (TOC_{initial}).

(Equation 4)

\[ C_{cell mass} = TOC_{initial} - TOC_{media} \]  

Although the TOC method is frequently used in environmental studies to analyze the behavior of bacteria or the health of an ecosystem in surface waters,\textsuperscript{40} many TOC analyzers, such as the TOC-V Series Organic Carbon Analyzer (Shimadzu, Kyoto, Japan) in the CEST laboratory facility, are extremely sensitive to high concentrations of salts, in particular phosphorous. These compounds can be corrosive or cause scaling and damage to the internal components such as the typically gold plated IR detector used to measure CO\textsubscript{2}.\textsuperscript{41} The machine in CEST is also not equipped to measure solid samples (cells).\textsuperscript{42}
Thus many samples obtained from bacteria laboratories and grown in rich, specialized media designed for high growth density cannot be measured because the carbon and salt concentrations are similar in these media. Although dilution would reduce the salt concentration to acceptable levels, it would also lower the carbon levels to near the detection limits, leading to an unreliable result.

In recent years, several TOC analyzers that can handle higher salt concentrations have been developed. The STIPTOC features a temperature range from 600°C to 900°C and an easily accessible salt trap that can be cleaned regularly. The higher temperatures reduce scaling, and the salt trap makes removal of salt scale and buildup easy, allowing slightly higher salt concentrations to be tolerated. The Elementar highTOC II features combustion temperatures up to 1200°C, and an injection zone temperature control to insure separation of interfering matrix elements such as high salts prior to combustion. This device can handle salt concentrations up to an extreme concentration of 280 g/l.

2.4 Materials and Methods

2.4.1 Media composition

Two media formulations were used in this work: RMPABA, an undefined, rich organic medium, and Minimal, a defined media with sodium malate as the sole carbon source. Adjustment of pH was made using 1 N HCl or 1 N NaOH. (See Appendix E for media recipes) The RMPABA media was sterilized by autoclave at 121°C for 20 minutes, and the Minimal Media was sterilized by filtration using Millipore Steritop GP Express PLUS 0.22 mm SCGPT05RE filters in order to avoid clouding. To maintain culture purity, 10 mL/L media of 1 mg/mL Rifampicin and 1 mL/L media of 10 mg/mL Kanamycin were added to the cultures.
The media chemicals CaCl$_2$*2H$_2$O, HCl, KH$_2$PO$_4$, K$_2$HPO$_4$, MgCl$_2$*6H$_2$O, MgSO$_4$*7H$_2$O, Na$_2$EDTA, NaOH, Bactopeptone and Yeast Extract, were obtained from Fisher or FisherBiotech, Fairlawn, NJ. The chemicals H$_3$BO$_3$, Cu(NO$_3$)$_2$*3H$_2$O, FeSO$_4$*7H$_2$O, MnSO$_4$*H$_2$O, NaMoO$_4$*2H$_2$O, (NH$_4$)$_2$SO$_4$, ZnSO$_4$*7H$_2$O, D,L-malic acid, Thiamine, $p$-amino benzoic acid ($p$ABA), Rifampicin, and Kanamycin were obtained from Sigma-Aldrich, St. Louis, MO. For all solutions E-Pure Water (18 MOhms) was used except where tap water is indicated.

2.4.2 Culture preparation

*B. viridis* cultures obtained from the Laboratories of James R. Norris in the Department of Chemistry at the University of Chicago were used in this work.$^{45,46}$ To maintain sterility, all culturing activities were performed in a Edge Guard model EG-4252 laminar flow hood (The Baker Company, Stanford, ME) in an air atmosphere. Cells were inoculated at 2% by volume into liquid RMPABA media and 20% by volume into Minimal media, sealed tightly and allowed to grow under constant illumination at 32°C ± 2°C. Illumination was provided by 25 W light bulbs arrayed around a shelving unit, with vessels placed 3 to 8 inches away from the bulbs. The average illumination was 186 lux. (See Appendix F) To maintain a live fresh inoculating culture, fresh RMPABA media was inoculated every 7 days. Five-day-old cells were utilized for inoculation of all growth experiments. Samples for analysis were harvested under sterile conditions, the vessel re-sealed, and placed into the incubator to continue growth.
2.4.3 UV-Vis spectrophotometry

Samples were prepared by pipetting approximately 0.7 mL of the bacterial broth to be measured into cuvette. The cuvettes used were Fisher Scientific Suprasil quartz microcuvettes with a 10.00 ± 0.01 mm pathlength, 45 mm height, and 1.0 mL capacity, which were transparent to light between 190nm and 2600nm. For dense solutions with an absorbance over 0.8 absorbance units, bacterial suspensions were diluted with E-Pure water, each time by a factor of two, and subsequent measurements were taken until two measurements below 0.8 absorbance units at 660nm were obtained. Suspensions with an absorbance less than 0.8 were diluted once, and measured again to insure a good reading. For suspensions with an absorbance less than 0.2, dilutions were not made. Complete spectrum UV Vis measurements were taken on a Shimadzu UV Mini-1240 spectrophotometer with a range from 190nm to 1100nm. The machine has an absorbance reading range of –3.99 – 3.99 absorbance units, with the least error between 0.1 and 0.8 absorbance units.

At the outset of each measurement set, the absorbance of water at each wavelength was measured in each cuvette as a full spectrum blank. The full spectrum blank typically did not exceed an absolute value of 0.01 absorbance units. The full spectrum blank was subtracted from the full spectrum sample data in order to obtain a corrected absorbance measurement for the sample.

Absorbencies at wavelengths 660nm and 1020nm, indicated by a black X on Figure 5, were extracted from the full spectrum data. These two wavelengths were selected based on their specific relevance. The absorbance at 660nm, or A660 avoids characteristic light absorption maxima associated with bacteriochlorophyll b: peaks near 400nm, 605nm, 835-850nm, and 1020-1030nm. It also avoids the 451nm and 483nm
peaks characteristic to the major carotenoids, 1,2-dihydroneurosporene and 1,2-dihydrolycopene, of *B. viridis*, and proteins located in the ultraviolet range of the spectrum, below 300 nm. The black square demonstrates the region most often used for quantifying photosynthetic bacteria. Initially it was assumed that the A660 measurement is proportional to the concentration of cells. In addition, the absorbance at wavelength 1020nm, or A1020, was also compared. This wavelength is characteristic of a particular pigmented protein complex, the light harvesting complex.

![Image](image-url)

**Figure 5: The Pigment Spectrum of Extracted Membranes from *B. viridis*.** A full spectrum scan of the pigment containing membranes extracted from whole cells. The characteristic maxima of these membranes are shown here marked with an X, near 400 nm, 605 nm, 835-850 nm, and 1010-1030 nm. The peaks 451 nm and 483 nm are attributed to carotenoids. The area between 630 nm and 730 nm, enclosed in a box, is devoid of any pigmentation, making this region an ideal area to take a measurement for cell culture density, because an independent change in an internal component of the cell will not have an effect. The wavelength 660 nm, marked by a black X in the middle of this region, is used to determine the density of the culture. The wavelength 1020 nm, labeled and marked by a black X, is the signature of bacteriochlorophyll *b*, which is the primary light harvesting pigment for this bacterium.
2.4.4 Colony Forming Unit (CFU) Counting

CFU counting was applied to aseptically sampled cells at various culture age, grown in RMPABA or Minimal media. To prepare samples for plating, 1 mL samples of a bacterial culture were obtained, and a 10-fold serial dilution was performed immediately. A second undiluted sample was obtained and the absorbance of the cell broth was measured and recorded as outlined in 2.2.3. Serial dilutions were performed by adding 1 mL of undiluted bacterial broth or a previous member of the dilution series to 9 mL of 10 mM Tris/1 mM Sodium EDTA buffer at pH 7.0 in sterile 50 mL Falcon tubes. (See Figure 2 for a 10-fold dilution scheme.) EDTA was added as an anticoagulant. The Falcon tube was then agitated by hand for approximately 30 seconds to achieve a well-mixed suspension. The tubes containing the desired dilutions were also re-agitated for 5-10 seconds immediately prior to sampling for plating the dilution.

Agar plates were prepared by mixing 20g fine agar powder (Sigma, St. Louis, MO) with 1 L RMPABA media. The mixture was autoclaved to simultaneously sterilize the solution and melt the agar. Approximately 20 mL of melted agar media was poured into a Fisherbrand polystyrene 100 mm x 15 mm disposable sterilized Petri dish (Cat No. 08-757-12) and allowed to cool, covered, in a sterile environment until solid. Poured plates were stored, if necessary, in sealed sterile plastic sheaths at 2-4°C until used, but not for longer than 4 weeks.

Plating was performed by sterile pipetting 100 μL of the diluted specimens most likely to contain an appropriate concentration onto a prepared agar plate. The bacterial solution was spread evenly by placing the plate onto a mechanized spinning wheel designed specifically for use in this work and rotating against an immobile sterilized bent
glass rod until the liquid was well-distributed and absorbed by the plate, approximately 30 seconds. (See Appendix A: Mechanized Spin Plating) Plates were then covered, sealed with Parafilm, and incubated at 32°C± 2°C under incandescent light bulb illumination for 48 hours, until colonies were large enough to be counted. Plates with between 3 and 300 discrete and well-formed colonies were counted, and plates with greater number of colonies were discarded as uncountable and unreliable. The number of colonies counted was multiplied by the dilution factor of the broth and 10 (to account for the 100 μL sample volume) to obtain the total CFUs per milliliter, and the assumed number of live cells per milliliter in the original broth.

In order to determine if a relationship between absorbance and live cell count existed for the entire growth curve, and what the parameters of that relationship were, cells were grown for 20 days. During those 20 days, cells were measured at least daily by both UV-Vis Absorption and plating with serial dilutions to obtain a CFU count. Cultures were grown in both minimal media as well as RMPABA media, in triplicate. To determine stationary only values, stationary cells of 5-6 days age from a separate culture were used to prepare cell broth dilutions of ¼, ½, ¾, and full concentrations of the initial broth. Each dilution was immediately plated and optical density was measured to obtain a curve for cells at stationary.
2.5 Results and Discussion

2.5.1 Determining the culture density range for most accurate UV-Vis measurement

In order to determine what range of measured absorbance at 660 nm, or A660 could be used to get an accurate measure of relative cell concentration, an optically dense stationary culture of 5 day old bacteria was measured and then subsequently diluted by half five times, and the absorbance of each dilution measured. The CFU/mL value was calculated based on a relationship determined in 2.4.2 and is displayed for reference only. The dilutions employed and their actual cell number and measured absorbance readings are listed in Table 1.

In order to determine the range in which calculated UV-Vis measurements, corrected for dilution factor, can be used as a reliable measure of relative cell density, a dilution corrected “Calculated A660” was compared to the measured A660 at each dilution. The 1/8 dilution measurement was 0.578 absorbance units. Based on the

<table>
<thead>
<tr>
<th>Sample Dilution</th>
<th>Cell Concentration (CFU/mL)</th>
<th>Measured A660 (a.u.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.9 x10^8</td>
<td>1.62 ± 0.08</td>
</tr>
<tr>
<td>1/2</td>
<td>1.4 x10^8</td>
<td>1.53± 0.08</td>
</tr>
<tr>
<td>1/4</td>
<td>7.2 x10^7</td>
<td>1.05± 0.05</td>
</tr>
<tr>
<td>1/8</td>
<td>3.6 x10^7</td>
<td>0.58± 0.03</td>
</tr>
<tr>
<td>1/16</td>
<td>1.8 x10^7</td>
<td>0.29± 0.01</td>
</tr>
<tr>
<td>1/32</td>
<td>9.0 x10^6</td>
<td>0.15± 0.01</td>
</tr>
</tbody>
</table>

NOTE: The cell concentration calculated using a relationship determined in 2.4.2 and is presented for reference only. It is subject to an error of 23.15%.
logarithmic behavior of light absorbance, error is minimized around a measurement of 0.4 absorbance units. Consequently, the 1/8 dilution was used to estimate what the absorbance should have been for each of the other dilutions, assuming no change in cell characteristics. (See Table 2 and Figure 6) The best correlation between measured and calculated absorbance were obtained for measured absorbance values below about 1.0 absorbance units. The 1/32 and 1/16 dilutions result in actual values very close to the expected 660, but the absorbance for the 1/32 dilution is near the lower end of machine sensitivity and subject to some noise, leading to a slightly increased deviation between the ideal and actual readings. At full concentration, the actual reading is only one third of the calculated value. These results are consistent with the logarithmic nature of absorbance, which indicates that the least error and most precise measurement would occur between absorbance readings of 0.2 to 0.8.

TABLE 2
THE DISCREPANCY BETWEEN ACTUAL AND EXPECTED SPECTROPHOTOMETER MEASUREMENTS OF A SERIES OF DILUTED CELL BROTH SAMPLES

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Measured A660</th>
<th>Expected 660 (Calculated)</th>
<th>%Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.620 ± 0.081</td>
<td>4.621</td>
<td>185%</td>
</tr>
<tr>
<td>½</td>
<td>1.532 ± 0.077</td>
<td>2.310</td>
<td>51%</td>
</tr>
<tr>
<td>¼</td>
<td>1.052 ± 0.053</td>
<td>1.155</td>
<td>10%</td>
</tr>
<tr>
<td>1/8</td>
<td>0.578 ± 0.029</td>
<td>0.578</td>
<td>Used to calculate Ideal</td>
</tr>
<tr>
<td>1/16</td>
<td>0.289 ± 0.014</td>
<td>0.289</td>
<td>0%</td>
</tr>
<tr>
<td>1/32</td>
<td>0.151 ± 0.008</td>
<td>0.144</td>
<td>-4%</td>
</tr>
</tbody>
</table>

NOTE: The “Ideal” value is determined mathematically by multiplying the reading in the most accurate range, a dilution factor of 8, by the factor that would correct it to the given dilution. Above a reading of approximately 1.0, significant error is encountered between the actual and “ideal” values. Any measurement above 1.0 should never be used to represent the culture.
The maximum A660 measurements obtained were in the range 1.5-1.6 absorbance units, above which no increase in measured absorbance is observed, even for cultures with a significantly higher ideal absorbance. This suggests that 1.5 absorbance units is an inherent limitation for *B. viridis* due to significant culture density. (Figure 7) In a dense sample, multiple reflections create artifacts in the measurement because light is redirected by many particles and may end up either being reflected away from the detector or being redirected toward the detector, and the measurement is compromised. In a dilute sample there are few particles, so light either passes through the sample to be recorded as “transmitted light” or reflects out of the sample to be recorded as “absorbed light” or “scattered light.” Since there are relatively few multiple reflections, the measurement is more reliable.

Figure 6: The Inherent Measurement Limitation for UV Vis Measurements of *B. viridis* cultures. The Ideal absorbance is linear WRT dilution of the sample. The Measured Absorbance is linear up to an absorbance of around 0.8-1.0, however above 1.0, there is significant non-linearity. The limits of the machine and the bacterial pigmentation prevent accurate measurement. Above about 1.5 absorbance units, the UV-Vis detector can observe no further increase in culture density.
In addition to the difficulties achieving the expected culture density measurements at absorbance values above 0.8, significant distortion across the entire spectrum was observed for all spectra in this range. This distortion can be seen when the full spectrum data is plotted by dilution factor. (See Figure 8) The sloping behavior of the spectrum between 400nm and 940nm is flattened. The peak at 1020nm experiences a break in continuity near the maximum caused by the intensity of the related pigment. The values from Table 2 are plotted here with the spectrum, Actual (■) and Calculated (or Expected) (●) A660 measurements. The actual measurements fall along the spectra, but the ideal measurements are of significantly higher value for the more dense samples. This suggests that the distortions are occurring where the inherent machine limitation is reached. In order to insure a reliable spectrum, and the mathematically extracted

Figure 7: The Effect of Dense and Dilute Cultures. In a dense sample, light is reflected multiple times, creating artifacts in the measurement. When there are many large particles, light is redirected multiple times and may end up either being reflected away from the detector or being redirected toward the detector, and the measurement is compromised. If there are few particles, this effect is limited to light either passing through the sample and being recorded as “transmitted light” or light being reflected and not passing through the sample, and being recorded as “absorbed light” or “scattered light.” In a dilute sample, there are relatively few reflections, and the measurement is more reliable.
measurements at wavelength 660 nm and 1020 nm, only spectra in which A660 is less than 0.8 should be utilized.

2.5.2 A Correlation for the Relationship between A660 and CFU count for *B. viridis*

An overall correlation describing the relationship between the optical density of the cells and the live cell count was determined by plotting Colony Forming Units per mL (CFU/mL) against the calculated absorbance at 660 nm, A660. A linear correlation existed for only a small portion of the dataset (See Figure 9). The boundary between well
Figure 9: The Relationship Between CFU Count and Calculated A660 for data collected for the entire growth cycle of *B. viridis*. At low cell density and culture absorbance, a well-correlated region is observed, within the dashed lines. A) For RMPABA media, the data above approximately $1.5 \times 10^8$ CFU/mL and 3.0 a.u. has high error and no correlation. B) For Minimal media, this poorly correlated range is above $1.5 \times 10^7$ CFU/mL and 1.3 a.u. This poorly correlated range is associated with the later stages of cell growth, when pigment changes, flocculation, contamination, and cell death potentially contribute to unstable measurements. See Below for analysis of the well correlated portions of the data.
and poorly correlated points is about $1.5 \times 10^8$ CFU/mL and 3.0 (dilution-corrected) absorbance units for RMPABA and $1.5 \times 10^7$ CFU/mL and 1.3 (dilution-corrected) absorbance units for Minimal media.

If the cells experience size or morphology changes during the growth phase or begin to clump or coagulate, the molar extinction coefficient, $\epsilon$, for the cells would change, affecting the relationship between $A_{660}$ and CFU. As the culture moves out of the exponential growth stage, where the primary effort of the cells is to replicate, significant variation in pigment per cell can also result due to changing intracellular composition, which changes the relationship between absorbance and cell concentration.

Considering that photosynthetic bacteria researchers, as a whole, rarely present a linear correlation based on the entire growth curve, it is likely that the pigmentation, specifically, affects the morphology or molar extinction coefficient of the culture over time. This suggests that the bacteria go through more than one phases of growth; the bacteria must both double their numbers and produce new pigment, and these two activities need not be coupled. The growth and pigment production behavior, based on the CFU count, $A_{660}$, and $A_{1020}$ is revisited in Chapter 3.

Cell flocculation at higher culture densities and contamination may affect CFU count directly. In plating, several flocculated cells would manifest as only one colony-forming unit, while the same number of independent cells would manifest as several colony-forming units. Although any cultures identified as contaminated are discarded immediately, even a slight amount of contamination can have a significant impact on CFU measurement. This is especially true since many contaminating species have a faster growth rate than *B. viridis*. Looking at all of these factors as a whole, it is clear
that data gathered later in the cell cycle, in the range where scatter and error is extreme cannot be considered reliable for monitoring cell number.

2.5.2.1 A Correlation for Minimal media

Data gathered over time from the growth curve of B. viridis in Minimal media was plotted with data gathered for cells as they reached stationary, approximately Day 5. (See Figure 10) Both curves group well together. This means that the cells maintain similar optical and plating characteristics both at early time (during exponential growth) and into stationary growth. A combined correlation was determined to accommodate the data, and the fitted equation is shown in Equation 5.

\[
\text{CFUs} = 7 \times 10^6 (A660) - 254482 \tag{5}
\]

The average percent deviation between the cell number calculated with this relationship and the actual cell number is about 8.5% for the region in which it is valid, between inoculation and the onset of stationary (about Day 5). This value is determined by averaging the absolute value of the percent difference observed for each time point in this region. (See Table 3) Beyond day 7, the error in the calculated value increases to around 75%, so this correlation cannot be used for cells beyond early stationary growth.
Figure 10: For Minimal Media, the Correlation Between Live Cell Count and Absorbance at Early Time and Stationary. The correlation is reasonable for data gathered between the time of inoculation and Day 5 (Early Time). Cells at a single time point at stationary, Day 5, also fit well with this correlation. This indicates that the correlation can be used for cells up to approximately Day 5 of culture growth, corresponding to a calculated A660 of less than 1.5 a.u.

TABLE 3
THE DIFFERENCE BETWEEN MEASURED VALUES AND VALUES CALCULATED USING THE CORRELATION FOR MINIMAL CELL GROWTH FROM EQUATION 5

<table>
<thead>
<tr>
<th>Day</th>
<th>% Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-3.4%</td>
</tr>
<tr>
<td>2</td>
<td>8.2%</td>
</tr>
<tr>
<td>3</td>
<td>-9.7%</td>
</tr>
<tr>
<td>4</td>
<td>11.8%</td>
</tr>
<tr>
<td>5*</td>
<td>9.3%</td>
</tr>
<tr>
<td>7 - 30</td>
<td>74.7%±10%</td>
</tr>
</tbody>
</table>

NOTE:
* Day 5 is an average of the deviation for Day 5 and stationary data from both datasets (7 measurements).
2.5.2.2 RMPABA Media

A reasonable correlation between absorbance and CFU counts could be determined for cells grown in RMPABA medium in the range below 2.0x10^8 live cells/mL and 3.0 absorbance units (See Figure 11 “Early Time” and Equation 6). Cells entering stationary phase, just after day 5, were evaluated as described in the previous section for Minimal media-grown cells and compared to the correlation determined from the growing cells at later time (See Figure 11 “Stationary” and Equation 7).

\[ \text{CFUs} = 5 \times 10^7 (A_{660}) - 3 \times 10^7 \]  \hspace{1cm} (6)

\[ \text{CFUs} = 9 \times 10^6 (A_{660}) - 3 \times 10^6 \]  \hspace{1cm} (7)

Figure 11: For RMPABA media, the Correlation for CFU and Absorbance at Early Time. The correlation is good for data gathered between the time of inoculation and Day 5. Cells at a single point, when stationary was reached, did not fit well with this correlation. Cells at early time were newly inoculated from a culture of similar stationary age.
The error between measured values and calculated values was determined using the correlation for RMPABA early time cells, Equation 6, and RMPABA stationary cells, Equation 7. The early time correlation specified the better overall relationship to the measured values. During the first 5 days of growth, about 21% deviation from the measured values occurs. (See Table 4) This is a significant improvement compared to the stationary correlation, which averages 62% error during the first five days. Beyond day 5, both correlations generated calculated values that deviated wildly from the measured value.

The slope of the CFU/absorption correlation for early time stage cells was more than five times as steep as the slope for stationary cells. Stationary cells either produce pigment faster than they reproduce, or else a greater proportion of the cells have died. To determine if the rate of pigment production is different, the relationship between

### TABLE 4


<table>
<thead>
<tr>
<th>Day</th>
<th>Early Time Correlation</th>
<th>Stationary (Day 5) Correlation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%Deviation</td>
<td>%Deviation</td>
</tr>
<tr>
<td>Stationary Cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>-290.1%</td>
<td>4.5%</td>
</tr>
<tr>
<td>Growth Curve/Early Time</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>22.4%</td>
<td>25.0%</td>
</tr>
<tr>
<td>2</td>
<td>29.2%</td>
<td>39.5%</td>
</tr>
<tr>
<td>3</td>
<td>19.5%</td>
<td>80.5%</td>
</tr>
<tr>
<td>4</td>
<td>7.5%</td>
<td>79.5%</td>
</tr>
<tr>
<td>5</td>
<td>27.3%</td>
<td>85.2%</td>
</tr>
<tr>
<td>7-30</td>
<td>60.6% ± 55%</td>
<td>75.7% ± 14.4%</td>
</tr>
</tbody>
</table>
absorbance at 1020 nm, or A1020 and cell number was compared to the relationship between absorbance at 660 nm, or A660 and cell number. (See Figure 12) The results showed that stationary data was nearly identical in slope. The early time data was fairly well grouped, but the number of cells counted per A660 absorbance unit was slightly higher than the number of cells counted per A1020 absorbance unit. This suggests that the rate of pigment production, or increase in A1020 relative to cell number, is not significantly higher than the rate of increase of cells, or A660. The very tight grouping in the stationary data indicates that no excess pigment is being produced per cell during stationary growth. This strongly suggests that the discrepancy in the data results from the onset of cell death. This would reduce the number of live cells, and therefore colony-forming units observed per absorbance unit, without affecting the pigment concentration.
2.5.2.3 Comparison of Minimal and RMPABA media correlations

As shown above, to determine a cell number, within an order of magnitude, during the first 5 days of Minimal or RMPABA media culture, the correlation determined with the early time cells can be employed. It is important to note, however, that this correlation is useful only as an approximation tool, and at least 10% error for Minimal and 20% error for RMPABA should be expected. Although cell cultures are most easily maintained on rich, undefined media, because it is undefined and allows relatively
unchecked growth, there is greater variability in cell counting. Use of RMPABA should be limited to maintaining cell cultures, creating cells for subsequent experimental use and for other purposes in which repeatability and defined parameters are not necessary, but not for the purpose of evaluating experimental conditions. Minimal Media should be employed for quantitative work. For Minimal Media cultures, A660 and CFU count were reasonably correlated so that only A660 required monitoring on a regular basis, while CFU count could be periodically monitored in order to insure ongoing compliance with the determined correlation. CFU count should also be employed for any growth experiments that go beyond the exponential and early stationary phase.

2.6 Conclusions

Considering the features of *B. viridis*, such as small size and pigmentation, bench scale volumes and salt rich media, UV-Vis Absorption/Optical Density and Live Cell Count by Plating were selected for as the most appropriate methods quantitative culture evaluation. It was determined that:

- UV-Vis Absorption/Optical Density measurements were accurate and repeatable in the range below an optical density of about 0.8 absorbance units.

- Comparison of absorbance and Live Cell Count by serial dilution plating could quantify the actual number of live cells, as well as reveal changes in the pigment content and status of the cell culture at different growth stages not possible with absorption measurements alone.

- For Minimal media, cell composition or morphology [as determined by correlation between Absorbance (A660) and Colony Forming Units (CFUs)] was constant during both the exponential and early stationary phase.

- For Minimal media, a correlation was determined which will allow the culture to be tracked on a regular basis using UV-Vis/Absorbance measurements alone. Occasional comparison with CFU count is advisable to insure the correlation remains accurate, but the correlation will allow a significant reduction in measurement time.
For RMPABA, cell composition or morphology [as determined by correlation between Absorbance (A660 & A1020) Colony Forming Units (CFUs)] was different during both the exponential and early stationary phase. The data suggest that the discrepancy is caused by the onset of cell death early in the growth cycle.

From this work it was concluded that further study of cell growth dependence on condition and toxin concentration should be performed in the more repeatable, quantifiable Minimal media to avoid complications.
2.7 References


2. Schuler, 379.


8. Instruction Manual UVmini-1240/UVmini-1240V, Chapter 4, (Kyoto Japan: Shimadzu Corporation Analytical Instruments Division, 2001)


10. Wright, 2069-2073.


23. Schuler, 150.


25. Torura, 176.


30. Schuler, 149.


34. Torura, 173.

35. Schuler, 379.


38. Schuler, 379.


42. Dennis Birdsell and Jon Loftus, Discussion of Faculties Use, Fall 2003, Center for Environmental Technology, University of Notre Dame, Notre Dame, IN.

43. Envitech, United Kingdom. <http://www.envitech.co.uk/Prod_StipTOC.html>


46. Drews, 255-262.

47. Schuler, 150.


52. Drews, 255-262.


60. Schuler, 151.
CHAPTER 3

STANDARD GROWTH BEHAVIOR OF B. VIRIDIS

3.1 Introduction

3.1.1 Previous work and the next step

UV-Vis Spectrophotometry and CFU count by plating a dilution series were identified as the two most useful and reliable methods based on investigation of a variety of methods and comparison of the results obtained by each method. (Chapter 2) A correlation between these two methods, and the range within which it was bounded, was determined. Defining the best methods of measurement of culture growth and their limitations is important to the long-term goal of designing a system for waste treatment and remediation. It is also critical to developing an understanding of the fundamental behavior difference of the bacterium under rich (ideal) and minimal (relatively poor) conditions. Knowledge of the range of behavior of the bacteria is necessary to have some standard against which to compare the responses to experimental conditions.

The investigation of methods in Chapter 2 also suggested that the culture growth response in Minimal media, preferred for quantitative experiments, was significantly less vigorous than in RMPABA media. If there is a significant depression in culture growth or pigment production (important for photosynthesis energy harvesting), then a reformulation of the media and modification of the applied growth conditions may be
required. The growth response under each condition was therefore quantified to provide information about the expected behavior of the culture throughout the growth curve. This was done in order to provide a standard against which to compare experimental results and to determine if media optimization is required.

3.1.2 Goals & Justification

In this chapter, the data for culture growth of *B. viridis* cells is presented in three ways, as CFU count, UV-Vis Absorbance at 660nm and 1020nm, and pH behavior, which reflects the behavior of the culture in terms of live cells, culture density, pigment production, and culture metabolic activity, respectively. In addition, the Two-Point method, developed for simplifying culture quantification by requiring only two measurements, is investigated and tested as it pertains to reliably quantifying the known culture behaviors.

The CFU count is a direct measure of live, active cells, which provides direct culture information throughout the time course. This measurement may be compromised late in the culture growth by contamination; as the culture ages and cells die or become dormant, the number of live, active cells may no longer be the total number of cells present. In Chapter 2, it was determined that a changing culture morphology or behavior made A660 measurements beyond approximately day 5, roughly corresponding to the end of exponential growth, difficult to correlate to a CFU count. For cultures in Minimal media, the onset of stationary was the boundary, and for RMPABA cultures, the correlation became questionable as soon as late exponential growth. Despite this limitation, a measure of absorbance can be useful, even beyond this range, because it
provides information about the changing optical density of the culture as well as information about the changing composition of the cell.

The absorbance at 660 nm measures total optical density of the culture, and takes into account even cells that are dormant or dead. Because A660 describes the density of the culture in a relatively featureless area of the spectrum, the ratio of absorbance at some other wavelengths of interest to the A660 can provide an internal measure of the shift in cell composition. In addition, knowledge of the typical absorbance behavior at key wavelengths throughout the time course is useful for comparative purposes. A shift in the absorbance behavior can indicate a number of relevant cell responses, such as lack of pigment formation, pigment damage, or a decrease in culture optical density, suggesting damage to the cells or inability of the cells to increase in size.

The pH behavior of the culture is important to quantify because these photosynthetic bacteria rely on a pH gradient to generate energy, and the performance of the culture has been linked to the pH environment. In addition, the Two-Point method was developed based on strategic difficulties in measuring replicates and dilutions of more than 50 samples on a daily basis. In practical terms, reducing the number of measurements required to obtain a reliable comparison makes this a more useful and powerful method for application in an industrial setting. By using each of these techniques, a complete picture of the expected culture behavior can be known and described, setting the stage for future work.
3.1.3 Background on plotting culture growth

Time course data for growing bacterial cultures is generally plotted in semi-log fashion.¹ A typical microbe undergoes five distinct phases: lag, exponential, decline, stationary, and death.² (See Figure 1) During lag phase, the microbe is adjusting to the new surroundings and preparing to grow. Next, the microbe enters the exponential phase. On a log scale, the exponential growth phase will appear linear; this growth is typically well represented by an exponential function. Growth then begins to slow during the decline and finally achieves and maintains its maximum growth, at stationary. After the stationary phase, cells begin to die and the number of live cells will decline.³ The death phase can only be seen using a method which quantifies the density of live cells, because

Figure 1: An Idealized Growth Curve for Microbes. The growth behavior of a microbe has 5 distinct phases: lag, exponential, decline, stationary, and death. Plotted on a logarithmic scale, the exponential growth phase will appear linear.
a method which counts only total cells, cell mass, or culture optical density does not
discriminate between live and dead cells.

Lag phase is largely determined by the physiological state of the cells when they
are inoculated into fresh media. This is a period of adaptation where cells prepare to
grow in a new medium. If there is a significant difference between the two medias, the
lag phase can be extensive. If cells are being inoculated into much the same conditions,
the lag phase may be almost non-existent, and cells will typically assume the same
growth rate in the same medium.

Exponential phase is the most important phase to characterize. The rate of
growth for a cell culture indicates its health, its ability to utilize its food and nutrient
sources, whether adequate food and nutrients are available, and demonstrates whether or
not it is growing in a repeatable fashion. The growth rate will often change for different
food sources. Bacterial culture growth also responds to changes in oxygen
concentration, light intensity, and temperature, among other environmental
factors. For growth in the exponential phase, the growth rate (μ) can be determined
from an exponential fit of cell density data plotted against time. Any measure of cell
density can be used, and the resulting rate will apply to that measure of cells. The change
in the cell concentration (dX) with time (dt) is the result of the rate of growth (μ) times
the cell concentration (X). This is subject to the boundary condition that at time zero,
the concentration of cells is the initial concentration (X₀). (Equation 1)

\[
\frac{dX}{dt} = \mu X, \quad X = X_0 \text{ at } t = 0
\]  

(8)
This equation can be integrated and rearranged to give a relationship for cell concentration (X) with respect to time (t). (Equation 2)

$$X = X_0 e^{\mu t}$$  \hspace{1cm} (9)

An exponential fit can be determined for the data, in the form $y = me^{\mu t}$, and the exponential growth rate, $\mu$, determined. This rate mathematically has the unit “time$^{-1}$.” The unit of measure, CFU or absorbance units (a.u) in this work, is incorporated into the final cell concentration, X, from the initial cell measurement, $X_0$. It is also the initial cell measurement, $X_0$, which adjusts the calculated cell number to the correct magnitude for the measurement method used. Ideally, the rate should be consistent regardless of measurement method and its order of magnitude, since the various methods are all used to describe the growth behavior of the same culture. A convention of writing either “CFU/time” or “a.u./time” to indicate the source of the dataset being used to determine the rate is employed in this work. However, this rate can not be used as a linear rate.

Once the growth rate is determined, it can also be used to calculate the doubling time ($\tau_d$) of the culture, which is a measure of the time required to double the microbial mass. This is calculated by assuming that $X = 2X_0$, or double the initial cell mass. In this case, $X_0$ will cancel out, leaving $2 = e^{\mu \tau_d}$, and a simple rearrangement gives the doubling time ($\tau_d$).18 (Equation 3)

$$\tau_d = \frac{\ln 2}{\mu} = \frac{0.693}{\mu}$$  \hspace{1cm} (10)
Growth rate and doubling time are interchangeable characteristics, but it is often useful to refer to one or the other for ease of comparison. These equations can be applied to data gathered by any of the methods for counting total and live cells in a culture described in Chapter 2 to determine the growth rate and doubling time of the culture.

After the growth rate slows in the deceleration phase, cells enter the stationary phase. In this phase, cells cease division and multiplication. This may occur for a number of reasons; food or nutrients run short, the cells have produced enough end product to block further growth, or cells stop growing while undergoing a shift to another metabolism. The primary characteristic useful in the stationary phase is the total amount of cell production that can be expected, which can also be affected by nutritional and physical culture conditions. This can be estimated from the growth curve, or by developing a mathematical average of the total culture density over several days prior to the death phase, but after the deceleration phase. The death phase is primarily visible using a measurement method that reports the number of live cells. In the death phase, the number of live cells begins to decrease. The death phase typically follows the same first order kinetics as the exponential phase, and analogous equations can be determined.

Death rate and behavior were not used as a characteristic of growth in this work.

3.2 Materials & Methods

The two primary medias, RMPABA and Minimal, were prepared as described in Chapter 2.2.1, following the recipe outlined in Appendix E. The measurement methods employed were UV Vis Spectrophotometry (Chapter 2.2.3) and CFU Count (Chapter
2.2.4). Cultures were prepared as described in Chapter 2.4.2, with any additional or altered treatments outlined below.

3.2.1 Culture and sample preparation

3.2.1.1 Comparison of RMPABA and Minimal medias

RMPABA and Minimal media were prepared, adjusted to pH 6.9, and sterilized by autoclave or sterile filtration, respectively. The 50/50 (v/v) media was prepared by measuring the respective sterile medias in sterile volumetric flasks, and mixing them in sterilized glassware. A standard inoculation of 20% by volume of a 5-day-old RMPABA grown culture, concentrated and resuspended in fresh culture medium to reduce the concentration of cell byproduct, was employed. This resuspension was employed to minimize the effect of byproducts in the inoculating cell culture on lag and early time behavior.

3.2.1.2 Determining the reproducibility of cultures grown in Minimal media

In each case, Minimal media was prepared, adjusted to pH 6.9, and sterilized by filtration. A standard inoculation of 20% by volume of a 5-day-old RMPABA culture, concentrated and resuspended in fresh Minimal culture medium was employed. Each data set was gathered from independently constructed experiments. These independent experiments used Minimal media mixed in different batches, and were inoculated at different times by different batches of inoculating cells. Five day old cells from the same line of inoculating culture were employed. Comparison of RMPABA grown cells, presented here only in summary, was performed using the same procedure.
3.2.2 Characterization of cell growth and health for all performed experiments

The behavior of *B. viridis* cultured in RMPABA, Minimal, and mixed medias was quantified by six primary features. (See Table 1) These can be divided into measured parameters and calculated ratios. The measured parameters include CFU count, Absorbance at 660nm (A660), Absorbance at 1020nm (A1020), and pH. The calculated ratios include A1020/CFU count and A1020/A660.

### 3.2.2.1 Measured parameters

During the first 5 days of growth, when the greatest changes in cell number were expected based on previous observations, the culture characteristics measured by CFU Count, UV Vis Spectrophotometry at 600 nm (A660) and 1020 nm (A1020), and pH were monitored every 6 to 9 hours. For the first 10 days, the culture was monitored every 12 hours, and thereafter, every 24 hours. This insured that any early time fluctuations in

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Measures</th>
<th>Significance</th>
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</thead>
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<tr>
<td>CFU Count, rate of increase</td>
<td>Live cells per volume, growth rate</td>
<td>Growth indicates success or failure of culture, rate allows comparison between cultures.</td>
</tr>
<tr>
<td>A660, rate of increase</td>
<td>Culture Density, growth rate</td>
<td>Growth indicates success or failure of culture, rate allows comparison between cultures.</td>
</tr>
<tr>
<td>pH</td>
<td>Base production</td>
<td>Base is produced during growth and energy production.</td>
</tr>
<tr>
<td>A1020</td>
<td>Pigment Production</td>
<td>Pigment is critical to energy harvesting.</td>
</tr>
<tr>
<td>A1020/CFU Count</td>
<td>Pigment per cell</td>
<td>Determines the physiological state of the cell.</td>
</tr>
<tr>
<td>A1020/A660</td>
<td>Pigment per cell</td>
<td>Determines the physiological state of the cell.</td>
</tr>
</tbody>
</table>

NOTE: Each characteristic of growth helps quantify and define the basic behavior of *B. viridis* in culture, and can be used to evaluate the effect of experimental conditions.
behavior during the growth phase would be noted. The pH of the culture, monitored using an Orion Model 410A pH meter, indicates whether or not physiological processes are proceeding as expected. The calculated parameters A1020/CFU Count and A1020/A660 were also determined to relate cell growth and pigment formation.

3.2.3 Developing and testing the Two Point Method

Based on the “standard” Minimal growth and the similar features of multiple batches of cells grown in Minimal media, the features of the curve were carefully considered. The bacteria show very little lag when grown in Minimal media with a 5-day old washed and resuspended inoculating culture. This suggests that they enter exponential growth immediately upon inoculation. They also tend to shift from exponential growth to stationary growth around day 5. Roughly, day 5 can be considered both the end of the exponential phase and an estimation of the maximum growth achieved at stationary. Based on these two points, the “Two-Point” method was developed.

UV-Vis data for the Two-Point method was collected just after culture inoculation, and again on the fifth day of growth. An exponential curve was then fitted using those two data points and an exponential rate of growth determined, based on Equation 2. In order to check the validity of this method, the data for cultures grown in Minimal media presented in this chapter as the standard was used to calculate a rate based on all data collected in the first five days of culture and on the first and fifth day measurements.
Error is calculated by using the minimum and maximum value obtained for initial culture density and final culture density for three identical specimens. An exponential fit, known to best characterize the exponential growth phase, is determined for the initial minimum and final maximum values, and for the initial maximum and final minimum value. From this, a growth rate can be determined. (See Figure 2) This provides the maximum and minimum possible growth rates which could be experienced by the culture, and the calculated range is reported as the error of the measurement. This slightly overestimates the error, because the minimum initial value tends to correspond to the minimum final value, and likewise for maximum values.

Figure 2: Error Calculation for Two-Point method. The reported value is calculated using the average of the initial and final cultures. (Solid line) The maximum initial value and minimum final value are used to calculate the minimum possible slope; the minimum initial value and maximum final value are used to calculate the maximum possible slope. (Dashed lines) This slightly overestimates, and therefore provides the maximum range of error expected. Values shown here are exaggerated to clearly demonstrate the principle.
3.3 Results & Discussion

The bacteria were grown in RMPABA media, Minimal media, and a 50/50 blend of the two medias in order to understand the contributions of each to the growth behavior. Using the characteristics of growth described above, the responses were recorded and examined in order to provide a baseline for understanding the growth. There was a significant difference in growth between cells inoculated into RMPABA media and Minimal media. This was most evident in a decreased density of cells and decreased pigment production. Based on the need for repeatability and the significant variability of RMPABA growth, Minimal media was ultimately determined to be the better choice for quantitative experimentation. In order to characterize the quantitative Minimal media system, two batches of cells grown in Minimal media were compared to determine the variability in the growth. The repeatability in Minimal media, the media of choice for quantification, of the total growth and culture density, the rate of growth, and the other defining characteristics were considered and discussed. This will provide a baseline for interpreting the significance of variations observed in response to an applied condition.

3.3.1 Growth curve for RMPABA, Minimal, and a 50/50 RMPABA and Minimal mix

In order to determine the expected response of *B. viridis* cells to RMPABA and Minimal medias, a growth curve, measured using the methods described above, was determined for RMPABA media, Minimal media, and a 50/50 mix of RMPABA and Minimal grown in 1-liter batches in triplicate. As expected, when cells were inoculated into fresh media, an increase in the number of live cells and increase in culture density was noted.
3.3.1.1 Increase in CFU count

According to the results of CFU counting, cells began at an initial concentration around 3x10^6 cells/mL and increased in cell number with no observable lag phase evident for any of the three medias offered, minimal. Minimal/RMPABA 50/50 or RMPABA. (See Figure 3) Cells in RMPABA media and 50/50 media exhibited extremely similar behavior and experienced a sharp increase in cell number during the exponential phase, at rate of 0.8375 CFU/day and 0.7294 CFU/day, respectively, which ended around day 7. A short stationary phase, with an average maximum of 2.45x10^8 cells/mL for RMPABA and 2.11x10^8 cells/mL for 50/50 media, was followed by slow decrease in the number of cells present. Cells in Minimal media experienced a nearly 1/3 slower growth rate, 0.2564 CFU/day, and entered stationary with about one fifth of the cell density of the RMPABA culture around day 7, near 4.0x10^7 cells/mL. In Minimal media, the culture remained in stationary throughout the remainder of the experiment.

3.3.1.2 Increase in absorbance at 660 nm

Along with the increase in the number of live cells, a corresponding increase in the optical density of the culture, A660, was noted. (See Figure 4) After inoculation, the cultures had a starting density of around 0.45 absorbance units (a.u). The RMPABA and 50/50 cultures grew with almost identical behavior, growth rates of 0.4176 a.u./day and 0.4039 a.u./day, respectively, until day 5. The deceleration period for RMPABA was extended, and a total average density of about 5.4 a.u. was obtained during the stationary phase. The culture in 50/50 media performed slightly less well, obtaining only a stationary average of 4.0 a.u. Minimal media cultures grew significantly slower, a
Figure 3: Growth Curves Monitored by CFU count for RMPABA, 50/50 Mix, and Minimal Media. Top: Data for the entire life of the culture through day 30. Bottom: Growth rates calculated from exponential fits based on the linear portion of the data. RMPABA and 50/50 media displayed similar growth rates: 0.8375 CFU/day and 0.7294 CFU/day respectively. Minimal media had a slower growth rate, 0.2564 CFU/day, and exhibited less growth, however growth in Minimal did remain in stationary for the duration of the experiment, while RMPABA and 50/50 cultures exhibited a slight decrease in cell number.
Figure 4: Growth Monitored by A660 for RMPABA, 50/50 Mix, and Minimal Medias. Top: RMPABA and 50/50 media yielded almost identical behavior throughout the entire growth curve, with slightly higher total culture density in the stationary growth phase in RMPABA. Minimal grown cultures exhibited less growth total growth. Bottom: The exponential behavior of RMPABA and 50/50 media varied by 3%, with growth rate 0.4167 a.u./day and 0.4039 a.u./day respectively. Minimal media had an almost 50% slower growth rate, only 0.2593 a.u./day.
growth rate of only 0.2593 a.u./day. Stationary was reached around day 5, with a lower average stationary culture density, 1.3 absorbance units.

3.3.1.3 Culture pH

The pH of the culture changes as the bacteria grow due to their metabolic processes and is indicative of the behavior and health of the culture. (See Figure 5) The rate of pH increase was calculated with the same method as growth rate, by fitting an exponential curve to the relevant portion of the curve. For RMPABA and 50/50 culture, an exponential increase rate of 0.0197 units/day and 0.0222 units/day, respectively, was observed. For Minimal cultures, a rate of pH increase of 0.0176 units/day was observed. These rates did not correlate to the growth rates, rather a steady linear increase of around 0.02 pH units/day was observed for cells in all medias until an intrinsic pH limit was reached, and then pH increase leveled off. A final pH of 8.0 seemed to be a marker by which the onset of stationary could be estimated for growth in RMPABA and 50/50 media. Although this pH point was not, in and of itself, an inhibitor of growth, (See Chapter 5.3.8) the physiological processes which generated a pH of 8.0 also generated inhibitors which were enough to terminate any further growth. A final pH around 7.7 was the standard maximum for cultures grown in Minimal media. Although less growth was achieved for Minimal, the maximum pH for the culture also seemed to occur simultaneously with the onset of stationary.

3.3.1.4 Pigment content of cells

Pigment concentration also increased with culture age, as expected.\textsuperscript{21,22,23,24} The pigments in photosynthetic bacteria mediate photosynthesis and energy generation, so
Figure 5: The pH Behavior of Cultures Grown in RMPABA, 50/50, and Minimal Medias. Top: All cultures were adjusted to pH 6.9 at the outset. RMPABA and 50/50 increased steadily until pH 8.0, at the onset of stationary. Minimal media had a maximum pH of 7.7, coinciding with the stationary phase. Bottom: Exponential rate of pH increase did not correlate directly to growth rates.
quantifying and following the pigment in the cells is important to understanding the health and the activity of the cell. The absorbance at 1020 nm, or A1020, is obtained by the same method described in Chapter 2.2.3 and used above to determine the absorbance at 660 nm, or A660. The spectrum is measured, with any necessary dilutions, and the absorbance at 1020 nm is extracted from the spectrum and corrected for dilution.

3.3.1.4.1 Increase in Absorbance at 1020 nm

Both RMPABA and 50/50 had nearly identically increases in A1020 until the deceleration phase, at which time the RMPABA surpassed the 50/50 media blend. (See Figure 6) During the exponential phase, RMPABA had an increase in A1020 of 0.4751 a.u./day and 50/50 was actually slightly higher during the exponential phase, with a rate of 0.4834 a.u./day. Deceleration, as evidenced by the end of the linear increase portion of the curve plotted on a semi-log scale, began around day 4, slightly sooner for A1020 than for growth measured by either A660 or CFU count. When stationary was achieved, the maximum average A1020 for RMPABA was higher, at 5.7 a.u., than that for 50/50, at 3.9 a.u. Minimal media had about half the rate of increase as RMPABA during the same exponential period, only 0.247 a.u./day, and achieved only 1/5 of the total pigment signature, 1.1 a.u.

3.3.1.4.2 Correlation of A1020 to CFU count and A660

In order to determine the relative concentration of pigment to cells, two different ratios were considered. The ratio of A1020 to CFU count gives a measure of the amount of pigment present to the number of live cells. (See Figure 7 Top) The ratio of A1020 to A660, (See Figure 7 Bottom) gives a ratio of the pigment signature to the culture density.
Figure 6: Pigment Increase Measured by A1020 for RMPABA, 50/50 Mix, and Minimal Media. Top: The pigment increase for RMPABA and 50/50 media cultures had similar behavior, but Minimal media had a slower rate and achieved only 1/5 of the total pigment signature. Bottom: RMPABA and 50/50 cultures showed almost identical exponential behavior, with A1020 increase rate of 0.475167 a.u./day and 0.4834 a.u./day, respectively. Minimal media exhibited only ½ of that, 0.247 a.u./day, achieved by RMPABA.
Figure 7: Ratios of Pigment to Cells for RMPABA, 50/50 Mix, and Minimal Media. Top: The ratio of CFUs to A1020 shows a significant decrease in the amount of pigment per cell for all cultures. Bottom: The ratio of A1020 to A660 demonstrates the most significant decrease in pigment per cell for Minimal media.
Because A1020 and A660 are measured simultaneously, the behavior of the ratio A1020/A660 is significantly smoother than the ratio for A1020/CFU count.

A decrease in the ratio of A1020/CFU indicates a decrease in the amount of pigment per cell. Figure 6 Top shows that on a scale divided by $10^{-8}$, the ratio of A1020 to cells is in the vicinity of $20\pm5$ at the outset of the experiment. This number is small because the absorbance is on the order 1 and CFU count is on the order $10^6$ to $10^8$. This ratio drops rapidly, and by day 3, RMPABA and 50/50 cultures are around 1 on the same scale. Minimal drops less quickly, but by day 10, it is around 2.5. This suggests that the amount of pigment per cell is decreasing. In physical terms, it is likely that as the cells replicate, the pigment is divided between the mother cell and the budded cell, and the focus is on creating more cells rather than on generating new pigment. For Minimal, which has a slower drop, it indicates that fewer cells are being generated, which is supported by the CFU count and the A660 measurement, and the pigment is not so severely divided.

For RMPABA and 50/50, an increase in pigment per cell can be noted around the onset of the stationary period. At approximately day 6, when the ratio value is just under 1, the ratio begins to climb. This increase continued steadily until around day 13, with a ratio value of around 5. This indicates that the amount of pigment per cell is increasing, suggesting that the cells, having reached stationary and stopped dividing, have shifted focus to generating new pigment. The pigment level does not rise at early stage in the culture, but the specific reason is not known. It seems as though the pigment production is decoupled from the cell production, suggesting that the cells are partitioning their efforts to the task of generating non-photosynthetic components, and photosynthetic
components and pigment sequentially. Recovery of pigment levels is not noted for Minimal media, which remains around 2 for the rest of the observed measurements. This suggests that while some new cells are generated in Minimal media, pigment formation is occurring at a relatively low rate. The A1020 data suggests that this rate is about half of the rate of pigment formation in the presence of yeast extract and bactopeptone. (See 3.3.1.4.1)

The ratio of A1020 to A660 for all cultures begins at about 1.1 (Figure 7 Bottom). The ratio for Minimal media experiences an almost immediate drop, ending around 0.8, indicating a significant decrease in the amount of pigment per cell. This value never increases or recovers. For RMPABA and 50/50 medias, no immediate behavior is noted, but a slight decrease in the ratio, to around 1.0, occurs after day 5. This supports the trend observed in the ratio of cell number to A1020, with two phases; first cells are generated, then pigment is produced. The ratio stays around 1.0 for some time, but toward the end of the culture, an increase to the initial value of 1.1 is noted for RMPABA and 50/50 media. A slight increase to 0.9 is noted for Minimal media, but recovery to the initial value was never observed. (Late time data not shown) This supports the ratio data based on cell number, which suggests that pigment production is severely impeded in Minimal media.

3.3.1.5 Discussion

3.3.1.5.1 The differences between RMPABA and Minimal medias

The growth in Minimal media differs from that observed in RMPABA media in several ways. First, the number of cells and culture density is reduced to approximately 1/3-1/5 of the growth observed in RMPABA media. Second, the pigment production is
significantly less, both in rate and in total pigment produced. The primary difference
between these Minimal and RMPABA medias is the presence or absence of Yeast Extract
and Bactopeptone. It has been noted for B. viridis in other studies that in the absence of
yeast extract, the total growth of cells is diminished, the content of bacteriochlorophyll b
is lowered, and the susceptibility to mechanical damage is increased.\textsuperscript{25} This increased
mechanical sensitivity may be due to a change in lipid composition, which could also
make the cell membranes more vulnerable to hydrophobic attack by a variety of
chemicals, especially cyclic hydrocarbons.\textsuperscript{26} Although no obvious mechanical sensitivity
was observed during handling, for example during centrifugation, the decrease in growth
and pigment production suggests that the cell’s viability was compromised. Without
these ingredients, the Minimal media typically achieves one fifth to one half of the
growth or pigment production observed in RMPABA. Because pigment production is
slowed, a better correlation between CFU count data and the A660 data can be obtained.

RMPABA and the 50/50 mix are extremely similar in behavior. (Table 2) Even
50\% of the total RMPABA concentration of yeast extract and bactopeptone provide the
nutrients necessary for vigorous, healthy growth of nearly the same intensity as
RMPABA growth at early times in the culture. The differences between RMPAMA and
50/50 typically occur late in the exponential phase, which indicates that the 50/50 culture
growth becomes nutrient limited at late time due to less yeast extract and bactopeptone.
TABLE 2
CHARACTERISTICS OF GROWTH
OBSERVED FOR B. VIRIDIS
IN RMPABA, 50/50, AND MINIMAL MEDIAS

<table>
<thead>
<tr>
<th>Characteristic of Growth</th>
<th>RMPABA</th>
<th>50/50 Mix</th>
<th>Minimal</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFUs</td>
<td>Maximum (CFU)</td>
<td>2.45x10^8</td>
<td>2.11x10^8</td>
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<tr>
<td></td>
<td>Growth Rate</td>
<td>0.8375</td>
<td>0.7294</td>
</tr>
<tr>
<td></td>
<td>(CFU/day)</td>
<td></td>
<td></td>
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<tr>
<td></td>
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<tr>
<td></td>
<td>(days)</td>
<td></td>
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<tr>
<td>A660</td>
<td>Maximum (a.u.)</td>
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<tr>
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<td>Value at Onset</td>
<td>Less than 1</td>
<td>Less than 1</td>
</tr>
<tr>
<td></td>
<td>of Stationary</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Recovered Value</td>
<td>Greater than 5</td>
<td>5</td>
</tr>
<tr>
<td>A1020/A660</td>
<td>Initial Value</td>
<td>1.1</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>Value at Onset</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>of Stationary</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Recovered Value</td>
<td>1.1</td>
<td>1.1</td>
</tr>
</tbody>
</table>
A practical approach to the reduction of growth observed in Minimal media

Growth in Minimal media, compared to RMPABA media, resulted in a 4/5 reduction in the number of live cells, and a significantly slower CFU derived growth rate. The final culture density, measured by A660, was also decreased by nearly ¾, with a corresponding reduction in absorbance derived growth rate. This reduction in growth rate is consistent with a nutrient/energetic limitation encountered by cells grown in Minimal media, which significantly impairs both cell replication and pigment production.

It is likely that there is a critical compound or compounds present in RMPABA, but absent in Minimal media. Based on the growth similarity of the 50/50 blend and the RMPABA media, it is likely that these compounds are present in at least 100% excess in RMPABA media. This compound is a carbon compound, a nitrogen compound, or a vitamin or growth factor, any of which could be readily available through the yeast extract and bactopeptone provided in the RMPABA media. Since yeast extract and bactopeptone are both undefined nutritional sources, direct determination of the missing factor is not possible. General carbon and nitrogen limitations can be tested by increasing the concentration of malate and ammonium sulfate in the Minimal media. The vitamin limitation can be tested by supplementing various vitamins to the Minimal media formulation. In general, one or more vitamins is required as a growth factor for members of the family Rhodospirillaceae; biotin, thiamine, niacin, p-ABA, pyridoxine, and B12 are the most common. The B vitamins, including Biotin, Pantothenate, Niacin, B-12, and Pyridoxine, are frequently supplied to cultures via yeast extract or bactopeptone, and all of these have been reported as useful to the growth of bacteria in the PNSB family.

Pucheau et al report that supplementation with p-ABA can significantly improve the formation of photosynthetic apparatus. Pantothenate, a precursor to
Coenzyme A, which plays a critical role in the Krebs Cycle, as well as a number of other bacterial metabolisms, is also recommended to enhance the growth of a number of organisms. This provides a good list of nutrients which might be missing when Yeast and Peptone are removed, and which can therefore be assayed to determine if vitamin supplementation is necessary.

Pigment production was also significantly hampered by the absence of yeast extract and bactopeptone. Minimal media had about half the rate of increase for pigment as RMPABA during the same exponential period, and achieved only 1/5 of the total pigment signature. In addition, no onset of pigment production, indicated by a slight increase in the ratio of pigment per cell some time after day 5, was observed after the onset of stationary for cells in Minimal media. This suggests that pigment formation is occurring at a much lower or entirely negligible rate. The A1020 data suggests that this rate is about half of the rate of pigment formation in the presence of yeast extract and bactopeptone. The nature of the A1020 measurement, however, based on optical density, allows for some additional observed signature due to the baseline scattering of light by newly formed cells and not new pigment formation. This means that the rate of pigment formation in Minimal media calculated by a spectrophotometric measurement could be slightly exaggerated. In the spectrophotometric ratio, A1020/A660, the ratio for Minimal media experiences an almost immediate drop, ending around 0.8, which never recovers significantly. This suggests that very little new pigment is being synthesized in Minimal media, and the culture was hindered from generating energy to drive metabolism and growth. By isolating the compound or compounds which lead to the reduction in growth, and supplementing them in purified form and measured concentration, the growth of the
cells in Minimal media can be enhanced without sacrificing the repeatability lost in the presence of the undefined yeast extract and bactopeptone.

3.3.2 Testing culture growth variability in Minimal media

To confirm that growth in Minimal media can be considered reliable from batch to batch, two batches of Minimal media cells grown at separate times were compared using the characteristics of growth defined earlier in this chapter.

3.3.2.1 CFU increase

In Minimal media, a similar inoculation of washed RMPABA cultured cells resulted in a nearly identical increase in the number of live cells for both cultures. (See Figure 8) The cells had 0.2564 CFU/day and 0.2692 CFU/day growth rates, respectively, during the exponential phase, between day 0 and about day 5. Both achieved stationary around day 10, at a live cell concentration of around 5.5x10^7 cells/mL. Almost no lag phase is evident, and there is no apparent death of the cells before about day 20 for either culture.
3.3.2.2 Increase in Absorbance at 660 nm

The absorbance at 660nm has similar characteristics. (See Figure 9) Both curves have similar exponential rates of growth, 0.2593 a.u./day and 0.2891 a.u./day respectively until about day 5, and experience deceleration leading to stationary around day 10. The second curve is consistently offset by approximately 0.4 absorbance units. This offset is most likely a result of the variation observed in RMPABA cultures, which were used for inoculation, or an instrumental error. This indicates that a control will be needed for every experiment to verify the intensity of the curve under the non-experimental conditions. Determining the multiplication factor needed to bring both controls to the same value can allow comparison of measurements in different experiments by
normalizing the data. Because the shift is constant throughout the curve, however, the growth rate would not be affected.

3.3.2.3 Culture pH

Cultures were inoculated in media adjusted to pH 6.9 and subsequently demonstrated almost identical pH behavior. (See Figure 10) Both cultures experienced a linear increase in pH of about 0.13 pH units/day until day 9, which coincides with the onset of the stationary phase, and then leveled off at pH 7.7. This characteristic pH of 7.7 pH units correlates with the onset of stationary for Minimal grown cells.
3.3.2.4 Increase in Absorbance at 1020 nm

The pigment behavior, as shown by A1020 mirrors the behavior of the two cultures at A660, except the shift is closer to 0.5 absorbance units for the pigment signature. (See Figure 11) Although the rates of pigment increase are still similar, they vary slightly more than the rates for A660. The first culture has a rate of pigment increase of 0.247/day and the second culture has a rate of pigment increase of only 0.172/day, approximately 2/3 of the first culture. This supports the possibility of a variation in initial culture pigmentation. Both cultures reach stationary at similar times, around day 7. The shift throughout the culture means that for cultures with similar behavior, the rate of pigment generation will be the same.
3.3.2.5 Ratio of pigment to cells

The behavior of the pigment ratios demonstrates that the two measurements of cell culture growth are well grouped and consistent in their pigment-cell behavior. The A1020/CFU count is very similar during the first 5 days of culture growth, with some divergence as the culture ages. (See Figure 12A) While the ratio calculated for the first set levels out around 2 on the given scale, the second batch continues to decrease. This indicates that the culture could be experiencing difficulties in producing pigment. Similar behavior is noted in the A1020/A660 behavior. (See Figure 12B) Although the cultures start with a similar ratio, which could be expected given their qualitatively similar but shifted curves for both measures, the second batch decreases more markedly to level at approximately 0.73, while the first batch exhibits a slightly higher pigment...
Figure 12: Pigment to Cell Ratios for Two Batches of Minimal Grown *B. viridis*. A) The ratio of CFUs to A1020 similar behavior at early time, but at later time, the second culture diverges and shows a lower pigment ratio. B) The ratio of A1020 to A660 begins at a similar value, however the second culture diverges lower between day 3 and day 15. At late time, the culture displays possible recovery behavior and the ratio increases to the 0.8 of the first culture.
ratio of 0.83 after leveling off. This supports the conclusion that data in the stationary portion of the curve should not be used.

3.3.2.6 Summary of Minimal media comparison results

Minimal media shows good repeatability from batch to batch. The number of live cells produced during two different sets of batch growth is nearly identical. Although they were started with concentrated inoculating broth prepared from different batches of RMPABA grown cells, the standardization method is adequate to produce consistent results. The pH behavior for both cultures was also similar, indicating comparable metabolic processes, and a maximum pH of 7.7 was the characteristic pH at which culture growth terminated for both cultures. The growth rate for both measurements and final concentration of cells determined by CFU count were tightly grouped for the respective batches, with no lag between the onset of cell replication and pigment production. This indicates that under these conditions A660 measurements and live cell counts can be expected to provide similar exponential growth rates from batch to batch. This bodes well for using Minimal media as a base media for further investigation, however there are still some areas of concern.

Despite similar live cell numbers, the spectroscopic data gathered shows a significant constant discrepancy between the two cultures. This indicates that the inoculating cultures, RMPABA grown cells, may have had some characteristic differences in culture density or pigment content which were observed throughout the Minimal growth period. If a control is prepared for each experiment, the A660 expected for normal conditions will be known, and the total growth can be compared within the experiment to indicate the relative effect of an applied condition. The control cultures
can also be compared to determine the factor by which they are related. This factor can be applied to the data gathered to make comparison of results between experiments possible and meaningful.

### 3.3.3 Validation of the Two Point Method

The Two Point method was developed to minimize the number of measurements required to characterize growth accurately. The two points measured to describe growth are at time zero, immediately following inoculation, and day 5. These are considered the best points because the bacteria show very little lag at the outset of growth, allowing initial time to be used to describe both the initial density of the culture and the beginning of the exponential growth phase, and day 5 describes both the end of the exponential phase the maximum growth achieved at stationary reasonably well. To check the validity of these selections, an exponential rate was calculated based on all data collected in the first five days of culture, and on the first and fifth day measurements (Figure 13). The upper line denotes the fit for the complete data, and resulted in a rate of 0.2285a.u./day. The lower line denotes the fit for only the two selected data points, a rate of 0.2306a.u./day. There is just less than 1% difference between these two rates, validating this two-point measurement strategy. The maximum density of this culture, calculated as the average of the values measured during the stationary growth phase, is 1.26 absorbance units, which diverges by 11.5% from the day 5 measurement of 1.13 absorbance units.

The two-point method gives a very good estimation of the growth rate, and it provides a reasonable estimation of the total growth. In addition, the two-point method
will be applied to compare results obtained within sets of data, which vary only by the defined varied condition. Even if there are inaccuracies due to deviations between the actual results and the two-point results, they will be consistent within the set. The control will also provide a means for standardization if two sets must be compared. The pigment behavior is analogous to the A660 behavior, and the same two-point method is applicable for calculating an exponential rate of pigment increase.

Figure 13: Minimal Growth Curve and Rate of Growth Two-Point Method. Minimal grown cultures experience exponential growth until approximately day 5, and at day 5, the culture is near its maximum expected growth. The growth rate calculated based on all exponential data, 0.2285/day differs by less than 1% from the growth rate calculated based on only initial and final data, 0.2306/day. Because the exponential rate is calculated using only two points, the $R^2$ value is 1 because it perfectly fits those two data points, and is therefore omitted.
3.4 Conclusions

Based on this data, Minimal media yields repeatable culture growth results, with minor or explainable discrepancies between each of the measured and calculated characteristic parameters introduced in this chapter. A fully characterized media composition would allow tracking of carbon sources, as well as comparison batch to batch. In this media, a correlation can also be determined between A660 data and CFU count data. The defined nature and repeatable results make Minimal media the media of choice for quantitative experiments when investigating the effects of applied conditions.

While RMPABA grown cells experience lush and rapid growth, Minimal media grown cells experience a significant reduction in growth. (See Table 3) Unlike RMPABA media, Minimal does not have a wide variety of carbon and nutrient sources to support a variety of metabolisms for survival and growth, and therefore offers only a limited number of metabolic options.38 The growth rate of cells in Minimal media is only one third to one half of that of RMPABA cells, and their exponential phase terminates earlier, resulting in less overall growth. They have only 16%-24% of the total growth of

### TABLE 3

**COMPARISON OF CHARACTERISTICS OF GROWTH OF *B. VIRIDIS* IN RMPABA AND MINIMAL MEDIA**

<table>
<thead>
<tr>
<th>Characteristic of Growth</th>
<th>RMPAMA</th>
<th>Minimal</th>
<th>Minimal % of RMPABA</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFUs Maximum (cells)</td>
<td>2.45X10^8</td>
<td>4.0X10^7</td>
<td>16.3%</td>
</tr>
<tr>
<td>Growth Rate (CFU/day)</td>
<td>0.8375</td>
<td>0.2564</td>
<td>30.6%</td>
</tr>
<tr>
<td>A660 Maximum (Abs)</td>
<td>5.4</td>
<td>1.3</td>
<td>24.1%</td>
</tr>
<tr>
<td>Growth Rate (a.u./day)</td>
<td>0.4176</td>
<td>0.2593</td>
<td>62.1%</td>
</tr>
<tr>
<td>A1020 Maximum (Abs)</td>
<td>5.7</td>
<td>1.1</td>
<td>19.3%</td>
</tr>
<tr>
<td>Increase Rate (a.u./day)</td>
<td>0.4751</td>
<td>0.2470</td>
<td>52.0%</td>
</tr>
</tbody>
</table>
RMPABA cells, and experience a significant reduction in pigment production, further impairing the cells.

Before Minimal media can be utilized for investigation of the inherent abilities of \emph{B. viridis} to break down environmental contaminants, it must be optimized to provide defined quantities of the nutrients and the growth enhancing conditions necessary for growth and pigment production. This optimization includes an investigation of the micronutrient, carbon and nitrogen requirements of \emph{B. viridis}, as well as the pH environment most conducive to their growth.

To facilitate the evaluation of many conditions quickly, the Two-point method, utilizing data from initial time and day five, was developed and tested. An exponential growth rate calculated with data gathered every 12-24 hours and a growth rate calculated using the Two Point method for the same data differ by less than one percent. The “total final growth” differed by about 10%. Error was determined for the Two-point method by calculating the rate of growth from the minimum initial data point to the maximum final data point and the maximum initial data point to the minimum final data point. This provides the greatest range in which the growth range can be expected and creates a boundary for the error of the averaged growth rate. The Two-point method and the corresponding error calculation provide a rapid and efficient way to survey a wide variety of conditions while obtaining reasonable and useful data.
3.5 References


2. Schuler, 154.


5. Blanch, 184.


8. Lang, 2827-2834.


15. Schuler, 156.
17. Bailey, 397.
18. Schuler, 156.


28. Ostafin, 53-68.


35. Campbell, 182-183.


CHAPTER 4

KNOWN GROWTH REQUIREMENTS OF B. VIRIDIS AND OTHER PNSB

4.1 Introduction

The long-term goal of work with B. viridis was to design a system for the treatment of difficult or environmentally problematic pollutants, with a focus on degradation of those compounds which are beyond the scope of current municipal waste capabilities or which are industrially generated and must therefore be dealt with onsite. In Chapter 2, methods for analysis and characterization of photosynthetic bacteria were discussed. In Chapter 3, the basic behavior of the bacteria in both rich and minimal medias was characterized in order to determine a baseline against which experimental work can be compared. Although significantly more growth was observed in the rich RMPABA media, the Minimal media, with a sole carbon source, was a superior choice for experimentation because growth could be correlated to specific nutrient availability and concentration, and limited to one set of metabolic pathways. This is critical to reliably characterizing the effect of added compounds of interest, because in RMPABA media the behavior of the bacteria can change for a variety of reasons unrelated to the experimental condition. The problem with using Minimal media is a severe limitation of growth, which appeared to be due to missing nutrients or inappropriate conditions. To remedy that problem while maintaining the defined character of Minimal media, optimizing the simple media to contain adequate growth factors, vitamins, or nutrients at specific and known concentrations was necessary.
To yield Minimal media, the recipe for RMPABA, inherited along with the bacteria,\(^1\) was modified by removing the yeast extract and bactopeptone. (See Appendix E for media formulations) When these heterogeneous compounds were removed, vitamins, growth factors, carbon and nitrogen sources were removed. The literature was surveyed to determine what is known about the growth requirements and conditions most supportive to the growth of not only \(B.\ viridis\), but also all varieties of Purple Non-Sulfur Bacteria related to \(B.\ viridis\). Using this information, an outline of the growth conditions most likely to support good growth was generated. These conditions included the effect of inoculation volume, density and handling, vitamin supplementation with pantothenate,\(^2,3\) biotin, folic acid, cysteine, niacin, pyridoxine, \(B_{12}, 4\), \(p\)-ABA and thiamine, carbon source and concentration,\(^5,6\) nitrogen assimilation using molecular nitrogen and ammonia, buffering, and pH environment. This focused list provided direction for optimizing the growth of \(B.\ viridis\) in the new media formulation. Good growth was defined as both prolific cell regeneration and ample formation of pigment.

4.2 Known Requirements for Growth

4.2.1 Inoculation volume, density and mechanical handling

The initial percent volume (and density) of the inoculation broth affects the resulting culture growth.\(^7\) An inoculation of 5%-10% by volume of stationary culture broth is common for many types of cells.\(^8\) Because the final culture density varies between cell types, the density of the inoculating broth is typically defined by the maximum density of the culture. For \(B.\ viridis\) in RMPABA, this maximum density is typically \(2.45 \times 10^8\) cells/mL or 5.4 a.u. Although adding more cells leads to more cell
doubling, other effects, such as quorum sensing\(^9\) and inhibition can influence culture growth when excessively high inoculation concentrations are used.

Mechanical processes involved in the preparation of inoculation broth, such as centrifugation and washing of cells prior to seeding, are used to remove artifacts of previous growth conditions, such as residual nutrients carried by the inoculating medium and byproducts of growth which act as growth inhibitors as they accumulate. These contaminants could lead to inhibition or poorly defined culture growth. On the other hand, excessive centrifugation or washing could irreversibly damage the cells and reduce the observed growth. Cells are generally washed by centrifugation at around 3000 RPM, the media is decanted, and fresh media is added. The cells are then agitated, either by hand or by placing briefly on a vortex, to resuspend. This procedure is repeated several times and concluded by resuspending the cells in fresh media to some fraction of their original volume. During centrifugation and vortexing, the cells can be mechanically damaged by shear, pressure or vibration, which can disrupt their cell membranes and cause them to rupture. \(B.\ viridis\) and other PNSB, which are gram-negative, are particularly susceptible to mechanical damage because they contain only a small amount of peptidoglycan, which forms the cell wall. Peptidoglycan forms chains, which confer structural stability.\(^{10}\) To avoid this potential damage, centrifuge speeds should be kept low, and vortexing or agitation should be as gentle as possible. During washing, solutions which do not have the correct osmolarity can cause the cells to either burst or shrivel as equilibrium is sought between internal and external solute concentration. To avoid this, it is important to use media or a buffer of similar osmolarity for washing.
Based on these factors, it is important to evaluate the protocols being used to insure that they are not causing damage or impairing the cells.

4.2.2 Vitamins

Normally in rich media, vitamins are provided by yeast extract\textsuperscript{11} and bactopeptone, commonly employed nutritional supplements in photosynthetic bacterial cultures,\textsuperscript{12,13} which also provide a variety of amino acids, carbohydrates, lipids, minerals, vitamins, growth factors, and other trace chemicals necessary for cell reproduction and metabolism.\textsuperscript{14,15,16} Yeast extract, in particular, is an excellent source of B-vitamins, which are crucial to metabolism.\textsuperscript{17} To compensate a defined medium for loss of nutritional complexity, vitamins can be supplemented to the culture.\textsuperscript{18,19,20} Vitamins are direct precursors for coenzymes, which are critical in regulating enzyme activity in cells. They are particularly important in organisms lacking a de novo synthesis pathway.\textsuperscript{21}

Essential vitamins required for \textit{Rhodospirillaceae} and other PNSB vary from species to species, and also from strain to strain.\textsuperscript{22} The first isolated strain of \textit{B. viridis} required \textit{p}-ABA, biotin, and vitamin B.\textsuperscript{23} For the strain of \textit{B. viridis} used in this work, the growth factors thiamine and \textit{para}-Aminobenzoic acid (\textit{p}-ABA) are needed in the media formulation.\textsuperscript{24,25} (See Appendix E: Media) Vitamins commonly required as a growth factor for members of the family Rhodospirillaceae also include niacin, pyridoxine, B\textsubscript{12}, and pantothenate.\textsuperscript{26,27} In addition, folic acid and cysteine are known to play crucial roles in regulating synthesis and metabolism. These vitamins play a variety of critical roles, summarized in Table 1.\textsuperscript{28,29,30,31,32,33}
4.2.3 Carbon metabolisms

Carbon is the fundamental compound required for all life. This means that in order to live and reproduce, *B. viridis* must obtain carbon from some source and be able to process it metabolically. The possibilities for PNSB are extremely diverse, since this group of bacteria is known to grow successfully using a variety of metabolisms. The most preferred metabolism for PNSB is photoheterotrophic, or light as an energy source and an organic carbon as an electron donor. These bacteria can also grow photoautotrophically, lithotrophically, or heterotrophically. The two primary enzyme pathways utilized for growth are the reductive pentose phosphate pathway and the citric
acid (TCA) cycle, either forward or in reverse. In the case of photosynthesis, where the cells utilize light to generate and store energy in a separate step, the reductive pentose phosphate pathway or the reverse TCA cycle are most likely. More information about photosynthesis can be found in Appendix D. Where sugars are being used, glycolysis reduces the sugar to pyruvate, which can be used either in the TCA cycle (aerobic) or in some fermentative pathway (anaerobic). (See Figure 5) In this section, the pentose phosphate pathway and the TCA cycle are discussed. In the next section, specific carbon compounds of interest are covered.

Figure 1: Glycolysis Converts Glucose to Pyruvate in a Series of Enzymatic Steps. Pyruvate can either be converted to acetate and used in the TCA cycle or utilized in a fermentative metabolism. (http://www.sirinet.net/~jgjohnso/cellularresp.html)
4.2.3.1 Reductive pentose phosphate pathway

The reductive pentose phosphate pathway, or Calvin Cycle, is the pathway through which CO$_2$ is autotrophically incorporated into cell mass.$^{34}$ (See Figure 6) This series of reactions is also known as the “dark reactions” of photosynthesis. For this pathway, the primary carbon incorporated would be carbon dioxide. For $B. viridis$, which prefers photoheterotrophic growth, any organic carbon utilized would participate primarily as an electron donor in the “light reactions,” or the reactions directly utilizing light energy, of photosynthesis.

![Calvin Cycle Diagram](http://www.bact.wisc.edu/Bact303/bact303metabolism)

Figure 2: The Calvin Cycle. The reductive pentose phosphate pathway allows the autotrophic incorporation of CO$_2$ and the generation of precursors to cellular components. (http://www.bact.wisc.edu/Bact303/bact303metabolism)
In this pathway, the NADPH and ATP generated by the light reactions of photosynthesis are utilized to drive incorporation of CO₂ and generation of cellular components. The cycle originates with ribulose bisphosphate, and relies on the enzyme ribulose bisphosphate carboxylase to cleave the molecule and incorporate carbon dioxide, giving 2 molecules of 3-phosphoglycerate. In order for this cycle to proceed and for cellular components to be generated, CO₂ must be available to the cycle. Without CO₂, the cells can harvest endless energy from light, but no new cellular components will be produced.

4.2.3.2 The citric acid cycle

The citric acid cycle, also known as the tricarboxylic acid cycle (TCA) or Krebs cycle, is the hub of the metabolic process in a wide variety of both eukaryotic and prokaryotic organisms.³⁵ (See Figure 7) This cycle can operate either catabolically, degrading complex carbons, or anabolically, building cellular precursors and storing energy. If the cells are utilizing complex organic carbons such as glucose or other sugars, the pyruvate generated via glycolysis is converted to acetyl- Co-A, which enters into the cycle by conversion into citrate by citrate synthase. It stepwise converts acetyl Co-A to CO₂ in a series of 8 steps resulting in 2 molecules of CO₂, 3 molecules of NADH, 1 molecule of FADH₂, 1 molecule of GTP, and a free CoA. The TCA cycle is also important because the generation of NADH and FADH₂ are critical in driving the synthesis of ATP, or chemical energy.³⁶ The TCA cycle in PSB provides biosynthetic intermediates and also functions as a source of reducing power.³⁷,³⁸ The cycle generates oxaloacetate in the last step, which is reacted with a new acetyl Co-A, renewing the cycle. While the cycle has end products and reactants, it is more important for its role in
Figure 3: The TCA and reverse TCA cycles. The reactions of the citric acid cycle begin with Acetyl-CoA being converted to citrate. Acetate is converted by acetate thiokinase acetyl-CoA. Fumarate and Malate are located toward the end of the cycle. Fumarase converts malate to fumarate. Malate is converted to oxaloacetate by malate dehydrogenase. In the reverse cycle, CO₂ is incorporated instead of released, and the conversions flow in the opposite direction. (http://www.bact.wisc.edu/Bact303/bact303metabolism)
generating a variety of precursors, and compounds may be utilized directly from or entered directly into the cycle at any point. When the pathway is operating anabolically, as is true for some types of photysynthetic bacteria, especially green photosynthetic bacteria,\textsuperscript{39} carbon dioxide is assimilated and energy is utilized. This energy is generated by the earlier reactions of the photosynthetic machinery. As with the catabolic pathway, intermediates can be removed at any point in order to support cellular regeneration.

4.2.4 Carbon forms of interest

There are several types of carbon which should be investigated. The first type is inorganic carbon, or CO\textsubscript{2}. In addition, sugars must be investigated as part of glycolysis and the catabolic TCA cycle. In this group, sucrose and glucose are good representatives. Finally, carbons which participate in the TCA cycle directly, either catabolically or anabolically should be investigated. In this group, malate, fumarate, acetate, and citrate are considered.

4.2.4.1 Carbon dioxide

To grow phototrophically, PNSB must be supplied with an exogenous electron donor and an electron acceptor. The detailed mechanism for phototrophic growth is given in Appendix D: Photosynthesis. \textit{B. viridis} typically prefers organic carbon as the electron donor and can also assimilate it as the carbon source. It can use a variety of compounds as the electron acceptor; carbon dioxide is one of the more commonly used. It can also assimilate carbon dioxide for production of biomass either via the reductive pentose phosphate pathway or the reverse TCA cycle. The flow of carbon is linked to the redox potential of the electron donor, and the more highly reduced the electron donor, the
more preferable it is for carbon dioxide to be assimilated for growth.\textsuperscript{40} This is because a more highly reduced electron donor can generate more energy during the “light reactions” of photosynthesis, and therefore provide more ATP, NADHP, and other energy storage molecules used to drive the CO\textsubscript{2} assimilation.

In nature, \textit{B. viridis} obtains CO\textsubscript{2} from the atmosphere. Because carbon dioxide is only 3.3 x 10\textsuperscript{-2} % of the atmosphere,\textsuperscript{41} under typical conditions the amount of carbon dioxide dissolved in atmosphere equilibrated water (or media) at 30°C is 0.1257 g/100 g. At 100% saturation, the concentration is around 30.2 mmol, but the actual saturation is often less. These levels may not be sufficient to sustain growth, so for this reason the organism has evolved to use organic carbon sources as electron acceptors as well as to grow under alternative non-photosynthetic metabolisms when necessary.\textsuperscript{42} In the laboratory when testing for CO\textsubscript{2} utilization, exogenous carbon dioxide can be added by supplementing the media with bicarbonate, which has been shown to assist where CO\textsubscript{2} is required for growth.\textsuperscript{43} If a greater supply of CO\textsubscript{2} leads to greater growth, then it suggests that the bacteria are growing using either the Calvin or reverse Krebs cycle via a photosynthetic pathway. If a greater supply of CO\textsubscript{2} has no effect, it indicates either that the bacteria are unable to fix CO\textsubscript{2} using either of these pathways, or that the bacteria are blocked from generating energy during the “light reactions” by lack of an acceptable exogenous electron donor or by damage to the photosynthetic apparatus and pigments.

4.2.4.2 Sugar metabolism: Glucose and sucrose

Most PNSB are able to grow on one or more common sugars, largely bypassing photosynthesis. The inability to use fructose is a common characteristic of many bacteria in this group, but most are able to use glucose.\textsuperscript{44} Glucose, a cyclic 6-carbon structure,
can undergo conversion to pyruvate in glycolysis and subsequent aerobic oxidation, funnelling into the TCA cycle, or anaerobic fermentation, bypassing the cycle completely.\textsuperscript{45} Sucrose, a more complex sugar, is a glucose and a fructose molecule joined by a glycosidic bond. Sucrose must be cleaved to glucose and fructose by hydrolysis of the glycosidic bond before it can be used in metabolism.\textsuperscript{46} This growth on sugars is observed under both aerobic chemotrophic conditions and phototrophic conditions,\textsuperscript{47} so while photosynthesis may occur and provide a slight amount of energy and carbon fixation, it is not necessary to this metabolism.

4.2.4.3 Carbons of the citric acid cycle: Malate, fumarate, acetate, citrate

Malate is universally utilized by nonsulfur purple bacteria, the only compound for which this is true,\textsuperscript{48} and it is commonly employed in their growth media.\textsuperscript{49} Heavy growth was observed on malate for the very first isolated strain,\textsuperscript{50} and it is preferentially used by a number of PNSB in the presence of several types of carbon.\textsuperscript{51} Malate occurs near the end of the catabolic TCA cycle so supplementing this compound could act as a metabolic boost. If TCA enzymes are missing from the genome of \textit{B. viridis} or inhibited by the presence of toxic compounds in remediation applications, then the presence of malate could act as a shunt, bypassing the requirement for these enzymes while allowing the production of biosynthesis intermediates. In the anabolic TCA cycle, malate occurs near the beginning of the pathway, so the presence of malate would help drive CO\textsubscript{2} assimilation and simultaneously allow for the production of biosynthetic components.

Photosynthetic bacteria have also been known to utilize malate through alternate pathways.\textsuperscript{52,53,54,55} Some species are known to catabolize malate to glyoxylate and acetyl-CoA by a reversal of the glyoxylate cycle.\textsuperscript{56} Malate can also act as an electron
donor in the photosynthesis light reactions, functioning to generate energy for the assimilation of CO₂, and later be salvaged as additional carbon. Evaluating the necessary concentration of this highly relevant compound is crucial to obtaining the optimum growth.

Fumarate is one enzymatic step away from malate; fumarase catalyzes the reaction from fumarate to malate by condensing a water molecule onto the fumarate molecule. Given their chemical similarity and closeness, this compound can act as malate, boosting the metabolic flow of the catabolic TCA cycle, or helping to initiate the anabolic cycle and drive CO₂ assimilation. It would also have similar chemical properties if utilized as an electron donor for photosynthesis. In addition, because there is only one enzymatic step separating these two compounds, fumarate can easily be converted to malate to participate in any alternative pathway.

Acetate is a precursor to the citric acid cycle; acetate thiokinase adds Coenzyme A to acetate to yield acetyl-CoA, which is the compound that funnels into the cycle. Acetate metabolism has been noted in purple non-sulfur bacteria, and is thought to result from the presence one or more of the citric acid cycle enzymes driving the acetyl-CoA through the cycle. Citrate is the first step in the cycle, and it is created when citrate synthase catalyzes the condensation of acetyl-CoA and oxaloacetate, the last compound in the cycle. For citrate to be assimilated and the cycle to be driven, the greatest number of TCA enzymes must be present. The ability to utilize this compound demonstrates that the complete catabolic pathway is present in an organism. There are a number of photosynthetic bacteria that are unable to utilize citrate, making a bypass with a compound from another part of the cycle particularly important. If citrate is utilized,
then the TCA cycle is likely to be complete and being used catabolically to generate energy. The inability to use citrate suggests that the bacteria are either using the TCA cycle in pieces to manufacture intermediates or not using the citrate cycle at all.

4.2.5 Nitrogen source and nitrogen fixation

With the removal of Yeast extract and Bactopeptone, a variety of rich organic compounds, some containing nitrogen, are no longer available for the growth of *B. viridis*. After carbon, nitrogen is one of the most important compounds for cell synthesis. In photosynthetic bacteria, there are several ways in which nitrogen might be obtained: by assimilating organic nitrogen, by utilizing ammonia, or by fixing molecular nitrogen from the atmosphere. Photosynthetic bacteria are known to fix molecular nitrogen to ammonia, using the enzyme nitrogenase. Photosynthetic bacteria are among the few types of bacteria containing the enzyme nitrogenase.64,65,66

By supplementing ammonia directly, nitrogenase can be repressed and the enzyme pathway blocked. This can be advantageous or problematic. If bacteria are employing the nitrogenase pathway, then they must devote energy to this energetically expensive process (20-30 mols of ATPs per mol NH₃), diverting it from other processes.67 In the case where the bacteria are being utilized for a specific reaction or degradation, supplementing ammonia directly will free up this energy to be used in the desired reaction. Blocking the pathway can become problematic because in some cases, this repression appears to be permanent.68 If the strain in use has had its nitrogenase permanently shut off, then adequate ammonia supplementation is critical to insure that the bacteria have a supply of readily incorporated ammonia, and without this
supplementation the bacteria will not grow. Particularly in *B. viridis*, a recovery of the enzyme is not observed following supplementation with ammonia.69

Nitrogenase has been a very popular enzyme for study in the last 20 years,70,71,72 on topics ranging from structure,73,74,75 to genetics,76 to regulation,77,78 to its universal presence or absence in particular families of bacteria.79 Nitrogenase is actually composed of two loosely associated proteins, an Fe-protein dimer and a MoFe-protein tetramer.80

(See Figure 8) Electrons flow through these two proteins together to generate adequate energy to break the N≡N high 947 kJ/mol bond energy of molecular nitrogen. The 64 kD Fe protein dimer contains two 289 residue subunits as well as a [4 Fe-4S] cluster and two ATP binding sites. (See Figure 4A) The 220 kD Mo-Fe protein, a \( \alpha_2\beta_2 \) tetramer of two loosely associated \( \alpha\beta \) dimers, contains two 491 residue \( \alpha \) subunits and two 522 residue \( \beta \) subunits, as well as a P-cluster pair and a Fe-Mo cofactor. (See Figure 4B)

The P-cluster pair contains two linked [4Fe-4S] clusters bridged by two Cys thiol ligands and a disulfide bond, (See Figure 4C) and the FeMo-cofactor contains a [4Fe-3S] cluster and a [1Mo-3Fe-3S] cluster bridged by three non-protein ligands, one of which is thought to be the binding location. (See Figure 4D) The energy to drive nitrogen fixation is generated either by photosynthesis or by oxidative electron transport from chemoheterotrophic activities. (See Figure 9) These electrons are then transferred to ferridoxin, which transfers the energy to the Fe-protein.81 Two ATPs are then used to drive the reaction transferring an electron to the MoFe Protein. This electron transfer must occur eight times, utilizing a total of 16 ATPs to drive a complete enzyme cycle and
Figure 4: The X-ray Crystallography Structure of the Fe-protein and FeMo-protein of Nitrogenase. The two loosely associated Nitrogenase proteins, an Fe-protein dimer (A) and a MoFe-protein tetramer (B). (A) The 64 kD Fe-protein dimer contains two 289 residue subunits, a [4 Fe-4S] cluster, and two ATP binding sites. (B) The 220 kD Mo-Fe protein, an $\alpha_2\beta_2$ tetramer, contains two 491 residue $\alpha$ subunits and two 522 residue $\beta$ subunits, as well as a P-cluster pair (C) and a Fe-Mo cofactor (D). (C) The P-cluster pair contains two linked [4Fe-4S] clusters bridged by two Cys thiol ligands and a disulfide bond. (D) The FeMo-cofactor contains a [4Fe-3S] cluster and a [1Mo-3Fe-3S] cluster bridged by three non-protein ligands, one of which is thought to be the binding location, denoted by N-N in the center. Voet and Voet, 1995.
convert N₂ to NH₃. The breaking of dinitrogen and subsequent assembly of the ammonia molecule occurs on the MoFe-protein in three distinct chemical steps, each breaking one of the three initial bonds. (See Figure 10) In addition to creating 2 molecules of NH₃, one molecule of H₂ is created with each complete MoFe-protein cycle. The overall reaction is

\[
N₂ + 8H^+ + 16 ATP + 16H₂O \rightarrow 2 NH₃ + H₂ + 16 ADP + 16 P_i.
\]

The inherent generation of H₂ by this reaction has become a topic of some interest as an alternate method of fuel generation in recent years.  

\[
\begin{align*}
2H^+ + 2e^- &\quad \rightarrow H\quad N-N = N-H \\
N≡N &\quad \rightarrow H-N=N-H \\
2H^+ + 2e^- &\quad \rightarrow N-N \\
&\quad \rightarrow 2NH₃
\end{align*}
\]

Figure 6: The Reduction of N₂ to NH₃ on the MoFe-protein during Nitrogenase Mediated Nitrogen Fixation.

\[
\begin{align*}
2H^+ + 2e^- &\quad \rightarrow H\quad N-N = N-H \\
N≡N &\quad \rightarrow H-N=N-H \\
2H^+ + 2e^- &\quad \rightarrow N-N \\
&\quad \rightarrow 2NH₃
\end{align*}
\]

Figure 5: The Flow of Electrons through Ferridoxin, Fe-protein, and MoFe-protein during Nitrogenase Mediated Nitrogen Fixation. Adapted from Rees, 1993.
Although the stoichiometric requirement is for 16 ATPs, under typical physiological conditions 20-30 ATPs per cycle, or per NH₃ fixed, are required due to side processes.⁸⁴ Because of this extremely high energy cost, most nitrogen fixers have elaborate mechanisms to shut off nitrogen fixation in an environment with a surplus of alternate nitrogen sources, such as NH₃.⁸⁵ In *Rhodospirillum rubrum*, complete repression of nitrogenase activity was observed with a 10 mM addition of NH₄⁺ to the culture medium.⁸⁶ In another study, two forms of nitrogenase were identified in *R. rubrum*, one constitutively active and one subject to regulation by an activating system. The forms seemed to be interchangeable without *de novo* synthesis, indicating that there was some other mechanism for conversion between the two forms. It was determined that the form of nitrogenase present was dependent on the prior culture conditions. The constitutively active form was present in cultures where no nitrogen source of any kind had been offered; the repressible form was found in cultures that had been provided with any type of nitrogen, molecular or organic nitrogen.⁸⁷ In *B. viridis*, growth with nitrogenase is lush, however ammonia-induced switch off activity has also been noted, and the cells experience a rapid halt in nitrogenase activity when supplemented with ammonia. Some *Rhodospirillaceae* experience a rapid recovery of nitrogenase activity when the ammonia levels drop below a certain limit, however this is not noted for all strains of *B. viridis*. Irreversible nitrogenase shut off has been noted for several strains of *B. viridis*, and it is therefore possible that extended culturing with ammonia,⁸⁸,⁸⁹ as is present in RMPABA media, can significantly reduce the nitrogen fixing capabilities of the culture. In addition, the high energy requirement of nitrogen fixation can divert energy from other processes and reduce the total growth rate observed.⁹⁰ In order to
determine the capabilities and optimum conditions for growth, the culture should be
tested both with and without the presence of ammonia to determine if nitrogenase is
present and active, and to determine if supplementation with ammonium allows
rechanneling of energy to increase growth.

4.2.6 Headspace gas and O₂

Photosynthetic bacteria are especially noted for their lack of oxygen tolerance.⁹¹,⁹² In addition to nitrogenase repression by the presence of ammonia, nitrogenase is also
quite sensitive to oxygen, commonly known as “oxygen stress.” The presence of oxygen
has been shown to control nitrogen fixation at the transcriptional level, in the
accumulation of the nitrogenase proteins, and by the cellular fixation activity.⁹³ In fact,
the oxygen repression of nitrogenase has been shown to be so strong that under oxygenic
conditions, ammonia based inhibition of the enzyme is not observed. The nitrogenase
complex is easily destroyed in the presence of oxygen, so many aerobic organisms have
developed protective mechanisms. Since anoxygenic bacteria typically inhabit oxygen
free environments, these mechanisms are not necessarily present in PNSB.⁹⁴ It is
important to look at the effect of oxygen free vs. oxygenic environment to understand the
capabilities of *B. viridis*.

Nitrogenase is not the only oxygen sensitive feature of the photosynthetic
bacterium. In addition to the effect of an oxygenic environment on nitrogen fixation, its
effect on the photosynthetic pigments can be significant. Although a number of
photosynthetic bacteria can grow in aerobic or microaerobic conditions in the dark, they
are typically unable to grow in the light in the presence of oxygen.⁹⁵ In photosynthetic
literature, this assumption is so ubiquitous that “phototrophic” conditions are defined as light present and oxygen absent, and “aerobic” conditions are defined as oxygen present and in darkness.\textsuperscript{96,97,98} The inability of these bacteria to grow in oxygenic conditions occurs because oxygen inhibits bacteriochlorophyll synthesis.\textsuperscript{99} In one strain of \textit{B. viridis}, growth in oxygen and light was not observed, and thylakoids, a photosynthetic structure, were not formed.\textsuperscript{100} Under “aerobic” conditions, another strain of \textit{B. viridis} produced no bacteriochlorophyll and very little carotenoid pigments, and the characteristic membrane structure typically observed was absent.\textsuperscript{101} In a third strain of \textit{B. viridis}, it was noted that the thylakoids became increasingly unstructured as the partial pressure of oxygen was increased. Since well ordered thylakoids are necessary to the energetics of photosynthesis, this decrease in order lead to a decrease in growth rate.\textsuperscript{102}

In order to verify the behavior of our strain under oxygenic conditions, it is important to consider both the growth as well as the pigment formation in the presence of oxygen.

4.2.7 The pH & osmolarity

The concentration of buffer and the osmolarity of the environment can have a significant effect on the ability of cells to grow and to perform their metabolic processes.\textsuperscript{103} It has also been noted that the pH optimum for growth on some substrates differs markedly from the pH optimum for growth on others in a number of \textit{Rhodospirillaceae}.\textsuperscript{104} Attention to the pH and osmolarity is especially important for photosynthetic bacteria because photosynthesis, in bacteria or in plant cells, works on the principle of creating a $\Delta$\textit{pH} across the thylakoid membrane.\textsuperscript{105,106} (See below and Appendix D: Photosynthesis for details) Light energy is used to generate this gradient,
and the gradient allows the light energy to be converted into chemical energy, or ATP. In this process, known as a “proton pump,” electrons are passed through the various photosynthetic pigments of the reaction center creating a charge imbalance and chemical potential that energizes the membrane-bound ATP-ase to pump protons out of the thylakoid.\(^{107}\) (See Figure 11) In chloroplast photosynthesis, this $\Delta pH$ is around 3.5 pH units; the internal pH falls to around 5 and the external pH rises to around 8.5, very close to the observed maximum pH for growth of \textit{B. viridis} under rich conditions.\(^{108}\) For chloroplasts, maintaining an internal pH in the range of 5.-5.5 is of critical importance for maximum electron flow and chemical energy generation.\(^{109}\) Photosynthetic bacteria have to establish a similar gradient across their thylakoid membranes in order to drive photosynthesis.

It is possible that when the media pH reaches 8.0, the proton pump can no longer function efficiently and further chemical energy cannot be generated, halting growth. At high pH it is more difficult to pump protons out of the thylakoid. Lowering the external...
pH would increase proton diffusion into the thylakoid and restore the ability of the cells to generate a gradient.\textsuperscript{110} The extracellular pH also affects the activity of other enzymes, and therefore the ability of bacteria to grow and flourish. Most enzymes typically have optimal range somewhere between pH 3 and pH 8. Many organisms attempt to regulate their own intracellular pH, however this is imperfect and requires energy that cannot be dedicated to growth.\textsuperscript{111} In the bacterium \textit{Sacrina ventriculi}, for example, the pH of the environment regulates the carbon and electron flow during fermentation.\textsuperscript{112} In the denitrifier \textit{Paracoccus denitrificans}, the pH of the environment determines which enzyme pathway is utilized in converting nitrate and nitrite to molecular nitrogen.\textsuperscript{113} In the photosynthetic bacterium \textit{Rhodobacter capsulatus}, the uptake of malate and other TCA cycle C4 compounds via transport proteins showed a marked increase at pH values greater than 7.\textsuperscript{114} This suggests that a shift in pH will significantly alter the metabolic functions of the bacteria. Since CO\textsubscript{2} usage by photosynthesis may require a lower media pH in order to allow the formation of a $\Delta$pH, this increase in transport at high pH may provide a mechanism to encourage use of the catabolic TCA cycle when CO\textsubscript{2} assimilation and photosynthesis can not take place. Conversely, if catabolic growth on complex carbon is preferred, a lowered pH may be necessary to shut off transport of TCA cycle compounds and encourage anabolic growth, or use of the reductive pentose phosphate pathway. Because pH can have such a significant effect on the growth and metabolic pathway behavior of \textit{B. viridis}, it is critical to understand the pH response and the pH optimum for growth in a less complex media.
4.2.8 Light intensity and vessel size

When the bacteria are growing photosynthetically, light is an integral part of the reaction. Because the bacterial suspension generally becomes dense, especially in later exponential growth, the penetration of light can be significantly reduced. Due to the pigment concentration of PSB, the light energy is expected to decrease quasi-exponentially as the penetration depth increases. In a dense culture, at a depth of 1.5 cm, only 7% of the incident light will penetrate, and growth and activity are diminished in the deeper parts of the culture.\(^{115}\) The reduction of light, however, is not necessarily an indication that the culture will perform poorly. Nakada et al. discovered that the deeper portions of the culture, receiving only tiny amounts of light energy, converted light to energy much more efficiently.\(^{116}\)

The light intensity the culture observes can have a significant impact on the growth and the response of the culture, but it is not just low light that alters the growth of a culture. *Rhodopseudomonas palustris* cells subtly modify their light harvesting complexes in response to low or high light conditions.\(^{117}\) The antenna complexes also provide a buffer to moderately high light intensities, preventing bleaching of the reaction center pigments. This leads to an observed decrease in the pigment per cell for *Rhodobacter capsulatus*.\(^{118}\) Under extremely high light intensities, the growth of new pigment is inhibited, and therefore the pigment per cell decreases.\(^{119}\) Knowledge of the optimum illumination and vessel depth is particularly relevant in the case of larger reaction vessels or scale up, because scale up can result in a greater depth of culture broth, reducing the available light. Though there is additional pigment formation in low light, this additional pigment does not compensate to bring the growth rate up to the level of more brightly lit cultures. The light must be adequate to encourage

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\(^{115}\) Nakada et al., 1983.
\(^{116}\) Nakada et al., 1984.
\(^{117}\) Nakada et al., 1985.
\(^{118}\) Nakada et al., 1986.
\(^{119}\) Nakada et al., 1987.
photosynthetic use of light energy for growth and other metabolic processes, but not too bright, causing the cells to inhibit their pigment formation or experience photobleaching. Since the pigment concentration decreases in high light, it is imperative to avoid over illumination. It is, then, a matter of discovering the proper intermediate light intensity.

4.3 Summary

There are a wide variety of factors involved in achieving good growth in a photosynthetic bacteria, especially in a simple, defined medium such as Minimal media. In order to insure that the culture is started well, the mechanical handling and processing techniques must be tested to confirm that they do not destroy the cells. In addition, the cells must be provided with all the necessary growth requirements. This includes

- vitamins and growth factors
- carbon in a metabolically useful form
- nitrogen, either as molecular nitrogen or as fixed nitrogen
- an appropriate headspace gas
- the correct pH and osmolarity
- a moderate level of light

The most important compound to building new cells and growth is carbon, and a variety of carbons provide potential growth materials. In addition, the carbon source employed gives a great deal of information about what metabolic pathway they are using for growth. Carbon dioxide, sucrose, glucose, malate, fumarate, citrate, and acetate will be assayed to determine which provides the greatest level of growth and to help in understanding what metabolic pathway is being employed. The nitrogen requirements
inform the state of the nitrogenase enzyme present in the bacteria. In the absence of nitrogenase, fixed nitrogen must be provided. In addition, fixed nitrogen may allow for significantly greater growth, an asset under certain culture conditions. The correct pH is essential for growth, because it is known to influence both the uptake and metabolism of carbons as well as the ability of photosynthetic organisms to use light to generate a chemical potential and harvest energy. These aspects are studied in Chapter 5 and Chapter 6.
4.4 References


2. Grula, 968-974.

3. Toennies, 707-713.


10. Tortora, 87-88.


20. Toennies, 707-713.


24. Ostafin, 53-68.


30. Scott, 1223-1376.


34. Voet, 649-659.

35. Voet, 538-562.
36. Voet, 540.


41. Ray, 433.

42. Tabita, 885-914.

43. Wright, 2069-2073.

44. Madigan, “Microbiology, physiology, and ecology of phototrophic bacteria,” 39-111.

45. Voet, 444.

46. Voet, 260.

47. Tabita, 885-914.


49. Shaw, 466-472.

50. Drews, 255-262.

51. Sojka, 707-718.

52. Voet, 538-562.

53. Shaw, 466-472.

54. Sojka, 707-718.
55. Truper, 299-312.


57. Voet, 554.

58. Voet, 557.


61. Tabita, 885-914.

62. Voet, 538-540, 549.

63. Sojka, 707-718.


68. Truper, 1672-1677.

69. Truper, 1672-1677.

70. Burris, 9339-9342.


80. Voet, 776-781.

81. Rees, 921-928.

82. Rees, 921-928.

83. Kim, 389-397.

84. Burris, 9339-9342.


87. Carithers, 779-789.


90. Yoch, 657-676.


93. Oelze, 219-225.


95. Keister, 849-856.


98. Lang, 2827-2834.

99. Cellarius, 234-244

100. Lang, 2827-2834.

103. Shuler, 163.
104. Sojka, 707-718.
106. Sojka, 707-718.
107. Voet, 634-638.
111. Shuler, 162-163.
114. Shaw, 466-472.
117. Gall, 5185-5190.

CHAPTER 5

OPTIMIZING THE GROWTH OF B. VIRIDIS IN MINIMAL MEDIA AT PH 6.9
AND EXPLORATION OF THE PH OPTIMUM FOR THIS BACTERIUM

5.1 Introduction

In order to quantify the ability of bacteria to grow in the presence of a target compound or to degrade that target compound, it is necessary to establish growth conditions that provide the best combination of defined nutrients and growth factors necessary for vigorous growth. Chapter 3 established that Minimal media is a good defined media for quantification and repeatability of culture growth measurements because it limits the bacteria to a single carbon source, resulting in a more repeatable measurement from run to run and a predictable growth curve. Because photosynthetic bacteria are facultative, or have a variety of metabolisms, these measurements, in an undefined rich media such as RMPABA, were less repeatable because of the potential for small variations in metabolic pathways from batch to batch. However, cells grown in Minimal media experienced a ~1/3 to 1/2 reduction in growth rate, achieved only 20% of the total growth yield, and suffered around 80% reduction in pigment production compared with identically-grown cells in RMPABA media. To determine the conditions for which the growth potential of the culture in Minimal media could be optimized, the effects of inoculation volume, density and handling, vitamin supplementation with pantothenate, biotin, folic acid, cysteine, niacin, pyridoxine, and B12, p-ABA and
thiamine, carbon source and concentration, molecular nitrogen and ammonia, buffering and pH environment were examined.  

5.2 Materials and Methods

5.2.1 Materials

The Minimal media was prepared as described in Chapter 2.4.1, following the recipe outlined in Appendix E, and filter sterilized. All solutions were prepared in 18 mOhm Epure water. Sodium malate and ammonium sulfate, the carbon and nitrogen sources found in the original Minimal media formulation, were added as the stock solutions described in Appendix E to achieve the desired concentration. The vitamins were obtained from Sigma-Aldrich and prepared as stock solutions of 0.5 mM concentration. The sugars, sucrose and glucose, were obtained from Sigma-Aldrich and added as solids. The sodium bicarbonate was obtained from Fisher and was added as a solid. The sodium fumarate was added as a stock solution of 861.5 mM concentration, generated by adding Fumaric acid from Aldrich and pH adjusting with sodium hydroxide obtained from Fisher. The gases nitrogen, air, and argon were bubbled through the media for a minimum of 30 minutes to achieve saturation. Nitrogen was generated with a Nitrox UHP Nitrogen Generator (UHPN1501, Domnick Hunter), and air was obtained from the house air line. Pure argon (UN1006) was obtained from Mittler. These were passed through filters and used for sparging experiments. Potassium phosphate buffer was prepared according to the recipe noted in Appendix E and added as described.
5.2.2 Measurement methods

Cultures were monitored using UV-Vis Spectrophotometry (Chapter 2.4.3) at Absorbance at 660nm (A660) and Absorbance at 1020nm (A1020) to measure growth and pigment formation, pH, and the calculated ratio A1020/A660. (See Chapter 3.2.2), gathered at the time of inoculation and on day 5. The Two Point Method analysis was applied to three identical specimens for each condition to obtain an average growth rate and standard deviation. (See Chapter 3.2.3) The increase in pigment expression in the sample (mainly corresponding to the sample’s light harvesting complex) was monitored by its characteristic absorbance at 1020 nm and correlated to the apparent absorbance at 600 nm as described in Chapter 3. CFU counts were determined every 4-8 generations (20-40 days) in order to confirm that the relationships determined for absorbance and CFU count remained accurate, but not used directly for quantification purposes. (Chapter 2.4.2.1) The pH measurements were made using an Orion Model 410A pH meter.

5.2.3 Preparation of samples for testing growth conditions

In order to examine the effect of an altered condition, altered Minimal media formulations were prepared without addition of the media component being tested so that varying the concentration could be achieved by simple addition of stepwise amounts. Each condition was prepared and measured in triplicate. The media was adjusted to pH 6.9, filter sterilized, and distributed to smaller vessels, either 250 mL or 50 mL. The headspace comprised approximately 22% of the total volume of the 250 mL vessels and 40% of the total volume of the 50 mL vessels. Each vessel was then amended with individually sterilized additives in order to achieve the desired experimental condition.
The remainder of the culture preparation follows the description outlined in 2.4.2. Unless indicated otherwise, a “standard 20% inoculation,” defined as a 20% by volume of a 5-day-old cell broth concentrated approximately 10X, to a final calculated OD of 25 a.u. was used. This optimum inoculation was determined based on the optimum inoculation volume experiment. (See 5.2.3.1 and 5.3.1) To prepare it, an adequate volume of 5-day grown culture to inoculate all experimental samples was first centrifuged at 3600 RPM to remove the spent media. The cells were then resuspended in approximately 1/10 of the initial volume of fresh prepared Minimal media at pH 6.9 so that its A660 was equal to 25 absorbance units. The culture was agitated well to insure complete mixing and sampled for spectrophotometric measurement. Following sampling, the culture was sealed, incubated under illumination at approximately 32°C, and allowed to grow a total of 5 days of culture growth, at which time the culture was removed from incubation and sampled.

5.2.3.1 Inoculation volume

To determine the best inoculation amount, identical 250 mL vessels containing Minimal media were inoculated with varying amounts of cells in the range 0.4% to 80% by volume prepared at the standard 10X concentration. The percent addition was based on the volume prior to concentration, so a 50% inoculation would be made by adding 5% of the total culture volume of the prepared inoculation broth. For inoculations with a final volume greater than 5% of the total culture volume, adequate media was removed to allow space for the addition.
5.2.3.2 Treatment of inoculation broth: concentration and washing

To test whether concentrating cells affected their ultimate growth potential, the cells were centrifuged in 50 mL Falcon tubes in a ThermoICE CentraCL2 centrifuge at 3600 RPM for 20 minutes without temperature control in order to pellet the cells. The media was then discarded and the cells were resuspended with approximately 1/10th of their previous volume in fresh Minimal media and agitated to insure that the pellet was fully dispersed and used for inoculation or subsequently washed. Washing was performed to remove any contaminants in the spent media or loosely attached to the cells. To wash the cells, the cells were resuspended in fresh Minimal media at pH 6.9, well agitated and then centrifuged again. This process was repeated a total of three times, and the cells were then resuspended as above in fresh Minimal media at pH 6.9 and used for inoculation of the experimental cultures. To compare the growth of cells subjected to concentration with those used directly from the inoculating culture broth, a volume of culture broth equivalent to the initial volume of the concentrated broth was used.

To determine the effect of the concentrating process on culture growth, identical 250 mL culture bottles were prepared for 6 conditions, performed in triplicate. For the unconcentrated samples, adequate media was removed to make room for the necessary addition. An inoculating broth subjected to concentration was prepared, and the resultant preparation had a calculated A660 of 24.8 absorbance units. Inoculations of 2%, the standard 20%, and 40 % by volume were made in triplicate for unconcentrated and concentrated inoculating broth. Percent volumes reflect total volume of pre-concentrated broth, compared to the volume of the culture being inoculated.

To determine whether washing to remove any traces of compounds in the spent media could affect the growth of the cells, adequate identical 250 mL culture bottles were
prepared for three conditions in triplicate. For one set of bottles, 20% of the volume was removed to make room for a large addition. Five day old cell broth was concentrated and then resuspended to a density of 25.1 absorbance units. The supernatant from the first centrifugation step was reserved. One third of the cells were subsequently washed three times and resuspended to a density of 26.0 absorbance units. A 20% by volume inoculation, adjusted for the difference in final density, was made to all cultures using the concentrated only cells for two of the sets, and the washed cells for the third in triplicate. To one of the concentrated only sets, a 20% by volume addition of the supernatant recovered from the first washing was made for comparison.

5.2.3.3 Intrinsic growth of cells

The effect of Minimal Media Components on culture growth was tested using 1) Minimal media, which contains buffer, carbon, nitrogen, and other nutrients as the control, 2) phosphate-buffered water utilizing the buffer recipe described in Appendix E, 3) buffered water plus sodium malate as a carbon source, 4) buffered water plus ammonium sulfate as a nitrogen source, and 5) buffered water plus both carbon and nitrogen sources. (See Table 1 and Appendix: E Media) To prepare these, water containing 6.142 M of potassium phosphate buffer was prepared, sterile filtered, and distributed to twelve 250 mL vessels for the four experimental conditions. Minimal media was also prepared and distributed to three 250 mL vessels for the control. Sterile malate and ammonium sulfate solutions were added as described in Table 1 to the individual vessels. The vessels were then inoculated with 20% by volume of concentrated cells, as previously described. The cultures were agitated to insure good
mixing, sampled for spectrophotometric and CFU analysis, and incubated for five days under illumination.

5.2.3.4 Vitamin supplementation

The effects of p-ABA, thiamine and Supersalts (See Table 2) were investigated to determine if these compounds were required, and at what concentration growth was optimized. In the standard Minimal media preparation, p-ABA, added as a single component stock solution, has a final concentration of 1.82 μM, and thiamine, added in solution with the Supersalts, has a final concentration of 2.08 μM in Minimal media. For this investigation, a Supersalts solution was prepared without thiamine, and thiamine and p-ABA were prepared as separate solutions.
To prepare the samples, sufficient 250 mL vessels to assay in triplicate were prepared with media stripped of various components, as described in Table 3. To the base medias described, adequate stock solutions of either p-ABA or thiamine were added to generate cultures with 0, 1, 4, 6, and 8 μM final concentration. For each compound, the zero served as a negative control.

Biotin, pantothenate, niacin, B12, pyridoxine, folic acid, and l-cysteine additives were also tested to determine if they could enhance culture growth. The 2 μM concentration of p-ABA and thiamine was used as a reference point around which to construct the investigation of folic acid, biotin, niacin, pyridoxine, B12, l-cysteine, and pantothenate. For each supplement, standard Minimal media was prepared according to formulation, sterile filtered, and distributed to 250 mL sterile bottles to investigate each

### Table 2
**The Composition of Supersalts Including Trace Elements and Its Concentration in the Final Broth**

<table>
<thead>
<tr>
<th>Supersalts</th>
<th>per 1000 mL Water</th>
<th>Conc. in Supersalts (mM)</th>
<th>Conc. in Broth (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na₂EDTA</td>
<td>0.4 g</td>
<td>1.27</td>
<td>44.55</td>
</tr>
<tr>
<td>MgSO₄*7H₂O</td>
<td>4.0 g</td>
<td>16.23</td>
<td>568.02</td>
</tr>
<tr>
<td>CaCl₂*H₂O</td>
<td>1.5 g</td>
<td>10.20</td>
<td>357.10</td>
</tr>
<tr>
<td>FeSO₄*7H₂O</td>
<td>0.2 g</td>
<td>0.72</td>
<td>25.18</td>
</tr>
<tr>
<td>Thiamine</td>
<td>20.0 mg</td>
<td>0.07</td>
<td>2.08</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Trace Elements (per 250 mL)</th>
<th>(μM for Trace)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cu(NO₃)₂*3H₂O</td>
<td>10 mg</td>
</tr>
<tr>
<td>MnSO₄*H₂O</td>
<td>397.00 mg</td>
</tr>
<tr>
<td>ZnSO₄*7H₂O</td>
<td>60.0 mg</td>
</tr>
<tr>
<td>H₃BO₃</td>
<td>700.0 mg</td>
</tr>
<tr>
<td>NaMoO₄*2H₂O</td>
<td>187.00 mg</td>
</tr>
</tbody>
</table>
Stock solutions of each vitamin were prepared at 0.5 mM, and supplemented to the Minimal media to achieve the desired final concentrations of 0.5, 1, 2, or 4 μM.

5.2.3.5 Carbon

*B. viridis* and other PNSB function under such a variety of possible metabolisms that it is necessary to explore several different carbon sources and types of usage.

Bicarbonate was added to supply exogenous CO₂ needed for photoautotrophic growth. (See 4.2.3.1) Malate, citrate, acetate, and fumarate are intermediates of the TCA cycle and organic carbon sources for photoheterotrophic growth. (See 4.2.3.2) Sucrose and glucose are sugars, common carbon sources for heterotrophic growth and fermentation (or anaerobic growth). To explore the carbon preferences of *B. viridis*, these carbon sources were assayed in four experiments: the effect of exogenous CO₂ by addition of sodium bicarbonate, the culture growth in the presence of various organic carbon sources

<table>
<thead>
<tr>
<th>Condition</th>
<th>Base Media</th>
<th>Added Stock Solutions</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Minimal</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>Negative Control Thiamine</td>
<td>SP Free</td>
<td>Thiamine Free Supersalts, p-ABA</td>
<td>1X, 1.82 μM</td>
</tr>
<tr>
<td>Negative Control PABA</td>
<td>SP Free</td>
<td>Supersalts</td>
<td>1X</td>
</tr>
<tr>
<td>Negative Control SS</td>
<td>SP Free</td>
<td>p-ABA</td>
<td>1.82 μM</td>
</tr>
<tr>
<td>Varied Supersalts</td>
<td>SS Free</td>
<td>Supersalts</td>
<td>0 – 3X Standard</td>
</tr>
<tr>
<td>Varied Thiamine</td>
<td>T Free</td>
<td>Thiamine</td>
<td>0 – 4 μM</td>
</tr>
<tr>
<td>Varied PABA</td>
<td>P Free</td>
<td>p-ABA</td>
<td>0 – 4 μM</td>
</tr>
</tbody>
</table>

NOTE: Standard Minimal Media contains Supersalts (Thiamine) and p-ABA

Adjusted Medias as follow:

SP Free: Minimal Media minus Supersalts (Thiamine) and p-ABA

SS Free: Minimal Media minus Supersalts (Thiamine)

T Free: Minimal Media minus Thiamine (including Thiamine Free Supersalts)

P Free: Minimal Media minus p-ABA
including TCA cycle intermediates and sugars, the ability of fumarate to support growth of the bacteria, and the optimum concentration of malate for growth of *B. viridis*.

**Sodium Bicarbonate**

Sodium bicarbonate was supplemented to Minimal media and NC media, defined as Minimal minus sodium malate solution. The purpose of NC media was to provide a carbon free media to determine if any bicarbonate effect was as a result of the bicarbonate alone or if it is a synergistic effect of bicarbonate and malate. Because the primary goal was to evaluate the effect of exogenous CO$_2$, the additions of bicarbonate were calculated based on the effective percent saturation of carbon dioxide. (See Table 4) To obtain this “Saturation Equivalent,” the molar concentration of carbon dioxide in 100% saturated water at 30°C was calculated to be 30.2 mM.$^7$ This value was calculated using temperature dependant solubility data for CO$_2$ in water from Perry’s Handbook. This molar concentration was then converted into grams of sodium bicarbonate.

Identical 250 mL vessels with Minimal or NC (carbon free) media were prepared for all conditions. The vessels were then inoculated with a standard 20% addition of cells. Initial samples of each culture were obtained for measurement of pH and absorbance, and bicarbonate was added, and the vessels were sealed immediately by

<table>
<thead>
<tr>
<th>Saturation Equivalent</th>
<th>Molar Concentration (mM)</th>
<th>Addition Per Liter (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (0%)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>25%</td>
<td>7.6</td>
<td>0.6</td>
</tr>
<tr>
<td>50%</td>
<td>15.1</td>
<td>1.3</td>
</tr>
<tr>
<td>100%</td>
<td>30.2</td>
<td>2.5</td>
</tr>
<tr>
<td>150%</td>
<td>45.2</td>
<td>3.8</td>
</tr>
<tr>
<td>200%</td>
<td>60.4</td>
<td>5.1</td>
</tr>
</tbody>
</table>
capping tightly with a plastic ring in place to minimize loss of gaseous CO₂ as the bicarbonate was absorbed into the media and CO₂ was released. The vessels were then incubated at 32°C under illumination for 5 days, and subsequently measured.

**Organic Carbon Sources**

To explore alternate sources of organic carbon, sucrose, glucose, citrate, and acetate were offered at 20 mM, both in conjunction with 20 mM malate and as alternatives to malate. Identical sterile 50 mL vessels for all conditions were prepared with NC media. To obtain a media with one of these compounds as a sole carbon source, NC Media (carbon free) was supplemented with solid compound or prepared solutions to achieve a 20 mM addition of the particular carbon source. (See Table 5) To achieve a media with 20 mM of the carbon source plus 20 mM of malate solution, 28 mL/L of malate plus the defined addition were added to the vessel. The total carbon available was 20 mM for sole carbon additions, and 40 mM for additions of malate plus carbon. Vessels were inoculated with a standard 20% inoculation and incubated.

**Fumarate**

To examine the growth of *B. viridis* on fumarate, and explore the possibility of utilizing this carbon as an alternative to malate, fumarate was offered at 10 mM, 20 mM,
and 30 mM concentrations in Minimal media and NC media (carbon free). In addition, a
carbon free control and a malate only control were employed. Adequate sterile vessels
were prepared, and fumarate was subsequently appended to the vessels as a prepared
861.5 mM solution in 18 mOhm water to the desired molar concentration. (See Table 6)
In NC media, the fumarate functioned as the sole carbon source, providing a total of 10
mM, 20 mM, or 30 mM available carbon. Minimal media, both the constant 20 mM
malate plus fumarate supplemented to a total of 30 mM, 40 mM, and 50 mM carbon were
available for assimilation. The vessels were inoculated using a standard 20% by volume
inoculation, closed, and incubated for 5 days under illumination, and then sampled for
measurement of absorbance and pH.

**Malate**

In Minimal media, 20 mM malate was offered as Sodium Malate solution. In
addition, malate was selected as the preferred carbon source for phototrophic growth of
*B. viridis* in the organic carbon investigation. (See 5.3.5.2) To determine the optimum
concentration of malate for growth of *B. viridis*, malate was added as a 745.8 mM

### TABLE 6

**FUMARATE AS A SOLE CARBON SOURCE OR COMBINED WITH MALATE**

<table>
<thead>
<tr>
<th>Final Fumarate (mM)</th>
<th>Media</th>
<th>Fumarate Addition (mL)</th>
<th>Malate (mM)</th>
<th>Total Carbon (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 mM</td>
<td>NC</td>
<td>0 mL</td>
<td>0 mM</td>
<td>0 mM</td>
</tr>
<tr>
<td>0 mM</td>
<td>Minimal</td>
<td>0 mL</td>
<td>20 mM</td>
<td>20 mM</td>
</tr>
<tr>
<td>10 mM</td>
<td>NC</td>
<td>2.9 mL</td>
<td>0 mM</td>
<td>10 mM</td>
</tr>
<tr>
<td>10 mM</td>
<td>Minimal</td>
<td>2.9 mL</td>
<td>20 mM</td>
<td>30 mM</td>
</tr>
<tr>
<td>20 mM</td>
<td>NC</td>
<td>5.8 mL</td>
<td>0 mM</td>
<td>20 mM</td>
</tr>
<tr>
<td>20 mM</td>
<td>Minimal</td>
<td>5.8 mL</td>
<td>20 mM</td>
<td>40 mM</td>
</tr>
<tr>
<td>30 mM</td>
<td>NC</td>
<td>10.6 mL</td>
<td>0 mM</td>
<td>30 mM</td>
</tr>
<tr>
<td>30 mM</td>
<td>Minimal</td>
<td>10.6 mL</td>
<td>20 mM</td>
<td>50 mM</td>
</tr>
</tbody>
</table>
solution to final concentrations in the media ranging from zero to 160 mM. Because the higher concentrations required a large volume addition, a corresponding amount of media was removed from the culture bottles in order to make room for the malate addition. (See Table 7) Prepared culture vessels were inoculated with a standard 20% inoculation and incubated under illumination.

5.2.3.6 Nitrogen

**Nitrogen Assimilation and Headspace Gas**

It was important to determine if, and how efficiently, *B. viridis* fixes nitrogen for use in growth, the effect of providing fixed nitrogen, and the oxygen sensitivity of the organism. Vessels with Minimal Media [(NH₄)₂SO₄ = 5.30 mM] and ammonium sulfate free Minimal media [(NH₄)₂SO₄ = 0 mM] were prepared and then steriley sparged for a minimum of 30 minutes with molecular nitrogen, air, or argon to achieve saturation. (See Table 7)
Argon was employed as a nitrogen- and oxygen-free control. Air was used to obtain an oxygenated culture. A standard 20% inoculation was made, and cultures were incubated under illumination.

**Fixed Nitrogen Concentration**

Since the presence of fixed nitrogen in the form of ammonia significantly improves the growth rate of *B. viridis*, (See 5.3.6.1) the relationship between growth and the concentration of ammonia in the media was determined. First, nitrogen free Minimal media (Minimal –(NH₃)₂SO₄) was prepared and distributed to twenty-one 50 mL vessels. Seven ammonia concentrations between zero and four times the standard Minimal media...

---

**TABLE 8**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Media</th>
<th>Nitrogen Source</th>
<th>Sparged Gas</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fixed + Molecular Nitrogen</td>
<td>Minimal (NH₃)₂SO₄ + N₂</td>
<td>(NH₃)₂SO₄ + N₂</td>
<td>N₂</td>
</tr>
<tr>
<td>Fixed + Molecular Oxygen</td>
<td>Minimal (NH₃)₂SO₄ O₂</td>
<td>(NH₃)₂SO₄ O₂</td>
<td>O₂</td>
</tr>
<tr>
<td>Fixed + Argon</td>
<td>Minimal (NH₃)₂SO₄ Argon</td>
<td>(NH₃)₂SO₄ Argon</td>
<td>Argon</td>
</tr>
<tr>
<td>Molecular Nitrogen</td>
<td>Minimal – (NH₃)₂SO₄</td>
<td>N₂</td>
<td>N₂</td>
</tr>
<tr>
<td>Molecular Oxygen</td>
<td>Minimal – (NH₃)₂SO₄</td>
<td>None</td>
<td>O₂</td>
</tr>
<tr>
<td>Argon</td>
<td>Minimal – (NH₃)₂SO₄</td>
<td>None</td>
<td>Argon</td>
</tr>
</tbody>
</table>

**TABLE 9**

<table>
<thead>
<tr>
<th>Name</th>
<th>Media Added 10% (NH₃)₂SO₄ Solution (mL)</th>
<th>Final (NH₃)₂SO₄ Conc (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrogen Free</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>¼ Std Nitrogen</td>
<td>0.438</td>
<td>1.33</td>
</tr>
<tr>
<td>½ Std Nitrogen</td>
<td>0.875</td>
<td>2.65</td>
</tr>
<tr>
<td>Std Nitrogen</td>
<td>1.75</td>
<td>5.30</td>
</tr>
<tr>
<td>2X Nitrogen</td>
<td>3.5</td>
<td>10.60</td>
</tr>
<tr>
<td>3X Nitrogen</td>
<td>5.75</td>
<td>15.90</td>
</tr>
<tr>
<td>4X Nitrogen</td>
<td>7</td>
<td>21.20</td>
</tr>
</tbody>
</table>

Table 8) Argon was employed as a nitrogen- and oxygen-free control. Air was used to obtain an oxygenated culture. A standard 20% inoculation was made, and cultures were incubated under illumination.

**Fixed Nitrogen Concentration**

Since the presence of fixed nitrogen in the form of ammonia significantly improves the growth rate of *B. viridis*, (See 5.3.6.1) the relationship between growth and the concentration of ammonia in the media was determined. First, nitrogen free Minimal media (Minimal –(NH₃)₂SO₄) was prepared and distributed to twenty-one 50 mL vessels. Seven ammonia concentrations between zero and four times the standard Minimal media...
concentration were employed, and these were prepared by adding 10% ammonium sulfate solution to the vessels. (See Table 9) For the 5.75 mL and 7 mL addition, media was removed to make room for the addition. In both cases, this caused a significant 10% or greater dilution. A concentrated inoculating broth prepared as described above was used to make a standard 20% inoculation, and the culture was incubated under illumination for 5 days and then measured.

5.2.3.7 Potassium phosphate buffer concentration

Since growth is known to be pH sensitive, stronger buffering is a way to increase the possible total growth before the cell’s growth is impaired by pH effect. Control of the system pH by means of a buffer is very important. In Minimal media, a potassium phosphate buffer (abbreviated KPO) is used. (See Appendix E) Minimal media minus the potassium phosphate buffer (Minimal –KPO) was prepared, adjusted to pH 6.9, and distributed to 250 mL experimental vessels to examine 5 concentrations in triplicate. The standard amount of buffer in the initial Minimal Media formulation was 3.1 M \( \text{KH}_2\text{PO}_4/3.6 \text{ M K}_2\text{HPO}_4 \) Both higher and lower concentrations of buffer were assayed by amending pH 6.9 adjusted buffer solution as described in Appendix E to the vessels to

TABLE 10
POTASSIUM PHOSPHATE BUFFER
ADDITIONS TO BUFFER FREE MEDIA

<table>
<thead>
<tr>
<th>Relationship to Standard</th>
<th>Media</th>
<th>Added KPO Buffer Solution (mL)</th>
<th>Final Concentration in Media (( \text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4 )) M</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (Negative Control)</td>
<td>Minimal –KPO</td>
<td>0</td>
<td>0 / 0</td>
</tr>
<tr>
<td>½</td>
<td>Minimal –KPO</td>
<td>1.315</td>
<td>1.535 / 1.809</td>
</tr>
<tr>
<td>1 (Control)</td>
<td>Minimal –KPO</td>
<td>2.625</td>
<td>3.071 / 3.617</td>
</tr>
<tr>
<td>2</td>
<td>Minimal –KPO</td>
<td>5.25</td>
<td>6.142 / 7.235</td>
</tr>
<tr>
<td>4</td>
<td>Minimal –KPO</td>
<td>10.5</td>
<td>12.283 / 14.469</td>
</tr>
</tbody>
</table>
achieve the desired buffer concentration. (See Table 10) The cultures were given a standard 20% inoculation with concentrated inoculating solution and incubated under illumination.

5.2.3.8 The acceptable pH range for growth

In order to determine the relationship between the initial culture pH and the growth behavior of the culture, media was prepared and adjusted to pH values between 4.5 and 9.5, approximately every 0.2 pH units. The growth in each of these pH values was followed over a course of four generations, with each generation inoculated from 5 day old cells grown at the same pH they were initially cultured in. For RMPABA, if no increase in cell density, defined by a significant positive change in absorbance, was observed, those cultures were not carried into the next generation. This selection process was significantly less rigorous for Minimal since lower cell yields were expected and therefore detection of small cell increases was difficult. Here, cultures were carried into the next generation unless the absorbance decreased after inoculation. (See Table 11) For Minimal, the profile of tested cultures does not represent the profile of a positive growth result.

Because the natural pH behavior in rich RMPABA media, unaffected by nutrient limitation, was unknown, the experiment was performed both in RMPABA and in Minimal media. For RMPABA, the media was mixed according to the recipe in Appendix E, distributed to 250 mL experimental vessels, pH adjusted for each condition, and sterilized by autoclave. A 2% by volume inoculation with unconcentrated 5-day-old cells grown in RMPABA was made to the prepared vessels. For the first generation, 5-day-old cells grown in RMPABA broth were used. In subsequent generations, cells of
### TABLE 11

**SELECTED PH VALUES IN THE RANGE 4.5 TO 9.5**

**CULTURED TO EXPLORE THE PH DEPENDANT GROWTH BEHAVIOR OF *B. VIRIDIS***

<table>
<thead>
<tr>
<th>pH Value</th>
<th>RMPABA</th>
<th>Minimal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Gen1</td>
<td>Gen 2</td>
</tr>
<tr>
<td>4.5</td>
<td>Y</td>
<td></td>
</tr>
<tr>
<td>4.7</td>
<td>Y</td>
<td></td>
</tr>
<tr>
<td>4.9</td>
<td>Y</td>
<td></td>
</tr>
<tr>
<td>5.2</td>
<td>Y</td>
<td></td>
</tr>
<tr>
<td>5.4</td>
<td>Y</td>
<td></td>
</tr>
<tr>
<td>5.6</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>5.8</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>6.2</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>6.5</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>6.9</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>7.3</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>7.5</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>7.7</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>7.9</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>8.1</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>8.3</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>8.5</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>8.7</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>8.9</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>9.1</td>
<td>Y</td>
<td></td>
</tr>
<tr>
<td>9.3</td>
<td>Y</td>
<td></td>
</tr>
<tr>
<td>9.5</td>
<td>Y</td>
<td></td>
</tr>
</tbody>
</table>

**NOTE:** In RMPABA, growth and the absence of growth was detected easily. In Minimal, the total growth was lower, making it more difficult to ascertain that growth was not observed. To be certain that all growth was continued to the next generation, the selection procedure for abandoning a culture condition was significantly less rigorous for Minimal. The culture selection profile does not reflect observed growth. **Contamination in pH 6.9 RMPABA prevented subsequent generations.**
corresponding pH were employed. After inoculation, the cultures were incubated under illumination.

For the Minimal media investigation, media was prepared as described in Chapter 2.2.1, with pH adjustment to the desired pH listed in Table 11 immediately prior to sterile filtration. In Minimal media, a standard 20% inoculation was used to inoculate Generation 1. Subsequent generations were inoculated with 20% by volume of 5-day-old cells from the prior generation grown at the same pH as the initial culture, listed in Table 11. The appropriate volume was concentrated to 1/10 the original volume and resuspended in media of the same pH to insure the correct final pH of the experimental vessels. The absorbance of the concentrated broth was not standardized to 25 a.u. (calculated) because the final cell concentrations were often too low to allow such a concentration to be achieved with approximately 20% by volume. In some cases, using the entire prior generation would not have provided adequate cells to obtain a meaningful measurement, much less to prepare a concentrated inoculating broth with calculated OD of 25 a.u., as described in 4.3.2.4.

5.3 Results and Discussion
5.3.1 Inoculation volume

The maximum growth, measured by absorbance, was achieved using a 20% by original volume inoculation with inoculating broth prepared by concentrating and resuspending the seed culture in fresh media. Concentration of the seed culture allowed a significant inoculation without creating a significant dilution. These results are presented in terms of doubling time. (See Figure 13) Recall from Equation 3.3 that doubling time is
an inverse function of the growth rate. The minimum doubling time corresponds to the maximum rate of growth.

For intermediate inoculation volumes, the standard deviation was small: never more than 10%, and less than 5% for most points. At extremely high inoculation, such as the 80% point, and extremely low inoculation, around 1%, the spread was significantly greater. For the 80% point, the standard deviation was nearly 50% of the value, and for very low inoculation, the standard deviation was on the order of 30% of the total value. At low volume, this error most likely resulted from magnification in pipetting error; because the inoculation was small, very few cells were present and a slight discrepancy in volume corresponded to a larger percent of the total. As the cells replicate, this discrepancy becomes more visible. At high volume, the error most likely results from the extremely high concentration of cells present, which could create a heterogeneous

Figure 1: The Growth Rate for Various Percent, by Volume, Inoculation. A best fit curve was plotted to draw attention to the trend. The most rapid doubling time, or fastest growth, occurs around 20% by volume inoculation. The data point at 80% was not included in this fit. At such a high inoculation, the error is extremely large. The error in results is also large for very small inoculations. Error was calculated based on the standard deviation of three repeats.
suspension in which cells clumped together due to space limitations. Clumped cells would go dormant in response to the significantly concentrated environment, and isolated cells would grow in response to fresh media. The response would vary dependent on the microenvironment encountered within the heterogeneous suspension.

The doubling time was greatest for cells inoculated at a moderately high concentration. At low concentration, the cells were not able to experience the cell-cell interactions thought to be necessary to trigger cell division. At extremely high concentrations, quorum sensing can cause the cells to become dormant in order to preserve the resources necessary. The shortest doubling time occurred in a midrange inoculation concentration, around 20% by volume.

The total change in optical density during the growth period was also considered (See Figure 14). This change was determined by subtracting the calculated initial A660 from the calculated final A660. Up to about 20% -30% inoculation, a greater inoculation results in a linearly greater change in culture density. Above about 30% inoculation, there is a deviation from this trend; no additional change in cell density is observed with additions of greater volumes of inoculating culture. This coincides with the decrease in doubling time demonstrated in Figure 1. Although the final total culture density was greater for those cases in which more cells were initially added, this additional density results only from the greater number of cells added at the outset, and does not represent additional new growth. The pH and pigment behavior followed the trends in rate and amount of growth noted.
From these results, it can be concluded that an inoculation of approximately 20% volume prior to concentration cell broth with an A660 of 25 ± 1 a.u. (defined as the “standard 20% inoculation”) will both optimize growth rate and stay in the range where additional cells contribute to increased total growth. Using this as a standardized inoculation protocol ensures that the experimental cultures will be similar at the outset of each experiment.

5.3.2 Treatment of inoculation broth: concentration and washing

To confirm whether concentration and washing protocol had an adverse effect on culture outcome, the effects of both the concentration process using centrifugation at 3600 RPM, and the washing of the cells via three sequential resuspension and

Figure 2: Change in Culture Density as Affected by Initial Inoculation Concentration. Up to 20%-30% inoculation, an increase in inoculation results in a linear increase in new cells generated, measured by change in culture density. Above this concentration, additional inoculation does not generate additional culture density.
centrifugation cycles in fresh, carbon free media were considered. The concentration experiment was repeated at 2%, 20%, and 40% inoculation. The 20% inoculation reflects the standard inoculation concentration defined as optimum above. The 2% inoculation is 1/10 of the standard 20% inoculation, and the 40% inoculation is 2X the standard. This spread of inoculation concentration was employed in order to determine if the effects are constant or vary based on the amount of inoculation. Concentration, washing, and the effect of spent RMPABA broth were considered only with a standard 20% inoculation.

The growth rates of cultures inoculated with concentrated and unconcentrated cells were extremely similar at both 20% and 40% inoculation concentrations. (See Figure 15) At 2% inoculation however, there is a nearly twofold difference in the rate of growth, from around 0.1 a.u./day to 0.2 a.u./day. At this low volume, it is likely that the extra nutrients present from the RMPABA broth introduced in the medium have a significant effect. In RMPABA media, vigorous growth could be obtained with as little as 1% inoculation, while a significant reduction in growth rate results from such a low inoculation for Minimal media. Except at very low inoculation volume, illustrated here with the 2% by volume inoculation, the standard deviation of the measurements for the cultures inoculated with unconcentrated cells was significantly greater. At low inoculation volume, the error was similar for both conditions; this could be attributed to the low density of the cell broth resulting from the presence of only a few cells. Because even a slight difference in cell density is a large percent of the total density, a higher error was likely for these cultures. At 20% and 40% by volume inoculation, the error of the measurement is as much as ten fold greater for unconcentrated cells. In the unconcentrated inoculations, 20%-40% of the culture medium is RMPABA media. In
addition, it is spent RMPABA media, which means that there are not only a wide variety of undefined nutrients present, but there are also a wide variety of metabolic side products present from the growth of the inoculating culture. Both the undefined nature of RMPABA and the end products of growth cause this increased variability in growth.

The rates of growth for concentrated cells and washed cells differed by less than one percent with a standard deviation of about 10%. (See Figure 4) The similar growth rates between concentrated and washed cells indicate the washing process is not detrimental to the cells. Concentrated cells with an added aliquot of spent RMPABA media equal to the original volume of the inoculating broth have a growth rate nearly 50% greater than either concentrated or washed cells, however the error inherent in the

Figure 3: Effect of Inoculation Concentration on Growth Rate of Cells. Except at low inoculation volume, there is very little difference in the average growth rate of cultures inoculated with concentrated and unconcentrated cells. There is significantly more variability in the unconcentrated cells, demonstrated by the increased error (dashed lines for unconcentrated cells, solid lines for concentrated cells). This variability likely stems from the presence of the spent media, which contains both extra nutrients, as well as inhibitors produced during the prior growth. Data points are offset by 0.5% volume to allow easier viewing.
rate increases to around 15%. It is likely that this increase in growth is a result of additional nutrients and carbon present in the spent media. Because this spent media is RMPABA, an undefined media, there is an increased degree of variability in the response. In addition, the media contains waste products and inhibitors generated by the bacteria during growth that can contribute to the increased error observed. This suggests that it is important to perform the concentrating step in order to remove spent RMPABA media and increase the repeatability of the results.

Figure 4: The Effect of Concentration, Washing, and Spent Media Inclusion on Growth Rate. The rate of growth for concentrated cells and washed cells differs by less than 1%. The rate of growth for cultures including the % volume amount of spent RMPABA media in addition to the resuspended cells is nearly 50% greater.
Based on these results, it can be concluded that:

- The mechanical stresses of concentration by centrifugation do not negatively impact the cells and the concentration protocol can be safely applied.

- The presence of spent RMPABA media clearly causes a difference in the growth rate and total growth of the cells, and increases the variability in the result.

- Spent RMPABA media must be removed in order to insure the best possible reproducibility and avoid the metabolic variations associated with RMPABA media.

- The process of washing did not significantly damage or impair the cells, nor does it significantly enhance the reliability of the experiment.

Based on these results, the standard procedure to prepare a cell broth was defined as concentrating the cells by centrifugation, removing all spent RMPABA media, and resuspending the cells to approximately $1/10^{th}$ of their initial concentration in carbon free minimal media, which will yield a cell broth with a calculated A660 of 25 absorbance units.

5.3.3 Intrinsic growth of cells

To evaluate the possibility that the cells experience an initial growth based on their physiological state or the intracellular nutrient content at the time of inoculation, an experiment was designed to look at the response of cells in buffered water, buffered water plus only a carbon source, buffered water plus only a nitrogen source, buffered
water plus both carbon and nitrogen sources, as well in Minimal media, which contains buffer, carbon, nitrogen, and other nutrients. (See Table 3 and Appendix E: Media)

While the cells do experience some growth in the negative control (Buffer Only), with a total rate less than 0.08 a.u./day, there is significantly more growth, 0.16 a.u./day, observed in the control condition, or inoculation into Minimal media. (See Figure 6) The small rate of growth observed in the negative control could be a result of either RMPABA metabolism continuing briefly or the cells using nutrients present intracellurally. It is not likely that this is a result of CO₂ assimilation, because although the cells are photosynthetic and can assimilate carbon dioxide, an electron donor is necessary for this process to proceed. This electron donor is typically an organic carbon for PNSB. In this case, the malate would serve as the electron donor.

![Figure 5: Response of Cells in Buffered Media plus Amended Carbon and/or Nitrogen. Cells with only buffer or nitrogen experience the most significant reduction in growth, doubling at less than half of the control rate. Cells missing nitrogen also experience a lesser reduction in growth, doubling only 1/3 as rapidly. Buffer supplemented with both carbon and nitrogen is indistinguishable from control media.](image-url)
Even in the presence of nitrogen, the growth is significantly reduced to around 0.07 a.u./day by the absence of malate as a carbon source (Buffer + Ammonium condition) to approximately the growth rate in buffered water only. This suggests that the presence of carbon was the most important factor in obtaining good growth. In the absence of nitrogen in the form ammonium sulfate, (Buffer + Malate), growth was reduced to two thirds of the control growth, around 0.1 a.u./day. This indicates that the presence of fixed nitrogen can also be important to the growth of the cell.

When both carbon and nitrogen are offered, even in the absence of minerals and minor nutrients, the growth is improved to approximately the same rate in the control media. There is less than 5% difference between the growth in control media and the Buffer + Malate + Ammonium media, which is less than the error in the respective measurements. It seems unlikely that the bacteria have no need of nutrients other than carbon and nitrogen. For pigment formation alone, photosynthetic bacteria require magnesium as the central atom in porphyrin formation.10(See Figure 7)

Figure 6: The General Structure for a Porphyrin, a precursor to a bacteriochlorophyll production. For bacteriochlorophyll, a Mg+ ion is located in the center of the ring.
There are two possibilities for the apparent lack of requirement for nutrient and vitamin supplementation. The first is that the bacteria cannibalize old pigments to form pigment in new cells. Since the A1020 signature and A1020/A660 ratio is not significantly different for control or nutrient free cells, this seems unlikely. If cells were cannibalizing pigment components in order to generate new cells, a significant reduction in the A1020/A660 ratio would be observed, suggesting a decreased pigment per cell. (See Figure 8) Since no difference in the pigment signature was noted, it is possible that the cells may carry within or firmly attached to themselves, a certain amount of nutrients, which may be sufficient for growth during the first 5 days. This suggests that the stunted growth observed in Minimal media results from the lack of some micronutrient or vitamin offered to the culture only through the presence of yeast extract or bactopeptone, making determination of the vitamin requirements for culture growth important.

![Figure 7: The Pigment Ratio for Various Media Conditions. No significant difference is noted in the pigment ratio for the various conditions. This indicates that pigment is not being cannibalized for nutrients in the conditions where trace elements are not present to provide the magnesium required to generate new pigment.](image-url)
5.3.4 Vitamin supplementation

5.3.4.1 p-ABA, thiamine, and Supersalts: nutrients in the current media formulation

To examine the effect of p-ABA and thiamine on culture performance, these vitamins were added to cultures at molar concentrations between zero and 10 μmol since they are distinct compounds. (See Figure 9) The effect of the Supersalts solution was also investigated. Because this is a stock solution of many components, it was added based on ratios between zero and 4 times the standard. (See Figure 10) There was no remarkable difference in the growth rate for varying additions of p-ABA, thiamine, or Supersalts. The pH change as a function of growth was similar for all cases, and the pigment behavior did not differ between cases.

Figure 8: The Effect of p-ABA and Thiamine on the Growth of B. viridis in Minimal Media. There is very little effect from either of these two additives. Even at 0 μm, there is no significant change in the response of the culture.
There are several possible reasons for this almost total lack of effect observed with additions of these three components on the growth of *B. viridis*:

- These vitamins and minerals are not necessary for growth.
- *B. viridis* carries some small amount of these nutrients intracellularly, which can sustain some growth on a fresh carbon source.
- Some other limitation to the growth exists, and additional *p*-ABA, thiamine, and minerals from Supersalts are not needed before the limitation is reached.

It is unlikely that vitamins are not necessary for growth, since there is rather voluminous agreement that these compounds are useful in the growth of photosynthetic bacteria and specifically *B. viridis*. (See 4.2.2) There is also little difference in growth between Minimal media and the *p*-ABA, thiamine, and Supersalts free medium (Buffer +

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**Figure 9:** The Effect of Varying Concentrations of Supersalts in Minimal Media on *B. viridis*. There is no discernible effect of varying the amount of Supersalts supplemented to the media, even when there is no supplementation at all.
Malate + Ammonium from 5.3.3). This supports the idea that there may be adequate nutrients intracellularly to support some growth, however the total growth is still limited compared to the RMPABA media growth. This indicates that there is some other growth limitation in the system. Potential culprits include:

- a missing critical nutrient or vitamin not present in the original media formulation, provided only via the undefined components Yeast Extract or Peptone, which creates an enzymatic bottleneck preventing normal cellular growth activity.
- the lack of adequate carbon or nitrogen, shutting down biosynthesis.
- some unfavorable growth condition such as insufficient buffering or improper pH causing the cells to be unable to grow and reproduce.

5.3.4.2 Effect of vitamins known to be useful in the growth of PNSB: folic acid, biotin, niacin, pyridoxine, B$_{12}$, l-cysteine, and pantothenate

The vitamins biotin, pantothenate, niacin, B$_{12}$, pyridoxine, folic acid, and l-cysteine were not included in the original media formulation, but are known to support growth in PSNB. These were added to Minimal media at the concentrations 0, 0.5, 1, 2, or 4 $\mu$M. In all cases, these additions had no effect on the growth rate or total growth. The pigment behavior and pH were nearly indistinguishable between the experimental conditions and the Minimal media control. Molar concentrations were added exactly, however in Figure 10, the data points are displaced just slightly to the left or right in order to make viewing both the datapoints as well as the error bars easier.
Figure 10: The Effect of Vitamin Addition on Growth of *B. viridis* in Minimal Media. Top: Biotin, Pantothenate, Niacin, and B12 show no significant effect on growth rate from the additions. Bottom: Pyridoxin, Folic Acid, and *l*-cysteine do not enhance the growth rate of *B. viridis*. Both: Concentrations are slightly displaced left or right to enhance viewing.
Why these vitamins, which are generally considered useful to the growth of photosynthetic bacteria, should cause no change in the growth behavior is unclear. The same logic discussed in 5.3.4.1 applies here. It is possible that none of these additional growth factors are necessary for the strain of *B. viridis* in use, or it is possible that other metabolic inhibitions prevent the cells from demonstrating an effect. Since biotin\textsuperscript{11} and pantothenate\textsuperscript{12} participate significantly in photosynthetic carbon assimilation and the TCA cycle, not using the supplemented compounds would mean the bacteria are capable of synthesizing these compounds from smaller simpler molecules or that carbon is not assimilated via photosynthesis or the TCA cycle. Since the majority of PNSB require these compounds for culture growth, it is likely that they do not possess a *de novo* synthesis pathway and the presence of these compounds should cause an observable improvement in growth.

The conclusion which must again be drawn is that there is a limitation based on some other growth requirement. Since carbon is the primary element in all life, it must be considered that the 20 mM of malate provided in the Minimal media formulation may be inadequate for any greater growth than is observed in the Minimal control. In that case, no amount of vitamin supplementation would encourage more growth. In an attempt to improve the growth, the next step is investigation of the requirement for additional carbon or a different carbon source.
5.3.5 Carbon

5.3.5.1 The addition of sodium bicarbonate

Supplementation with bicarbonate to provide exogenous CO$_2$ does not enhance growth in Minimal media. (See Figure 11) In carbon free media, indicated by the pink squares (the lower of the two sets), there is a slight upwards trend in growth from 0.07 a.u./day to 0.075 a.u./day as the concentration of CO$_2$ equivalents increases to saturation. Above the equivalent of 100% saturation, (see Methods Section 5.2.3.5, Table 4 for a review of the condition preparations), the growth rate in both Minimal and NC media is decreased in the presence of bicarbonate supplementation. In NC media, very little growth occurs, but even this miniscule amount is reduced slightly by the presence of more than a 100% saturation equivalent of bicarbonate. The effect on pigment

![Figure 11: The Effect of Exogenous CO$_2$ Supplementation on Growth Rate of B. viridis. The growth rate in Minimal media is not affected by bicarbonate additions up to a 50% saturation equivalent. For carbon free (NC) media, there may be a slight increase in growth rate, however the difference is within the error of the measurement. At and above 100% saturation, there is a decrease in the rate of growth.](image-url)
production behavior mirrors the effect on growth rate behavior. This was an unexpected result, because CO2 can be utilized during photoautotrophic growth. The supplementation was expected to increase the ability of the cells to grow and to engage in their metabolism. Cultures of *B. viridis* are known to be sensitive to high pH, and Minimal cultures are known to halt growth around 7.7 pH units, so pH behavior of the culture grown with added bicarbonate is instructive in understanding why this unexpected result was obtained.

Because the vessels had to be sealed immediately after the addition of bicarbonate to insure the greatest retention of the CO2, an initial pH could not be measured. To estimate the likely initial pH, sodium bicarbonate was added to Minimal and NC media at pH 6.9, according to the concentrations given in Table 5.4 and the pH change was recorded. This was performed in an open vessel, so the values for saturation equivalents over 100% are an imperfect measure of the pH shift in the culture condition. The results are displayed in terms of pH shift due to CO2 saturation equivalent. (See Figure 12) The pH increased in direct relationship to the amount of sodium bicarbonate added. At saturation, the pH had increased by about 0.7 pH units. If we assume a starting media pH of 6.9, this suggests that the media pH at 100% saturation is 7.6 pH units. Since Minimal media cultures have been observed to slow their growth around 7.7 pH units, bicarbonate additions at and above the 100% equivalent leave relatively little room for new growth before the pH limit is reached.
When we study the final pH of the cultures, measured on Day 5, several trends are visible. (See Figure 13) The final pH in NC cultures increases steadily as the amount of added bicarbonate increases, however this pH increase is directly correlated to the increase in media pH based on bicarbonate addition, suggesting that there was almost no pH change based on growth in NC media. Since only minute growth was observed in the NC media, it suggests that CO₂ alone cannot act as a carbon source for bacterial growth.

For cells grown in Minimal media, a pH increase of 0.6 pH units to a final pH of approximately 7.5 is observed in when no bicarbonate is added. The final pH for the cells grown in Minimal media increases steadily as the addition of bicarbonate increases for cultures with a final pH less than 7.7 units, corresponding to cultures with 50%
5.3.5.2 Organic carbon sources: small organic carbon compounds

The addition of alternative carbon sources had a variety of effects on the growth of *B. viridis*. (See Figure 14) They are summarized here and will be discussed subsequently.
Figure 14: The Response of *B. viridis* to Alternative Carbon Sources. A) Compounds were added at 20 mM as sole carbon sources. Sucrose and glucose were both readily assimilated in the absence of malate. Citrate and acetate were not readily assimilated, and a significant reduction in rate was observed. For acetate alone, the rate was reduced below that of previously observed carbon free growth rates. B) 20 mM of each compound was supplemented to 20 mM malate, giving a total carbon content of 40 mM. The growth rate with glucose was not significantly changed from its growth as a sole carbon source. The addition of sucrose did not significantly alter the rate of growth from control. The growth rate for citrate and acetate was improved in the presence of malate, but still less than control, indicating that these compounds may act to inhibit growth.
• Sucrose and glucose, either in the absence or presence of malate, increased the culture growth rate compared to malate alone.

• Both citrate and acetate in the presence or absence of malate led to a reduced growth rate compared to the control, but to a lesser extent with malate.

• The pH change in the culture was consistent with the observed growth rates.

• The pigment ratio was fairly consistent with the growth.

• Glucose or sucrose will support growth of *B. viridis*. The growth rates without malate for each of these sugars was slightly higher than with malate.

The observed growth rates for malate only and malate plus sucrose are similar, suggesting that malate is the preferentially utilized compound. Despite this apparent preference for malate, sucrose can still successfully support chemoheterotrophic growth.

The observed rate of growth for glucose and glucose plus malate cultures is similar, suggesting preferential use of glucose, even in the presence of malate. While glucose supplementation does assist in generating a greater growth rate than malate cultured cells, an increase of approximately 0.05 a.u./day, and more overall growth. The pigment ratio is reduced to 0.81, lower than the control malate grown culture, 0.86. This strain of *B. viridis* can grow chemoheterotrophically, reducing the need for the photosynthetic apparatus and causing a decrease in the amount of pigment produced. Therefore, the decrease in pigment ratio suggests a non-photosynthetic metabolism. The photosynthetic abilities of the bacteria are what make them attractive; they can drive unfavorable reactions with their supplemental energy generation. In this case, gaining additional growth by adding a very easily usable carbon source would not support the goal of generating a working system for the destruction of environmental contaminants.
Neither citrate nor acetate leads to improved bacterial growth. The enzyme isocitrate lyase, which participates in the assimilation of citrate, is present only in exceedingly small concentration in \textit{B. viridis}.\textsuperscript{13} This would make the assimilation of citrate sluggish. Since acetate requires several prior enzymatic steps and then funnels through the same enzymatic pathway, the low level of isocitrate lyase can create a bottleneck for assimilation of both carbon sources. (See Figure 4.3)

5.3.5.3 Fumarate as a malate alternative

Fumarate is viable a substitute for malate. In carbon free media (0 mM fumarate, NC media), the growth rate for unsupplemented media was extremely low. (See Figure 15) For NC media supplemented with fumarate, the growth rate was similar to the growth rate for 20 mM malate (0 mM fumarate, Minimal media) for all concentrations. The pigment behavior was consistent with the growth rates and showed no remarkable differences as a function of fumarate concentration in either Minimal Media or NC media. The pigment ratio, A1020/A660, was around 0.85 for all cases. The pH of the culture was also tracked as before. There was no significant variation in the culture pH between conditions, except that no pH change was observed in the carbon free control. This lack of pH change was consistent with the significantly reduced rate of growth.

Because fumarate can be used for growth, it is a confirmation that the TCA cycle enzymes fumarase and malate dehydrogenase are functional in \textit{B. viridis}. (See Figure 4.3 and Section 4.3.2.3) In addition, since it is a viable substitute for malate, fumarate can be readily exchanged for malate if material costs, culture conditions, or upstream processes make the use of fumarate more convenient than malate.
5.3.5.4 Optimum concentration of malate

Malate is a common carbon source for all bacteria, and is the only compound universally utilized by purple non-sulfur bacteria.\textsuperscript{14,15,16,17,18,19} Malate provides results roughly equivalent or superior to all other carbon sources assayed in this work and supports photosynthetic growth. Supplementing malate can act as a metabolic boost, bypassing the requirement for TCA enzymes not present in the genome or not expressed by \textit{B. viridis}. Since a number of photosynthetic bacteria are unable to utilize citrate, including \textit{B. viridis}, as demonstrated here, this bypass is particularly important.\textsuperscript{20} In addition to growth employing the TCA cycle, photosynthetic bacteria have also been

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure15.png}
\caption{The Effect of Varying Concentrations of Fumarate in Minimal and NC Media on the Growth of \textit{B. viridis}. The carbon free control has a significant reduction in growth, to less than half of the Minimal control. Supplementation with fumarate in carbon free media brings the growth rate up to around the same level as the malate containing control. A slight decrease in growth rate is observed at the 30 mM concentration for both media types.}
\end{figure}
known to utilize malate through alternate pathways. Some species are known to catabolize malate to glyoxylate and acetyl-CoA by a reversal of the glyoxylate cycle. Evaluating the necessary concentration of this highly relevant compound is crucial to obtaining the optimum growth.

As shown in Figure 16, malate supports growth at intermediate concentrations and inhibits growth at high concentrations. Up to approximately 50 mM concentration, increasing additions of malate contribute to greater growth. Above 50 mM, the rate of growth experienced by the culture begins to decrease, and by 160 mM malate, the growth rate is less than that for carbon free media. A second order polynomial can be fit to the data, and integration indicates that the function is maximized at 50 mM. (See Equation 1)

\[
\mu = -1 \times 10^{-5} [\text{Malate}]^2 + 0.001 [\text{Malate}] + 0.1422
\]

\[
R^2 = 0.913
\]

Figure 16: The Relationship between Growth Rate of *B. viridis* and Malate Concentration. Up to approximately 50 mM, increasing the malate concentration leads to a greater rate of growth. Above 50 mM, the rate of growth begins to fall off. At 150 mM, the rate of growth is less than the rate of growth with no carbon source provided, indicating that at high concentrations, malate can act as an inhibitor.
\[ A_{660} = -1 \times 10^{-3} [\text{Malate}]^2 + 0.001 [\text{Malate}] + 0.1422 \] (11)

Despite pH adjustment of the malate solution to 6.9, its addition in large volumes caused a slight decrease in initial pH, relative to the amount added. Below 150 mM, the effect was minor, causing less than 0.1 pH units of change, and no notable effect on growth rate. At 160 mM malate, the initial pH of the culture was decreased by nearly an entire pH unit to 5.9, and the final pH was unchanged relative to the initial measurement indicating only minor growth. The pigment behavior mirrors the growth behavior, and there is no distinct change in relative pigment production with a change in malate concentration. Based on these results, the following it can be concluded that the optimum malate addition is 50 mM.

For cultures with an extremely high concentration, the volume of malate solution added produced a significant dilution. This reduces the concentration of all the other nutrients in the media, limiting their availability and could cause a reduction in growth. This dilution could also lead to nitrogen starvation by reducing the final concentration of ammonium in the culture media. In addition, the pH was reduced by nearly a pH unit at the highest malate additions, and acidic pH could have a significant inhibitory effect on growth. Several experiments have demonstrated significant pH sensitivity for this bacterium. Although a given optimum pH of 6.9 was true in RMPABA, the acceptable pH range for optimum growth and the sensitivity to a pH shift in Minimal media is unknown. To explore these potential reasons for growth inhibition, the effects of nitrogen source and added fixed nitrogen, as well as buffering and pH environment were tested.
5.3.6 Nitrogen

Without yeast extract and bactopeptone, a variety of rich organic compounds, some containing nitrogen, are no longer available for the growth of *B. viridis*. After carbon, nitrogen is one of the most important compounds for cell synthesis. In photosynthetic bacteria, nitrogen can be obtained by assimilation of organic nitrogen, utilization of ammonia, or fixing molecular nitrogen from the atmosphere. Photosynthetic bacteria are among the few types of bacteria containing the enzyme nitrogenase which fixes molecular nitrogen.²²,²³ By supplementing ammonia, nitrogenase can be repressed and the enzyme pathway blocked. In some cases, this is reversible, and in other cases, it appears to be permanent. Particularly in *B. viridis*, a recovery of the enzyme is not observed if prior generations of the culture have been supplemented with ammonia.²⁴ If nitrogenase has been permanently repressed in the cultures used in this work, an adequate ammonia addition is critical. In addition, nitrogen fixation is an extremely energetically involved process; providing fixed nitrogen will reduce the energetic burden on the cells, allowing them to devote energy to other processes such as degradations.

In addition to nitrogenase repression by the presence of ammonia, “oxygen stress” can also repress nitrogenase formation.²⁵ The presence of oxygen has been shown to control nitrogen fixation at the transcriptional level, in the accumulation of the nitrogenase proteins, and by the cellular fixation activity.²⁶ The nitrogenase complex is easily destroyed in the presence of oxygen, so many aerobic organisms have developed protective mechanisms. Since anoxygenic bacteria are extracted from oxygen free environments, these protective mechanisms are not necessarily present for PSB.²⁷ Further, while a number of photosynthetic bacteria can grow chemoheterotropically in
aerobic or microaerobic conditions in the dark, they are typically unable to grow photosynthetically in the light in the presence of oxygen\textsuperscript{28} because oxygen inhibits bacteriochlorophyll synthesis.\textsuperscript{29} In photosynthetic literature, this assumption is so ubiquitous that “phototrophic” conditions are defined as light present and oxygen absent, and “aerobic” conditions are defined as oxygen present and in darkness.\textsuperscript{30,31,32}

To quantify the effect of nitrogen and oxygen on \textit{B. viridis}, several factors were investigated. First, the effect of ammonia supplementation at the standard Minimal media concentration on the growth of \textit{B. viridis} was considered, both in the presence of nitrogen and in the presence of other atmospheres. In addition, this growth was compared to growth in the absence of fixed nitrogen in both nitrogen and oxygen atmospheres. The pigment production was also compared under oxygen and nitrogen equilibrated media. Second, to insure that the lack of growth observed in Minimal media was not a result of nitrogen shortage caused by the removal of the undefined organic components, ammonium sulfate was provided at a variety of molar concentrations to study the effect of fixed nitrogen supplementation at varied concentrations.

5.3.6.1 Molecular nitrogen assimilation and headspace gas

\textit{B. viridis} was grown in the presence and absence of fixed nitrogen, in the form ammonium sulfate, to media sparged for 30 minutes with headspace gas of molecular nitrogen (N\textsubscript{2}), argon, or air and then capped. No significant difference based on the type of gas sparged was noted for cultures growth in the absence of fixed nitrogen. In the absence of fixed nitrogen, the growth rates were significantly reduced, to around 0.05 a.u./day, close to the base growth expected in carbon and nitrogen free media.(See 5.3.3). (See Figure 17) A significantly reduced rate is noted by other researchers for other cells
as well; for *Rhodopseudomonas palustris*, the growth rate is reduced by 50% and the cell yield is significantly reduced when fixing nitrogen compared to when it was provided with fixed nitrogen as NH$_4^+$. The poor growth of cells in the presence of N$_2$ gas suggests that the culture has experienced inhibition of its nitrogenase, which is either permanent or may require several generations in the absence of fixed nitrogen to be induced.

In the presence of fixed nitrogen, the growth rates were universally on the order of three times higher than without fixed nitrogen. The rate of growth and the total growth observed were nearly 20% greater in the presence of oxygen accessible in the air sparged media compared to either the presence of sparged nitrogen or sparged argon. The final

![Figure 17: The Effect of Nitrogen, Argon, or Air as Sparged Gases in the Presence and Absence of Fixed Nitrogen. Growth is significantly reduced in the absence of fixed nitrogen, to around 0.05/day, only slightly more than growth in carbon free media. In the presence of ammonia, growth rates are on the order of three times greater, however the unexpected result of a greater growth rate in the presence of Air vs. Nitrogen or Argon was noted.](image-url)
density of the air sparged condition was around 0.4 absorbance units higher than the nitrogen sparged condition. There was a very slight decrease in the final pigment ratio for the cells grown in air sparged media, 1.12, compared to the final ratio for cells grown in nitrogen sparged media, 1.17, suggesting that while there is some difference in total growth, there is very little physiological difference in pigment formation based on the headspace gas, or the presence of oxygen.

Observation of phototrophic growth in ammonia supplemented air sparged media was surprising; the literature suggests that photosynthetic growth of *Rhodospirillaceae* and their pigment production is inhibited by oxygen. (See Chapter 4.2.6) The slightly decreased pigment ratio in the presence of oxygen was too small to indicate significant inhibition of pigment production and exclude photosynthetic growth. This result confers an advantage to *B. viridis* in process development. Because it is not susceptible to significant oxygen damage, and growth is, in fact, slightly enhanced by the presence of oxygen, maintaining strictly anoxygenic conditions is not necessary. This facilitates its use in conjunction with currently existing systems, where maintaining strict anoxygenic conditions is difficult or impossible without a major investment in new infrastructure.

### 5.3.6.2 Fixed nitrogen (ammonium) concentration

Supplementation of ammonium, as ammonium sulfate, at any concentration improved the growth rate of *B. viridis* by around 30%. (See Figure 18) The cultures were prepared in an air environment, but not sparged, based on the previous finding that oxygen was not a factor in pigment damage. The amount of supplementation did not seem to be as important as the presence of supplementation. The pigment composition of the cultures was nearly identical in all cases except no supplementation, and displayed
similar A1020/A660 ratios. Without supplementation, the final pigment measurement was lower, but consistent with the noted reduction in growth rate. The final pH of the cultures was similar for all conditions. In the nitrogen free condition, which had no ammonium sulfate addition, the final pH was similar to the other cultures despite slower growth, suggesting that the ammonium sulfate may help with buffering the culture.

This indicates that nitrogen in the standard Minimal media formulation is present in excess with relation to the growth observed under the current standard condition. While lack of fixed nitrogen further suppresses the growth, in order to improve the growth in Minimal media, some other causative factor for the low growth must be determined.

![Figure 18](image_url)

Figure 18: The Effect of Varying Concentrations of Ammonium Supplementation on the Growth Rates of *B. viridis*. Supplementation at any concentration gives significantly improved results compared to nitrogen free media, however there is no specific optimum concentration, and as long as some concentration of nitrogen is provided growth is enhanced. This suggests that as little as 1 mM is adequate to provide fixed nitrogen to the culture during the first 5 days of growth.
5.3.7 Potassium phosphate buffer concentration

Control of the system pH by means of a buffer is very important.\textsuperscript{34} We have seen this demonstrated with \textit{B. viridis} by the intrinsic pH limit of the culture and the response to a pH shift from bicarbonate addition. Growing \textit{B. viridis} cultures produce base, and using more concentrated buffer counteracts this effect and neutralizes the base. Once the buffering capacity is spent, no further buffering is observed, and the pH begins to increase. In this experiment, maintenance of pH near the optimum, around 6.9, was the goal, so the pH results are presented in their entirety in addition to growth rate result.

Buffering had a significant positive impact on the growth of \textit{B. viridis}. Up to a concentration of 12 mM, an increase in buffer concentration was correlated with a higher growth rate. (Figure 19) This concentration of buffer, double the standard, was sufficient to maximize the growth rate, but additional buffer offered added control over the culture pH. The highest concentration of buffer leads to the smallest pH change during the course of the culture’s generation of base. (Figure 20, A and B) With the exception of unbuffered media, which shifted to an initial pH of 7.5 during sterilization and preparation, all cultures started at pH 6.9 after adjustment.

It was interesting to note that the highest pH achieved, even in the unbuffered condition, was 8.5, which is noted as the optimum external pH for chloroplasts.\textsuperscript{35} Chloroplasts are membrane-based structures found in photosynthetic eukaryotes, similar to thylakoids in bacteria; both contain chlorophyll compounds to harvest light. The light energy is used to drive the production of a H\textsuperscript{+} gradient across the membranes, which can be used to produce chemical energy, which is later stored in the form of ATP. This H\textsuperscript{+} gradient affects the local pH, and there must be a ΔpH across the membrane in order to
generate energy. Given the structural and functional similarities between chloroplasts and thylakoids, it is possible that once the culture media reaches pH 8.5, the ΔpH, or pH gradient, can no longer be efficiently formed within the thylakoids, and energy harvesting is shut down completely. This would significantly impair the ability of the bacteria to grow with any metabolism requiring photosynthetic activity and would also reduce the growth and growth rate.

Figure 19: The Growth Rate of *B. viridis* in the Presence of Varying Concentration of Potassium Phosphate Buffer. Above a doubled concentration, 12 M total buffer, no additional growth rate enhancement was observed with additional buffering. At double and above the standard concentration, a growth rate enhancement of about 15% was observed, bringing the total growth rate for these cultures to around 0.2/day. At lower buffer concentrations, a decrease in growth was observed, to only 2/3 of the normal growth rate, 0.1 a.u./day, in the absence of buffer.
Figure 20: The Final pH (A) and pH Change (B) after 5 days of Growth in Varying Concentration of Potassium Phosphate Buffer. In the unbuffered and half standard concentration, the final pH reached nearly 8.5, a total change of 1 and 1.5 pH unit respectively. The unbuffered media experiences a significant increase in initial pH after sterilization, to initial pH 7.5. For the remaining cultures, there was a steady, nearly linear decrease in the final pH as well as the total change in pH for the culture with increasing buffer concentration. This demonstrates that with increased buffering, the culture conditions are maintained closer to the desired pH.
5.3.8 The acceptable pH range for growth

The pH of the culture has been demonstrated peripherally in other experiments to have a critical impact on the ability of the culture to grow and flourish. First, the optimum pH for culture growth must be determined. Second, because Minimal media is a modification of the rich growth conditions observed in RMPABA and wild type growth, it must be determined how this change in media formulation affects the optimum pH range. The response of the culture to varying pH in RMPABA was studied as a control. With the RMPABA data for comparison, the response of the culture to varying pH in Minimal media was studied to determine the optimum pH under the more restricted media composition, and these changes are discussed to try to understand the significant changes observed.

5.3.8.1 RMPABA

Growth in RMPABA media is observed between pH 5.6 and pH 8.5, with the optimum growth observed for pH 6.2-6.5. (See Figure 21) There is a slightly increased growth in each subsequent generation, especially where growth is vigorous (pH 5.8-pH 8.0). The growth below pH 5.6 drops off sharply from nearly maximum growth to no growth observed within 0.4 pH units. For pH values above the optimized growth point, growth drops off slowly, almost linearly, until no further growth is observed above about 8.5. Below 5.6 and above 8.9, growth was not observed in the first generation and it was determined that the cells were no longer living because no growth was observed upon inoculating into standard rich media. Cultures that did not grow were not carried into subsequent generations.
The final pH of RMPABA cultures was determined and the maximum pH achieved for any culture, regardless of initial pH, was in the vicinity of 8.5 pH units. (See Figure 22) This seems to be a fundamental limit for the strain, because negligible growth is recorded in cultures that are initiated at values above this. Below 5.6 and above 8.9, no growth was observed in the first generation, and cultures were therefore not carried into subsequent generations.

Figure 21: The Total Growth, Measured by A660, Observed in RMPABA Media Adjusted to Various pH Values. Growth is observed between pH 5.6 and pH 8.5, with the maximum growth observed in the vicinity of pH 6.5. There is a slightly increased growth in subsequent generations, especially where growth is vigorous (pH 5.8-pH 8.0). Below 5.6 and above 8.9, no growth was observed in the first generation, and cultures were therefore not carried into subsequent generations.

The final pH of RMPABA cultures was determined and the maximum pH achieved for any culture, regardless of initial pH, was in the vicinity of 8.5 pH units. (See Figure 22) This seems to be a fundamental limit for the strain, because negligible growth is recorded in cultures that are initiated at values above this. Below 5.6 and above 8.9, the data falls along a 45-degree line, indicating that there is no net change in pH. The acidic pH values increase slightly, and the basic pH values decrease slightly from initial. This minor shift is most likely due to the buffer in the media, as there is no growth activity from the bacteria that would induce a change in pH.
The change in pH was loosely correlated with the change in culture density. (See Figure 23) This behavior was consistent in all generations of growth, and confirms base release. This base release is most likely tied to the chemical gradient generated during photosynthesis. While this correlation is not tight and can not be used to quantify growth, it does demonstrate the behavior of the bacterium: more growth is associated with a greater change in pH. More pigment production was observed for cultures that generated more total biomass. Except for cultures that were significantly damaged (below 5.6 and above 8.9), the pigment ratio was similar for all cultures.

Figure 22: The Final pH for Cultures Based on Their Initial pH. The maximum pH achieved, regardless of initial pH, was in the vicinity of 8.5. Where no growth was observed, below 5.6 and above 8.9, a change in pH was not noted, and the values fall along a 45-degree line.
5.3.8.2 Minimal media

For Minimal Media, the growth is presented as the ΔA660, defined as A660final – A660initial, or change in the absorbance at A660, because a 20% inoculation shifts the baseline significantly, making visualization of final culture density (A660) more difficult. In addition, in some of the less vigorous cultures, even collection of cells from the entire vessel would not allow a significant inoculation to be made. By presenting the change in culture density instead of the total culture density, this effect is normalized. The first generation of growth, though reduced in total growth, demonstrated similar characteristics as RMPABA growth. Growth in the first generation is observed between pH 5.4 and pH 8.5, with the optimum growth observed for pH 6.2-6.5. (See Figure 24)

Figure 23: A Loose Correlation between Growth and pH Change for RMPABA Grown Cells. The growth of the bacteria is loosely correlated to a pH change in; the greater the pH change, the greater growth obtained in RMPABA. Because of the fundamental pH limitation of 8.5 for RMPABA, the lower the initial pH, the greater culture growth can be obtained.

\[ y = 1.5599x + 1.0153 \]
\[ R^2 = 0.6728 \]
This mimics the wide pH window for growth observed in RMPABA media, indicating that the first generation inoculated in Minimal media from RMPABA culture is still functioning in the same metabolism as used in RMPAMA media. After 5 days, the culture was subcultured to fresh media and the pH range in which growth was detected narrowed significantly. After the metabolic shift, growth was observed only between pH 5.4 and pH 6.2. In the range where growth occurs, more growth was observed in each subsequent generation.

Figure 24: The Change in Culture Density for Cultures Grown in Varied pH Minimal Media. Growth in minimal media is strongly pH dependant. In the first generation, the behavior is similar to that of RMPABA and the good growth area spans 3 pH units, indicating that the culture remains in the same metabolism for some time after subculturing. In the second and subsequent generations, the pH envelope narrows and growth is limited to one pH unit with a maximum at 5.6. The red lines on the graph indicate this region and optimum.
The growth rate behavior of the cultures demonstrate the same trends as the total growth behavior, as would be expected. (See Figure 25) The cultures achieved growth rates as high as 0.2 a.u./day at their maximum, and the rates decreased significantly at non-optimal pH values. In some of the later generations, the culture can actually be seen to decrease in total density slightly at pH values below 5.2 and above 6.2, giving negative growth rates. This suggests that the cells are decomposing at these pH values. The total pigment increase, and pigment ratios are consistent with the growth noted, and do not show any unexpected behavior. Where more growth is observed, more pigment
formation was observed. Within the same generation, the pigment ratios were similar for all cultures in which growth was noted.

The maximum pH achieved in the first generation was about 8.0, which is similar to the fundamental limit for the RMPABA strain. (See Figure 26, Compare to Figure 22) The final pH was somewhat affected by the initial pH; cultures with a higher initial pH generally had a higher final pH. In the subsequent generations, where the pH optimum shift was noted, the maximum pH obtained for cultures that demonstrated growth is in the

![Figure 26: The Final pH of Cultures Grown in Minimal Media Based on Initial pH. The first generation behaved similarly to RMPABA growth. In subsequent generations, a lower maximum pH around pH 7.0 was obtained. Where no growth was observed, a significant change in pH was not noted, and data points fell approximately along a 45-degree angle.](image-url)
vicinity of pH 7, and no change in pH is noted above this value. At some of the higher pH values, a slight decrease in pH was noted, and values fell below the 45-degree line in the figure. Since no growth was noted in these cultures, this is most likely a result of the buffer present in the media acting over time. The shifted pH maximum may be a fundamental value for culture growing in Minimal media, or it may be a result of the less vigorous culture growth noted in Minimal media.

Figure 27: The Correlation of pH Change to Change in Culture Density, as Measured by A660. An increase in pH change is loosely correlated with greater change in culture density, however there is a shift in magnitude between the first and subsequent generations. The slope for all generations is similar, but each generation has fewer and fewer pH values at which growth is observed, making it difficult to definitively identify a trend in later generations. Values along the Y-axis zero experienced no growth, and therefore pH change is not tied to a change in A660.
As expected from the example of RMPABA media, a greater change in pH typically corresponded to a greater amount of cell production, however even a loose correlation could not be determined for this data. (See Figure 27) Due to the significantly smaller pH range in which growth is observed, a considerable number of data points represented cultures that experienced no growth; these cultures fall along the X axis and were not considered in determining a correlation. This leaves relatively few points with which to define a trend. The data in later generations suggests that the increase in cell density per unit of pH change is approaching levels near the behavior observed while the cells were still in their original metabolism.

5.3.8.3 Discussion of pH behavior and changes in response to media

In RMPABA, growth over a wide range of pH values was observed. In this media, there are many available carbon options, and many possible metabolic pathways. The greatest total growth was achieved by adjusting the starting pH to between 6.2 and 6.5. This is lower than the pH 6.9 optimum suggested for the RMPABA media formulation, however it provides the bacteria a wider window of growth before they produce too much base and bring the culture above pH 8.5.

In Minimal media, optimum growth in successive generations was observed within a 1 unit window around pH 5.6. This range is a significantly narrower envelope than the 3 unit envelope around the optimum pH 6.5 observed for RMPABA media. In contrast, growth in Minimal media was not sustained beyond the first generation at pH 6.9, the suggested optimum pH for growth in RMPABA media. This is significant,
because it suggests that a different metabolism may be favored when cultures are restricted to the nutrients in Minimal media. Assimilation of malate, the sole carbon source available, for photoheterotrophic or photoautotrophic growth appears to be optimized around pH 5.6. A pH optimum shift can occur for different pathways based on enzyme pH optimums. Malate dehydrogenase is the primary enzyme interacting with malate to form oxaloacetate in the TCA cycle. Fumarate hydratase, often called fumarase, generates malate by condensing fumarate with water, but can also allow the reverse reaction in the anabolic TCA cycle. A thorough investigation of two these two enzymes, which interact with malate in the TCA cycle, in the enzyme databases BRENDA\textsuperscript{36} and EXPASY\textsuperscript{37} indicated that both of these enzymes function over a wide range of pH from 4-9. Ubiquinol-cytochrome-c reductase, a photosynthesis enzyme, also function over this range. This suggested that enzyme limits were not a direct factor in the pH shift observed in the Minimal media growth of \textit{B. viridis}.

In photoheterotropic conditions, malate can be used both as a carbon source and electron donor, however if the carbon is in its acid form, and therefore relatively reduced, an electron sink such as CO\textsubscript{2} is required to maintain redox balance.\textsuperscript{38} When CO\textsubscript{2} is dissolved in water, the molar concentration of carbonic acid, H\textsubscript{2}CO\textsubscript{3}, is approximately 10\textsuperscript{-5}M. In the case of a sodium bicarbonate addition, the equilibrium would be shifted toward a more basic pH as CO\textsubscript{2} is released. The chemical equilibrium of CO\textsubscript{2} in water is governed by Equation 2 and Equation 3.
Based on the equilibrium, the concentration of H\(^+\) ions is approximately 2.2x10\(^{-6}\), which results in a pH of 5.6\(^{39}\). This acidic pH is the pH of acid rain and the subsequently acidified lakes, from which many species of *Rhodospirillaceae* are isolated\(^{40}\). This is also exactly the pH at which the growth of *B. viridis* is optimized, and can be sustained from generation to generation, suggesting that *B. viridis* requires CO\(_2\) as an electron sink to be able to sustain photoheterotrophic growth on Minimal media using malate.

As the pH of water changes, different forms of inorganic carbon predominate\(^{41}\) (See Figure 28). The growth maximum coincides with the range of pH where carbonic acid and free dissolved carbon dioxide predominate. The narrow window of growth for *B. viridis* in Minimal media can be explained by considering both the inorganic carbon behavior and the RMPABA behavior. If CO\(_2\) must be available, growth cannot occur until the pH drops below around 6.2 and CO\(_2\) becomes predominant, establishing an upper pH limit for growth in Minimal media. The growth in RMPABA, which displays a wide pH range, probably taking advantage of multiple metabolic possibilities, drops off sharply below about 5.4, suggesting a fundamental limitation for the species. This establishes a lower pH limit for the species. In this experiment, growth of *B. viridis* in

\[
\text{H}_2\text{CO}_3(aq) \rightleftharpoons K_1 \text{H}^+(aq) + \text{HCO}_3^-(aq) \\
K_1 = 4.2 \times 10^{-7}
\]

\[
\text{HCO}_3^-(aq) \rightleftharpoons K_2 \text{H}^+ + \text{CO}_3^{2-}(aq) \\
K_2 = 4.8 \times 10^{-11}
\]

(12) (13)
Minimal media was observed in the range 5.4 to 6.2, which corresponds to the boundaries suggested by the inorganic carbon behavior and the fundamental limitations for the species. This also suggests that in media shifted to pH 5.6, supplementation with carbon dioxide or bicarbonate is needed because the only remaining metabolic pathway is photoautotrophic growth. Now that the culture pH conditions for photoautotrophic growth are known, optimization of nutrient concentrations and the addition of vitamins need to be reevaluated for their effect on photoautotrophic metabolism to determine if the

![Figure 28: The Behavior of Inorganic Carbon Dissolved in Water at Various pH Values. Saturated carbon dioxide in water has a pH around 5.6. Below that, dissolved CO₂ and carbonic acid predominate. Between pH 6 and pH 10, bicarbonate predominates, and above pH 10, carbonate predominates. The vertical dashed line indicates pH 5.6, the growth maximum for B. viridis in Minimal media. This coincides with the region where free CO₂ predominates.](image_url)
limited growth observed for Minimal media can be improved by instead optimizing the media formulation at pH 5.6

5.4 Conclusions

The growth of *B. viridis* in Minimal media was investigated with the purpose of improving the growth rate and total growth and bringing it from its original 20% of the maximum observed in RMPABA to something much closer to the maximum. Improving the pigment ratio and making the bacteria more capable of photosynthesis was also desired. The treatment of the cells, vitamin supplementation, carbon source, nitrogen source, and buffering and pH were considered. The major outcomes and conclusions for the optimization of *B. viridis* growth in Minimal media at pH 6.9 and the exploration of its pH optimums are listed below:

- To establish maximum growth potential, a larger inoculation using a standardized, concentrated culture with waste media removed, of A660 = 25 a.u. was necessary for Minimal media grown cultures; about 20% by volume produced the optimum growth rate and total growth. The process of washing and concentrating caused no significant damage to the cells or their subsequent growth.
- The concentration of *p*-ABA, thiamine, Supersalts and the presence of folic acid, biotin, niacin, pyridoxine, B12, cysteine, pantothenate, bicarbonate did not influence culture growth at pH 6.9.
- An optimum concentration of malate, the preferred carbon source for photoheterotrophic growth, was identified as 50 mM. Glucose, sucrose, and
fumarate can be used in the place of malate with similar growth potential. Sodium acetate and sodium citrate inhibited growth regardless in the absence and presence of malate. Exogenous CO₂ did not improve the growth rate of *B. viridis*.

- Cells were found to be incapable of fixing molecular nitrogen, and were able to utilized fixed nitrogen even in air-sparged cultures. Ammonia (as little as 1 mM) enhanced growth three-fold over cells which had only molecular nitrogen available. The presence of an aerobic environment caused no detectable damage to the photosynthetic pigments.

- *B. viridis* is significantly pH sensitive. Additional buffering improves growth. Multigenerational growth in Minimal media was significant in the pH range 5.4-5.8 with an optimum at pH 5.6, whereas in RMAPA growth occurs from 5.6 to 8.5 with an optimum pH in the range 6.4 to 6.5. The range of 5.4-5.8 was correlated to higher concentrations of free dissolved carbon dioxide.

5.5 References


2. Toennies, 707-713.


8. Shuler, 163.


17. Shaw, 466-472.


29. Cellarius, 234-244.
30. Tae Ho Lee, 91-96.
31. Moschettini, 302-309.
32. Lang, 2827-2834.
33. Yoch, 657-676.
34. Shuler, 163.
36. BRENDA, The Comprehensive Enzyme Information System, Release 5.1 <www.brenda.uni-koeln.de/>
CHAPTER 6

OPTIMIZING THE GROWTH OF B. VIRIDIS IN MINIMAL MEDIA AT PH 5.6

6.1 Introduction

In Chapter 5, the effort to optimize the photosynthetic growth of B. viridis in Minimal media led to the conclusion that the primary factor limiting sustainable culture growth was the pH environment of the media. In Minimal media adjusted to pH 6.9, culture survival could not be supported for more than a single generation (See Chapter 5.3.8.2). Although the first generation growth rate in Minimal media adjusted to pH 5.6, was just over 0.15 a.u./day, slightly less than the first generation growth rate in Minimal Media at pH 6.9, (See Figure 5.25) stable growth at this rate was obtained in at least 20 generations.

In Chapter 5, the mechanical effects of concentration were established as safe and non-damaging to the cells, and removing the spent media was determined sufficient to avoid artifacts from previously present nutrients or waste products. (See 5.3.2) Those results were considered sufficient, and the same mechanical processing of inoculating cultures was employed. In addition, the effect of oxygen caused no significant decrease in pigment formation. (See 5.3.6.1) Since the basic structural composition of the cell is not expected to change as various enzyme pathways are invoked, it was anticipated that
pigments would continue to resist oxygen damage. The preparation of vessels was subsequently performed in an air atmosphere.

In this chapter, those conditions presented in Chapter 4 and explored in Chapter 5 with some expected impact on PNSB cell growth are revisited for Minimal media at pH 5.6. To account for any changes in cell signaling based on the shifted metabolism, the effect of inoculation volume was considered. In addition, the same vitamins and micronutrients explored in Chapter 5 were revisited here; thiamine, p-ABA, and the mixture “Supersalts” were investigated at three concentrations and the vitamins biotin, pantothenate, niacin, pyridoxin (vitamin B₆), folic acid, l-cystein, and vitamin B₁₂ were investigated at the single concentration 2μM. The carbons malate, bicarbonate, and fumarate were investigated as likely participants in photoheterotrophic growth, and sucrose, a sugar, was investigated to determine whether heterotrophic growth was occurring. Having established that nitrogenase is not active in this strain (See 5.3.6.1), the optimum concentration of ammonia was explored. Since the pH is recognized as a primary factor in growth, the concentration and type of buffering system was considered; phosphate (useful in the range pH 5.9- pH 8.0), carbonate (useful in at neutral or basic pH, with pKₐs 6.36 and 10.32 respectively), and acetate (useful in the range of pH 3.7-pH 5.8) buffers were examined. Because the bacteria are speculated to employ a primarily phototrophic metabolism, two new factors were investigated: 1) the total culture volume and its resultant surface area, and 2) the light flux incident on the culture surface.
6.2 Materials and Methods

The methods utilized in the optimization of media adjusted to pH 5.6 were similar to the methods used in Chapter 5. Culture preparation was done in a sterile air atmosphere. The materials used were identical to those employed thus far. (See 5.2.1) Minimal media was prepared, adjusted to pH 5.6, sterile filtered, distributed to smaller experimental vessels of 50 mL or 250 mL, and subsequently inoculated using a concentrated broth. The headspace comprised approximately 22% of the total volume of the 250 mL vessels and 40% of the total volume of the 50 mL vessels. Because the shift to pH 5.6 allowed consecutive generations of B. viridis to be cultured in Minimal media, a Minimal 5.6 maintained inoculating culture was established and used in all inoculations. Concentration of these cells was performed as described in Chapter 5 (See 5.2.3.1 and 5.3.1). Cells were centrifuged to remove spent media and then resuspended at 1/10th of their initial concentration, which typically yielded an inoculating broth with a density of 10-30 a.u., and inoculations were adjusted to achieve the optimum total cell inoculation, an initial culture density of 0.15-0.25 a.u. (typically 20%-30% by volume), determined as optimum in this chapter. (See 6.3.2 and 6.3.3) Where a current component of the media or an alternative carbon source was investigated, the component of interest was left out of the media batch and amended as a sterile pH 5.6 adjusted solution to achieve the desired experimental concentration. Each condition was repeated in triplicate to insure repeatability. The full spectrum absorbance, including A660 and A1020, and pH were measured at the time of inoculation and again on day 12, unless otherwise noted.
6.2.1 Baseline growth behavior determination

To provide a baseline against which growth behavior could be compared, a growth curve for *B. viridis* in Minimal media adjusted to pH 5.6 was determined. Three liters of Minimal media were prepared according to the initial formulation and adjusted to pH 5.6. The media was then sterile filtered to three one-liter vessels and inoculated with 20% by initial volume concentrated cells grown in Minimal 5.6. The spectrum, pH, and live cell count were taken approximately every 24 hours until stationary growth was achieved, around 30 days. From the spectrum, the wavelengths 660 nm and 1020 nm were extracted to give a measure of the culture density and the pigment formation. The characteristics of growth, summarized in Table 3.1, were either recorded or determined by calculation as before.

6.2.2 Modification of the Two-Point Method

The Two-Point technique was developed to allow large-scale screening, and discussed in Chapter 3. (See 3.2.3 and 3.3.3) Based on a growth curve determined for Minimal media adjusted to pH 5.6, the Two-Point technique was modified to observe the most relevant portion of the growth curve. In Minimal media adjusted to pH 5.6, the culture underwent an extended lag period of approximately 5 days before increasing in culture density. Since the lag phase is a period of negligible change in culture density, the density measurement obtained on the first day of growth was assumed to be roughly equivalent to the density of the culture on the fifth day of growth. In order to observe the curve during the most relevant period, the growth rate was calculated as if measured between the 5th and 12th day of culture. To minimize the required operational time, the
cultures were inoculated and immediately measured, and then subsequently measured on
the 12th day of growth. The error was calculated as described in Chapter 3.2.3, using the
minimum and maximum initial and final data to determine the slowest and fastest
possible rates of growth to give boundaries on the averaged growth rate.

6.2.3 Inoculation concentration

To determine the effect of varying inoculation concentration, cells growing in
Minimal 5.6 media were concentrated by centrifugation, separated from the spent media,
and resuspended at 1/10th of their initial volume, a broth with optical density at 660nm of
approximately 10 a.u. Concentrations between 1% and 80% by culture volume, based on
the original volume of inoculating broth, were used to inoculate the experimental vessels.
In addition, unconcentrated cells were also used to inoculate vessels at 2%, 20%, and
40% by volume to contrast the effect of including spent media. The unconcentrated broth
had an absorbance of 1.1 a.u. at 660nm.

Identical sterile vessels were prepared, and adequate fresh media was removed as
necessary to allow space for larger volume inoculations, above 20% concentrated, as well
as 20% and 40% unconcentrated. Absorption at A660 was measured at the time of
inoculation, and again on day 12. Because of the discrepancy in the final density of the
inoculating solution, the results are considered both in terms of concentration by percent
pre-concentration volume, as well as by the initial density of the culture.
6.2.4 Vitamin supplementation

To investigate the effect of vitamins, two separate methods were employed. First, Supersalts and p-ABA free Minimal media (SS/p-ABA Free) was made and adjusted to pH 5.6. This media was distributed to experimental vessels and supplemented with Supersalts, Thiamine Free Supersalts, and p-ABA in order to achieve 6.0μM concentrations of thiamine and p-ABA in the experimental condition or a zero concentration of one vitamin in the negative control. (See Table 1, Numbers 2-5), Supersalts solution was added at twice the standard concentration in the Minimal media formulation, 70 mL/L, or left out as a negative control (See Table 1, Numbers 6-7). The other vitamins were added as stock solutions to a final concentration of 2.0μM. (See Table 1, Number 8-14) Following the preparation of these media, a concentrated

TABLE 12
VITAMIN ADDITIONS TO BASE MEDIA
TO INVESTIGATE THE EFFECT OF VITAMIN SUPPLEMENTATION ON THE GROWTH OF B. VIRIDIS

<table>
<thead>
<tr>
<th>#</th>
<th>Condition</th>
<th>Base Media</th>
<th>Additives</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>Minimal</td>
<td>None</td>
</tr>
<tr>
<td>2</td>
<td>No Thiamine</td>
<td>SS/ p-ABA Free</td>
<td>Thiamine Free Supersalts, p-ABA</td>
</tr>
<tr>
<td>3</td>
<td>3X Standard Thiamine</td>
<td>SS/ p-ABA Free</td>
<td>Thiamine, Thiamine Free Supersalts, p-ABA</td>
</tr>
<tr>
<td>4</td>
<td>No p-ABA</td>
<td>SS/ p-ABA Free</td>
<td>Supersalts</td>
</tr>
<tr>
<td>5</td>
<td>3X Standard p-ABA</td>
<td>SS/ p-ABA Free</td>
<td>p-ABA, Supersalts</td>
</tr>
<tr>
<td>6</td>
<td>No Supersalts</td>
<td>SS/ p-ABA Free</td>
<td>p-ABA</td>
</tr>
<tr>
<td>7</td>
<td>2X Standard</td>
<td>SS/ p-ABA Free</td>
<td>p-ABA, Supersalts</td>
</tr>
<tr>
<td>8</td>
<td>Biotin</td>
<td>Minimal</td>
<td>Biotin Solution</td>
</tr>
<tr>
<td>9</td>
<td>Pantothenate</td>
<td>Minimal</td>
<td>Pantothenate Solution</td>
</tr>
<tr>
<td>10</td>
<td>Niacin</td>
<td>Minimal</td>
<td>Niacin Solution</td>
</tr>
<tr>
<td>11</td>
<td>Pyridoxin (B6)</td>
<td>Minimal</td>
<td>Pyridoxine Solution</td>
</tr>
<tr>
<td>12</td>
<td>Folic Acid</td>
<td>Minimal</td>
<td>Folic Acid Solution</td>
</tr>
<tr>
<td>13</td>
<td>l-cystein</td>
<td>Minimal</td>
<td>l-Cystein Solution</td>
</tr>
<tr>
<td>14</td>
<td>Vitamin B12</td>
<td>Minimal</td>
<td>Vitamin B12 Solution</td>
</tr>
</tbody>
</table>
inoculating broth with a calculated absorbance of 26.1 a.u. was used to inoculate the cultures, with volumes adjusted to achieve an initial culture volume of approximately 0.20 a.u.

6.2.5 Carbon and nitrogen effects

6.2.5.1 Malate

In order to determine the optimum concentration of malate for the growth of *B. viridis* cells, NC media, free of any carbon source, was prepared and distributed. Malate was then added as a 15 M solution, pH adjusted to 5.6, to each vessel as necessary to generate vessels for 12 concentrations ranging between zero and 160 mM malate. The vessels were subsequently inoculated to achieve an initial density of 0.20 a.u. using a concentrated inoculating broth with A660 equal to 16.9 absorbance units. The culture was measured at the time of inoculation and on day 12.

6.2.5.2 Bicarbonate

To determine if preparation of the media at pH 5.6 alters the effect of a bicarbonate addition, bicarbonate was offered at six concentrations. (See Table 2, and 5.2.3.5 Bicarbonate for discussion of “saturation equivalent.”) The cultures were prepared using standard Minimal media, pH adjusted to 5.6 using HCl. A concentrated inoculating broth with an A660 of 15.4 a.u. was used to inoculate the cultures and achieve an initial culture density of 0.20 a.u. Cultures were sampled and bicarbonate was added just prior to sealing the bottles in order to retain the maximum CO2 saturation possible by preventing carbon dioxide release into the atmosphere. The initial pH measurement was measured only for media not supplemented with bicarbonate. The
measurements necessary to evaluate the growth and pigment behavior of these cultures were taken at initial time, as well as day 5, 10, 13, 16, and 20 to establish the course of the growth.

6.2.5.3 Alternate carbon sources: fumarate and sucrose

To determine what other carbon sources can be utilized by *B. viridis* growing in pH shifted media, three sets of three vessels with NC media were prepared and 20 mM concentrations of pH adjusted malate, fumarate, or sucrose solutions were added to each. A carbon free NC set constituted the negative control, while the malate supplemented set was considered the positive control. The vessels were inoculated with concentrated inoculating broth of A660 equal to 17.6 a.u. to achieve an initial culture density of 0.20 a.u. A660 measurements were made at initial time, and again on day 12.

6.2.5.4 Concentration of ammonia supplementation

The effect of nitrogen concentration was determined by preparing nitrogen free Minimal media at pH 5.6 and adding ammonium sulfate concentrations between zero and four times the standard Minimal media concentration \[\{(NH_4)_2SO_4 = 5.30\text{ mM}\}\] using a pH

<table>
<thead>
<tr>
<th>Saturation Equivalent</th>
<th>Addition Per Liter (g)</th>
<th>Molar Concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (0%)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>25%</td>
<td>0.6</td>
<td>7.5705</td>
</tr>
<tr>
<td>50%</td>
<td>1.3</td>
<td>15.093</td>
</tr>
<tr>
<td>100%</td>
<td>2.5</td>
<td>30.187</td>
</tr>
<tr>
<td>150%</td>
<td>3.8</td>
<td>45.233</td>
</tr>
<tr>
<td>200%</td>
<td>5.1</td>
<td>60.374</td>
</tr>
</tbody>
</table>
adjusted 10% ammonium sulfate solution. For 4 times the standard, 7 mL of media was removed to make room for the addition. The cultures were inoculated to an approximate initial culture density of 0.20 a.u. with a concentrated inoculating broth of 26.1 a.u. The cultures were measured at the time of inoculation and again on the 12th day of growth.

6.2.6 Buffer type and concentration

Phosphate, Acetate, and Carbonate buffer solutions of 1.4 M concentration were prepared and adjusted to pH 5.6. Each buffer was added to buffer free Minimal media prepared at pH 5.6. Five final concentrations in the media, between zero and 30 mM, were used for each buffer. The majority of the concentrations were in the range 0 mM to 15 mM, the range in which a positive effect of buffer was noted for cells grown in Minimal 6.9 media. (See Chapter 5.3.7) Once the cultures were prepared, they were inoculated to an initial culture concentration of 0.2 a.u. with a concentrated culture with A660 equal to 18.3 a.u. They were measured again on day 12 after incubation under illumination.
6.2.7 Vessel volume and illuminated surface area considerations

To determine the effect of the culture volume and the ratio of surface area to volume, vessels of 50 mL, 250 mL, 1 L, and 2 L were used to grow cultures. (See Table 3) The height of the bottle was measured from the bottom of the vessel to the maximum volume mark, where the top of the culture broth would stop. The total surface area of the culture was approximated by adding the circumference times the height to twice the area of the vessel bottom. This was considered a reasonable approximation because the vessels were cultured on wire shelves in a space illuminated from all directions. The entire culture surface would therefore be illuminated. In addition, the bottles were glass, so light would be transmitted through the glass and headspace to the top surface of the culture. These vessels were sterilized by autoclave, filled to their listed volume with Minimal 5.6 media, and inoculated to a initial culture density of approximately 0.2 a.u. with a concentrated inoculating broth with a density of 16.9 a.u. The vessels were incubated under illumination for 12 days and subsequently measured.

<table>
<thead>
<tr>
<th>Volume</th>
<th>Diameter</th>
<th>Height</th>
<th>Surface Area (SA) cm²</th>
<th>SA/Vol cm²/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>mL</td>
<td>cm</td>
<td>cm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>4.3</td>
<td>4.4</td>
<td>88.483</td>
<td>1.7697</td>
</tr>
<tr>
<td>250</td>
<td>6.25</td>
<td>8.35</td>
<td>225.31</td>
<td>0.9012</td>
</tr>
<tr>
<td>1000</td>
<td>9.3</td>
<td>14.8</td>
<td>568.27</td>
<td>0.5683</td>
</tr>
<tr>
<td>2000</td>
<td>12.4</td>
<td>17</td>
<td>903.77</td>
<td>0.4519</td>
</tr>
</tbody>
</table>
6.2.8 Light intensity

To determine the effect of the intensity of light, 15 identical vessels were prepared and inoculated to 0.2 a.u. initial culture density with the same broth used in Section 6.2.7. The vessels were sampled for measurement, then placed at varying distances from a single 25-Watt incandescent bulb. (See Figure 29) Three vessels were wrapped in tin foil to prevent light exposure and placed in the same enclosure to insure otherwise similar temperature and treatment. The incident light on each row and placement was measured several times during the course of growth using an Extech Instruments Foot Candle /Lux Meter P/N Q017255. The averaged values were used to describe the incident light on the culture. (See Table 4) The lower light intensities (below 200 lux) experienced less than 20% fluctuation in intensity between measurements. The intermediate intensities fluctuated around 30% from measurement to measurement. High light intensities, above 1000 lux, fluctuated as much as 40% between measurements. Vessels were measured again on day 12.

<table>
<thead>
<tr>
<th>Position</th>
<th>Left (lux)</th>
<th>Center (lux)</th>
<th>Right (lux)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Row 1</td>
<td>1654</td>
<td>2233</td>
<td>966</td>
</tr>
<tr>
<td>Row 2</td>
<td>391</td>
<td>440</td>
<td>240</td>
</tr>
<tr>
<td>Row 3</td>
<td>223</td>
<td>173</td>
<td>183</td>
</tr>
<tr>
<td>Row 4</td>
<td>102</td>
<td>87</td>
<td>72</td>
</tr>
<tr>
<td>Wrapped</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
6.2.9 Validation of Optimized media

Once each condition was investigated to determine the individual optimum, a composite optimized media was designed, incorporating the results of each experiment. (See Table 7, in Section 6.4.1) To validate the net improvement in culture growth and performance, a growth experiment was performed using the initial Minimal 5.6 media,

Figure 1: The Arrangement of Vessels for Varied Light Intensity.
and the Optimized Minimal 5.6. Both medias were prepared in three-liter batches, and three one-liter sterile vessels were prepared with each media. One liter vessels were used in order to have adequate volume to sample approximately every 24-48 hours throughout the growth, though after stationary was reached, measurements were sometimes taken at less frequent intervals. The vessels were inoculated to an initial culture density of around 0.2 a.u. with a concentrated inoculating broth with A660 11.7 a.u., sealed, and placed under light of 500-750 lux.

6.3 Results and Discussion

6.3.1 Baseline culture behavior

6.3.1.1 Growth of cells

In pH 5.6 adjusted media, a lag period was noted in the change in optical density of the culture. (See Figure 2) This lag was not noted in the increase of live cells, or CFUs, demonstrating that the increases in number of live cells and the culture optical density, or absorbance, were decoupled. In RMPABA and Minimal at pH 6.9, the growth was not decoupled. (See Chapter 3 for standard growth behavior at pH 6.9 in both medias.) The CFU count is a measure of the number of live cells present; absorbance measures the number of cells present based on a presumed extinction coefficient. Based on the Beer-Lambert Law (See Equation 2.3), if the absorbance is changing while the path length and concentration stay the same, then the molar extinction coefficient for the culture must be changing, indicating a morphological change in the size or pigmentation of the cells. This suggests that the cells first undergo reproduction, doubling their
The number of live cells increased at an exponential rate of 0.3371 CFU/day during the first 5 days of culture, and entered stationary with an average maximum of approximately 4.5x10^7 CFU/mL. The absorbance at 660 nm, A660, began to increase exponentially after day 5, with a rate of 0.3418 a.u./day from around day 5 to around day 12, and a maximum culture density of around 3.0 absorbance units was achieved after a deceleration period lasting until approximately day 20. The rate of cell growth and the rate increase of culture optical density at A660 differed by less than 2%.  

numbers by splitting into smaller, less pigmented cells (during the period of live cell number increase), and second, increasing their size and pigmentation (during the period of A660 increase).  

Figure 2: The Growth and Culture Density Increase of Cells Grown in pH Adjusted Minimal Media. The formation of new live cells and the increase in culture density were decoupled in pH adjusted media, however the growth rates differed by less than 2%. First, live cells increased at a rate of 0.3371 cfu/day, followed by an increase in culture density at a rate of 0.3418 a.u./day. Error for A660 is between 2% and 10%. Error bars are shown.
6.3.1.2 Photosynthetic pigment behavior

To determine whether the decoupling of A660 and CFU increase pattern was related to a delay in photosynthetic pigment production, the pigment production, characterized by absorbance at A1020, was examined as a function of time. The lag period for pigment production was approximately 5 days as well, and pigment then increased at a rate of 0.3719 a.u./day until about day 12. (See Figure 3) The deceleration phase of pigment production continued until around day 20, when an average final pigment density of 2.6 was obtained.

![Graph showing the increase in pigment measured by A1020 for cells grown in pH 5.6 Minimal Media. The increase in pigment is slightly more rapid than the increase in cell number or cell density, with a rate of 0.3719 a. u./day. A final A1020 of around 2.6 was obtained. Error is between 3% and 11%. Error bars are shown.]

\[
y = 0.0062e^{0.3719x} \\
R^2 = 0.9849
\]
The similar delays in both A660 and A1020 increase suggests that the lag in culture optical density at A660 is tied to changes in the pigment content of the cells. The ratios describing the relationship between cell number and pigment support this. The ratio of A1020/A660 is relatively constant up to day 5, during the period when cells are replicating, and increases by approximately 20% between day 7 and day 15, when constant growth rate is achieved. (See Figure 4A) This suggests that after day 7 there is an increase in the concentration of pigment relative to the concentration of cells. The ratio between CFU/A1020 begins around 3.0, and rapidly drops to less than 1.0 during the lag phase. (See Figure 4B) This indicates that while the number of cells is increasing rapidly, the relative production of pigment is low, suggesting a phase where cells are increasing without concomitant production of pigment. When a stationary cell density is reached, the pigment per cell ratio increases dramatically to around 10, suggesting a second phase of pigment production. Both analyses suggest a growth phase when cells increase at the expense of pigment concentration, followed by a pigmentation phase when pigment production occurs.

6.3.1.3 pH behavior

The culture begins with the pH adjusted to 5.6, but rises roughly linearly throughout the lag, exponential, and deceleration culture periods (See Figure 5). The maximum pH, around 7.5, is reached around day 20 or 25, which marks the end of the deceleration and the onset of stationary culture density.
Figure 4: The Pigment to Cell Ratio of *B. viridis* Cells Grown in pH 5.6 Minimal Media. A) The ratio of A1020/660 begins around 0.8, and remains in that vicinity for the first five days, and then increases to around 1 beyond day 12 and then begins to slowly decrease back to the initial ratio of 0.8. B) The Ratio of A1020/CFU begins around 3.0, drops rapidly to less than 1.0 during the lag phase, and subsequently increases dramatically to around 10. In both of these cases, the behavior indicates that pigment formation is occurring after the increase in live cells is observed. Through day 10, error on A1020/A660 is on the order of 2%. The error for A1020/CFU is on the order 10%
6.3.1.4 Comparison to Minimal media at pH 6.9

The growth observed for cells grown in Minimal 5.6 is slightly improved over the growth in the initial Minimal media adjusted to pH 6.9 (See Table 16 and Chapter 3.3.1 for Minimal 6.9 results). The rate of generation of new cells, increase in optical density at A660, and pigment production at A1020 are all higher for Minimal at pH 5.6. The final live cell concentration is 0.5x10^7 CFU/mL, greater for cells cultured in pH shifted media, and the total culture density and final pigment signature are around 130% greater in Minimal 5.6 than in Minimal adjusted to pH 6.9. This demonstrates a significant improvement in cell growth, in addition to the previously determined ability to sustain multigenerational growth. (See 5.3.8.2)
TABLE 5
CHARACTERISTICS OF GROWTH
OBSERVED FOR B. VIRIDIS
IN MINIMAL MEDIAS AT pH 6.9 AND 5.6

<table>
<thead>
<tr>
<th>Characteristic of Growth</th>
<th>Minimal at pH 6.9 (See Chapter 3)</th>
<th>Minimal at pH 5.6</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFUs</td>
<td>Maximum (CFU) 4.0x10^7</td>
<td>4.5 x 10^7</td>
</tr>
<tr>
<td></td>
<td>Growth Rate (CFU/day) 0.2564</td>
<td>0.3371</td>
</tr>
<tr>
<td></td>
<td>Doubling time (days) 2.6</td>
<td>2.1</td>
</tr>
<tr>
<td>A660</td>
<td>Maximum (a.u.) 1.3</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td>Growth Rate (a.u./day) 0.2593</td>
<td>0.3418</td>
</tr>
<tr>
<td></td>
<td>Doubling time (days) 2.6</td>
<td>2.0</td>
</tr>
<tr>
<td>pH</td>
<td>Initial pH 6.8</td>
<td>5.6</td>
</tr>
<tr>
<td></td>
<td>Final pH 7.7</td>
<td>7.5</td>
</tr>
<tr>
<td>A1020</td>
<td>Maximum (a.u.) 1.1</td>
<td>2.6</td>
</tr>
<tr>
<td></td>
<td>Increase Rate (a.u./day) 0.2470</td>
<td>0.3719</td>
</tr>
<tr>
<td></td>
<td>Doubling time (days) 2.8</td>
<td>1.9</td>
</tr>
<tr>
<td>A1020/CFUs (on X10^-8 scale)</td>
<td>Initial Value 20</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Value at Onset of Stationary 2.5</td>
<td>Less than 1</td>
</tr>
<tr>
<td></td>
<td>Recovered Value 2 (decrease in pigment content)</td>
<td>10 (increase in pigment content)</td>
</tr>
<tr>
<td>A1020/A660</td>
<td>Initial Value 1.1</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>Value at Onset of Stationary 0.8</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>Recovered Value 0.9, overall decrease</td>
<td>0.85, no overall decrease</td>
</tr>
</tbody>
</table>
Following the exponential increase of CFU, a large increase in the pigment content of each cell occurs that was not noted for the cultures grown in Minimal media at pH 6.9, which actually experienced a decrease in pigment content per cell over time. In Minimal 5.6, there was a slight decrease in pigment ratio during the lag phase, while the number of cells increased, but the total pigment ratio recovered and exceeded the initial concentration following the exponential phase. This indicates that pigment production is able to proceed at pH 5.6. In pigmented cells, the ability to harvest light and generate chemical energy is available to the metabolism; this may contribute significantly to the ability of cells in media adjusted to pH 5.6 to sustain multi-generational growth.

In Minimal 5.6 cultures, the rate of CFU increase, and the rates of culture density increase and pigment induction are decoupled but similar in magnitude. Monitoring A660 can represent the number of cells, but when it is changing during a constant cell number, as it is here, absorbance data can be considered an indicator of the relative metabolic health of the cells, where healthier cells will be larger and more pigmented. Combining this information with the pigment signature of the cells, A1020, can provide information about the relative pigmentation of the cells and the pigment behavior. Metabolically challenged cells or those unable to produce pigment will achieve a smaller increase in culture density, and therefore demonstrate a slower growth rate. For the purposes of rapid and efficient screening of culture conditions to optimize the growth media, the culture density, as measured by A660, the pigment behavior, as measured by A1020, and the pH can be used to provide practical information about the growth, pigmentation, and health of the culture.
6.3.2 Validation of Two-Point Method Modification for cultures in pH 5.6

The Two-Point method was validated and used for cultures growing in Minimal media adjusted to pH 6.9. In Minimal 5.6, the growth patterns shifted and it was therefore necessary to evaluate the growth behavior based on the portion of the absorbance curve which experienced the greatest change in density. The A660 increase for cells in Minimal 5.6 over time is shown, along with the data points gathered immediately following inoculation and the data point gathered closest to exactly day 12 (288 hours). (See Figure 6) The rate calculated for the complete exponential portion of the growth curve was 0.3418 a.u./day. Using the initial data as T = 5 days, and the data point closest to T = 12 days, a Two-Point growth rate of 0.3282 a.u./day was determined. These two rates differed by approximately 4%. The initial time absorbance, 0.072 absorbance units, was comparable to the actual 5-day data, 0.079 absorbance units. These two values varied by less than 10%, but since the values were both so small, the actual shift in the behavior of the growth curve due to the difference was minor. The modification, using initial time data as day 5 and day 12 data, provided an accurate representation of the actual A660 induction rate. Because this method was employed for experiments featuring the same batch of media, inoculated at the same time with the same concentrated inoculation solution, any slight deviations encountered were be consistent throughout the set. This allowed comparison even if the growth rate calculation deviated from the actual growth rate due to some behavioral shift of the bacteria.
6.3.3 Inoculation concentration

The optimum rate of increase in A660 values was achieved between 10% and 30% by volume inoculation, or initial culture densities corresponding to 0.1 to 0.25 absorbance units. (See Figure 7) Below that range, negligible A660 growth was observed, and above that range, the growth rate began to drop significantly. Cultures inoculated with unconcentrated cells achieved only about half of the growth rate observed in cultures inoculated with concentrated cells. Unlike the addition of spent RMPABA media, which provided a variety of nutrients and carbon compounds even after some culture growth, spent Minimal 5.6 contained many fewer nutrients and was therefore less likely to bring in nutrients that would enhance growth and more likely to bring in end products that would slow the growth. It was therefore important that the cells undergo
concentration to remove the spent media. The final culture density increased steadily
with increased initial inoculation until an initial culture density of 0.3 absorbance units,
or a 30% by volume inoculation. (See Figure 8). Based on these results, an inoculation to
achieve an initial culture density of around 0.2 a.u. was used in all subsequent
experiments. This corresponded to roughly a 20% by volume inoculation of a ten-fold
concentration of cells in the inoculating culture. This ten-fold concentration was
achieved by centrifuging the cells and resuspending in fresh media at 1/10th the initial
concentration. The concentrated inoculating broth was in the vicinity of 10 a.u. at 660
nm, and the inoculation volume used was adjusted for greater or lesser concentration.
Figure 7: The Effect of Initial Inoculation on Growth Rate for Cells in Minimal 5.6 Media. A) The rate of growth is maximized between 10% and 30% by volume inoculations, and drops off above that range. Below 10% by volume inoculation, negligible growth is observed. B) When the initial culture density is between 0.1 and 0.25 a.u., the highest rates of growth are obtained. At densities below 0.1 a.u., growth is not observed. Above 0.3 a.u., the rate of growth decreases.
Figure 8: The Effect of Initial Inoculation on Final Culture Density. A) There is a steady increase in final culture density up to 30% inoculation, when the culture reaches a density of 3.0 absorbance units. Beyond this limit, no further growth is observed as a result of additional inoculation. B) The limit to this increase is around 0.25 a.u. as the initial culture density.
6.3.4 Vitamin supplementation

The presence or absence of \( p \)-ABA thiamine or supersalts did not have a major effect of the A660 increase of the culture (See Figure 9). The growth rate was around 0.19 a.u./day regardless of the presence or absence of \( p \)-ABA. Tripling the concentration of thiamine caused a 25\% increase in the growth rate, from 0.1882 a.u./day to 0.2366 a.u./day. The negative control for thiamine also displayed 8\% more growth than the control, however these two conditions have significantly overlapping error. The addition of twice the standard concentration of Supersalts caused 9\% increase in the growth rate, up to 0.2056 a.u./day from the control, however the absence of Supersalts caused a 10\% reduction in A660 growth rate compared to the control.

![Figure 9: The Effect on Growth Rate of Modifying the Concentration of Vitamins and Supersalts Present in the Standard Media Formulation. The effects of modifications are mild, except in the case of increasing the thiamine concentration, which causes a 25\% increase in growth rate. The effect of doubling Supersalts is not significant compared to the control, but there is a significant difference between doubled Supersalts, indicating that there is a relationship between increased Supersalts concentration and increased growth.](image-url)
In addition to the vitamins and minerals provided in the original Minimal media formulation, 2μM concentrations of biotin, pantothenate, niacin, pyridoxine, folic acid, l-cystein, and vitamin B$_{12}$ were added to the pH 5.6 Minimal media (See Figure 10).

The addition of biotin had the most significant effect on the growth of the culture, increasing the growth rate by over 50% from the control. Pantothenate and vitamin B$_{12}$ also enhance the growth rate by more than 10%. Niacin, pyridoxine, and folic acid differ from the control by less than 5%, and l-cysteine causes a 10% decrease in the growth rate. The pH increases and pigment behavior noted for these cultures are all typical for the growth observed, except in the case of supplementation with biotin. With biotin, the final pH of the culture is 0.6 pH units higher than control, and the pigment ratio is

![Figure 10: The Effect of Vitamin Supplementation on the Growth Rate of B. viridis in Minimal 5.6 Media. The most significant effect is noted with the addition of Biotin, which increases the rate of growth by over 50%. Pantothenate and vitamin B12 also have mild enhancing effects, around 12% and 14% improvement, respectively. L-cysteine decreases the rate of growth by 10%. The other vitamins have 5% or less difference from control.](image-url)
increased by 19% over the final value of the control (See Figure 12). For all other vitamin additions, there is negligible change between the initial and final pigment ratios.

The six most effective supplements are summarized in Table 17. The significance of these compounds is reviewed in Chapter 4.2.2, and summarized in Table 4.1. The vitamin which had the greatest impact on overall cell health was biotin, which increased the growth rate by over 50% and caused a 20% increase in the ratio of pigment to culture density. This indicates that biotin has a significant effect on pigment formation. Biotin is known to enhance the assimilation of malate and to assist in the synthesis of fatty acids, 5, which contributes directly to the formation of the thylakoid membranes. The resulting

Figure 11: The Pigment Ratios of Cultures Supplemented with Various Vitamins. There is a 19% increase in the pigment ratio for biotin compared to control. (Highlighted in maroon) In all other conditions, there is negligible change between generations. This indicates that biotin has a significant enhancing effect on the ability of the culture to form pigment.
increase in the optical density of the culture at 660 nm may be a direct effect on the number of cells or result from the increase in pigment concentration, which allowed the cells to gather more energy through photosynthesis for use in growth and metabolism. Pantothenate and vitamin B₁₂ also have a mild enhancing effect. Increasing the concentration of thamine also caused a significant enhancement to growth. There was a small but clear positive effect of increasing Supersalts concentration.

6.3.5 Carbon and nitrogen effects

6.3.5.1 Malate

There is a significant effect on the growth rate caused by changes in the malate concentration. (See Figure 13) At very low concentrations, there was almost no detectable growth, and the growth increased almost linearly with concentration up to about 40 mM. Above about 80 mM, the growth rate decreases with increasing concentration of malate. The data was fitted with a binomial curve. (See Equation 1)

\[
\text{Growth Rate (a.u./day)} = -5 \times 10^{-5}[\text{Malate}]^2 + 0.0072[\text{Malate}] + 0.0422
\] (14)

\[
\frac{dC}{dt} = \frac{Q - dC}{V} - kC
\]

\[
\frac{dC}{dt} = \frac{dC}{dt} + kC
\]
This equation is maximized at 72 mM, with a growth rate of approximately 0.32 a.u./day, which is more than a 60% increase with respect to the standard concentration of 20 mM.

The pH behavior of this culture was consistent with the growth, with more growth generating higher final pH values. For some of the very high growth rates, between 40 mM and 100 mM, the final pH values were as high as 8.4, exceeding the typical 7.7 limit expected for Minimal cultures. The pigment behavior of these cultures was typical of the growth observed, and no unusual increases or decreases in pigment ratio were observed.

In Minimal 5.6, the concentration of malate has a significant effect. There was over 60% difference between the response to the standard concentration and the optimized concentration. The optimized concentration was rounded up and set at 75 mM.
In addition to providing a round number, this provided more carbon to the cells without exceeding the 80 mM concentration above which a sharp decline in growth rate was observed. There was no significant shift in the initial pH due to the malate, which was added as a pH adjusted solution. At the extremely high concentrations of malate, there was also a significant dilution of the mineral components of the broth. For 160 mM, the malate solution comprised one fifth of the culture broth by volume. However the major reason for the observed dependence of growth on malate concentration was likely to be NAD$^+$ shortage inhibition of the TCA cycle and other enzymatic reactions. (See 4.2.3.2) Three reactions in the TCA cycle, pyruvate dehydrogenase, and other cellular reactions rely on NAD$^+$ as a cofactor. The reaction catalyzed by malate dehydrogenase begins with the binding of NAD$^+$ to the enzyme, causing a major structural rearrangement of the enzyme, followed by binding of malate to the enzyme.$^6$ In the presence of high concentrations of malate, the NAD$^+$ pool would be diminished and the NAD$^+$/NADH ratios altered. This would prevent it from acting as a cofactor in other reactions, and could effectively shut down cell growth at high malate concentrations.

The high final pH values observed in cultures with very high growth rates was not characteristic of previously observed Minimal grown cultures, at either pH 5.6 or pH 6.9; however the increase in total growth and rate of growth was significant for the cultures near the optimum. This high pH value suggested that the cultures were actively engaging in metabolic processes and photosynthesis, thereby producing larger concentrations of base. This was a positive indicator that the growth of the culture was significantly improved both by the pH shift, which allowed metabolic processes, as well as by the presence of increased concentration of malate.
6.3.5.2 Bicarbonate

The addition of bicarbonate significantly reduced the lag phase preceding the increase in A660 measurement in contrast to the control cells, which experienced a significant lag phase, and did not enter exponential until just after day 13. The lack of culture density was visually obvious, and measurements were taken at approximately 5 day intervals until day 20. (See Figure 13A) The cases with bicarbonate supplementation experienced a 7-day reduction of the lag phase, and entered exponential growth just after day 5. The growth behavior of the supplemented conditions was remarkably similar, which was visible in their growth curves. The growth rate for the control, calculated during the same period as the experimental conditions, was negligible. The growth rate calculated after the lag phase ended as consistent with the rates calculated for cultures supplemented with up to 100% saturation equivalent of bicarbonate, or 30 mM concentration of bicarbonate. (See Figure 13B) The culture growth rates for the control condition and for 30 mM bicarbonate were both in the vicinity of 0.3 a.u./day, and differed by less than 2.5%. Above 30 mM, there was a 30% reduction in the growth rate. The pigment behavior of the culture as measured by A1020 followed similar trends.

The trend in pH over time in the culture did not conform to the expected behavior of a Minimal 5.6 culture. (See Figure 14) The initial pH prior to the addition of bicarbonate was 5.6. Bicarbonate addition shifts the pH to basic by approximately 0.7 units at 7.6 mM, 1 unit at 15 mM, 1.3 units at 30 mM, and 1.6 units above 30 mM concentration. Subsequent culture behavior, as measured between days 5 and 20, indicated that each culture experienced the same qualitative increase in pH, however the curve was displaced upwards for greater concentrations, or saturation equivalents, of
Figure 13: The Effect of Bicarbonate Supplementation on Growth of *B. viridis*. A) There is a 7 day increase in the lag phase for the control condition, which has no bicarbonate addition. This lag is not observed in any of the cases where bicarbonate supplementation is offered. B) The growth rate after the lag phase (■) is roughly equivalent to the rates observed in cultures supplemented with up to 100% saturation. Above 100% saturation, the growth rate decreases by around 30%.
bicarbonate. The control condition increased from around 5.6 to 7.7 by the end of the culture period. The bicarbonate conditions, on the other hand, increased to between 6.5 (25%) and 7.5 (200%) by day 5. Less absolute change in pH was observed in the more heavily supplemented conditions because the initial pH was higher, close to 8.0, which was the intrinsic pH maximum of the culture.

Bicarbonate up to 30 mM concentration can significantly reduce the lag phase for *B. viridis*, and achieve similar growth rates as those observed in the control. When bicarbonate was added to the pH 5.6 cultures, a significant amount of carbon dioxide was released as the equilibrium shifted, providing CO₂ to the culture. The optimum condition to reduce the lag phase and increase the growth rate was a 100% saturation equivalent addition.

![Figure 14: The pH Behavior of Cultures Supplemented with Bicarbonate. The higher the concentration of bicarbonate, the higher the pH is shifted. The control pH ends at around 7.7, which is typical for Minima 5.6 growth. At very high bicarbonate additions, the pH increases to well above 7 by day 5, and subsequently increases to over 8.0. The large blue square indicates the initial pH of Minimal media prior to addition of bicarbonate.](image)
6.3.5.3 Alternate carbon sources: fumarate and sucrose

Malate is known to be a good source of carbon for photosynthetically growing *Rhodospirillaceae*, so malate at moderate concentrations is considered the control. For *B. viridis* growing in Minimal 5.6 media, 20 mM malate resulted in a growth rate of around 0.2 a.u./day. (See Figure 15) When no carbon was supplemented, the rate of growth dropped 100 fold, to around 0.02 a.u./day, which was nearly negligible growth. Replacing the malate with fumarate resulted in a growth rate only about 3% less, functionally identical. This did not occur when the malate was replaced with 20 mM sucrose, in which case the growth rate dropped to the level of the carbon free control. The pH of these cultures was consistent with the observed growth. The pigment behavior for fumarate was similar to the behavior of the control, and consistent with the expected

![Figure 15: The Effect of Alternate Carbon Sources on the Growth of B. viridis in Minimal 5.6 Media. Malate and Fumarate both support roughly equivalent growth, around 0.2/day. These two carbon sources differ by only 3%. Sucrose can not support growth, and the culture behaves similarly to a carbon free control.](image)

258
Minimal 5.6 culture behavior. There was a significant decline in the pigment ratio of both the carbon free control, about 14% less than the control, and the sucrose condition, about 16% less than control.

The bacteria are reliant on photosynthetic growth when they are growing in Minimal 5.6 media. This is supported by their need for exogenous CO$_2$, provided as bicarbonate, and the improvement in growth and sustainability with a pH shift that makes carbon dioxide more chemically accessible. Malate was able to support photosynthetic growth, and fumarate, which is only one enzymatic step away (See Figure 4.3), was equally able to support growth. This is most likely either because fumarate is enzymatically converted to malate by hydration of the double bond or because they are structurally similar compounds. (See Figure 16) These compounds can both function as electron donors, or accept a proton to drive the photosynthesis reactions. This indicates that these two compounds could be used interchangeably if there was a economic or supply reason to prefer fumarate over malate. In addition, fumarate is implicated in one of the primary pathways of toluene degradation known in Rhodospirillaceae.$^7$ The presence of fumarase hydratase suggests that bacteria growing in the presence of malate could reverse the pathway to generate fumarate for use in degradative metabolism if necessary. Malate was maintained as the optimum sole carbon source for growth in Minimal 5.6, however the utility of fumarate should be noted. If degradations do not proceed in the presence of malate, it can be readily exchanged for fumarate.

Sucrose is a significantly more complex molecule and a relatively common carbon source for secondary consumers and organisms using a fermentative metabolism. In order to participate in fermentation or the TCA cycle, it must first undergo glycolysis.
to be broken down into the usable form pyruvate. (See Figure 4.1) Glycolysis is not available to *B. viridis* when it is limited to a photosynthetic metabolism. Since sucrose is an uncharged molecule and unable to act as an electron donor, it can not participate in photosynthesis. This demonstrates that the bacteria are engaged in photosynthetic growth when they are cultured in Minimal 5.6 media.

### 6.3.5.4 Fixed nitrogen concentration

The presence of fixed nitrogen in the form of ammonium sulfate is important for growth of *B. viridis*, since nitrogenase seems to be irreversibly repressed in this strain rendering them unable to fix molecular nitrogen. (See 5.3.6) Once fixed nitrogen was present, however, there was not an extreme difference between various concentrations. (See Figure 17) The initial Minimal formulation contains 5.3 mM ammonium sulfate, and the highest growth rates occurred at and above this concentration, but not above 15 mM, with a binomial fit indicating an optimum in the vicinity 12.5 mM. (See Equation 2)

\[
\text{Growth Rate (a.u./day)} = -0.0006[NH_3]^2 + 0.0152[NH_3] + 0.1047
\]  

\text{(15)}
As the concentration went above about 15 mM, the rate declined very slightly. The increase in pH was consistent with the growth observed. The pigment ratio of the bacteria was enhanced around 10% for cultures that had any concentration of fixed nitrogen, compared to the nitrogen free culture, but there was no particular concentration at which the pigment production was optimized.

The optimized concentration of ammonium sulfate was in the range 5 mM, near the standard concentration in the original Minimal media formulation, to 15 mM. Below these concentrations, the cells had a slower growth rate and a lower total growth, possibly because they were experiencing a nitrogen limitation. The maximum of growth occurred at 12.5 mM. Within the 5 mM to 15 mM range of ammonium sulfate concentrations,

![Graph](image.png)  
**Figure 17:** The Growth Rate of *B. viridis* as a Function of Fixed Nitrogen Concentration. There is a gentle curve optimized around 12.5 M ammonium sulfate, however any concentration greater than above 5 mM and below about 15 mM gives growth in the maximum range. As the concentration is increased above 15%, the growth rate begins to decline.

\[ y = -0.0006x^2 + 0.0152x + 0.1047 \]

\[ R^2 = 0.851 \]
there was no significant decrease in pigment concentration, and the culture pH was well within the expected range of for typical culture growth.

6.3.6 Buffer type and concentration

The three buffers assayed were phosphate (useful in the range pH 5.9- pH 8.0\(^8\)), carbonate (useful at neutral or basic pH, with pK\(_a\)s 6.36 and 10.32 respectively\(^9\),\(^10\)), and acetate (useful in the range of pH 3.7- pH 5.8\(^11\)). The intention was to give a wide spread of pH ranges and buffer types. Of the three buffers assayed, phosphate buffer was the best choice for promoting growth.(See Figure 18) The carbonate buffer, adjusted to 5.6 ± 0.1, had no real effect at any concentration, and the acetate buffer adjusted to 5.6 ± 0.1 actively decreased the ability of the cells to grow, especially at higher concentrations. Above about 15 mM concentration of acetate buffer, the density of the culture decreased slightly with time.

The response of the phosphate buffer (KPO) could be fitted with a binomial equation (See Equation 3), optimized at 12.7 mM. Close to optimum growth could be obtained above about 25 mM and below around 100 mM. There was only 3% difference between the maximum observed rate, at 12.7 mM and the rate observed at 6.7 mM.

\[
\text{Growth Rate (a.u./day)} = -0.0003[KPO]^2 + 0.0067[KPO] + 0.1614
\]  
(16)

The acetate buffer was the most effective at maintaining a pH in the vicinity of 5.6, and above 10 mM concentration, no change in pH was observed. (See Figure 19) Phosphate buffer was able to maintain pH in the range of 6.7 for concentrations corresponding to the optimum growth range. Any concentration of carbonate buffer resulted in a final pH that was generally in the vicinity of 7 pH units, indicating that carbonate was not very effective at buffering the media.
Figure 18: The Effect of Varying Concentrations of Phosphate, Carbonate, and Acetate Buffers on Growth of *B. viridis*. Phosphate was clearly the best buffer to improve growth, and the optimum concentration was in the vicinity of 12.7 mM. Carbonate buffer caused very little effect at any concentration, and acetate buffer actively decreased the rate. At high acetate concentrations, the culture density decreases, demonstrated by the negative rates.

\[ y = -0.0003x^2 + 0.0076x + 0.1614 \]

\[ R^2 = 0.9445 \]

Figure 19: The Final pH of Cultures Buffered with Phosphate, Carbonate, and Acetate. Acetate was the most effective at maintaining the initial pH. Phosphate maintained a pH around 6.7 for concentrations corresponding to the maximum growth rates. Carbonate provided the least buffering, allowing the pH to reach around 7 and above.
The pigment ratio of the cultures was roughly similar for phosphate and carbonate buffers. (See Figure 20) Especially for phosphate buffer, the ratio was $0.86 \pm 0.04$ for all buffer concentrations. Carbonate buffer did not have a significant impact, but significantly more fluctuation was evident. Up to about 6 mM, acetate buffer did not significantly modify the pigment ratio, however above 6 mM, significant bleaching occurred. By the maximum concentration used, 26.8 mM, the ratio of pigment to culture concentration was reduced by nearly 50%.

The best choice for buffering was potassium phosphate buffer. It allowed the most growth while keeping the pH below the 8.0 range at which growth slows or ceases. Although the acetate buffer was the most effective at maintaining the initial pH, if the

![Figure 20: The Effect of Phosphate, Carbonate, and Acetate Buffers on Pigment Formation in B. viridis. There is no significant effect relative to control for either phosphate or carbonate buffer. Acetate buffer causes significant bleaching, and by 26.8 mM, nearly 50% reduction in pigment ratio is noted for acetate buffer. Phosphate buffer at 6.7 mM is the Minimal 5.6 control.](image-url)
culture does not grow, it is not an effective buffer. In addition to reducing the growth rate, acetate also caused a significant reduction in the pigment concentration. The effect was so severe that it could be identified by visual inspection; the cultures appeared pale compared to the control. The carbonate buffer released a large amount of gas upon pH adjustment. Instructions for carbonate buffer also specify that it must be made immediately prior to use because the release of carbon dioxide over time will shift the pH equilibrium and reduce the buffering capacity. It was possible that the carbonate buffer had lost its buffering capacity by release of CO₂ during pH adjustment, and therefore was ineffective. That would account for the largely indifferent response to varying additions.

The usefulness of bicarbonate addition suggested that addition of carbonate buffer to the system under the right conditions could be helpful to culture growth because it would provide an exogenous carbon source, however these results indicated that the primary buffer should be the phosphate buffer. Carbon dioxide was more effectively provided by an addition of solid bicarbonate, as performed in 6.2.5.2. The primary goal was to provide adequate buffer without overshooting and causing a reduction in growth rate. In the range 5mM - 20 mM phosphate buffer, good growth was observed, and the optimum concentration was around 12.7 mM,

6.3.7 Volume and surface area considerations

The effect of both volume and surface area per volume on culture growth and pigment formation was significant. Vessels with smaller volumes and greater surface area per volume had significantly greater growth. There was around a 40% decrease in growth rate between either a 50 mL or 250 mL vessel and the 1 or 2 Liter vessels. (See
Figure 21A) The effect on the pigment ratio was reversed. As the volume of the vessel increased, the pigment ratio increased. (See Figure 21B) The pigment ratio for the larger vessels was more than 30% greater than that for the smaller vessels. The surface area to volume ratio was a significant factor in this effect. (See Figure 22) An increase of only 1 cm$^2$/mL caused a nearly 40% increase in growth rate, however this change also caused a corresponding 30% decrease in the ratio of pigment to culture density.

The effect of volume or surface area/volume ratio made it clear that smaller bottles are better, and if large volumes must be cultured, improved growth could be obtained by using a modified geometry. Since the concentrations of nutrients and the subsequently produced waste were constant between vessels, and it had been established that there was no significant difference between cultures exposed to either pure nitrogen or air (See 5.3.6.1), this preference was most likely associated with the small depth penetration of light in a dense culture, preventing light from reaching the entire culture.

As the bacterial suspension became dense, especially in later exponential growth, the penetration of light was significantly reduced. Due to the pigment concentration of PNSB, the light energy was expected to decrease quasi-exponentially as the penetration depth increased. In a dense culture, by a depth of 1.5 cm, only 7% of the incident light will penetrate, and growth and activity are diminished in the deeper parts of the culture.\textsuperscript{12} In larger bottles, which have a diameter of 9.3 cm (1L) to 12.4 cm (2L), the penetrating light in a dense culture was reduced significantly at the later stages of growth. It was therefore likely that the differences observed in bottle size performance were due to the effects of light penetration and total light incident on the cells.
Figure 21: The Growth of *B. viridis* in Bottles of Varying Volume. A) As the volume of the vessel increased, the growth rate decreased exponentially. The 2 L volume had a rate over 45% slower than the 50 mL vessel. B) The pigment per cell increased significantly as the size of the vessel increased. The ratio was over 30% higher for the 2 L vessel than for the 50 mL vessel.
Figure 22: The Effect of Surface Area/Volume on Growth (A) and Pigment/Cell Ratio (B) for *B. viridis*. An increase in surface area per volume caused a nearly 30% increase in the growth rate (A), however it also caused a corresponding 30% decrease in pigment ratio (B).
Gentle agitation of the culture could allow circulation of the cells so that all cells have some exposure to light, however several groups have proposed flat panel reactors, which would increase the surface area/volume of the culture vessel and also insure more complete penetration of light and cell exposure on a constant basis.\textsuperscript{13,14,15} In scale up, such a reactor would be critical to encouraging good growth of \textit{B. viridis}.

It was interesting to note that the pigment per cell increased as surface area/volume ratio decreased. This suggested that \textit{B. viridis} can respond to its environment, similar to the cultures of \textit{Rhodobacter sphaeroides} used by Nakuda et al., which increased their efficiency at greater depths.\textsuperscript{16} The increase in pigment concentration under light limited conditions, such as would be encountered in the depths of a larger vessel, was similar to the behavior of other \textit{Rhodospirillaceae}, which increase their pigment concentration in reaction to low light conditions.\textsuperscript{17} These results also indicate that the light intensity was of great import to the culture behavior, and a study of light intensity in identical vessels is an important undertaking. Based on these results, experiments should be limited to either 50 mL or 250 mL vessels when possible, and scale up must include a provision to maintain a high surface area to volume ratio.

6.3.8 Light intensity

The cultures required light for growth, however too little or too much light significantly reduced the growth rate of the cultures. (See Figure 23) There was a steady increase with increasing light to around 500 lux, and there was no detectable growth in cultures which were wrapped in foil and received no light. At high intensities of light, above about 1000 lux, the growth rate decreased. Above 2000 lux, cultures were almost
as inhibited as if no light were available. The data can be fitted to a binomial equation (See Equation 4), and the optimum was at 1167 lux of incident light.

$$\text{Growth (a.u./day)} = -3 \times 10^{-7} I^2 + 0.0007I + 0.0423$$

(17)

The light intensity the culture observed has a significant impact on the growth and the response of the culture. In part, the decreasing growth rates at high light intensity were due to photobleaching, which occurs when the antenna light harvesting complexes (LHCs) absorb photons of light and are photooxidized to their excited state. In moderate light conditions, they rapidly transfer these photons of energy to the reaction center (RC) and return to their ground state. (See Appendix D: Photosynthesis) Under high light...
intensity, the LHCs become saturated and must dissipate excess excitation. In this way, the LHCs buffer the reaction center pigments against bleaching for short term variations in light intensity.\textsuperscript{18} Over the long term, cells will respond to high or low light intensity with structural changes; \textit{Rhodopseudomonas palustris} cells, for example, subtly modify their light harvesting complexes in response to low or high light conditions.\textsuperscript{19} This was apparent as a difference in the pigment quantity or quality.

Under ordinary light conditions, which are around 200-500 lux, the pigment ratio was 0.8. This was consistent with the expected behavior of the culture in Minimal 5.6. (See Figure 24) There was a slight increase, compared to the cultures grown in the dark, for the low light cultures, which had a ratio in the vicinity of 1. There was a steady decrease in the A\textsubscript{1020}/A\textsubscript{660} ratio above 500 lux. By an intensity of 1500-2000 lux, the ratio decreased to below 0.6, which could not sustain good growth. This suggests that it is not merely a matter of adding more light to generate more growth and photosynthesis. There was a significant decrease in pigment per cell observed for \textit{B. viridis} at very high light intensities. The inhibition of new pigment formation under high light conditions was observed in other photosynthetic bacteria as well, and therefore the pigment per cell decreased\textsuperscript{20, 21} making it imperative to avoid over illumination. Nakada et al. discovered that the deeper portions of the culture, receiving only small amounts of light energy, converted light to energy much more efficiently.\textsuperscript{22} Although additional pigment formation, indicated by a higher A\textsubscript{1020}/A\textsubscript{660}, was noted for \textit{B. viridis} in low light, this additional pigment did not adequately compensate to bring the growth rate up to the level of moderately lit cultures. It was a matter of discovering the appropriate intermediate
light intensity to obtain the best combination of pigment formation and total growth. The optimum light intensity for growing *B. viridis* was in the range 500 lux to 750 lux.

6.4 Conclusions: Optimized Media

6.4.1 Optimized media formulation

Based on the results presented here, an optimized media was formulated combining each of the maximizing conditions identified. (See Table 18) For the optimized media, the ammonium sulfate, Supersalts, and potassium phosphate buffer concentrations were increased by about a factor of two. Thiamine was increased by a factor of three, and in order to make this addition, it was removed from the Supersalts.
formula, and added as a separate solution. The carbon, malate, was increased by a factor of four. The vitamins biotin, B12, and pantothenate were added to the formulation at a final concentration of 4x10^{-6} M, 2x10^{-6} M, and 2x10^{-6} M, respectively.

The physical conditions for the optimized culture were also adjusted and specified. (See Table 19) The pH 5.6 is maintained, however a bicarbonate addition just prior to vessel closure is known to shift the pH equilibrium toward basic. After being sealed, the cultures are placed under light between 500 and 750 lux, and culture vessels with a high surface area to volume ratio are preferentially employed.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Minimal 5.6</th>
<th>Optimized</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH goal</td>
<td>5.6</td>
<td>5.6*</td>
</tr>
<tr>
<td>Addition of Bicarbonate</td>
<td>NONE</td>
<td>2.536 g/ L</td>
</tr>
<tr>
<td>Light Intensity</td>
<td>N.D.</td>
<td>500-750 lux</td>
</tr>
<tr>
<td>Optimum Bottle</td>
<td>N.D.</td>
<td>50mL or 250 mL</td>
</tr>
</tbody>
</table>

**NOTE: *Shifted after initial measurement by Bicarbonate Addition***
6.4.2 Validation of Optimized media

6.4.2.1 Growth of cells

The live cell increase and the increase in culture density continued to be slightly decoupled, however the lag phase in culture absorbance was reduced for both the original formulation and the optimized formula. (See Figure 25) The exponential increase in culture density did not begin until day 4, but prior to that, there was a slow increase during the lag phase not noted in the initial Minimal 5.6 culture. (See 6.3.1.1) The Minimal culture experienced a growth rate of 0.1705 a.u./day, less than half of the 0.3816 a.u./day rate observed for the optimized formula. The final culture density of the optimized condition, 7.3 absorbance units, was almost double that of the pre-optimized media, 4.0 absorbance units.
The growth observed by measurement of CFUs was similar for both cultures. (See Figure 26) Both the Optimized media and the Minimal 5.6 media gave a growth rate of approximately 0.25 CFU/day, and differ by less than 3%. Both conditions led to a final cell concentration in the vicinity of $7.5 \times 10^7$ CFU/mL, with the cell density in Minimal media just 4% less than in Optimized media. There was no lag phase, and cells began to increase immediately, leveling off and remaining roughly constant after about day 6. The growth rates determined by A660 and CFU count were within 25% for the Minimal 5.6 media, however the rates differ by more than 50% for Optimized media.

Figure 25: The Culture Density Behavior of *B. viridis* Grown in Minimal 5.6 Media and Optimized Media. The growth rate for the optimized condition was over twice the rate for the pre-optimized media, and final culture density was almost double for the optimized condition.
6.4.2.2 Pigment behavior

The pigment increase, described by A1020, began around day 4, and was even more rapid than the increase in cell density. (See Figure 27) For the Minimal 5.6 condition, there was only a slight 5% increase in rate over the cell density increase, for a total rate of 0.179 a.u./day. In the Optimized media, the rate of pigment increase, 0.4371 a.u./day, was about 15% greater than the increase in culture density. Comparatively, the Optimized media had a pigment increase over 80% faster than the pre-optimized Minimal media. The exponential increase in pigment concentration slowed and stopped by about day 10 for the Optimized media, but the exponential phase lasted much longer, until about day 16, for the Minimal 5.6 media. The final A1020, representing pigment, was nearly three times as dense for Optimized media, 8.9 absorbance units, as it was for Minimal 5.6 media, 3.1 absorbance units.
The ratios of pigment to both cells and total culture density indicated that cells were producing more pigment in Optimized media than in Minimal 5.6 media. (See Figure 28) The pigment to CFU ratio increased in the Optimized media to a ratio of around 20, compared to Minimal 5.6 cells, which only increased to 10. (See Figure 28A) The A1020/A660 ratio began around 0.8 for both cultures. In Minimal 5.6 media, the ratio remained unchanged, however for Optimized media, it increased 56% to around 1.25 and remained high during the culture period. (See Figure 28B) This was the highest ratio of A1020 to A660 observed for *B. viridis*, including cultures grown in RMPABA, which rose briefly to 1.2, but then decreased. (See Chapter 3.3.1.4, Figure 3.6)
Figure 28: The Ratio of Pigment to Cells for *B. viridis* in Optimized and Minimal 5.6 Medias.  A) The Ratio of A1020/CFU began around 8.0, and dropped rapidly to 1.0 during the lag phase for both conditions. The Minimal 5.6 condition increased to around 10 by stationary. The Optimized condition demonstrated an increase to over 20 by the end of stationary. This behavior indicated that pigment formation was significantly greater for cells grown in Optimized media than in Minimal 5.6 media. B) The ratio A1020/A660 began around 0.8 for both conditions, and remained in that vicinity for Minimal 5.6 media. For Optimized media, the ratio increased steadily until about day 7 and reached 1.25.
6.4.2.3 pH behavior

Although both cultures were initially adjusted to pH 5.6, the Optimized culture shifted to around 6.8 almost immediately due to the addition of bicarbonate. (Figure 29)

The maximum pH for both cultures was reached after the cells enter the stationary phase. The final pH was 7.7 for Minimal 5.6 cells, which was the expected range. Optimized cells reached a final pH around 8.0, which was typical of RMPABA cells, and indicated that they were close to the fundamental pH limit for photosynthesis.23

![Figure 29: The pH Behavior of B. viridis grown in Minimal 5.6 and Optimized Medias. Both conditions experienced an increase in pH, however Optimized media jumped to 6.8 by the end of the first day. Minimal 5.6 experienced the expected increase to around 7.7. Cells grown in Optimized media increased in pH until around 8.0.](image)
6.5 Conclusions

The Optimized media formulation was a significant improvement over Minimal media at either pH value. The live cell count was increased by a factor of nearly 20 compared to cells grown in Minimal pH 6.9 media, and brought into the same range as the RMPABA growth observed in Chapter 3. (See Table 3.2) The increase in cell number and the increase in culture density and pigment in Minimal 5.6 and Optimized Minimal medias, both measured by spectrophotometry, remained decoupled, however absorbance data at both wavelengths provided a clear picture about the relative health of the cells. The number of live cells was similar for both pH 5.6 media conditions, but the health of the cells was clearly improved by the Optimized media with respect to the Minimal 5.6 media. (See Table 20)

Indicators of the increased health resulting from the media optimization, compared to the pre-optimized Minimal media include:

- A reduction of lag time, observed as a rapid and vigorous response to subculture
- A nearly doubled rate of increase in culture density
- A nearly 2 fold increase in final culture density, measured as A660
- A nearly 3 fold increase in pigment signature, measured as A1020, greater even than the pigment signature observed for cultures grown in RMPABA

The pigmentation of the cells was an area of particular improvement. Biotin had a particularly striking effect on increasing the pigment ratio of the cells. This additional pigment production allowed the cells to harvest more light energy through photosynthesis for use in metabolic processes and growth. Another critical alteration was the supplementation of exogenous CO₂ as bicarbonate in concert with the adjusted pH.
Without the CO₂, the photosynthesis reaction could not be driven and the weak growth observed in Minimal 6.9 media resulted. It seems most plausible that the pH shift was important because it allowed the cells to access CO₂ in the most useful form. When bicarbonate was supplemented, the pH increased, however the supplementation provided a greater reserve of CO₂.

**TABLE 9**

**CHARACTERISTICS OF GROWTH OF *B. VIRIDIS*:
A COMPARISON OF PRE-OPTIMIZED MINIMAL 5.6 MEDIA AND OPTIMIZED MEDIA
BASED ON THE EXPERIMENTAL RESULTS PRESENTED IN 6.4.2**

<table>
<thead>
<tr>
<th>Characteristic of Growth</th>
<th>Minimal pH 5.6 Pre-Optimized</th>
<th>Optimized Minimal pH 5.6</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFUs</td>
<td>Maximum (CFU) 7.2X10⁸</td>
<td>7.5X10⁸</td>
</tr>
<tr>
<td></td>
<td>Growth Rate (CFU/day) 0.2461</td>
<td>0.2537</td>
</tr>
<tr>
<td></td>
<td>Doubling time (days) 2.8 days</td>
<td>2.7 days</td>
</tr>
<tr>
<td>A660</td>
<td>Maximum (a.u.) 4.03</td>
<td>7.30</td>
</tr>
<tr>
<td></td>
<td>Growth Rate (a.u./day) 0.1705</td>
<td>0.3816</td>
</tr>
<tr>
<td></td>
<td>Doubling time (days) 4.1</td>
<td>1.8</td>
</tr>
<tr>
<td>pH</td>
<td>Initial pH 5.6</td>
<td>5.6 (6.8 after bicarbonate addition)</td>
</tr>
<tr>
<td></td>
<td>Final pH 7.7</td>
<td>8.0</td>
</tr>
<tr>
<td>A1020</td>
<td>Maximum (a.u.) 3.1</td>
<td>8.9</td>
</tr>
<tr>
<td></td>
<td>Increase Rate (a.u./day) 0.179</td>
<td>0.4371</td>
</tr>
<tr>
<td></td>
<td>Doubling time (days) 3.9</td>
<td>1.6</td>
</tr>
<tr>
<td>A1020/CFUs (on X10⁻⁸ scale)</td>
<td>Initial Value 8</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Value at Onset of Stationary 1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Recovered Value 10</td>
<td>20+</td>
</tr>
<tr>
<td>A1020/A660</td>
<td>Initial Value 0.8</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>Final Value 0.8 (No change)</td>
<td>1.25</td>
</tr>
</tbody>
</table>
With these two additions, the cells were much more capable of driving photosynthesis, allowing them to generate more energy. This additional energy meant they were able to drive more metabolic processes and experience more growth. In addition, increased pigment formation and energy generation gave the cells more energy to drive unfavorable reactions such as degradation of unwanted and sometimes recalcitrant compounds. The Optimized media formulation was a defined media which generated cell growth that was both repeatable and healthy. These cells were ideally suited to degradation of compounds because they were using a primarily photosynthetic metabolism; they were already generating energy that could be used in alternative functions such as degradations.
6.6 References

5. Yoshio, 786.
6. EXPASY Enzyme Database, EN 1.1.1.38 <http://www.expasy.org/enzyme/1.1.1.38>
18. Garcia, 205-209.
7.1 Introduction

The primary goal in bioremediation is to degrade waste or contaminant chemicals using a biological agent. In this case, the biological agent is the bacterium \textit{B. viridis}. Achieving this goal requires two things. First and foremost, the bacteria must be capable of resisting damage in the presence of the compound of interest. Many of the common wastes and contaminants are not readily biodegraded and actively damage living cells. If the bacteria are incapable of surviving in the presence of the compound, then there is no second step in developing a successful biodegradation. Once it is determined that the cells can survive in the presence of a compound, their potential for degradation can be investigated.

There are several ways that a compound might be degraded. Some compounds experience active uptake and are utilized as a carbon source directly. In other cases, the compound is directed through a series of enzymatic manipulations to make it suitable for use. Some compounds are degraded co-metabolically as a side reaction of an enzyme being used for a primary metabolism. Finally, genetic manipulation to introduce the components of a non-native metabolic pathway can be done.
7.1.1 Capabilities of PNSB

The group of bacteria known as Purple Non-Sulfur Bacteria (PNSB) have a wide variety of capabilities in degradation of contaminant compounds. They are known to degrade C-1 compounds, organic acids, amino acids, fatty acids, alcohols, carbohydrates,\(^1\) and a wide variety of aromatic compounds considered non-degradable prior to 1988.\(^2\) (See Table 1-10) Although many members of this family have been investigated, the bulk of the findings are based on work with \textit{R. palustris}. This species is known to degrade caffeate, cinnamaldehyde, cinnamate,\(^3\) cyclohexanecarboxylate,\(^4\) cyclohexaneprionate, \(\Delta-1\)- and \(\Delta-3\)-cyclohexenecarboxylate, ferulate, hydrocaffeate, hydrocinnamaldehyde, 4-hydroxybenzaldehyde, 4-hydroxybenzoate, 4-hydroxybenzoylformate, 4-hydroxycinnamate, 4-phenylbutyrate, 3-phenylpropionate, 5-phenylvalerate,\(^5\) indole, pyridine, nicotinic acid, 4-dimethyl amino pyridine, inidazole, captan, pyrazine, 2-amino pyrazine, quinoline, 8-hydroxy quinoline, guanine, uracil, and carbenxazim.\(^6\) As of 2002, \textit{R. palustris} had additionally been shown to degrade phenol, phloroglucinol, benzyl alcohol, benzaldehyde, phenyl acetate, 3-chloro-benzoate, 2-hydroxybenzoate, 4-coumarate, vanillate, and pimelate.\(^7\) Based on work with \textit{R. palustris}, a pathway of degradation was proposed which proceeds through either benzoyl-Co A or 4-Hydroxybenzoyl-CoA, which indicates that in addition, benzoate and 4-hydroxybenzoate would be degraded, which was supported.\(^8\) Phenol is thought to be degraded through this pathway as well, however degradation through 4-hydroxyphenylacetate has also been observed.\(^9\)

In addition to \textit{R. palustris}, a number of other photosynthetic bacteria have been shown to have potential as degraders, however less work has been done with these
bacteria. The presence of the same enzymatic pathway through benzoyl-CoA in *Rhodomicrobium vannieli* has been confirmed, and this species was observed to grow well and utilize benzoate, benzyl alcohol, syringate, and vanillate. For syringate and vanillate, a 1/6- and 1/3-fold reduction, respectively, in bacteriochlorophyll content is noted. Weak growth was noted for some strains on 3-hydroxycinnamate, N-benzyolglycine, 3-chlorobenzoate, 3,4-dimethoxybenzoate, N-benzyolglycine, 4-hydroxybenzoate, 3,4,5-trimethoxybenzoate and phthalate.10 *Rhodobacter sphaeroides* has been observed to grow on 4-dimethyl amino pyridine, pyrazine, 2-amino pyrazine, and captan.11 Although they have not been observed to grow significantly on indole, *Rb. sphaeroides* also passively reduces the culture concentration of indole. This is thought to be a result of a side reaction from which the bacteria cannot gain carbon or nitrogen.12 *Rb. capsulatus* can grow on acetic acid, propionic acid, isobutric acid, n-butyric acid, isovaleric acid, n-valeric acid,13 and hippurate.14 *Rhodospirillum photometricum* and *Rs. rubrum* were capable of growth on the halogenated acids chloroacetic, 2-bromopropionic, 2-chloropropionic, and 3-chloropropionic.15 Many of these bacteria share common compounds, and the wide variety of compounds which have been reported suggest that the PNSB as a group will be capable of functioning in many biodegradative roles.

The most closely related bacterium to *B. viridis* is *B. sulfoviridis*; these two bacteria share a 98.7% sequence similarity.16 While adequate research has not been done on *B. sulfoviridis* to generate a list as impressive as that for *R. palustris*, this species has a large number of compounds to its credit. The degradations of toluene, trans-cinnamate, and benzylsuccinate, an intermediate in the expected toluene biodegradation pathway, have been observed.17 *B. sulfoviridis* strains which use ethanol, glycerol, propionate,
benzoate, cinnamate, crude oil, butyrate, acetate, caprylate, and 4-hydroxybenzoate have also been identified. This range of compounds suggests a wide range of metabolic abilities, and since these bacteria are so closely related, it is highly likely that *B. viridis* would have the same wide range. Because of its strong ability to degrade toluene, which is relatively unique among the PNSB, *B. sulfoviridis* has been used particularly in elucidating the mechanism of toluene degradation. These mechanisms of degradation contribute to an enhanced understanding of both the process of degradation and ways of improving or adding degradations.

7.1.2 Anaerobic and aerobic pathways for chemical degradation by PNSB

A wide variety of anaerobic and aerobic pathways for chemical degradation have been either determined or postulated. The most prevalent and noteworthy pathway researched for photosynthetic bacteria is that related to the anaerobic degradation of aromatic compounds. This pathway goes through the central compound benzoyl-CoA and is thought to have entry points for the majority of aromatic compounds. (See Figure 30) Most compounds which contain an –OH substituent are degraded first to 4-hydroxybenzoate and then to benzoyl-CoA. Chlorobenzoate, benzyl alcohol, toluene, and ethylbenzene are degraded to various compounds which are converted to benzoyl-CoA directly. Typically, compounds are degraded to a benzoyl-CoA analogue which features the original substituent, and that substituent is subsequently cleaved. Recent work has focused around defining the steps in these pathways.
The pathways for toluene, ethylbenzene, and phenol are well known. The degradation of toluene begins by the condensation of toluene with fumarate by benzylsuccinate synthase to yield benzylsuccinate.\(^{20}\) (See Appendix G) The next step adds a CoA unit to the benzylsuccinate. It is thought that the CoA group improves the ability of the compound to be degraded via the enzymatic pathway.\(^{21}\) The benzylsuccinyl-CoA is then dehydrogenated to \(E\)-phenylitaconyl-CoA, and then there are two additional hypothesized intermediates before the conversion to benzoyl-CoA.\(^{22}\) Ethylbenzene is degraded to benzoyl acetate before the CoA thioester is added.\(^{23}\) The benzyol acetate is then condensed with a second CoA thioester and cleaved to yield benzoyl-CoA and Acetyl-CoA.\(^{24}\) (See Appendix H) There are one or more initial steps in
the phenol degradation to convert the phenol to 4-hydroxybenzoyl.\textsuperscript{25} The 4-
hydroxybenzoyl is then converted to benzoyl-CoA by addition of a CoA thioester by 4-
hydroxybenzoate-CoA ligase and cleavage of the –OH group by 4-hydroxybenzoate-CoA
reductase.\textsuperscript{26} (See Appendix I) For each of these pathways, Benzoyl-CoA is then degraded
via a 13-step pathway to Acetyl-CoA, which enters the TCA cycle.(See Appendix I and
Figure 4.3)

The ability to degrade aromatic compounds is fairly well distributed among many
anaerobic bacteria in addition to PNSB.\textsuperscript{27} Degradation of a wide variety of aromatic
compounds has been observed in \textit{R. palustris}, \textit{Rhodomicrobium vanniellii}, \textit{Rhodobacter
sphaeroides}, \textit{Rb. capsulatus}, \textit{Rhodospirillum photometricum} and \textit{Rs. rubrum}. Growth on
benzoate, the central bottleneck of the pathway, has also been noted for \textit{Rhodospirillum
fulvum}\textsuperscript{28} and \textit{Rhodocyclus purpureus};\textsuperscript{29} which suggest that the main pathway is fairly
ubiquitous among the PNSB. Although toluene and ethylbenzene have been successfully
degraded, and their entry to the pathway is fairly well known, there has been no noted
complete utilization of benzene or m-xylene by photosynthetic bacteria.\textsuperscript{30} (See Figure 2)
These two compounds are typically degraded by mineralization with a consortium of
bacteria and do not go through benzyol-CoA.\textsuperscript{31} Elements of the anaerobic aromatic
pathway have also been discovered in \textit{Azoarcus} sp. strain EbN1\textsuperscript{32,33} and EB1\textsuperscript{34}
(ethylbenzene catabolism to benzoyl-CoA), \textit{Azoarcus} sp. strain T (toluene via
benzylsuccinate,\textsuperscript{35} general xylenes, monoflrorotoluenes, benzaldehyde, and 1-methyl-
cyclohexene to benzylsuccinate analogues,\textsuperscript{36} and \textit{m}-Xylene oxidation to 3-
 methylbenzoyl-CoA,\textsuperscript{37}), \textit{Thauera aromatica}\textsuperscript{38} and \textit{Thauera} sp. (toluene through benzoyl-
CoA, chloro-, fluoro- and methyl-analogues through the same pathway to analogous

290
benzoyl-CoA compounds), sulfur reducing strain PRTOL1 (conversion of toluene to benzylsuccinate), and *Xanthomonas malophila* SU1 (toluene, m-xylene, and p-xylene via benzylsuccinate).

Recent research suggests that elements of the aerobic aromatic degradation pathway may also be present in some PNSB, particularly *R. palustris*. (See Appendix K) This set of pathways has primarily been identified in aerobic pseudomonads, and maps typically start with the compound toluene. The first step in aerobic toluene degradation involves an oxygenase of some kind, which adds one or more hydroxyl groups to the toluene, reducing the resonance structure of the ring compound. The *meta*
cleavage pathway goes through the compound catechol, and begins with either 2- or 3-
monooxygenase or toluene dioxygenase. An alternate toluene degradation pathway
starts with toluene side-chain monooxygenase, which generates benzylalcohol, and goes
through the intermediate benzoate. A final route starts by degrading toluene to 4-
hydroxytoluene with toluene 4-monooxygenase, and this pathway goes through the
intermediate compound 4-hydroxybenzoate.

Anaerobic enzyme pathways for a wide variety of other compounds are known as
well. Pathways relevant to this work are available for compounds dichloroethane,
dimethylsulfoxide, MEK, and tetrahydrofuran. The dichloroethane pathway ends at
glycolate, which is only one enzymatic step from glyoxylate, part of the TCA cycle via
the intermediary metabolism. Methyl ethyl ketone is degraded to ethyl acetate, which is then cleaved to acetate and ethanol. The ethanol is degraded by an
NADP dependant alcohol dehydrogenase to acetaldehyde, which is general for several
alcohols. (See Appendix M) Both acetate and acetaldehyde can be readily utilized
through the TCA cycle, entering as acetyl-CoA. The pathway for the degradation of
tetrahydrofuran is still unproven. (See Appendix N) In the study on which the pathway
was based, not all of the intermediates were detected, and it was thought that spontaneous
isomerization of 2-hydroxytetrahydrofuran might mean that the reaction may not require
an enzyme. Later work, however, confirmed a similar pathway for 2,5-
dimethyltetrahydrofuran, supporting the pathway as hypothesized. The pathway ends at
4-hydroxybutanoate, which can be converted to succinate semialdehyde and then
succinate and used in the TCA cycle. Bacteria capable of growing on tetrahydrofuran
were also found to be capable of degrading dioxane. The dimethyl sulfoxide pathway
breaks compounds down in just 3 enzymatic steps to hydrogen sulfide, which is a commonly assimilated electron donor for photosynthetic bacteria.\textsuperscript{53,54} (See Appendix O) This constitutes a wide variety of metabolisms which have been found in bacteria and classified, suggesting that many compounds can be biodegraded if the correct enzymes are found and applied.

7.1.3 Mechanisms of toxicity and solvent tolerance in bacteria

Many organic solvents are highly toxic to bacteria. For many of these compounds, especially polar and lipophilic ones, the mechanism of this toxicity is based on the ability of these compounds to accumulate in the membranes of cells.\textsuperscript{55} When the compounds infiltrate the cellular membrane, they decrease the structural integrity of the membrane, causing the cell to become leaky which allows proteins, lipids and ions to escape. This significantly disrupts the membrane potential of the cells, critically damaging their ability to do photosynthesis, which relies on a potential across the membrane in order to generate chemical energy from light.\textsuperscript{56}

Photosynthetic bacteria are gram-negative bacteria, with both a cytoplasmic membrane and an outer lipid membrane, which confers some solvent resistance. The cytoplasmic membrane resists attack by polar and charged molecules, and the outer membrane resists apolar molecules.\textsuperscript{57} In addition, the outer membrane can undergo changes in its composition in response to the surroundings. This typically includes altering the polar head groups of phospholipids, increasing the percentage of saturated fatty acids, and converting \textit{cis}-unsaturated fatty acids to \textit{trans}-unsaturated fatty acids. Each of these actions either renders the outer membrane even less susceptible to
disruption or increases the stiffness of the membrane, allowing more solvent incorporation before the membrane looses structural integrity. Altering the head groups of the membranes can alter the charge of the lipid, helping to maintain charge balance, even with the incorporation of solvent molecules.\textsuperscript{58}

A strain of \textit{Pseudomonas} containing an active solvent pump has also been identified, suggesting that this strategy may be another protective method used by microbes. Where changing the membrane structure is a matter of the bacteria adapting to damaging conditions, actively removing solvent from the membrane is a proactive approach allowing the cell to modify the apparent concentration of solvent, and therefore the concentration of solvent in the membranes. These pumps are thought to be related to the pumps utilized in antibiotic resistance. The solvent pump is energy dependant, and the presence of energy production inhibitors significantly increases the content of solvent in the membranes.\textsuperscript{59} This means that bacteria possessing this ability, as well as an energetic advantage such as photosynthesis, would be better able to drive the pump and protect themselves from high solvent concentrations.

7.2 Materials and Methods

To screen the tolerance and degradation abilities of \textit{B. viridis}, the culture performance was tested in the presence of either 10 mM or 2 mM concentrations of 17 families of chemicals commonly produced as industrial waste. (See Table 21) The structures, molecular weights, and densities are shown in Appendix P. The applied concentrations were determined either from the body of literature described in 7.1.1 or by pre-screening to identify the range of tolerated concentration. Alcohols were offered at
<table>
<thead>
<tr>
<th>Group (S#)</th>
<th>Chemical Family</th>
<th>Compounds Included</th>
<th>Concentration Applied</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (Alcohols/OH)</td>
<td>Alcohols</td>
<td>Methanol, Ethanol, 1-propanol, 2-propanol (isopropanol), 1-butanol, t-butanol, isoamyl alcohol</td>
<td>10 mM</td>
</tr>
<tr>
<td></td>
<td>Phenols</td>
<td>Phenol</td>
<td></td>
</tr>
<tr>
<td>2 (Aromatics)</td>
<td>Phenols</td>
<td>Phenol</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BTEX</td>
<td>Benzene, Toluene, Xylenes</td>
<td>2 mM</td>
</tr>
<tr>
<td></td>
<td>Aromatic Acid</td>
<td>Benzoic Acid/Benzoate</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Aromatic Aldehyde</td>
<td>Benzaldehyde</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Organic Nitrogen</td>
<td>Indole</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Oxygenated 5 Member Ring</td>
<td>Tetrahydrofuran</td>
<td></td>
</tr>
<tr>
<td>3 (Mixed)</td>
<td>Aldehyde</td>
<td>Gluteraldehyde, Valeraldehyde, Formaldehyde</td>
<td>2 mM</td>
</tr>
<tr>
<td></td>
<td>Ketone</td>
<td>Methyl Ethyl Ketone</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ester</td>
<td>Amyl acetate</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Haloalkane</td>
<td>Methylene Chloride</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Multiple Carbon Ring</td>
<td>Potassium Biphthalate, Ninhydrin</td>
<td></td>
</tr>
<tr>
<td>4 (Mixed)</td>
<td>Sulfoxide</td>
<td>Dimethyl Sulfoxide</td>
<td>2 mM</td>
</tr>
<tr>
<td></td>
<td>Amide</td>
<td>Dimethyl Formamide</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Amine</td>
<td>n-butylamine, n-dodecylamine, n-Hexadecylamine</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Haloalkane</td>
<td>1,2-Dichloroethane</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Nitrile</td>
<td>Acetonitrile</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Oxygenated Arene</td>
<td>Dioxane</td>
<td></td>
</tr>
</tbody>
</table>
10 mM, based on considerable evidence from prior work in this laboratory that *B. viridis* has a higher tolerance to alcohols than to other compounds, such as aromatics. For the remainder of the chemicals, 2 mM concentration was utilized. This lower concentration was selected based on literature suggesting that many of the more troublesome compounds are most easily handled at concentrations in the range 1-3 mM.\textsuperscript{60,61}

To prepare each experiment, Minimal 5.6 media and NC media adjusted to pH 5.6 were prepared as before (See Appendix E for formulations) and subsequently distributed to experimental vessels of either 250 mL or 50 mL volumes. Once the vessels were prepared, they were inoculated with a standard 20% inoculation to an initial optical density of approximately 0.2 a.u. The inoculating broth concentrations were of A660 equal to 18.3 a.u. (S1), 13.1 a.u. (S2), 13.7 a.u. (S3), and 10.8 a.u. (S4). For each set, a Minimal 5.6 control and a carbon free (NC media) control were prepared. The compounds were divided into 4 groups, as noted in Table 1. The appropriate volume or weight of chemical was determined, and added after inoculation and immediately before sealing the vessel. This was to prevent volatilization and to insure that any chemicals that were not highly water-soluble would not create a surface layer through which the bacteria would pass and thereby become excessively coated in the chemical.

The culture density was measured at initial time and on day 12 using absorbance. The modified Two-Point method was used because it was considered likely that some chemicals could cause an extended lag phase. By employing the modified Two-Point method, any growth following an extended lag period would be observed, but any immediate growth would also be accounted for, because the initial and final data points would approximate the culture density at the beginning and end of exponential and the
number of days between the two points would be approximately the same as for the unmodified method. Therefore a growth rate could be determined with fair accuracy for any culture beginning exponential growth on or before day five and terminated exponential growth before or around day 12. The growth and growth rate as measured by absorbance at A660, pigment behavior, and culture pH were considered in comparing the cultures to control. As before, pigment and pH are reported only if they differ significantly from the expected behavior.

7.3 Results and Discussion

There were two primary goals for this work. The first was to determine the ability of \textit{B. viridis} to survive in the presence of these compounds. The ability of the bacteria to tolerate the presence of these compounds would be indicated by several factors. The first is a growth rate of similar magnitude. If the cells could grow at a similar rate, then they were not significantly disrupted by the presence of the compound. If cell growth was inhibited by a compound, it indicated that the compound can not be used to support growth, and that the compound decreased the ability of the bacteria to grow or survive. In addition, the pigment signature provided significant information about the effect of the compound. If the pigment production or ratio was reduced, it indicated that the compound either prevented pigment from being produced or it significantly damaged the pigment after it has been produced. Since many compounds are known to damage membranes, the thylakoid membranes that contain the pigments are particularly susceptible to damage, which would be reflected by a change in the UV-Vis absorption spectrum, notably a decrease in the A1020/A660 pigment ratio.
The second goal of this work was to determine whether any compounds enhanced the growth of the bacteria. If additional growth were observed, or if growth was observed in carbon free media, it would indicate that the cells might be able to utilize the compound as a carbon source. If growth were observed in carbon free media, then the compound would be capable of being a sole carbon source. If more than the control growth were observed in Minimal 5.6 media, then the compound would be capable of being utilized concurrently with malate or acting as an enhancer to growth. It is likely, though not certain, that the compound was being degraded and utilized as a carbon source to generate the additional cell mass. Therefore any compounds displaying an enhanced growth response are prime candidates for further exploration because they had a good chance of resulting in a successful degradation.

7.3.1 Screen 1: Alcohols

7.3.1.1 Results

In general, alcohols were not only well tolerated, but acted as growth enhancers when provided in the presence of malate. The rate of growth for Minimal 5.6 cultures supplemented with 10 mM alcohol was increased compared to the control except in the case of isoamyl alcohol and phenol. (See Figure 3) No growth was observed in NC/carbon free media for any of these compounds. The total growth, and total pigment signature were consistent with the varying growth rates for the majority of these alcohols.

Where there was more growth compared with the control, more pigment was also observed. The ratio of pigment to cell density was similar for all cultures, and indicated that there was no particular pigment enhancement or inhibition caused by these compounds.
The final pH of the growing cultures was typically in the vicinity 7.0 – 7.5 for all the conditions that showed equal or better growth than the control. The isoamyl alcohol and phenol cultures had less pH change, consistent with the reduced growth rate and total growth. The carbon free conditions had no change from their starting pH 5.6. (See Figure 4) Compared with previous studies where additional growth and faster growth rates coincided with greater pH changes, for all but two of the spiked chemicals, the final pH observed was less than what would have been expected based on the observed additional growth. The increase in growth rate was on the order of 30% for most cultures, however the difference in pH was not more than around 6%. (See Table 2)
Figure 4: The Final pH Observed for *B. viridis* Cultures Supplemented with Various Alcohols or Phenol. The pH was generally in the range 7-7.5 for cultures grown in Minimal media, and very little change from the initial pH of 5.6 was observed for cultures grown in carbon free media.

**TABLE 22**

**COMPARISON OF GROWTH RATE AND FINAL pH OF *B. VIRIDIS* CULTURES SUPPLEMENTED WITH ALCOHOLS WHICH CAUSED GROWTH ENHANCEMENT COMPARED TO THE MINIMAL 5.6 CONTROL**

<table>
<thead>
<tr>
<th>Compound</th>
<th>% Change in Growth Rate (Compared to Control)</th>
<th>% Change in Final pH (Compared to Control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol</td>
<td>32.3%</td>
<td>-2.2%</td>
</tr>
<tr>
<td>Ethanol</td>
<td>24.1%</td>
<td>5.5%</td>
</tr>
<tr>
<td>1-Propanol</td>
<td>8.0%</td>
<td>-3.9%</td>
</tr>
<tr>
<td>2-Propanol</td>
<td>42.7%</td>
<td>6.1%</td>
</tr>
<tr>
<td>1-Butanol</td>
<td>22.1%</td>
<td>1.6%</td>
</tr>
<tr>
<td>t-Butanol</td>
<td>44.3%</td>
<td>-0.3%</td>
</tr>
</tbody>
</table>
7.3.1.2 Discussion

Ethanol is one of the most commonly applied antimicrobial compounds, used ubiquitously as an antiseptic.\(^6^2\) In the case of \textit{B. viridis}, the growth observed in the presence of 10 mM alcohol indicates that many small chain alcohols can actually function as growth enhancers, contrary to their typical use as antimicrobials. Both methanol and ethanol are extremely short chain alcohols, with only one and two carbons respectively. These caused about 30\% enhancement to the growth rate. It is possible that NADP dependant alcohol dehydrogenase was present and participated in the degradation of ethanol and methanol.\(^6^3\)

Alcohols with substituent groups contributed to more growth than straight chain alcohols. (See Appendix P for structures) Both 2-propanol and t-butanol experienced more than 40\% growth enhancement. The straight chain analogues 1-propanol and 1-butanol experienced only 8\% and 22\% growth enhancement, respectively. Isoamyl alcohol, similar to 1-butanol with an additional methyl group on the second carbon of the straight chain, resulted in a 50\% decrease in growth rate.

Since the isomers 1- and 2- propanol and 1- and t- butanol carry the same amount of carbon, it must be assumed that the difference in growth enhancement reflects the ability of the bacteria to break down and utilize the molecule. Both 2-propanol and t-butanol contain a methyl substituent group, however isoamyl alcohol also has a methyl group but experiences a 50\% decrease in growth. In both 2-propanol and t-butanol, the primary carbon has at least one methyl group as well as one -OH substituent as well. The likely site of attack for these two chemicals is at the primary carbon. (See Figure 5) This structure either fits an enzymatic process present in the bacterium, or the electron
distribution created around the primary carbon, because of the two substituents, is particularly easy to break.

For methanol and ethanol, this site is not present, and the good growth observed suggested that *B. viridis* shares with *B. sulfoviridis* the ability to degrade short chain alcohols,\textsuperscript{64} possibly by an NADP dependant alcohol dehydrogenase, which is general for several alcohols and could therefore participate in the degradation of several of these successful degradations.\textsuperscript{65}

The longer chains, such as isoamyl alcohol, are more non-polar in nature, and may therefore interact in a disruptive way with the cellular membranes. Phenol, which is non-polar by function of its aromatic ring, may also interact to disrupt the cells. Because it contains an aromatic group, it was also assayed at a lower concentration, along with other aromatic compounds.
7.3.2 Screen 2: Aromatic compounds

7.3.2.1 Results

Aromatic compounds, or compounds which contain a benzene ring, were not as universally well tolerated as alcohols. (See Appendix P for structures) Even at the small concentration of 2 mM, many acted as inhibitors. (See Figure 6) Phenol, here offered at only 20% of the concentration employed in 7.3.1, and tetrahydrofuran were among the only compounds that did not cause a decrease in the rate of growth. Both of these compounds displayed growth rates within 5% of the control. Benzene and toluene caused 22% and 42% inhibition to growth, respectively. Xylenes caused 107% inhibition, or a decrease in the total density of cells with respect to the initial culture density. Both benzaldehyde and benzoate reduced the rate of growth to approximately that of the carbon free control, even though malate was present. Indole also significantly inhibited growth in the presence of malate, however the indole growth in the absence of malate was 103% of the growth in the carbon free control.

A significant result is that there is a very high variability in the response of the culture to phenol, benzene, and toluene. The error for these three compounds was as much as 50% of the average growth rate. This was observed as the error in this experiment, among these repeats, but similar variation was also observed on a consistent basis in pre-screening work. This suggested a significant sensitivity to even minute fluctuations in concentration due to pipetting error and volatilization, and that 2 mM was near the maximum concentration that does not inhibit growth, and indicated that tight control of concentration would be necessary in a waste system containing these components.
The pigment production mirrored the growth behavior in most cases, the ratio of A1020/A660 was similar to the control in most cases. (See Figure 7) In the case of both phenol and tetrahydrofuran, there was a slight increase in the pigment ratio, 12% and 5% respectively, in Minimal media, and no change in carbon free media. Benzene, toluene, and xylenes reduced the ratio, 4%, 15%, and 41%, respectively, in Minimal media, with corresponding decreases in pigment in carbon free media, which would cause significant impairment of photosynthesis. Both benzaldehyde and benzoate were not significantly effected in their pigment ratio in Minimal media, however since growth was not observed, it is likely that the pigment was not being actively used in photosynthesis. Benzaldehyde had a significant effect on the pigment ratio in the absence of carbon.
causing a 30% decrease compared to the carbon free control. The pigment ratio for indole was decreased around 25% both in the presence and absence of carbon. The pH behavior of the culture mirrored the growth rate, except in the presence of benzaldehyde.

In Minimal media, the pH was reduced from the initial 5.6 to 5.5, and in carbon free media, the pH was reduced to 4.4 pH units.

7.3.2.2 Discussion

Growth and response instability in the response to the presence of aromatic compounds is very common, due to damage to the cells at higher concentrations. In P. 

_pudita_ mt-2, the addition of 1% by volume toluene killed 99% of the cells, but 0.3% by volume toluene resulted in the survival of 99% of cells. At 2 mM benzene, toluene, and
xylenes all caused inhibition with respect to the control, but the significant fluctuations at that concentration suggested that 2 mM was near a cutoff above which significant inhibition occurs and below which the compound is tolerated.

It is known that substituents on the benzene ring reduce the thermodynamic stability of the ring, and create a point of attack, making it easier for microbes to break down the chemical. In this survey, however, the inhibition of growth increased as the aromatic ring became more highly substituted. It was possible that in the case of toluene and xylenes, the increased polarity of the compounds, which results from the substitution of the benzene ring, allowed them to interact with the membranes of the bacteria causing them to be more leaky and therefore disrupt growth or damage the cells. The increased reduction in the ratio A1020/A660 with increased substitution supported this, because it demonstrated that there is membrane damage occurring in relationship to the number of substituents present.

One of the primary routes of degradation of toluene begins with condensation of toluene and fumarate to benzylsuccinate by benzylsuccinate synthase and goes through benzoyl-CoA. (See Appendix G) Since fumarate was not provided, this reaction may have been unable to proceed. In some bacteria, malate is adequate to allow benzylsuccinate formation, however this ability is not universal. For malate to support growth, the enzyme benzylsuccinate synthase would either need to be nonspecific enough to utilize malate or the bacteria would need to convert malate to fumarate for use. Alternately, \textit{B. viridis} may lack a crucial enzyme in the pathway. Bacterial growth was inhibited 42\% by the presence of toluene, and 85\% by the presence of benzoate. This suggested that there might be elements missing in the benzoyl-CoA pathway. If this were
benzoate CoA ligase, it would have a major impact on degradation of benzoate, which was observed, but no impact on the degradation of other aromatic compounds passing through benzoyl-CoA. This suggested either another enzyme is not present, or there was some other effect being observed.

If the concentration of these compounds was too high, the toxic nature of the compounds could affect the cells at a faster rate than they are degrading them. Toluene is known to significantly permeabilize membranes by becoming incorporated among the polar molecules which compose them. At higher concentrations, the toluene could cause loss of membrane integrity, which would inhibit growth. In the case of *B. viridis*, there are two targets for this type of damage; the inner and outer cell membranes, composed of phospholipids and lipopolysaccharides, and the photosynthetic membranes, composed of lipids. The ability of toluene to incorporate into membranes may also significantly damage the photosynthetic membranes, evidenced by a reduction in pigment signature, which would make the membranes leaky and hamper the ability of the bacterium to create a gradient across the membrane.

Benzoate did not significantly reduce the pigment content per cell. Benzoic acids have been known to reduce the pH gradient across membranes. Since the pigment ratio was not reduced, the formation of the photosynthetic apparatus was not impaired. The use of the apparatus to create a pH gradient and generate energy from photosynthesis for growth and metabolism, however, appeared to be significantly impaired.

The growth of *B. viridis* in carbon-free media with indole indicated that it can be used sparingly as a carbon source. Rajasekhar et al. discovered that inorganic nitrogen sources could significantly enhance the biodegradation of indole with other organisms, so
the presence of ammonia might further assist in growth.\textsuperscript{74} The growth in minimal media was less than the control, but it was similar to the growth rate observed in carbon free media supplemented with indole. This suggested that, unlike previous cases where the presence of malate helped improve growth of the culture, no similar effect was observed in the case of indole. Perhaps indole was inhibiting the use of malate due to preferential uptake of indole or some other mechanism, but indole was also being utilized for moderate growth. Rajasekhar et al. observed 50\% growth inhibition when 1 mM indole was present and malate or other carbons were provided. They also observed a significant 1/3\textsuperscript{rd} - 1/6\textsuperscript{th} decrease in the production of pigment,\textsuperscript{75} which is similar to the 25\% reduction observed for \textit{B. viridis}. This suggests that the two organisms experienced the presence of indole in a similar way and may share nearly identical metabolic pathways, yet to be elucidated, for indole degradation.

7.3.3 Screen 3: Aldehydes, ketones, and other common solvents

7.3.3.1 Results

Within the third group of chemicals, there are none that enhance the growth of \textit{B. viridis} in the presence of Minimal 5.6 media. (See Figure 8) There was very little effect on the total growth for MEK, Methylene Chloride, or amyl acetate, suggesting that \textit{B. viridis} could tolerate the presence of these solvents, but not necessarily utilize them as a carbon source in place of malate. Reduced growth was observed in the presence of valeraldehyde and potassium hydrogen phthalate, and no growth was observed in the presence of gluteraldehyde, formaldehyde, and ninhydrin. In the case of gluteraldehyde, formaldehyde, and ninhydrin, there was also a significant decrease (30\%) in the pigment level compared to the control. (See Figure 9) Final pH was consistent with the
growth observed, except in the case of potassium hydrogen phthalate, which caused a significant decrease in pH in the carbon free media from the initial 5.6 to 4.9.

7.3.3.2 Discussion

There were no compounds in this group of solvents that actively enhanced the growth of cells in the presence of malate, and aldehydes, ninhydrin, and potassium hydrogen phthalate were extremely inhibiting. That aldehydes were inhibiting to growth was not extremely surprising because it was already observed that benzaldehyde led to a more significant decrease in growth rate than benzene or toluene. This accumulated evidence suggested that the cells might be particularly susceptible to damage by
compounds with an aldehyde group, possibly due to the high affinity of the double bond for biological molecules.

For ninhydrin, the growth in the absence of malate was improved compared to growth in the presence of malate, but in both cases the growth was minor and there was a significant reduction in the pigment ratio for cells in the presence of ninhydrin. This suggested that the mechanism of toxicity for ninhydrin is related to a damaging effect on the pigment, and therefore a reduction in photosynthesis.

The growth and pigment formation in Minimal 5.6 in the presence of methyl ethyl ketone or methylene chloride was within 1% of the control, suggesting that neither of these two compounds is damaging to the growth or pigment formation. For methylene chloride, there was a small amount of growth observed in carbon free media, however it

Figure 9: The Pigment Ratio for Cells in Minimal 5.6 and NC (Carbon Free) Media, Supplemented with 2 mM of Various Common Solvents. Significant decrease in the ratio was evident for gluteraldehyde, formaldehyde, and ninhydrin. MEK=Methyl Ethyl Ketone, KPH=Potassium Hydrogen Phthalate.
was significantly less than the growth observed in either control or supplemented Minimal media. This suggested that while methylene chloride is not disruptive to growth, it is not utilized to any great extent as a carbon source. In the absence of malate, a small amount of growth was observed, which suggested that methylene chloride is sparingly assimilated when presented as a sole carbon source.

7.3.4 Screen 4: N, S, and halogen containing compounds and miscellaneous pollutants

7.3.4.1 Results

There was no significant inhibitory effect noted for any of the chemicals assayed in this screen, except in the case of n-dodecylamine, which caused a decrease in the final culture concentration with respect to the initial concentration. (See Figure 10) The presence of n-butylamine increased the rate of growth by about 13%, and acetonitrile and dioxane reduced the rate of growth by 7% and 12% respectively. In all other cases, the difference between the experimental condition and the control was less than 5%, well within the possible error of growth determination. No growth was observed in any of the carbon free conditions. Both n-dodecylamine and n-hexadecylamine were added to the broth as solids, and these did not dissolve in the broth. Both underwent a morphological change from large white solids to smaller solids with brown coloration around the edges, but a significant portion of the added chemical remained at the conclusion of the experiment. This suggested that the bacteria may have adhered to the chemical, but there was inconclusive evidence to suggest either that the bacteria intentionally adhered in order to use the compounds for growth, or that the bacteria became chemically bound to the compound and damaged.
Most of the compounds considered were fairly well tolerated by the bacteria, and caused no significant change in the pigment content. (See Figure 11) The presence of n-dodecylamine inhibited pigment formation, causing a 36% reduction in the pigment ratio. This decrease was in addition to a corresponding decrease in culture density. The decrease in the ratio therefore indicated that while the culture was damaged by the compound, the pigments were damaged relatively more. There was a slight decrease in the pigment production per cell in the case of acetonitrile and dioxane, but these were in the range of 10%, so the cell was not significantly inhibited. The presence of n-hexadecylamine actually caused a 10% enhancement in the pigment production of B. viridis.
The pH behavior of the cultures largely mirrored the growth, except in the case of n-butylamine, which started 0.4 units higher than control after addition of the chemical. It had a final pH 0.5 units higher than the control, despite only marginally greater increase in culture optical density. (See Figure 12) This higher pH throughout was also reflected in the carbon free control, where no growth was observed. The other straight chain amines also caused an increase in the final pH of the carbon free conditions where no growth was observed. They did not, however, cause a significantly higher final pH in the Minimal media condition, where growth was observed in the presence of n-hexadecylamine.

Figure 11: The Effect of N, S, and Halogen Containing Compounds and Common Pollutants on the Final Pigment Ratio of B. viridis. N-dodecylamine caused a 36% decrease in the pigment ratio, and acetonitrile and 1,4-dioxane caused smaller 10% and 8% decreases in the ratio. The presence of n-hexadecylamine caused a slight 10% increase in pigment ratio. DMSO= N,N-Dimethyl Sulfoxide, DMF= Dimethyl Formamide, A=amine, Cl=Dichloro
7.3.4.2 Discussion

The majority of the compounds investigated in this group neither inhibited nor enhanced growth. The only compound to cause significant inhibition was n-dodecylamine. It was interesting that this compound completely inhibited growth, but both a longer and a shorter straight chain amine did not cause such inhibition. This suggested that there might be two different mechanisms of protection or assimilation in use, and the twelve carbon compound bypasses both. Alternatively, the chemical structure could affect the rate at which the compound dissolves in water, and therefore its effect on the cells. The solubility of long chain amines falls off as the chain length increases.

Figure 12: The Final pH of Cultures of B. viridis Amended with Various Common Pollutants. In general, the pH is consistent with the observed growth. In the case of the amines, there is an observable increase in pH. The cultures amended with n-butylamine experienced approximately 0.5 pH unit increase in final pH. This pH increase was observed in the carbon free cultures as well, even in the presence of negligible growth.
increases, so the solubility of dodecylamine in water is greater than that of hexadecylamine in water, less than 0.1 g/100 mL at 22°C. This would increase the bioavailability of dodecylamine in the water compared to hexadecylamine.

Dioxane is a chemical of significant interest because it is a carcinogen and has been identified in at least 27 of the EPA’s 1,674 National Priorities List sites.\textsuperscript{77} Degradation of dioxane is limited, and it is toxic to many organisms. Until recently, advanced oxidation processes (AOPs) were the only proven technology for 1,4-dioxane treatment. 1,4-Dioxane was believed to be very resistant to both abiotic and biologically mediated degradation due to its heterocyclic structure with two ether linkages. Recent work, however, has shown some ability of undefined consortiums to degrade dioxane.\textsuperscript{78} Work with some photosynthetic bacteria has shown that treatment with dioxane can lead to the formation of colorless alternatives to the 820 nm absorbing pigments.\textsuperscript{79} It is promising that \textit{B. viridis} was not significantly inhibited by its presence, nor were the pigments degraded. Because \textit{B. viridis} has a different set of pigments, including bacteriochlorophyll \textit{b}, than most photosynthetic bacteria, they may have some resistance against the mechanism of damage that caused the formation of colorless pigments in bacteriochlorophyll \textit{a} containing PSB.

\textbf{7.4 Conclusions}

The screen of compounds has demonstrated several possible degradations by \textit{B. viridis}. In the presence of malate, the compounds methanol, ethanol, propanol, and butanol enhanced the growth of \textit{B. viridis} by around 30%, and reduced the total change in pH relative to total growth, suggesting that a longer growth period might be observed.
The branched alcohols contributed to greater growth than the straight chain alcohols. This and the inability of *B. viridis* to grow in the presence of isoamyl alcohol suggested a possible site of attack on the molecule at the branch site on the primary carbon. The additional growth suggested that the alcohols were being degraded and utilized, which indicated that *B. viridis* may contain an NADP dependant alcohol dehydrogenase, used for alcohol degradation in other bacteria. At 10 mM, phenol caused severe inhibition to the growth of the cells, but at 2 mM, a great variation in response was noted. This suggested that phenol may be degraded and utilized, but that the concentration is critical. A concentration slightly lower than 2 mM might provide the best possible condition for degradation. Indole allowed for a 100% increase in growth over the carbon free control when provided as the sole carbon source, which suggested that *B. viridis* may be able to degrade and utilize this compound. MEK and methylene chloride also appeared to be either tolerated or degraded for further growth. These chemicals deserve significant further attention to determine if they are actually degraded and incorporated into cell mass, or if some other mechanism is enhancing the growth.

The BTEX chemicals surveyed all caused significant inhibition of the growth at 2 mM. In addition, benzoate caused a greater level of inhibition than any of the BTEX compounds. Since benzoate is an intermediate in the known BTEX degradation pathway, there is a possibility that the cells cannot degrade these compounds, or that they would be degraded via an unknown pathway. Alternately, the cells may participate in portions of the pathway but are missing key enzymes to complete the pathway. This lack of enzyme could be genetic or could indicate a critical nutrient or cofactor is missing which does not allow the enzyme to function correctly. The vitamins assayed in earlier chapters can
Contribute significantly to enzyme function as cofactors, so the next step in the investigation would provide these and assay degradation potential. If the compounds are not degraded, they can cause serious membrane damage, which would account for the decrease in both growth and pigment signature noted. A critical investigation would include assay of these chemicals in the presence of fumarate, as well as in the presence of various B vitamins in an attempt to enhance the abilities of any enzymes present.

No other compounds displayed significant growth enhancement, but B. viridis tolerated the presence of most of the chemicals examined in screen 4, including dioxane, which is highly relevant. If B. viridis can be encouraged to degrade dioxane either directly or co-metabolically, it would be of great use to many industries. This possibility deserves significant attention, and there is much potential for further exploration with this species.
7.5 References


38. Leutwein, 517-524.


47. Renhao Li, 1,2-Dichloroethane Pathway Map. University of Minnesota, July 15, 2002 <http://umbbd.ahc.umn.edu/dce/dce_map.html>


52. Bernhardt, 120-123.


54. Voet, 628.


59. Isken, 6056-6058.


61. Wright, 2069-2073.


64. Zengler, 204-212.

65. Eubanks, 1133-1143.


67. Ramos, 3887-3890.


70. Zengler, 204-212.


73. Sikkema, “Mechanisms of Membrane Toxicity of Hydrocarbons,” 201-222.

74. Rajasekhar, “Photometabolism of Indole,” 39-44.

75. Rajasekhar, “Photometabolism of Indole,” 39-44.


8.1 Introduction

There are two main areas of focus for future work with *B. viridis*: to continue optimization of the media and culture conditions to insure the maximum possible growth and pigment production, and to better understand the cultures in the presence of toxins.

The current Optimized media at pH 5.6 demonstrates a significant improvement with respect to the baseline media, Minimal media at pH 6.9; however, there are several vitamin additions such as Biotin, Thiamine, Vitamin B_{12}, Pantothenate, and \( p \)-ABA, which have not been tested at a wide range of concentration. Through this research, the external conditions under which the culture will perform best have been defined, and it is clear that the incident and penetrative light conditions have a significant impact on the performance of the culture. A new bioreactor design to allow a higher surface area to volume ratio would improve the success of scaled up culture growth.

Use of *B. viridis* as a tool for remediation and treatment of waste is the second area of focus of much greater magnitude. A brief screen of chemicals was performed to determine families of chemicals that enhance, do not affect, or inhibit growth. Based on the screen, three things were inferred:
• Decreased growth demonstrated the chemical at offered concentration was either an inhibitor or was damaging to the bacteria. In some cases, a change in chemical concentration or culture conditions might allow utilization of these chemicals;

• Unchanged growth demonstrated that the chemical did not actively damage the bacteria, but was not necessarily assimilated. Identification of chemicals which may be co-metabolically degraded or degraded under different conditions is needed;

• Enhanced growth suggested that the bacteria are able to assimilate carbon from the compound for growth or that the compound was able to act as an enhancer in some other way.

Which of these is true for each chemical compound needs to be evaluated, either by isolation of enzymes induced during exposure to specific compounds, or by genetic screening.

8.2 Suggestions for Future Work
8.2.1 Optimizing media formulation and culture conditions

The Optimized media improved culture growth and pigment formation by a factor of two and three, respectively. The most effective vitamin in improving both culture density and pigmentation was biotin; however, thiamine, vitamin B12, and pantothenate also had a moderate effect. (See Chapter 6.3.4) Except for thiamine, each of these vitamins was tested at a single concentration, $2 \times 10^{-6}$ M. Thiamine was tested at zero, $2 \times 10^{-6}$ M, and $6 \times 10^{-6}$ M. To more closely identify the optimal concentration of these vitamins, *B. viridis* should be cultured in the presence of a wider range of concentrations,
between 0 M and 10x10^{-6} M. It is expected that the growth and health of the culture will improve with increasing concentration to some level, but above that concentration, additional supplementation will not be correlated to additional growth or pigment formation. The concentration corresponding to the best growth results should be utilized in the revised media formulation.

A redesign of the light exposure experiment could enhance the understanding of the light intensity requirements of the bacteria. Due to the logarithmic nature of light and the experimental design, a great many values between 0 and 500 lux were measured, and several additional values around 1000 lux and 2000 lux were obtained. (See Chapter 6.2.8) A careful design with a modified spatial layout could provide a greater number of samples in the range 500 lux – 2000 lux. Light intensity is based on inverse square law with respect to distance, so this would indicate a greater number of cultures 10-20 centimeters from a 25 watt bulb. In the layout used before, where the bulb was recessed about 10 centimeters from the aperture of the housing, this would suggest placement between 1 and 10 centimeters from the bulb housing to achieve this light range. The area available is highlighted in yellow in Figure 13.

In addition to determining the optimum incident light, the results of this research indicated that the penetration of light is limited and better growth is observed when the majority of the culture is in the “shallow” portion of the vessel. (See Chapter 6.3.7) Because growing entirely in 50 mL batches is not an efficient way to utilize these bacteria, flat panel reactors have been designed by several groups to increase the surface area/volume of the culture vessel, insuring more complete penetration of light, while allowing large culture volume.\textsuperscript{1,2,3}
There are two separate goals for a design. The first goal is to design a small bench scale vessel for evaluation of growth conditions and degradative potential. This vessel would most be most effective between 250 mL and 1 L, so that several conditions can be assayed simultaneously, and should maintain a surface area/volume ratio greater than 1. In addition, this vessel design should be inexpensive so that many can be created in order to be able to screen a wide variety of conditions or compounds simultaneously.

Figure 1: The Arrangement of Vessels to Achieve 500-2000 lux Light Intensity.
A simple initial change could be the use of flat polystyrene culture bottles which are commercially available, however some of the compounds may cause a decrease in their structural integrity so care must be taken. For application to industrial waste streams, it would be simple to generate a flat panel reactor that met the volume requirements for the remediation task. (See Figure 14) This would incorporate several design features: addition of malate, ammonium sulfate, bicarbonate, and critical vitamins, pH adjustment of the waste stream to 5.6 prior to contacting the bacteria, a surface to volume (S/V) ratio of greater than 1, and a recycle stream that would insure inoculation of the incoming stream. The addition of growth enhancers would also serve to dilute the incoming concentrations of chemicals to 2 mM or below for aromatics and many solvents) and around 10 mM for alcohols. Ideally, it would be positioned so that a light flux of approximately 1000 lux is incident on the surface, and a sampling port would allow aseptic sampling of the effluent so the health of the bacteria and concentration of target components could be monitored.

Figure 2: Proposed Design of Flat Panel Reactor
8.2.2 Toxicity

The LD$_{50}$ (50% of lethal dose) toxicity of a chemical compound is normally reported as an overall concentration. There are four possible outcomes of contact with various concentrations of the target compound. (See Figure 15) As in A, the increasing concentration of the compound could cause poorer and poorer growth because it is an inhibitor and cannot be assimilated. As in B, an increasing concentration could enhance the growth to a certain concentration because it can be utilized as a carbon source, and then cause damage to the cells because concentration causes cell damage faster than the compound can be assimilated. As in C, the compound could cause greater enhancement to the growth as the concentration is increased, because the compound is assimilated as a carbon source at all concentrations. Finally, as in D, it is possible that the compound could be severely toxic and at any concentration and prevent cell growth.

![Figure 3: Four Possible Outcomes of Contact with Various Concentrations of the Target Compound. A) increased concentration of the compound causes poorer growth. B) increased concentration enhances the growth to a certain concentration, and then causes cell damage. C) increased concentration causes unlimited enhancement to growth D) at any concentration, cells will not grow.](image-url)
In addition to the simple concentration of toxin in the media, the concentration of cells, or the concentration of toxin per cell, also seemed to have an impact on the effect of the toxin. To evaluate the difference in cell concentration on the effect of a toxic compound, a moderate concentration of compound can be added to cultures in the exponential growth phase, adjusted to various cell densities with sterile, fresh media.

8.2.3 Degradation confirmation and pathways

To definitively determine the degradation of a compound, the concentration of the compound must be tracked over time. If the concentration can be readily assayed by a chemical or spectrophotometric method, then its presence can simply be tracked. Alternatively, radiolabeled compounds can be used to track the fate of the compounds. To do this, a radiolabeled compound would be added to the culture, and the spent media passed through HPLC to achieve separation. Identification of the various radiolabeled peaks, by the use of standards of known intermediates, would indicate the presence and relative concentration of degraded forms of the compound. Small molecules, which would indicate complete mineralization, would elute in the void volume, and the decrease in the parent peak would indicate the occurrence of degradation. In addition, the cell mass could be separated from the culture broth and counted using liquid scintillation counting to determine how much of the compound had been incorporated into the cell mass. To minimize the use of radiolabel and reduce the costs associated with their purchase and disposal, the radiolabel should be provided at micromolar concentrations, along with the balance of cold compound to the desired total concentration, to act as a marker to determine the relative distribution of the compound. In addition, these assays should be done in the smallest culture volumes possible, not greater than 50 mL.
8.2.3.1 Alcohols

When malate is present, methanol, ethanol, propanol, and butanol enhanced the growth of \textit{B. viridis} by around 30\%. (See Chapter 7.3.1) In addition, the final pH was significantly lower than the control. This suggests that a longer growth period might be possible, and could lead to a more complete degradation of the alcohols present. To test this hypothesis, a growth curve measured daily for more than 20 days could demonstrate a change in the length of the growth phase.

Based on the response pattern, it seemed likely that branched alcohols are more easily degraded and result in more growth than straight chain alcohols. A likely site of attack is on the primary carbon if has both an –OH group and a methyl or other substituent group. (See Figure 7.5) Since growth on isoamyl alcohol was not observed, it seems that a branch site alone does not contribute to the increased growth. In order to determine more definitively the site of attack and the pattern of degradation among the alcohols, longer chain alcohols with the proposed site as well as their straight chain counterparts should be offered and assayed for growth. Alternatively, alcohols radiolabeled on various carbons could be employed. By assaying the reaction products to determine how the molecules are broken, the site at which the alcohol is cleaved can be confirmed.

8.2.3.2 Aromatics

The presence of the benzoate pathway was not confirmed. Benzoate itself caused a significant inhibition in growth, and toluene resulted in a nearly 50\% inhibition in growth. Alternatively, a particular nutrient or cofactor may be missing for the enzymes, rendering the pathway ineffective. An example would be the lack of Coenzyme A,
required for benzoate CoA ligase to generate benzoyl CoA. (See Appendix J) CoA is synthesized from pantothenate. If inadequate pantothenate was offered, the benzoate could not be converted to benzoyl-CoA, and the degradation could not proceed.

For toluene, there are a number of additional modifications which could encourage growth. First, the assay for growth and degradation should be performed in the presence of fumarate, which is required in the first step. (See Appendix G) The addition of malate is adequate for benzylsuccinate formation in *Azoarcus* sp. strain T, however this ability may not be universal. It would also require supplementation with pantothenate to insure adequate Coenzyme A. In addition, providing additional exogenous CO2 should be explored because exogenous CO2 can act as an electron donor and help in the creation of chemical energy, or ATP, which is used to drive many of the reactions in the pathway. In addition, CO2 has a significant role in many degradations because it is condensed with the aromatic compound during the pathway reactions. In a denitrifying bacteria strain T, the addition of ATP or CoA caused variation in the products observed, which demonstrates that providing the appropriate requirements can significantly improve the efficiency and effectiveness of the pathway. An excess of magnesium ions has also been shown to decrease the lag phase for cells grown in high concentrations of toluene. It is thought that this occurs because magnesium participates in membrane repair, and therefore high concentrations of magnesium contribute to reducing the impact of solvent damage to the membranes. With knowledge of the enzymatic steps involved in the pathway and the mechanism of toxicity, the critical factors for the degradation of each compound can be determined and assayed.
Another important task is to insure that all the enzymes of the pathways are present. If the additions do not enhance growth and lead to degradation, then the bacteria may not code some enzyme or set of enzymes. In that case, the missing enzymes must be determined. One way to isolate the likely enzymes that are missing is to provide the substrate for each enzymatic step and assaying for the product, either in whole cells or in cell extracts. Alternatively, known amino acid or RNA sequences for important enzymes can be used to probe for the presence of the enzymes in \textit{B. viridis}.

8.2.3.3 Other compounds

The degradation of other compounds can be assayed using similar techniques to those described above. Of particular importance was the ability of \textit{B. viridis} to tolerate the compound dioxane, which is of significant current interest. Since the presence of dioxane is tolerated, unlike many other organism which find it highly toxic, even if the compound can not be assimilated, there may be a cometabolic reaction which would cause degradation while the bacteria grew on another carbon compound presented. This possibility deserves significant attention.

8.2.4 Alternate uses of the bacteria

\textit{B. viridis} cells contain both a cellular membrane, and membranes associated with photosynthesis. The molecular response of membranes to hydrophobic and aromatic chemicals, especially those that negatively affect growth, can be used to absorb lipophilic components of waste streams in the absence of actual chemical degradation. The accumulation of aromatic compounds in the cellular membrane has been observed to cause a significant decrease in the extracellular concentration of these compounds.\footnote{9}
Although the results are extremely preliminary, in an examination of the toluene spiked media at early time intervals after contact with cells, the HPLC results indicate that toluene is removed almost immediately upon contacting the bacteria. (See Figure 16) After two passes through the cell layered filter, nearly 75% of the initial toluene concentration has been removed. Experiments in which the cells were contacted with toluene spiked media via a well mixed solution also demonstrated a similar effect, and after 90 minutes, only 12% of the initial toluene signature remained. This would cause significant damage the bacterium, preventing it from being able to grow or regenerate in the presence of high concentrations of compound. In this case the bacteria should be thought of as similar to activated charcoal- an additive which removes contamination but must be regenerated; regeneration would take the form of generating cultures for use separate from the waste stream. Further investigation into this use could demonstrate how efficiently the bacteria can remove lipophilic compounds and how much could be removed before they were spent.
Using Bacteria to Filter Toluene from an Aqueous Base

Figure 4: The Removal of Toluene By Filtering With B. viridis Cell Mass. A slight decrease is noted in the control broth, which passes only through the filter support. In the broth passed through a filter layered with bacteria, only 40% of the initial toluene signature remains. After 2 passes, only 25% of the initial toluene remains.

8.3 References


2. Hoekema, 1331-1338.


4. Voet, 825-826.


8. Pinkart, 4219-4226.

WORKS CITED


Birdsell, Dennis and Loftus, Jon. Discussion of Facility Use, Fall 2003, Center for Environmental Technology, University of Notre Dame, Notre Dame, IN.


Hiraishi, Akira. “Transfer of the Bacteriochlorophyll b-Containing Phototrophic Bacteria Rhodopseudomonas viridis and Rhodopseudomonas sulfoviridis to the Genus


Irschik, H. and Oelze, J. “The Effect of Transfer from Low to High Light


Kniemeyer, Olaf and Heider, Johann. “(S)-1-Phenylethanol dehydrogenase of *Azoarcus* sp. strain EbN1, an enzyme of anaerobic ethylbenzene catabolism.” *Archives of Microbiology* **176** (2001): 129-135.


Kuver, Jan; Xu, Yohong; and Gibson, Jane. “Metabolism of cyclohexane carboxylic acid by the photosynthetic bacterium Rhodopsedomonas palustris.” Archives of Microbiology 164 (1995): 337-345.


Larimer, Frank W.; Chain, Patrick; Hauser, Loren; Lamerdin, Jane; Malfatti, Stephanie; Do Long; Land, Miriam L.; Pelletier, Dale A.; Beatty, J. Thomas; Lang, Andrew S.; Tabita, F. Robert; Gibson, Janet L.; Hanson, Thomas E.; Bobst, Cedric; Torres y Torres, Janelle L.; Peres, Caroline; Harrison, Faith H.; Gibson, Jane; and Harwood, Caroline S., “Complete genome sequence of the metabolically versatile photosynthetic bacterium Rhodopseudomonas palustris.” Nature Biotechnology 22 no.1 (Jan 2004): 55-61.


Leutwein, Christina and Heider, Johann. “(R)-Benzylsuccinyl-CoA dehydrogenase of Thauera aromatica, an enzyme of the anaerobic toluene catabolic pathway.” Archives of Microbiology 178 (2002): 517-524.


Madigan, Michael. “Microbiology, Physiology, and Ecology of Phototrophic Bacteria.”


Nakada, Eiju; Asada, Yasuo; Takaaki, Aria; and Miyake, Jun.“Light Penetration into Cell Suspensions of Photosynthetic Bacteria and Relation to Hydrogen...


Rabus, Ralf; Kube, Michael; Beck, Alfred; Widdel, Friedrich; and Reinhardt, Richard. “Genes involved in the anaerobic degradation of ethylbenzene in a denitrifying bacterium, strain EbN1.” Archives of Microbiology 178 (2002): 506-516.


Zengler, Karsten; Heider, Johann; Rossello-Mora, Ramon; and Widdel, Friedrich. “Phototrophic utilization of toluene under anoxic conditions by a new strain of Blastochloris sulfoviridis.” Archives of Microbiology 172 (1999): 204-212.


BRENDA, The Comprehensive Enzyme Information System, Release 5.1
www.brenda.uni-koeln.de/>

Elementar Americas, Inc. Mt. Laurel, NJ, USA <http://www.chnos.com/hightoc.htm>

Envitech. United Kingdom. <http://www.envitech.co.uk/Prod_StipTOC.html>


The University of Minnesota Biocatalysis/Biodegradation Database  
<http://umbbd.ahc.umn.edu/index.html>

NIOSH Document #705005


Personal Communication: R.L. Irving, Biochemical Engineering Class, Department of Civil Engineering, Spring 2002, University of Notre Dame, Notre Dame, IN.


TRI On-site and Off-site Reported Disposed of or Otherwise Released (in pounds), for facilities in All Industries, for All Chemicals, U.S., 2003 Data Update as of June 8, 2005. <http://www.epa.gov/triexplorer/>
APPENDIX A

SPIN PLATE DEVICE

Introduction

Spread plating is an established microbiology method that has been employed to obtain a count of the number of live microbes in a culture.\textsuperscript{1,2} It is used in many different disciplines including medical and pharmaceutical research,\textsuperscript{3} waste water treatment and remediation,\textsuperscript{4} food science,\textsuperscript{5,6} bioprocessing, and quality control.\textsuperscript{7} The spreading of the cell broth and rotation of the plate are both highly irregular since the procedure is usually performed manually. This means that the results, which depend on the speed with which the plate is rotated, are highly dependant on the technician’s familiarity with the procedure, strength, and muscle fatigue. This muscle fatigue can lead to variations in the speed of rotation over time when large amounts of plating is performed, and it is also problematic for the technician, since it can cause muscle injury and even nerve damage due to repetitive stress injury.

In 1994, Filley and Hill proposed a makeshift electric turntable for culture plating.\textsuperscript{8} In their device, a used centrifuge was employed to rotate the culture plate. Electric operation was attempted but the researchers noted that caution against excessive speed was required, and suggested that the rotor could also be operated manually. They noted that the results were “satisfactory” but did not quantify the results against the
traditional tabletop method. Here, we present and evaluate an easily constructed, foot
treadle operated device which reduces technician fatigue and standardizes the plate
rotation speed. When the plate is rotated while in contact with a flame-sterilized glass
spreading rod until all liquid is evenly spread and absorbed into the plate (approximately
30 seconds), this method reduces the deviation in the counts obtained while maintaining a
value equivalent to that obtained with the traditional hand rotation method. Operator error
that could lead to contamination, such as slipping and touching the surface of the plate
while rotating the turntable, is minimized since intrusion into the sterile work zone is
avoided. This device also significantly reduces technician fatigue, which allows the work
to be completed more easily and with less interruption. This device can be constructed
simply and inexpensively using existing spin plating and other equipment found in a
typical biological laboratory and materials readily obtainable from a hardware store.

Device Construction

The three primary items used to construct this device were 1.) a manual spin
plating turntable Fisher (Catalog No. 08-758-10), 2.) a 115 volt, 22 watt stirrer motor
with maximum rotation speed approximately 300 RPM obtainable through EH Sargent &
Co, Chicago, IL, and 3.) a variable speed foot treadle (Cat No 980-SC3) obtained from
Linemaster Switch Corporation. (See Figure 5) Other model turntables, stirring motors,
and foot treadles may be substituted.
The plan for assembly of the device is rather simple. (Figure 6) The device was constructed by mounting both the plate (Figure 1a) and the motor (Figure 1b) (inverted) such that the top of the motor shaft is aligned with the rotatable portion of the turntable. The turntable is mounted on a horizontally movable screw shaft so that it can be moved into contact with the motor shaft. (See Figure 2 and Figure 3) A discarded 30.5 cm x 12 cm x 11 cm prefabricated metal electrical components box (Varian Extrion Division Assembly No. EF 2698-1 Rev. F S/N W-24812) was reused to house the device, securing the motor in an inverted and immobile position and raising the rotating portion of the turntable level with the shaft of the motor. Any alcohol sterilizable metal or plastic may also be used for the housing. A rubber cap was added to the end of the shaft to increase the friction and widen the shaft to improve the operability of the device. The power cord from the motor shaft was passed through a hole in the side of the box and spliced into the foot treadle (Figure 1c) output and the foot treadle power cord connected to a wall outlet. For the experiments in which variable speeds are employed, a Variac voltage regulator was employed in line between the foot treadle power cord and the wall outlet. The produced device (Figure 7) was readily mobile easily sterilizable.
Figure 2: The Simple Design of the Spin Plating Device. The motor is mounted inverted, and a soft rubber cap is aligned with the turntable. The power cord to the motor is connected to a foot treadle so the device can be operated hands-free.

Figure 3: The Final Design for the Device. The design was implemented using the noted parts and a recycled electrical housing box. The device is made of parts which can be alcohol sterilized and it is light enough that it can be readily moved into the hood for plating and removed to provide more room for other operations. In experiments where speed adjustments were made, the device was plugged into a Variac voltage regulator, and the Variac was plugged into the wall outlet.
Culture Preparation

*B. viridis*, obtained from the Laboratories of James R. Norris at the University of Chicago, Department of Chemistry was used in this experiment. To demonstrate device performance, 7-day old cultures of *B. viridis*, a microaerophilic photosynthetic bacterium, were inoculated at 2% by volume into liquid RMPABA media (See Appendix E) and allowed to grow to an absorbance at 660 nm of 1.5 a.u., approximately 5-7 days, corresponding to the late exponential growth phase. Absorbance measurements were taken on a Shimadzu UV Mini-1240 spectrophotometer. Approximately 0.7 mL of the culture suspension was pipetted into a Fisher Scientific Suprasil quartz microcuvette with a 10.00 ± 0.01 mm pathlength, 45 mm height, and 0.5 mL capacity. For very dense solutions, bacteria were diluted with Epure Water (18 MOhms), each time by a factor of two, and subsequent measurements were taken until the suspension absorbance at 660 nm was below 0.8 absorbance units. Measurements were corrected for dilution factor before analysis.

Agar Plate Preparation

Sigma fine agar powder (Sigma, St. Louis, MO) was used to prepare culture plates. Agar plates were prepared manually by mixing 20 g agar powder per 1 L RMPABA media with 20 g/L of agar powder. The mixture was autoclaved to simultaneously sterilize the solution and melt the agar. Approximately 20 mL of melted agar media was poured into each Fisherbrand polystyrene 100 mm x 15 mm disposable sterilized Petri dishes (Cat No. 08-757-12) and allowed to cool, covered, in a sterile
environment until solid. Poured plates were stored in sealed sterile plastic sheaths at 2-4°C until used, but not for longer than 4 weeks.

Serial Dilution

The cell suspension was first measured to determine the A660, and the approximate number of cells present in the suspension estimated. This estimate was used to determine the range of dilutions necessary. The undiluted cell suspension was prepared for plating by serial dilution by a factor of 10. (See Figure 2.2) Serial dilutions were performed by adding 1 mL of diluted bacterial broth or previous well-mixed dilution to 9 mL of 10 mM Tris buffer plus 1 mM NaEDTA to prevent coagulation at pH 7.0 in sterile 50 mL Falcon tubes. The Falcon tube was then agitated by hand for approximately 30 seconds to achieve a well-mixed suspension. The tube was also re-agitated immediately prior to sampling. This factor of 10 dilution was repeated until 3-4 dilutions in the estimated vicinity of 30-3000 cells per mL (3-300 cells/100 μL plated volume) were obtained.

Plating of Cultures

Plating was performed by pipetting 100 μL of the desired dilution onto the center of a pre-prepared spinning agar plate that had been allowed to equilibrate to room temperature. A flame-sterilized bent glass rod was rested on top of the agar and held stationary. The wheel was then rotated by hand for control experiments, or by the mechanized apparatus. The added fluid was generally visually well distributed and absorbed into the agar within 30 seconds. Total spin time was 30 seconds for the varied
rotation speed experiments, and 5, 15, or 30 seconds in the varied time experiments, when increments of time were used. The timed period was started when the foot treadle was depressed (or when the first manual rotation was made) and at the end of the time period, the glass rod was lifted from the surface of the agar. Plates were then sealed with Parafilm, and placed under incandescent light bulb illumination of approximately 170±30 lux, at a temperature of 32°C for 48 hours, at which time the colonies formed were counted. Four different dilutions with 5 plates per dilution were plated for each condition, for a total of 20 plates. Plates with between approximately 3 and 300 visible colonies were counted. In general, plates with greater number of colonies were discarded as uncountable and unreliable. There were, in general, two countable dilutions, yielding approximately 10 countable plates per condition. The number of colonies counted per plate was multiplied by the dilution factor of the broth and 10 (to account for the 100 μL sample volume) to obtain the total number of live cells per milliliter in the starting cell suspension. Incidences of smudging and contamination were noted, and those plates severely affected were not included in the statistics.

Counting Colonies

Colony forming units (CFUs) are generally used as a measure of the number of bacteria in the cell suspension. Because cells are microscopic, and it can not be insured that each colony starts from a single cell, each colony is said to represent one colony forming unit from the initial suspension. For most cultures, each CFU corresponds to approximately one cell, however if the cells are prone to flocculation, each CFU can represent two or more cells.\textsuperscript{11} \textit{B. viridis} are fairly well segregated cells not known to
flocculate, and with gentle agitation in the dilutions, one cell per CFU is a reasonable assumption. CFUs were counted by placing the plate against a black surface and visually counting each colony, after taking a picture. In order to insure that each colony was only counted once, counted colonies were marked with a dot after being counted.

Results and Discussion

In order to determine the optimal speed of continuous rotation, 20 plates for each condition (5 plates for each of 4 dilutions) were prepared using 100 μl of each dilution. The plated volume was spread for 30 seconds at each of three speeds: slow, medium, and maximum and by hand. Maximum speed was approximately 300 RPM and was the upper limit of the motors capability (Setting 90 on the Variac). “Slow” was approximately 40 RPM, and was determined by lowering the voltage on the Variac unit until the motor would just barely rotate (Variac setting 55). “Medium” was approximately 110 RPM, and was determined by selecting a Variac setting of 72, between that for “Maximum” and “Slow.” The optimum speed to obtain results consistent with the traditional manual method was the maximum speed, or 300 RPM. The optimal time for spreading was evaluated by preparing 20 plates each at 300 RPM spread for 5 seconds, 15 seconds, or 30 seconds to determine the necessary time to obtain a plate consistent with the traditional method.

At the maximum speed of the rotation (300 RPM) applied for 30 seconds, the average count was closest to the count obtained by the traditional manual method (Figure 4A), and the standard deviation in the count was reduced significantly. Compared to slower speeds, the standard deviation was reduced by 67% and 80% respectively. The
standard deviation was reduced by 56% over the manual method. It estimated that the manual method provides a speed of rotation approximately similar to the “maximum” speed setting, however the nature of the rotation is less regular due to the intermittent operation of the turntable when it is operated by hand. At slower speeds, fewer rotations were completed and a larger amount of fluid remained unabsorbed at the completion of the spreading period. These plates (at “slow” and “medium” speed) tended to have smudges instead of discrete colonies. It is likely that movement of bacteria across the wet surface of the agar prevented the reproducible formation of discrete CFUs. Cell division also could have occurred in the time between the completion of the spin and the eventual absorption of the bacterium into the agar, leading to both a possibly greater overall count and a much larger standard deviation. The total time of the spin spreading was also critical in obtaining reproducible CFU counts. (Figure 4B) The minimum time to obtain good results was approximately 30 seconds. Although the average number of colonies counted was fairly consistent (within approximately 1X10^5 CFUs), the total error associated with the measurement was 3 and 4 times greater for 15 second spin and 5 second spin, respectively. At 5 seconds, the variability is unacceptably large; the error is larger than the measurement to which it is applied. In the traditional hand method, plates are typically spun until fluid is well spread and absorbed. In the mechanized method, fluid is typically well spread and absorbed by 30 seconds, meeting the traditional criteria. In order to reduce operator time and increase efficiency, greater lengths of time beyond meeting the traditional criteria were not explored.

Another consequence of manual spinning or spinning at slow non-optimal speed is an increase in the number of incidences of either colony smearing or plate
Figure 4: Effects of Rotation Speed and Spreading Time A) At the maximum speed of the rotation (300 RPM) applied for 30 seconds, the average count was closest to the count obtained by the traditional manual method, and the standard deviation in the count was by around 70% compared to slower speeds and by 50% compared to the manual method. Since the traditional method has a non-quantified and variable speed, it is displayed in bar format. B) The total time of the spin spreading was also critical in obtaining reproducible CFU counts. The minimum time to obtain good results was approximately 30 seconds. The average number of CFUs was fairly consistent, however the total error associated with the measurement was 3 and 4 times greater for 15 second spin and 5 second spin, respectively. At 5 seconds, the error is larger than the measurement to which it is applied.
contamination. Smearing was defined as growth which was spread across a wide area, and not in a discrete colony. (Figure 5A, C, D) It is likely that the smearing resulted from poorly planted cells which would be able to diffuse and create smudges, rather than discrete colonies. Contamination, for this purpose, was defined as any growth on the plate not consistent with the known morphology of the cells being used in the application. (Figure 5B, C) Typical *B. viridis* colonies at two days are circular, slightly mounded, opaque, off white, smooth, and shiny. Colonies which were irregularly shaped or textured, unusual colors, or dull are considered contamination.

In this work, 20 plates (5 plates for each of 4 dilutions) were prepared for each condition. The results were calculated using all countable plates, which included 2

![Figure 5: Examples of Contamination and Smearing. This occurred more frequently in the slower and shorter spin conditions. A) Smearing B) Contamination by a irregular morphology C) smudging and irregular morphology D) colonies all appear diffuse and are slightly smudged.](image)
dilutions. This resulted in a total of 10 plates. Contamination and smudging incidence was recorded for all 20 countable and uncountable plates. Plotted in Figure 6A and 6B are the sum of all incidences of contamination or smudging on each plate, for the varied speeds of rotation and rotation by hand, and for the total time of spreading using the optimum mechanized speed, 300 RPM, respectively. Values greater than 20 (the total number of plates) indicate that both contamination and smudging were noted on the same plate. Mechanized spinning at the optimum speed reduces the incidence of contamination and smudging by a factor of 4 over the traditional hand spun method. At slower speeds and at shorter spreading times, significantly greater smudging and contamination is noted, likely because there is significant fluid remaining on the plate, which allows the cells greater motility prior to the cells imbedding in the agar.

The mechanized device, when used at the optimum speed, 300 RPM, with a spreading interval of 30 seconds, improves the distribution of the colonies across the plate surface in comparison to both hand spun results as well as slower speeds and shorter spinning times. (See Figure 7A) In this condition, colonies are much more evenly distributed and discrete across the surface of the plate. For the plates spun at slower speed and shorter intervals, the distribution is non-uniform and colonies are more difficult to distinguish. Plates spun by hand display similar characteristics. This poor distribution displays in two primary ways. One is colony clumping, where a large number of colonies are located almost on top of one another. (Figure 7B) The other is uneven distribution across the plate, where colonies might be frequent in one quadrant of the plate, but entirely absent from another. (Figure 7C) In the plate shown, spun for a 15 second interval, 70% of all colonies appear in the upper two quadrants and no colonies
Figure 6: Effect of Speed of Rotation and Spreading time on Contamination. A) The speed of rotation has a significant effect on the behavior of the colonies. The total amount of smudging or contamination is reduced by a factor of 4 when mechanized spinning at the optimum speed replaces spinning by hand. Since the traditional method has a non-quantified and variable speed, it is displayed in bar format. B) Using the optimized mechanized speed of 300 RPM, various lengths of spin time were explored. At short spin times, a much greater incidence is noted. This is likely a result of non-imbedded colonies in the fluid which remains on the plate at the conclusion of the spread time.
appear in the lower right quadrant. Although no direct correlation between smudging and
distribution was determined, both occur with greater frequency in the hand spun, slower
rotation speed, and shorter spin time conditions.

Repetitive stress injuries (RSI) are listed by OSHA as one of the leading causes of
injury in workplaces, and laboratory work is not exempt from this pattern, which is
estimated to cost $20 billion annually in lost time alone.\textsuperscript{12} NIOSH has determined that
repetitive, forceful, or prolonged exertions of the hands, pushing, pulling, and prolonged
awkward postures significantly increase the risk of RSIs.\textsuperscript{13} In 1994, there were 705,000
injuries due to poor ergonomic conditions.\textsuperscript{14} Manually spinning the turntable involves
these motions. Working in a sterile laminar flow hood also requires that the arms be held
forward with the shoulders rounded forward, adding strain to an already high risk task.\textsuperscript{15}
Shoulder injuries have been substantiated in cases of highly repetitive work including
cyclical flexion and repeated or sustained shoulder postures with greater than 60 degrees
of flexion, which is required for working with the body outside of the sterile area and the
hands and arms inside the sterile area.\textsuperscript{16} Carpal Tunnel Syndrome and other wrist

Figure 7: Examples of Colony Distribution. A) In the optimum condition, 300 RPM for
30 seconds, colonies are well distributed across the plate. B) Clumping of colonies
occurs in hand spun, slower speed, and short time conditions. C) In the non-optimum
conditions, many plates appear to have poor distribution with most colonies occurring in
only one or two sectors of the plate surface. No colonies appear in the lower right
quadrant, and over 70% of colonies are in the upper half.
injuries have been strongly associated with repetitive work, poor wrist posture, and forceful work. Combination of several high risk activities increases the risk of musculoskeletal injury. Because the manual operation of a turntable and working in a sterile hood are both activities which place the worker at high risk for RSI, it is imperative to reduce the detrimental impact as much as possible. The mechanized device does this by minimizing the repetitive nature of the task and requiring less exertion on the part of the operator.

In order to reduce the risk of injury while using the mechanized device, several poor postures and habits must be avoided. First, the device should be positioned, as much as possible, at a height that requires neither hunching or raising the arms significantly and exceeding 60 degrees of flexion. Also, the spreader should be held against the plate with the arm extended into the sterile area with the arm straight. (See Figure 8A) Due to the strain of working in a sterile area, it is easy to drop the elbow, causing the wrist to bend. (Figure 8B). This leads to an increase in the risk of musculoskeletal injury. It is also important to insure that the spreader does not rest on

Figure 8: The Postures Used with the Mechanized Device can Improve its Ergonomic Friendliness. A) Maintaining a straight wrist posture will reduce the chance of injury. B) Allowing the wrist to bend increases the chance of injury. Note that gloves are neglected here ONLY for the purpose of demonstrating more clearly the hand positioning. The agar plate and area is sterile and unused.
the edge of the plate. This increases the contact area and the likelihood of contamination, and it also causes a vibration, which may lead to stress injury. While working, the operator should be sure to sit up straight and not hunch the shoulders. The foot treadle should be placed in front of the chair, such that the leg need not be extended to operate it, and simply resting the foot on the floor will trigger the machine. This will help to insure good posture and no strain on the foot or leg.\textsuperscript{19}

The mechanized spin plating device described here is superior for several reasons. The mechanized wheel allowed for the liquid to be spread more evenly than if the plate was turned by hand. This results in a lower deviation in CFU count from plate to plate, reduces smudging and contamination, and provides an enhanced distribution of countable colonies. A mechanized wheel also reduces operator fatigue and operator error. This allows for a more consistent application as well as happier operators. By reducing the need to manually spin the plate, the likelihood of repetitive stress injury is reduced. The mechanized turntable described here gives improved ergonomics while simultaneously improving results.
References

1. Tortora, 166-168.


11. Tortora, 166-168.


13. NIOSH Document #705005

and Low Back (Cincinnati, OH: U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES, 1997), 1-2, 3-1, 3-2, 5a-1, 5b-a.


16. Bernard, 1-2, 3-1, 3-2, 5a-1, 5b-a.

17. Bernard, 1-2, 3-1, 3-2, 5a-1, 5b-a.

18. Bernard, 1-2, 3-1, 3-2, 5a-1, 5b-a.

19. Bernard, 1-2, 3-1, 3-2, 5a-1, 5b-a.
## APPENDIX B

### TABLE 1

TOTAL RELEASES FOR 2003 IN THE US

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<td>30,778</td>
</tr>
<tr>
<td>488 TOLUENE</td>
<td>57,974,725</td>
</tr>
<tr>
<td>489 TOLUENE DIISOCYANATE (MIXED ISOMERS)</td>
<td>48,838</td>
</tr>
<tr>
<td>490 TOLUENE-2,4-DIISOCYANATE</td>
<td>19,405</td>
</tr>
<tr>
<td>491 TOLUENE-2,6-DIISOCYANATE</td>
<td>3,361</td>
</tr>
<tr>
<td>492 TOXAPHENE</td>
<td>2,374</td>
</tr>
<tr>
<td>493 TRADE SECRET CHEMICAL</td>
<td>13,800</td>
</tr>
<tr>
<td>494 TRANS-1,3-DICHLOROPROPENE</td>
<td>1,062</td>
</tr>
<tr>
<td>495 TRANS-1,4-DICHLORO-2-BUTENE</td>
<td>45</td>
</tr>
<tr>
<td>496 TRIADIMEFON</td>
<td>1</td>
</tr>
<tr>
<td>497 TRIALLATE</td>
<td>914</td>
</tr>
<tr>
<td>498 TRIBENURON METHYL</td>
<td>22</td>
</tr>
<tr>
<td>499 TRIBUTYL Tin METHACRYLATE</td>
<td>10</td>
</tr>
<tr>
<td>500 TRICHLORFON</td>
<td>21,919</td>
</tr>
<tr>
<td>501 TRICHLOROACETYL CHLORIDE</td>
<td>1</td>
</tr>
<tr>
<td>502 TRICHLOROETHYLENE</td>
<td>7,175,496</td>
</tr>
<tr>
<td>503 TRICHLOROFLUOROMETHANE</td>
<td>224,045</td>
</tr>
<tr>
<td>504 TRICLOPYR TRIETHYLMONIUM SALT</td>
<td>0</td>
</tr>
<tr>
<td>505 TRIETHYLAMINE</td>
<td>1,522,781</td>
</tr>
<tr>
<td>506 TRIFLURALIN</td>
<td>48,796</td>
</tr>
<tr>
<td>507 TRIFERINE</td>
<td>147</td>
</tr>
<tr>
<td>508 TRIPHENYL Tin CHLORIDE</td>
<td>10</td>
</tr>
<tr>
<td>509 TRIPHENYL Tin HYDROXIDE</td>
<td>10</td>
</tr>
<tr>
<td>Chemical</td>
<td>Total On- and Off-site Disposal or Other Releases</td>
</tr>
<tr>
<td>------------------------------------------------------------------------</td>
<td>---------------------------------------------------</td>
</tr>
<tr>
<td>510 TRIS(2,3-DIBROMOPROPYL) PHOSPHATE</td>
<td>313</td>
</tr>
<tr>
<td>511 TRYPAN BLUE</td>
<td>5</td>
</tr>
<tr>
<td>512 URETHANE</td>
<td>127,982</td>
</tr>
<tr>
<td>513 VANADIUM (EXCEPT WHEN CONTAINED IN AN ALLOY)</td>
<td>1,908,095</td>
</tr>
<tr>
<td>514 VANADIUM COMPOUNDS</td>
<td>52,134,295</td>
</tr>
<tr>
<td>515 VINOCOZOLIN</td>
<td>0</td>
</tr>
<tr>
<td>516 VINYL ACETATE</td>
<td>3,113,177</td>
</tr>
<tr>
<td>517 VINYL CHLORIDE</td>
<td>693,967</td>
</tr>
<tr>
<td>518 VINYLIDENE CHLORIDE</td>
<td>156,037</td>
</tr>
<tr>
<td>519 WARFARIN AND SALTS</td>
<td>26</td>
</tr>
<tr>
<td>520 XYLENE (MIXED ISOMERS)</td>
<td>40,575,531</td>
</tr>
<tr>
<td>521 ZINC (FUME OR DUST)</td>
<td>38,663,057</td>
</tr>
<tr>
<td>522 ZINC COMPOUNDS</td>
<td>696,378,844</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>4,443,166,690</strong></td>
</tr>
</tbody>
</table>

Reference:

1. TRI On-site and Off-site Reported Disposed of or Otherwise Released (in pounds), for facilities in All Industries, for All Chemicals, U.S., 2003 Data Update as of June 8, 2005. (http://www.epa.gov/triexplorer/)
APPENDIX C

THE WASTEWATER TREATMENT FACILITY AND REACTOR EQUATIONS

A typical wastewater treatment plant generally uses the following basic schematic with variations required by the specific conditions of the water being treated. The following types of treatment are generally included in the wastewater treatment facility. This outlines a basic description of the unit operations and their function.

Preliminary Treatment

Preliminary treatment is generally concerned with removing the components of wastewater that are large or damaging to the facility equipment. (See Figure 1) These operations generally include a bar screen (Unit 1), a comminutor (Unit 2), and a grit

![Figure 1: A Typical Wastewater Plant](image-url)
chamber (Unit 3). The bar screen is designed to remove large objects such as tree limbs, dead animals, shopping carts, and other refuse from the waste stream. Bar screens are either coarse or fine; a coarse screen has steel bars with spacing between 1 cm and 6 cm, a fine screen is woven wire mesh. The comminutor is a circular grinder designed to grind any solids remaining in the waste stream into 3 cm to 8 cm sized pieces. After the comminutor, a grit chamber slows the flow to allow sand and grit to settle out. This is a relatively easy operation because sand has a specific gravity about 2.5 times that of the organics contained in the wastewater, so the retention time of the grit chamber is generally between 1 and 15 minutes. Occasionally, the grit chamber will be aerated in order to keep organics afloat as well as aerate deoxygenated wastewater. In these cases, the retention time is often extended to 20 minutes. The grit is generally removed from the bottom of the grit chamber by a screw conveyor or mechanical scrapers. The recovered grit may have organics with it, so it must be washed before disposal to avoid odor or health problems.

Primary Treatment

Primary treatment is designed to remove the organics and any remaining solid matter. This is often done with a settling or clarification tank (Unit 4), in which turbulence is kept to a minimum, allowing the materials to flocculate together. There are two types of organic wastes. The first type is organic material slightly heavier than water that will sink slowly to the bottom, known as raw sludge. This material is removed by scraping it along the bottom to a collection area where it can be removed. The second type is organics that are lighter than water, and those will float to the surface where they
can be removed with a skimming arm. The retention time for this unit operation is
generally 1.5h – 2.5h. In the past, this was considered complete and sufficient treatment
of wastewater, however current standards require secondary treatment for both the water
and the sludge fraction leaving this unit.

Secondary Treatment

The water fraction leaving the top of the primary clarifier has generally lost much
of the suspended organics, but still has a significant amount of dissolved organics. These
organics create a significant BOD (or biological oxygen demand) because aerobic
microbes will attack them. At this point, biological treatment is generally used to reduce
the amount of organics and the oxygen demand they would create if the water were
discharged directly. There are several requirements for this operation (Units 5 and 6).
One is that the water be significantly aerated to allow oxygenic organisms to grow and
use the dissolved organics. The second is that the organisms have a high degree of
contact with the water.

There are several types of biological reactors used. These include suspended
growth reactors and fixed film reactors. A suspended growth reactor (Unit 5) is a vat or
channel in which the microorganisms are suspended in the water and aerated by bubbling
oxygen through the water. This unit can be designed and modeled either as a CSTR or a
PFR (see below). This is also known as an “activated sludge system” and is the most
widely used type of wastewater treatment in the United States. In this process, microbes
grow in an aerated reactor. They break the organic wastes into CO₂, water, and biomass.
The liquid is then passed on to a secondary clarifier (Unit 6) where the microbes settle to
the bottom and the clarified water is passed on to a disinfection unit (Unit 7). The settled microbes are both recycled to the aeration tank and disposed of with the sludge gathered in earlier processes.

Fixed film reactors are an alternative to suspended growth reactors, and provide surfaces for microorganisms to attach to as a biofilm, and the wastewater flows over these surfaces. The most common type of fixed film reactor is a “trickling filter” reactor, in which stones or other man-made blocks with high surface area provide the surface to which the microbes attach. The water is then sprayed over the packed beds and allowed to trickle downwards. A newer development is a rotating contactor, where several large disks or drums are covered with the biofilm and rotated slowly through the water and air, allowing the microbes to obtain the necessary oxygen. In these fixed film reactors, microbes must be periodically “sloughed off” in order to prevent a buildup. In general, water leaving the fixed film reactors is analogous to water leaving the secondary clarifier and can be passed to the disinfectant unit. In the disinfection unit, chlorine or another disinfectant is used to ensure that no pathogenic organisms leave the wastewater treatment plant.

Sludge Treatment and Disposal

There are two sources of sludge in the wastewater treatment plant. One is the sediment produced in the primary clarifier (Unit 4), known as “raw sludge”, “a name that doesn’t do justice to the undesirable nature of this stuff.” [Vesilind, 1997] The second source is the excess microbes produced in the biological treatment of the water(Unit 6).
The disposal of this sludge is cited as being one of the most difficult problems in wastewater treatment by all of the texts consulted.

There are several options for sludge disposal. One is to combine the sludge from the primary clarifier (Unit 4) and the activated sludge (Unit 6) so that microbes can degrade the organics in the raw sludge. This is generally done in a digestor (Unit 8), which can be anaerobic or aerobic. This reduces the total sludge volume. The sludge is then dewatered. This can be done by gravity thickening, centrifugation, pressing, or drying on sand beds. The water from these processes is either returned to the water treatment plant or returned to the environment through evaporation. The sludge must then be disposed of. One disposal method, which can be applied even with wet sludge, is to apply it to agricultural land. The problem with this is a buildup of both heavy metals and of the non-degradable organics, and the use is often limited to crops not intended for human consumption. The dried sludge is also incinerated or dumped in landfills. Both of these methods cause a heavy environmental load, either through greenhouse gasses or filling of landfill space. An additional method to reduce this environmental load or to make the sludge safer for land application would both valuable and practical.

**Equations for Working with Reactors**

The primary type of reactor used for continuous cultivation of cells is the Continuous-flow Stirred Tank Reactor (CSTR). (See Figure 2) This type of reactor allows the easiest mixing of live culture and incoming sterile media. This is often the ideal scale up from batch reactions, and will be evaluated for use with PSB. It is likely that ideal chemostat conditions will not be achieved with the final reactor determined for
PSB because of its flat panel geometry, however this may be able to be modeled as CSTRs in series. Because live cells are highly sensitive to their surroundings, pH and dissolved gas controls are often included in the design. For aerobic cultures, dissolved oxygen is monitored, and oxygen can be bubbled through the reactor to increase the oxygen concentration. In an anaerobic culture, this can be replaced with nitrogen to maintain an oxygen free environment or carbon dioxide to provide an additional carbon source.

For cell concentration in a chemostat of a given volume \(V\), a steady state material balance can be made on the cell concentration \(X\), the substrate concentration \(S\), or the product of the reactor \(P\), if there is one. Shown below is a mass balance on cell concentration, with a constant flow rate \(F\). This balance can be readily performed on substrate or product concentration. The equation can also be easily converted to equations for substrate concentration using \(Y_{x/s}\), the biomass to substrate yield coefficient or to equations for product concentration using a biomass to product yield coefficient, \(Y_{x/p}\). For a well mixed CSTR, the concentration of \(X, S,\) or \(P\) in the reactor is also the outflow concentration.

![A Continuous Stirring Tank Reactor](image)

Figure 2: A Continuous Stirring Tank Reactor
For this derivation, we assume steady state and that there is no incoming biomass. The dilution rate, \( D = \frac{F}{V} \), is the reciprocal of residence time, and by dividing by volume, it appears in the equation. Generally, there is no incoming biomass in a steady state chemostat, either because it is filtered out or because it can be considered negligible with respect to the total biomass in the reactor \( X_o << X \). In most cases, \( k_d \) can be assumed negligible in comparison to the rate of growth \( \mu \). The operating limits of the reactor are defined by the reaction rate, equal to the dilution rate. Where the dilution rate is greater than the maximum reaction rate, \( D > \mu_{\text{max}} \), the reactor will flow too quickly for the bacteria to replenish themselves and wash out. The maximum dilution rate can be calculated by calculating \( D \) with \( \mu_{\text{max}}, K_s, \) and \( S = S_0 \).

Plug flow reactors (PFRs) are not commonly used for cell culture, however awareness of their geometry is applicable to flow through a flat bed reactor. (See Figure 3) The difficulties with an ideal PFR are the lack of backmixing and the inability to adjust the reactor conditions. In a live culture situation, there must be contact between the growing portion of the stream and the sterile portion of the stream, so the perfect PFR

\[
V \frac{dX}{dt} = FX_o - FX + V\mu X - Vk_d X
\]

divide by \( V \), \( D = \frac{F}{V} \)

\[
D \frac{dX}{dt} = DX_o - DX + \mu X - k_d X
\]

Assume Steady State, \( X_o = 0 \)

\[
0 = -DX + \mu X - k_d X
\]

\[
D = \mu - k_d
\]

If \( k_d \) is negligible, \( D = \mu \)

Where \( \mu \) is based on Monod Kinetics,

\[
D = \mu = \frac{-\mu_m S}{K_s + S}
\]
would end up with a sterile stream. To solve this problem, a PFR must be used with a recycle stream in order to allow contacting of sterile and live portions of the stream. The need for maintaining reactor conditions consistent with the culture requirements is almost impossible to solve while maintaining ideal plug flow, causing another typical difficulty in using plug flow with bacterial cultures. For modeling purposes, a PFR can be considered a series of batch reactors because each discrete slice of fluid moving through the PFR has no contact with the segments on either side of it. The mass balance is generally made on this thin slice, \( \Delta \). In the diagram below, \( F \) is used to denote flow rate. \( F_r \) is the flow of the recycle, \( F_0 \) is the influent flow, and \( F_e \) is the effluent flow. \( X \) is for the biomass concentration. \( X_r \) is the recycled biomass, \( X_o \) is the incoming biomass, often negligible, and \( X_e \) is the amount of biomass in the outflow. \( S \) is the substrate concentration, where the same subscripts apply. \( L \) is the total length of the reactor. The reactor can be modeled by assuming that the change in flow and biomass is equal to the total reaction rate. Where the fluid is of constant density, \( F \) is constant and can be removed from the equation. The equation is often also modified by using a characteristic time constant, \( \theta \), which reflects the length divided by the flow, or the total time that the liquid will be in the reactor. The equation simplifies to a differential equation where the change in biomass with characteristic time is equal to the known growth rate \( (\mu) \). For bacterial growth, this growth rate is equal to that determined for Monod Kinetics.
Here, integration is based on the Boundary Conditions
\[ z = 0, \theta = 0, X = X_0, S = S_0 \]
\[ z = L, \theta = L/F, X = X_e, S = S_f \]

Another common method used is attached culture systems or trickle bed “filter” where bacteria create a biofilm on a solid medium such as rocks or synthetic surface. (See Figure 4) The media is then passed over the culture bed contacting the bacteria with nutrients and substrate. This is most often used with aerobic cultures because of the relatively high diffusion of air into a thin film, making an anaerobic system difficult.
Oxygen diffusion, however, is often the limiting factor in the aerobic metabolisms found in these systems. Modeling this type of system is difficult and largely empirical.

Equations to keep in mind when considering this type of system are passive diffusion,

\[ J_s = K_p (C_E - C_I) \], where diffusion is equal to the diffusion coefficient times the internal and external concentration difference, and the Damkohler number, \( D_a \), which is the ratio between maximum rate of bioconversion and the maximum rate of diffusion.

By determining the \( D_a \) number, it is possible to determine if bioconversion or diffusion is rate limiting.

\[
D_a = \frac{\text{Max Bioconversion Rate}}{\text{Max Diffusion Rate}} = \frac{r_{\text{max}}}{(D_e / \delta)S_o}
\]

- \( D_e \) = Effective Diffusivity of Substrate
- \( \delta \) = Thickness of Path
- \( S_o \) = Bulk Concentration

![Figure 4: Model of a Trickle Bed Reactor](image-url)
Adapted from


APPENDIX D
PHOTOSYNTHESIS

Bacterial photosynthesis is a process in which organisms possessing a chlorophyll molecule can convert light energy, or photons, into chemical energy in the form of energy storage molecules such as NADH and ATP. Bacterial photosynthesis is simpler than plant photosynthesis, and is based only one photosystem. There are two sets of reactions, the light reactions, and the dark reactions. The light reactions include the harnessing of light energy by pigments and transfer of electrons, and subsequent generation of a pH gradient, across the photosynthetic membrane. The dark reactions, not discussed here, consist of the subsequent assimilation of either CO₂ or organic carbon using the energy generated in the light reactions and either the Calvin cycle or the reverse TCA cycle.

The light harvesting complexes (LHCs) and associated pigments are the most important components of photosynthesis because they confer the ability to trap light energy. The spectrum for each type of bacteriochlorophyll and bacteria is unique, and can be used to identify a photosynthetic bacterium. (See Table 23) B. viridis displays a unique spectrum based on its characteristic pigments and light harvesting complexes; peaks near 400 nm, 605 nm, 835-850 nm, and 1010-1030 nm are associated with this bacteria. The peaks 451 nm and 483 nm are attributed to carotenoids, and the peak at 1020 nm is the signature of bacteriochlorophyll b, the primary light harvesting pigment for this bacterium. (See Figure 8)
Figure 9: The Pigment Spectrum of Extracted Membranes from *B. viridis*. This is a full spectrum scan of the pigment containing membranes extracted from whole cells. These membranes have characteristic maxima, shown here as peaks marked with an X, near 400 nm, 605 nm, 835-850 nm, and 1010-1030 nm. The peaks 451 nm and 483 nm are attributed to carotenoids. The wavelength 1020 nm is the signature of bacteriochlorophyll *b*, which is the primary light harvesting pigment for this bacterium.

### TABLE 1

**THE BACTERIOCHLOROPHYLLS AND THEIR CHARACTERISTIC WAVELENGTHS AND THE BACTERIA ASSOCIATED WITH EACH**

<table>
<thead>
<tr>
<th>Bacteriochlorophyll</th>
<th>Wavelength</th>
<th>Bacteria Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>825-890</td>
<td>Purple, some green</td>
</tr>
<tr>
<td>B</td>
<td>1020-1040</td>
<td>Some purple</td>
</tr>
<tr>
<td>C</td>
<td>745-755</td>
<td>Some green</td>
</tr>
<tr>
<td>Cs</td>
<td>740</td>
<td><em>Chloroflexus aurantacus</em></td>
</tr>
<tr>
<td>D</td>
<td>705-740</td>
<td>Some green</td>
</tr>
<tr>
<td>E</td>
<td>719-726</td>
<td>Green (brown colored)</td>
</tr>
<tr>
<td>G</td>
<td>788</td>
<td><em>Heliospirillum, Heliobacillus</em></td>
</tr>
</tbody>
</table>

Figure 9: The Pigment Spectrum of Extracted Membranes from *B. viridis*. This is a full spectrum scan of the pigment containing membranes extracted from whole cells. These membranes have characteristic maxima, shown here as peaks marked with an X, near 400 nm, 605 nm, 835-850 nm, and 1010-1030 nm. The peaks 451 nm and 483 nm are attributed to carotenoids. The wavelength 1020 nm is the signature of bacteriochlorophyll *b*, which is the primary light harvesting pigment for this bacterium.
The bacteriochlorophyll (Bchl) pigments, or LHCs, are the principal photoreceptors and gather light. Bacteriochlorophylls gather different wavelengths of light depending on their exact structure. The structural differences in Bchl \( b \) shift the primary absorbance wavelength of this molecule to 1020 nm. The LHCs pigments are arrayed in a highly ordered formation, as antenna complexes to the reaction center (RC), and the gathered energy is passed through these to the reaction center where the light energy is converted to chemical energy. (See Figure 10)

The harvested energy and light energy incident on the reaction centers drive the transfer of an electron from an electron donor to an electron acceptor, however these two chemicals never interact directly. Instead, the electron passes through a chain of membrane proteins and structures in the photosynthetic reaction center and cytochrome. (See Figure 11) These work as a proton pump, driven by the electron exchange, creating a proton gradient, or \( \Delta pH \), across the membrane. This \( \Delta pH \) can be used to generate

![Figure 2: The Arrangement of Antenna LHCs and a RC. Light energy is transferred among the antenna complex (green) and finally to the RC (purple). The antenna pigments allow more light to be harvested and used. (Blankenship, 1996)](image)
chemical energy from the light energy the bacteria receives when the proton gradient is reversed through membrane bound ATPase to drive ATP synthesis.\textsuperscript{4}

In plant photosynthesis, two photosystems are present, which generate a significant reducing power, and water is typically used as the electron donor. It is from the breaking of this water molecule that plant photosynthesis generates oxygen. Because the bacteria have only one photosystem, which does not generate the same reducing power and therefore cannot break water, they need an alternative electron donor, and sulfide or reduced sulfur compounds or organics such as malate or succinate are often used in this capacity.\textsuperscript{5} Because they do not break water, they do not generate oxygen as a byproduct of photosynthesis. Carbon dioxide is often employed as the electron acceptor,
and is also assimilated as a carbon source in the Calvin Cycle or the Reverse TCA cycle. (See 4.2.3)

The generalized net photosynthesis reaction is:
\[ \text{CO}_2 + \text{H}_2\text{A} + \text{Light Energy} \rightarrow [\text{CH}_2\text{O}] + 2\text{A} + \text{H}_2\text{O} \]
Where A represents a generalized electron donor in its reduced (H₂A) and oxidized (2A) states.

This is often broken into two reactions:

1. \[ 2\text{H}_2\text{A} \rightarrow 2\text{A} + 4[\text{H}^+] \]

   This reaction is driven by light energy, and is the source of the proton gradient generated during photosynthesis. In plant photosynthesis, this reaction is the source of oxygen, because the two oxidized oxygen atoms bond to form the molecule O₂. In bacterial photosynthesis, it generates oxidized sulfur, and small organic compounds that can be assimilated for growth.

2. \[ 4[\text{H}^+] + \text{CO}_2 \rightarrow (\text{CH}_2\text{O}) + \text{H}_2\text{O} \]

   This describes the dark reactions in which CO₂ is assimilated as a carbon source via the Calvin cycle or the Reverse TCA cycle. The reducing power of the [H⁺] can be converted to either ATP via ATPase, or NADH by driving NADH dehydrogenase in reverse to reduce NAD⁺ to NADH.
References


5. Voet, 627-659.
## APPENDIX E

### MEDIA FORMULATIONS

### TABLE 1

THE FORMULATION FOR MEDIAS USED IN THIS WORK

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>RMPABA /L</th>
<th>Minimal /L</th>
<th>NC /L</th>
<th>Optimized /L pH 5.6</th>
</tr>
</thead>
<tbody>
<tr>
<td>10% (NH₄)₂SO₄</td>
<td>7.0 mL</td>
<td>7.0 mL</td>
<td>7.0 mL</td>
<td>16.5 mL</td>
</tr>
<tr>
<td>NaMalate Soln</td>
<td>28.0 mL</td>
<td>28.0 mL</td>
<td>28.0 mL</td>
<td>120.0 mL</td>
</tr>
<tr>
<td>Potassium Phosphate Solution</td>
<td>10.5 mL</td>
<td>10.5 mL</td>
<td>10.5 mL</td>
<td>117.5 mL</td>
</tr>
<tr>
<td>Supersalts (SS)</td>
<td>35.0 mL</td>
<td>35.0 mL</td>
<td>35.0 mL</td>
<td>70.0 mL*</td>
</tr>
<tr>
<td>Bactopeptone</td>
<td>0.9 g</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Yeast Extract</td>
<td>0.9 g</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1% MgCl₂*6H₂O</td>
<td>9.744 mL</td>
<td>9.744 mL</td>
<td>9.744 mL</td>
<td>9.744 mL</td>
</tr>
<tr>
<td>1 M CaCl₂*2H₂O</td>
<td>0.3 mL</td>
<td>0.3 mL</td>
<td>0.3 mL</td>
<td>0.3 mL</td>
</tr>
<tr>
<td>50 μg/mL PABA</td>
<td>0.5 mL</td>
<td>0.5 mL</td>
<td>0.5 mL</td>
<td>0.5 mL</td>
</tr>
<tr>
<td>Thiamine*</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>12.0 mL</td>
</tr>
<tr>
<td>Biotin*</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>8.0 mL</td>
</tr>
<tr>
<td>Vitamin B₁₂*</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>4.0 mL</td>
</tr>
<tr>
<td>(cyanocobalamin)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>4.0 mL</td>
</tr>
<tr>
<td>Pantothenate*</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>4.0 mL</td>
</tr>
<tr>
<td>Distilled Water*</td>
<td>~800 mL</td>
<td>~800 mL</td>
<td>~800 mL</td>
<td>~520 mL</td>
</tr>
<tr>
<td>Tap Water</td>
<td>120 mL</td>
<td>120 mL</td>
<td>120 mL</td>
<td>120 mL</td>
</tr>
<tr>
<td>Addition of NaHCO₃</td>
<td>NO</td>
<td>NO</td>
<td>NO</td>
<td>2.536 g</td>
</tr>
</tbody>
</table>

**NOTE:**

* Supersalts is added Thamine-free in Optimized media
* Vitamin stock solutions are 0.5 mM unless otherwise indicated
* Fill to 1 Liter
### TABLE 2
THE FORMULATION OF MALATE SOLUTION USED IN MAKING MEDIA

<table>
<thead>
<tr>
<th>NaMalate Soln (per 1000 ml)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>DL-malic acid</td>
<td>100.0 g</td>
</tr>
<tr>
<td>NaOH</td>
<td>60.0 g</td>
</tr>
</tbody>
</table>

### TABLE 3
THE FORMULATION OF POTASSIUM PHOSPHATE BUFFER USED IN MAKING MEDIA

<table>
<thead>
<tr>
<th>Potassium Phosphate Buffer (per 1000 ml)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>KH₂PO₄</td>
<td>40.0 g</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>60.0 g</td>
</tr>
</tbody>
</table>

### TABLE 4
THE FORMULATION OF SUPERSALTS SOLUTION USED IN MAKING MEDIA

<table>
<thead>
<tr>
<th>SuperSalts (per 1000 ml)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Na₂EDTA</td>
<td>0.4 g</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>4.0 g</td>
</tr>
<tr>
<td>CaCl₂·H₂O</td>
<td>1.5 g</td>
</tr>
<tr>
<td>FeSO₄·7H₂O</td>
<td>0.2 g</td>
</tr>
<tr>
<td>Thiamine</td>
<td>20.0 mg</td>
</tr>
<tr>
<td>Trace Elements</td>
<td>20.0 ml</td>
</tr>
</tbody>
</table>

### TABLE 5
THE FORMULATION OF TRACE ELEMENTS SOLUTION USED IN MAKING SUPERSALTS

<table>
<thead>
<tr>
<th>Trace Elements (per 250 ml)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Cu(NO₃)₂·3H₂O</td>
<td>10 mg</td>
</tr>
<tr>
<td>MnSO₄·H₂O</td>
<td>397.00 mg</td>
</tr>
<tr>
<td>ZnSO₄·7H₂O</td>
<td>60.0 mg</td>
</tr>
<tr>
<td>H₃BO₃</td>
<td>700.0 mg</td>
</tr>
<tr>
<td>NaMoO₄·2H₂O</td>
<td>187.00 mg</td>
</tr>
</tbody>
</table>
APPENDIX F

ARRANGEMENT OF 25-WATT BULBS IN THE LIGHT BOX USED TO INCUBATE *B. VIRIDIS*

![Figure 1: The Light Box. Shown above, 250 mL vessels being incubated on the top shelf, and 1 Liter inoculating culture vessels being incubated on the third shelf.](image)

**TABLE 1**

<table>
<thead>
<tr>
<th>Shelf</th>
<th>Left Side</th>
<th>Center</th>
<th>Right Side</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>Top</td>
<td>236</td>
<td>156</td>
<td>249</td>
<td>213.7</td>
</tr>
<tr>
<td>Second</td>
<td>(No Shelf)</td>
<td>134</td>
<td>133</td>
<td>133.5</td>
</tr>
<tr>
<td>Third</td>
<td>193</td>
<td>173</td>
<td>201</td>
<td>189.0</td>
</tr>
<tr>
<td>Bottom</td>
<td>200</td>
<td>263</td>
<td>166</td>
<td>209.7</td>
</tr>
</tbody>
</table>

NOTE: The average light intensity is $186.5 \pm 36.9$
50 mL vessels were placed 3-6 inches from bulbs
250 mL vessels were placed 4-8 inches from bulbs
APPENDIX G:

ANAEROBIC TOLUENE DEGRADATION PATHWAY
Figure 1: Anaerobic Toluene Degradation Pathway. Prepared by Ryan McLeish and Eva Young © University of Minnesota, 2005.
<http://umbbd.ahc.umn.edu/tol2/tol2_image_map.html>
APPENDIX H:

ANAEROBIC ETHYLBENZENE DEGRADATION PATHWAY
APPENDIX I:

ANAEROBIC PHENOL DEGRADATION PATHWAY

Figure 1. Anaerobic Phenol Degradation Pathway. Prepared by Stephen Stephens © University of Minnesota, 2005.
<http://umbbd.ahc.umn.edu/phe/phe_image_map.html>
APPENDIX J:

ANAEROBIC BENZOATE DEGRADATION PATHWAY
Figure 2: The Degradation Pathway from 3-Hydroxypimelyl-CoA to Acetyl-CoA. This is a continuation of the Benzoate pathway, leading to the TCA compound Acetyl-CoA. Prepared by Dong Jun Oh. © University of Minnesota, 2005. <http://umbbd.ahc.umn.edu/benz/benz_image_map2.html>
APPENDIX K:

AEROBIC TOLUENE DEGRADATION VIA THE META CLEAVAGE PATHWAYS
Figure 1. Three Potential Pathways of Aerobic Degradation of Toluene to Acetaldehyde and Pyruvate. Show are pathways starting with toluene 2- or 3-monoxygenase or toluene dioxygenase. Prepared by Dong Jun Oh. © University of Minnesota, 2005. <http://umbbd.ahc.umn.edu/tol/tol_image_map2.html>
Figure 2. Two Potential Pathways of Aerobic Degradation of Toluene to Benzoate or 4-Hydroxybenzoate. Show are pathways starting with toluene side-chain monooxygenase or toluene 4-monoxygenase. The continuation of these pathways is shown in Figure 3 and 4 (Benzoate) and Figure 5 (4-Hydroxybenzoate). Prepared by Yuemo Zeng. © University of Minnesota, 2005. <http://umbbd.ahc.umn.edu/tol/tol_image_map1.html>
Degradation Continues in Vanillin Pathway
(See Figure X Below)

Degradation Continues in Nitrobenzene Pathway
(See Figure 4 Below)

Figure 3: Aerobic Benzoate Degradation: Continuation of Toluene Degradation from Benzoate through Catechol. Also shown are enzymatic steps from benzoate to 4-hydroxybenzoate and 2-Chlorobenzoate to catechol. Degradation from catechol shown in Figure 4. Prepared by Jingfeng Feng. © University of Minnesota, 2005.
<http://umbbd.ahc.umn.edu/benz2/benz2_image_map.html>
Figure 4: The Aerobic Nitrobenzene Degradation Pathway. Aerobic benzoate degradation continues from catechol, shown here one step below nitrobenzene in the right hand pathway. Pathway assembled by Hugh McTavish and Dave Roe. © University of Minnesota, 2005. <http://umbbd.ahc.umn.edu/nb/nb_image_map.html>
Figure 5. The Aerobic Vanillin Degradation Pathway. The aerobic degradation of 4-hydroxybenzoate continues along the pathways shown here. Degradation of 3-Carboxy-\textit{cis},\textit{cis}-muconate and 2-Hydroxy-4-carboxymuconate semialdehyde continues via the intermediary metabolism. (KEGG cycle) Pathway prepared by Dong Jun Oh and Stephen Stephens. © University of Minnesota, 2005. <http://umbbd.ahc.umn.edu/van/van_image_map.html>
APPENDIX L:

ANAEROBIC 1,2-DICHLOROETHANE DEGRADATION PATHWAY

Figure 1: The Anaerobic 1,2-Dichloroethane Degradation Pathway. Dichloroethane is broken down in a series of steps to glycolate. Pathway prepared by Trung Bui. ©Univ. of Minnesota, 2005. <http://umbbd.ahc.umn.edu/dce/dce_image_map.html>
APPENDIX M:

METHYL ETHYL KETONE DEGRADATION PATHWAY

Figure 1: Methyl Ethyl Ketone Degradation Pathway. MEK is degraded to acetaldehyde, which can be utilized in the TCA cycle. Pathway prepared by Jingfeng Feng. ©University of Minnesota, 2005. <http://umbbd.ahc.umn.edu/mek/mek_image_map.html>
APPENDIX N:
TETRAHYDROFURAN DEGRADATION PATHWAY
Figure 1: The Degradation Pathway for Tetrahydrofuran. Tetrahydrofuran is degraded to 4-Hydroxybutanoate in 4 enzymatic steps. Pathway prepared by Jodi Bjerke. © Univ. of Minnesota, 2005. <http://umbbd.ahc.umn.edu/thf/thf_image_map.html>
APPENDIX O:

DIMETHYL SULFOXIDE AND ORGANOSULFIDE CYCLE

Methanol
Molecular Weight = 32.04 g/mol
Specific Gravity = 0.792

Ethanol
Molecular Weight = 46.07 g/mol
Specific Gravity = 0.789

1-propanol
Molecular Weight = 60.0956 g/mol
Specific Gravity = 0.803

2-propanol (isopropanol)
Molecular Weight = 60.0956 g/mol
Specific Gravity = 0.785

1-Butanol
Molecular Weight = 74.12 g/mol
Specific Gravity = 0.810 sp gr

t-butanol
Molecular Weight = 74.12 g/mol
Specific Gravity = 0.779 sp gr

Isoamyl alcohol
Molecular Weight = 88.1492 g/mol
Specific Gravity = 0.809

Phenol (Used Solid Form)
Molecular Weight = 94.1128 g/mol
(Specific Gravity = 1.07)
(using crystals)

Benzene
Molecular Weight = 78.1134 g/mol
Specific Gravity = 0.8786 sp gr

Toluene
Molecular Weight = 92.14 g/mol
Specific Gravity = 0.867 sp gr
Xylenes
Molecular Weight = 106.17 g/mol
Specific Gravity = 0.862 sp gr

Benzaldehyde
Molecular Weight = 106.13 g/mol
Specific Gravity = 1.044 sp gr

Benzoic Acid
Molecular Weight = 122.1232 g/mol
(solid)
Benzoate Stock: 751 mM

Indole
Molecular Weight = 117.15 g/mol
(solid)

Tetrahydrofuran
Molecular Weight = 72.1066 g/mol
Specific Gravity = 0.886

Gluteraldehyde
Molecular Weight = 100.177 g/mol
Specific Gravity = 1.06 sp gr

Valeraldehyde
Molecular Weight = 86.1334 g/mol
Specific Gravity = 0.818 sp gr

Formaldehyde
Molecular Weight = 30.0262 g/mol
(37% solution)
Specific Gravity = 1.09

Methyl Ethyl Ketone
Molecular Weight = 72.1066 g/mol
Specific Gravity = 0.805 sp gr

Amyl acetate
Molecular Weight = 130.1864 g/mol
Specific Gravity = 0.876
Methylene Chloride
Molecular Weight = 84.9328 g/mol
Specific Gravity =  1.3255 sp gr

Potassium Biphthalate
Molecular Weight = 204.2151 g/mol
(Solid)

Ninhydrin (Used Solid Form)
Molecular Weight = 178.144 g/mol
Specific Gravity =  0.862

Dimethyl Sulfoxide
Molecular Weight = 78.1288 g/mol
Specific Gravity =  1.096 sp gr

N,N-Dimethyl Formamide
Molecular Weight = 73.0944 g/mol
Specific Gravity =  0.944 sp gr

n-butylamine
Molecular Weight = 73.1376 g/mol
Specific Gravity =  0.741 sp gr

n-Dodecylamine
Molecular Weight = 185.36 g/mol
(solid)

n-Hexadecylamine
Molecular Weight = 241.4592 g/mol
(solid)

1,2-Dichloroethane
Molecular Weight = 98.9586 g/mol
Specific Gravity =  1.253 sp gr

Acetonitrile
Molecular Weight = 41.0524 g/mol
Specific Gravity =  0.7857

Dioxane
Molecular Weight = 88.106  g/mol
Specific Gravity =  1.033 sp gr