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PRODUCTION OF POLYHYDROXYALKANOATES BY *RALSTONIA EUTROPHA* USING CONDENSED CORN SOLUBLES

BY

PANCHALI CHAKRABORTY

A thesis submitted in partial fulfillment of the requirements for the

Masters of Science

Major in Biological Sciences

Food and Biomaterial Processing Specialization

South Dakota State University

2007

PRODUCTION OF POLYHYDROXYALKANOATES BY *RALSTONIA EUTROPHA* USING CONDENSED CORN SOLUBLES

This thesis is approved as a creditable and independent investigation by a candidate for the Masters of Science and is acceptable for meeting the thesis requirement for this degree. Acceptance of this does not imply that the conclusions reached by the candidate are necessarily the conclusion of the major department.

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ABSTRACT

PRODUCTION OF POLYHYDROXYALKANOATES BY *RALSTONIA EUTROPHA* USING CONDENSED CORN SOLUBLES

PANCHALI CHAKRABORTY

2007

Polyhydroxyalkanoates (PHA) are biodegradable polymers that have widespread applications in pharmaceutical and food industry. The high cost of PHA production and extractions has limited its applications. The objective of this study was to use condensed corn solubles (CCS), a low value byproduct of corn ethanol production, as an inexpensive medium for growth of *R. eutropha* ATCC 17699 and production of polyhydroxyalkanoates at a reduced cost. This research study was divided into three parts. The first objective was to develop the low cost CCS as a suitable medium for optimal growth of *Ralstonia eutropha*. Various concentrations of the CCS medium (80, 240, 400, and 700g/L wet basis) were tested and compared to the control media (nutrient broth and defined medium). Trials were conducted in 1 L aerated shake flasks (800 ml medium) incubated at 30 deg C, an initial pH of 7, and 250 rpm. 240g/L CCS medium was found to be the optimum medium for growth of *R. eutropha*. Both the highest cell count ($3.23 * 10^9$ cfu/ml) and growth rate ($0.29\ h^{-1}$) in 240g/L CCS medium were significantly different ($P<0.05$) from those obtained with the 80 g/L CCS medium. The organism did not grown in 400g/L and 700g/L CCS formulations. To optimize the nitrogen content in the medium, CCS medium (240g/L) was supplemented with four different levels of ammonium bicarbonate to achieve C:N ratios of 30:1, 50:1, 70:1, and

90:1. Both the highest cell number and growth rate in media with C:N ratio of 50:1, were significantly different ($P<0.05$) from the media with C:N ratio of 30:1 and 90:1 but was not significantly different to the medium with C:N ratio of 70:1. HPLC analysis of the fermentation samples showed a more rapid utilization of the carbon sources (i.e., carbohydrates, glycerol, and volatile fatty acids) in the medium with C:N ratio of 50:1 as compared to the all other nitrogen supplemented media. Thus condensed corn soluble medium containing 240g/L CCS and a C:N ratio of 50:1 was used as the growth medium for pure culture of *R. eutropha* ATCC 17699.

For the second part of the study the organism was fed additional carbon sources (volatile fatty acids) to trigger polyhydroxyalkanoate production when it had reached optimum growth. PHA accumulates in granules and serves as a carbon or energy storage material. The polymer is produced under nitrogen, oxygen or phosphorous limitation in the presence of excess of carbon sources.

Volatile fatty acids namely acetic, butyric and propionic acids were fed individually at 1g/L, 3g/L, or 5g/L at 48h whereas lactic acid was added at 2g/L, 4g/L or 8g/L. The trials were carried out in aerated shake flasks and the acids were fed after the organism has reached stationery phase of growth and nitrogen became limiting. It was found that 5g/L acetic acid, 5g/L butyric acid, 8g/L lactic acid and 5g/L propionic acid gave best results in terms of acids consumption, fermentation efficiencies, high PHA productivity, concentration and PHA content.

In the third study the objective was to determine the PHA production by *Ralstonia eutropha* when grown in the optimum CCS medium in the bench top fermentor. However

the volatile fatty acids namely acetic, butyric, lactic, and propionic acids were fed simultaneously as a mixed acid solution called Artificial Rumen Fluid (ARF) in the ratio of 10:2:15:20. The ARF was fed using three different feeding strategies. In the first treatment, ARF was fed at an interval of 24 h from 48 h to 96 h at a volume of 124 ml at each feeding time. The second treatment consisted of feeding of ARF at every 3 h interval from 48 h to 78 h, resumed at 88 h and continued to 109 h at a volume of 19.5ml at each feeding. The third treatment consisted of feeding ARF continuously at 7.75 ml/h. It was found that mixed acid feeding following the second strategy gave best results in terms of maximum cell population, overall acid consumption rate, fermentation efficiency, dry weight, PHA concentration, content and productivity. However there was no significant difference in dry weight, PHA productivity and content using three different feeding strategies. When shake flask and fermenter trials were compared there was an increase in maximum cell population, growth rate, and PHA production.

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LIST OF ABBREVIATIONS

AA	: Acetic Acid
ADM	: Archer Daniel's Midland Company
ADP	: Adenosine Diphosphate
ARF	: Artificial Rumen Fluid
ATCC	: American Type Culture Collection
ATP	: Adenosine Triphophate
BA	: Butyric Acid
C	: Centigrade
C/N / C:N	: Carbon: Nitrogen Ratio
CCS	: Condensed Corn Solubles
CFU	: Colony Forming units
CoA	: Coenzyme A
DCW	: Dry Cell Weight
Deg	: Degree
DSC	: Differential Scanning Calorimetry
FE	: Fermentation Efficiency
g	: Multiplication Factor Corresponding to Earth's Gravitational Field
GC	: Gas Chromatography
GC-FID	: Gas Chromatography with Flame Ionization Detector
GC-MS	: Gas Chromatography with Mass Spectroscopy
GPC	: Gel permeation Chromatography

GRAS	: Generally Regarded As Safe
HB	: Hydroxybutyrate
HHx	: Hydroxyhexanoate
HO	: Hydroxyoctanoate
HPLC	: High performance Liquid chromatography
HV	: Hydroxyvalerate
LA	: Lactic Acid
MCG	: Mitsubishi Gas Chemicals
Mcl	: Medium Chain Length
NADPH	: Nicotinamide Dinucleotide Phosphate
NCIMB	: National collection of Industrial, Food and Marine bacteria
NCPM	: Non- cellular Polymer Material
NMR	: Nuclear Magnetic Resonance
P(HB)	: Polyhydroxybutyrate
P(HV)	: Polyhydroxyvalerate
P(HB-CO-HV)/P (HB-HV)	: Copolymer of Polyhydroxybutyrate and Polyhydroxyvalerate
PA	: Propionic Acid
PHA	: Polyhydroxyalkanoate
RPM	: Revolutions Per Minute
SA	: Succinic Acid
Scl	: Short Chain Length

SDS	: Sodium Dodecyl Sulphate
SFE	: Supercritical Fluid Extraction
TCA	: Tricarboxylic Acid Cycle
TSA	: Tryptic Soy Agar
UV	: Ultra Violet
VA	: Valeric Acid
VFA	: Volatile Fatty Acid
VVM	: Volume/Volume/Minute

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Chapter 1

Introduction and Background

1.1 Introduction

Synthetic polymers became popular in the 1940's, and have been used in a wide range of products since then (Lee 1996b). They have favorable mechanical and thermal properties, and are also very stable and durable (Poirer et al. 1995). A major use of synthetic polymers is in packaging materials; however this results in major waste disposal problems (Morikawa et al. 1981). These synthetic polymers can also have harmful effects on wildlife and degrade the aesthetics of cities and forests (Nishida et al. 1992).

As a result of the disadvantages of synthetic polymers, there has been a growing public and scientific interest regarding the development and use of biopolymers. Biodegradable polymers are made from renewable resources such as agricultural wastes, corn, cassava, tapioca, whey, etc., and thus do not lead to depletion of finite resources. The most studied of the biodegradable polymers include polyesters, polylactides, aliphatic polyesters, polysaccharides and various copolymers (Byrom 1992). These biopolymers have many of the desirable physical and chemical properties of conventional synthetic polymers (Amass et al. 1998). To this point high production costs have limited the use of biopolymers, however if these costs can be reduced, there would be widespread economic interest (Nishida and Tokiwa 1992).

The focus of this project is production of the biopolymer polyhydroxyalkanoate (PHA). PHA is actually a term used to describe a diverse family of polymers that are composed of 3-hydroxy fatty acid monomers. The carboxyl group of one monomer forms

an ester bond with hydroxyl group of the neighboring monomer. The PHAs generally are in the form of (R)-β-hydroxyl fatty acids, where the pendant group (R) varies from methyl (C1) to tridecyl (C13). Polyhydroxybutyrate (PHB) is the most widely studied member of the PHA family. The structure of PHB and polyhydroxyvalerate (PHV) differ in the composition of their R group. The R group in PHB is methyl while it is ethyl in PHV. The side chains of PHA can be modified by cross linking of unsaturated bonds. This results in difference in the length and composition of side chains, results in diversity of PHA polymer family (Gorenflo et al. 2001, Madison and Huisman 1999, Nishida and Tokiwa 1992).

PHA was first discovered in *Bacillus megaterium* (Lemoigne 1926), where it accumulates in granules and serves as a carbon or energy storage material (Choi and Yoon 1994). The polymer is produced under nitrogen, oxygen or phosphorous limitation in the presence of excess of carbon sources (Du et al. 2001, Du and Yu 2002). Bacteria mainly produce short chain length and medium chain length PHAs (Anderson and Dawes 1990) and the polymer can be separated from the cells by aqueous or solvent extraction. (Lee 1996a).

Microorganisms can subsequently colonize the surface of the biopolymers, secrete enzymes and degrade them into corresponding monomers, which are then used for growth (Lee 1996a). The end products of aerobic PHA degradation are carbon dioxide and water, compared to methane following anaerobic degradation (Doi at al. 1987).

1.2 Review of Literature

1.2.1 Nomenclature and Morphology

Ralstonia is named after E. Ralston, an American bacteriologist who first described *Pseudomonas pickettii* and suggested a taxonomic relationship to *Pseudomonas. solanacearum* based on DNA homology. Li et al. (1993) proposed the existence of two related genera within this homology group II of pseudomonads, and the generic name *Burkholderia* was proposed (Yabuuchi at al. 1995). There were phenotypical and phylogenetic similarity among *Burkholderia pickettii, Burkholderia solanacearum* and *Ralstonia eutropha*, yet three *Ralstonia* species were not able to assimilate galactose, mannitol, mannose and sorbitol whereas eleven species of *Burkholderia* could. Analysis of 16S rRNA also supported two genera, viz *Burkholderia* and *Ralstonia*. The *Ralsonia* branch is comprised of *R. picketti, R. solanacearum and R. eutropha* (Yabuuchi at al. 1995). The G+C ratio of the three species are 64, 66.6, and 66.5 respectively.

R. eutropha is a gram negative organism, and was formerly known as *Alcaligenes eutrophus*. They are straight rods with peritrichous flagella and well rounded ends. They occur singly, in pairs, and often in chains of 5 to10 cells (Yabuuchi at al. 1995). Figure 1 shows an electron micrograph of 48 h old *R. eutropha* cells containing PHA granules.

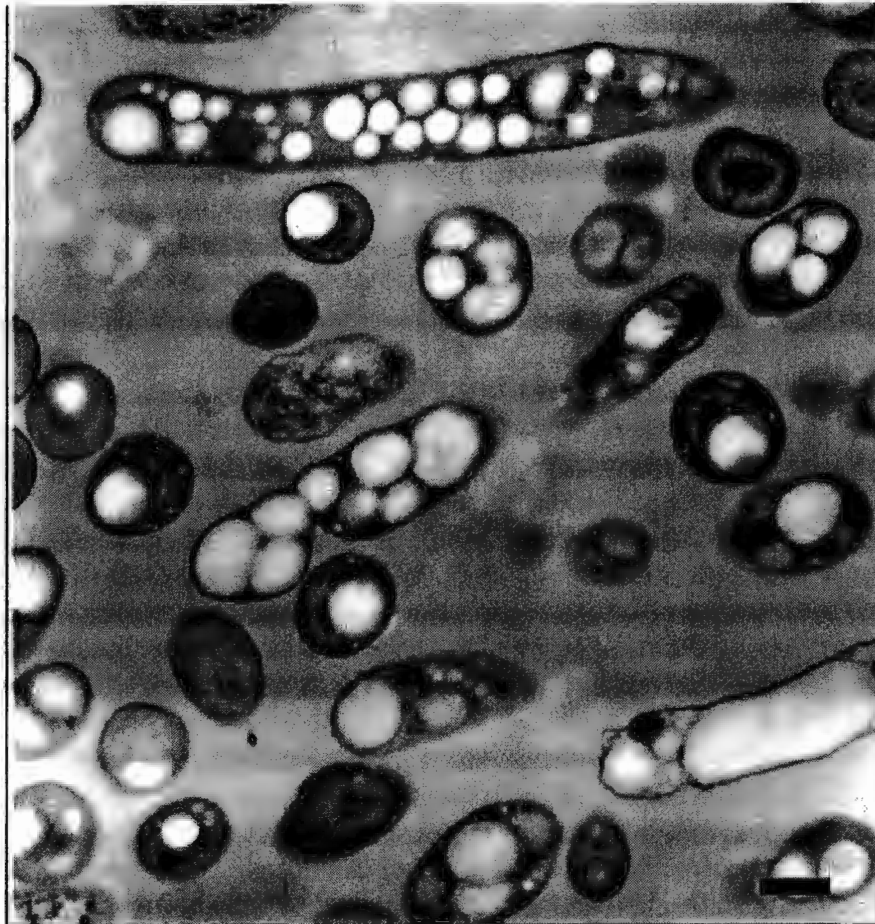

Figure 1.1 Morphology of PHA laden cells of *R. eutropha* (Tian et al. 2005).

1.2.2 Growth Requirements

The optimal temperature for both growth and PHA production of *R. eutropha* is 30 deg C. (Byrom 1994, Shioya 1992, Shimazu et al. 1993). Optimal pH is 7.0 for efficient cell growth; however the optimal pH for PHA production is less well defined. Some studies have maintained a pH of 7.0 throughout fermentation (Choi and Lee 2000, Guocheng at al. 2001, Lee 1996b), while others consider pH 8.0 as best for PHA production (Shioya 1992, Shimazu et al. 1993, Jedlinski et al. 1999). In these studies the media pH at inoculation was normally maintained around 7.0, but rose to about 8.5 by the

end of fermentation (Wang and Yu 2001, Yu et al. 2002). The increase in pH was mainly caused by consumption of the volatile acids present in the media (Yu et al. 2002).

In shake flasks, agitation rate was usually maintained at 150-250 rpm. In fermentors, agitation rates are normally set at 950-1,000 rpm to prevent oxygen limitation (Shimizu et al. 1999), which would be detrimental to growth. In addition, aeration rates of 1 v/v/min were used to maintain dissolved O_2 at 20 % of saturation (Shang et al. 2003a). Under oxygen limitation, pyruvate was excreted and less PHB was accumulated when compared to nitrogen limitations (Scandola et al. 1988). The use of pure oxygen (instead of air) resulted in higher cell densities and greater PHB production in fed batch fermenters (Shang et al. 2003b). However, this resulted in a greater accumulation of carbon dioxide, and levels in outflow gas. CO_2 is inhibitory to both cell growth and PHB production, even though *R eutropha* is an autotrophic microbe that can use CO_2 as a carbon source.

To study the inhibitory effect of CO_2, two different methods were used. McIntye and McNeil (1997) used an external pulse injection of CO_2 and noted inhibition of both cell growth as well as PHB synthesis. The level of inhibition was directly related to CO_2 concentration and pulse duration. However, the sudden increase in CO_2 concentration did not represent a true fermentation process; therefore they evaluated the autogenous method of CO_2 feeding. In this method, microbes were grown under a higher CO_2 concentration just by lowering the gas flow rate. There was no artificial feeding of CO_2 in the fermenter. Inhibition of cell growth rate and PHB concentration was observed, but was less than pulse injection method (Shang et al. 2003b).

1.2.3 Nutritional Requirements

R. eutropha (wild-type strain H16) grows on a variety of carbon sources, including several sugars, but it cannot metabolize glucose (Madison and Huisman 1999). The organism is also capable of using organic acids such as acetate, propionate, lactate, butyrate, valerate at low concentrations (Kunioka et al. 1989c). Nitrogen is an important nutrient in the growth phase for anabolic reactions, but nitrogen deficiency is typically used to trigger PHA production when excess carbon is present (Kim et al. 1994). It is also important to maintain phosphorous and magnesium levels at 0.35 g/L and 10 mg/L, respectively (Asenjo et al. 1995). This broadens the molecular weight distribution of PHB and also lowers the molecular weight of PHB. Oxygen is essential for growth, but low dissolved oxygen triggers PHA accumulation. The concentration of all nutrients should be maintained at the optimal level so as to avoid change in the metabolism of the organism due to over- or under-feeding of any nutrient (Shang et al. 2003a).

1.2.3.1 Carbon

R. eutropha can assimilate and oxidize a range of monosaccharides, disaccharides and polyols as sole sources of carbon and energy (Smith 1914). However, the wild-type strain cannot metabolize glucose, galactose, mannose, mannitol, or sorbitol (Byrom 1987). A glucose utilizing mutant NCIMB 11599 has been developed. *R. eutropha* does not produce any acid by the fermentation of carbohydrates (Madison and Huisman 1999). *R. eutropha* can utilize a range of organic acids for PHA synthesis (Yu et al. 2002). Additional details are provided in the following sub-section. *R. eutropha* can use

previously stored intracellular PHB for growth in the absence of any other carbon source (H16). The organism can also grow chemolithotrophically in CO_2, H_2 and air mixtures.

The range of metabolizable substrates can be increased by recombinant DNA technology. For example, substrate utilization genes can be inserted in PHA producers, or PHA biosynthesis genes can be inserted in organisms that have wide range of utilizable substrates. Molecular engineering can also be used to improve metabolic pathways so as to increase PHA synthesis and/or production of new types of PHAs. Since the carbon source is a major contributor to PHA cost, inexpensive sources of carbon are important. However, the yield of PHA per gram of carbon source provided should also be high (Lee 1996b).

Carbon source limitation can also trigger rapid accumulation of PHB in cells of *R. eutropha* (NCIMB 11599) as the TCA cycle slows, and levels of free CoASH decrease so ketoacyl thiolase is not inhibited (Shang et al. 2003a, Guocheng et al. 2001). Shang et al. (2003a) discovered that β-ketoacyl-coA thiolase condensed the accumulating acetyl-coA into acetoacyl-coA. This was then reduced to (R)-3-hydroxybutyryl CoA by ketoacyl-CoA reductase, which was then polymerized to poly (3-hydroxybutyrate) (PHB). As the concentration of carbon increases, there is an increase in cell growth. However if other nutrients become limiting, growth slows down and carbon is diverted to PHB production (Shang et al. 2003a).

1.2.3.1.1 Volatile Fatty Acids

R. eutropha can metabolize several fatty acids, but these can be toxic, depending on concentration and pH. Toxicity is due to the presence of undissociated lipophilic molecules that penetrate the cell membrane and acidify cell cytoplasm (Salmond et al. 1984). This results in loss of proton gradient across membrane, increase in cell osmotic pressure, and decline in acid utilization rate, growth rate and yield (Lawford and Rousseau 1993, Axe and Bailey 1995).

When grown on a mixture of acetate, lactate, and butyrate *R. eutropha* uses lactate first, because a large amount of ATP is required for transportation of acetate and butyrate. However, the highest yield of P (3-hydroxybutyrate) (PHB) is obtained from butyrate. This finding has been confirmed through the metabolic flux analyses developed by Shi et al. (1997). Flux ratios are used to compare the effects of carbon sources on PHB synthesis.

The glyoxalate pathway is required for growth of *R. eutropha* on butyrate, and it competes for butyrate with the PHB biosynthetic pathway. Shi et al. (1997) found that when the nitrogen source was depleted, *R. eutropha* shifts from growth to PHB production. The lack of nitrogen blocks amino acid synthesis pathways and NADH consumption rate decreases. However, there is no significant difference in flux of butyrate into the TCA cycle, where isocitrate dehydrogenase generates NADH. *R. eutropha* can direct 67 % of butyrate into the TCA cycle, whereas only 33 % of either acetate or lactate can enter. The accumulating NADH is redirected to PHB biosynthesis

(Huidong et al. 1997), where it is used to convert acetoacetyl coA to (R)-3-hydroxybutyryl-coA. These results demonstrate that butyrate is more energetically efficient than acetate and lactate for PHB production. The results also indicate that the maximum PHB yield may be limited by the available NADH.

Propionate utilization is important for production of copolymers containing hydroxyvalerate (PHV) units. However, acetic acid is also required since valerate is a (5-carbon atom) C5 monomer that results from condensation of acetic and propionic acids. When propionic acid was used alone, it was diverted to growth, meaning that less propionate would be available during PHA synthesis to incorporate HV units into the copolymer (Yu et al. 2002). When mixtures of acetic and butyric versus acetic and propionic acids were compared, consumption rates of butyric and propionic acids were similar, but PHA productivity was higher in the acetate and butyrate mixture (Yu et al. 2002).

When valeric acid was fed alone, a copolymer of P(HB-HV) was formed (Madison and Huisman 1999). However, since valeric acid has toxic effects on cell growth, polymer productivity was low. Therefore, Shang et al. (2004) developed an alternative feeding strategy that involved providing glucose as one carbon source to support growth and synthesis of the homopolymer PHB. Then valerate was added to synthesize a copolymer of P (3hydroxybutyrate-co-3-hydroxyvalerate) and increase the fraction of hydroxyvalerate in the copolymer. The authors concluded that synthesis of the copolymer P(HB-HV) and degradation of the homopolymer PHB occurred

simultaneously in *R. eutropha* when valerate was provided as the only carbon source during the final stages of fermentation. (Shang et al. 2004).

Since 4-hydroxy valeric acid is not commercially available, levulinic acid or 4-ketovaleric acid can be used to produce copolymers with HV units (Gorenflo et al. 2001). A combination of octanoic acid and levulinic acid can also be used as a carbon source to synthesize copolymer containing 3-hydroxybutyric acid (3HB), 3-hydroxyvaleric acid (3HV), and traces of 3-hydroxyhexanoic acid (3HHx) and 3-hydroxyoctanoic acid (3HO). However, octanoic acid is toxic to *R. eutropha* when used in higher concentrations, so it is usually added as the sodium salt in low levels (Gorenflo et al. 2001). Since fatty acids such as propionic and valeric acids are toxic at higher concentrations, amino acids (threonine, valine, isoleucine) were used to produce copolymers of 3-hydroxybutyrate and 3-hydroxyvalerate under nitrogen limiting conditions. This is because these amino acids are degraded through propionyl – CoA intermediate (Madison and Huisman 1999, Steinbuchel and Pieper 1992).

The costs of volatile fatty acids (VFAs) are high, compared to sugars. However, the use of an artificial rumen to generate VFAs may be cost effective. The second part of this project was to explore the conversion of whole stillage (a byproduct of the ethanol industry) to VFAs by use of an artificial rumen. The end products of the artificial rumen were then was fed to *R. eutropha* in a second reactor for production of PHA.

1.2.3.2 Nitrogen

Nitrogen is important for both cell growth and production of PHB (Handrick et al. 2000). Low nitrogen levels in the growth stage result in relatively low cell dry mass, as

well as a reduction in PHB synthetic activity. However, the PHB content of the cells are comparatively high. When nitrogen is in abundance, cell growth increases and most of the NADH generated is used for amino acid biosynthesis, resulting in low PHB content.

Following the growth stage, when nitrogen is limiting, NADH is used as a coenzyme of acetoacetyl coenzyme A reductase to convert acetoacetyl CoA to (R)-3-hydroxybutyryl CoA (a precursor of PHB). High yields of P (3HB) (121 g/L) and productivity (2.42 g/L/h) were obtained from a 70 g/L cell mass of *R. eutropha* when nitrogen was limited (Kim et al. 1994).

Haywood et al. (1989) found that in a nitrogen-free environment, and in the presence of alternative carbon source such as valeric acid, PHB was degraded and new polymer, composed of 3HV units, was synthesized. Similar effects were seen with butyric acid as well. However, no such phenomenon occurred in a steady state nitrogen limiting condition.

R. eutropha is capable of utilizing nitrogen from a variety of ammonium compounds such as ammonium sulphate, ammonium chloride, ammonium carbonate and bicarbonate and also different from complex nitrogen sources such as corn steep liquor, green grass juice, silage juice, yeast extract or fish peptone (Lee 1996b, Loo et al. 2005, Madison and Huisman 1999, Marangoni et al. 2001, Shimuzu et al. 1999, Wang and Yu 2001). Nitrogen and phosphate limitation are most commonly used to trigger polymer production by *R. eutropha*. During nitrogen limitation, NaOH is typically used to control pH (Ryu et al. 1997).

The mole ratio of carbon to nitrogen in the medium is also important for polymer production. Low (<20:1) C:N ratios stimulate biomass production and limit PHB productivity and yield (Shimuzu et al. 1999). This occurs because carbon sources are directed more towards energy generation and anabolic reactions. At high C:N ratio (20-200:1), growth rates were lower, but there was an increase in both PHB productivity and yield. The maximum PHA productivity was found at the C:N ratio of 75:1 (Shimuzu et al. 1999).

C:N ratios also affect the molecular size of PHA. Wang and Yu (2001) found that in general, the molecular weight is larger when cells are grown under low (4:1) C:N ratio. At high (72:1) C:N ratios, the size of polymer size was reduced by 58 %, but the amount of polymer per unit biomass increased by 140 %. They postulated that nitrogen limited conditions (high C:N ratio) create more polymer synthesis centers, resulting in the pool of monomers being distributed to a larger number of elongating polymers of smaller size (Wang and Yu 2001). However, even during PHA production stage, nitrogen concentration in the media must be within a certain critical range, since total elimination of nitrogen might suppress metabolic activity (Suzuki et al. 1988).

1.2.3.3 Phosphorous

The most commonly used phosphorus sources in laboratory media include Na_2HPO_4, K_2HPO_4 and KH_2PO_4, however complex phosphate sources such as green grass juice and silage have been used (Loo et al. 2005, Madison and Huisman 1999,Ryu et al. 1999, Shimuzu et al. 1999, Wang and Yu 2001). Initially a certain amount of

phosphate is fed to the fermenter to stimulate desired cell growth. After a certain length of time, phosphate becomes limiting and PHA accumulation begins (Ryu et al. 1999). When phosphate limitation is used to induce polymer production, additional cell growth can take place, since phosphate does not participate directly in protein synthesis. In addition, this element helps in the recycling of ATP/ADP, which leads to an increase in polymer production. Also under phosphate limitation it is not necessary to substitute NH_4OH for NaOH for pH control (Ryu et al. 1997). NaOH is a toxic compound that in high concentration might result in cell lysis, which in turn might result in low cell growth and PHB production (Ryu et al. 1999).

Phosphate feeding during the polymer production phase has resulted in high polymer accumulation by *R. eutropha* (Ryu et al. 1997). At the end of 27 h, polymer accumulation was 63 % of the total cell mass (Squio et al. 2004). Maximum yield of P(3HB) was obtained when pH was controlled using ammonium hydroxide and phosphorous was made the limiting factor instead of nitrogen (Squio et al. 2004). Using an initial phosphate concentration of 5.5 g/L, the fed-batch fermentation resulted in a final cell concentration of 281 g/L, a PHB concentration of 232 g/L, and a PHB productivity of 3.14 g/L/h, which are the highest values ever reported to date (Ryu et al. 1997). Monsanto (USA) has reported commercial production of P(3HB-co3HV) in a fed batch culture of *R. eutropha* under phosphate limitation (Byrom 1987). The report also suggests that oxygen, sulphur, magnesium and potassium could be used as limiting nutrients (Lee 1996b, Byrom 1987, Kim 1996).

1.2.4 Media

1.2.4.1 Laboratory Media

Shimuzu et al. (1999) grew *R. eutropha* in a medium containing 10 g/L yeast extract, 10 g/L polypeptone, 5 g/L meat extract, and 5 g/L ammonium sulphate. The medium was used to determine the effect of butyric and valeric acid on PHA production and to evaluate the effects of different C:N ratios. The C:N ratio was maintained at constant value by adjusting the levels of fatty acids and ammonium sulphate (Shimuzu et al. 1999). Du et al. (2001) utilized a similar medium that contained 10 g/L yeast extract, 10 g/L peptone, 10 g/L yeast extract, and 5g/L ammonium sulfate for cultivation of *R. eutropha.*

In a larger two-stage continuous system, the growth medium consisted of 10 g/L glucose and 2.5 g/L ammonium sulfate, along with 1 ml of a mineral solution. This mineral solution contained per liter: 0.2 mg $MgSO_4$, 10 mg $CaCl_2.2H_2O$, 6 mg ferrous ammonium citrate, 9.92 g $Na_2HPO_4.7H_2O$, and 0.83 g of KH_2PO_4. In the first stage, a 50 g/L glucose solution was fed at 0.14 h^{-1} to support cell growth, while a 500 g/L glucose solution was fed at 0.075 h^{-1} in stage two to support PHB production (Guocheng et al. 2001).

A medium developed by Wang and Yu (2001) was composed of 5 g/L yeast extract, 5 g/L peptone, 2.3 g/L beef extract, and 2.3 g/L ammonium sulfate. After inoculation, the culture was agitated at 180 rpm at 30 deg C for 24 h (Guocheng et al. 2001). The cell mass was harvested by centrifugation and suspended in a mineral medium consisting of: 3.8 g/L of Na_2HPO_4, 2.65 g/L of K_2HPO_4, 0.4 g/L $MgSO_4.7H_2O$ and 1 ml

trace element solution. Magnesium and phosphate source were used to broaden the molecular weight distribution and lower the molecular weight of polymers (Asenjo et al. 1995). The trace element solution consisted of 200 mg/L $FeNH_4SO_4$, 6 $ZnSO_4 . 6H_2O$, 6 $MnCl_2 . 4H_2O$, 2 $CuSO_4.5H_2O$, 2 $NaB_4O_2 . 10H_2O$, and 2 $NaMoO_4.2H_2O$ (Wang and Yu 2001). This mineral medium mainly helps in synthesis of different metalloenzymes (cofactor include molybdenum, a [NiFe] site, copper centers or iron-sulfur clusters) such as membrane bound hydrogenase, periplasmic nitrate reductase, nitrite oxide reductase by the organism. All these three enzymes are encoded in *R. eutropha* H16 genes (Bernhard et al. 2000). The same medium was also used by Yu et al. (2002) to determine the inhibition and utilization kinetics of acetic butyric and propionic acids when they were added as mixed acids (Yoon et al. 1995).

Jendrossek (2005) found that *R. eutropha* can be grown in Lauria-Bertini medium, nutrient broth or tryptic soy broth (Jendrossek 2005).

1.2.4.2 Current Industrial Media

A glucose salts medium has been widely for PHA production (Byrom 1987, Du et al. 2001, Madison and Huisman 1999), but the medium cost was very high. Therefore, a variety of lower costs carbon substrates have been evaluated for PHA production (Lee 1996b). Soy wastes from soy milk, dairy byproducts, and malt wastes from beer brewery plants have been used as carbon sources (Yu et al. 1999). Soybean oil is yet another carbon source. On soybean oil, the wild type strain of *R. eutropha* produced dry weights as high as 118-126 g/L and a PHB content of dry cell at as high as 72-76 % (Kahar et al. 2004). *R. eutropha* is reported to produce PHA from plant oils or tapioca hydrolysate,

waste plant oils and waste tallow (Lee 1996b). Waste plant oils usually yielded PHB but a copolymer of P(HB-HV) was produced from tallow. Up to 80 % accumulation of PHA was found in the cells (Taniguchi et al. 2003). *R. eutropha* was grown in palm kernel oil (5g/L) by Loo et al. (2005) for synthesis of P (3-hydroxybutyrate-3-hydroxyhexanoate). The accumulation of PHA was 87% of dry weight (Loo et al. 2005).

Koller et al. (2005) grew *R. eutropha* on complex corn steep liquor and casamino acids. Complex nitrogen and phosphorous sources such as green grass juice and silage juice were supplemented. The biomass concentration obtained was between 7.0 and 7.44 g/L and PHA productivity was about 0.60-0.65 g/L/h (Koller et al. 2005). PHA has also been produced from food scraps using *R. eutropha*. The food scraps were digested in an anaerobic reactor producing acetic, lactic, propionic and butyric acid. The acids were transferred to the culture. A copolymer of P (3hydroxybutyrate-3hydroxyvalerate) was produced. The final dry cell weight obtained was 22.7 g/L and the PHA per cell dry weight was 72.6 % (Du and Yu 2002).

Marangoni et al. (2001) showed that glucose utilizing mutant of *R. eutropha* can grow in hydrolyzed lactose and can utilize galactose. The biomass obtained was 1.2 g/L and the yield of PHA was 0.5 g/g of carbon source when grown in hydrolyzed lactose. This also suggested that the organism was capable of growing in whey (Marangoni et al. 2001). In this study corn steep liquor was used as a nitrogen source. When *Escherichia coli* harboring genes of *R. eutropha* was grown in whey, final cell and PHB concentrations of 119.5 and 96.2 g/L, respectively, were obtained in 37.5 h, which resulted in PHB productivity of 2.57 g/L/h (Woo et al. 2000).

Tobella et al. (2005) reported that PHA can be synthesized by growing *Wautersia* sp. in cellulose paper pulp mill effluents containing 2.4.6-trichlorophenol. The organism was able to grow in toxic compounds and convert them to biodegradable polymers, which provided an alternative to toxic compound removal (Tobella et al. 2005).

1.2.4.3 Proposed Alternative Industrial Medium

The research described in this thesis is part of a larger effort focused on developing a dual substrate, dual fermentation process to produce PHA. In the first bioreactor *R. eutropha* will be rapidly grown to a high cell density on a low cost medium. In the second bioreactor a mixed culture of rumen microbes will metabolize biomass into volatile fatty acids (VFAs). Those VFAs will subsequently be fed to the *R. eutropha* cell mass. The focus of this thesis is developing a process to rapidly grow *R. eutropha* cells, then determining the rate at which VFAs can be fed to produce PHA.

The substrate to be evaluated in this study for the growth phase of *R. eutropha* is condensed corn solubles (CCS), which is a low-value byproduct of the dry-mill, corn ethanol industry. In the dry mill process, the whole corn is milled, mixed with water, and enzymatically hydrolyzed to convert starch to glucose, which is converted to ethanol by fermentation. After distillation to remove ethanol, the larger corn particles are recovered by centrifugation as distiller's wet grains. The supernatant is condensed in multiple-effect evaporators to give condensed corn solubles (Jerke 2001).

The moisture content of CCS ranges from 45-75 %, and on a dry matter basis it consists of approximately 29 % protein, 9 % fat and 4 % fiber. The high fat level may inhibit the growth of some microorganisms, whereas higher level of protein can enhance

growth. Corn-milling byproducts are typically marketed as animal feed because of their high protein content. However these byproducts may also contain residual carbohydrates, which might be utilized by microbial fermentation to produce industrial biopolymers. The solubles are an excellent source of vitamins and minerals, including phosphorous and potassium (Anonymous 2001).

1.2.5 Metabolism

1.2.5.1 Synthetic Pathway

Production of PHA involves three enzyme-catalyzed steps (Figure 1.2). In the first step, β-ketoaceyl CoA thiolase condenses two acetyl CoA molecules into acetoacetyl –CoA. This molecule is then reduced to R-3-hydroxybutyryl CoA by acetoacetyl CoA dehydrogenase / reductase. In the final step, R-3-hydroxybutyryl CoA monomers are polymerized to P(3-hydroxybutyrate) molecules by the action of P(3HB) polymerase / synthase (Madison and Huisman 1999).

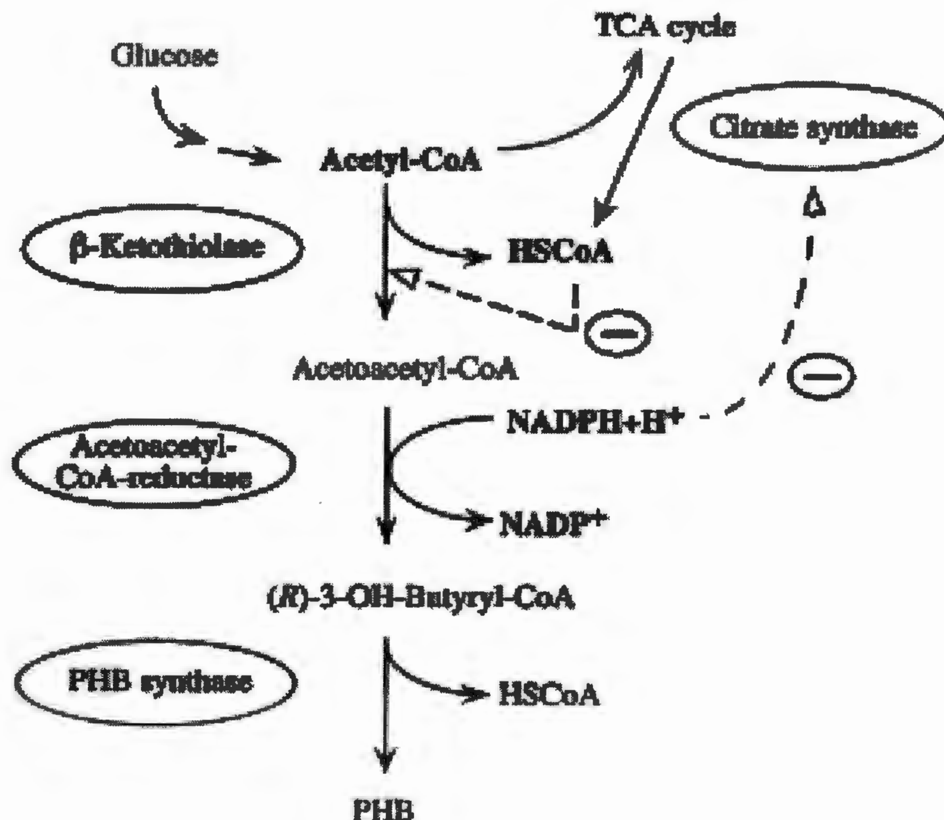

Figure 1.2 Biosynthetic pathway of PHB for *R. eutropha* (Kessler and Witholt 2001).

1.2.5.2 Initiation of PHA Formation

There are two theories to describe the initiation of PHA formation in the cell. According to the micelle formation model, PHA synthase enzymes are distributed randomly in the cytoplasm, and as PHA is formed it aggregates into water insoluble micelles. In the budding model, PHA synthase is bound to the cytoplasmic membrane, and the PHA polymer grows between the phospholipid bilayers. The completed PHA granule is then released in the cytoplasm (Stubbe and Tian 2003). The study of early formation of granules by fluorescence microscopy complies with the budding model of PHA formation (Jendrossek 2005).

The principle of PHA production is to grow the desired organism to a high cell density in a balanced medium, then limit one of the essential nutrients to trigger PHA synthesis. One of the critical considerations is the concentration of this limiting nutrient, since each strain of bacteria has its own optimum triggering level (Lee 1996b). If nutrient limitation occurs prematurely, the final cell count will be low and PHA productivity will be reduced, even though the cells will have a high PHA content. On the other hand, if nutrient limitation is delayed the cell population will be maximized, but the cells will have a lower polymer content and low PHA productivity (Lee 1996b).

R. eutropha does not produce PHA until the stationary phase (Hanggi 1990, Hrabak 1992). PHAs are accumulated as granules inside the cells, with about 8-13 granules per cell and diameters ranging from 0.2-0.5 um (Byrom 1994). PHA accumulating microorganisms can be easily identified by staining with Sudan black or Nile blue (Ostle and Holt 1982).

1.2.5.3 Products

Polyhydroxyalkanoate (PHA) is general term used to describe a family of polymers. The polymers are composed of 3-hydroxy fatty acid monomers. The carboxyl group of one monomer forms an ester bond with hydroxyl group of the neighboring monomer. The PHAs generally are in the form of (R)- β- hydroxyl fatty acids, where the (R) group varies in composition. The polymers with hydroxyl group incorporated in γ, δ and ε positions are also found. Differences in the length and composition of side chains results in diversity of PHA polymer family (Gagnon et al. 1994, Madison and Huisman 1999, Ojumu et al. 2004).

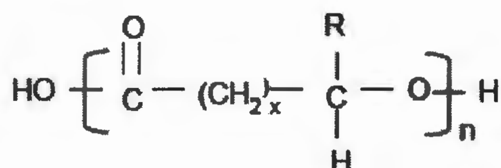

Figure 1.3 General Structure of Polyhydroxyalkanoates (Lee 1996b).

Table 1.1 Structural Formulae of Some Representative Members of Polyhydroxyalkanoate family.

x	R	Products	Abbreviations
1	hydrogen	poly (-3-hydroxypropionate)	P(3HP)
	methyl	poly (-3-hydroxybutyrate)	P(3HB)
	ethyl	poly(3-horoxyvalerate)	P(3HV)
	propyl	poly (-3-hydroxyhexanoate)	P(3HHx)
	pentyl	poly (-3-hydroxyoctanoate)	P(3HO)
	nonyl	poly (-3-hydroxydodecanoate)	P(3HDo)
2	hydrogen	poly (-4-hydroxybutyrate)	P(4HB)
	methyl	poly (-4-hydroxyvalerate)	P(4HV)
3	hydrogen	poly (-5-hydroxybutyrate)	P(5HB)
	methyl	poly (-5-hydroxyvalerate)	P(5HV)

P (3HB) is produced when the bacteria are fed on one carbon substrate such as glucose, butyrate, acetate, lactate etc. The co-feeding of substrates such as propionate and valerate with other carbon sources such as glucose, fructose, acetate, butyrate etc. resulted in formation of copolymers containing HV monomer units (Madison and Huisman 1999, Shimuzu et al. 1999). The use of valerate alone as the substrate produced copolymer of HB and HV units (Shimuzu et al. 1999). But valerate can yield even better ratio of HV when glucose or mixed carbon source are used as growth substrates (Anderson and Dawes 1990). The organism can form copolymer of P (3HB-3HV) when the acetate and propionate are both present in the growth medium (Doi et al. 1987). HV content of the copolymer is dependent on the ratio of propionic acid fed to the organism.

HV units are also generated though at a lesser concentration when propionic acid is used alone (Shimuzu et al. 1999). However when propionate and valerate are used at high concentration, they can be toxic to the organism.

R. eutropha is also capable of incorporating 3HV when supplemented with different amino acids such as valine, isoleucine, leucine and threonine. However the amount of incorporation and toxicity depends on the type and concentration of the respective amino acids (Steinbuchel and Pieper 1992, Yoon et al. 1995). *R. eutropha* can also accumulate copolymer with up to 7 % HV on fructose, succinate, acetate, and lactate. Co-polyesters of 3HB and 4HB were formed when mixture of butyrate and 4HB or 4chlorobutyrate, 1,4-butanediol or γ-butyrolactone were fed to *R. eutropha* H16 (Kunioka et al. 1989b). Recombinant strain of *R. eutropha* produced P(3-HB-co-3-HHx) from hexanoate and octanoate (Kichise et al. 1999). Terpolymer of P(3 HB-4HB-3HV) was accumulated when butyrate, valerate and 4HB were used as substrates (Kunioka 1989c). Terpolymer of 3HB, 3HV and 5HV was formed when mixture of 5-chlorovalerate and valerate were used as carbon sources (Doi et al. 1988a).

1.2.6 Lab Scale PHA Production

1.2.6.1 Batch Culture

The cell culture of *R. eutropha* was grown in the laboratory media (1.2.4.1) in shake flasks at 30 deg C and agitated at 180 rpm (Wang and Yu 2001). Sodium acetate and NH_4Cl were then added to study the effect of nutrient nitrogen on acetate utilization, cell growth, and PHB formation and also to study the effect of acetate concentration. The results showed that that the acetate concentration (3 g/L and 6 g/L) in the media did not

affect the acetate utilization rates. But the C:N ratio affect the acetate utilization rate. At a C:N ratio of 76:1, acetate was only used for PHB production, cell activity maintenance, and energy for polymer synthesis. But under C:N ratio of 30:1, acetate was also used for biomass production initially and thus the rate of utilization of acetate was higher (Wang and Yu 2001).

Batch cultures of *R. eutropha* was used to study the HV content in the copolymer of P(HB-HV) by feeding mixed acids (acetic, propionic, and butyric) (Yu et al. 2002). Kunioka's medium was used for the growth of the organism in 500 ml flasks. C:N ratio was fixed at 80:1. When propionic and acetic acids were used, copolymer was produced, but when butyric and acetic acid were used only homopolymer (PHB) was produced. When propionate was used alone, the copolymer consisted of only 40 % HV but when used as mixed acids, 80 % HV units were formed (Yu et al. 2002).

R. eutropha (H16) was used for the biosynthesis of P(3HB-4HB) by two stage cultivation (Yoon et al. 1995). The organism was grown in 500 ml aerated shake flask for 24 hr in Kunioka's medium. The cells were harvested by centrifugation. The harvested cells were transferred to 500 ml flask containing the mineral medium and trace element solution. Butyric acid, 4-hydroxybutyric acid, and ammonium sulphate were added to the mineral medium. The cells were cultivated to about 78 h, centrifuged, washed and lyophilized. The co-polyesters were then extracted from lyophilized cells (Yoon et al. 1995).

1.2.6.2 Fed Batch Culture

Fed-Batch fermentations in a 5 L bioreactor were conducted by Lefebvre et al. (1997). pH was controlled using HCl and NH_4OH. Gas flow rate was set at 1 volume/volume/min (vvm), with an agitation rate of 950 rpm. The concentration of feeding solution was 700 g glucose/L (Shang et al. 2003a). Cell density up to 100 g/L could be achieved when the mutant strain of *R. eutropha* (NCIMB 11599) was grown in glucose. When nitrogen was limited, P (3HB) was produced to 121 g/L. The productivity of PHB was 2.42 g/L/h and the yield was 75 % of the biomass (Kim et al. 1994). A copolymer of P (3HB-3HV) was also produced using fed batch fermentation using glucose and propionate as carbon sources under nitrogen limitation and low dissolved oxygen (21-29 %) (Lefebvre et al. 1997).

Shimuzu et al. (1999) conducted fed batch experiments in 5 L fermenter using *R. eutropha* (H16). Butyric and valeric acids were used as carbon sources during the PHA accumulation phase. The volume of the medium, agitation and aeration were 2 L, 300 rpm, and 2 L/min respectively. The effect of change in concentration of volatile fatty acids and the mole ratio of C:N as well as BA:VA on P(HB-HV) were studied. The production of copolymer was more than 50 % of the dry weight, when the total concentration of fatty acid was 1.5 g/L and the ratio of BA:VA was 1:1. The C:N mole ratio was set at 42:1 (Shimuzu et al. 1999).

Shang et al. (2004) cultivated *Ralstonia eutropha* NCIMB 11599 in 5 L fermenter. Glucose and valerate were fed in the PHA accumulation phase to produce P(HB-HV) with a high fraction of HV. The volume of the medium was 1.5 L initially.

The temperature, pH and agitation were 30 deg C, 6.7, and 900 rpm respectively. By sequential feeding of glucose followed by valeric acid dry cell weight and copolymer production reached 110.2 g/L and 37.8 g/L respectively. Valeric acid was fed using a pH stat when dry cell weight reached 100 g/L. However in the whole experiment the concentration of valeric acid was kept below 5 g/L due to its toxic effect. The HV content reached 62.7 mole % of the total copolymer produced (Shang et al. 2004).

1.2.6.3 Continuous Culture

Alcaligenes. latus and *Pseudomonas. Oleovorans* are the two organisms that have been successfully grown in continuous culture. When grown continuously on sucrose, *Alcaligenes. latus* gave a cell concentration of 16.2 g/L and productivity of 2.55 g/L/h. *Pseudmonas. Oleovorans* was grown in a two stage continuous culture on 15 % octane. At a steady state cell concentration, the organism grew to a cell concentration of 11.6 g/L and PHA productivity of 0.58 g/L/h. But if the dilution rate of the feed was increased (0.09 h^{-1} to 0.46 h^{-1}) there was a decrease in cell concentration and PHA yield (Lee 1996b).

Two stage chemostats were used for the production of PHA by *R. eutropha* since they were 1.7 times more productive than single stage continuous cell cultures (Lee 1996b). At a dilution rate of 0.064 h^{-1}, a cell concentration of 75 g/L and a PHA productivity of 2.86 g/L/h were reached (Lee 1996b).

For continuous production of PHB from *Ralstonia eutropha* (WSH3), Du et al. (2001) used a two stage system. In the first stage, growth rate was maximized by providing high rates of aeration (1.5 v/v/min), agitation (600-1000 rpm), and dissolved

oxygen saturation (20 %). Temperature was set at 30 deg C, while pH 7.0 was maintained using ammonia water (the source of nitrogen). The maximum dilution rate was 0.21 h^{-1}, with 50 g/L glucose serving as the carbon source. The medium from the first stage was continuously transferred to the second stage. Residual ammonium ions from the first stage carried over to the second stage, and to maintain a higher, constant C:N ratio, additional carbon (500 g/L of glucose) was fed into the second reactor at 0.054 h^{-1}. Higher dilution rates in the second reactor decreased PHB productivity. At a dilution rate of 0.075 h^{-1}, maximum PHB productivity (1.23 g/L/h), PHB content (30.5 g/L), 72.1 % of dry cell weight was reached (Guocheng et al. 2001).

1.2.7 Industrial Scale PHA Production

The most important cost factors for PHB production include the substrate cost and productivity/yield potential of the microbe. The genera most commonly considered for PHB production include *Azotobacter sp*., *Methylobacterium sp. Pseudomonas oleovorans, Alcaligenes latus,* Recombinant *Escherechia coli* and *R. eutropha.* These bacteria have been selected because they can grow to high cell densities and high PHA content resulting in high PHA productivity (Anderson and Dawes 1990).

Gorenflo et al. (2001) scaled up the cultivation of the cells of recombinant *R. eutropha* and *Pseudomonas. putida* to 500 L using glucose, gluconic acid, and octanoic acid as the carbon sources. The aeration and agitation were 300 L/min and 300 rpm respectively. The temperature and pH of growth were 30 deg C and 7.0 respectively. Octanoic acid, gluconic acid or glucose was added during the growth phase and levulinic acid was added during the PHA accumulation phase. NH_4OH or NH_4Cl was added as the

nitrogen source. At the end of the fermentation, 19.7 g/L cell density was reached. The PHA content of the cell reached to about 50 %. The PHA produced composed of 34.6 mol % 4HV, 63.7 mol % 3HV and 1.7 mol % 3HB (Gorenflo et al. 2001).

Byrom (1987) outlined the large scale production of PHA. He suggested that *R. eutropha* could be grown in a fed-batch mode in a 35,000 L air lift fermentor on a glucose salt medium with phosphate as the growth limiting factor. After 60 h the growth phase stopped and PHA accumulation phase began. At this point phosphate became limiting and glucose was added, resulting in a final PHB level of 75 % of the total cell dry weight at the end of fermentation (total fermentation time of 110-120 h) (Byrom 1987). If instead of glucose, a mixture of glucose and propionic acid were fed, a copolymer of hydroxyvalerate was produced. A high productivity (232 g/L) and concentration (3.14 g/L/h) of P (3HB) was obtained under optimal condition (Ryu et al. 1997).

1.2.8 PHA Characteristics

PHA is an umbrella term, used to describe a family of polymers composed of 3 hydroxy fatty acid monomers. The composition of PHA is related to the substrate used for growth, the producing microbe, and the method used for recovery. Polymers typically consist of subunits two carbons shorter than the substrate used (Piorer et al. 1995). Higher molecular weight polymers are obtained when they are extracted using neutral solvents rather than sodium hypochlorite (Dawes and Senior 1973). PHAs can serve as a cellular reservoir for carbon and energy (Senior and Dawes 1971). Specific types of PHAs are described in the following section.

1.2.8.1 Structure

PHAs are macromolecules composed of 3 hydroxy fatty acid monomers, linked by ester bonds between the carboxyl terminal and the hydroxyl terminal ends of adjacent monomers (Abe et al. 1990, Choi and Yoon 1994). There are many differences in length and composition of the side chains, and thus the polymer molecular weight, depending on the microbe, substrate, and method of isolation.

PHAs can also be classified as short or medium chain length, depending on carbon chain length of the monomer (Doi et al. 1992). Short chain length (scl) PHAs are composed of C3-C5 3-hydroxy fatty acids, and are typically crystalline, brittle, and stiff. Medium chain length (mscl) PHAs are composed of C6-C16 3-hydroxy fatty acids and have low crystallinity, tensile strength, and melting point (Nishida and Tokiwa 1992).

Figure 1.3 shows the generic formula for PHAs where x varies 1 through 4. R is a pendant group that can be hydrogen or hydrocarbon chains of up to around C13 in length (Madison and Huisman 1999, Lee 1996b). The main members of the PHA family are the homopolymers poly (3-hydroxybutyrate), P(3HB), which is the above generic formula with R=(methyl), and poly(3-hydroxyvalerate), P(3HV), generic formula with R=(ethyl). Various types of PHA copolymers are possible. Copolymers of PHAs vary in the type and proportion of monomers arranged in random sequence. For example poly (3-hydroxybutyrate-co-3-hydroxyvalerate), P(3HB-co-3HV), is composed of monomer units, R=(methyl) and R=(ethyl) that are arranged randomly. This is sold under the trade name of BIOPOL. Poly (3-hydroxybutyrate-co-3-hydroxyhexanoate), P(3HB-co-3HHx), consists of the monomers R=(ethyl) and R= (propyl). This is a polymer of commercial

significance and is sold under the NODAX^{TM} family of copolymers (Steel and Notron-Berry 1986, Green et al. 2001).

According to Steinbüchel and Lütke-Eversloh (2003), 4HB-containing PHAs are also very promising in terms of mechanical properties and can be obtained from cheap carbon sources such as glucose and 1, 4-butanediol by employing engineered organisms (Crank et al. 2004).

1.2.8.2 Physical Properties

The molecular masses of PHAs are generally within the range of 1,000-1,000,000 Da (Madison and Huisman 1999). PHAs are biodegradable polymers that have properties similar to polypropylene. The melting temperature of PHAs is 170 deg C and a degradation temperature of 185 deg C (Madison and Huisman 1999). PHA based co-polymers may be blended with inorganic compounds such as $CaCO_3$, talc and mica to improve stiffness and strength, enhance barrier properties and increase the opacity (Jedlinski et al. 1999, Anderson and Dawes 1990).

PHB has been studied in most detail. PHB granules are usually in a mobile amorphous state. Dry PHB granules have a non-crystalline core and a crystalline shell (Hahn et al. 1995). PHB has good oxygen impermeability, moisture resistance, water insolubility, and optical purity (Lindsay 1992, Holmes 1988). Young's modulus and tensile strength of PHB are similar to polypropylene, but elongation at break is 6 % as opposed to that of 400 % for polypropylene (Ojumu et al. 2004). It has good UV resistance, but poor resistance to acids and bases (Helleur 1988). The oxygen permeability is very low, making P (3HB) a suitable material for use in packaging

oxygen-sensitive products. P (3HB) has low water vapor permeability compared to other bio-based polymers but higher than most standard polyolefins and synthetic polyesters (Lindsay 1992, Hanggi 1990).

P (3HB) is free from even traces of catalysts and is toxicologically safe. The monomer and the polymer are natural components and metabolites of human cells (Jedlinski et al. 1999, Kim et al. 2000). P (3HV) has similar properties to that of P(3HB).

Copolymers are usually produced, so as to reduce the stiffness and brittleness observed in homopolymers. P (3HB-co-3HV) is the most widely studied copolymer (Ojumu et al. 2004). P (3HB-co-3HV) with a HV content of 30 % has a melting temperature of 143 deg C and at 40 %, the melting temperature is about 75 deg C (Mergaert et al. 1994), there is no decrease in degradation temperature. This allows thermal processing of the copolymer without degradation (Ojumu et al. 2004). P (3HB-co-3HV) is comparable to polypropylene in terms of water and gas barrier properties (Marangoni et al. 2001). As the co-monomer content increases, there is an increase in toughness, flexibility, elongation to break, and melting temperature (Lee 1996a). It does not degrade under normal conditions and is indefinitely stable in air (Marchessault 1996). A copolymer of P (3HB) and P (4HB) was formed by *R. eutropha* when fed on 4-hydroxybutyric acid or 4-chlorobutyric acid (Doi et al. 1988b). There was a decrease in crystallinity and melting point (150 deg C) when the 4HB fractions increase. At 40 % 4HB in the monomer, the polymer behaved like an elastic rubber (Kunioka et al. 1989a).

1.2.8.3 Degradation

Microbes are capable of producing short and medium chain length PHAs (Brandl et al. 1988, Lageveen et al. 1988). Degrading microorganisms are present in the range of $3.5 * 10^4$ - $1.8 * 10^7$ cfu/gm of soil (Tansengco and Tokiwa 1998). The microorganisms localize on the surface of the PHB films, secrete enzymes that degrade the polymer into basic monomers (Tokiwa and Calabia 2004). Low molecular weight, melting temperature and crystallinity increase the rate of enzymatic biodegradation (Tokiwa and Suzuki 1978, Nishida and Tokiwa 1992).

PHAs can be completely degraded to CO_2 and water by microbial enzymes under aerobic conditions, and methane under anaerobic conditions (Hankermeyer and Tjeerdema 1999 , Jendrossek et al. 1996 , Williams and Peoples 1996). Co-polymers containing the PHB monomer units degrade more rapidly than any other copolymer or homopolymer (Doi et al. 1989). PHB degraders are distributed among *Pseudonocardiaceae, Micromonosporaceae, Thermonosporaceae, Streptosporangiaceae* and *Streptomycetaceae* (Tokiwa and Jareret 2003).

PHAs can also be degraded by thermal decomposition (Kunioka and Doi 1990, Doi et al. 1990). The end product of thermal degradation is typically crotonic acid, secondary products such as propylene, ketones, acetaldehydes and CO_2 were also formed (Billingham et al. 1978).

1.2.9 PHA Recovery

The first step in all PHA recovery process is to recover the cells via centrifugation, typically followed by a wash step and additional centrifugation to remove

media components. The cell pellet is then typically dried prior to extraction. Extraction of the PHA from the cell mass can be done using different techniques. Some of the widely used compounds and techniques used for PHA extraction are discussed below.

1.2.9.1 Chloroform

Pure PHB was extracted using this method (Hahn et al. 1995). The cell broth was centrifuged at 4000 x g for 15 min at 25 deg C and washed twice with distilled water and freeze dried. Hot acetone wash of the freeze dried cells was done for 20 min. The cells were dried and treated with chloroform (50 volumes) for 48h at 30 deg C. The mixture was centrifuged and clear chloroform solution containing PHB was filtered. Then non-solvent precipitation of the material was done using a mixture of methanol and water (70:30), which was further filtered to obtain pure PHB (Hahn et al. 1995). Methanol was used to separate non-PHA fatty acids and also to make the cells more permeable for PHA extraction using chloroform.

In another method the cell mass from the fermentation broth was harvested by centrifugation at 10000 x g for 10 min (Wang and Yu 2001). The dry cell mass was then washed with distilled water and lyophilized. 0.1 g of DCW was dispersed in 10 ml chloroform, sealed and kept at 60 deg C for 24 h. After the sample had cooled to the room temperature, chloroform was filtered through a glass fiber filter. The solvent was vaporized and the amount of PHB obtained was measured (Wang and Yu 2001).

Copolymer (P3HB-3HV) was extracted from freeze dried cells with chloroform containing 2 % H_2SO_4 and precipitated in ice cold methanol (Shang et al. 2004). The

precipitated polymer was redissolved in chloroform and the precipitation step was repeated. The copolymers were then air dried and stored at 4 deg C (Shang et al. 2004).

Fractionation can be used to separate the monomer units of a copolymer (Green et al. 2001). Chloroform along with n-hexane or diethyl ether can be used for fractionation of PHA copolymers. In the method described by Green et al. (2002) the harvested cells were washed with 0.1 M NaCl and 50 mM Tris-HCl. Chloroform was refluxed into the dry cells (50:1) for about 5 h and thus PHA was extracted. The extract was filtered using Whatman filter paper no. 4 and dried under nitrogen into minimal volume. 10 ml of diethyl ether/hexane (3:1) was added to the partially dried PHA extract and was precipitated. The precipitate was once again centrifuged and washed with diethyl ether/hexane and dried under vacuum. Further fractionation of PHA copolymer was done by boiling the dried solid diethyl ether/hexane precipitate PHA copolymer in acetone for 5 h (Green et al. 2001).

1.2.9.2 Hypochlorite

Hypochlorite can be used to digest the non PHA cellular material. But this results in degradation of PHA. If treatment with hypochlorite is preceded by surfactant pretreatment, higher quality yield is produced (Ramsey et al. 1990). There was less degradation of PHB if the cells were freeze dried and then treated with aqueous solution as an irreversible conversion of PHB to crystalline state occurred (Hahn et al. 1995).

The extraction by hypochlorite involved harvesting cells by centrifugation of the fermentation broth, addition of 1 % hypochlorite solution to the biomass, and mixing at 25 deg C for 1 h (Hahn et al. 1995). Hypochlorite digestion resulted in dissolution of non-

PHA cellular material in the aqueous solution, which was separated from PHA using centrifugation. The extraction with sodium hypochlorite followed by treatment with SDS resulted in lesser degradation of PHA as compared to that with sodium hypochlorite alone (Hahn et al. 1995).

A dispersion of chloroform and sodium hypochlorite was also found to recover PHA with less degradation (Hahn et al. 1994). In this method, the harvested cells containing PHA were treated with dispersion of chloroform and hypochlorite solution. The treatment was carried out at 30 deg C for 90 min. The phase separation of hypochlorite (light) and chloroform containing PHA cells (heavy) occurred. The light phase was removed and the heavy phase was filtered. The filtrate was precipitated by methanol and water (7:3). The precipitate was again filtered. The PHA obtained was washed with water and spray dried (Choi and Lee 1997).

1.2.9.3 Supercritical Fluid Extraction

The large amount of toxic solvent used for extraction makes the conventional methods undesirable (Hampson and Ashby 1999). Supercritical fluids have high densities and low viscosities that make them suitable for extraction. CO_2 is most widely used for SFE because of its low toxicity, cost, inflammability and moderate critical pressure and temperature. SFE was also used to purify the PHA from lipid impurities present in the cell when grown on lard and tallow (Hampson and Ashby 1999).

Hejazi et al. (2003) developed a method to extract PHA using SFE. The cells of *R. eutropha* were first harvested by centrifugation, washed using distilled water, and resuspended in 4 ml Tris-HCL buffer. Methanol was added to increase polarity of

supercritical CO_2. The cell mixture (18 g/L) was put into an 8ml extraction vessel. The temperature and pressure of CO_2 was increased over critical point (P_c-72.8 atm and T_c-31 deg C) After the cells were saturated with supercritical CO_2, pressure was released suddenly causing disruption of cell membrane and release of PHB granules into suspension. 2 ml of this PHB containing suspension was mixed with 4 ml of chloroform in a capped tube and the mixture was shaken for 15 min. It was then centrifuged at 4000 x g for 15 min. The mixture separated into three phases. The bottom layer consisted of a mixture of PHB in chloroform, which was removed with an automatic pipette and analyzed (Hejazi et al. 2003).

1.2.9.4 Sodium Hydroxide

Choi et al. (2000) successfully use NaOH for extraction of copolymer P(3HB-3HV). At the end of the fermentation the cells were harvested by centrifugation of fermented broth. NaOH was added to 50 g/L biomass and the final concentration of NaOH was made to 0.2 N. NaOH was mixed at 30 deg C for 1 h. Copolymer of P(3HB-3HV) was separated from the solution containing dissolved non-cellular-polymer-material (NCPM) by centrifugation (Choi and Lee 2000).

Ethylene carbonate, propylene carbonate, and 1M-HCl-chloroform extraction were used at large scale for PHA extraction (Scandola et al. 1988).

1.2.10 PHA Analysis

Several methods have been used for PHA analysis. Spectrophotometric methods were widely used in the past (Law and Slepecky 1961), but have been replaced by gas chromatographic or high performance liquid chromatographic methods (Braunegg et al.

1978). Literature has been found on the use of ion-exclusion high-pressure liquid chromatography (HPLC) for the rapid assay of PHA (Carr et al. 1983). Currently GC-MS and NMR analysis are two most widely used methods for PHA analysis (Findlay and White 1983, Helleur 1988, Morikawa and Marchessault 1981).

1.2.10.1 Gas Chromatography

GC analysis can be used to quantify PHA production, but it does not provide qualitative information regarding the presence of co-monomers or their percent composition (Braunegg et al. 1978, Comeau et al. 1988). Mass spectrometry, flame ionization detection and nuclear magnetic resonance techniques are used to quantitate the levels of monomers. Gel Permeation Chromatography has been used to determine average molecular weight and molecular weight distribution (Green at al. 2001).

Braunegg et al. (1978) had extracted polyhydroxybutyrate that was converted to hydroxybutyric acid by methanolysis. The methyl esters were analyzed using PU4410 Phillips gas chromatograph with a packed glass column (10 PEG 20 M, 4 mm x 1.5 m) and a flame ionization detector. Nitrogen was used as carrier gas (30 ml/min). The injector and detector temperatures were 180 deg C and 200 deg C respectively. The analysis was started at 100 deg C for 3 min, and then the temperature was increased (at a rate of 8 deg C/min) to 150 deg C for 1 min (Braunegg et al. 1978, Hejazi et al. 2003).

Hewlett-Packard 6890 gas chromatograph flame ionization detector was also used to analyze PHA copolymers by Green et al. (2001). The column was Permaphase PEG 25 Mx capillary column (25 m by 0.32 mm). A 2 ul portion of the organic phase was analyzed after split injection (split ratio, 1:40).Helium (32 ml/min) was used as the carrier

gas. The temperature of the injector and detector were 230 and 275 deg C, respectively. A temperature program that was used was increasing the temperature to 120 deg C and holding for 5 min then at a temperature increase of 8 deg C/min to 180 deg C for 12 min).Under these conditions different retention times were obtained for different monomer units (Timm and Steinbuchel 1990).

1.2.10.2 Nuclear Magnetic Resonance

NMR is a physical phenomenon based upon the magnetic property of an atom's nucleus. The most often-used nuclei are hydrogen -1 and carbon -13. NMR spectroscopy is the only technique that can provide detailed information on the exact three-dimensional structure of biological molecules in solution. Green et al. (2001) used Proton NMR to quantitate the level of co-monomer in the copolymer. Varian Unity Plus 600 spectrometer was used to isolate the different monomers of PHA (Green et al. 2001).

In another experiment by Hahn et al. (1995), the molecular structure of PHB was determined using Bruker model AMX –FT NMR spectrophotometer. 500 MHz C-NMR spectrum of PHB was obtained at 25 deg C with a pulse repetition time of 3 s (Hahn et al. 1995).

Ishida et al. (2001) used JEOL GSX-270 spectrometer for H NMR and C NMR analyses. 270 MHz (HNMR) spectra was recorded at 18 deg C in $CDCl_3$ solution of polyester with 4s pulse repetition time. 67.8 MHz was recorded for carbon NMR spectra with 1 s pulse repetition and other conditions remaining same. The 4HB co-monomer fractions were estimated from intensities of resonances (Ishida et al. 2001).

1.2.10.3 Differential Scanning Calorimetry

Differential scanning calorimetry can be used to measure a number of characteristic parameters of a sample. Using this technique it is possible to observe fusion and crystallization events as well as glass transition temperatures (T_g) and melting temperature (T_m). DSC30 of Mettler Instrument Corporation was used for DSC measurements of PHA polymers (Green et al. 2001). In this experiment, the sample was heated to a temperature above 30 deg C of the melting temperature in an aluminum dish and was held at this temperature for 3 min. The sample was then cooled to -80 deg C and reheated to a temperature of the melting temperature at a rate of 10 deg C/min. There was a step like increase in the DSC graph and the mid point of this step was recorded as glass transition temperature. The sample was allowed to crystallize. When heated, an endothermic peak was observed and the temperature of the peak was taken as melting temperature (Green et al. 2001).

Hahn et al. (1994) measured the crystallinity of the PHB granules using DSC, Dupont model 2000. The enthalpy of fusion for 100 % crystalline sample was 146 J/g. From the enthalpy of fusion, the crystallinity of PHB sample was determined (Hahn et al. 1994).

1.2.11 Applications

Since PHB is toxicologically safe, it can be used for articles which come into contact with skin, feed or food (Biomer is in the process of registering its PHA products for food contact) (Crank et al. 2004). In the food industry, PHA has a wide application as edible packaging material, coating agent, flavor delivery agent and as dairy cream

substitute (Williams and Peoples 1996, Woo et al. 2000). It can also be used for making bottles, cosmetics, containers, pens, golf tees, films, adhesives and non-woven fabrics, toner and developer compositions, ion- conducting polymers and as latex for paper coating applications (Baptist 1963a, Baptist 1963b, Rutherford et al. 1997, Steel and Notron- Berry 1986). It can be used to make laminates with other polymers such as polyvinyl alcohol. P(HB-HV) can thus be used in reconstructive surgery. Also the degradation products of PHB are found in large concentrations in human blood plasma, so it will not be toxic for human use (Lee 1996a).

1.2.12 Commercialization of PHA

ZENECA bioproducts commercially produced PHA using a fed-batch culture of *R. eutropha*. It manufactured about 1000 tons PHA per year (Byrom 1994). P (3HB) was produced using glucose as the substrate. For the manufacturing of the copolymer, both glucose and propionic acid were used. Large scale production of a PHA copolymer P(3HB-co-3HV) was sold under the trade name of BIOPOL. The cost was $16 /kg, compared to $1 /kg for polypropylene (Lee 1996b).

In 1996, Zeneca sold its Biopol business to Monsanto, which in 2001 sold its Biopol to Metabolix, a U.S. biotechnology company (Crank et al. 2004, Tokiwa and Calabia 2004). Today, Metabolix is producing PHAs through fermentation of commercial-grade corn sugar in a 50 cubic meter fermenter in overall fermentation times of less than 40 hours and final PHA concentration of 100 g/L.

Procter & Gamble (P&G) has engaged in research and development efforts to develop and commercialize the PHAs named Nodax™ (Noda et al. 2004). For

commercial viability, the targeted PHA concentrations were to reach 60-80 g/L by 2006 (Crank et al. 2004). The biotechnology company Biomer, located in Krailling, Germany produces PHAs on a small-scale commercial basis for specialty applications.

Another company planning to enter the bulk PHA market is PHB Industrial, São Paulo, Brazil. This is a joint venture between sugar and alcohol producer Irmãos Biagi and the Balbo Group. The project is currently at pilot plant stage, producing 50 tons per annum. The company planned to construct a 10,000 tons per annum (PHA blends and composites) plant for startup in 2006 (PHB IND, 2003).

In Japan, Mitsubishi Gas Chemicals (MCG) has made an in-depth development study of the production of P(3HB) from methanol fermentation (trade name Biogreen™). Archer Daniels Midland Company (ADM) and Metabolix have selected Clinton, Iowa as the location for the first commercial plant for PHA natural plastics in March 2006. This plant will have an annual capacity of 50,000 tons per year, and will produce high performance natural plastics that are environment friendly and manufactured from renewable resources. The PHA plant will be located adjacent to ADM's wet corn mill in Clinton. The plant will utilize starch from the mill's existing corn grind capacity as raw material for PHA production (Crank et al. 2004, Anonymous 2006).

The keys to lowering PHA production costs are to develop more productive bacterial strains by recombinant DNA technology, reduce substrate costs, improve fermentation processes, and develop more efficient recovery process (Lee 1996 b). Recombinant technology has the potential to increase PHA yield and productivity (Guocheng et al. 2001). Processes are being developed to produce low cost feedstocks for

PHA production, for example by fermenting agricultural wastes to mixed organic acids (Shimuzu et al. 1999). In the future these techniques may lower the price of the polymer to $4 /kg. Lee (1996b) suggested that PHA can be produced from transgenic plant, *Arabidopsis thaliana* harboring the genes of *R. eutropha*. P(3HB) content was 14 % of the dry cell weight. However more work needs to be done to study plant biochemistry and genetics so as to increase the productivity and yield of PHA. Thus aim of this research is to reduce the cost of PHA to that of starch, 20 cents/kg (Lee 1996b).

1.3 Objectives

The overall goal of this research was to develop a system to grow *Ralstonia eutropha* to high cell concentrations, and then fill these cells with PHA as a means of producing a value added product from the byproducts of ethanol industry. This research is significant from an industrial perspective because if low cost biopolymers can be generated, they have many potential uses in food and pharmaceutical industries.

To generate high concentrations of *R. eutropha*, specific objectives included:

1. Compare various media for their ability to support rapid growth of *R. eutropha.*
2. Determine the optimum concentration of condensed corn solubles (CCS) to serve as a basal medium,
3. Determine the proper carbon:nitrogen ratio to maximize growth, and subsequently trigger PHA production.

The C:N ratio is important, because PHA is produced under nitrogen limitation in the presence of excess carbon sources. The first three objectives are met in chapter 2.

The carbon source for PHA production would be volatile fatty acids (VFAs) produced by growing a rumen culture in whole stillage. The VFAs would be drawn from this rumen culture and fed to the *R. eutropha* cell mass produce PHA.

To produce PHA within the *R. eutropha* cell mass specific objectives include:

4. Determine the rates and efficiencies at which four volatile fatty acids (acetic, butyric, lactic and propionic) are utilized and individually converted to PHA by *R. eutropha*.

The results of this study are discussed in chapter 3.

5. Determine the rate and efficiency at which a mixture of the four volatile fatty acids (acetic: butyric: lactic: propionic at 10:2:15:20) is used and converted to PHA.
6. Determine the rate and efficiency at which a mixture of the four volatile fatty acids are converted to PHA during fed-batch or continuous addition using different feeding strategies.

These results are discussed in chapter 4.

Chapter 2

Development of Low Cost Medium for *Ralstonia eutropha*

2.1 Abstract

Condensed corn solubles (CCS), a low value byproduct of corn ethanol production, was used as a base to develop an inexpensive medium for growth of *Ralstonia eutropha* ATCC 17699. Both nutrient broth and a Kunioka's medium were used as controls. Trials were conducted in 1 L aerated shake flasks (800 ml medium) incubated at 30 deg C, an initial pH of 7.0, and 250 rpm. Inocula consisted of a 1 % volume grown for 24 h in the same medium. Initial trials in the defined medium evaluated the tolerance of *R. eutropha* to various antifoams. Cognis Clerol FBA 5059, at 1ml/50ml media was least inhibitory and provided adequate foam control. Various concentrations of the CCS medium (80, 240, 400, and 700 g/L wet basis) were next compared to the control media. The Kunioka's medium produced $6.3 * 10^9$ cfu/ml, while nutrient broth yielded $3.3 * 10^9$ cfu/ml. The 240g/L CCS medium produced $2.6 * 10^9$ cfu/ml, with a growth rate of 0.29 h^{-1}. The 80 g/L CCS medium results were significantly lower than the 240 g/L CCS medium, and *R. eutropha* did not grown in 400g/L and 700g/L CCS formulations. Trials in 80 and 240 g/L CCS media then evaluated the effects of continually maintaining pH 7 throughout the growth phase. The maximum cell population and growth rates were not significantly different, perhaps due to the intrinsic buffering capacity of CCS. The CCS medium was then supplemented with four different levels of ammonium bicarbonate to achieve C:N ratios of 30:1, 50:1, 70:1, and 90:1. The best results ($P<0.05$) were obtained in the CCS media with 50:1 or 70:1 C:N ratio. At 50:

1 ratio, the maximum cell population was 4.63 * 10^9 cfu/ml, while the maximum growth rate was 0.25 h^{-1}. HPLC analysis of the fermentation samples showed a more rapid utilization of the carbon sources (i.e., carbohydrates, glycerol, and volatile fatty acids) in the medium with C:N ratio of 50:1.

2.2 Introduction

Polyhydroxyalkanoates (PHAs) are macromolecules produced by approximately 250 microbial species, however, only a few can produce PHA in high concentrations. These include *Alcaligenes latus* (Yabuuchi et al. 1995), *Pseudomonas oleveorans* (Brandl et al. 1988), *Ralstonia eutropha* (Kim 1994) and recombinant *Escherichia coli* (Lee and Lee 1994). PHAs accumulate as intracellular granules when a bacterium is grown under unbalanced nutritional conditions (i.e., excess carbon and some limiting factor). PHA synthesis can be triggered by nitrogen and phosphorous limitation (Madison and Huisman 1999) and to a lesser extent oxygen limitation (Byrom 1987). PHAs function as a carbon and energy reserve for cells, (Anderson and Dawes 1990) and simultaneous synthesis and degradation occurs in *R. eutropha* under nitrogen free conditions.

PHAs are composed primarily of 3-hydroxy fatty acid monomers, in which the carboxyl group of one monomer is esterified by the hydroxyl group of the next monomer (Madison and Huisman 1999). The general structure of PHA, shown in Figure 2.1, consists of (R) β-hydroxyl fatty acids and the R group is called the pendant group. The number of carbon atoms of R varies from 3 to 13. The alkyl side chain may be saturated, unsaturated, halogenated, aromatic or branched monomers (Abe et al. 1990, Choi and Yoon 1994, Doi et al. 1990).

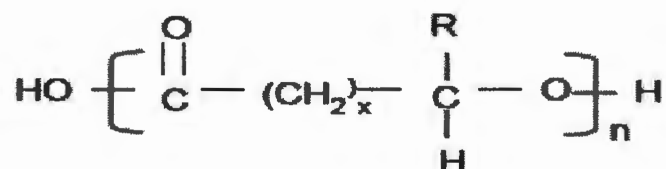

Figure 2.1 General structure of Polyhydroxyalkanoate (Lee 1996b).

PHA can be extracted from bacterial cells by various means, and used to replace plastics in many applications. PHAs have similar physical properties to many conventional plastics, but have the advantage of being completely biodegradable to water and carbon dioxide by microbial enzymes (Lee 1996a, Lee 1996b). The chief factor limiting commercialization of PHAs as plastic replacements has been the cost of production.

In this study we attempt to develop a condensed corn solubles (CCS) based medium as a low cost alternative to support growth of *R. eutropha*. This bacterium, formerly known as *Alcaligenes eutrophus,* is a Gram negative aerobe that has been investigated for production for PHA. Its optimal growth temperature is 20-37 deg C, and its optimal pH is reported to be 7.0 (Yu et al. 2002, Guocheng et al. 2001). The wild-type strain of *R. eutropha* does not metabolize glucose, however a glucose-utilizing mutant was created to produce poly (3-hydroxybutyrate-co- 3-hydroxyvalerate), P(3HB-3HV) (Byrom 1992). Our first objective was to determine if CCS would support growth of *R. eutropha*. Then we identified the optimal concentration of CCS and evaluated different levels of nitrogen supplementation to maximize cell population and growth rate. We also identified an effective antifoam agent and evaluated the effect of pH control.

2.3 Materials and Methods

2.3.1 Culture Type and Propagation

The ATCC 17699 type strain of *R. eutropha* was used. The culture was routinely transferred to nutrient broth, and incubated on a reciprocating shaker (250 rpm) at 30 deg C for 24 h. For short term maintenance the culture was stored on tryptic soy agar slants covered with mineral oil and stored in the refrigerator. Lyophilization was used for long term storage.

2.3.2 Media

Nutrient broth and a defined medium (Kunioka et al., 1989) were used as controls. Kunioka's medium was prepared by adding 2.5 g beef extract, 5 g yeast extract, 5 g ammonium sulphate and 5 g peptone to 1 L deionized water. The basal CCS media were prepared by mixing various amounts of CCS in deionized water, adjusting the pH to 7.0 using 10 M NaOH, then centrifuging at 11,000 rpm for 7 min at 15-25 deg C. The supernatant was then filtered through Whatman filter paper #113 and dispensed into appropriate vessels prior to autoclaving. Table 2.1 lists the composition of the different CCS media.

Table 2.1 Composition of CCS based media.

Medium Concentration (g/L)	Volume CCS (ml)	Volume Water (ml)
80	76.3	923.7
240	229	771
400	381.7	618.3
700	667.9	332.1

2.3.3 Experimental Design

Inoculum for all trials was prepared in a stepwise manner, by transferring the culture from TSA plates into 100 ml of the various media, then incubating the flasks for 24 h on a rotary shaker (250 rpm) at 30 deg C. These cultures were subsequently used to inoculate the same media in the respective trials. An inoculum rate of 1 % (v/v) was used.

Experimental trials were conducted in 1 L conical flasks that contained 800 ml of medium. Filter sterilized air (1 L/L/min) was provided through a glass sparger inserted through the rubber stopper, with a companion filtered vent tube. Flasks were incubated for a minimum of 48 h at 30 deg C and 250 rpm. Three replications of each trial were performed to assess growth rate and maximum cell population.

Due to the importance of aeration and associated problems of foaming, tolerance trials were conducted with four different antifoams (Cognis Clerol FBA 3107, Cognis Clerol FBA 5059, Cognis Clerol FBA 265, and Cognis Biosupmex 36 K). *R. eutropha* was grown in Kunioka's medium (Kunioka et al. 1989a, Kunioka et al. 1989b, Kunioka et al. 1989c), and at 24 h replicate plate counts were performed to establish the baseline cell population. Samples of the broth (50 ml) were then dispensed into test tubes, to which 1 ml of the individual antifoams were added. Two replications were performed for each antifoam, with two replication of the control (that lacked antifoam). Tubes were incubated for 3 h, followed by plating on TSA agar. After incubation for 2 days, counts were compared to assess the effect of antifoams on cell viability. Because the Cognis Clerol FBA 5059 was found to be least inhibitory, it was used at a rate of 2 ml/800 ml of the media in subsequent trials to control foaming.

The next set of experiments compared two concentrations of CCS media (80 g/L and 240 g/L), without pH control. Subsequent trials evaluated the effect of controlling pH in the optimal range (7.5-8.5) by addition of 10 N H_2SO_4 to these media. After the optimal CCS concentration was found to be 240 g/L, this medium was supplemented with a 178 g/L stock solution of filter-sterilized nitrogen (NH_4HCO_3) to bring the C:N ratio to 30:1, 50:1, 70:1 and 90:1 (see Table 2.2). In these trials the pH was not controlled, as the previous trials indicated the CCS medium had adequate buffering capacity.

Table 2.2 Composition of 240 g/L CCS media supplemented with nitrogen.

C:N Ratio	Volume of NH_4HCO_3 Solution (ml)	Volume of CCS Medium (ml)
30:1	34	766
50:1	20.4	779.6
70:1	14.3	785.7
90:1	11.3	788.7

2.3.4 Analytical Methods

Samples were collected at regular intervals during incubation, and pH was measured using an Acumet 950 pH meter. Viable cell counts were done with TSA. In latter trials, samples were also analyzed via a Waters HPLC system for content of sugars, organic acids and glycerol. These samples were first filtered through a non-sterile 0.2 um filter to remove solids then frozen until analysis. An Aminex HPX87H column, operated at 65 deg C with a helium-degassed, 4 mM H_2SO_4 mobile phase at a flow rate of 0.6 ml/min was used. Peaks were detected using a refractive index detector. Standard solutions of maltose, glucose, lactic acid, acetic acid, propionic acid, succinic acid and glycerol (at 3 and 30 g/L) were used to calibrate the integrator.

2.3.5 Statistical Analysis

All trials were performed in triplicate. Maximum cell populations were analyzed using randomized complete block design to determine the least significant differences between treatments. Data were analyzed using the PROC GLM procedure of SAS software to determine F values and LS means. Growth rates were determined from the exponential regression equations of individual curves, then calculating the average of replications. The growth rates were statistically analyzed by ANCOVA to test homogeneity of slopes. Statistical data were analyzed at the significant level of $P<0.05$.

2.4 Results and Discussion

Trials were first conducted to study the effects of different antifoams on the growth of *R. eutropha* in Kunioka's medium. Cultures containing the antifoam Cognis Clerol FBA 5059 achieved a maximum cell population of $3 * 10^9$ cfu/ml, which was similar to the control which lacked antifoam ($3.1 * 10^9$ cfu/ml). Cognis Clerol FBA 3107, Cognis Clerol FBA 265, and Cognis Biosupmex 36 K yielded maximum populations of $1.76 * 10^9$ cfu/ml, $2 * 10^9$ cfu/ml and 2.1 cfu/ml respectively. Therefore Cognis Clerol FBA 5059 was used in subsequent trials.

In order to develop a low cost medium that would support the rapid growth of *R. eutropha* to a high cell population (which would subsequently be used to produce PHA from organic acids) we evaluated condensed corn solubles (CCS) as nutrient base. Gibbons et al. (1997) have found that CCS, which costs $160/ton, was much less expensive than conventional media, and still supported rapid growth of *Clostridium*

thermoaceticum. Using a low cost substrate could significantly reduce the total production costs of PHA (Lee 1996b).

In these experiments nutrient broth and Kunioka's medium were used as a control, against which to compare four concentrations of the CCS medium, without pH control. The organism did not grow in the CCS media at concentrations of 400 g/L and 700 g/L. This was likely due to catabolite inhibition, since these formulations contained high concentrations of both glucose and glycerol, and the H16 strain of *R. eutropha* cannot metabolize glucose or glycerol (Madison and Huisman 1999). For example, the 400 g/L CCS medium contained approximately 5 g/L glucose and 70 g/L glycerol, compared to 2 g/L and 24 g/L, respectively, in the 240 g/L CCS medium. Therefore, data for the higher CCS concentration media are not shown.

Figure 2.2 shows the average growth curves for these trials, while Table 2.3 provides maximum growth rates and cell populations. For all media, the highest cell populations occurred at approximately 24 h, with Kunioka's medium yielding the highest cell counts, followed by significantly lower ($P<0.05$) levels for both nutrient broth and CCS 240 g/L. The cell population in the 80 g/L CCS medium was lower yet. Growth rate in the Kunioka's medium and nutrient broth were not significantly different from 240 g/L CCS media. This may have been due to the presence of smaller utilizable compounds present in the media as a result of enzymatic degradation and byproducts of yeast fermentation. The lowest growth rate was observed in the 80 g/L CCS medium. This might have been due to carbon limitation.

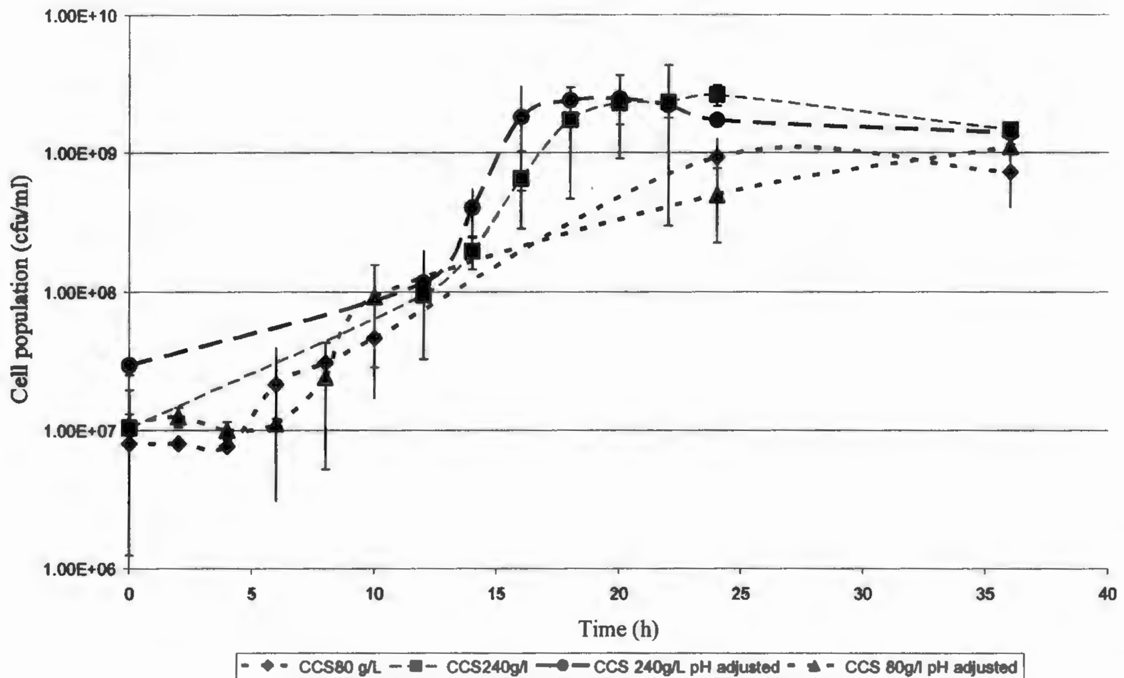

Figure 2.2 Growth curves of *R. eutropha* in different media without pH control.
* Error bars represent one standard deviation.

Table 2.3 Comparison of growth rates and maximum cell population of *R. eutropha* in different media without pH control.

Medium	Maximum Growth Rate (h^{-1})	Maximum Biomass (CFU/ml)
Defined medium	0.29[a]	6.3E+09[a]
Nutrient Broth	0.24[a]	3.3E+09[b]
CCS medium 80g/L	0.21[b]	9.3E+08[c]
CCS medium 240g/L	0.29[a]	2.6E+09[b]

[a,b,c] **Means within column not sharing common superscript differ significantly ($P<0.05$)**

The differences between the CCS and control media may have been due to changes in pH. The optimal pH of *R. eutropha* is 7.0, although its pH tolerance extends to 8.5. Figure 2.3 shows the pH of the four media throughout the incubation period. At 24 h, pH levels in Kunioka's and the 240 g/L CCS media were approximately 8.2, while pH

was 8.5 for the nutrient broth and 8.7 for the 80 g/L CCS media. These high pH levels may have limited cell growth, or resulted in the subsequent decline in cell numbers. Some studies suggest that media pH be maintained around 7.0 for both the growth and PHA accumulation phases (Choi and Lee 1999, Gorenflo et al. 2001, Guocheng et al. 2001, Shang et al. 2004). Therefore, trials were subsequently performed with pH control to evaluate these media.

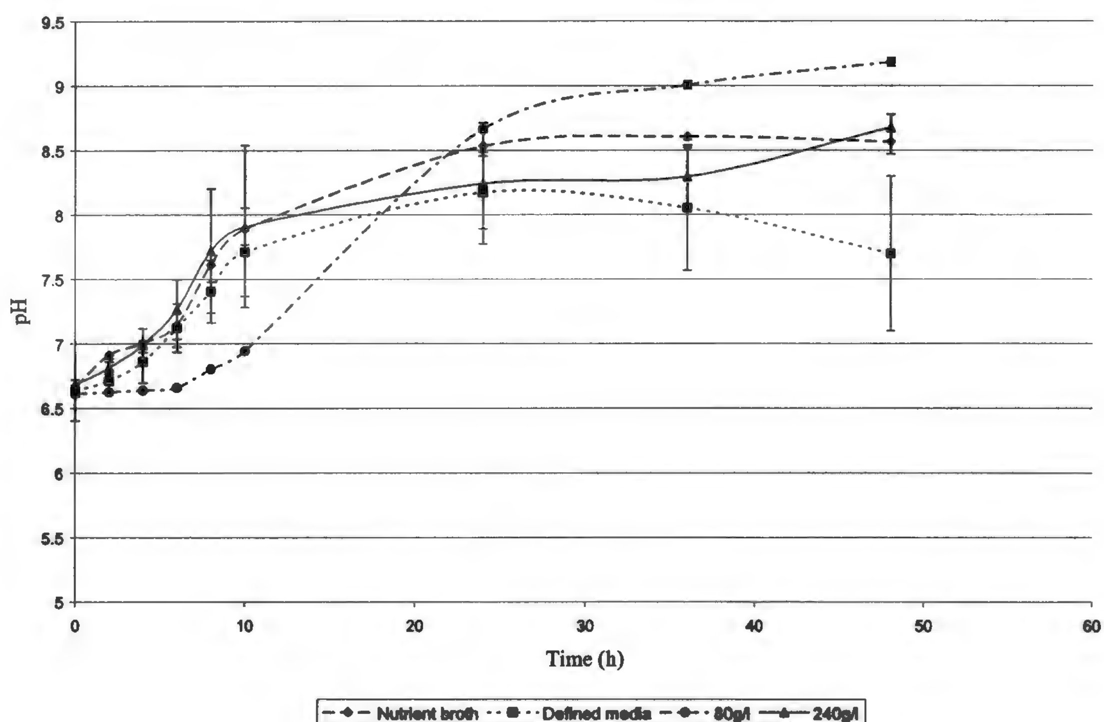

Figure 2.3 Change in pH during growth of *R. eutropha* on defined media, nutrient broth and basal CCS media at concentration of CCS at 80 g/L and 240 g/L.
* Error bars represent one standard deviation.

The trend of increasing pH during incubation (Figure 2.3) was, in part, due to the utilization of organic acids present in the CCS medium (Tsuge et al. 2001). Figure 2.4 shows the average growth curves for trials in which pH was controlled at 7.5-8.0

throughout incubation for the 80 and 240 g/L CCS media. Table 2.4 provides maximum growth rates and cell populations for these trials. The maximum cell populations and growth rates were again significantly higher for the 240 g/L CCS medium trials compared to the 80 g/L medium. However for each type of medium, there was no significant difference for either parameter, with or without pH control. Thus, we concluded that pH was not the critical factor for lower growth rate in 80 g/L CCS. Moreover, the 240 g/L CCS medium was considered as the optimal medium for growth of *R. eutropha*, with pH control not necessary.

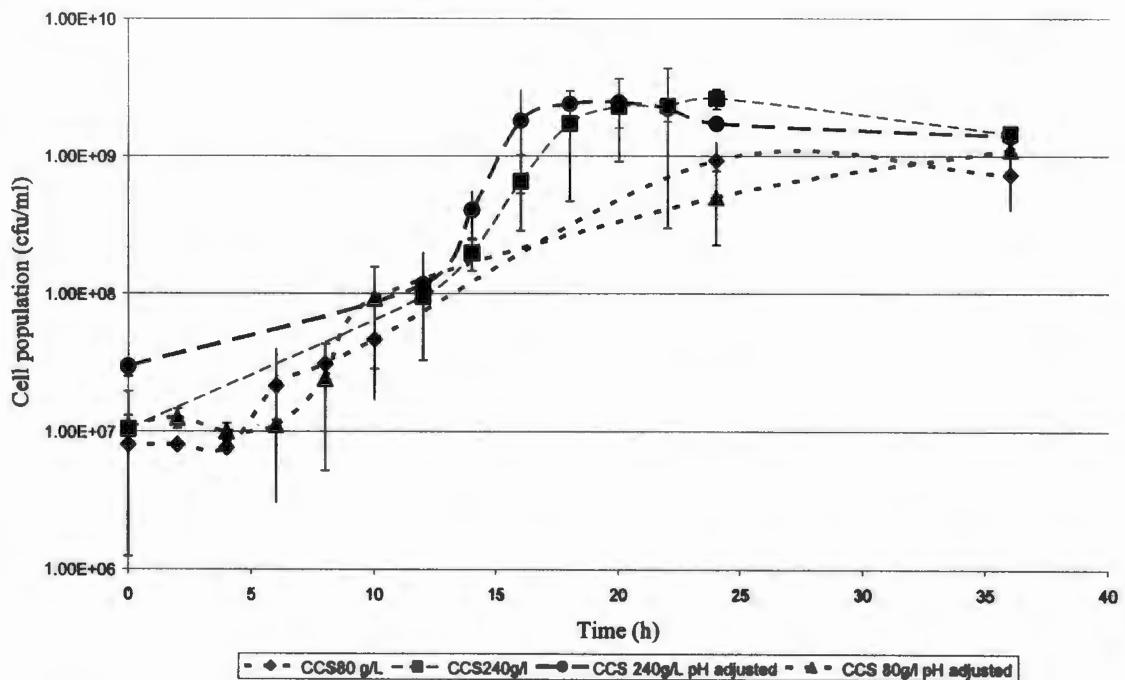

Figure 2.4 Growth curves of *R. eutropha* in basal CCS media (80 g/L and 240 g/L) with or without pH adjustment.
* Error bars represent one standard deviation.

Table 2.4 Comparison of growth rates and maximum cell population of *R. eutropha* when grown in different concentration of CCS media with or without pH control.

Media Type	Maximum Growth Rate (h^{-1})	Maximum Biomass (CFU/ml)
CCS medium 240g/L w pH control	0.24^a	$2.5E+09^a$
CCS medium 80g/L w pH control	0.18^b	$1.1E+09^b$
CCS medium 240g/L wo pH control	0.29^a	$2.6E+09^a$
CCS medium 80g/L wo pH control	0.23^a	$9.3E+08^b$

^{a,b} Means within column not sharing common superscript differ significantly ($P<0.05$)

The next series of experiments evaluated the effect of supplementing the 240 g/L CCS medium with different levels of nitrogen (as NH_4HCO_3), to determine the optimal C:N ratio (30:1, 50:1. 70:1 and 90:1). In these trials the pH was not controlled, but did remain in the 8-8.5 level throughout, which was at the upper level of the desired range (Yu et al. 2002, Wang and Yu 2001). This was likely due to the intrinsic buffering capacity of the compounds present in CCS, combined with the additional buffering effect of NH_4HCO_3.

Figure 2.5 shows the average growth curves for these trials, while Table 2.5 provides maximum growth rates and cell populations. In the prior trials (Figure 2.2) we observed that cell populations in the CCS media without nitrogen supplementation peaked at 24 h. However, when nitrogen was added to the medium, cell numbers continued to increase until 48 h, peaking at higher levels. Thus, nitrogen was a limiting factor in the basal CCS medium. The highest growth rates and cell populations were achieved at the 50:1 and 70:1 C:N levels. Though there were no significant difference in the two levels in terms of growth rate and cell population between the two media, both growth rate and cell population was slightly higher when C:N ratio was 50:1. Nitrogen

depletion slightly reduced cell populations at the 90:1 ratio, while ammonia toxicity could have reduced growth in the 30:1 trials.

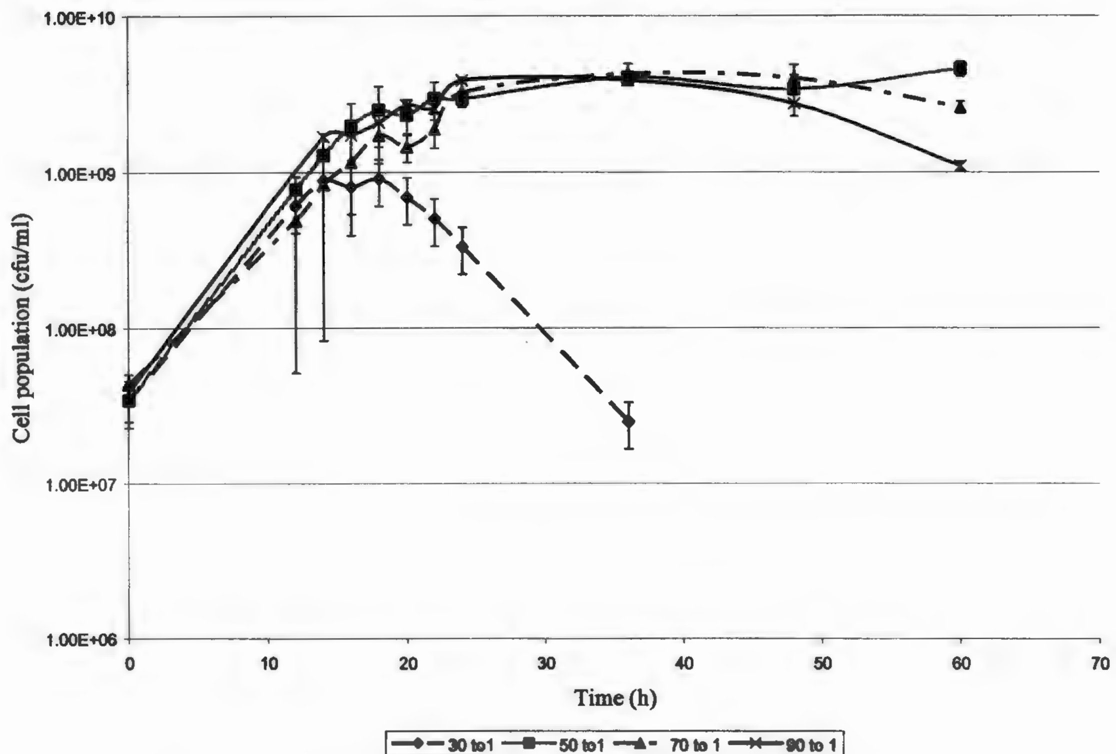

Figure 2.5 Growth curves of *R. eutropha* in 240 g/L CCS medium supplemented with different levels of nitrogen.
* Error bars represent one standard deviation.

Table 2.5 Comparison of growth rates and maximum cell population of *R. eutropha* when grown in 240 g/L CCS medium supplemented with different levels of nitrogen.

Media Type	Maximum Growth Rate (h^{-1})	Maximum Biomass (CFU/ml)
CCS medium 240g/L C:N ratio 30:1	0.19[a]	6.95E+08[a]
CCS medium 240g/L C:N ratio 50:1	0.25[b]	4.63E+09[b]
CCS medium 240g/L C:N ratio 70:1	0.20[ab]	4.28E+09[b]
CCS medium 240g/L C:N ratio 90:1	0.19[a]	2.46E+09[c]

[a,b,c] Means within column not sharing common superscript differ significantly ($P<0.05$)

HPLC samples were also collected from these trials to determine organic acid utilization rates. Organic acids are present in the basal CCS medium as a result of prior metabolism of yeast or bacterial contaminants during ethanol fermentation. Lactic acid was present in higher amounts (2.4 g/L) in one lot of CCS, but was typically about 1.5 g/L. The lactic acid present in the all media was completely utilized by 12 h for media with C:N ratios of 50:1 and greater, and by 18 h in the 30:1 medium.

Succinic acid was present in the CCS media at an initial level of 0.8 g/L, while acetic acid levels were 0.7 g/L. Utilization rates for both acids followed the same trend as noted previously for lactic acid, with the 30:1 C:N ratio medium showing slower acid utilization rates compared to the other media. In all cases, both succinic and acetic acid were depleted by 12-18 h.

It was found that lactic acid was used at a faster rate i,e $6.32 * 10^{-3}$ mol/g cells.h, whereas acetic acid was used at $2.88 * 10^{-3}$ mol/g cells.h. When both the acids were fed as a mixture, a diauxic growth curve was observed which showed that the utilization of acetic acid started only after the depletion of lactic acid (Tsuge et al. 2001).

Propionic acid was present in the CCS medium at 0.5 g/L in most cases, however in the 30:1 C:N trial there was higher initial amount of propionic acid, since it was prepared from a different batch of CCS. This might also be the reason for the presence of higher concentration of lactic acid in 30:1 (C:N) medium. Propionic acid utilization rates were much slower than that observed for the other acids, with little change in concentrations until after 36 h. In the media containing 50:1 and higher C:N levels, propionic acid was depleted by 48 h. Propionic acid is better utilized at about a pH of 7.5

(when added individually) due to a decrease in the concentration of undissociated propionic acid as an uncoupler. This might have resulted in longer retention of the propionic acid in the media (Kim et al. 2005).

Table 2.6 Organic acid utilization rates of *R. eutropha* when grown in CCS media with different C:N ratios.

240 g/L CCS Medium, C:N Ratio	Acid Utilization Rate (g/L/h)			
	Acetic	Lactic	Propionic	Succinic
30:1	0.033	0.133	0.007	0.039
50:1	0.044	0.125	0.008	0.061
70:1	0.039	0.125	0.008	0.060
90:1	0.044	0.116	0.008	0.045

Growth rates, maximum cell populations, and acid utilization rates were statistically similar for the 240 g/L CCS medium, when supplemented with nitrogen at either the 50:1 or 70:1 C:N level. We did, however, observe slightly higher growth rates and cell populations at the 50:1 level, helping us to conclude that this medium was best for the growth of *R. eutropha*. Efficiency of utilization of volatile fatty acids is important because these acids can be fed to *R. eutropha* to trigger PHA formation and accumulation.

2.5 Conclusions

Condensed corn solubles can be used as an inexpensive medium for growth of *R eutropha*. We found the 240g/L level to be the optimum in terms of specific growth rate and maximum cell growth. At higher CCS concentrations, growth was inhibited, while at lower CCS levels growth was limited by carbon level. Furthermore, we found that the buffering capacity of CCS was sufficient to maintain pH in an appropriate range without active pH control. When nitrogen supplementation was evaluated, C:N ratios of 50:1 and

70:1 were found to result in optimal growth rate, maximum cell numbers, and acid utilization rates, although the 50:1 level was slightly better. Therefore, we concluded the optimum medium formulation to be CCS at 240g/l with C:N ratio of 50:1 with an initial pH of 7.

Chapter 3

Conversion of Volatile Fatty Acids into Polyhydroxyalkanoate by *Ralstonia eutropha*

3.1 Abstract

A condensed corn soluble medium containing 240 g/L CCS and a C:N ratio of 50:1 was used as the growth medium for pure culture of *Ralstonia eutropha* ATCC 17699. Cultures were incubated in 1 L aerated flasks (500 ml medium) at 250 rpm, 30 deg C, with an initial pH of 7.0 for 120 h. Volatile fatty acids (acetic, butyric, lactic, and propionic) were fed individually at 1, 3, or 5 g/L at 48 h, while lactic acid was added at 2, 4, or 8 g/L at 48 h. In terms of PHA concentration, productivity and PHA content, optimal levels for each acid were 5 g/L acetic acid, 5 g/L butyric acid, 8 g/L lactic acid and 5 g/L propionic acid. An overall comparison of the volatile acids indicated that butyric and propionic acids provided the best results. Butyrate achieved the highest fermentation efficiency (95.6 %), PHA concentration (4.6 g/L), and productivity (0.037 g/L/h), however only the fermentation efficiency was significantly different ($P<0.05$) from propionic acid. Meanwhile, propionic acid resulted in the highest cell population ($6.67 * 10^9$cfu/ml), but it was not significantly different from that obtained with butyric acid. However, lactic acid resulted in the highest acid consumption rate (0.08 g/L/h) and PHA content (40.7 %), while butyric and propionic acids were lower.

3.2 Introduction

Polyhydroxyalkanoate (PHA) synthesis is a two stage process (Lee 1996b). Cells are first grown to a high cell density in a nutrient balanced medium, then at least one

essential nutrient is limited, while excess carbon is provided. This unbalanced nutritional state triggers intracellular accumulation of PHA. Many different carbon sources can be used for PHA production, with some microbes able to use volatile fatty acids (VFAs) such as acetic, butyric, lactic, and propionic (Madison and Huisman 1999).

Polyhydroxybutyrate (PHB) is the most widely studied PHA. Metabolic flux analyses were done to study conversion of different volatile fatty acids into PHB (Huidong et al. 1997). The authors determined that PHB production is dependent on NADH generation, which provides the energy essential to convert acetoacetyl-coA to (R)-3-hydroxybutyryl-CoA. Butyrate was found to be more suitable for PHB production, since 67 % of butyrate was directed to the TCA cycle to generate the required NADH. However, only 33 % of acetate and lactate were directed to the TCA cycle, producing insufficient levels of NADH.

Various fatty acids result in PHAs with different composition of monomer units. When butyric acid was used as carbon source, only PHB was formed. However, when pentanoic, propionic, valeric, and oleic acids were used, a copolymer of poly hydroxybutyrate-co-hydroxyvalerate, P(HB-HV) was formed (Doi et al. 1990). Production of HV units depends not only on the availability of propionate, but also on acetate, since HV monomers are formed from condensation of acetic and propionic acids (Yu et al. 2002). Copolymers are often desired, because they have better mechanical properties, melting properties, and biodegradability compared to pure PHB, and thus are a better replacement for polyethylene (Lee 1996a, Shimuzu et al. 1999).

Although some microbes can use VFAs, these acids can be inhibitory or toxic depending on pH and acid concentration. Undissociated lipophilic molecules of fatty acids can easily pass through the cell membrane, and then dissociate to acidify the cytoplasm (Salmond et al. 1984). As a result, the proton gradient cannot be maintained, and energy generation and transport systems are disrupted (Lawford and Rousseau 1993). The accumulation of anions also increases osmotic pressure (Roe et al. 1998). High initial VFA concentrations thus result is low growth rate, acid utilization rate, and yield of PHA (Axe and Bailey 1995). However, as VFAs are gradually consumed, cell activity resumes. At low initial VFA levels, substrate limitation can also result in slow acid utilization rates (Yu et al. 2002). However, when VFAs are present at appropriate levels, the undissociated fatty acids molecules enter the cytoplasm, are activated, and metabolized into CO_2, cell biomass, or PHA (Yu et al. 2002).

Production of PHAs also depends on the C:N ratio. Low C:N ratios divert more carbon to energy formation and anabolic reactions for cell growth, leaving less for PHA production. However, at high C:N ratio, PHA productivity is increased due to insufficient supply of nitrogen for growth (Shimuzu et al. 1999). In the current study, nitrogen was supplemented initially to maximize cell growth in a condensed corn solubles (CCS) medium. When nitrogen became limiting at 48h, VFAs (acetic, butyric, lactic and propionic acid) were added to determine acid utilization rates, fermentation efficiency, PHA concentration, and PHA productivity.

3.3 Materials and Methods

3.3.1 Culture, Maintenance, and Inoculum Propagation

The ATCC 17699 type strain of *R. eutropha* was used. The culture was routinely transferred to nutrient broth, and incubated on a reciprocating shaker (250 rpm) at 30 deg C for 24 h. For short term maintenance the culture was stored on tryptic soy agar slants covered with mineral oil and stored in the refrigerator. Lyophilization was used for long term storage. Inoculum for all trials was prepared in a stepwise manner, by transferring the culture from TSA plates into 100 ml of the CCS medium (described below), then incubating the flasks for 24 h on a rotary shaker (250 rpm) at 30 deg C. These cultures were subsequently used, at a 1 % (v/v) rate, to inoculate aerated shake flasks experimental trials.

3.3.2 Medium

A low-cost medium based on condensed corn solubles (CCS) was developed in a prior study (chapter 2). This medium, containing 240 g CCS/L and a C:N ratio of 50:1 was the best medium for the growth of *R. eutropha*. The medium was prepared by mixing 230 ml CCS with 770 ml deionized water, adjusting the pH to 6.5 using 10M NaOH, then centrifuging at 11,000 rpm for 7 min at 15-25 deg C. The supernatant was then filtered through Whatman filter paper #113 and autoclaved. A filter sterilized 178 g/L NH_4HCO_3 stock solution was then added to adjust the C:N ratio to 50:1. The pH was further adjusted to 7.0 by adding 10 N H_2SO_4 before inoculation.

3.3.3 Experimental Design

Experimental trials were conducted in 1 L conical flasks that contained 800 ml of the CCS medium. Filter sterilized air (1 L/L/min) was provided through a glass sparger inserted through a rubber stopper, with a companion filtered vent tube. A few drops of antifoam (Cognis Clerol FBA 5059) were added to the medium before inoculation. Flasks were incubated at 30 deg C and 250 rpm for 48 h, since prior research had shown *R. eutropha* to reach its maximum population by this time. At that point, individual volatile fatty acids (acetic, butyric, lactic and propionic) were added at three different levels. Stock solutions of these acids were prepared and filter sterilized before use. For acetic, butyric, and propionic acids, levels of 1, 3, and 5 g/L were used, while 2, 4, and 8 g/L of lactic acid were added. Incubation was continued for an additional 72 h. Three replications were performed for each level of each fatty acid to determine the effect of individual fatty acids on cell viability, acid utilization, and PHA production.

3.3.4 Analytical Methods

Samples were collected at regular intervals during incubation, and pH was measured using an Acumet 950 pH meter. Viable cell counts were done with tryptic soy agar (TSA). Samples were also analyzed via a Waters HPLC system for content of sugars, organic acids and glycerol. These samples were first filtered through a non-sterile 0.2 um filter to remove solids, then frozen until analysis. An Aminex HPX87H column, operated at 65 deg C with a helium-degassed, 4 mM H_2SO_4 mobile phase at a flow rate of 0.6 ml/min was used. Peaks were detected using a refractive index detector. Standard solutions of maltose, glucose, lactic acid, acetic acid, propionic acid, succinic acid and

glycerol (at 3 and 30 g/L) were used to calibrate the integrator. Samples collected at 0, 72, and 120 h were also tested for ammonia and phosphate using Hach Ammonium and Phosphate Unicell tests.

At 24 and 120 h, 50 ml samples were collected to determine cell dry weights. To determine cell dry weight samples were centrifuged and the precipitate was dried in a hot air oven at 80 deg C for 2 days. An additional 50 ml sample was collected at 120 h to determine PHA levels. To measure PHB, 50 ml samples of broth were centrifuged, and pellets were then lyophilized and ground using a mortar and pestle. The method developed by Braunegg et al. (1978) was used to simultaneously extract and derivatize PHB to the 3-hydroxyalkanoate methyl esters of the monomers. In this method, 20-30 mg of ground cells were digested by adding 5 ml of digest solution and incubating at 90-100 deg C for 4 h. The digest solution contained 50 % chloroform, 42.5 % methanol, 7.5 % sulfuric acid (v/v %). After cooling, sample was washed with 2 ml of water, and the bottom layer (containing the chloroform and methyl esters of PHB) was collected and placed in a GC vial with anhydrous sodium sulfate (to remove residual water). Vials were frozen until analysis.

PHB was quantified using a Hewlett-Packard 5890 Series II gas chromatograph with a flame ionization detector (GC-FID) (Braunegg et al. 1978, Comeau et al. 1988). Split injection was used onto a Supelco SSP-2380 capillary column (30 m x 0.25 mm I.D. with 0.20 um film). The inlet head pressure was 28 psi and the temperature program started at 50 deg C for 4 min, then increased by 3 deg C/min to a final temperature of 146 deg C for 4 min. The injector and detector temperatures were 230 deg C and 240 deg C,

respectively. Purified poly-(3-hydroxybutyric acid co-3-hydroxyvaleric acid) obtained from Sigma-Aldrich was used for a standard calibration. The co-polymer consisted of 88 % 3-hydroxybutyric acid and 12 % 3-hydroxyvaleric acid. Co-polymer concentrations of 2-10 mg/ml chloroform were digested as above, then analyzed by GC-FID. Retention times were 14.9 min for methylated 3-hydroxybutyric acid, and 17.8 min for methylated 3-hydroxyvaleric acid.

3.3.5 Statistical Analysis

All trials were performed in triplicate. Maximum cell populations and fermentation efficiencies were analyzed using randomized complete block design with volatile fatty acids added at different levels as the treatments and time as blocks. Data were analyzed using the PROC GLM procedure of SAS software to determine F values and LS means. Growth rates and acid utilization rates were determined from the exponential regression equations of individual curves, then calculating the average of replications. The growth rates and acid utilization rates were statistically analyzed by ANCOVA to test homogeneity of slopes. Statistical data were analyzed at the significant level of $P<0.05$.

3.4 Results and Discussion

The objective of this study was to determine the effects of adding various volatile fatty acids to a 48 h culture of *R. eutropha*, which had been grown to a high cell density on a condensed corn solubles (CCS) medium. Of primary interest were factors including cell viability (and potentially additional growth), PHA production, and the utilization rates and fermentation efficiency of the individual acids. Since the initial 48 h of all trials

were replicates, that data was pooled to show the average growth response of *R. eutropha* on the CCS medium. Figure 3.1 is an average fermentation curve, showing cell populations and VFA utilization curves for *R. eutropha* in CCS medium during the initial 48 h. Table 3.1 provides rates of cell growth and VFA, ammonia, and phosphate utilization during the first 48 h.

The exponential phase lasted approximately 16 h, however cell numbers continued to increase at a lower rate reaching $2.6 * 10^9$cfu/ml by 48 h. *R. eutropha* achieved a maximum growth rate of 0.13 h^{-1} on the CCS medium in the aerated shake flasks. Since the wild type strain of *R. eutropha* (H16) was used, the 1.5-2.0 g/L of glucose present in the CCS medium was not metabolized (Madison and Huisman 1999). The CCS medium also contained 20-23.5 g/L of glycerol, and this was reduced to 11-13 g/L by 48 h (data not shown). Cell growth was also supported by the consumption of the organic acids present in CCS. Lactic acid was consumed at the fastest rate (0.054 g/L/h), followed by acetic, butyric, succinic and propionic. Butyric acid was not metabolized until stationary phase. Over 94 % of lactic acid was used, likely because less energy is needed to transport lactate to the central metabolic pathway (Huidong et al. 1997). At least two-thirds of the acetic, butyric, and succinic acids were also consumed within 48 h, while only 35 % of the propionic acid was utilized. At 48 h only minimal amounts of lactic, acetic, and butyric acids were present, while approximately 0.5 g/L of propionic and succinic acids were present.

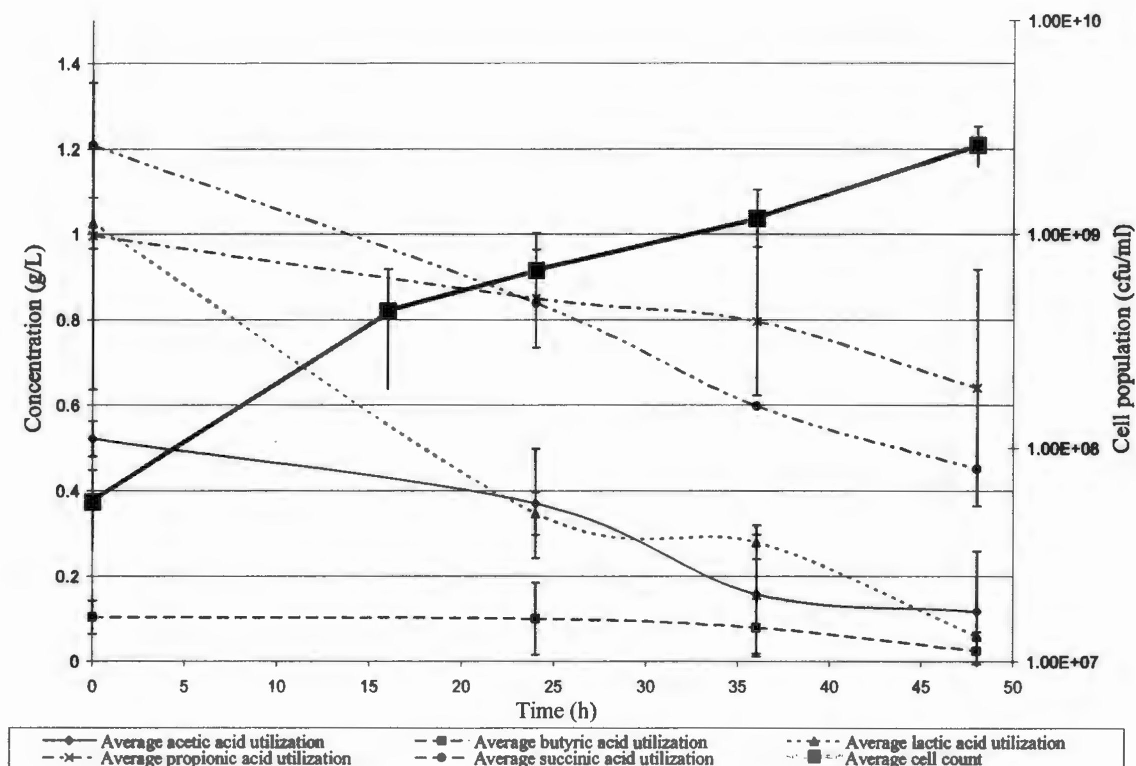

Figure 3.1 Growth and organic acid utilization by *R. eutropha* in the CCS medium though 48 h.

* Error bars represent one standard deviation.

Table 3.1 Growth and nutrient utilization rates of *R. eutropha* in CCS medium through 48h.

Maximum Cell Population (cfu/ml)	Maximum Growth Rate (h^{-1})	Ammonia Utilization Rate (g/L/h)	Phosphate Utilization Rate (g/L/h)
2.6E+09	0.13	0.17	0.22

VFA Utilization Rate (g/L/h)				
Acetic	**Butyric**	**Lactic**	**Propionic**	**Succinic**
0.033	0.026	0.054	0.010	0.021
VFA Fermentation Efficiency (%)				
Acetic	**Butyric**	**Lactic**	**Propionic**	**Succinic**
77.6	76.2	94.2	35.6	62.7

Yu et al. (2002) cultivated *R. eutropha* (H16) in a medium containing yeast extract, beef extract, peptone and ammonium sulphate. After 50 h of growth at 30 deg C in agitated 500 ml flasks, they achieved a dry cell mass of 5 g/L, compared to the 2 g/L level we achieved in CCS (data not shown). Other researchers have observed higher growth rates of *R. eutropha* when grown on various substrates or under different conditions. For example, Shimazu et al. (1999) obtained a growth rate of 0.26 h^{-1} when *R. eutropha* (H16) was grown in an aerated 5 L fermentor using Kunioka's defined medium, that contained yeast extract and meat extract as the carbon sources (Shimuzu et al. 1999). Marangoni et al. (2001) found that the glucose utilizing mutant strain of *R. eutropha* (DSM 545) achieved a growth rate of 0.26 h^{-1} on an invert sugar medium in 1 L Erlenmeyer flasks that were agitated at 150 rpm. When grown in glucose and galactose, growth rates were 0.2 h^{-1} and 0.11 h^{-1}, respectively. Utaker et al. (2002) found that the generation time of *R. eutropha* was 4.76 h when grown under autotrophic conditions in flasks filled with a basal medium and atmosphere containing 20 % O_2, 5 % CO_2, and 75 % H_2. The generation time calculated from the growth of the organism in the CCS medium was 5.3 h.

As noted by Tsuge et al. (2001), Tsuge (2002), and Lee (1999), inexpensive carbon sources generally result in lower growth rates of PHA producing organisms, due to the inefficient use of certain nutrients. In the present study the wild type strain of *Ralstonia eutropha* was not able to utilize the glucose and did not efficiently use the high amount of glycerol present in the CCS medium. To optimize the growth rate, Tsuge et al. (2001), Tsuge (2002), and Lee (1999), suggested the use of recombinant microbes that

could utilize the nutrients more efficiently. The presence of high concentrations of organic acids can also reduce growth rates, and levels below 1 g/L are recommended (Gorenflo et al. 2001, Tsuge et al. 2001). Therefore, most studies that use VFAs as additional carbon sources only add the organic acids after the organism had reached optimum growth, to trigger PHA granule formation (Yu et al. 2002, Shimuzu et al. 1999, Guocheng et al. 2001, Wang and Yu 2001). The total VFA concentration of CCS was about 4 g/L, and this might have resulted in the lower growth rates.

Ammonia and phosphate levels were also monitored during the initial growth phase. Ideally, one or both of these nutrients becomes limiting at the end of exponential phase, to trigger the shift from reproductive metabolism to PHA synthesis (Madison and Huisman 1999). Ammonia levels fell from 18 g/L to 6 g/L by 72 h, with a utilization rate of 0.17 g/L/h, while phosphate levels fell from 26.7 g/L to 10.6 g/L, with a utilization rate of 0.22 g/L/h. Because nitrogen and phosphate were not depleted until 96 h, this could have contributed to the continued increase in cell numbers observed after 48 h (data shown later). It is likely that at least some of the VFAs fed at 48 h were utilized for growth, until the point at which nitrogen became limiting.

3.4.1 Effects of Acetic Acid Addition

Figure 3.2 shows the average of three fermentation trials where 5 g/L acetic acid was added at 48 h. Trends were similar when either 1 or 3 g/L acetic acid were added, with cell numbers continuing to rise through 72 h, and acetic acid levels falling immediately after addition. Succinic acid utilization was not affected by addition of acetic acid, but propionate utilization was repressed. Propionic acid utilization is pH

dependent, and as other acids were used, the pH increased, allowing better utilization of propionic acid (Kim et al. 2005).

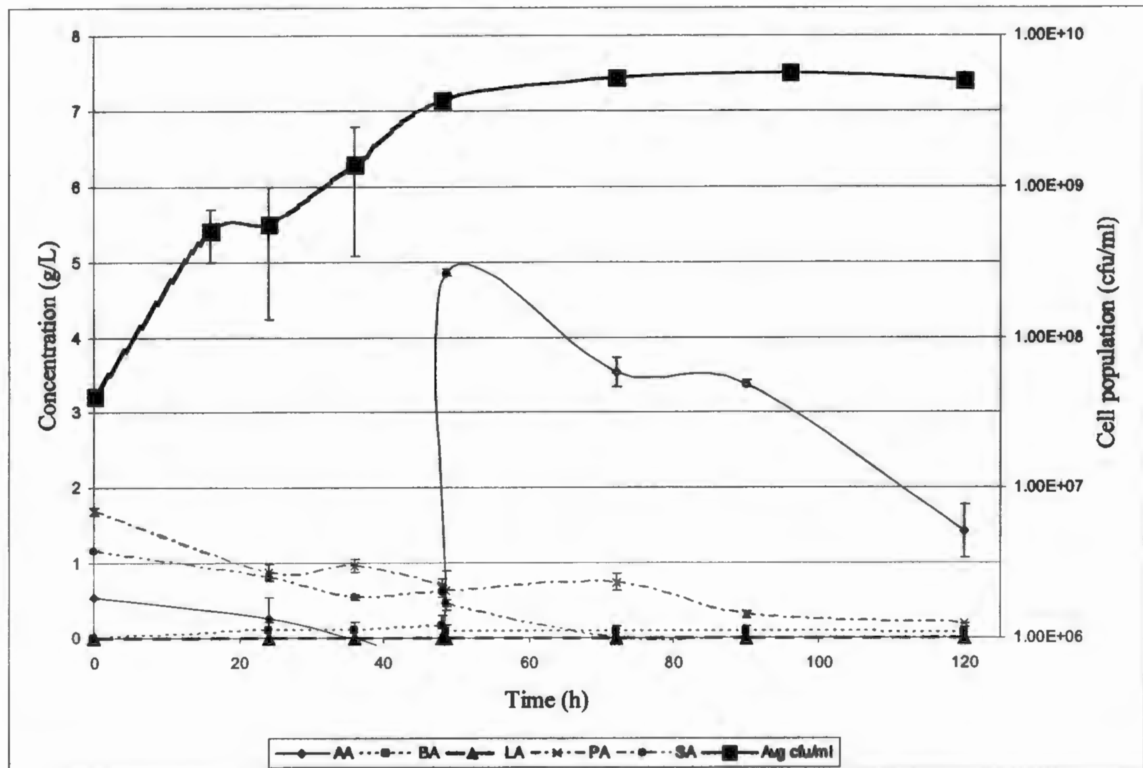

Figure 3.2 Growth and VFA utilization curve by *R. eutropha* when acetic acid fed at 5 g/L.
* Error bars represent one standard deviation.

Table 3.2 summarizes the acetic acid addition trials. The maximum cell population was directly related to the level of acetate fed to the culture. However, there was no significant difference in maximum cell population between the 3 and 5 g/L trials, perhaps due to the eventual limitation of nitrogen at 96 h. Acid utilization rates followed a similar trend, with higher rates observed at higher acid levels. The reduced fermentation efficiency at the 5 g/L level was due to fermentation being halted at 120 h, since the trend line in Figure 3.2 suggests that acetic acid would have been depleted by 140 h. The

ammonia and phosphate present at 3.9 g/L and 4.8 g/L at 72 h, respectively, were almost depleted by 96 h. This corresponded with the lack of additional growth after 72 h. As expected it was found that the utilization rates of both ammonia and phosphate was higher in the first 72 h since the nutrients were directed towards anabolism (data not shown).

Table 3.2 Comparison of maximum cell population, fermentation efficiency and nutrient utilization rates of *R. eutropha* when acetic acid was fed at different levels.

Acetic Acid Added (g/L)	Maximum Cell Population (CFU/ml)	Fermentation Efficiency (%)	Acid Utilization Rate (g/L/h)	Ammonia Utilization Rate (g/l/h)	Phosphate Utilization Rate (g/l/h)
1	3.28 E +09[a]	100[a]	0.018[a]	0.13[a]	0.17[a]
3	4.97 E +09[b]	100[a]	0.039[b]	0.10[a]	0.13[b]
5	5.70E +09[b]	70.6[b]	0.048[b]	0.13[a]	0.20[a]

[a,b] Means within column not sharing common superscript differ significantly ($P<0.05$)

At the end of each trial, samples were processed to determine PHA content, and Table 3.3 provides the PHA production data when acetic acid was fed at different levels. The highest PHA concentration, dry cell weight, and PHA productivity were obtained when acetic acid was fed at 5 g/L, but there were no significant difference between treatments. PHA contents were also statistically similar.

Table 3.3 Comparison of Dry Weight and PHA concentration, productivity and yield of *R. eutropha* when acetic acid was fed at different levels.

Acetic Acid Added (g/L)	PHA Concentration (g/L)	Cell Dry Weight (g/L)	PHA Productivity (g/L/h)	PHA Content (%)
1	1.7[a]	5.4[a]	0.013[a]	30.79[a]
3	1.9[a]	6.5[a]	0.016[a]	29.2[a]
5	2.9[a]	9.9[a]	0.024[a]	29.3[a]

[a,b] Means within column not sharing common superscript differ significantly (P<0.05)

Wang and Yu (2001) grew *R. eutropha* (ATCC 17699) in shake flasks in a basal medium containing yeast extract, beef extract, peptone and ammonium sulphate. Acetic acid was fed as the additional carbon source (5-6 g/L), with different C:N ratios (15:1, 30:1, 45:1 and 76:1). PHA productivity was in the range of 0.031-0.046 g/L/h, while PHA content from 20-55 % depending on concentration of carbon sources and the C:N ratio of the medium. Lee (1999) reported that purified carbon sources resulted in better PHA yields, productivities, and concentration as compared to low cost medium. He also noted that the recombinant strain of *R. eutropha* (H16), that contained glucose-utilizing genes of *E. coli*, had a PHA productivity of 3.1 g/L/h and a PHA concentration of 95.9 g/L when grown in a glucose medium. When a recombinant *E. coli* (harboring the *R. eutropha* gene) was grown in the same medium, the PHA productivity was 4.63 g/L/h and concentration was 141.6 g/L. However, when the same strain of *E. coli* was grown in low cost whey medium, PHA productivity declined to 0.87g/L/h and PHA concentration fell to 43 g/L (Lee 1996a).

In this study the wild-type strain of *R. eutropha* was grown on an inexpensive medium, and the combination might have resulted in low PHA concentration and productivity. But from an economical point of view, the use of purified media to increase PHA yield will significantly increase the production cost. Thus, use of recombinant strains that have better capacity to metabolize inexpensive carbon sources, and corresponding improvements to fermentation strategies need to be developed to increase the PHA productivity and lower production costs. Based on trials with the CCS basal medium, feeding 5 g/L acetic acid provided the best results. Although fermentation efficiency was highest at 3 g/L, acetic acid would also have also been completely utilized had the 5 g/L trial been extended.

3.4.2 Effects of Butyric Acid Addition

Figure 3.3 shows the average of three fermentation trials where 5 g/L butyric acid was added at 48 h. The trends were similar in trials when either 1 or 3 g/L butyric acid were added, with cell numbers continuing to rise through 48 h, and butyric acid levels falling immediately after addition. Succinic and propionic acid utilization followed similar trends as observed during acetic acid addition trials.

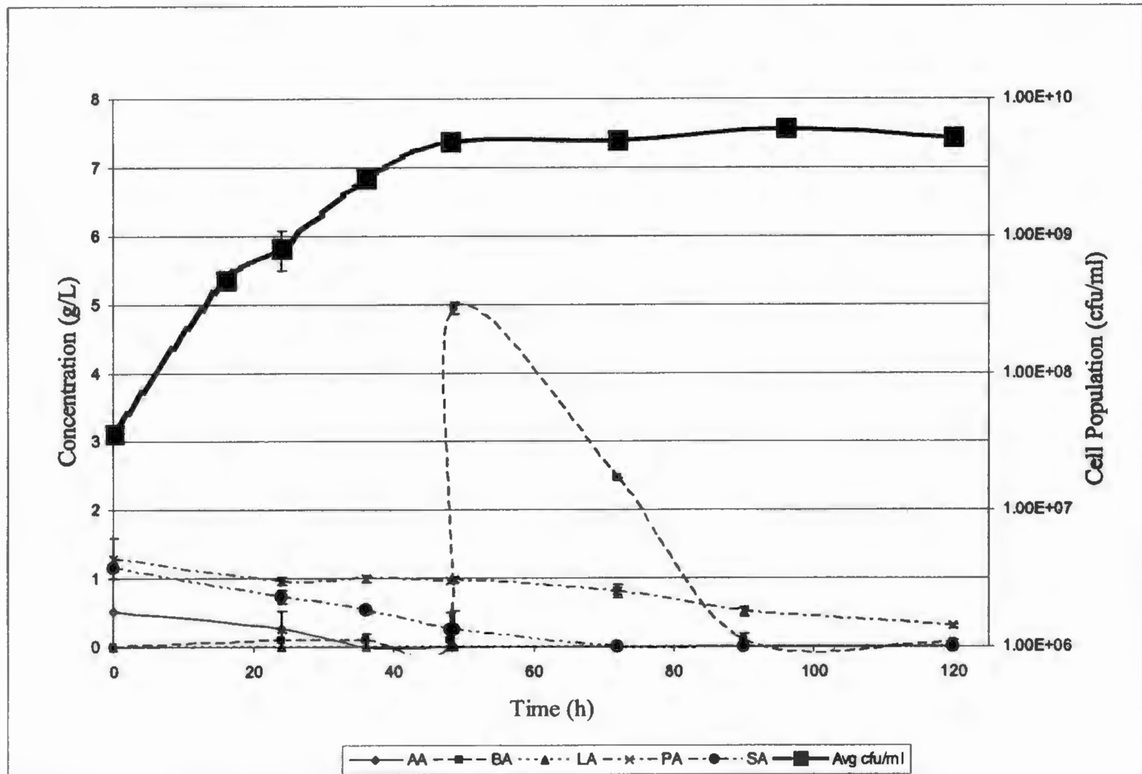

Figure 3.3 Growth and VFA utilization curve by *R. eutropha* when butyric acid fed at 5 g/L.
* Error bars represent one standard deviation.

Table 3.4 summarizes the results when butyric acid was fed at different levels. The highest cell population was reached when the organism was fed butyric acid at 5 g/L, as expected. Surprisingly the maximum cell count was statistically similar when the organism was fed 1 g/L butyric acid, but was lower when 3 g/L butyric acid was fed. Butyric acid consumption rates were significantly higher at the 5 g/L feeding rate ($P<0.05$), but fermentation efficiency were similar at the 1 and 5 g/L feeding rate. Ammonium and phosphate utilization rates decreased as the cells reached stationary phase, and followed the same trends as observed in the acetic acid feeding trials.

Table 3.4 Comparison of maximum cell population, fermentation efficiency and nutrient consumption rates of *R. eutropha* when butyric acid was fed at different levels.

Butyric Acid Added (g/L)	Maximum Cell Population (CFU/ml)	Fermentation Efficiency (%)	Acid Utilization Rate (g/L/h)	Ammonia Utilization Rate (g/L/h)	Phosphate Utilization Rate (g/L/h)
1	5.33E +09[a]	100[a]	0.022[a]	0.14[a]	0.11[a]
3	4.42E +09[b]	95.6[b]	0.041[a]	0.14[a]	0.16[b]
5	6.17E +09[a]	98.9[ab]	0.068[b]	0.13[a]	0.17[b]

[a,b] Means within column not sharing common superscript differ significantly (P<0.05)

Table 3.5 shows that PHA concentration, productivity, PHA content, and dry cell weight were directly proportional to the amount of butyric acid fed, although dry cell weight was not significantly different. The acid was almost completely utilized at the 5 g/L feeding level, resulting in the highest rate and extent of PHA production. The PHA concentration and productivity were significantly higher (P<0.05) at 5 g/L. Shimizu et al. (1999) reported that 5 g/L PHA was obtained when *R. eutropha* (H16) was grown in a defined medium (containing 10 g/L yeast extract, 5 g/L meat extract) with a C:N ratio of 42:1, and butyric and valeric acids both fed at 1.5 g/L. Tsuge (2002) reported that the theoretical yield of PHA from butyric acid is 0.65-0.98 g/g substrate. The PHA yield in this study (0.82 g/g) was comparable to the theoretical yield, but in addition to the butyric acid, 0.6 g/L of propionic acid was also consumed during the PHA accumulation phase.

Table 3.5 Comparison of Dry Weight and PHA concentration, productivity and yield of *R. eutropha* when butyric acid was fed at different levels.

Butyric Acid Added (g/L)	PHA Concentration (g/L)	Dry Weight (g/L)	PHA Productivity (g/L/h)	PHA Content (%)
1	0.4[a]	9.8[a]	0.003[a]	3.9[a]
3	1.5[a]	8.8[a]	0.013[b]	17.4[b]
5	4.6[b]	14.5[a]	0.037[c]	31.9[b]

[a,b] Means within column not sharing common superscript differ significantly ($P<0.05$)

3.4.3 Effects of Lactic Acid Addition

Figure 3.4 shows the average of three fermentation trials where 8 g/L lactic acid was added at 48 h. The trends were similar when either 2 or 4 g/L lactic acid were added, with cell numbers rising through 72 h, and lactic acid levels falling immediately after addition. One difference with the prior acetic and butyric trials was that the CCS medium used in these trials contained higher initial levels of lactic acid, such that the total initial organic acid concentration was 4.9 g/L. This reduced the growth rate, so that the 48 h cell population was lower. Tsuge et al. (2001) previously observed that high presence of other organic acid levels (1-3.5 g/L) reduced the growth rate (from 0.45 h^{-1} to 0.12 h^{-1}) and dry cell weight (from 27.4 g/L to 2.4 g/L). Succinic and propionic acid utilization were both repressed until the majority of lactic acid was consumed.

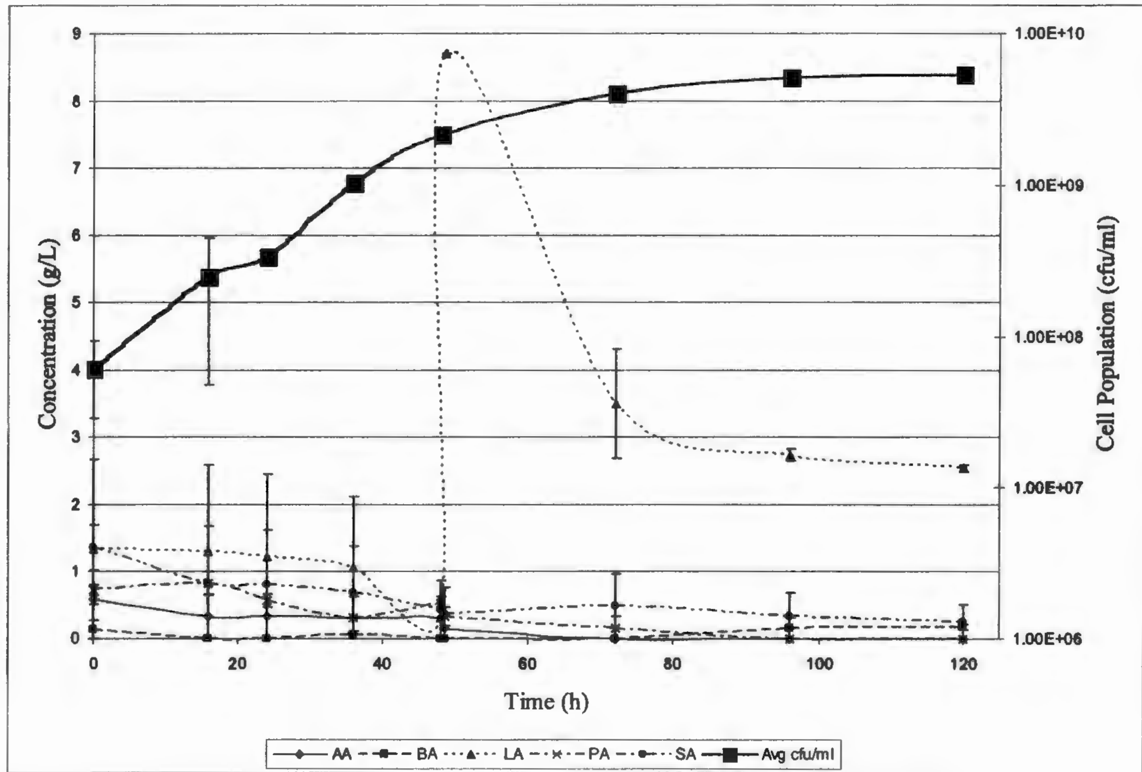

Figure 3.4 Growth and VFA utilization curve by *R. eutropha* when lactic acid fed at 8 g/L.
* Error bars represent one standard deviation.

Table 3.6 summarizes the results when lactic acid was fed at different levels. The highest cell population was reached when the organism was fed 4 g/L lactic acid. The 8 g/L level may have resulted in metabolic inhibition that reduced growth (Tsuge et al. 2001). When lactic acid was fed at 2 and 4 g/L, it was completely used by the end of the trial, compared to 70 % fermentation efficiency at 8 g/L. Again, the high acid level in the 8 g/L trial appears to have inhibited metabolism. Lactic acid consumption rates were similar at the 4 and 8 g/L levels. The rates of ammonia and phosphate utilization were somewhat slower than those observed when acetic and butyric acids were fed.

Table 3.6 Comparison of maximum cell population, fermentation efficiency and nutrient consumption rate of *R. eutropha* when lactic acid was fed at different levels.

Lactic Acid Added (g/L)	Maximum Cell Population (CFU/ml)	Fermentation Efficiency (%)	Acid Utilization Rate (g/L/h)	Ammonia Utilization Rate (g/L/h)	Phosphate Utilization Rate (g/L/h)
2	4.03E +09[a]	100[a]	0.037[ab]	0.10[a]	0.13[a]
4	6.83E +09[c]	100[a]	0.055[bc]	0.11[a]	0.10[a]
8	5.32E +09[b]	70.7[b]	0.080[c]	0.10[a]	0.10[a]

[a,b,c] Means within column not sharing common superscript differ significantly (P<0.05)

PHA concentrations and productivities, along with cell dry weights, were substantially lower for the 2 and 4 g/L lactic acid levels (Table 3.7), compared to acetic and butyric acid. The 8 g/L addition level showed improvement, especially in PHA content, even though the low cell numbers limited PHA concentration. The PHA concentration, productivity and PHA content were significantly higher (P<0.05) at 8 g/L addition. These data suggest that the optimal level of lactic acid falls between 4 and 8 g/L. Tsuge (2001) grew *R. eutropha* ATCC 17697 in a chemically defined medium with pH control, and then fed lactic acid at a rate of 0.8 g/L for 42 h (33.6 g/L total lactic acid addition). He obtained a PHA concentration of 54.8 g/L, PHA content of 73.1 %, and a productivity of 1.3 g/L/h (Tokiwa and Calabia 2004). By feeding lactic acid at a slow rate, Tsuge (2001) was able to maintain total acid levels below inhibitory levels. In our trials the higher acid levels appeared to have lowered cell growth and consequently repressed PHA accumulation. Thus low cell concentration might have lowered the PHA concentration and productivity even though the PHA content might have been high (Lee 1996b).

Table 3.7 Comparison of Dry Weight and PHA concentration, productivity and yield of *R. eutropha* when lactic acid was fed at different levels.

Lactic Acid Added (g/L)	PHA Concentration (g/L)	Dry Weight (g/L)	PHA Productivity (g/l/h)	PHA Content (%)
2	0.1[a]	3.5[a]	0.001[a]	4.0[a]
4	0.8[a]	4.0[a]	0.006[a]	17.0[b]
8	2.4[b]	6.0[a]	0.020[b]	40.7[c]

[a,b,c] Means within column not sharing common superscript differ significantly ($P<0.05$)

3.4.4 Effects of Propionic Acid Addition

Figure 3.5 shows the average of three fermentation trials where 5 g/L propionic acid was added at 48 h. Similar trends were observed in the trials when either 1 or 3 g/L propionic acid were added. The cell numbers increased through 72 h, and propionic acid was used immediately after it was fed. Utilization of acetic and succinic acids was repressed by the addition of propionic acid, but the other organic acids present in CCS were utilized.

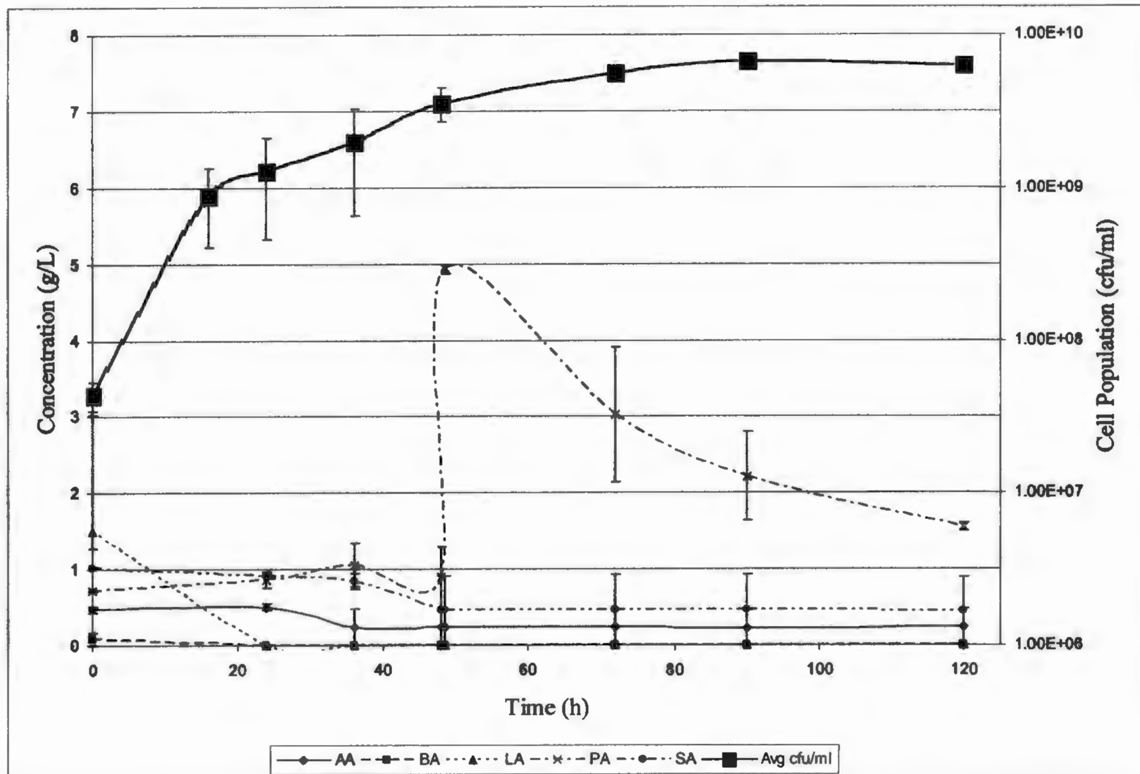

Figure 3.5 Growth and Acid utilization curve by *R. eutropha* when propionic acid fed at 5 g/L.
* Error bars represent one standard deviation.

The results obtained by feeding different levels of propionic acid, shown in Table 3.8, indicate that the highest cell population and acid utilization rate were reached at the 5 g/L propionic acid level. However, the 5 g/L propionic acid level resulted in a significantly ($P<0.05$) lower fermentation efficiency than that at 1 g/L and 3 g/L. Figure 3.5 suggests that propionic acid would have been completely utilized with an additional 24 h fermentation time. Ammonia and phosphate utilization were again somewhat reduced, as in the lactic acid trials.

Table 3.8 Comparison of maximum cell population, fermentation efficiency and nutrient consumption rate of *R. eutropha* when propionic acid was fed at different levels.

Propionic Acid Added (g/L)	Maximum Cell Population (CFU/ml)	Fermentation Efficiency (%)	Acid Utilization Rate (g/L/h)	Ammonia Utilization Rate (g/L/h)	Phosphate Utilization Rate (g/L/h)
1	2.09E +09[a]	93.3[a]	0.024[a]	0.08[a]	0.13[a]
3	4.02E +09[b]	96.3[a]	0.042[b]	0.09[a]	0.10[a]
5	6.67E +09[c]	68.6[b]	0.046[b]	0.09[a]	0.13[a]

[a,b,c] Means within column not sharing common superscript differ significantly ($P<0.05$)

As seen from Table 3.9, PHA concentration, productivity, PHA content and cell dry weights were highest at 5 g/L of propionic acid addition. The final dry weights obtained were not significantly different between the 3 and 5 g/L treatments. Kim et al. (1992) obtained a PHA content of 70 % from *R. eutropha* (H16) grown on fructose and propionic acid, when propionic acid was maintained below 1.5 g/L using a pH stat method. Shang et al. (2003) reported a PHA productivity of 1.8 g/L/h when propionic acid was fed to *R. eutropha* (NCIMB 11599) in a chemically defined medium containing glucose. Tsuge et al. (2002) found that when propionic acid was fed to *Alcaligenes eutrophus* at a concentration of 3 g/L in a two stage pH controlled system, the final dry weights attained was 64 g/L and the total polymer content was 55 %. While the 5 g/L propionic acid addition level was considered the best in this study using a basal CCS medium, the results are far below those described in the studies above.

Table 3.9 Comparison of Dry Weight and PHA concentration, productivity and yield of *R. eutropha* when propionic acid was fed at different levels.

Propionic Acid Added (g/L)	PHA Concentration (g/L)	Dry Weight (g/L)	PHA Productivity (g/L/h)	PHA Content (%)
1	0.7^{a}	6.0^{a}	0.005^{a}	10.2^{a}
3	2.1^{a}	10.0^{ab}	0.020^{b}	20.7^{b}
5	4.3^{b}	14.0^{bc}	0.036^{c}	29.3^{c}

[a,b,c] Means within column not sharing common superscript differ significantly ($P<0.05$)

One of the major problems of using inexpensive carbon sources for PHA production is the relatively low growth rate of the bacteria, which in turn lowers PHA productivity (Tsuge 2002). The CCS medium used in this study contained glucose, organic acids and glycerol. The H16 strain of *Ralstonia eutropha* was not able to utilize glucose and partly utilized the glycerol. The presence of high concentration of organic acids in the growth phase might have lowered the growth rate (Lee et al. 1999). Moreover the trials were carried out in 1 L shake flasks, in which did not permit precisely controlled aeration. The cumulative effect of these factors might have lowered the PHA concentration and productivity.

3.4.5 Comparison of Acetic, Butyric, Lactic and Propionic acids

The best levels of acetic, butyric, lactic and propionic acids were compared, based on the parameters shown in Table 3.10. Yu et al. (2002) found that when both acetate and propionate were present, acetate was utilized preferentially for biomass production. Propionate was used primarily for generation of hydroxyvalerate monomer units in the copolymer of P(3HB-co3HV) in the PHA accumulation stage. Butyrate was used in

preference to propionate for formation of PHB, because it is energetically more favorable and thus gave best results in terms of fermentation efficiency, PHA concentration and productivity (Yu et al. 2002). In this study butyrate yielded best results in terms of all the parameters. Propionic acid yielded similar results, except that fermentation efficiency was lower. Lactic acid yielded the lowest cell population, dry cell weight, and PHA concentration but the cells rapidly used the acid and achieved the highest PHA content. This might be because of the presence of highest concentration of the organic acids in the media initially in the lactic acid trials. Acetic acid resulted in intermediate cell population, dry cell weight, acid consumption rate and PHA content. But PHA concentration and productivity were low. The overall PHA concentration, PHA content and productivity for all the acids were lower than those reported in the literature. The combination of factors such as use of low cost medium as opposed to purified carbon sources and use of wild type strain of *R. eutropha* might have resulted in lower PHA production (Lee et al. 1999).

Table 3.10 Comparison of optimal levels of four volatile fatty acids on key fermentation parameters.

Acid and Level (g/L)	Maximum Cell Population (CFU/ml)	Dry Weight (g/L)	Fermentation Efficiency (%)	Acid Utilization Rate (g/L/h)	PHA Conc. (g/L)	PHA Prod. (g/L/h)	PHA Content (%)
Acetic (5)	5.70E +09[a]	9.9[ab]	70.6[a]	0.048[a]	2.9[ab]	0.024[ab]	29.2[a]
Butyric (5)	6.17E +09[ab]	14.5[b]	95.6[b]	0.041[a]	4.6[a]	0.037[a]	31.9[a]
Lactic (8)	5.32E +09[a]	6[a]	70.7[a]	0.080[b]	2.4[b]	0.020[b]	40.7[a]
Propionic (5)	6.67E +09[b]	14.7[b]	68.6[a]	0.046[a]	4.3[a]	0.036[a]	29.3[a]

[a,b,c,d] Means within column not sharing common superscript differ significantly ($P<0.05$)

There were no significant differences between the ammonia utilization rates for the different treatments, but acetic and butyric acids additions showed somewhat faster utilization rates for phosphates (data not shown). In all trials there were only small amounts of ammonia or phosphate left in the media at the end of each trial. As noted by Shimizu et al. (1999) and Wook et al. (1999), the complete lack of nitrogen may suppress enzyme activity in PHA synthesis. Thus a small amount of ammonia in the media (1-3 g/L) might have been necessary to trigger PHA synthesis. Asenjo et al. (2004) reported that to broaden the molecular weight distribution of PHA produced, phosphate levels should be at least 0.7 g/L. So the presence of residual phosphate (3-5 g/L) in the media might help in better PHA characteristics.

3.5 Conclusions

Butyric acid at 5 g/L showed best results in terms of PHA production followed by propionic acid at 5 g/L. Lactic acid resulted in lowest PHA concentration whereas acetic acid showed intermediate results. Ammonia and phosphate present in the media were used at similar rates for all the individual acids and were completely utilized by 96 h. It can be concluded that none of the volatile fatty acids are inhibitory provided they are added at the stationary phase of growth of the organism. The volatile fatty acids can be added at high levels once the organism attained its maximum growth.

Chapter 4

PHA Productivity and Yield of *Ralstonia eutropha* when Intermittently or Continuously Fed a Mixture of Volatile Fatty Acids

4.1 Abstract

This study evaluated three strategies of feeding a mixture of volatile fatty acids to *R. eutropha* ATCC 17699 for the purpose of producing PHA. The culture was grown to a high cell density ($2.3 * 10^{10}$ cfu/ml) in a nitrogen supplemented, condensed corn solubles (CCS) medium in a 5 L Bioflo 3 bioreactor. After 48 h of incubation at 30 deg C, pH 7.0, 500 rpm agitation, and aeration of 1L/L/min, a mixture of volatile fatty acids was added to the bioreactor. This acid mixture (referred to as artificial rumen fluid, ARF) contained acetic, butyric, lactic and propionic acids in the ratio of 10:2:15:20. The feeding strategies included: 1) addition of 124 ml at 48, 72, and 96 h, 2) addition of 19.5 ml every 3 h, from 48-96 h and again from 88-109 h. and 3) continuous addition of 7.75 ml/h from 48-96 h. The 3 h feeding strategy gave the best results in terms of maximum cell population ($5.68 *10^{10}$ cfu/ml), overall acid consumption rate (0.25 g/L/h), fermentation efficiency (100 %), PHA concentration (8.37 g/L), PHA content (39.52 %) and productivity (0.0697 g/l/h). However there was no significant difference in PHA concentration, productivity and PHA content using three different feeding strategies.

4.2 Introduction

R. eutropha can produce polyhydroxyalkanote (PHA) under certain nutritional conditions. Typically, PHA is accumulated when excess carbon is present and another nutrient, such as nitrogen, phosphorous or oxygen, is limiting (Madison and Huisman

1999). However, Shang et al. (2003) found that carbon source limitation can also result in accumulation of polyhydroxybutyrate (PHB). The lowest concentration (2.5 g/L) of glucose favored PHB accumulation, with more than 50 % of PHB accumulated before phosphate limitation occurred, as opposed to 25 % PHB accumulation for glucose concentrations of 4 and 9 g/L (Shang et al. 2003a). They postulated that carbon limitation might slow the TCA cycle, decrease coenzyme A, and direct acetate to production of PHB.

PHA production generally occurs during stationary phase, hence cells are first grown to high density, after which a key nutrient is limited to trigger PHA synthesis (Lee 1996b). Because of this dual phase process, PHA production lends itself to fed-batch, as well as, continuous operation. In the fed-batch mode, cells are grown through exponential phase in a balanced medium to maximize growth rate. This medium is formulated to run out of a key nutrient when the maximum cell population is achieved, then additional carbon is added to produce PHA (Madison and Huisman 1999). This follows Pontryagin's maximum principle, which is an optimal feeding strategy for fed-batch fermentation (Shioya 1992). The key to this principle is determining the optimum switching time (T_c). The maximum growth rate (u_{max}) should be initially maintained, then switched to the critical growth rate (u_c) at T_c to maximize the specific product production rate (ρ_{max}). Since growth rate is affected by C:N ratio, it should also be changed at T_c.

Shimizu et al. (1999) compared this optimal feeding strategy to a constant growth rate strategy which maintained the growth rate of *R. eutropha* uniformly at u_c. The organism was grown in a 5 L fed batch fermentor with butyric and valeric acids as the

carbon sources. They found that P(HB-HV) productivity was higher using the optimal feeding strategy, due to the significantly higher cell biomass levels achieved. They noted that the calculation of optimum switch time can be applied to longer fermentation times and different carbon sources as well (Shioya 1992).

For continuous production of PHB from *R. eutropha* (WSH3*)*, Du et al. (2001) used a two stage system. In the first stage, growth rate was maximized by providing high rates of aeration (1.5 v/v/min), agitation (600-1000 rpm), and dissolved oxygen saturation (20 %). Temperature was set at 30 deg C, while pH 7.0 was maintained using ammonia water (the source of nitrogen). The maximum dilution rate was 0.21 h^{-1}, with 50 g/L glucose serving as the carbon source. The medium from the first stage was continuously transferred to the second stage. Residual ammonium ions from the first stage carried over to the second stage, and to maintain a higher, constant C:N ratio, additional carbon (500 g/L of glucose) was fed into the second reactor at 0.054 h^{-1}. Higher dilution rates in the second reactor decreased PHB productivity (Guocheng et al. 2001) and the amount of PHA produced by continuous fermentation was lower than in comparable fed batch fermentation. Additional work is needed to improve PHA productivity in continuous systems (Lee 1996b).

A variety of carbon sources have been used for production of PHA using both fermentation strategies. Carbohydrates, oils, alcohols, acids, and hydrocarbons are potential carbon sources for PHA production. Cane and beet molasses, cheese whey, plant oils, hyrolysates of corn, cellulose, hemicellulose, palm oil, soybean oil, tallows, corn steep liquor, casamino acids, and food scraps had been used to produce PHA using

different organisms (Du and Yu 2002, Kahar et al. 2002, Koller et al. 2005, Loo et al. 2005, Marangoni et al. 2001, Taniguchi et al. 2003). Chang et al. (1994) and Lee (1996b) noted that fatty acids would be expensive carbon sources for PHA production, and that this would only be economical when the fatty acids were produced from agricultural wastes by other organisms. In this present study, condensed corn solubles (a byproduct of fuel ethanol production) was used as the primary growth medium. A mixture of acetic, lactic, butyric and propionic acid was then fed to this cell mass to trigger PHA production.

4.3 Materials and Methods

4.3.1 Culture, Maintenance and Inoculum Propagation

The ATCC 17699 type strain of *R. eutropha* was used. The culture was routinely transferred to nutrient broth, and incubated on a reciprocating shaker (250 rpm) at 30 deg C for 24 h. For short term maintenance the culture was stored on tryptic soy agar slants covered with mineral oil and stored in the refrigerator. Lyophilization was used for long term storage. Inoculum for all trials was prepared in a stepwise manner, by transferring the culture from TSA plates into 100 ml of the CCS medium (described below), then incubating for 24 h on a rotary shaker (250 rpm) at 30 deg C. The cultures were tested for purity by performing different biochemical tests. The pure cultures were subsequently used, at a 1 % (v/v) rate, to inoculate bioreactor trials.

4.3.2 Medium

A low-cost medium based on condensed corn solubles (CCS) was developed in a prior study. This medium, containing 240 g CCS/L, with a C:N ratio of 50:1 was the best

medium for the growth of *R. eutropha*. The medium was prepared by mixing 1,370 ml CCS with 4,630 ml deionized water, adjusting the pH to 6.5 using 10 M NaOH, then centrifuging at 11,000 rpm for 7 min at 15-25 deg C. The supernatant was then filtered through Whatman filter paper #113 and autoclaved. A filter sterilized 178 g/L NH_4HCO_3 stock solution was then added to adjust the C:N ratio to 50:1. The pH was further adjusted to 7.0 by adding 10 N H_2SO_4 before inoculation.

4.3.3 Experimental Design

Experimental trials were conducted in 5 L New Brunswick, Bioflo III bioreactor that contained 4 L of CCS medium. Filter sterilized air (1 L/L/min) was provided through an air supply port. A few drops of antifoam (Cognis Clerol FBA 5059) were added to the medium before inoculation. Bioreactors were incubated at 30 deg C and 500 rpm for 48 h, since prior research had shown *R. eutropha* to reach its maximum population by this time. At that point, mixed volatile fatty acids (acetic, butyric, lactic and propionic) were added using three different strategies. At 48 h, a mixture of volatile fatty acids (total volume of 372 ml over 48-50 h) was added using three different strategies. The volatile fatty acid mixture contained, by volume: 10 g/L acetic acid (3.72 ml), 2 g/L butyric acid (0.74 ml), 15 g/L lactic acid (5.59 ml), and 20 g/L propionic acid (7.45 ml). In the first set of experiment, 124 ml of the mixed acid solution was added at 48, 72, and 96 h. In the second set of experiments, approximately 19.5 ml the mixed acid solution was added at 48 h and every 3 h thereafter until 78 h. Supplementation was resumed at 88 h and continued every 3 h through 109 h. In the third set of experiments, acid addition was done continuously from 48 to 96 h by use of a peristaltic pump, which added the mixed acid

solution at a rate of 7.75 ml/h. Incubation was continued until 144 h. Two replications were performed for each feeding strategy to determine the effect of mixed fatty acids on cell viability, acid utilization, and PHA production.

4.3.4 Analytical Methods

Samples were collected at regular intervals and pH was measured using an Acumet 950 pH meter. Viable cell counts were done with tryptic soy agar (TSA). Samples were also analyzed via a Waters HPLC system for content of sugars, organic acids and glycerol. These samples were first filtered through a non-sterile 0.2 um filter to remove solids, then frozen until analysis. An Aminex HPX 87H column, operated at 65 deg C with a helium-degassed, 4 mM H_2SO_4 mobile phase at a flow rate of 0.6 ml/min was used. Peaks were detected using a refractive index detector. Standard solutions of maltose, glucose, lactic acid, butyric acid, acetic acid, propionic acid, succinic acid and glycerol (at 3 and 30 g/L) were used to calibrate the integrator. Samples collected at 0, 72, and 120 h were also tested for ammonia and phosphate using Hach Ammonium and Phosphate Unicell tests. At 72, 96 and 144 h, 50 ml samples were collected to determine cell dry weights. Samples were centrifuged and the precipitate was dried in the hot air oven at 80 deg C for 2 days.

To measure PHB during bioreactor trials, 50 ml samples of broth were collected at 72, 96 and 144 h and centrifuged. The pellets were then lyophilized and ground using a mortar and pestle. The method developed by Braunegg et al. (1978) was used to simultaneously extract and derivatize PHB to the 3-hydroxyalkanoate methyl esters of the monomers. In this method, 20-30 mg of ground cells were digested by adding 5 ml of

digest solution and incubating at 90-100 deg C for 4 h. The digest solution contained 50 % chloroform, 42.5 % methanol, 7.5 % sulfuric acid (v/v %). After cooling, sample was washed with 2 ml of water, and the bottom layer (containing the chloroform and methyl esters of PHB) was collected and placed in a GC vial with anhydrous sodium sulfate (to remove residual water). Vials were frozen until analysis.

PHB was quantified using a Hewlett-Packard 5890 Series II gas chromatograph with a flame ionization detector (GC-FID) (Braunegg etal. 1978, Comeau et al.1988). Split injection was used onto a Supelco SSP-2380 capillary column (30 m x 0.25 mm I.D. with 0.20 um film). The inlet head pressure was 28 psi and the temperature program started at 50 deg C for 4 min, then increased by 3 deg C/min to a final temperature of 146 deg C for 4 min. The injector and detector temperatures were 230 deg C and 240 deg C, respectively. Purified poly (3-hydroxybutyric acid co-3-hydroxyvaleric acid) obtained from Sigma-Aldrich was used for a standard calibration. The co-polymer consisted of 88 % 3-hydroxybutyric acid and 12 % 3-hydroxyvaleric acid. Co-polymer concentrations of 2-10 mg/ml chloroform were digested as above, and then analyzed by GC-FID. Retention times were 14.9 min for methylated 3-hydroxybutyric acid, and 17.8 min for methylated 3-hydroxyvaleric acid.

4.3.5 Statistical Analysis

All trials were performed in duplicate. Maximum cell populations, fermentation efficiencies, PHA productivities, yields and concentrations were analyzed using randomized complete block design with volatile fatty acids added at different levels as the treatments and time as blocks. Data were analyzed using the PROC GLM procedure

of SAS software to determine F values and LS means. Growth rates and acid utilization rates were determined from the exponential regression equations of individual curves, then calculating the average of replications. The growth rates and acid utilization rates were statistically analyzed by ANCOVA to test homogeneity of slopes. Statistical data were analyzed at the significant level of $P<0.05$.

4.4 Results and Discussion

The objective of this study was to determine the effects on cell viability and PHA production of adding mixed volatile fatty acids (to simulate artificial rumen fluid) to a 48 h culture of *R. eutropha*. In all bioreactor trials, the organism was incubated under similar condition for the first 48 h, and this data was relatively uniform. Therefore, Figure 4.1 and Table 4.1 show the average cell population, growth rate, acid utilization, and ammonia and phosphate uptake rates during this growth period.

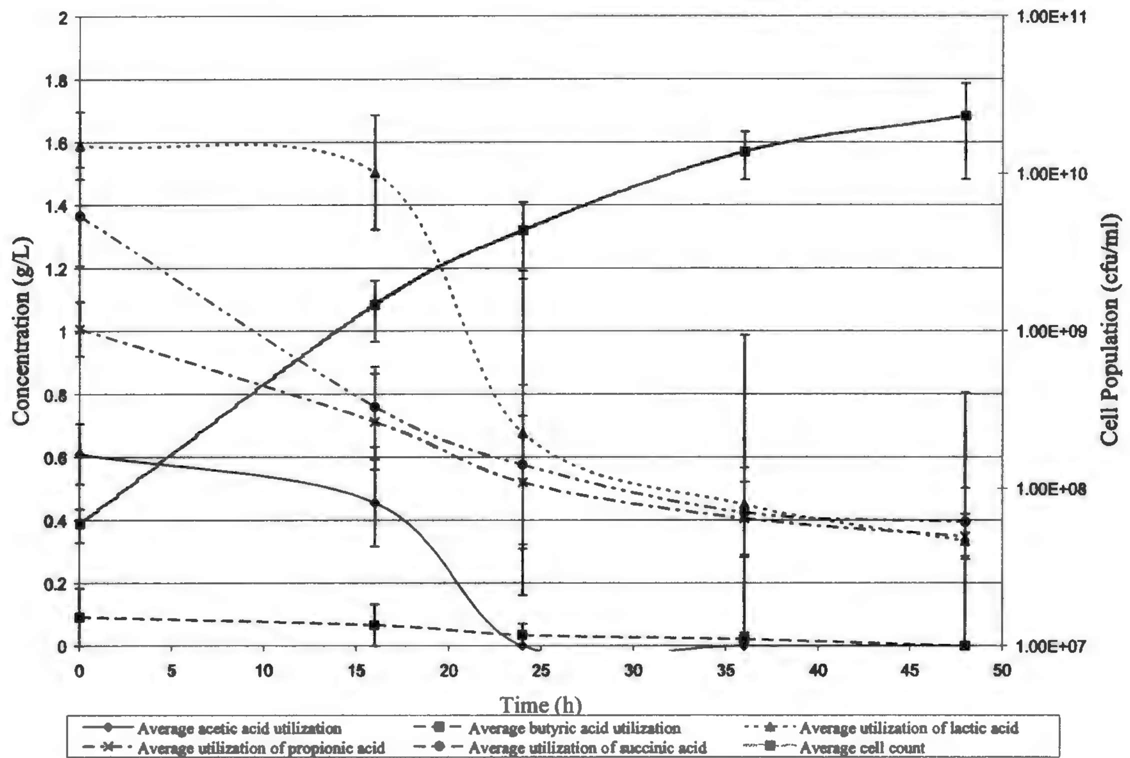

Figure 4.1 Average growth rate and organic acid utilization during the initial 48 h incubation in the CCS medium.

* Error bars represent one standard deviation.

Table 4.1 Growth and nutrient utilization rates of *R. eutropha* during the initial 48 h incubation in the CCS medium.

Maximum Cell Population (cfu/ml)	Growth Rate (h^{-1})	Ammonia Utilization Rate (g/L/h)	Phosphate Utilization Rate (g/L/h)
2.3E+10	0.20	0.23	0.23

Acid Utilization Rate (g/L/h)				
Acetic	**Butyric**	**Lactic**	**Propionic**	**Succinic**
0.051	0.026	0.046	0.023	0.027
Acid Consumption (%)				
Acetic	**Butyric**	**Lactic**	**Propionic**	**Succinic**
100	100	81.25	65.5	71.2

Compared to shake flasks trials discussed in Chapter 3, the maximum cell population in bioreactor trials was almost 10 fold higher. Likewise, the growth rate of *R. eutropha* was also higher in the bioreactor (0.20 h^{-1}) compared to the aerated shake flasks (0.13 h^{-1}). The difference in growth was likely due to the improved aeration and agitation provided in the bioreactor, along with more consistent pH control. Organic acid utilization rates were generally similar for all trials, except for lactic acid. It was consumed slower than acetic acid in the bioreactor. The preferences of acids were acetic, lactic, succinic, butyric and propionic. The acid utilization rates were higher in the bioreactor (except lactic acid), due to the higher cell population. Percentage acid consumptions were also higher in the bioreactor with the exception of lactic acid. Ammonia and phosphate usage rates were higher in bioreactor trials, again due to the higher cell population.

4.4.1 Effects of different VFA feeding strategies on cell growth, acid utilization, and PHA production

Three VFA feeding strategies were compared in this study. In the 24 h interval method, the artificial rumen fluid (ARF) was added at a rate of 124 ml to the fermentation broth at 48, 72, and 96 h. In the second method, approximately 19.5 of ARF was added at 3 h intervals, from 48-78 h, and then continuing from 88-109 h. In method three, the ARF was fed continuously at 7.75 ml/h from 48-96 h. Thus a total of 372 ml of ARF was fed in all the three treatments.

Figures 4.2-4.4 shows that the ARF feeding method either resulted in no change or a slight increase in cell populations. As expected, the 24 h addition method (Figure 4.2) resulted in the highest spikes in VFA concentration, with acid levels then falling as

R. eutropha metabolized the acids to PHA. However acetic (3.5 g/L), and to a lesser extent propionic acids, accumulated over time. These acids also accumulated in the continuous ARF feeding method (Figure 4.4). There was no apparent accumulation of lactic, butyric, or succinic acids for all the three feeding strategies. With the exception of acetic acid in the 24 h method, the VFAs were almost completely utilized by 144 h. The highest PHA concentration was observed for the 3 h strategy (Figure 4.3).

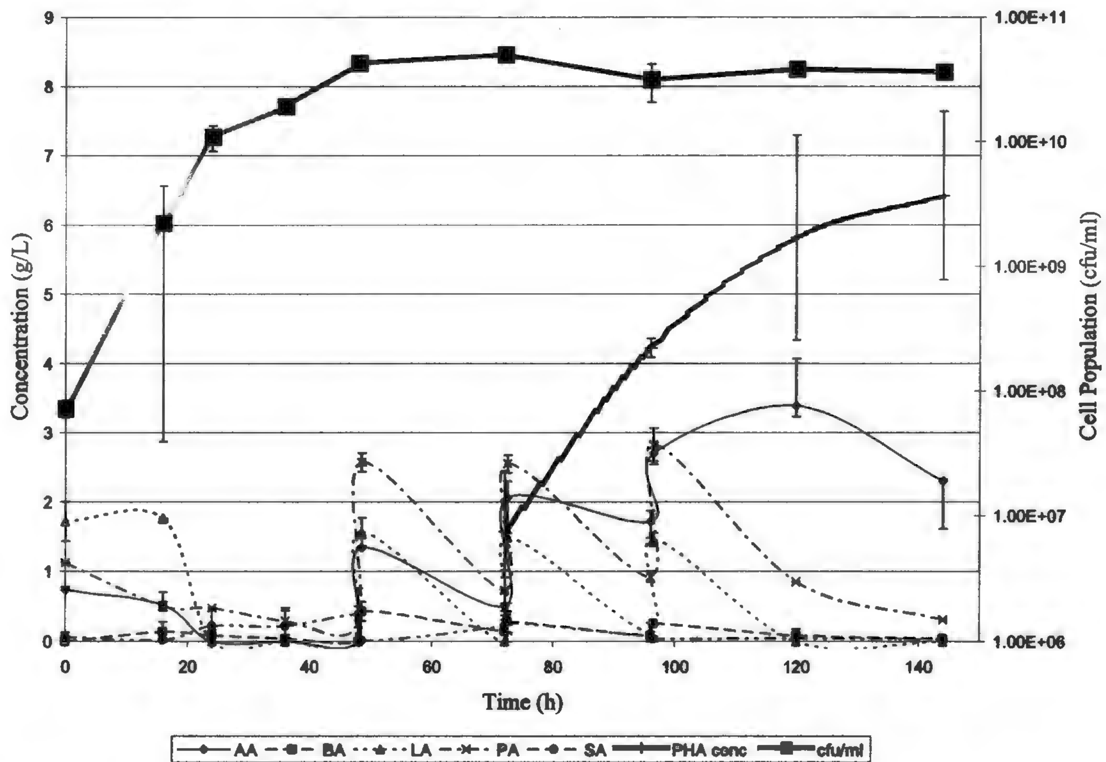

Figure 4.2 Acid utilization and growth of *R. eutropha* with 24 h interval additions of ARF.
* Error bars represent one standard deviation.

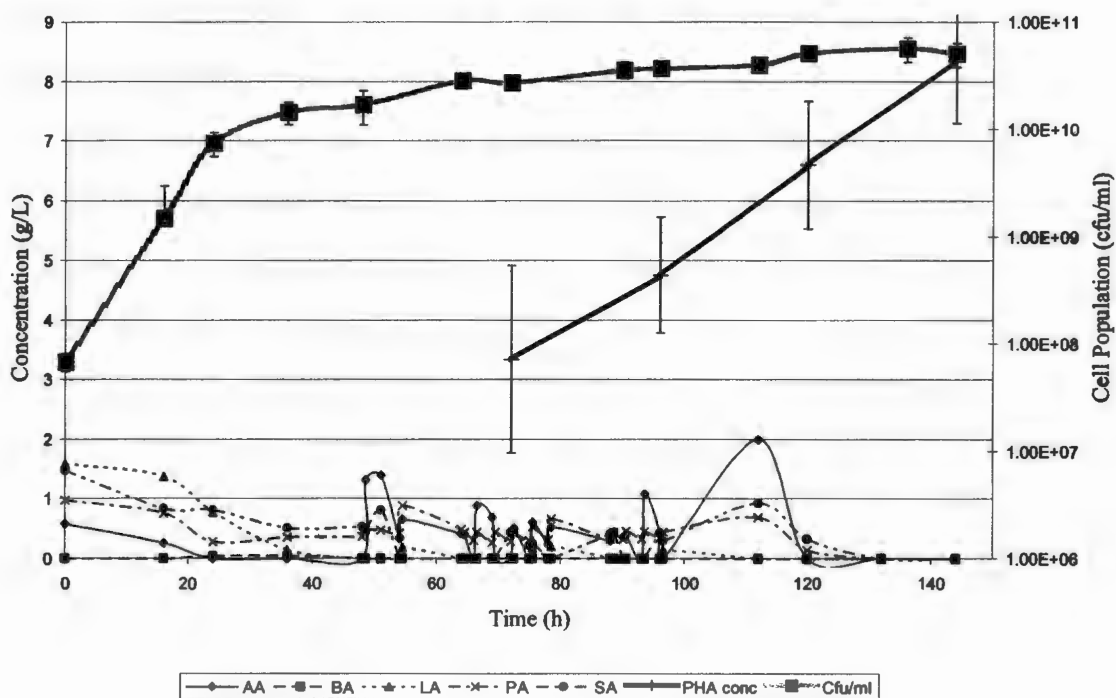

Figure 4.3 Acid utilization and growth of *R. eutropha* with 3 h interval additions of ARF.

* Error bars represent one standard deviation.

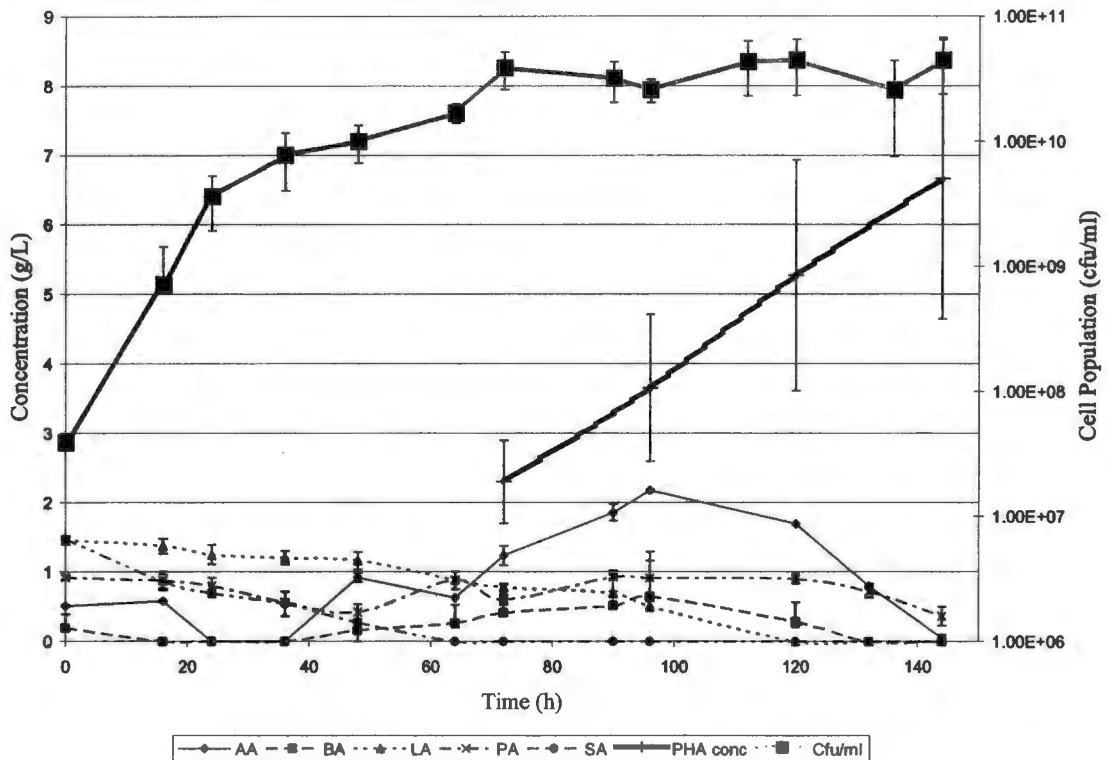

Figure 4.4 Acid utilization and growth of *R. eutropha* with continuous ARF addition.
* Error bars represent one standard deviation.

The effects of the different feeding strategies on maximum cell population, and ammonia and phosphate utilization rates are shown in Table 4.2. In all cases, the cell population continued to rise after ARF additions began, suggesting that the acid levels were not inhibitory. The maximum cell populations were not significantly different between the three treatments. This growth was supported by the residual nitrogen (5.03 g/L) and phosphorus (8.1 g/L) present in the medium at 72 h. By 96 h most of the ammonia and phosphorous present in the medium were used. The rates of ammonia utilization and phosphate utilization were similar for all feeding strategies.

Table 4.2 Comparison of maximum cell population, ammonia and phosphate utilization rates under different ARF feeding strategies.

Feeding Strategy	Cell Population at 48 h (CFU/ml)	Maximum Cell Population (CFU/ml)	Ammonia Utilization Rate (g/L/h)	Phosphate Utilization Rate (g/L/h)
24 h Addition	4.28E+10[a]	5.03E+10[a]	0.12[a]	0.10[a]
3 h Addition	1.70E+10[b]	5.68E+10[a]	0.10[a]	0.13[a]
Continuous Addition	1.01E+10[b]	4.47E+10[a]	0.11[a]	0.11[a]

[a,b,c] Means within column not sharing common superscript differ significantly ($P<0.05$)

Utilization rates of the four VFAs during the final 96 h of fermentation (48 to144 h), along with the combined acid utilization rates, are shown in Table 4.3. The combined utilization rate also includes consumption of succinic acid that was already present in the CCS medium. The highest combined acid utilization rates were observed in the 3 h and continuous ARF addition methods, with a lower rate in the 24 h addition method (likely due to the periodic spikes in VFA concentrations). If VFA concentrations are too high, acid utilization and cell activity can decrease. Undissociated fatty acids can pass through the cell membrane, and then dissociate to acidify the cytoplasm (Salmond et al. 1984). As a result, the proton gradient cannot be maintained as desired, and energy generation and transport system dependent on proton gradient are disrupted (Lawford and Rousseau 1993). This can also cause an increase in osmotic pressure due to the accumulation of anions (Roe et al. 1998). Acid utilization rates might have also been reduced by the depletion of certain acids at the end of each 24 h phase.

Table 4.3 Comparison of acid consumption rates under different ARF feeding strategies.

Feeding Strategy	Acid Utilization Rate (g/L/h)				Combined Acid Utilization Rate (g/L/h)
	Acetic	Butyric	Lactic	Propionic	
24 h Addition	0.029[a]	0.009[a]	0.074[a]	0.077[a]	0.20[a]
3 h Addition	0.052[b]	0.010[a]	0.080[a]	0.083[a]	0.25[b]
Continuous Addition	0.047[b]	0.014[b]	0.078[a]	0.080[a]	0.24[b]

[a,b,] Means within column not sharing common superscript differ significantly ($P<0.05$)

The trend of higher acid utilization rates with the 3 h and continuous ARF addition methods was also evident for the individual acids, however the differences were only significant for acetic and butyric acids. Propionic and lactic acids were used most rapidly, followed by acetic and butyric acid.

In comparison to the prior aerated shake flasks trials (Chapter 3) which were fed with individual volatile fatty acids, the addition of mixed acids to the bioreactor resulted in more rapid uptake of propionic acid and slower utilization of butyric acid. Propionate utilization rate rose from 0.046 g/L/h in aerated shake flasks to approximately 0.08 g/L/h when added as a part of the ARF mixture in the bioreactor trials. This was likely due to the higher cell populations achieved in the bioreactor trials, coupled with the fact that proprionate utilization by *R. eutropha* is energetically favorable (Yu et al. 2002). Moreover, addition of propionic acid in small doses might have controlled the change in pH and thus resulted in better utilization. The decline in the utilization rate of butyric acid, from 0.068 g/L/h when fed individually in aerated shake flasks to 0.009 g/L/h in

bioreactor trials, may have been due to the additional NADH needed to utilize this acid (Shang et al. 2003a).Utilization rates for lactic and acetic acids were similar.

Table 4.4 shows the fermentation efficiencies of the four VFAs, along with the combined fermentation efficiency. The 3 h and continuous ARF feeding strategies resulted in the highest combined fermentation efficiency, while the lowest occurred with the 24 h addition method. This is consistent with the lower acid utilization rates observed with the 24 h feeding strategy.

Table 4.4 Comparison of fermentation efficiencies under different ARF feeding strategies.

Feeding Strategy	Fermentation Efficiency (%)				Combined Acid Fermentation Efficiency (%)
	Acetic	Butyric	Lactic	Propionic	
24 h Addition	58.8^{a}	94.7^{a}	100^{a}	95.7^{a}	82.7^{a}
3 h Addition	100^{b}	100^{b}	100^{a}	100^{b}	100^{b}
Continuous Addition	98.7^{b}	100^{b}	100^{a}	95^{a}	97.8^{b}

^{a,b,c} Means within column not sharing common superscript differ significantly ($P<0.05$)

In comparing the individual acids, lactic acid was consumed completely, with greater than 95 % utilization of propionic and butyric acids. The high fermentation efficiencies for lactic and propionic acids are consistent with the metabolic preference of *R. eutropha*, especially considering that the ARF contained 15 and 20 g/L of these acids, respectively. The high butyric acid consumption was due to the fact that ARF only contained 2 g/L of this acid. We had previously noted that *R. eutropha* least preferred acetic acid (Chapter 3), therefore its lower fermentation efficiency was not surprising.

Fermentation efficiencies were higher for all the acids in the bioreactor trials, compared to the prior shake flask trials.

Cell dry weights and PHA production parameters for the different ARF feeding strategies are provided in Table 4.5. The 3 h feeding strategy resulted in the highest cell dry weight, PHA concentration, productivity, and PHA content, but the values were not significantly different from the other feeding strategies. Cell dry weight and PHA production were much higher than that obtained during the shake flask trials. The highest PHA concentration in the cells in the shake flask trials was 4.6 g/L. Whereas in the fermentor trials the PHA concentration of the cells was about 1.6 times than that of the shake flask trials.

Yu et al. suggested (2002), that an average yield of PHA was 0.39 g/g of carbon sources (acetic, butyric and propionic). In the current study the highest yield of about 0.2 g/g was observed when the second feeding strategy was used.

Table 4.5 Comparison of cell dry weight and PHA production under different ARF feeding strategies.

Feeding Strategy	Cell Dry Weight (g/L)	PHA Concentration (g/L)	PHA Productivity (g/L/ h)	PHA Content (%)
24 h Addition	17.6[a]	6.42[a]	0.0537[a]	36.23[a]
3 h Addition	21.13[a]	8.37[a]	0.0697[a]	39.52[a]
Continuous Addition	17.3[a]	6.67[a]	0.056[a]	37.78[a]

4.5 Conclusions

Bioreactor trials yielded better results than prior shake flask trials due to better aeration/agitation and more controlled growth conditions. Higher cell number, cell mass,

acid utilization, and PHA production were observed when the organism was cultured in the bioreactor.

Preference of use of the organic acids in the bioreactor was in the order of propionic acid, lactic acid, acetic acid and butyric acid. Acetic and butyric acids were better utilized when added individually since there was no competition among carbon sources. However lactic showed comparable rate of usage when added as mixed acid or added individually. Propionic acid actually fared better when added as mixed acid. This was because these acids were used in preference to other acids. So there was no noticeable difference in behavior of acid utilization when added as mixed acid. Fermentation efficiencies in mixed acid addition were higher because of the smaller dosage of additions than individual additions.

Highest cell population, cell mass, overall acid consumption, fermentation efficiency, and PHA production were obtained when the organism was grown in the fermenter and was fed mixed acid using the second feeding strategy. In the first strategy the sudden rise in the VFA concentrations must have lowered the pH by accumulation of acid. This might have caused decrease in acid consumption, fermentation efficiency and PHA productivity. Though the dosages of acids were small for the continuous feeding, continuous addition might have lowered the efficiency of the process. The smaller dosage and the fed batch mode of the 3h strategy might have resulted in better control of pH and of catabolite repression. So the optimized growth conditions (as discussed before) for the organism and second feeding strategy of mixed acid addition was considered the best to obtain optimum PHA production by the organism.

Chapter 5

Conclusions

From the present study it can be concluded that condensed corn solubles can be successfully used as growth medium for *R. eutropha.* PHA can be produced by feeding a mixture of volatile fatty acids (acetic, butyric, lactic and propionic) after the organism has reached maximum cell population in the CCS medium. The ratio of the mixed acids (10:2:15:20) obtained as a byproduct from fermentation of whole stillage by rumen bacteria, were not inhibitory to the growth or PHA production ability of *R. eutropha.*

Specific conclusions of the investigation are pointed below:

1. CCS medium with a concentration of 240 g/L with C:N ratio of 50:1 best supported the growth of *R. eutropha.*
2. When the volatile fatty acids were fed individually, 5 g/L of acetic, butyric and propionic and 8 g/L of lactic acid showed best results in terms of cell growth, acid use and PHA production.
3. When fed as mixed acid, acetic, lactic and propionic acids could be added at higher levels than that observed in individual addition study. But the dosage of addition is a major factor that determined the acid use and the fermentation efficiency of acids. The organism yielded better growth, cell numbers, acid use, fermentation efficiency and PHA production when optimal dosage of mixed acid was used. Fed batch culture with small dosage yielded better results in terms of all the parameters when compared to continuous culture.

These results show that CCS could serve as a potential commercial medium for PHA production using *R. eutropha* by fed batch fermentation. The use of recombinant strain instead of the wild strain of the organism and an optimal fermentation strategy may increase the PHA productivity and yield.

Chapter 6

Suggestions for Future Research

Prior research has shown that *R. eutropha* has great potential for PHA production, however, a major limitation is feedstock cost. The focus of this study was to develop a low cost medium to rapidly grow cells of *R. eutropha* to a high cell mass, then feed this cell mass VFAs to fill the cells with PHA. The next step would be to increase the PHA production and yield by using recombinant strains that utilizes all the nutrients present in the CCS medium. It is also recommended to find out if the polymer produced as well as the organism has the GRAS status so that it can be used in food applications. Achieving GRAS status will establish its potential use as fat mimetic as well as flavor encapsulating agent in food. The application of the biopolymer as a fat mimetic can be tested by spray drying the biopolymer as extremely small sized particles and then redispersed in the non-fat food matrix. Textural and sensory characteristics of the particular food can be tested in comparison with original fat-rich version.

Encapsulation and controlled release are considered as emerging technologies. Ingredients in 'micro' or ' nano' sizes can be entrapped and delivered to target sites at optimum time. However using this technology requires extensively understanding the physico-chemical properties of the encapsulating material, ingredient as well as the target. This technology can be used to deliver flavors, probiotic and neutraceutical actives, anti-microbial actives etc.

From the current research it has been proved that the organism is able to utilize butyric and propionic acid at high rates to produce PHA. If instead of artificial rumen

flora, butyric acid and propionic acid producing bacteria in whey media are used to produce the volatile fatty acids, then the end product will be more suitable used for food applications. The reason is that both these organisms have already attained the GRAS status.

Chapter 7

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Chapter 8

APPENDICES

8.1 ANOVA Table for comparison of growth rates and maximum cell population when the organism is grown in different media.

Treatment	Parameters	Sources of variation	DF	MS	F Values	
Media (nb, dm, 80 and 240)	Maximum cell count	Treatment	3	14.5	13.54	**
		Error	8	1.07		
Media (nb and dm)	Growth rate	Treatment	1	0.01	0.27	
		Error	24	0.05		
Media (240 and 80)	Growth rate	Treatment	1	0	0	
		Error	24	0.05		
Media (nb and 240)	Growth rate	Treatment	1	11.6	21	**
		Error	24	0.55		
Media (nb and 80)	Growth rate	Treatment	1	2.11	35.19	**
		Error	24	0.06		
Media (dm and 80)	Growth rate	Treatment	1	15.8	37.61	**
		Error	24	0.53		
Media (dm and 240)	Growth rate	Treatment	1	2.37	39.5	**
		Error	29	0.06		

* and ** represent values significant at p=0.05 and p=0.01, respectively.
Df =degrees of freedom.
MS=mean square.

8.2 ANOVA Table for comparison of growth rates and maximum cell population when the organism is grown in different concentration of CCS media with or without pH control.

Treatment	Parameters	Sources of variation	DF	MS	F Values	
Media (80, 240, 80p and 240p)	Maximum cell count	Treatment	3	37.8	8.14	**
		Error	11	4.50		
Media (240 and 80)	Growth rate	Treatment	1	2.36	3.42	
		Error	29	0.69		
Media (240p and 80)	Growth rate	Treatment	1	1.48	2.96	
		Error	29	0.5		
Media (240p and 240)	Growth rate	Treatment	1	0.05	0.87	
		Error	29	0.05		
Media (240p and 80p)	Growth rate	Treatment	1	1.56	29.50	**
		Error	29	0.05		
Media (80p and 80)	Growth rate	Treatment	1	1	20	**
		Error	29	0.05		
Media (80p and 240)	Growth rate	Treatment	1	2.55	40.71	**
		Error	29	0.06		

* and ** represent values significant at p=0.05 and p=0.01, respectively.
Df =degrees of freedom.
MS=mean square.
p= pH adjustment

8.3 ANOVA Table for comparison of growth rates and maximum cell population when the organism is grown in media with different C:N ratio

Treatment	Parameters	Sources of variation	DF	MS	F Values	
Media (C:N-30,50,70,90)	Maximum cell count	Treatment	3	2.98	35.45	**
		Error	8	2.72		
Media (30 and 50)	Growth rate	Treatment	1	0.10	5.63	*
		Error	26	0.02		
Media (50 and 90)	Growth rate	Treatment	1	0.81	20.96	**
		Error	27	0.04		

Media (30 and 90)	Growth rate	Treatment	1	0.19	4.25	
		Error	26	0.04		
Media (70 and 90)	Growth rate	Treatment	1	3.9	4.03	
		Error	35	0.9		
Media (70 and 50)	Growth rate	Treatment	1	0.01	0.11	
		Error	29	0.08		

* and ** represent values significant at p=0.05 and p=0.01, respectively.
Df =degrees of freedom.
MS=mean square.
C:N= carbon isto nitrogen ratio at 30:1, 50:1, 70:1 and 90:1

8.4 ANOVA Table for comparison of maximum cell population, growth rate, fermentation efficiency and consumption rate when acetic acid was fed at different levels.

Treatment	Parameters	Sources of variation	DF	MS	F Values	
Acids	Maximum cell count	Treatment	11	48.6	17.94	**
		Error	24	2.71		
Acetic Acid (1 and 3)	Consumption rate	Treatment	1	1.55	15.15	**
		Error	20	0.1		
Acetic Acid (5 and 3)	Consumption rate	Treatment	1	0.07	0.45	
		Error	20	0.16		
Acetic Acid (5 and 1)	Consumption rate	Treatment	1	2.30	17.23	**
		Error	20	0.13		
Acids	Fermentation efficiency	Treatment	1	458.0	70.98	**
		Error	23	6.45		

* and ** represent values significant at p=0.05 and p=0.01, respectively.
Df =degrees of freedom.
MS=mean square.
1,3,5= three different levels of acetic acid
Acids=acetic, butyric, lactic and propionic

8.5 P values for maximum cell population and fermentation efficiencies when acetic acid was added at different levels.

Maximum cell population			
	Acetic acid (1)	Acetic acid (3)	Acetic acid (5)
Acetic acid (1)		0.0006 **	<0.0001 **
Acetic acid (3)	0.0006 **		0.0985
Acetic acid (5)	<0.0001**	0.0985	
Fermentation Efficiency			
	Acetic acid (1)	Acetic acid (3)	Acetic acid (5)
Acetic acid (1)		1	<0.0001**
Acetic acid (3)	1		<0.0001**
Acetic acid (5)	<0.0001**	<0.0001**	
* and ** represent values significant at p=0.05 and p=0.01, respectively.			

8.6 P values for dry weights, PHA productivity, yield, ammonia and phosphate utilization when acetic acid was added at different levels.

Dry Weights			
	Acetic acid (1)	Acetic acid (3)	Acetic acid (5)
Acetic acid (1)		0.7089	0.1438
Acetic acid (3)	0.7089		0.2602
Acetic acid (5)	0.1438	0.2602	
PHA Concentration			
	Acetic acid (1)	Acetic acid (3)	Acetic acid (5)
Acetic acid (1)		0.7310	0.1593
Acetic acid (3)	0.7310		0.2731
Acetic acid (5)	0.1593		
PHA productivity			
	Acetic acid (1)	Acetic acid (3)	Acetic acid (5)
Acetic acid (1)		0.7311	0.1593
Acetic acid (3)	0.7311		0.2731
Acetic acid (5)	0.1593	0.2731	
PHA Yield			
	Acetic acid (1)	Acetic acid (3)	Acetic acid (5)
Acetic acid (1)		0.8324	0.8322
Acetic acid (3)	0.8324		0.9997
Acetic acid (5)	0.8322	0.9997	
Ammonia utilization			
	Acetic acid (1)	Acetic acid (3)	Acetic acid (5)
Acetic acid (1)		0.7182	0.0882
Acetic acid (3)	0.7182		0.4460
Acetic acid (5)	0.0882	0.4460	
Phosphate utilization			
	Acetic acid (1)	Acetic acid (3)	Acetic acid (5)
Acetic acid (1)		0.0309*	1.000

Acetic acid (3)	0.0309*		0.0309*
Acetic acid (5)	1.000	0.0309*	
* and ** represent values significant at p=0.05 and p=0.01, respectively.			

8.7 ANOVA Table for comparison of maximum cell population, growth rate, fermentation efficiency and consumption rate when butyric acid was fed at different levels.

Treatment	Parameters	Sources of variation	DF	MS	F Values	
Acids	Maximum cell count	Treatment	11	48.5	17.94	**
		Error	24	2.71		
Butyric Acid (1 and 3)	Consumption rate	Treatment	1	1.05	3.01	
		Error	20	0.35		
Butyric Acid (5 and 3)	Consumption rate	Treatment	1	5.26	10.27	**
		Error	20	0.51		
Butyric Acid (5 and 1)	Consumption rate	Treatment	1	11.01	30.09	**
		Error	20	0.36		
Acids	Fermentation efficiency	Treatment	1	458.0	70.98	**
		Error	23	6.45		

* and ** represent values significant at p=0.05 and p=0.01, respectively.
Df =degrees of freedom.
MS=mean square.
1,3,5= three different levels of butyric acid
Acids=acetic, butyric, lactic and propionic

8.8 P values for maximum cell population and fermentation efficiencies when butyric acid was fed at different levels.

Maximum cell population			
	Butyric acid (1)	Butyric acid (3)	Butyric acid (5)
Butyric acid (1)		0.0411*	0.0609
Butyric acid (3)	0.0411*		0.0004**
Butyric acid (5)	0.0609	0.0004**	
Fermentation Efficiency			
	Butyric acid (1)	Butyric acid (3)	Butyric acid (5)
Butyric acid (1)		0.0446*	0.6267

Butyric acid (3)	0.0446*		0.1164
Butyric acid (5)	0.6267	0.1164	
* and ** represent values significant at p=0.05 and p=0.01, respectively.			

8.9 P values for dry weights, PHA productivity, yield, ammonia and phosphate utilization when butyric acid was fed at different levels.

Dry Weights			
	Butyric acid (1)	Butyric acid (3)	Butyric acid (5)
Butyric acid (1)		0.7342	0.1283
Butyric acid (3)	0.7342		0.0709
Butyric acid (5)	0.1283	0.0709	
PHA Concentration			
	Butyric acid (1)	Butyric acid (3)	Butyric acid (5)
Butyric acid (1)		0.2041	0.0005**
Butyric acid (3)	0.2041		0.51
Butyric acid (5)	0.0005	0.51	
PHA productivity			
	Butyric acid (1)	Butyric acid (3)	Butyric acid (5)
Butyric acid (1)		0.0204*	0.0005**
Butyric acid (3)	0.0204*		0.0051**
Butyric acid (5)	0.0005**	0.0051**	
PHA Yield			
	Butyric acid (1)	Butyric acid (3)	Butyric acid (5)
Butyric acid (1)		0.0407*	0.0015**
Butyric acid (3)	0.0407*		0.0554
Butyric acid (5)	0.0015**	0.0554	
Ammonia utilization			
	Butyric acid (1)	Butyric acid (3)	Butyric acid (5)
Butyric acid (1)		0.9308	0.6550
Butyric acid (3)	0.9308		0.5943
Butyric acid (5)	0.6550	0.5943	
Phosphate utilization			
	Butyric acid (1)	Butyric acid (3)	Butyric acid (5)
Butyric acid (1)		0.0437*	0.0261*
Butyric acid (3)	0.0437*		0.0877
Butyric acid (5)	0.0261*	0.0877	
* and ** represent values significant at p=0.05 and p=0.01, respectively.			

8.10 ANOVA Table for comparison of maximum cell population, growth rate, fermentation efficiency and consumption rate when lactic acid was fed at different levels.

Treatment	Parameters	Sources of variation	DF	MS	F Values	
Acids	Maximum cell count	Treatment	11	48.5	17.94	**
		Error	24	2.71		
Lactic Acid (2 and 4)	Consumption rate	Treatment	1	1.52	1.87	
		Error	20	0.81		
Lactic Acid (8 and 4)	Consumption rate	Treatment	1	2.7	1.62	
		Error	20	1.67		
Lactic Acid (8 and 2)	Consumption rate	Treatment	1	8.28	5.85	*
		Error	20	1.41		
Acids	Fermentation efficiency	Treatment	1	458.0	70.98	**
		Error	23	6.45		

* and ** represent values significant at p=0.05 and p=0.01, respectively.
Df =degrees of freedom.
MS=mean square.
2,4,8= three different levels of lactic acid
Acids=acetic, butyric, lactic and propionic

8.11 P values for maximum cell population and fermentation efficiencies when lactic acid was fed at different levels.

Maximum cell population			
	Lactic acid (2)	Lactic acid (4)	Lactic acid (8)
Lactic acid (2)		<0.0001**	0.0059**
Lactic acid (4)	<0.0001**		0.0015**
Lactic acid (8)	0.0059**	0.0015**	
Fermentation Efficiency			
	Lactic acid (2)	Lactic acid (4)	Lactic acid (8)
Lactic acid (2)		1	<0.0001**
Lactic acid (4)	1		<0.0001**
Lactic acid (8)	<0.0001**	<0.0001**	

* and ** represent values significant at p=0.05 and p=0.01, respectively.

8.12 P values for dry weights, PHA productivity, yield, ammonia and phosphate utilization when lactic acid was fed at different levels.

Dry Weights			
	Lactic acid (2)	Lactic acid (4)	Lactic acid (8)
Lactic acid (2)		0.8649	0.4019
Lactic cid (4)	0.8649		0.5002
Lactic acid (8)	0.4019	0.5002	
PHA concentration			
	Lactic acid (2)	Lactic acid (4)	Lactic acid (8)
Lactic acid (2)		0.5538	0.0206*
Lactic cid (4)	0.5538		0.0261*
Lactic acid (8)	0.0206*	0.0261*	
PHA productivity			
	Lactic acid (2)	Lactic acid (4)	Lactic acid (8)
Lactic acid (2)		0.5539	0.0206*
Lactic cid (4)	0.5539		0.0629
Lactic acid (8)	0.0206*	0.0629	
PHA Yield			
	Lactic acid (2)	Lactic acid (4)	Lactic acid (8)
Lactic acid (2)		0.0218*	0.0002**
Lactic cid (4)	0.0218*		0.0046**
Lactic acid (8)	0.0002**	0.0046**	
Ammonia utilization			
	Lactic acid (2)	Lactic acid (4)	Lactic acid (8)
Lactic acid (2)		0.9033	0.6344
Lactic acid (4)	0.9033		0.5517
Lactic acid (8)	0.6344	0.5517	
Phosphate utilization			
	Lactic acid (2)	Lactic acid (4)	Lactic acid (8)
Lactic acid (2)		0.3698	0.7990
Lactic cid (4)	0.3698		0.5519
Lactic acid (8)	0.7990	0.5519	
* and ** represent values significant at p=0.05 and p=0.01, respectively.			

8.13 ANOVA Table for comparison of maximum cell population, growth rate, fermentation efficiency and consumption rate when propionic acid was fed at different levels.

Treatment	Parameters	Sources of variation	DF	MS	F Values	
Acids	Maximum cell count	Treatment	11	48.5	17.94	**
		Error	24	2.71		
Propionic Acid (1 and 3)	Consumption rate	Treatment	1	2.82	15.01	**
		Error	20	0.19		
Propionic Acid (5 and 3)	Consumption rate	Treatment	1	0	0	
		Error	20	0.33		
Propionic Acid (5 and 1)	Consumption rate	Treatment	1	2.92	13.36	**
		Error	20	0.22		
Acids	Fermentation efficiency	Treatment	1	458.0	70.98	**
		Error	23	6.45		

* and ** represent values significant at p=0.05 and p=0.01, respectively.
Df =degrees of freedom.
MS=mean square.
1,3,5= three different levels of propionic acid
Acids=acetic, butyric, lactic and propionic

8.14 P values for maximum cell population and fermentation efficiencies when propionic acid was fed at different levels.

Maximum cell population			
	Propionic acid (1)	Propionic acid (3)	Propionic acid (5)
Propionic acid (1)		0.0148*	<0.0001**
Propionic acid (3)	0.0148*		<0.0001**
Propionic acid (5)	<0.0001**	<0.0001**	
Fermentation Efficiency			
	Propionic acid (1)	Propionic acid (3)	Propionic acid (5)
Propionic acid (1)		0.1695	<0.0001**
Propionic acid (3)	0.1695		<0.0001**
Propionic acid (5)	<0.0001**	<0.0001**	

* and ** represent values significant at p=0.05 and p=0.01, respectively.

8.15 P values for dry weights, PHA productivity, yield, ammonia and phosphate utilization when propionic acid was fed at different levels.

Dry Weights			
	Propionic acid (1)	Propionic acid (3)	Propionic acid (5)
Propionic acid (1)		0.2472	0.0146*
Propionic acid (3)	0.2472		0.1283
Propionic acid (5)	0.0146*	0.1283	
PHA Concentration			
	Propionic acid (1)	Propionic acid (3)	Propionic acid (5)
Propionic acid (1)		0.0583	0.0009**
Propionic acid (3)	0.0583		0.0396*
Propionic acid (5)	0.0009**	0.0396*	
PHA productivity			
	Propionic acid (1)	Propionic acid (3)	Propionic acid (5)
Propionic acid (1)		0.0058**	0.0009**
Propionic acid (3)	0.0058**		0.0396*
Propionic acid (5)	0.0009**	0.0396*	
PHA Yield			
	Propionic acid (1)	Propionic acid (3)	Propionic acid (5)
Propionic acid (1)		0.0151*	0.0162*
Propionic acid (3)	0.0151*		0.0230*
Propionic acid (5)	0.0162*	0.0230*	
Ammonia utilization			
	Propionic acid (1)	Propionic acid (3)	Propionic acid (5)
Propionic acid (1)		0.2936	0.8060
Propionic acid (3)	0.2936		0.4150
Propionic acid (5)	0.8060	0.4150	
Phosphate utilization			
	Propionic acid (1)	Propionic acid (3)	Propionic acid (5)
Propionic acid (1)		0.9034	0.3755
Propionic acid (3)	0.9034		0.3163
Propionic acid (5)	0.3755	0.3163	
*** and ** represent values significant at p=0.05 and p=0.01, respectively.**			

8.16 ANOVA Table for comparison of maximum cell population, growth rate, fermentation efficiency and consumption rate when different acids were fed at optimum levels.

Treatment	Parameters	Sources of variation	DF	MS	F Values	
Acids	Maximum cell count	Treatment	11	4.85	17.94	**
		Error	24	2.71		
Acetic Acid (5) and Butyric acid (5)	Consumption rate	Treatment	1	4.31	1.32	
		Error	20	3.3		

Butyric acid (5) and Propionic Acid (5)	Consumption rate	Treatment	1	2.88	1.98	
		Error	20	0.41		
Lactic Acid (8) and Propionic Acid (3)	Consumption rate	Treatment	1	4	5.9	*
		Error	20	0.68		
Acetic Acid (5) and Propionic Acid (5)	Consumption rate	Treatment	1	1.0	4.09	
		Error	20	0.21		
Acetic Acid (5) and Lactic Acid (8)	Consumption rate	Treatment	1	13.14	21.91	**
		Error	20	0.6		
Butyric acid (5) and Lactic Acid (8)	Consumption rate	Treatment	1	11.13	11.42	**
		Error	20	0.80		
Acids	Fermentation efficiency	Treatment	1	458.0	70.98	**
		Error	23	6.45		

* and ** represent values significant at p=0.05 and p=0.01, respectively.
Df =degrees of freedom.
MS=mean square.
1,3,5= three different levels of acetic, butyric and propionic acid
2,4,8= three different levels of lactic acid
Acids=acetic, butyric, lactic and propionic

8.17 P values for maximum cell population and fermentation efficiencies when different acids were fed at optimum levels.

Maximum cell population				
	Acetic acid (5)	Butyric acid (5)	Lactic Acid (8)	Propionic acid (5)
Acetic acid (5)		0.9401	0.2100	0.0343*
Butyric acid (5)	0.9401		0.0531	0.1123
Lactic Acid (8)	0.2100	0.0531		<0.0001**
Propionic acid (5)	0.0343*	0.1123	<0.0001**	
Fermentation Efficiency				
	Acetic acid (3)	Butyric acid (5)	Lactic Acid (4)	Propionic acid (3)
Acetic acid (3)		0.0267*	0.9000	0.0874
Butyric acid (5)	0.0267*		0.0347*	0.0209*
Lactic Acid (4)	0.9000	0.0347*		0.0874
Propionic acid (3)	0.0874	0.0209*	0.0874	

* and ** represent values significant at p=0.05 and p=0.01, respectively.

8.18 P values for dry weights, PHA productivity, yield, ammonia and phosphate utilization when butyric acid was added at different levels.

Dry Weights				
	Acetic acid (5)	Butyric acid (5)	Lactic Acid (8)	Propionic acid (5)
Acetic acid (5)		0.1358	0.2002	0.1211
Butyric acid (5)	0.1358		0.0120*	0.9457
Lactic acid (8)	0.2002	0.0120*		0.0106*
Propionic Acid (5)	0.1211	0.9457	0.0106*	
PHA Concentration				
	Acetic acid (5)	Butyric acid (5)	Lactic Acid (8)	Propionic acid (5)
Acetic acid (5)		0.0918	0.6125	0.1210
Butyric acid (5)	0.0918		0.0365*	0.8728
Lactic acid (8)	0.6125	0.0365*		0.0491*
Propionic Acid (5)	0.1210	0.8728	0.0491*	
PHA Productivity				
	Acetic acid (5)	Butyric acid (5)	Lactic Acid (8)	Propionic acid (5)
Acetic acid (5)		0.0918	0.6124	0.1210
Butyric acid (5)	0.0918		0.0365*	0.8728
Lactic acid (8)	0.6124	0.0365*		0.0491*
Propionic Acid (5)	0.1210	0.8728	0.0491*	
PHA Yield				
	Acetic acid (5)	Butyric acid (5)	Lactic Acid (8)	Propionic acid (5)
Acetic acid (5)		0.6993	0.1187	0.9874
Butyric acid (5)	0.6993		0.2231	0.7109
Lactic acid (8)	0.1187	0.2231		0.1219
Propionic Acid (5)	0.9874	0.7109	0.1219	
Ammonia utilization				
	Acetic acid (5)	Butyric acid (5)	Lactic Acid (8)	Propionic acid (5)
Acetic acid (5)		0.2357	0.0557	0.1234
Butyric acid (5)	0.2357		0.4150	0.6969
Lactic acid (8)	0.0557	0.4150		0.6654
Propionic Acid (5)	0.1234	0.6969	0.6654	
Phosphate utilization				
	Acetic acid (5)	Butyric acid (5)	Lactic Acid (8)	Propionic acid (5)
Acetic acid (5)		0.0063**	0.0057**	0.1909
Butyric acid (5)	0.0063**		0.9613	0.0917
Lactic acid (8)	0.0057**	0.9613		0.0839
Propionic Acid (5)	0.1909	0.0917	0.0839	

*** and ** represent values significant at p=0.05 and p=0.01, respectively.**

8.19 ANOVA Table for comparison of Dry Weight, productivity, yield, ammonia and phosphate utilization when different acids were fed individually.

Treatment	Parameters	Sources of variation	DF	MS	F Values	
Individual Acids	Dry Weight	Treatment	11	27.10	3.27	*
		Error	12	8.28		
	Productivity	Treatment	11	0	5.63	**
		Error	12	0		
	Yield	Treatment	11	276.53	5.93	**
		Error	12	46.65		
	Concentration	Treatment	11	4.13	5.63	**
		Error	12	0.73		
	Ammonia Utilization	Treatment	14	0	1.67	
		Error	15	0		
	Phosphate Utilization	Treatment	14	0.01	9.13	**
		Error	15	0		

*** and ** represent values significant at p=0.05 and p=0.01, respectively.**
Df =degrees of freedom.
MS=mean square.
1,3,5= three different levels of acetic, butyric and propionic acid
2,4,8= three different levels of lactic acid
Acids=acetic, butyric, lactic and propionic

8.20 ANOVA Table for comparison of maximum cell population, growth rate, fermentation efficiency and consumption rate when different feeding strategies of mixed acid were used.

Treatment	Parameters	Sources of variation	DF	MS	F Values	
Feeding Strategies (24,3 and C)	Maximum cell count	Treatment	2	7.87	4.04	
		Error	6	1.95		
3 and C	Consumption rate	Treatment	1	0	0.09	
		Error	4	0.03		
24 and 3	Consumption rate	Treatment	1	10.42	2884.32	**
		Error	4	0		
24 and C	Consumption rate	Treatment	1	10.10	333.83	**
		Error	4	0.03		
Feeding Strategies (24,3 and C)	Fermentation efficiency	Treatment	2	131.48	1052.81	**
		Error	3	0.12		

*** and ** represent values significant at p=0.05 and p=0.01, respectively.**
Df =degrees of freedom.
MS=mean square.

24,3,c= three different feeding strategies of mixed acids in the fermenter

8.21 P values for maximum cell population and overall fermentation efficiencies when different feeding strategies of mixed acid were used.

Maximum cell Population			
Strategies	24	3	C
24		0.5891	0.0776
3	0.5891		0.0657
C	0.0776	0.0657	
Overall Fermentation Efficiency			
Strategies	24	3	C
24		<0.0001**	<0.0001**
3	<0.0001**		0.0861
C	<0.0001**	0.0861	

* and ** represent values significant at p=0.05 and p=0.01, respectively.

8.22 P values for acid utilization rates for acids when different feeding strategies of mixed acid were used.

Acetic Consumption Rate			
Strategies	24	3	C
24		0.0378*	0.0236*
3	0.0378*		0.7957
C	0.0236*	0.7957	
Butyric Consumption Rate			
Strategies	24	3	C
24		0.9830	0.0349*
3	0.9830		0.0481*
C	0.0349*	0.0481*	
Lactic Consumption Rate			
Strategies	24	3	C
24		0.9940	0.9887
3	0.9940		0.9978
C	0.9887	0.9978	
Propionic Consumption Rate			
Strategies	24	3	C
24		0.9292	0.9052
3	0.9292		0.9180
C	0.9052	0.9180	

* and ** represent values significant at p=0.05 and p=0.01, respectively.

8.23 P values fermentation efficiencies of acids when different feeding strategies of mixed acid were used.

Acetic Fermentation Efficiency			
Strategies	24	3	C
24		<0.0001**	0.0231*
3	0.0231*		0.2304
C	0.0231*	0.2304	
Butyric Fermentation Efficiency			
Strategies	24	3	C
24		0.0003**	0.0003**
3	0.0003**		1.000
C	0.0003**	1.000	
Lactic Fermentation Efficiency			
Strategies	24	3	C
24		1.000	1.000
3	1.000		1.000
C	1.000	1.000	
Propionic Fermentation Efficiency			
Strategies	24	3	C
24		0.0009**	0.5567
3	0.0009**		0.0026**
C	0.5567	0.0026**	
* and ** represent values significant at p=0.05 and p=0.01, respectively.			

8.24 P values for comparison of dry weights, PHA productivity, yield, ammonia and phosphate utilization when mixed acid was fed using different strategies.

Dry weights			
Strategies	24	3	C
24		0.2638	0.9227
3	0.2638		0.2270
C	0.9227	0.2270	
PHA Productivity			
Strategies	24	3	C
24		0.1368	0.8544
3	0.1368		0.1862
C	0.8544	0.1862	
PHA Yield			
Strategies	24	3	C
24		0.2363	0.2464
3	0.2363		0.3683
C	0.2464	0.3683	
PHA Concentration			
Strategies	24	3	C
24		0.4259	0.9146
3	0.4529		0.4809

C	0.9146	0.4809	
Ammonia utilization			
Strategies	24	3	C
24		0.0500	0.9769
3	0.0500		0.0500
C	0.9769	0.0500	
Phosphate utilization			
Strategies	24	3	C
24		0.8795	0.3693
3	0.8795		0.4525
C	0.3693	0.4525	
*** and ** represent values significant at p=0.05 and p=0.01, respectively.**			