POLYHYDROXYALKANOATE SYNTHESIS BY RALSTONIA EUTROPHA FROM MULTIPLE SUBSTRATES

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TABLE OF CONTENTS

LIST OF TABLES ........................................................................................................................................ 4
LIST OF FIGURES ........................................................................................................................................ 5
CHAPTER 1. INTRODUCTION AND OBJECTIVES ...................................................................................... 7
  1.1 Introduction ........................................................................................................................................ 7
  1.2 Objectives .......................................................................................................................................... 13

CHAPTER 2. MATERIALS AND METHODS ............................................................................................ 14
  2.1 Chemicals, media, and cultures ........................................................................................................... 14
  2.2 Bioreactor Cultures ............................................................................................................................ 15
  2.3 Analysis of cells, PHA, and culture medium ......................................................................................... 15
  2.4 Crude enzyme preparation .................................................................................................................. 16
  2.5 In vitro bioconversion of levulinic acid ............................................................................................... 16

CHAPTER 3. GROWTH AND PHA FORMATION IN MULTIPLE SUBSTRATE CULTURES .... 18
  3.1 Batch cultures on glucose and fructose ............................................................................................... 18
    Influence of the C/N ratio on growth and PHA synthesis ................................................................. 18
    The C/N ratio effect on sugar substrate consumption ......................................................................... 18
  3.2 Batch cultures on levulinic acid .......................................................................................................... 19
    LA toxicity ............................................................................................................................................. 19
    Influence of C/N ratio on PHA formation from LA ........................................................................... 20
  3.3 Batch cultures on glucose, fructose, and levulinic acid ..................................................................... 21
    Different LA feeding amounts .............................................................................................................. 21
    Different LA feeding times .................................................................................................................. 22
  3.4 Fed-Batch fermentations ..................................................................................................................... 23
    Glucose and fructose ............................................................................................................................ 23
    Glucose, fructose, and LA .................................................................................................................... 24

CHAPTER 4. SUBSTRATE UTILIZATION MODELS .................................................................................. 26
  4.1 Individual substrates with excess nutrients ....................................................................................... 26
4.2 Multiple substrates with excess nutrients ................................................................. 28

*Glucose and Fructose* ........................................................................................................ 28

*Glucose, Fructose, and LA* .......................................................................................... 30

4.3 Glucose and Fructose with limited nitrogen ............................................................... 31

CHAPTER 5. IN VITRO BIOCONVERSION OF LEVULINIC ACID ...................................... 34

5.1 Cell disruption with sonication .................................................................................... 34

5.2 Acetyl-CoA formation by acetyl-CoA synthetase ....................................................... 34

5.3 Location of ACS in cells ............................................................................................. 35

5.4 Substrates of crude enzyme grown on acetate ......................................................... 36

5.5 Location of the LA-specific activation enzyme ......................................................... 37

5.6 LA adsorption by sonicated crude pellets ............................................................... 38

5.7 Continuous conversion of AcCoA and PrpCoA ....................................................... 38

5.8 The in vitro bio-conversion of LA into acetyl-CoA and propionyl-CoA ....................... 40

CHAPTER 6. FURTHER RESEARCH ................................................................................. 43

6.1 Regulation of initial fructose metabolism ................................................................. 43

6.2 Further analysis of LA to PHBVV pathway .............................................................. 43

6.3 Isolation of LA-specific activation enzyme .............................................................. 43

REFERENCES .................................................................................................................. 44
LIST OF TABLES

Table 1 – PHA formation after 48 hrs in R. eutropha grown on LA at different C/N ratios. ..................21
Table 2 – The formation of PHBV by R. eutropha at different total LA feeds. .................................21
Table 3 – PHA formation of R. eutropha at different LA feeding times. ........................................23
Table 4 – R. eutropha kinetic parameter for glucose, fructose, and LA. ..........................................28
Table 5 – Comparison of glucose and fructose Vmax and Km as single substrates and multi-
substrates. ........................................................................................................................................30
Table 6 – Comparison of glucose and fructose $V_{max}/K_M$ values at different C/N ratios. .................33
Table 7 – The localization of ACS activity in R. eutropha. .................................................................36
Table 8 – The substrate specificity of crude supernatant sonicate from acetate cultures. .................37
Table 9 – The substrate specificity of crude pellet sonicate from acetate cultures. ..........................37
Table 10 – The localization of the LA-specific activation enzyme. .....................................................38
Table 11 – AcCoA and PrpCoA further conversion. ............................................................................40
LIST OF FIGURES

Figure 1 – The PHA synthesis pathway from glucose, fructose, and LA in R. eutropha.................................8
Figure 2 – Glucose and fructose metabolism via the Entner-Doudoroff pathway. ........................................9
Figure 3 - Structures of the PHA polymers poly-3-hydroxybutyrate (PHB), poly-3-hydroxybutyrate-3-
hydroxyvalerate (PHBV) and poly-3-hydroxybutyrate-3-hydroxyvalerate-4-hydroxyvalerate (PHBVV). .................................................................10
Figure 4 – The structure of levulinic acid (LA), a substrate for PHA copolymers.............................11
Figure 5 – A possible initial reaction in the metabolism of LA by R. eutropha. .......................................12
Figure 6 – The metabolism of PrpCoA by the methylcitric acid cycle. .....................................................13
Figure 7 – Chromatogram of organic acids under in vitro assay conditions. .........................................17
Figure 8 – Chromatogram of CoA, AcCoA, and PrpCoA in a standard solution of pure CoA, AcCoA, and PrpCoA. .........................................................................................................................17
Figure 9 – The C/N ratio influences the growth and PHA synthesis of R. eutropha.................................18
Figure 10 – The consumption of glucose and fructose at different total C/N ratios...............................19
Figure 11 – R. eutropha growth in cultures containing different initial concentrations of LA..............20
Figure 12 – Glucose and fructose utilization in flask cultures with different amounts of total LA...........22
Figure 13 – Glucose, fructose and LA utilization in cultures with different LA feeding times..............23
Figure 14 – Fed-batch fermentation of R. eutropha grown on glucose and fructose from cane molasses hydrolysate.........................................................................................................................24
Figure 15 – Fed-batch fermentation of R. eutropha grown on glucose, fructose, and LA..................25
Figure 16 – Determination of µmax, Km, and Vmax for glucose. ..............................................................27
Figure 17 – Batch fermentation of R. eutropha grown on glucose and fructose.................................28
Figure 18 – Batch fermentation of R. eutropha grown on glucose, fructose, and LA.........................31
Figure 19 – Linear cell growth observed under nitrogen-limited conditions. .........................................33
Figure 20 – Under nitrogen-limited conditions and low sugar concentrations, Km >> S and, therefore, the plot of substrate concentration vs. specific substrate rate is linear.................................................................33
Figure 21 – Sonication level determination by peroxidase activity analysis...........................................34
Figure 22 – The conversion of acetic acid and CoA into AcCoA by ACS in the assay containing enzyme or no enzyme........................................................................................................35
Figure 23 – LA absorption in the membrane of the sonicated crude pellet........................................38
Figure 24 – The concentration decline of AcCoA and PrpCoA by the sonicated LA crude pellet.......39

Figure 25 – The formation of acetyl-CoA and propionyl-CoA from LA by the sonication pellet solution from LA cultured cells. ...........................................................................................................................................41

Figure 26 – The chromatogram of CoA, AcCoA, and PrpCoA in the LA bio-conversion assay at 0 and 3.5 hrs. ...............................................................................................................................................................................................................41

Figure 27 – LA molar mass balance in the in vitro bio-conversion. The formation and continuous conversion is accounted for in the molar mass % PrpCoA. .................................................................................................................................42
CHAPTER 1. INTRODUCTION AND OBJECTIVES

1.1 Introduction

Polyhydroxyalkanoates (PHAs) are a class of polyesters formed in bacteria as energy reserve (Naik, Venu Gopal et al. 2008). The PHA granules accumulate in cells, and can be recovered and processed into bioplastics (Loo, Lee et al. 2005). PHA plastics show potential as a major contributor to reduce petrochemical-based plastic consumption. Unlike petrochemical-based plastics, the starting materials for PHA plastics are renewable and therefore sustainable. Depending on environmental conditions, PHA plastics have a half life of 20-70 days (Volova, Gladyshev et al. 2006). Petroleum plastics have been known to decompose in 10 years or longer (Gross and Kalra 2002).

*Ralstonia eutropha* is a representative organism for PHA research. *R. eutropha* can grow to high cell densities, produce and accumulate PHA in a very high polymer content (50-80%) of cell mass, and has high tolerance towards growth inhibitors, which is important when utilizing inexpensive feedstock and acid substrates (Khanna and Srivastava 2005; Pereira, Sánchez et al. 2008). The bacterium can use various substrates for cell growth or PHA synthesis (Figure 1). When culture conditions are nutrient-rich, the cells utilize substrates for growth and maintenance. Under nutrient-limited conditions, such as nitrogen-limitation, the cells have an increased carbon flow towards PHA synthesis. The PHA yield from the carbon source is therefore increased. For example, a carbon/nitrogen ratio (C/N) of 10-20 favors cell growth, while a C/N of 30-40 favors PHA formation. Consequently, PHA cultures are often performed in two phases: cell growth phase, followed by PHA accumulation phase.
Figure 1 – The PHA synthesis pathway from glucose, fructose, and LA in R. eutropha. PhaA, β-ketothiolase A; CoA, coenzyme-A; ATP, adenosine triphosphate; BktB, β-ketothiolase B; NADPH, nicotinamide adenine dinucleotide phosphate; PhaB, NADPH-dependent acetoacetyl-CoA reductase; PhaC, PHA synthase.

Although PHA plastics are environmentally friendly, they currently cannot compete with petro-chemical based plastics economically. The feedstock accounts for a large amount of the PHA production cost. Pure substrates, such as glucose, are expensive and impractical as feed for mass production of PHA plastics (Page, Manchak et al. 1992). An alternative feedstock should be cheap and contain a large amount of carbohydrates. Sugar cane molasses is an inexpensive by-product of sugar refinery plants and contains a high sucrose content (~50% w/w). Sucrose is a legitimate carbon source for many bacteria, but R. eutropha cannot breakdown the disaccharide (Ramsay, Lomaliza et al. 1990). In one possible process, sucrose is hydrolyzed into glucose and fructose before the molasses can be used as a feedstock for R. eutropha (Khanna and Srivastava 2005; Pereira, Sánchez et al. 2008). Investigating R. eutropha growth, substrate utilization, and PHA content in molasses cultures can provide additional insight into the potential of molasses as feedstock.

Glucose and fructose are two major monosaccharides in sugar cane molasses hydrolysates. The metabolic pathways of glucose and fructose have been extensively studied. Interestingly, Ralstonia eutropha H16 strain, an original isolation from a lake sludge, cannot consume glucose most likely due to inadequate transportation into the cells (Pohlmann, Fricke et
al. 2006). The strain synthesizes glucose-specific catabolic cytosol enzymes, but limited glucose-specific membrane transport proteins. Mutant strains, such as *Ralstonia eutropha* B8562, have been genetically modified to utilize glucose (Volova, Trusova et al. 2006). Fructose is easily transported into the H16 strain via an ATP-binding cassette transporter (Pohlmann, Fricke et al. 2006). Surprisingly, the strain does not contain phosphofructokinase, which converts fructose-6-phosphate to fructose-1,6-bisphosphate, a key reaction in the most common glycolysis pathway, Embden-Meyerhoff. In the H16 strain glucose and fructose are eventually catabolized to 2-keto-3-deoxy-6-phosphogluconate (KDPG) via the Entner-Doudoroff glycolysis pathway (Pohlmann, Fricke et al. 2006). In the Entner-Doudoroff, glucose and fructose converge at glucose-6-phosphate (Figure 2). The H16 strain synthesizes gluconate kinase, which can convert glucose to glucose 6-phosphate. Fructose is converted to fructose-6-phosphate by fructokinase, and then fructose-6-phosphate is converted to glucose-6-phosphate by glucose-6-phosphate isomerase (Figure 2). The glucose-6-phosphate is eventually converted to acetyl-CoA. There is little knowledge on simultaneous glucose and fructose utilization for cell growth and PHA synthesis. Investigation of glucose and fructose utilization in PHA cultures might enhance the knowledge of *R. eutropha* carbohydrate metabolism.

![Figure 2 – Glucose and fructose metabolism via the Entner-Doudoroff pathway. GK, gluconate kinase; FRK, fructokinase; GPI, glucose 6-P isomerase; GPD, glucose-6-phosphate 1-dehydrogenase; PGL, 6-phosphogluconolactonase; PGD, phosphogluconate dehydratase; KDPG, KDPG aldolase; PD, pyruvate dehydrogenase.](Image)
Poly-3-hydroxybutyric acid (PHB), a PHA mono-polyester, is synthesized by *R. eutropha* when grown on glucose and/or fructose (Figure 3). PHB consists of the monomer 3-hydroxybutyric acid (3HB), a four carbon carboxylic acid containing a hydroxyl group on the third carbon (Figure 3). The mono-polyester exhibits thermal and mechanical properties similar to polypropylene, but is a hard brittle material with low ductility (Ojumu, Yu et al. 2004). *R. eutropha* can be induced to synthesize PHA copolymers with diverse material properties. The incorporation of small amounts of five carbon or longer hydroxy-acid monomers into PHA can increase the polymer's ductility (Luzier 1992; Pereira, Sánchez et al. 2008). 3-Hydroxyvalerate (3HV) and 3 hydroxyhexanoate (3HHx) are representative monomers with five and six carbons, respectively. A widely used method by which the second or third monomers can be introduced into PHA backbone is to feed the cells with precursor substrates such as valeric acid and levulinic acid. For example, the addition of levulinic acid or valeric acid to *R. eutropha* cultures will induce synthesis of the PHA copolymer, poly-3-hydroxybutyrate-3-hydroxyvalerate (PHBV) (Figure 3) (Page, Manchak et al. 1992; Chung, Choi et al. 2001; Pereira, Sánchez et al. 2008). PHBV is more flexible and shows less thermal degradation than PHB, making it more suitable for commercial application (Luzier 1992; Page, Manchak et al. 1992).

![Structures of PHA polymers](image)

*Figure 3 - Structures of the PHA polymers poly-3-hydroxybutyrate (PHB), poly-3-hydroxybutyrate-3-hydroxyvalerate (PHBV) and poly-3-hydroxybutyrate-3-hydroxyvalerate-4-hydroxyvalerate (PHBVV).*
The amount and feeding time of the organic acids are important factors to consider for cell growth and PHA copolymer formation. Most organic acids inhibit cell growth at high concentrations, so they have to be administered in small amounts (Page, Manchak et al. 1992). The introduction of co-substrates to cultures at different concentrations and times effects cell growth and PHA copolymer composition. Chung et al showed the cell mass and PHA content increased by 150% and 200%, respectively, when levulinic acid was fed four times in culture to a total of 4 g/L compared to introducing LA in culture once to a total of 4 g/L (Chung, Choi et al. 2001). Page et al illustrated that Azotobacter vinelandii had a delay incorporation of 3HV into PHBV when valerate was added to a glucose culture at time zero (Page, Manchak et al. 1992). When valerate was introduced at 12 hrs to the glucose culture, Azotobacter vinelandii incorporated 3HV into PHBV immediately.

![Figure 4 – The structure of levulinic acid (LA), a substrate for PHA copolymers.](image)

Levulinic acid (LA) is an interesting substrate for PHA copolymer production. LA, also known as 4-ketovaleric acid, is a five carbon carboxylic acid with a ketone group on the fourth carbon. The organic acid is readily formed from biomass waste and the conversion of simple carbohydrates, and therefore inexpensive compared to other acid co-substrates (Bozell, Moens et al. 2000). LA has less inhibitory effects on cell growth and PHA accumulation compared to other organic acids, such as propionate and valerate (Chung, Choi et al. 2001; Yu, Chen et al. 2009). In *R. eutropha* cultures, LA induces synthesis of the PHA tri-polymer poly-3-hydroxybutyrate-3-hydroxyvalerate-4-hydroxyvalerate (PHBV) (Figure 3, Figure 1) (Yu, Chen et al. 2009). Although there has been research on cultures with glucose and LA as substrate, there is no analysis on cultures with molasses sugars and LA as substrate (Chung, Choi et al. 2001; Keenan, Tanenbaum et al. 2004; Yu, Chen et al. 2009). Page et al utilized molasses and valerate together as substrate in fed-batch fermentation to induce production of PHBV by Azotobacter vinelandii, but LA was not analyzed in their work (Page, Manchak et al. 1992). Understanding *R. eutropha* growth, PHBV content, and substrate utilization in molasses sugar and LA cultures may further explain *R. eutropha* metabolism.
In the metabolic pathway of levulinic acid to PHBVV, LA is assumed to be activated via coupling with Coenzyme A (CoA) (Reinecke and Steinbüchel 2008; Yu, Chen et al. 2009). The coupling of acetic acid and CoA to form acetyl CoA is a similar reaction to the coupling of LA and CoA and it has been extensively studied (Beinert, Green et al. 1953). Acetyl-CoA synthetase (ACS) catalyzes the reaction with the energy of ATP and the enzyme activity can be enhanced by divalent cations, such as Mg²⁺ (Von Korff 1953; Webster 1956). It has been reported that a probable fatty acid-CoA synthetase is up-regulated in *R. eutropha* when exposed to LA in culture (Lee, Li et al. 2009). There is little knowledge on the fate of the activated LA. It seems that the ketone group on C-4 of LA should be reduced to 4-hydroxyvalerate (4HV) and assembled in a co-polyester of 4HV, but less than 2% 4HV is incorporated into the PHA backbone when *R. eutropha* is grown on LA. The two main monomers in the PHA co-polymers are 3HB and 3HV at roughly 55% and 43%, respectively (Yu, Chen et al. 2009). Based on the large LA carbon flow towards 3HB and 3HV in PHBVV synthesis, the activated LA may be split into propionyl-CoA (PrpCoA) plus acetyl-CoA (AcCoA), which has been suggested (Reinecke and Steinbüchel 2008; Yu, Chen et al. 2009) (Figure 5, Figure 1). In PHA metabolism, AcCoA and PrpCoA are condensed via β-ketothiolase B to form 3-ketovaleryl-CoA (Doi, Kunioka et al. 1987; Slater, Houmiel et al. 1998). β-ketothiolase B has broad substrate specificity and may be responsible for a reverse split reaction of activated LA into AcCoA and PrpCoA (Slater, Houmiel et al. 1998; Yu, Chen et al. 2009). In this case, AcCoA and PrpCoA are converted to 3-ketovaleryl-CoA, which is ultimately incorporated into PHBVV as 3HV. Alternatively, two AcCoA can condense via β-ketothiolase A to form acetoacetyl-CoA for 3HB incorporation into PHBVV. This may explain the high content of 3HB and 3HV formed in PHA of *R. eutropha* grown on LA. In growth metabolism, PrpCoA can be metabolized via the 2-methylcitric acid cycle (Horswill and Escalante-Semerena 1997) (Figure 6). *R. eutropha* strain HF39, which contains mutations in the 2-methylcitric acid cycle, cannot metabolize LA, suggesting also that propionyl-CoA may be an LA intermediate (Bramer and Steinbuchel 2001) (Figure 6). Demonstration of the intermediates in the initial reactions of the LA metabolic pathway may explain the PHA composition from *R. eutropha* grown only on LA.

![Figure 5](image_url) – A possible initial reaction in the metabolism of LA by *R. eutropha*. 
Figure 6 – The metabolism of PrpCoA by the methylcitric acid cycle. The bold cross-bars indicate metabolic blocks due to DNA/enzyme mutation. LA metabolism is inhibited when any of these enzymes are mutated.

1.2 Objectives

The overall objective of this thesis is to analyze *R. eutropha* utilization of glucose, fructose, and LA individually and in multi-substrate cultures. There are two core objectives:

1. By using a Monod model, determine the kinetics of individual substrates. Also, compare the consumption rates of glucose and fructose in single and multiple substrate cultures, and at different C/N ratios.

2. Reveal the initial key reactions in the metabolic pathway of LA utilization by *in vitro* bio-conversion and HPLC analysis.
CHAPTER 2. MATERIALS AND METHODS

2.1 Chemicals, media, and cultures

Glucose, fructose, levulinic acid, propionic acid, α-phenylenediamine dihydrochloride (OPD), coenzyme A, acetyl-CoA, propionyl-CoA, and acetyl-CoA synthetase were purchased from Sigma Aldrich. Acetic acid was purchased from Fisher Scientific.

A laboratory strain of *Ralstonia eutropha* was maintained on slant cultures containing 2 g/L NaH$_2$PO$_4$, 0.25 g/L MgSO$_4$-7H$_2$O, 2 g/L (NH$_4$)$_2$SO$_4$, 3.67 g/L K$_2$HPO$_4$, 1 g/L yeast extract, 15 g/L agar, 6 g/L glucose, and 1 mL/L trace solution. The slant cultures were kept at 4°C. For inoculum preparation, cells were collected from the slant culture and transferred to a 20 mL test tube containing 5 g/L yeast extract, 5 g/L peptone, 2.5 g/L meat extract, and 2 g/L (NH$_4$)$_2$SO$_4$. The test tube culture was incubated at 30°C for 48 hrs. The 5 mL test tube culture was then transferred to a 250 mL baffled flask containing 100 mL medium of 10 g/L substrate, 94% v/v mineral solution, and 0.1% v/v trace solution. The 100 mL seed flask culture was shaken at 30°C and 200 rpm in an orbital rotary incubator for 24 hrs. Depending on the experiment, the substrates were glucose, fructose, levulinic acid, and acetic acid. For example, if an experiment involved glucose and fructose in culture, the 100 mL seed flask culture would contain 5 g/L glucose and 5 g/L fructose to a total substrate concentration of 10 g/L. In the case of levulinic acid and acetic acid, the 100 mL seed flask culture contained 9 g/L glucose and 1 g/L organic acid to avoid growth inhibition from organic acid toxicity. Unless otherwise stated, the mineral solution contained 2 g/L NaH$_2$PO$_4$, 0.5 g/L MgSO$_4$-7H$_2$O, 2 g/L (NH$_4$)$_2$SO$_4$, and 7.34 g/L K$_2$HPO$_4$. When noted, the (NH$_4$)$_2$SO$_4$ concentration is changed to control the C/N ratio. Trace mineral solution contained 700 μM Fe(NH$_4$)$_2$SO$_4$, 17 μM ZnSO$_4$-7H$_2$O, 25 μM MnCl$_2$-4H$_2$O, 8 μM CuSO$_4$-5H$_2$O, 7.2 μM Na$_2$B$_4$O$_7$-10H$_2$O, and 8.3 μM NaMoO$_4$-2H$_2$O. The 100 mL seed flask culture was then used as inoculum for experimental cultures.

Unless stated otherwise, in shake flask experiments, cultures were grown in 500 mL baffled flask containing a 200 mL medium composed of 8-10 mL *R. eutropha* inoculum (from 100 mL seed flask culture), a specified substrate concentration, 94% v/v mineral solution, and 0.1% v/v trace solution. Cultures were shaken at 30°C and 200 rpm in an orbital rotary incubator. Aliquots of 50 mL culture medium were collected for analysis. In Chapter 5, crude enzyme solutions were prepared from levulinic acid or acetic acid shake flask cultures. Levulinic acid or acetic acid was equally fed five times to culture at 0, 12, 24, 36, and 48 hours to a total of 10 g/L to avoid growth inhibition from organic acid toxicity. The organic acids were fed 2 hours before cell harvest to increase the concentration of LA-specific enzymes in crude extracts.
2.2 Bioreactor Cultures

Batch and fed-batch fermentation were performed in a 3 L bioreactor (New Brunswick Scientific Co., Inc., Edison, N.J., U.S.A.). The temperature, pH, and dissolved oxygen probes were used to monitor and control the fermentations. Culture conditions were maintained at 30°C and pH 6.8. Agitation speeds were 200 RPM when the dissolved oxygen was above 10% maximum. When dissolved oxygen was below 10% maximum, the agitation speed increased accordingly. Cultures were inoculated with 100 mL seed flask culture mentioned above. The feeds are specified in the experiments below. Aliquots of 10-15 mL culture medium were collected for analysis.

2.3 Analysis of cells, PHA, and culture medium

The optical density of culture medium was measured at 620 nm to monitor cell growth with a Beckman DU530 Life Science UV/Vis Spectrophotometer. Aliquots of culture medium were centrifuged at 7000 rpm for 10 min to separate the supernatant (culture solution) from wet pellets (cells and PHA). The supernatant was analyzed by HPLC to measure the substrate concentrations as mentioned below. The wet pellets were washed by suspension in mineral solution to 50 mL and re-centrifuged. The washed pellets were frozen at -20°C and freeze-dried to determine the cell mass concentration and PHA content as mentioned below.

Residual sugar concentrations, residual organic acid concentrations, and PHA content and composition were analyzed by HPLC. Supernatant solutions from centrifuged culture medium were analyzed by HPLC to determine the residual substrate concentrations. Samples 20 µL were introduced into HPLC and eluted with a water-sulfuric acid carrier (pH 2.0) at 0.8 mL/min through a Rezex ROA-Organic Acid column (150 x 7.8 mm) maintained at 60°C (Phenomenex). To determine the concentration of glucose and fructose simultaneously, a 20 µL sample was introduced into HPLC and eluted with a water-potassium phosphate carrier (15mM K2HPO4) at 0.5 mL/min through a Supelco K column (300 x 7.8 mm) maintained at 80°C (Phenomenex). Sugars were detected with an RID detector and the organic acids were detected with a UV detector at 210 nm.

PHA content and composition in cells were determined after methanolation of freeze-dried cell mass in methanol (3.2% v/v sulfuric acid) at 100°C for 8 hours. The methyl hydroxyalkanoates were hydrolyzed to hydroxyalkanoic acid by adding 10 M NaOH solution for HPLC analysis. Samples were centrifuged and the supernatant was analyzed by HPLC. The hydroxyalkanoic acid samples were eluted in the same HPLC configuration as the organic acid samples above.
2.4 Crude enzyme preparation

The washed pellets from 50 mL cell harvests were re-suspended in 5 mL phosphate buffer consisting of 60 mM K$_2$HPO$_4$ and 2 mM MgSO$_4$. The solution was pipetted to 1 mL in 1.5 mL micro-centrifuge tubes for sonication. Sonication was performed with a Fisher Scientific Sonic Dismembrator Model 100. The sonicate 1 mL suspension was pulsed for 3 cycles of 30 seconds each at 40 output watts. The suspension was put on ice in-between sonication cycles. The time and cycle was selected to reduce heat from sonication. Peroxidase enzyme activity of sonicates were analyzed by the o-phenylenediamine dihydrochloride (OPD) reaction to determine the sonication output (watts). Peroxidase converts OPD to 2,3-diaminophenazine, which can be read spectrophotometrically at 450 nm. The Beckman DU530 Life Science UV/Vis Spectrophotometer was used to analyze peroxidase activity. The membrane-bound enzymes and water-soluble enzymes of the sonicated suspension were separated by centrifugation at 10000 rpm for 10 min. The supernatant (containing water-soluble enzymes) was kept at -20°C for later use. The wet pellet (containing membrane-bound enzymes) was suspended in phosphate buffer and re-centrifuged. The wet pellet was re-suspended in 0.9 mL phosphate buffer and kept at -20°C for later use.

2.5 In vitro bioconversion of levulinic acid

All reactions were performed in 1.5 mL micro-centrifuge tubes at 30°C and 200 rpm in an orbital rotary incubator. A typical reaction solution consists of 1 mM LA, 0.7 mM coenzyme A, 1.5 mM adenosine tri-phosphate (ATP), 30 mM K$_2$HPO$_4$, and 1 mM MgCl$_2$. The reaction was initiated by addition of crude enzyme (either from centrifuged pellet or supernatant) to 10% vol/vol. Levulinic acid was also substituted by acetic acid and/or propionic acid in the assay.

The organic acids were analyzed by HPLC as described in section 2.3. The organic acids in the in vitro bioconversion, however, were detected with a RID detector because of ATP peak interference with the organic acids in the UV detector (Figure 7). CoA, AcCoA, and PrpCoA were analyzed by HPLC. LA-CoA was not observed in the in vitro assay and this is discussed in section 5.8. 20 μL samples were eluted with a mobile phase consisting of 150 mM citrate buffer at pH 5.0 and 9% vol/vol acetonitrile. The samples were eluted through a Shimadzu Premier C18 column, 5 μm particle diameter, 250 mm x 4.6 mm ID. CoA, AcCoA, and PrpCoA were detected with a UV detector at 261 nm. The concentrations of CoA-containing compounds were calculated from individual HPLC standard curves of pure CoA intermediates obtained from Sigma-Aldrich. Under these conditions, the retention times were as follows: CoA, 4.0; acetyl-CoA, 5.7 min; propionyl-CoA, 10.7 min (Figure 8).
Figure 7 – Chromatogram of organic acids under *in vitro* assay conditions.

Figure 8 – Chromatogram of CoA, AcCoA, and PrpCoA in a standard solution of pure CoA, AcCoA, and PrpCoA.
CHAPTER 3. GROWTH AND PHA FORMATION IN MULTIPLE SUBSTRATE CULTURES

3.1 Batch cultures on glucose and fructose

*Influence of the C/N ratio on growth and PHA synthesis*

PHA synthesis was stimulated by nitrogen limitation. In shake flask cultures, the initial C/N ratio was controlled by adjusting the initial (NH₄)₂SO₄ concentrations. The carbon sources were glucose and fructose and the initial sugar concentration was 10 g/L. At 10 C/N, the PHB % in cells was 16% and the cell metabolism was shifted towards growth. At 20 C/N and above, the PHB % in cells accumulated to over 50% and the cell metabolism was shifted towards PHA synthesis. The C/N ratio may also influence other components of *R. eutropha* metabolism.

![Figure 9](image)

**Figure 9** – The C/N ratio influences the growth and PHA synthesis of *R. eutropha*.

*The C/N ratio effect on sugar substrate consumption*

Sugar consumption was analyzed by limiting nitrogen in pure sugar flask cultures. Shake flask cultures were prepared with different concentrations of (NH₄)₂SO₄ to produce C/N ratios of 13, 25, 51, and 101 (Figure 10). Cultures consisted of 5 g/L glucose, 5 g/L fructose, 94% mineral solution, and 0.1% trace solution. The glucose and fructose concentrations were monitored for 24 hrs. In the 13 C/N culture, the glucose and fructose consumption were similar. Fructose consumption was slightly faster than glucose consumption. When the initial C/N ratio was increased (nitrogen limitation), there is overall sugar consumption limitation, but fructose consumption is limited significantly more than glucose consumption. Based on the Entner-Doudoroff, fructose is catabolized in two reactions before it converges with the glucose metabolic
pathway. The two reactions are catalyzed by fructokinase first and glucose-6-phosphate isomerase second. Nitrogen limitation and/or the shift towards PHA synthesis may inhibit these enzymes.

![Graphs showing consumption of glucose and fructose at different C/N ratios](image)

Figure 10 – The consumption of glucose and fructose at different total C/N ratios.

3.2 Batch cultures on levulinic acid

**LA toxicity**

The toxicity of LA on *R. eutropha* was analyzed to avoid growth inhibition in further experiments. Flask cultures were prepared with initial LA concentrations of 2.5, 5.0, 7.5, and 10 g/L. Cultures consisted of 94% v/v mineral solution and 0.1% v/v trace solution. Cell densities were determined at 0 and 12 hrs by measuring optical densities at 610 nm. After 24 hrs, growth inhibition can be seen in the cultures containing LA concentrations of 5 g/L or higher and there is no growth observed at LA concentrations above 7.5 g/L. The cell growth in the 5 g/L LA culture declined by 50% of the 2.5 g/L LA culture cell growth. To reduce toxicity effects, LA was fed at increments of no more than 3.5 g/L in further experiments.
Influence of \(\text{C}/\text{N}\) ratio on PHA formation from LA

Flask cultures with LA as the sole carbon source were prepared with different nitrogen concentrations to analyze the influence of \(\text{C}/\text{N}\) ratios on PHA formation. Nitrogen was added to culture as \((\text{NH}_4)_2\text{SO}_4\) to concentrations of 0.6, 0.8, 1.2, or 2 g/L. Cultures contained 0.6, 0.8, 1.2, or 2 g/L \((\text{NH}_4)_2\text{SO}_4\), which produced \(\text{C}/\text{N}\) ratios of 42, 31, 21, or 13, respectively. LA was added to culture at 0, 12, 24, and 36 hrs to a total concentration of 10 g/L. Cultures consisted of 95 % v/v mineral solution, and 0.1 % v/v trace solution. Cultures were harvested after 48 hrs and analyzed. Cell growth was limited at \(\text{C}/\text{N}\) ratios above 30 due to nitrogen limitation (Table 1). Cultures at 13 and 21 \(\text{C}/\text{N}\) showed no growth limitation and reached 4 g/L cell density after 48 hrs. The PHA content decreased as the \(\text{C}/\text{N}\) ratio decreased because of carbon directed towards cell growth rather than PHA synthesis. This phenomenon was illustrated in the glucose and fructose cultures (Figure 9). It indicates LA is a carbon source for both cell growth and PHA synthesis. Interestingly, the 3HV content increased when the \(\text{C}/\text{N}\) ratio was decreased, changing the PHA composition (Table 1). While the 3HV concentration increased slightly when the \(\text{C}/\text{N}\) ratio decreased, the overall PHA and 3HB concentration decreased significantly. In the 3HB pathway, two acetyl-CoA are condensed by \(\beta\)-ketothiolase A to eventually produce 3HB. When nitrogen is in excess, there may be increased utilization of acetyl-CoA for cell growth and/or \(\beta\)-ketothiolase A may be inhibited. Based on the 3HV concentration, the activity of the LA to 3HV pathway is largely unaffected by the \(\text{C}/\text{N}\) ratio change. The results illustrate the pathways of LA to 3HB and LA to 3HV are affected differently when \textit{R. eutropha} is subject to nitrogen limitation.
Table 1 – PHA formation after 48 hrs in R. eutropha grown on LA at different C/N ratios.

<table>
<thead>
<tr>
<th>C/N (w/w)</th>
<th>Cell Density (g/L)</th>
<th>Residual Cell Mass (g/L)</th>
<th>PHA Content (% w/w)</th>
<th>3HV/PHA (% w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>42</td>
<td>3.0 ± 0.0</td>
<td>1.1</td>
<td>64 ± 1</td>
<td>29 ± 0</td>
</tr>
<tr>
<td>31</td>
<td>3.5 ± 0.0</td>
<td>1.1</td>
<td>69 ± 5</td>
<td>30 ± 2</td>
</tr>
<tr>
<td>21</td>
<td>4.0 ± 0.1</td>
<td>1.8</td>
<td>55 ± 4</td>
<td>40 ± 1</td>
</tr>
<tr>
<td>13</td>
<td>3.9 ± 0.1</td>
<td>2.3</td>
<td>41 ± 1</td>
<td>51 ± 0</td>
</tr>
</tbody>
</table>

3.3 Batch cultures on glucose, fructose, and levulinic acid

**Different LA feeding amounts**

The influence of different LA amounts on PHA formation by *R. eutropha* was investigated in multiple substrate cultures. Equal amount of LA was added to flask cultures at 0, 12, 24, and 36 hours. From the sum of LA added, flask cultures contained LA concentrations of either 2.5 g/L, 5.0 g/L, 7.6 g/L, or 10.1 g/L. Cultures consisted of 10 g/L cane molasses sugars (glucose and fructose), 90 % v/v mineral solution, and 0.1% trace solution. The cell density and PHA content increased from cultures with 2.5 g/L total LA to cultures with 7.6 g/L total LA (Table 2). This is expected because the carbon source and C/N ratio is increased in culture. At 10.1 g/L total LA, the cell density and PHA content decreased slightly due to growth inhibition at increased LA concentrations. LA is incorporated into PHA as both 3HB and 3HV in *R. eutropha*, while glucose and fructose are incorporated into PHA as 3HB. This is illustrated by the increase in 3HV content with an increase in LA feed (Table 2). Furthermore, the fructose consumption was significantly inhibited at increased LA concentrations, while glucose consumption was only slightly altered (Figure 12). Nitrogen limitation and LA toxicity both inhibit the ability of *R. eutropha* to metabolize fructose, suggesting harsh environmental conditions, in general, reduce the activity of enzymes involved in fructose catabolism.

Table 2 – The formation of PHBV by *R. eutropha* at different total LA feeds.

<table>
<thead>
<tr>
<th>Total LA (g/L)a</th>
<th>Cell Density (g/L)</th>
<th>PHA Content (% w/w)</th>
<th>3HV/PHA (% w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5</td>
<td>5.4 ± 0.0</td>
<td>33 ± 2</td>
<td>21 ± 5</td>
</tr>
<tr>
<td>5.0</td>
<td>6.2 ± 0.1</td>
<td>34 ± 0</td>
<td>27 ± 1</td>
</tr>
<tr>
<td>7.6</td>
<td>6.5 ± 0.2</td>
<td>36 ± 0</td>
<td>30 ± 2</td>
</tr>
<tr>
<td>10.1</td>
<td>6.4 ± 0.1</td>
<td>31 ± 3</td>
<td>32 ± 1</td>
</tr>
</tbody>
</table>

*a* Equal amount of LA was added at 0, 12, 24, and 36 hrs to the concentrations noted.

After 48 hrs, cultures were analyzed as described in Materials and Methods.
**Different LA feeding times**

The PHA formation by *R. eutropha* was further assessed in multiple substrate cultures by administering LA at different times. LA was added once at 0, 12, 24, or 36 hrs to a total concentration of 3.5 g/L. Cultures consisted of 10.5 g/L cane molasses sugars (glucose and fructose), 90 % v/v mineral solution, and 0.1% trace solution. After 48 hr cultivation, the cell density and PHA content increased from the 0 hr LA feed culture to the 24 hr LA feed culture (Table 3). Due to LA toxicity, growth was inhibited when LA was introduced at 0 and 12 hrs (Table 3, Figure 13). The 36 hr LA feed culture had a reduced cell density and PHA content because LA was not completely consumed in the 12 hour period (Figure 13). Also, the PHA content in the 0, 12, and 36 hr LA feed cultures was lower because not all the carbon sources were consumed in 48 hours, which lowers the C/N ratio (Figure 13). Interestingly, the 3HV content was significantly higher in the 12 hr LA feed culture, at 35 % 3HV (Table 3). Page et al have shown that the most active time of PHA biosynthesis in a valerate fed *Azotobacter vinelandii* batch culture was 12 hrs, in which the highest HV incorporation was observed (Page, Manchak et al. 1992).
Table 3 – PHA formation of *R. eutropha* at different LA feeding times.

<table>
<thead>
<tr>
<th>LA feed time (hr)</th>
<th>Cell Density (g/L)</th>
<th>PHA Content (% w/w)</th>
<th>3HV/PHA (% w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5.6 ± 0.4</td>
<td>41 ± 5</td>
<td>10 ± 1</td>
</tr>
<tr>
<td>12</td>
<td>5.3 ± 0.1</td>
<td>39 ± 2</td>
<td>35 ± 1</td>
</tr>
<tr>
<td>24</td>
<td>6.0 ± 0.0</td>
<td>48 ± 2</td>
<td>19 ± 1</td>
</tr>
<tr>
<td>36</td>
<td>5.0 ± 0.2</td>
<td>39 ± 1</td>
<td>11 ± 1</td>
</tr>
</tbody>
</table>

* a LA was added once to 3.5 g/L at the times noted. After 48 hrs, cultures were analyzed as described in Materials and Methods.

Figure 13 – Glucose, fructose and LA utilization in cultures with different LA feeding times. Arrows indicate the time of LA addition.

3.4 Fed-Batch fermentations

**Glucose and fructose**

Glucose and fructose from cane molasses hydrolysate were utilized as co-substrates by *R. eutropha* in fed-batch fermentation. The cane molasses hydrolysate feedstock contained 119 g/L glucose and 125 g/L fructose. The initial glucose and fructose hydrolysate concentration in culture was 9.0 g/L and 9.5 g/L, respectively. Nitrogen was supplied at the beginning of culture
with \((\text{NH}_4)_2\text{SO}_4\) (2 g/L) in the mineral solution and followed with ammonia as base for pH control. The batch phase occurred between 0-20 hrs and the cane molasses hydrolysate feeding occurred between 20-72 hrs. The maximum cell density was 37 g/L at 64 hrs. During the batch phase, glucose and fructose were consumed equally (Figure 14). Once the fed-batch phase began at 20 hrs, glucose was preferentially consumed over fructose. Also, the increase in C/N ratio and PHB content coincided with the decline in fructose consumption. The nitrogen limitation in fed-batch fermentation further illustrates the inhibition in the initial fructose pathway.

![Figure 14 – Fed-batch fermentation of R. eutropha grown on glucose and fructose from cane molasses hydrolysate.](image)

Glucose, fructose, and LA

Glucose, fructose, and LA were utilized as multi-substrates by \(R.\ eutropha\) in fed-batch fermentation. The sugars were supplied by sugar cane molasses. Two feedstock solutions were prepared: one with 250 g/L sugars (glucose and fructose) and one with 230 g/L sugars and 70 g/L LA. The LA feedstock was introduced to culture at 24.5 hrs, immediately after the batch phase. As the C/N ratio was increased (24.5 to 52.0 hrs), the LA feedstock was continuously fed into culture. Nitrogen was supplied at the beginning of culture with \((\text{NH}_4)_2\text{SO}_4\) (2 g/L) in the mineral solution and followed with ammonia as base for pH control. The maximum cell density was 37 g/L at 69 hrs. When the LA feedstock was introduced, the HV content increased and reached a maximum of 28% at 40.5 hrs. The HV content declined thereafter due to the feeding of the sugar-only feedstock and the depletion of the LA feedstock. The final C/N ratio was 24 and the PHA content reached 66% at 69 hrs. The decline in fructose consumption was also observed after the batch phase (Data not shown).
Figure 15 – Fed-batch fermentation of R. eutropha grown on glucose, fructose, and LA.
CHAPTER 4. SUBSTRATE UTILIZATION MODELS

4.1 Individual substrates with excess nutrients

Batch fermentations of *R. eutropha* were prepared with glucose, fructose, or LA as limiting substrate. All substrate concentrations were below 2 g/L and other nutrients were available in excess. A Monod model (Eq. 1) is used to simulate the kinetics of cell growth and substrate utilization. The model parameters, $\mu_{\text{max}}$ and $K_m$, are used to monitor *R. eutropha* behavior under nutrient rich or limited conditions.

$$\mu = \frac{\mu_{\text{max}} S}{K_m + S} \quad \text{(Eq. 1)}$$

Growth and substrate concentrations were measured to obtain values of $\mu_{\text{max}}$ and $K_m$ for each substrate. The Langmuir plot (Eq 2) was used to calculate the $\mu_{\text{max}}$ and $K_m$.

$$\frac{S}{\mu} = \frac{K_m}{\mu_{\text{max}}} + \frac{S}{\mu_{\text{max}}} \quad \text{(Eq. 2)}$$

The Langmuir equation is a linearized form of the Monod equation. The plot allows calculation of $\mu_{\text{max}}$ and $K_m$ from the slope and y-intercept with minimal distortion from experimental error (Doran 1995). The specific consumption rate ($V$) for each substrate was determined by calculating the substrate consumption rates at each point and dividing the rate by the cell density at that point (Figure 16a, b). Regression lines (up to 3) were used to obtain smooth fitting curves throughout the culture. For example, the glucose consumption rate was determined with 3 regression equations: a polynomial equation at the beginning and end, and a linear equation in the middle (Figure 16b). The derivative of each equation allowed for estimation of the consumption rate at each point. When regressions overlapped at time points, the rates were averaged. The same method was used for fructose and LA.

Under these conditions, fructose was the best substrate for *R. eutropha* growth. The carbon source had the highest $\mu_{\text{max}}$, maximum consumption rate ($V_m$), and overall cell yield ($Y_{X/S}$) at 0.30 hr$^{-1}$, 0.80 hr$^{-1}$, and 0.51, respectively (Table 4). Surprisingly, glucose was the worst substrate for *R. eutropha* growth under these conditions. The carbon source had the lowest $\mu_{\text{max}}$, $K_m$, and $V_m$ at 0.18 hr$^{-1}$, 0.08 g/L, and 0.46 hr$^{-1}$, respectively. From the multiple substrate cultures in Chapter 3, it seemed as though glucose was the preferred substrate over fructose, but those cultures were not at optimal condition and the sugar concentrations were much higher (above 10 g/L). Based on research on original *R. eutropha* species, fructose genes may be native to this laboratory strain, while glucose genes are most likely present due to gene modification (Pohlmann, Fricke et al. 2006; Reinecke and Steinbüchel 2008). This may explain why glucose is
not metabolized as efficiently as fructose, and why glucose metabolism in *R. eutropha* is not as responsive to harsh conditions as fructose metabolism (Table 4, Figure 14). LA proved to be a good substrate for *R. eutropha* at low concentrations. Of all substrates, *R. eutropha* had the highest affinity for LA with a $K_m$ of 0.01 g/L (Table 4). The organic acid also had a high $\mu_{\text{max}}$ and $V_m$ at 0.26 hr$^{-1}$ and 0.79 hr$^{-1}$, respectively. Interestingly, LA had a $Y_{X/S}$ of 0.36, which was much lower than glucose and fructose. LA is much more toxic to *R. eutropha* than sugar substrates and may still have toxic effects on the bacteria at low concentrations to cause a low cell yield.

![Figure 16](image-url)

Figure 16 – Determination of $\mu_{\text{max}}$, $K_m$, and $V_{\text{max}}$ for glucose. (a.) The time course of cell density. (b.) The time course of residual glucose concentration. (c.) The specific growth rate ($\mu$) versus residual glucose concentration. (d.) The specific glucose utilization (g/g·hr) versus residual glucose concentration.
Table 4 – R. eutropha kinetic parameter for glucose, fructose, and LA.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$\mu_{max}$ (hr$^{-1}$)</th>
<th>$K_m$ (g/L)</th>
<th>$V_m$ (hr$^{-1}$)</th>
<th>$Y_{X/S}$ a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>0.18</td>
<td>0.08</td>
<td>0.46</td>
<td>0.48</td>
</tr>
<tr>
<td>Fructose</td>
<td>0.30</td>
<td>0.05</td>
<td>0.80</td>
<td>0.51</td>
</tr>
<tr>
<td>LA</td>
<td>0.26</td>
<td>0.01</td>
<td>0.79</td>
<td>0.36</td>
</tr>
</tbody>
</table>

a The overall yield ($Y_{X/S}$) was determined from the slope of the cell density versus residual glucose concentration.

4.2 Multiple substrates with excess nutrients

**Glucose and Fructose**

A batch fermentation was prepared with glucose and fructose as limiting substrates to assess the consumption rates and growth of \textit{R. eutropha} with both sugars present. The $\mu_{max}$ on the mixed sugars was 0.25 hr$^{-1}$, which was determined from an exponential regression line of the cell density vs. time plot (Figure 17). The $\mu_{max}$ of \textit{R. eutropha} grown on two sugars is in between the $\mu_{max}$ on individual sugars (Table 4). The $\mu_{max}$ of 0.25 hr$^{-1}$ is actually closer to the $\mu_{max}$ of fructose because the fructose is consumed more than the glucose. The specific consumption rate ($V$) of glucose and fructose reached in culture was 0.18 hr$^{-1}$ and 0.45 hr$^{-1}$, respectively (Figure 17). The overall yield ($Y_{X/S}$) of the fermentation was 0.49, which is in between the $Y_{X/S}$ of each individual substrate (Table 4).

![Figure 17 – Batch fermentation of R. eutropha grown on glucose and fructose. (a.) The time course of cell density. (b.) The specific substrate utilization (g/g·hr) versus the residual substrate concentration.](image)

A modified form of the Monod equation was used to determine the $V_{max}$ and $K_m$ for glucose and fructose in multi-substrate culture. The $Y_{X/S}$ is expressed as gram cell over gram substrate, but it can also be expressed in rates (Eq. 3).
Based on the yield, $\mu$ and $\mu_{\text{max}}$ can be substituted for $V$ and $V_{\text{max}}$ in the Monod equation (Eq. 4).

$$V = \frac{V_{\text{max}}S}{K_m + S}$$  \hspace{1cm} (Eq. 4)

The modified Monod equation was linearized into the Lagmuir plot to calculate the $V_{\text{max}}$ and $K_m$ of each substrate (Eq. 5, Table 5).

$$\frac{S}{V} = \frac{K_m}{V_{\text{max}}} + \frac{S}{V_{\text{max}}}$$  \hspace{1cm} (Eq. 5)

Based on the $V_{\text{max}}$ and $K_m$ of glucose, the glucose consumption is reduced in the presence of fructose because of an increased $K_m$ relative to the $K_m$ in batch fermentation with glucose alone (Table 5). The glucose $K_m$ is increased by more than 10 times in the multi-substrate culture, while the $V_m$ is basically unchanged. The increased $K_m$ suggests fructose competitively inhibits glucose consumption in \textit{R. eutropha}. The competitive inhibition may occur where glucose and fructose converge at glucose-6-phosphate in the Entner-Doudoroff pathway (Figure 1). Based on the $V_{\text{max}}$ and $K_m$ of fructose, the fructose consumption is reduced in the presence of glucose due to both a decrease in $V_{\text{max}}$ and increase in $K_m$ relative to the $V_{\text{max}}$ and $K_m$ in batch fermentation with fructose alone (Table 5). The fructose $V_{\text{max}}$ and $K_m$ in the multi-substrate culture are 65% and 220% of the single substrate culture, respectively. The decrease in the fructose $V_{\text{max}}$ suggests glucose noncompetitively inhibits fructose catabolism in \textit{R. eutropha}. Glucose may indirectly inhibit fructose-associated enzymes through a feedback loop in \textit{R. eutropha}, a mechanism seen in other organisms (Rand and Tatum 1980). The increase in the fructose $K_m$ in the multi-substrate culture implies glucose also competitively inhibits fructose catabolism. The competitive inhibition of fructose by glucose may also occur at glucose-6-phosphate in the Entner-Doudoroff pathway (Figure 1). The increase in the glucose $K_m$ due to the presence of fructose is much more significant than any of the glucose inhibitory effects on fructose. Therefore, under low substrate concentration and excess nutrient conditions, fructose is the preferred substrate over glucose because \textit{R. eutropha} has a higher affinity for fructose.
Table 5 – Comparison of glucose and fructose Vmax and Km as single substrates and multi-substrates.

<table>
<thead>
<tr>
<th>Batch Fermentation Type</th>
<th>Single Substrate</th>
<th>Multi-Substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vmax (hr⁻¹)</td>
<td>Km (g/L)</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.46</td>
<td>0.08</td>
</tr>
<tr>
<td>Fructose</td>
<td>0.80</td>
<td>0.05</td>
</tr>
</tbody>
</table>

Glucose, Fructose, and LA

A batch fermentation was prepared with glucose, fructose, and LA as limiting substrates to examine the behavior of *R. eutropha*. The $\mu_{max}$ of *R. eutropha* was 0.29 hr⁻¹, which is similar to the $\mu_{max}$ of fructose and LA as individual substrates (Table 4). Interestingly, *R. eutropha* consumed LA almost exclusively with all three substrates present (Figure 18b). Even under optimal conditions, glucose and fructose consumption was minimal with specific consumption rates below 0.1 hr⁻¹. The maximum specific LA rate reached was 0.53 hr⁻¹, which is 67% of the $V_m$ of LA as the sole substrate. The specific glucose rate actually surpasses the specific fructose rate between 3-4 hrs (Figure 18c). The specific glucose rate is lower than the specific fructose rate at specific LA rates below 0.5 hr⁻¹. Alternatively, the specific glucose rate is higher than the specific fructose rate at specific LA rates above 0.5 hr⁻¹. The presence of LA may regulate or inhibit the metabolic pathways of glucose and fructose. For example, the intermediates from LA metabolism may converge on the sugar pathways. Since LA is metabolized at a higher rate, the sugar pathways will be halted because there is already a sufficient concentration of intermediates present. The overall yield in the fermentation was 0.52, which is similar to the yields of *R. eutropha* grown on sugars (Table 4). A yield of 0.52 is surprising because the yield of LA alone was 0.36. The higher yield may be due to the initial LA concentration in the multiple substrate culture was a third of the initial LA concentration in the single substrate culture, which would reduce the toxic effect of LA.
4.3 Glucose and Fructose with limited nitrogen

Batch fermentations with glucose and fructose were prepared with limited nitrogen to analyze the effect on the sugar consumption rates. Consumption rates were examined at C/N ratios of 20, 30, and 40. In all cultures, the glucose and fructose concentrations were below 1 g/L each. Under these conditions, the cell growths observed were linear (Figure 19). The initial specific growth rates of the 20, 30, and 40 C/N cultures were 0.196 hr⁻¹, 0.103 hr⁻¹, and 0.075 hr⁻¹, respectively. Also, the glucose and fructose specific consumption rates were linear relative to the respective concentrations (Figure 20). For linear cell growth, the Monod model indicates that under these conditions, the $K_m$ value is much greater than the substrate concentration, $K_m \gg S$. Therefore, equation 4 can be modified into a linear plot (Eq. 6).

$$ V = \frac{V_{max} S}{K_m} \quad \text{(Eq. 6)} $$

Based on equation 6, the $\frac{V_{max}}{K_m}$ values could be obtained from the slope of the primed substrate concentration vs. specific substrate rate for glucose and fructose at each C/N ratio.
(Figure 20, Table 6). The primed substrate concentration \((S')\) is the adjusted substrate concentration to exclude a \(y\)-intercept, which is irrelevant in equation 6 and under these conditions. The \(\frac{v_{\text{max}}}{K_m}\) value can reflect the overall consumption of a substrate. For example, a higher \(\frac{v_{\text{max}}}{K_m}\) suggests better substrate consumption than a lower \(\frac{v_{\text{max}}}{K_m}\). The glucose \(\frac{v_{\text{max}}}{K_m}\) values in the multiple-substrate culture were all below 1 \(\text{L hr}^{-1} \text{g}^{-1}\) and fluctuated (Table 6). In the single substrate culture, the glucose \(\frac{v_{\text{max}}}{K_m}\) was much higher than the \(\frac{v_{\text{max}}}{K_m}\) of the multi-substrate culture, illustrating the competitive inhibition of fructose. Interestingly, the \(\frac{v_{\text{max}}}{K_m}\) at 20 C/N is higher than any C/N ratio in the multiple-substrate culture, even at 3 C/N with excess nutrients. At 20 C/N, the overall cell yield was less than 0.4, while the yields for all other cultures were between 0.45 and 0.5. The reason for the low yield and high \(\frac{v_{\text{max}}}{K_m}\) in the 20 C/N culture may be due to cell metabolism at a transition phase between growth and PHA synthesis. At the transition phase, substrate utilization may not be as efficient due to conflicting growth and PHA metabolic pathways. Based on the \(\frac{v_{\text{max}}}{K_m}\) values, the nitrogen limitation on glucose consumption was overshadowed by the fructose competitive inhibition. The fructose \(\frac{v_{\text{max}}}{K_m}\) values were less affected by the glucose inhibition and more affected by the nitrogen limitation (Table 6). The \(\frac{v_{\text{max}}}{K_m}\) values of fructose demonstrated a reciprocal relationship with the C/N ratio. With an increasing C/N ratio or nitrogen limitation, the \(\frac{v_{\text{max}}}{K_m}\) or the overall fructose consumption logically decreased. Under low substrate concentrations and C/N ratios below 40, the results illustrate fructose is the preferred substrate over glucose by \(R.\ eutropha\). At C/N ratio above 40 C/N, batch fermentations may prove glucose is consumed preferentially over fructose.
Figure 19 – Linear cell growth observed under nitrogen-limited conditions.

Figure 20 – Under nitrogen-limited conditions and low sugar concentrations, $K_m > S$ and, therefore, the plot of substrate concentration vs. specific substrate rate is linear.

Table 6 – Comparison of glucose and fructose $\frac{v_{max}}{K_m}$ values at different C/N ratios.

<table>
<thead>
<tr>
<th></th>
<th>Single Substrate</th>
<th>Multiple Substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>3 C/N</td>
<td>3 C/N</td>
</tr>
<tr>
<td></td>
<td>5.67</td>
<td>0.45</td>
</tr>
<tr>
<td>Fructose</td>
<td>15.09</td>
<td>9.09</td>
</tr>
<tr>
<td></td>
<td>0.67</td>
<td>0.38</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.21</td>
</tr>
</tbody>
</table>
CHAPTER 5. IN VITRO BIOCONVERSION OF LEVULINIC ACID

5.1 Cell disruption with sonication

The sonication level (output watts) must be high enough to break down cell membrane, but small enough to prevent enzyme degradation. The peroxidase activity of cell suspensions was analyzed at 20, 40, 60, 80, and 100 sonication output watts using the cycles and times mentioned in Materials and Methods. The maximum peroxidase activity observed was 0.016 absorbance (ABS)/min at 40 output watts (Figure 21). At 20 watts, peroxidase activity was 0.004 ABS/min, 25% of the activity at 40 watts. The enzyme activity decreased significantly at watts greater than 40. Therefore, all cell samples prepared for in vitro experiments were sonicated at 40 output watts.

![Figure 21 – Sonication level determination by peroxidase activity analysis.](image)

5.2 Acetyl-CoA formation by acetyl-CoA synthetase

Acetyl-CoA synthetase (ACS) converts acetic acid and CoA into acetyl-CoA with the energy of ATP (Eq. 6). The reaction catalyzed by ACS was utilized to establish the monitoring method of CoA intermediates in vitro. The assay solution consisted of 450 μM acetic acid, 600 μM CoA, 800 μM ATP, and 55 Units/L ACS in a 60 mM K₂HPO₄ and MgCl₂ buffer. The same reaction without ACS was prepared as a negative control. The concentrations of acetate, CoA, and AcCoA were monitored with HPLC. The reaction with ACS reached equilibrium at 4-5 hours (Figure 22). In 4 hrs, the concentration changes of CoA and AcCoA in the ACS reaction were 357 μM and 332 μM, respectively. Therefore, AcCoA accounted for 93.0% of the CoA concentration decline. In the negative control, the CoA concentration also decreased, but to a
lesser extent. The CoA concentration decline in the negative control illustrates that CoA is degraded due to experimental conditions and, therefore, a small amount of CoA in the positive experiment is degraded and not converted to AcCoA by ACS. This explains why the acetyl-CoA yield from CoA in the ACS reaction is 93.0%.

\[
\text{Acetic Acid} + \text{CoA}^{\text{ACS+ATP}} \rightarrow \text{AcCoA} 
\]  

(Eq. 6)

![Figure 22](image)

Figure 22 – The conversion of acetic acid and CoA into AcCoA by ACS in the assay containing enzyme or no enzyme. (a.) The time course of acetate conversion. (b.) The time course of CoA conversion and AcCoA formation.

5.3 Location of ACS in cells

Wet pellet and supernatant, containing crude enzymes, were separated from sonicated cell mass grown on acetate to locate the ACS activity in \textit{R. eutropha}. The assay solution consisted of phosphate buffer containing 12.5% v/v pellet or supernatant sonicate solution, 0.7 mM acetate, CoA, and ATP. A solution containing no sonicate was used as a negative control.
The sonicate solution was introduced at time zero. The acetate, CoA, and AcCoA concentrations were measured at 0 and 2 hrs. With the supernatant, the acetate was depleted immediately, illustrating high AcCoA synthetase activity. With the pellet, the acetate concentration was 0.67 mM at 0 hrs and was 0 mM at 2 hrs. At 2 hrs, the supernatant sonicate and pellet sonicate reactions contained 201 µM CoA and 237 µM AcCoA, and 209 µM CoA and 216 µM AcCoA, respectively (Table 7). The formation of acetyl-CoA in the supernatant and pellet sonicate illustrates that the acetyl-CoA synthetase activity exist in the cytosol and membranes. The AcCoA formation only accounted for 30% of the acetate converted in both the supernatant and pellet assay. Since these are crude extracts, other enzymes are converting acetyl-CoA to further downstream products, which may account for the rest of the converted acetate. To verify the AcCoA synthetase activity in the pellet wasn’t from residual supernatant, the pellet was washed a second time and used as catalyst in the assay. The supernatant after the first and second washed pellet were substituted for the double washed pellet in the experiment. The acetate was measured at 0 and 2 hrs. The double washed pellet was the only solution which converted acetate. No ACS activity was observed in the washed supernatant solutions. This fact reveals that the ACS in the membrane is tightly associated with the lipids.

Table 7 – The localization of ACS activity in *R. eutropha*.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Supernatant</th>
<th>Pellet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetate (µM)</td>
<td>0 hrs 670 ± 2</td>
<td>2 hrs 0 ± 0</td>
</tr>
<tr>
<td>CoA (µM)</td>
<td>0 hrs 430 ± 5</td>
<td>2 hrs 201 ± 7</td>
</tr>
<tr>
<td>AcCoA (µM)</td>
<td>0 ± 0</td>
<td>237 ± 10</td>
</tr>
</tbody>
</table>

5.4 Substrates of crude enzyme grown on acetate

The supernatant from acetate culture sonication was tested for the ability to also activate propionate and LA. Acetate, propionate, or LA at 1.5 mM each were used as substrate in a phosphate buffer containing 10% v/v supernatant sonicate solution, ATP, and CoA. Reactions with no enzyme were used as negative control. The concentrations of acetate, propionate, and LA were measured at 0 and 2 hrs. After 2 hrs of incubation, 73% acetate and 77% propionate were removed, while only 5% LA was removed (Table 8). The results illustrate the supernatant from acetate culture sonication can activate both acetate and propionate, but not LA. The ability of ACS to activate propionate has been shown previously in *Salmonella typhimurium* (Horswill and Escalante-Semerena 1999). The LA concentration change in the assay with enzyme was not significantly different from the assay without enzyme (Table 8). These results indicate that the
crude supernatant enzymes from the sonicated *R. eutropha* culture grown on acetate cannot activate LA.

Table 8 – The substrate specificity of crude supernatant sonicate from acetate cultures.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Crude Supernatant</th>
<th>No enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 hrs</td>
<td>2 hrs</td>
</tr>
<tr>
<td>LA (mM)</td>
<td>1.5 ± 0.0</td>
<td>1.5 ± 0.0</td>
</tr>
<tr>
<td>Acetate (mM)</td>
<td>1.3 ± 0.1</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>Propionate (mM)</td>
<td>1.3 ± 0.7</td>
<td>0.3 ± 0.1</td>
</tr>
</tbody>
</table>

The ACS activity in the crude pellet from acetate cultures was also tested for its ability to activate LA and propionate. Acetate, LA, or propionate at 1.2 mM each were used as substrate in a phosphate buffer containing 25% v/v pellet sonicate solution, ATP, and CoA. The acetate, LA, and propionate concentrations were measured at 0 and 2 hrs. After 2 hrs, 50% acetate and 38% propionate were removed, while only 1% LA was removed (Table 9). Similar to the acetate crude supernatant, the acetate crude pellet activates acetate and propionate, but not LA. Neither the crude supernatant nor pellet from sonicated acetate cultures can activate LA suggesting the LA-specific activation enzyme is induced in the cell by the presence of LA in solution.

Table 9 – The substrate specificity of crude pellet sonicate from acetate cultures.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Crude Pellet</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 hrs</td>
</tr>
<tr>
<td>LA (mM)</td>
<td>1.2 ± 0.0</td>
</tr>
<tr>
<td>Acetate (mM)</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>Propionate (mM)</td>
<td>1.2 ± 0.1</td>
</tr>
</tbody>
</table>

5.5 Location of the LA-specific activation enzyme

The acyl-CoA synthetase that is responsible for activation of LA may be a membrane-bound enzyme or a water-soluble enzyme. Cell mass was harvested from cultures on LA. Pellet sonicate or supernatant sonicate were used as crude enzyme in a phosphate buffer containing 1.5 mM LA, CoA, and ATP. A reaction containing no enzyme was utilized as negative control. LA concentrations were measured at 0 and 4 hrs. After 4 hrs, the crude pellet and crude supernatant sonicates removed 1.36 mM and 0.10 mM LA, respectively. The results demonstrate that the LA-specific activation enzyme is located at the membrane and is most likely a membrane-bound enzyme. Based on the results of the crude pellet from sonicated acetate cultures, the LA-specific activation enzyme is inducible, not constitutive.
Table 10 – The localization of the LA-specific activation enzyme.

<table>
<thead>
<tr>
<th>Crude enzyme</th>
<th>LA (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 hrs</td>
</tr>
<tr>
<td>Pellet</td>
<td>1.46 ± 0.01</td>
</tr>
<tr>
<td>Supernatant</td>
<td>1.43 ± 0.00</td>
</tr>
<tr>
<td>No enzyme</td>
<td>1.48 ± 0.01</td>
</tr>
</tbody>
</table>

5.6 LA adsorption by sonicated crude pellets

To quantify the LA conversion to AcCoA and PrpCoA, a possible LA sink should be considered. LA is more hydrophobic than shorter organic acid such as acetate, so a large portion of LA removed in the assay may be adsorbed by the crude sonication pellet. To quantify LA adsorption, assay solutions were prepared without CoA. The reaction solution consisted of 917 µM LA, 25% v/v sonicate pellet solution, and either 800 µM or no ATP in a phosphate buffer. The LA concentration was monitored over a 2 hr period. The LA concentration declined in both the ATP and no ATP assays (Figure 23). In the ATP assay, 36% of the LA was physically adsorbed at a rate of 200 µM/hr (Figure 23). The LA decline in the reaction with no ATP indicates that LA is not converted to non-CoA related products by ATP associated enzymes. Therefore, LA is most likely adsorbed in the membranes of the crude pellets.

![Figure 23 – LA absorption in the membrane of the sonicated crude pellet.](Image)

5.7 Continuous conversion of AcCoA and PrpCoA

A crude sonication solution was used as catalyst for LA bio-conversion. Other enzymes in the pellet may further convert the AcCoA and PrpCoA derived from LA. AcCoA and PrpCoA were used as substrate in a sonicated crude pellet reaction. The reaction consisted of 25% v/v
sonicate pellet solution, 188 µM AcCoA, 140 µM PrpCoA, and ATP in a phosphate buffer solution. In 1 hr, the AcCoA and PrpCoA concentrations declined by 49 µM and 60 µM, respectively at rates of 49 µM/hr and 60 µM/hr (Figure 24). These rates were used to quantify the LA conversion to AcCoA and PrpCoA. Interestingly, when AcCoA or PrpCoA were used individually as substrate with crude sonicate pellet, very little AcCoA was removed, while PrpCoA was, to a large extent, converted to CoA and AcCoA (Table 11). The overall CoA pool yields after 1 hour for the AcCoA only assay, PrpCoA only assay, and AcCoA and PrpCoA assay were 97%, 94%, and 99%, respectively. PrpCoA is converted into AcCoA, which explains why PrpCoA is converted at a higher rate than AcCoA when both intermediates are present in the assay (Figure 24, Table 11). AcCoA is significantly decreased only when PrpCoA is present, suggesting that the crude sonicated pellet contains enzymes that combine AcCoA and PrpCoA into a product but cannot convert AcCoA alone. AcCoA and PrpCoA are known to condense to form 3-ketovaleryl-CoA (Doi, Kunioka et al. 1987; Slater, Houmiel et al. 1998), but this reaction requires NADPH, which is not provided in the reaction. Also, the CoA, AcCoA, and PrpCoA combined concentrations were 328 µM at 0 hrs and 4 hrs, which leaves 100% of the CoA pool accounted for. Therefore, 3-ketovaleryl-CoA cannot be present. The AcCoA alone cannot be converted by the crude pellet, but may be converted by the crude supernatant because AcCoA is a major compound in metabolic pathways, such as the TCA cycle.

![Figure 24](image_url)  
**Figure 24** – The concentration decline of AcCoA and PrpCoA by the sonicated LA crude pellet.
Table 11 – AcCoA and PrpCoA further conversion.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>AcCoA Only</th>
<th>PrpCoA Only</th>
<th>AcCoA + PrpCoA</th>
</tr>
</thead>
<tbody>
<tr>
<td>CoA (µM)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 hr</td>
<td>0 ± 0</td>
<td>7 ± 2</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>1 hr</td>
<td>39 ± 2</td>
<td>0 ± 0</td>
<td>105 ± 2</td>
</tr>
<tr>
<td>AcCoA (µM)</td>
<td>0 ± 0</td>
<td>100 ± 1</td>
<td>87 ± 2</td>
</tr>
<tr>
<td>0 hr</td>
<td>0 ± 0</td>
<td>3 ± 1</td>
<td>117 ± 3</td>
</tr>
<tr>
<td>1 hr</td>
<td>39 ± 2</td>
<td>49 ± 1</td>
<td>146 ± 2</td>
</tr>
<tr>
<td>PrpCoA (µM)</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>0 hr</td>
<td>0 ± 0</td>
<td>49 ± 1</td>
<td>146 ± 2</td>
</tr>
<tr>
<td>1 hr</td>
<td>39 ± 2</td>
<td>49 ± 1</td>
<td>146 ± 2</td>
</tr>
</tbody>
</table>

5.8 The in vitro bio-conversion of LA into acetyl-CoA and propionyl-CoA

The conversion of LA into AcCoA and PrpCoA by the sonicate pellet was analyzed in a 3.5 hr reaction. The in vitro reaction consisted of 25% v/v sonicate pellet solution, 0.71 mM LA, CoA, and ATP in a phosphate buffer solution. The concentration of LA, CoA, AcCoA, and PrpCoA were monitored at 30 min intervals. After 2 hrs, the initial 710 µM LA was depleted from the reaction (Figure 25). In 3.5 hrs, the CoA concentration decreased by 212 µM, while AcCoA and PrpCoA increased by 93 µM and 50 µM, respectively (Figure 26). The formation of AcCoA and PrpCoA is unequal due to the conversion of PrpCoA into AcCoA, as seen in section 5.7. The LA-CoA was not detected in the assay. Two reactions must occur for LA conversion to AcCoA and PrpCoA: binding of CoA to LA and LA-CoA split into AcCoA and PrpCoA (Figure 27). Based on the results, the two reactions may be catalyzed by the same enzyme, with LA-CoA not being released until another CoA is supplied and LA-CoA is split.

The rates of LA adsorption and continuous conversion PrpCoA was used to quantify the conversion of LA to AcCoA and PrpCoA (Figure 27). The LA membrane adsorption rate was 200 µM (Figure 23). Therefore, 400 µM LA was adsorbed by the membrane in 2 hours, which accounts for 56% of the LA (Figure 27). This leaves 44% LA for conversion to AcCoA and PrpCoA. In the conversion of LA into PrpCoA and AcCoA, 1 mole LA is converted to 1 mole AcCoA and 1 mole PrpCoA (Figure 27). Therefore, the molar formation and continuous conversion of either AcCoA or PrpCoA is equal to the molar conversion of LA. Since AcCoA was formed from both LA and PrpCoA (Table 11), PrpCoA was used to quantify the mass balance of LA. The formation of 50 µM PrpCoA accounted for 7% of the total LA. The continuous conversion rate of PrpCoA from Section 5.7 was used to calculate the remaining LA mass balance. Therefore, the PrpCoA conversion rate was calculated as 60 µM/hr. Within the 3.5 hr, 210 µM PrpCoA was converted to downstream products. Therefore, 30% PrpCoA of the total LA was continuously converted (Figure 27). The combined formation and continuous conversion of PrpCoA accounted for 84% of the 310 µM LA not adsorbed by the membrane/lipids (Figure 27). The remaining 7% LA was unaccounted. The AcCoA and PrpCoA yield in the LA bioconversion
illustrates these CoA intermediates are major initial intermediates in the metabolism of LA by *R. eutropha*.

**Figure 25** – The formation of acetyl-CoA and propionyl-CoA from LA by the sonication pellet solution from LA cultured cells.

**Figure 26** – The chromatogram of CoA, AcCoA, and PrpCoA in the LA bio-conversion assay at 0 and 3.5 hrs.
Figure 27 – LA molar mass balance in the *in vitro* bio-conversion. The formation and continuous conversion is accounted for in the molar mass % PrpCoA.
CHAPTER 6. FURTHER RESEARCH

6.1 Regulation of initial fructose metabolism

Based on research of glucose and fructose multi-substrate cultures, fructose metabolism by *R. eutropha* is inhibited during harsh culture conditions, significantly more than glucose metabolism (Figure 10, Figure 12, Figure 13, Figure 14). In the Entner-Doudoroff glycolysis pathway, fructose is catabolized in just two reactions to glucose 6-phosphate, the first mutual intermediate of glucose and fructose pathways (Figure 1). At least three core enzymes are involved in the initial reactions of fructose: a transport protein, fructokinase, and glucose-6-phosphate isomerase (Figure 1). Over-expressing one of these enzymes in *R. eutropha* may reduce the inhibition on fructose metabolism and improve PHA fermentations. Other enzymes may be involved that activate or inhibit the core enzymes, isolating the enzymes associated with fructose catabolism and analyzing their interactions may prove beneficial in understanding *R. eutropha* further.

6.2 Further analysis of LA to PHBVV pathway

From this thesis, it has been determined that LA is initially converted to AcCoA and PrpCoA. AcCoA and PrpCoA are then eventually converted to 3HBCoA and 3HVCoA. Based on the system and methods utilized above, the overall LA to PHBVV pathway can possibly be further analyzed. For example, β-ketothiolase B is known to condense AcCoA and PrpCoA into 3-ketovaleryl-CoA with the energy of NADPH (Slater, Houmiel et al. 1998; Yu, Chen et al. 2009). The 3-ketovaleryl-CoA formation may be detected with introduction of NADPH to the reaction solution. These experiments may provide further insight into the overall LA to PHBVV pathway.

6.3 Isolation of LA-specific activation enzyme

LA is split into AcCoA and PrpCoA by an activation enzyme located in the membrane of *R. eutropha*. Future research involves isolating, sequencing, and modeling the LA-specific activation enzyme. With this information, the mechanism of LA conversion to AcCoA and PrpCoA, and the LA binding location can be determined. The LA-specific activation enzyme is inducible, so there must be enzymes that regulate the synthesis of this enzyme. Exploring the pathway can expand the knowledge of regulation metabolic pathways.
REFERENCES


