ACRYLATE METABOLISM IN *MEGASPHAERA ELSDENII*

by

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(Under the Direction of Mark Eiteman)

ABSTRACT

Acrylic acid (CH_2=CH-COOH) is a commodity chemical with an annual production of about four million tonnes. Thioester derivatives of acrylate are metabolic intermediates in organisms using the "acrylate pathway". *Megasphaera elsdenii* and *Clostridium propionicum* metabolize lactate into propionate via the acrylate pathway by catabolizing lactate sequentially through lactyl-CoA, acrylyl-CoA, propionyl-CoA and propionate. The first and last reactions are catalyzed by a single CoA transferase, propionyl-CoA transferase. The goal of this study was to characterize these bacteria in the presence of acrylate. These organisms or the enzymes involved in acrylate metabolism could be important routes to industrial acrylate production.

INDEX WORDS: *Megasphaera elsdenii*, *Clostridium propionicum*, acrylate, carbon balance, metabolic flux, steady-state, redox, metabolic pathway

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DEDICATION

I want to dedicate my research to my parents, Mr. Shirish Prabhu and my mother Mrs. Yamuna Prabhu for their immense support they provided me throughout my student life. My grandfather Gajanan Prabhu and grandmother Vinaya Prabhu have been a great source of inspiration in my life to whom I would also like to dedicate the success of my work. Lastly, I'd like to dedicate my work to my sister Sejal Prabhu and my best friend Ankit Agrawal.

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Chapter 1

Introduction and Literature Review

Acrylic acid (CH_2=CH-COOH) is a commodity chemical with an annual production of about 4 million tons (Danner *et al.*, 1998). Acrylic acid and its esters are used in various industries such as paints, coatings, textiles, adhesives, plastics, detergents and super absorbent materials such as baby diapers (Danner *et al.*, 1998). Currently, acrylic acid is manufactured only from petroleum, mostly by direct oxidation of propene.

A driving force of this research has been developing a process for the production of acrylic acid from carbohydrate-based renewable resources. Since petroleum is a nonrenewable source of energy, renewable resources such as corn starch would be a preferred substrate for the production of biochemicals. Processes involving petroleum-derived products furthermore add to global CO_2 emissions making petroleum-based products environmentally unfriendly.

Metabolic pathways in bacteria and metabolic flux

Microorganisms have evolved pathways that allow them to metabolize various nutrients and thrive under different environmental conditions (Tang *et al.*, 2009). Variations in the type and amount of protein present in the cell accordingly alters the regulatory and metabolic network of the different bacterial species (Kelley *et al.*, 2003).

A metabolic pathway is simply a sequence of biochemical reactions connecting the various inputs, intermediates and output metabolites. Calculating metabolic flux is an important parameter in studying the cell metabolism and metabolically engineering strains for production of industrial products (Iwatani *et al.*, 2008). Metabolic flux is defined as the rate at which

compounds proceed through a metabolic pathway. Studying metabolic flux bears great importance in studying the cell physiology and interaction between the various intermediates in the metabolic pathway. The flux through a pathway or enzyme is liable to change with perturbation in its environment or with respect to genetic manipulation (Stephanopoulos, 1999).

The growth of organisms involves the balancing of the carbon flux during which the substrates get converted into diverse products. Flux analysis depicts the metabolic events taking place within the cell by measuring the utilization of the substrates and the generation of products (Holms, 1996) . While calculating fluxes, even products such as carbon dioxide and hydrogen are important in order to account for the redox and carbon balance. Metabolic fluxes, however, fail to explain the regulatory mechanisms of particular pathways within the cells (Holms, 1996). Another limitation can be the inability to distinguish between fluxes going through a splitting branch point of a pathway (Stephanopoulos, 1999).

Fluxes through a metabolic pathway also indicate the degree of involvement of the different enzymes present in the cell. Even when enzymes are present, in a cell there might be little or no flux going through that particular enzymatic step. Hence, we can also use the knowledge of pathway flux to extrapolate the involvement of the enzymes present in the pathway (Stephanopoulos, 1999).

In order to calculate metabolic fluxes one should have a metabolic model for an organism, taking into account all the major steps of the pathway (Nissen *et al.*, 1997). Several organisms use more than one pathway under different metabolic conditions. By calculating metabolic fluxes under different experimental conditions, it may be possible to determine which pathway is active (Nissen *et al*., 1997). Metabolic flux analysis can provide detailed information of intracellular fluxes through the reactions in central metabolism of the organism. *In vivo* the

flux of incoming substrates must be balanced by outgoing products in order to balance the stoichiometry of the various reactions taking place in the cell (Wiechert, 2001).

Acrylate metabolism in bacteria

Acrylate is expected to be toxic to most bacteria due to its highly reactive nature. The presence of the conjugated double bond acts as an electron withdrawing group and makes acrylate a strong oxidizing agent in cells (Freidig *et al.*, 1999). Acrylate can be potentially harmful to bacteria as it is capable of withdrawing electrons from other compounds in the cell and disturbing the equilibrium of the cell (Straathof, 2005). Acrylic acid and its derivatives are lethal to various bacterial species such as *Lactobacillus arabinosus* and *Leuconostoc mesenteroides* (Furst *et al.*, 1951). Acrylic acid is produced in marine ecosystems as a by-product of the dimethylsulfoniopropionate (DMSP) pathway. However, even nanomolar quantities of acrylic acid are detrimental to the metabolism of a majority of sea water cultures and natural bacterial consortia (Slezak *et al.*, 1994).

Microbial resistance to carboxylic acids can be improved by selective pressure (Steiner & Sauer, 2003) or metabolic engineering (Patnaik *et al.*, 2002). Commercially desirable titres for many other carboxylic acids have been achieved, and the chemical structure of acrylate should not be a limitation in its biological production, as various acids with a conjugated double bond such as fumarate and itaconate have been produced using fermentation technology (Straathof, 2005). For example, resting cells of *Clostridium propionicum* have been shown to metabolize acrylate at concentrations of 35 g/L, although they could not grow in this condition (Sinskey *et al.*, 1981). Also, cell-free extracts from *C. propionicum* harvested in the late log-phase catalyze the dehydration of (R)-lactate to acrylate, although the concentration of free acrylate formed from 75 mM (R)-lactate did not surpass 0.35-0.45 mM (Schweiger and Buckel, 1985). The

coenzyme A (CoA) ester of acrylate is a common biochemical intermediate, and free acrylate is rarely observed (Hodgson & McGarry, 1968, Ladd & Walker, 1959). Whole cells of *C. propionicum* ferment acrylate, lactate or alanine to either propionate or acetate (Kuchta, 1985). Washed cell suspensions of *M. elsdenii* were also capable of metabolizing lactate and acrylate. The products formed were hydrogen, carbon dioxide, acetate, propionate and volatile fatty acids (Ladd, 1959). Thus, bacteria are able to metabolize acrylate, although quantification of this degradation is incomplete.

Biological pathways involving acrylic acid

Two pathways exist for the production of propionic acid: the succinate pathway and the acrylate pathway (Wood, 1956). The succinate pathway has been demonstrated in the bacteria belonging to genus *Propionibacterium* (Kuchta & Abeles, 1985). *Clostridium drakei, Clostridium propionicum, Clostridium scalatogenes* and *Megasphaera elsdenii* are believed to use the acrylate pathway and hence will be the organisms we use in our study (Baldwin, 1965; Kusel, 2000). Acetate and propionate are the major end products of the acrylate pathway. The products formed are able to attain a redox balance for these anaerobic bacteria (Boyaval & Corre, 1995).

Several organisms belonging to the genus *Clostridium* use acrylyl-CoA as an intermediate or are capable of metabolizing acrylate. The obligate anaerobe *C. scatologenes* was isolated from an acidic coal mine pond (Kusel *et al.*, 2000). This bacterium utilizes H_2 and produces acetate as the main end product. It metabolizes several compounds such as vanillate, ferulate, ethanol, propanol, 1-butanol, glucose, lactate, and pyruvate (Kusel, 2000). *C. scatologenes* reduces the acrylate group of various aromatic compounds such as ferulate to support growth (Kusel, 2000).

The anaerobe *C. neopropionicum* was isolated from a wastewater treatment plant, and ferments ethanol to carbon dioxide, propionate, acetate and propanol (Tholozan, 1992). Carbon labeling demonstrated the absence of the succinate pathway, suggesting the occurrence of the acrylate pathway. Cell-free extracts of the organism grown on ethanol indicated several enzymes of the acrylate pathway (Tholozan, 1992).

C. homopropionicum was isolated from an anoxic sewage plant on 2-hydroxybutyrate as the sole carbon source. 2-,3-, and 4-hydroxybutyrate, crotonate, vinylacetate and pyruvate are fermented to acetate and butyrate, while lactate and acrylate are converted to acetate and propionate by this organism (Dorner, 1990). This result is similar to observations with *C. propionicum* and *Megasphaera elsdenii*.

M. elsdenii is an anaerobic rumen microorganism and belongs to the phylum Firmicutes. *M. elsdenii* is a large coccus in shape and mainly non-motile and non-sporeformer (Rogosa, 1971). *M. elsdenii* is the only rumen bacterium known to ferment DL-lactate to propionate using the acrylate pathway. As a result of rumen microbial fermentations, there is an accumulation of lactate and other reduced end-products (Counotte *et al.*, 1981). The lactate thus formed is further fermented to propionate by *M. elsdenii* making it an important component of rumen ecology (Counotte et al., 1981).

Although *M. elsdenii* is mainly a DL-lactate fermenting organism, it can utilize several sugars and organic acids as an energy source (Marounek *et al.*, 1989). Propionate is observed only as an end product from lactate fermentation and not from glucose. The absence of propionate results from the absence of lactate racemase in glucose fermenting strains, as this enzyme must be induced by lactate (Hino *et al.*, 1994). When grown in presence of both glucose

and lactate, *M. elsdenii* exhausts the lactate before glucose consumption commences (Hino et al., 1994). Thus, lactate is the preferred substrate, and this organism is a natural propionate producer.

The obligate anaerobe *C. propionicum* was isolated from the black mud of San Francisco Bay as an alanine fermenting bacterium (Cardon & Barker, 1946). *C. propionicum* is motile due to the presence of 3-5 peritrichous flagella. Although this organism is capable of forming spores, spores are not readily formed by all cells (Cardon & Barker, 1946). Alanine is essential for the growth of *C. propionicum* and this substrate yields propionate, acetate, ammonia and carbon dioxide. Additionally, cells require a complex nutrient such as yeast extract. Glucose is not utilized by *C. propionicum* to carry out cell metabolism (Cardon & Barker, 1946).

Acrylate pathways in bacteria

Lactate reduction to propionate in bacteria is either carried out by the "succinate pathway" or the "acrylate pathway" (Johns, 1952). Most bacteria that produce propionate follow the succinate pathway. In the succinate pathway, pyruvate is first carboxylated to oxaloacetate which is subsequently reduced to succinate and converted to succinyl-CoA. The succinyl-CoA formed is then decarboxylated to propionate and carbon dioxide (Swick & Wood, 1960).

As shown in Figure 1.1, the acrylate pathway generates acrylyl-CoA as an intermediate (Kuchta & Abeles, 1985). In this pathway, lactate is reduced to propionate, and lactate is also oxidized to acetate and carbon dioxide (Johns, 1952). The production of acetate is necessary for the generation of ATP, and the sink for the NADH produced from the acetate branch lies in the propionate branch, wherein acrylyl-CoA is reduced to propionyl-CoA by the enzyme acrylyl-CoA reductase (Schwieger & Buckel, 1984). Thus, the cells require both pathways to balance redox.

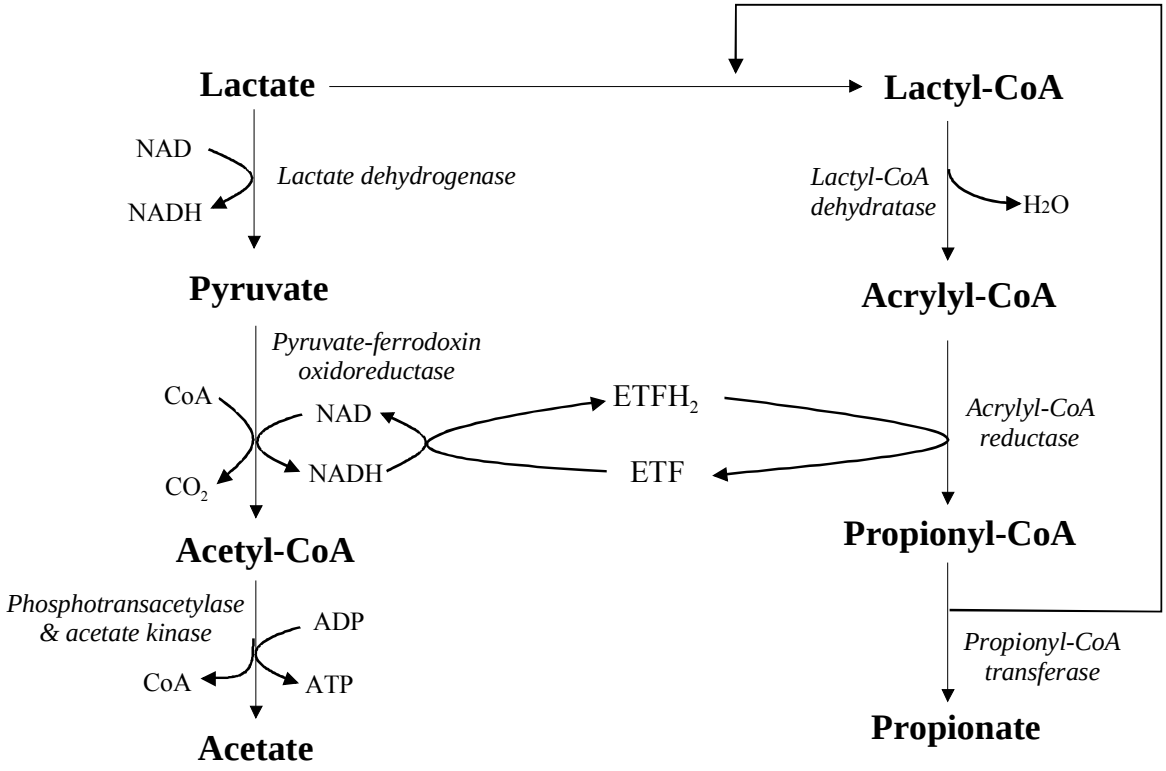

Figure 1.1: Acrylate pathway as demonstrated in *C. propionicum*

The simple stoichiometry for the pathway is:

3 Lactate + ADP + Pi → 2 Propionate + 1 Acetate + 1 CO_2 + 1 ATP + 2 H_2O

In the propionate branch of the pathway, lactate is converted to lactyl-CoA by an acyl transferase (Figure 1.1). Lactyl-CoA is dehydrated to acrylyl-CoA by the lactyl-CoA dehydratase (EC 4.2.1.54). The acrylyl-CoA formed is then reduced to propionyl-CoA by acryl-CoA reductase, and then finally propionyl-CoA transferase (EC 2.8.3.1) catalyzes the formation of propionate (Schwieger & Buckel, 1984). The Coenzyme A transferred from propionyl-CoA by the transferase can be donated to lactate and continue the cycle of propionate formation.

Enzymes of the acrylate pathway

i) Lactyl-CoA dehydratase (EC 4.2.1.54)

The pathway for propionate formation from lactate has been well established. One key step is the dehydration of lactyl-CoA to acrylyl-CoA catalyzed by the enzyme lactyl-CoA dehydratase (Hofmeister, 1992). The enzyme is made up of two components, EI and EII. EI has a molecular mass of 27 kDa and is oxygen sensitive (Kuchta, 1985). EI is irreversibly inactivated in the presence of oxygen with $t_{½}$ less than 60 s. The extreme oxygen sensitivity of EI may be due to the presence of a highly reduced metal or a radical in the component (Kuchta, 1986). Unlike EI, EII contains Fe-S clusters as part of the protein (Kuchta, 1986). EII is made up of two subunits having molecular masses 41 kDa and 48 kDa. It also contains equal amounts of riboflavin and flavin mononucleotide. The EI component of the enzymes is thought to be an activator protein whereas the EII component imparts the catalytic activity (Kuchta, 1985).

Lactyl-CoA dehydratase has been purified from *C. propionicum* grown on alanine as the carbon source. Partial purification of the enzyme from *M. elsdenii* was reported; however, the activity was very low and dehydration of lactyl-CoA was not clearly demonstrated (Kuchta, 1985).

ii) Acrylyl-CoA reductase

Acrylyl-CoA reductase catalyses the irreversible NADH-dependent formation of propionyl-CoA from acrylyl-CoA. The enzyme is a heterohexadecameric $(\alpha_2\beta\gamma)_4$ complex having a molecular mass of about 600 kDa. It is composed of a propionyl-CoA dehydrogenase and an electron transferring flavoprotein (ETF) responsible for oxidizing NADH to NAD with simultaneous reduction of acrylyl-CoA to propionyl-CoA (Hetzel, 2003). Acrylyl-CoA

reductase has recently been purified from *C. propionicum* cells. The N-terminal sequencing of the β and γ subunits revealed sequence similarity to the ETF from *M. elsdenii* (Hetzel, 2003).

In the pathway of lactate to propionate, the inactivation of acrylyl-CoA reductase blocks the formation of propionate from lactate. However, inactivation of acrylyl-CoA reductase does not block the hydration of acrylate to lactate (Kuchta, 1985), suggesting the reversible nature of the lactyl-CoA dehydratase.

Although the conversion of acrylyl-CoA to lactyl-CoA has been observed in cell extracts of *C. propionicum*, the reverse reaction has not been demonstrated. Despite evidence for conversion of lactate to propionate, the presence of acrylyl-CoA as an intermediate has not been detected. Either acrylyl-CoA is very short-lived because of the relative kinetics of the two enzymes, or lactyl-CoA dehydratase and acrylyl-CoA reductase form a complex so that the intermediate acrylyl-CoA is instantly passed onto the reductase to form propionyl-CoA (Kuchta, 1985). The free enzyme has a very low apparent K_M (2 μM) for acrylyl-CoA, so that the steady state concentration of the reactive acrylyl-CoA would be very low (Hetzel *et al.*, 2003). Hence, the enzyme does not make use of the high binding energy of CoA derivatives in order to increase the turnover number. Physiologically, the low turnover of acrylyl-CoA reductase (k_{cat} 4.5 s^{-1} for acrylyl-CoA) is balanced by large quantity of the enzyme present in the cell (Hetzel, 2003).

iii) Propionyl-CoA transferase

The conversion of lactate to propionate proceeds through coenzyme A thiol esters rather than the free carboxylates. Thus, in the propionate branch of the pathway the first step is conversion of (R)-lactate to its (R)-lactyl-CoA derivative. This reaction is catalyzed by the enzyme propionate:acetyl-CoA CoA-transferase (EC 2.8.3.1), also known as propionate-CoA transferase (Selmer, 2002). Physiologically, propionate-CoA transferase activates (R)-lactate

using either propionyl-CoA or acetyl-CoA as the coenzyme A donor (Selmer, 2002). The enzyme is homotetrameric (α_4), and the subunit has a molecular mass of 67 kDa. The enzyme exhibits broad substrate specificity for monocarboxylic acids including acrylate, propionate and butyrate, dicarboxylic acids are not attacked by this enzyme (Selmer, 2002).

iv) Hydrogenase

Some other organisms that utilize the acrylate pathway like *M. elsdenii* possess hydrogenases and thus are capable of evolving hydrogen gas however, *C. propionicum* lack hydrogenases (Adams, 1990, Ladd & Walker, 1959). Hydrogenases are a group of enzymes capable of catalyzing the reversible reaction,

$H_2 \leftrightarrow 2H^+ + 2e^-$

In this reaction hydrogen gas acts as an electron donor. H_2 evolution or H_2 oxidation has been encountered in several bacterial species, and hydrogenases from about 20 species have been purified. Hydrogenase activity has been observed in diverse groups of bacteria such as rumen bacteria, methanogens, sulfate reducers, photosynthetic bacteria, anaerobic fermenters and aerobic hydrogen and nitrogen-fixing bacteria (Adams, 1990). The gene encoding the [Fe]-hydrogenase of *M. elsdenii* has been characterized and cloned (Atta & Meyer, 2000).

Anaerobic fermentative bacteria like *M. elsdenii* and *Clostridium pasteurianum* acquire their energy from substrate level phosphorylation, and hydrogenases help balance the excess reductant by generating hydrogen gas (Adams, 1990).

Continuous Culture

Continuous cultures or chemostats have been used for several decades as a tool to study the physiology of different species under different growth conditions. Microbial physiologists have gained insight into cellular processes by studying metabolism at exponential balanced

growth when the growth rate is maintained constant (Koch, 1997). The advantage of a chemostat over a simple batch process is that cells are maintained at a constant physico-chemical environment. When one or more nutrients become limiting in a batch culture, cell growth stops. In a chemostat, because the nutrients are continuously supplied, the cell growth is determined by the rate at which the limiting nutrient is supplied. Because the volume of the bioreactor is maintained, the culture grows at a constant, steady-state specific growth rate (Hoskisson & Hobbs, 2005). Continuous culture is widely used for studying microbial physiology as a single parameter can be changed while keeping others constant (Ferenci, 2006).

Objectives

The general goal of this research is to study acrylate metabolism in bacteria that are capable of growing in the presence of acrylate. First, various bacterial strains believed to possess the acrylate pathway will be screened to select the best acrylate consumers. Then, the metabolic fluxes of those selected strains will be compared in batch culture to determine how acrylate affects growth and product distribution. Finally, analysis of cultures in chemostats will provide an understanding of how the selected strain partitions carbon under steady-state conditions in the presence of acrylate. Understanding acrylate metabolism may provide insights about the organism and the potential ability to synthesize acrylate biologically.

Specific studies

1) To compare different strains of genera *Clostridium* and *Megasphaera* and select organisms that grow in the presence of acrylate (Chapter 2).

2) To complete carbon balances of selected strains growing in the presence of acrylate under batch conditions (Chapter 2).

3) To compare metabolic fluxes in carbon limited chemostats carried out in the presence and absence of acrylate, including carbon balances and enzyme activity measurements at steady state (Chapter 3).

Hypotheses

1) Strains are not only capable of growing in presence of acrylate but are able to metabolize it and convert it to propionate and other volatile fatty acids.

2) Propionyl-CoA transferase is capable of transferring the CoA moiety to externally added acrylate and convert it to acrylyl-CoA which then enters the propionate pathway. Hence, in presence of acrylate the flux of acrylate to acrylyl-CoA will be greater than the flux of lactate to lactyl-CoA mediated by the enzyme propionyl-CoA transferase.

3) The presence of acrylate will affect the activity of enzymes propionyl-CoA transferase and acrylyl-CoA reductase. The increase in acrylate flux will match an increase flux of propionyl-CoA transferase activity.

4) The presence of lactate is indispensable for the growth of *M. elsdenii*.

5) Lactyl-CoA dehydratase is necessary for cell growth on lactate. However, growth will be restored in the presence of acrylate.

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Chapter 2

Selection and Characterization of Acrylate Degrading Bacteria

Introduction

Acrylic acid (CH_2=CH-COOH) is a commodity chemical with an annual production of about 4 million tons (Danner *et al.*, 1998). Acrylic acid and its esters are used in various industries such as paints, coatings, textiles, adhesives, plastics, detergents and super absorbent materials such as baby diapers (Danner et al., 1998). Production of acrylic acid (acrylate) biologically from renewable resources is of great interest to reduce reliance on petroleum. Several bacteria generate the thioester intermediate acrylyl-CoA as a part of their metabolic pathways (Hodgson & McGarry, 1968, Ladd & Walker, 1959). One pathway containing acrylyl-CoA involves the reduction of lactic acid to propionic acid and is referred to as the "acrylate pathway" (Counotte *et al.*, 1981). In this pathway, lactyl-CoA undergoes dehydration to acrylyl-CoA by the enzyme lactyl-CoA dehydratase (EC 4.2.1.54). Several organisms use the acrylate pathway, including *Clostridium propionicum* (Schweiger & Buckel, 1985) and *Megasphaera elsdenii (*Counotte *et al.*, 1981*).* Acrylyl-CoA is also associated with several other bacteria including *Clostridium scatalogenes* (Kusel *et al.*, 2000), *Clostridium neopropionicum* (Tholozan *et al.*, 1992), and *Clostridium drakei* (Gossner *et al*., 2008).

Since the conversion of lactate to propionate via acrylyl-CoA is a reduction, anaerobic microorganisms which use the acrylate pathway must also oxidize lactate when it is the sole carbon source. To accomplish this oxidation, lactate is oxidized to acetate and carbon dioxide with the simultaneous production of hydrogen, butyrate and small amounts of larger fatty acids

(Johns, 1952). Reducing equivalents such as NADH generated from the formation of acetate are balanced by their consumption from the formation of propionate (Schwieger & Buckel, 1984). Specifically, lactate is oxidized to pyruvate by lactate dehydrogenase (EC 1.1.1.27), generating a reducing equivalent such as NADH. The pyruvate formed is further oxidized and decarboxylated to acetyl-CoA by pyruvate ferredoxin oxidoreductase (EC 1.2.7.1) with the simultaneous reduction of two electron transferring flavoproteins (ETF) (Janssen, 1991). The ETFs are replenished by reducing acrylyl-CoA to propionyl-CoA by the enzyme acrylyl-CoA reductase (Hetzel *et al.*, 2003). In *M. elsdenii*, the ETFs isolated and purified are derivatives of FAD and FMN (Mayhew *et al.*, 1974). *M. elsdenii* also possesses a ferredoxin hydrogenase (EC 1.12.7.2) as a means to consume the excess reductant formed (NADH) by evolving hydrogen gas (Adams, 1990). Balancing redox reactions is a very important aspect of both anabolic and catabolic processes in living cells. During carbon metabolism redox reactions occur in which electrons or hydrogen atoms are transferred between various donor and acceptor molecules (Green & Paget, 2004). The role of electron carriers such as FAD and NAD is important in shuttling them to electron acceptors (Green & Paget, 2004).

A metabolic pathway is a sequence of biochemical reactions connecting the various inputs, intermediates and output metabolites. The flow rates of materials (fluxes) through a pathway respond to perturbations in cell environment, change of nutrients or as a result of genetic modifications (Stephanopoulos, 1999). Conservation of mass and cellular constraints require that the carbon and redox fluxes be balanced. Metabolic fluxes are important for studying cell metabolism and for developing metabolically engineered strains to generate industrial products (Iwatani *et al.*, 2008). Flux analysis depicts the metabolic events taking place within the

cell by measuring the utilization of the substrates and the generation of products formed (Holms, 1996).

In order to calculate fluxes, a metabolic model must be established using the metabolites of the pathway and the reaction stoichiometries (Kauffman *et al.*, 2003). The incoming fluxes to a metabolite are balanced by outgoing fluxes (Wiechert, 2001). Metabolic flux analysis has been employed as a method for studying shifts in intracellular carbon flux with respect to modifications in the environment.

When an organism encounters a change in its environment it responds by altering its metabolism accordingly. For example, when *E. coli* is grown on acetate instead of glucose, the cells decrease the flux through glycolysis with a simultaneous increase of flux through gluconeogenesis (Nielsen, 2003). *Clostridium sporogenes* does not produce formic acid when grown at a neutral pH, however, a change to lower pH stimulates this organism to accumulate formic acid (Montville *et al.*, 1985). A detailed study of metabolic fluxes addressing how acrylate affects metabolism has not been conducted for organisms capable of metabolizing acrylate. Understanding the metabolic fluxes in the presence of acrylate might assist the development of biological routes to the production of acrylate.

Materials and Methods

Strains and growth media

Table 2.1 shows a list of microorganisms used.

All of the *Clostridia* spp. strains were grown on Alanine medium and the *M. elsdenii* were grown on Lactate medium at their respective growth temperatures (see Table 2.1).

Table 2.1: List of strains used in the study

Strain	Reference	Medium	Growth temp °C
Clostridium drakei (DSMZ 12750)	Gossner *et al*., 2008	Alanine	30
Clostridium drakei (DSMZ 14470)	Gossner *et al*., 2008	Alanine	25
Clostridium propionicum (DSMZ 1682)	Cardon and Barker, 1947	Alanine	37
Clostridium propionicum (DSMZ 6251)	Cardon and Barker, 1947	Alanine	30
Clostridium homopropionicum (ATCC 51426)	Dorner and Schink, 1990	Alanine	37
Clostridium neopropionicum (DSMZ 3847)	Tholozan *et al*., 1992	Alanine	30
Clostridium scatologenes (ATCC 25775)	Kusel *et. al*., 2000	Alanine	37
Megasphaera elsdenii (ATCC 17753)	Elsden *et al*., 1956	Lactate	37
Megasphaera elsdenii (ATCC 17752)	Elsden *et al*., 1956	Lactate	37
Megasphaera elsdenii (ATCC 25940)	Elsden *et al*., 1956	Lactate	37

Alanine medium contained (per L) : 7.7 g L-alanine, 3.0 g peptone, 4.0 g yeast extract, 0.3 g cysteine·HCl, 0.1 g $MgSO_4 \cdot 7H_2O$, 0.018 g $FeSO_4 \cdot 7H_2O$, 5.0 mL of 1M potassium phosphate buffer pH 7.0 (final concentration is 50 mM potassium buffer), 2.5 mL saturated $CaSO_4$, and 0.1 mg resazurin. The gas atmosphere was 100% N_2. 1 M potassium phosphate buffer contained 10.7188 g K_2HPO_4 and 5.2324 g KH_2PO_4 per 100 mL. Lactate medium was identical to Alanine medium except 7.7 g D, L-lactate was used in place of L-alanine.

Growth rate measurements

For determining the growth rates of the different strains, the organisms were first grown for about 15 h (OD ~ 0.5–0.7) using either Alanine medium or Lactate medium. Then, 0.5 mL of this culture was used to inoculate 10 mL of identical fresh medium, and the optical density (OD) was measured every hour. The maximum specific growth rate (μ_{MAX}) was obtained from the slope of natural logarithm of OD versus time.

For determining the growth rates of the different strains in the presence of acrylate, when the second culture attained an OD of 0.1, acrylate (5 mM, 10 mM, 20 mM, 30 mM) was added to the tubes anaerobically, and the OD was measured every 15 min for 2 hours.

Metabolic fluxes

Metabolic fluxes were calculated in the presence and absence of acrylate. After the cultures attained an OD of 0.1, 5 mM acrylate was added to the samples for flux measurements in the presence of acrylate while no acrylate added to samples for flux measurements without acrylate. Samples were taken every 15 minutes and stored at -20°C for substrate and product analysis. Several chemical analogues of acrylate and lactate were also examined: 2-methyl propenoate, 3 butenoate, crotonoate, 3-hydroxybutyrate and (R)-2-hydroxybutyrate. For these compounds, metabolic fluxes were calculated as described above using 5 mM of each analogue.

Metabolic fluxes were calculated by fitting the substrate consumption rates (lactate and acrylate) and product formation rates (acetate, propionate, butyrate) using a quadratic equation.

The flux calculation for each strain was performed in triplicate, and the average of the three independent experiments was reported. To calculate metabolic fluxes for the various substrates and products culture OD of 0.2 was used.

Analysis

The optical density of the culture at 600 nm was used to monitor cell growth (Thermo Scientific Genesys 20, Madison, WI.). For measuring the concentrations of organic acids, high performance liquid chromatography was used as previously described (Eiteman & Chastain, 1997) using 16 mN H_2SO_4 as the mobile phase and an oven temperature of 40°C.

Results

Growth rates in the presence and absence of acrylate

Acrylate can be toxic to bacterial metabolism as the double bond in acrylate acts as an electron withdrawing group, reacting with essential cellular components and depriving the cell of these components (Straathof *et al.*, 2005). We were interested in determining the effect that acrylate had on the growth rates of organisms which might use the acrylate pathway (Table 2.1). As an initial screen, these strains were grown either on Lactate medium or Alanine medium in the presence or absence of 5 mM acrylate (Table 2.2).

Compared to medium without acrylate, the presence of 5 mM acrylate consistently decreased the maximum specific growth rate. Several strains such as *Cl. drakei, Cl. neopropionicum, Cl. homopropionicum* and *Cl. scatologenes* were unable to grow in the presence of even 5 mM acrylate (Table 2.2). Because these organisms were unable to grow in the presence of this relatively low acrylate concentration, they were not considered for further study.

Table 2.2: Growth characteristics of strains measured with and without 5 mM acrylate.

Strain	**Medium**	**Growth rate (without acrylate) h^{-1}**	**Growth rate (with 5 mM acrylate) h^{-1}**
DSMZ 12750 *Cl. drakei*	Alanine	0.246	0.225
DSMZ 14470 *Cl. drakei*	Alanine	0.211	Did not grow
DSMZ 1682 *Cl. propionicum*	Alanine	0.566	Did not grow
DSMZ 6251 *Cl. propionicum*	Alanine	0.152	0.181
DSMZ 3847 *Cl. neopropionicum*	Alanine	0.241	Did not grow
ATCC 51426 *Cl. homopropionicum*	Alanine	0.313	Did not grow
ATCC 25775 *Cl. scatalogenes*	Alanine	0.125	Did not grow
ATCC 17753 *M. elsdenii*	Lactate	0.725	0.535
ATCC 17752 *M. elsdenii*	Lactate	0.536	0.525
ATCC 25940 *M. elsdenii*	Lactate	0.812	0.518

Strains able to grow on 5 mM acrylate were subjected to higher acrylate concentrations, and their maximum specific growth rates were measured. As shown in Table 2.3, generally the specific growth rate (h^{-1}) decreased as the acrylate concentration increased. Also, the higher acrylate concentrations generally led to a longer lag phase, which created experimental challenges in accurate measurement of growth rates.

Although most *Clostridium* strains were unable to grow at an acrylate concentration greater than 5 mM, the three *M. elsdenii* strains, ATCC 17753, ATCC 17752 and ATCC 25940, each grew in the presence of 5 mM and greater acrylate concentration (Tables 2.2 and 2.3). The specific growth rates measured were calculated from an average of three experiments and for strain ATCC 17753, the specific growth rates spanned from 0.593 h^{-1} to 0.142 h^{-1} with acrylate concentrations ranging from 0 mM to 30 mM (Table 2.3 and Figure 2.1). ATCC 17753, ATCC 17752 and ATCC 25940 were able to grow in the presence of 30 mM of acrylate but with a much lower growth rates.

Table 2.3: Specific growth rate of several strains in the presence of acrylate.

Strain	Specific Growth Rate (h^{-1})				
	0 mM acrylate	**5 mM acrylate**	**10 mM acrylate**	**20 mM acrylate**	**30 mM acrylate**
M. elsdenii ATCC 17753	0.593	0.535	0.513	0.371	0.142
M. elsdenii ATCC 17752	0.606	0.556	0.537	0.371	0.098
M. elsdenii ATCC 25940	0.464	0.473	0.234	0.406	0.025
Cl. propionium DSMZ 6251	0.152	0.181	0.134	Did not grow	Did not grow
Cl. drakei DSMZ 12750	0.246	0.225	Did not grow	Did not grow	Did not grow

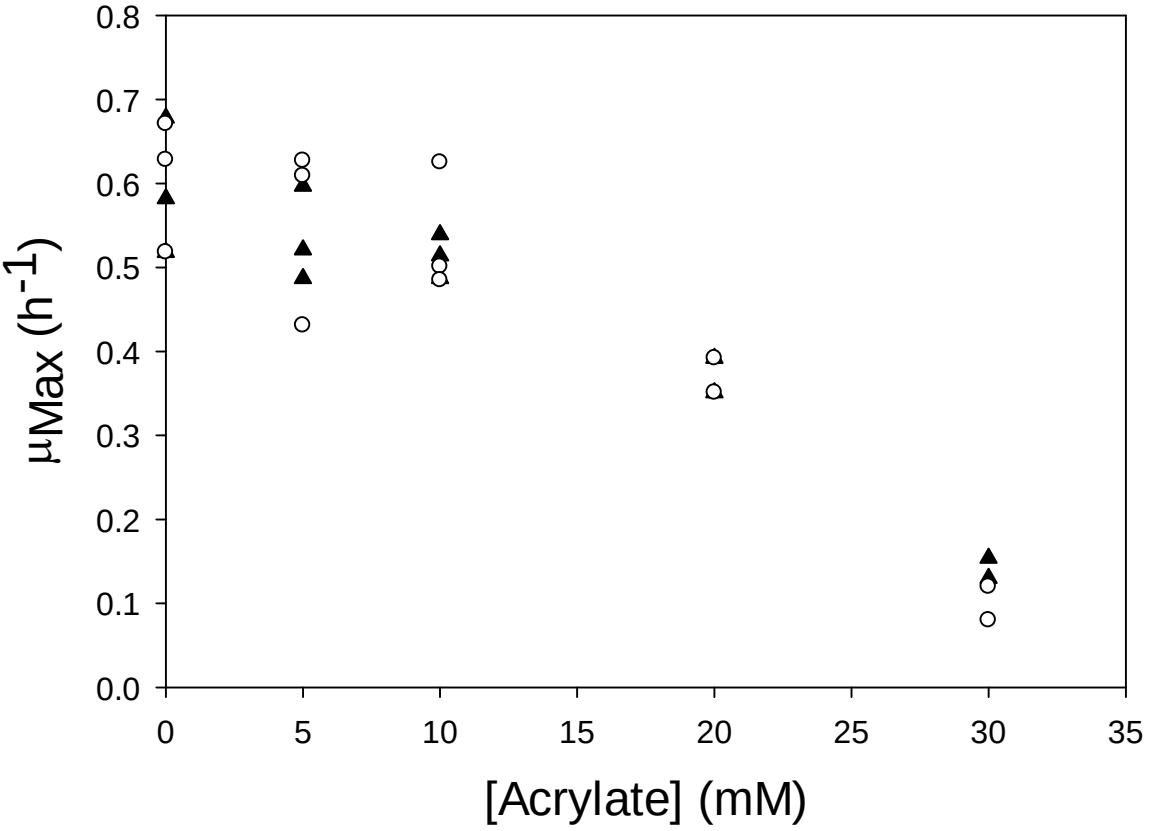

Figure 2.1: Maximum specific growth rate versus acrylate concentration for *M. elsdenii* ATCC 17752 (O) and ATCC 17753 (▲).

The strains ATCC 17752 and ATCC 17753 showed a similar growth profile with increasing acrylate concentration. At 30 mM acrylate, ATCC 17753 had a specific growth rate of 0.142 h^{-1} while ATCC 17752 had a slightly lower specific growth rate of 0.098 h^{-1} (Figure 2.1). Next we wanted to determine the extent to which these strains metabolized acrylate in the medium and how the presence of acrylate affected the metabolic fluxes of *M. elsdenii*.

Unsteady-state flux in the presence and absence of acrylate

To determine the rate at which acrylate was metabolized by *M. elsdenii*, the three strains of *M. elsdenii* were grown in batch culture in a medium containing both lactate and acrylate, and samples were collected to determine substrate and product concentrations. Acrylate was simultaneously metabolized during lactate consumption (Figure 2.2). The products formed were acetate, propionate and minute amounts of butyrate.

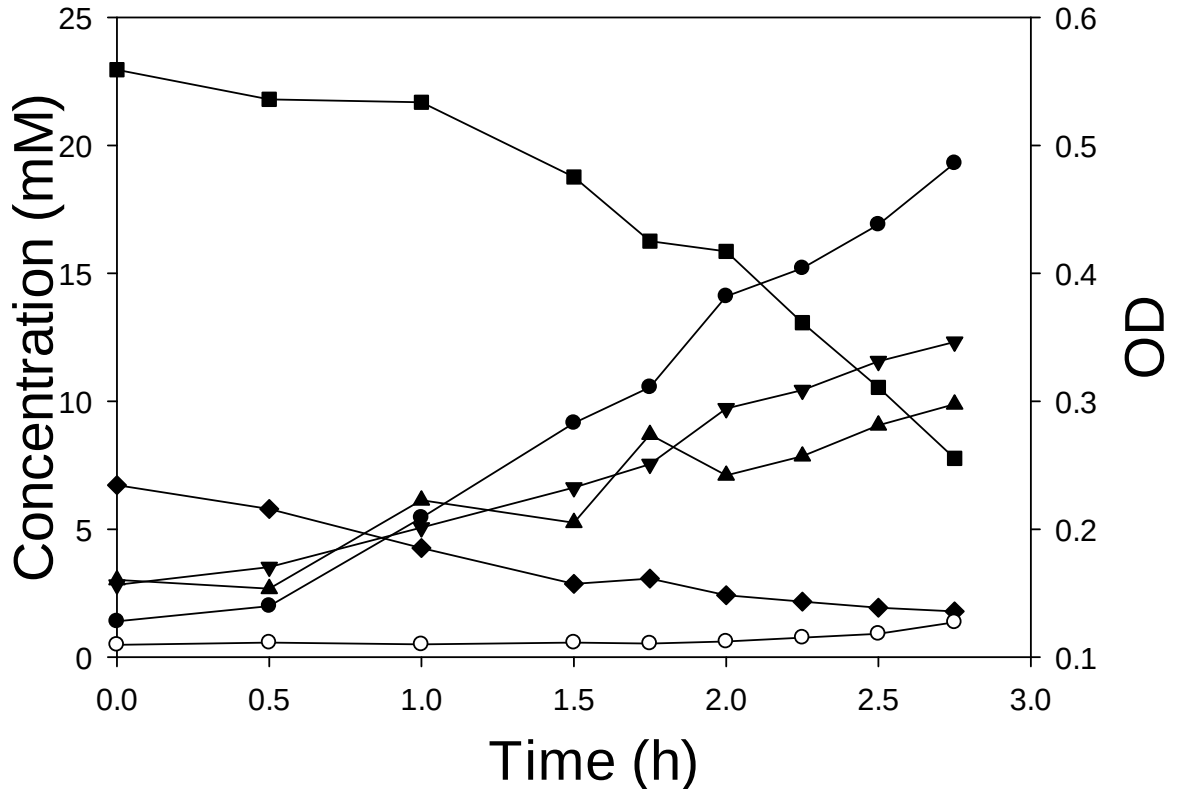

Figure 2.2: Batch culture of *M. elsdenii* ATCC 17753 on medium with 35 mM lactate (■) and 5 mM acrylate (◆) producing acetate (▲) propionate (▼) and butyrate (○) The OD (●) of the culture was also measured.

The metabolic fluxes were calculated in the presence and absence of acrylate for the strains ATCC 17752, ATCC 17753 and ATCC 25940 (Table 2.4). For ATCC 17753 and ATCC

25940, the flux of lactate is less in the presence of acrylate even though the propionate flux remains the same between the two conditions (Table 2.4). Thus, acrylate appears to replace lactate as the precursor of propionate. For example, for ATCC 17553 in the absence of acrylate the rate at which lactate is consumed is 82.9 mmol/gh, and the rate of propionate generation is 52.2% of the lactate flux. In the presence of acrylate, the rate at which lactate+acrylate is consumed is 72.4 mmol/gh, and the rate of propionate generation is 62.2% of this total flux. This observation for these strains supports the hypothesis that as more acrylate is converted to propionate and less lactate is metabolized to propionate. Three experiments were completed for each condition, and the results showed significant inconsistency (high standard deviation), and for several conditions the carbon balance, calculated as the ratio of carbon generated divided by carbon consumed, did not close. The quantity of carbon in yeast extract and peptone was over 1.0 g/L and its conversion toward acetate, propionate, or butyrate would have led to a calculated carbon balance greater than 100%.

Table 2.4: Unsteady-state metabolic flux calculated for *M. elsdenii* ATCC 17752, ATCC 17753 and ATCC 25940. The standard deviation of 3 measurements appears in parentheses.

Strain	Acrylate in Medium	Flux Lactate (mmol/gh)	Flux Acrylate (mmol/gh)	Flux Propionate (mmol/gh)	Flux Acetate (mmol/gh)	Flux Butyrate (mmol/gh)	Carbon Balance (%)
ATCC 17752	No	-54.1 (27.5)	0.0 (0.0)	30.4 (3.2)	29.4 (4.5)	0.9 (0.8)	114
ATCC 17753	No	-82.9 (23.1)	0.0 (0.0)	43.3 (5.3)	35.3 (2.7)	1.8 (0.8)	99
ATCC 25940	No	-61.6 (34.3)	0.0 (0.0)	47.0 (31.4)	51.0 (29.9)	3.0 (0.8)	169
ATCC 17752	Yes	-61.6 (11.5)	-22.0 (11.5)	30.8 (14.4)	32.3 (12.2)	1.3 (1.7)	79
ATCC 17753	Yes	-41.7 (11.0)	-30.7 (16.3)	45.4 (8.1)	33.1 (3.9)	3.5 (4.6)	118
ATCC 25940	Yes	-49.2 (30.1)	-45.8 (33.4)	31.9 (18.5)	26.8 (9.5)	-0.2 (1.4)	61

Acrylate and lactate analogues

The 3 strains of *M. elsdenii* were examined for their ability to grow in the presence of several chemical analogues of lactate and acrylate (Table 2.5). Each chemical analogue was added to the lactate-containing medium just as acrylate had been added.

Table 2.5: Metabolism of acrylate and lactate analogues.

Analogue	Analogue of acrylate/lactate	Metabolized
2-methyl propenoate	Acrylate	Yes
3-butenoate	Acrylate	Yes
crotonoate	Acrylate	No
(R)-2-hydroxy-butyrate	Lactate	No
3-hydroxy-butyrate	Lactate	No

The three *M. elsdenii* strains behaved the same. They each metabolized 2-methyl propenoate to isobutyrate and 3-butenoate to butyrate. However, crotonate, (R)-2-hydroxy-butyrate and 3-hydroxy-butyrate were not metabolized by the *Megasphaera* strains. Figure 2.3 shows the metabolism of lactate and 2-methyl propenoate to acetate, propionate and isobutyrate by ATCC 17753.

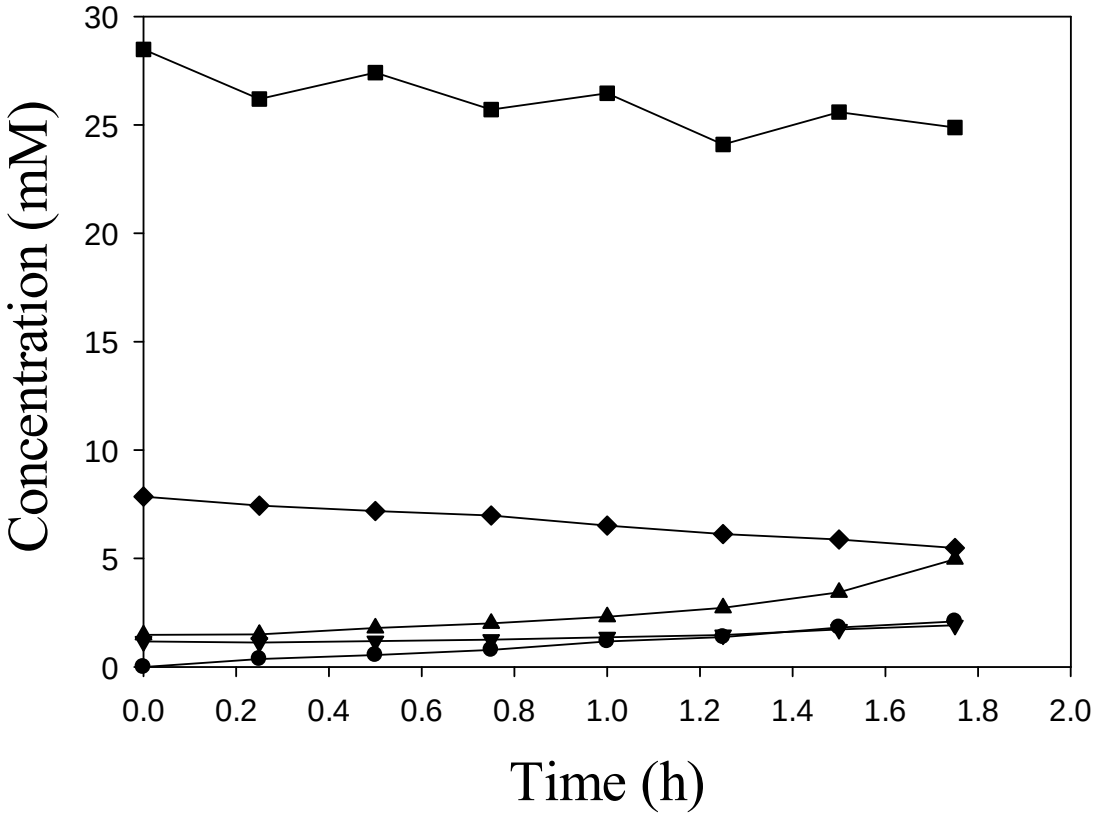

Figure 2.3: *M. elsdenii* ATCC 17753 metabolizing lactate (■) and 2-methyl propenoate (◆) to produce acetate (▲), propionate (▼) and isobutyrate (●) as products.

Discussion

Acrylate is toxic to most living cells due to its oxidizing nature (Freidig *et al.*, 1999), and the appearance of free acrylate in biological systems is not common. Instead, acrylyl-CoA has been suggested to be an intermediate in biochemical pathways (Ladd & Walker, 1959); (Hodgson & McGarry, 1968). *Clostridium* spp. and *Megasphaera elsdenii* use the acrylate pathway to reduce lactate into propionate in order to balance the ATP-generating oxidation of lactate to acetate. One enzyme in the acrylate pathway, propionyl-CoA transferase, is a general CoA-transferase which acts on several CoA thioesters, including acetyl-CoA, butyryl-CoA and

propionyl-CoA (Schulman & Valentino, 1976b). Propionyl-CoA transferase transfers the CoA group onto lactate using propionyl-CoA or acetyl-CoA as a CoA donor (Schwieger & Buckel, 1984). The enzyme has a fairly broad substrate specificity and is able to use acrylate as a substrate (Selmer *et al.*, 2002). A second enzyme, acrylyl-CoA reductase, not only serves as a sink for the NADH generated from acetate formation, but has a low apparent K_M of 2 μM for acrylyl-CoA (Hetzel et al., 2003). These two factors contribute to the low intracellular concentration of acrylyl-CoA. The third enzyme involved in the acrylate pathway is lactyl-CoA dehydratase, which has low activity in cells (Baldwin *et al.*, 1965), allowing the product acrylyl-CoA to be maintained at a low intracellular concentration and drive the flux forward. We reasoned that the unnatural substrate acrylate should be readily converted to acrylyl-CoA by the transferase, thus being incorporated into cell metabolism directly and bypass lactyl CoA dehydratase. We sought to determine the impact of acrylate on lactate metabolism in organisms using the acrylate pathway.

Our first objective was to determine the acrylate tolerance of several organisms under the assumption that those with greatest tolerance would be most likely to metabolize this compound. The presence of acrylate affected the maximum specific growth rate of the strains tested (Tables 2 and 3). *Clostridium* strains were unable to grow in greater than 20 mM acrylate, while the three *M. elsdenii* strains grew slowly even in the presence of 30 mM acrylate. The specific growth rate (h^{-1}) was indirectly proportional to the acrylate concentration for the different strains investigated.

Under batch conditions, acrylate is used as an electron acceptor, allowing a diversion of lactate increasingly to acetate (Table 2.4). Specifically, in the presence of acrylate, the flux of lactate through acetate increases with a simultaneous decrease in the flux of lactate going

towards propionate (Observed for ATCC 17753 and ATCC 17752). The likelihood of acrylate being converted to propionate is further strengthened by the observation that *M. elsdenii* grown on glucose lacks the ability to generate propionate. However, *M. elsdenii* grown on glucose supplemented with acrylate is capable of generating propionate (Hino & Kuroda, 1993). Acrylate is metabolized into propionate and possibly some other volatile fatty acids by rumen bacteria (Whanger & Matrone, 1967).

The cell also has flexibility in maintaining a redox balance. In order to balance the redox within a cell, the electron donating reactions have to be balanced by the electron accepting reactions (Brockman & Wood, 1975). Lactate and pyruvate oxidation generate reducing equivalents in *M. elsdenii* (Brockman and Wood, 1975), and these reducing equivalents are subsequently consumed during the conversion of acetyl-CoA to butyryl-CoA, acrylyl-CoA to propionyl-CoA, or during the production of hydrogen gas (Brockman & Wood, 1975, Baldwin & Milligan, 1964). Based on the flux calculations we performed on *M. elsdenii* (Table 2.4), the organism is probably metabolizing acrylate to propionate as proposed in Figure 2.4. In the absence of acrylate, lactate is the sole substrate responsible for producing both acetate and propionate to balance the reducing equivalents. However, when acrylate is present, it is converted to propionate, consuming NADH in the process (Figure 2.4). This causes lactate to synthesize only acetate, generating NADH to balance the redox of the cell (Figure 2.4). For ATCC 11753, the flux of lactate decreases by half in the presence of acrylate while maintaining the flux of propionate constant (Table 2.4), suggesting acrylate takes the place of lactate . The presence of acrylate does not impact generation of ATP (presumably the flux to acetate is proportional to ATP generation). These factors together indicate a larger fraction of lactate is directed towards acetate in the presence of acrylate, with the conversion of acrylate to propionate

becoming essentially a self-regulating branch. The two branches namely, lactate to acetate and acrylate to propionate are almost decoupled however are tied together to balance the redox of the cell.

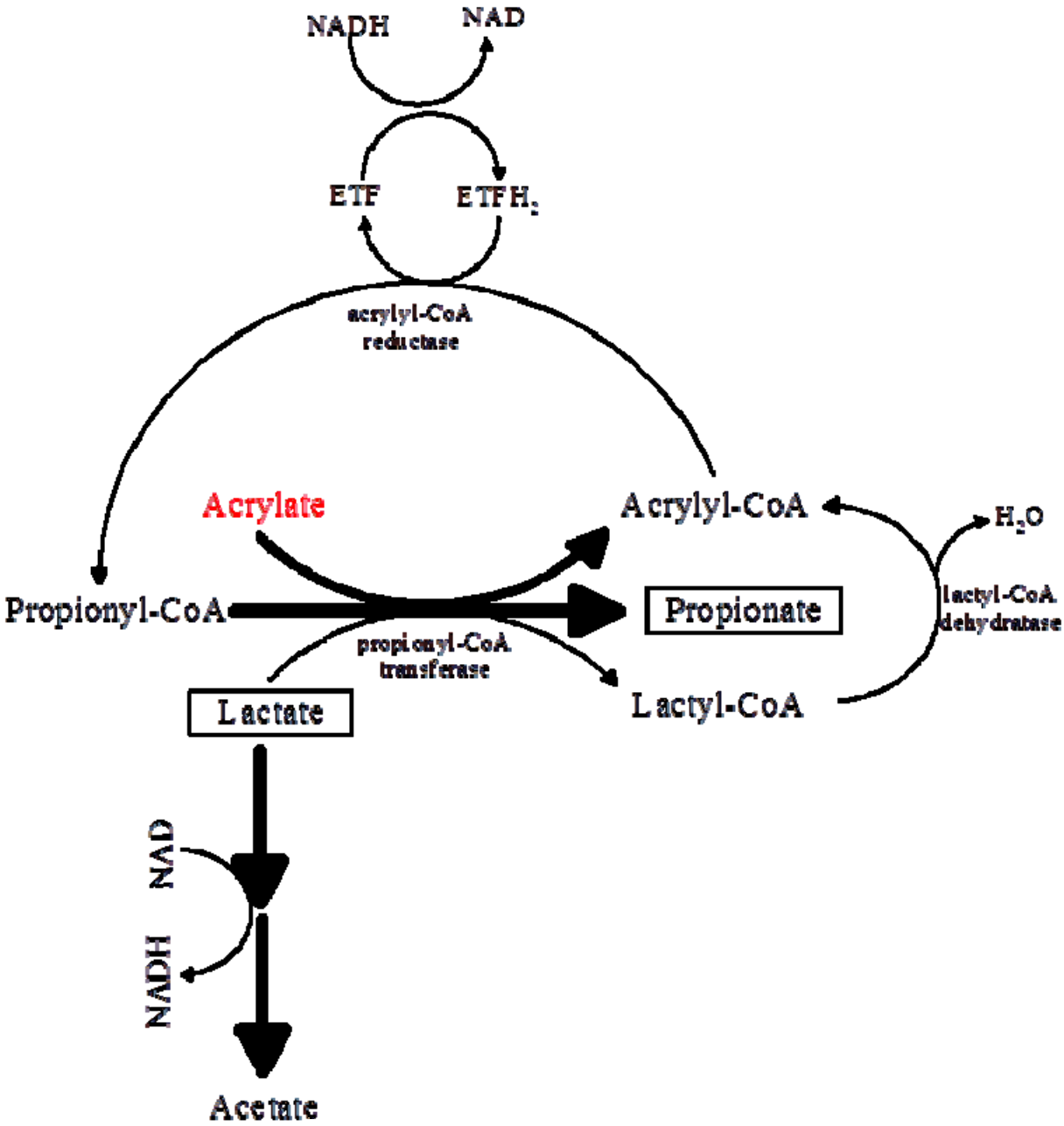

Figure 2.4: Hypothesized pathway exhibited by *M. elsdenii* in the presence of acrylate.

Analogues which are structurally similar to acrylate can also be used as electron acceptors by *M. elsdenii*, but analogues of lactate are not metabolized (Table 2.5). Like acrylate or lactate, the analogues might first be converted into their thioester before being further metabolized. The enzyme likely to carry out that transformation, propionyl-CoA transferase, is known to have a broad specificity which can mediate reactions of straight chain, branched chain, unsaturated and α,β-hydroxyl carboxylic acids (Tung & Wood, 1975), but the enzyme does not act on crotonate (Schulman & Valentino, 1976a). In our study, the two compounds with the vinyl group (a double bond on the terminal carbon), 2-methyl propenoate and 3-butenoate, were probably converted to their thioesters via propionyl CoA transferase (Figure 2.5). In contrast, crotonate, which differs from 3-butenoate only in having the C-C double bond between the α and β carbons, was not metabolized. These results also suggest that acrylyl CoA reductase, which normally reduces acrylyl CoA, reduces 3-butenoyl CoA and 2-methyl propionyl CoA, with propionyl CoA transferase mediating the final conversion to the acids butyrate and isobutyrate. The metabolism of each of these vinyl acids bypasses lactyl CoA dehydratase, but still provides the cells with a reduction step to balance the electrons generated in the conversion of lactate to acetate. Although propionyl-CoA transferase can mediate the transesterfication of hydroxy carboxylic acids including 2-hydroxy butyrate and 3-hydroxy butyrate (Tung & Wood, 1975), these substrates were not metabolized by *M. elsdenii*. Since lactyl-CoA dehydratase has a low specific activity (Kuchta & Abeles, 1985), in these cases metabolism is likely prevented by the specificity of lactyl-CoA dehydratase, which dehydrates the 3-carbon α-hydroxyl lactyl CoA to acrylyl-CoA, but appears not to dehydrate 4-carbon analogues having the hydroxyl group on either the α or β carbon.

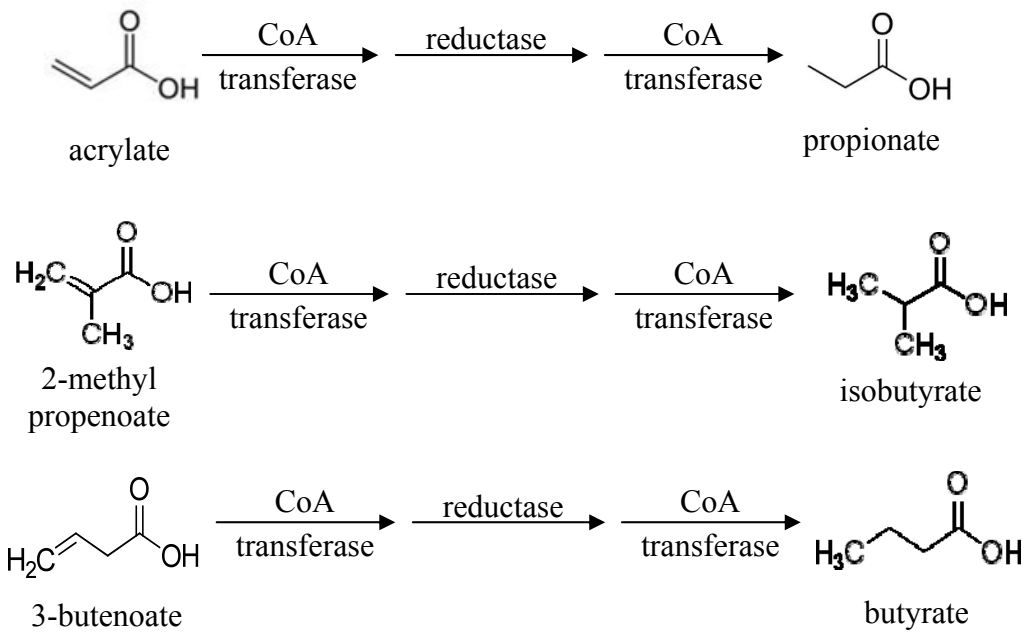

Figure 2.5: Formation of isobutyrate from 2-methyl propenoate

and formation of butyrate from 3-butenoate by *M. elsdenii* ATCC 17753.

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Chapter 3

Acrylate Metabolism by *M. elsdenii* Under Steady State Conditions

Introduction

Megasphaera elsdenii is a rumen bacterium capable of metabolizing DL-lactate to propionate and acetate as end products (Counotte *et al.*, 1981). Lactate is metabolized to propionate via the acrylate pathway, which produces acrylyl-CoA as an intermediate (Whanger & Matrone, 1967). While lactate is reduced to propionate in the acrylate pathway, the organism must simultaneously oxidize lactate to acetate and carbon dioxide with small amounts of butyrate (Johns, 1952). The acetate branch produces NADH and ATP while the propionate branch consumes the NADH in order for redox balance (Schwieger & Buckel, 1984).

The simple balanced stoichiometry of the two pathways is as follows:

$$3 \text{ Lactate} + \text{ADP} + \text{Pi} \longrightarrow 2 \text{ Propionate} + 1 \text{ Acetate} + 1 \text{ } CO_2 + 1 \text{ ATP} + 2 \text{ } H_2O$$

As noted above, *M. elsdenii* also produces butyrate as one of the products of fermentation of lactate (Hashizume *et al.*, 2003). Furthermore, as another means to balance the redox of the cell, *M. elsdenii* has the ability to evolve hydrogen gas (Hino *et al.*, 1991).

Washed cell suspensions of *M. elsdenii* ferment lactate and acrylate to yield acetate and propionate as products (Ladd & Walker, 1959). *M. elsdenii* is also able to grow on sugars like glucose and sucrose. However, propionate is not the end product formed from these sugar fermentations (Hino *et al.*, 1994).

A wide variety of strategies have been suggested to biologically synthesize acrylic acid using organic substrates (Danner *et al.*, 1998). Recently applications of enzymatic processes and

biological systems to synthesize various organic compounds have gained momentum (Yamada & Shimizu, 1988). With its ability to ferment acrylate, *M. elsdenii* is thought to be a good contender for this synthesis (Ladd & Walker, 1959). Studying this organism's metabolic pathways in the presence of acrylate will give us an insight into various enzymes of the pathway. In order to synthesize a product biologically and assess the feasibility of the process in terms of yield etc., carrying out a metabolic flux analysis is of utmost importance (Straathof *et al.*, 2005). Determination of *in vivo* metabolic flux is known as metabolic flux analysis. In addition to providing rates of cell metabolism, flux analysis also indirectly helps us determine the involvement of the various enzymes in the pathway (Stephanopoulos, 1998).

Continuous cultures or chemostats have been used for several decades as a tool to study the physiology of different species under various growth conditions (Koch, 1997). Cells in a chemostat attain a steady physico-chemical environment since the nutrients are constantly supplied at a predetermined rate. The cell growth is dictated by the limiting nutrient. Continuous culture is widely used for studying microbial physiology as a single parameter can be changed while keeping the others constant (Ferenci, 2006).

Calculating metabolic fluxes involves using a metabolic model which incorporates the major intracellular reactions, thus making possible the intracellular flux calculations obtained from the mass balances of the various intracellular metabolites (Avignone Rossa *et al.*, 2002). Metabolic flux analysis has been employed as a method for studying shifts in intracellular carbon flux with respect to modification in the environment (Sridhar & Eiteman, 2001).

There has not been a detailed calculation of the metabolic flux of *M. elsdenii* under steady state condition in the presence and absence of acrylate. We investigated the change in flux and the activity of key enzymes in the presence of acrylate by this organism.

Materials and Methods

Strains and growth media

Megasphaera elsdenii strain ATCC 17753 was used in this study.

Complex medium contained (per L): 3.00 g DL-lactate, 3.00 g Peptone, 4.00 g yeast extract, 0.3 g cysteine·HCl, 0.1 g $MgSO_4 \cdot 7H_2O$, 0.018 g $FeSO_4 \cdot 7H_2O$, 5.0 mL 1M Potassium phosphate buffer pH 7.0 (resulting in a final concentration of 50 mM), 2.5 mL $CaSO_4$ (saturated solution), 0.1 mg Resazurin, under an atmosphere of 100% N_2.

1M Potassium buffer: K_2HPO_4 10.7188 g, KH_2PO_4 5.2324 g per 100 mL.

Chemostat medium was similar to medium of (Soto-Cruz *et al.*, 2002) and contained (per L): 5.00 g lactate, 0.6 g yeast extract, 0.5 g cysteine·HCl, 40 mL mineral solution, and 1 mL vitamin solution. The mineral solution contained (per L): 0.20 g $CaCl_2$, 0.20 g $MgSO_4 \cdot 7H_2O$, 1.00 g K_2HPO_4, 1.00 g KH_2PO_4, 10.0 g $NaHCO_3$ and 2.00 g NaCl. The vitamin solution contained (per L): 0.5 mg biotin, 20 mg pyridoxine, 20 mg calcium pantothenate. The medium pH was adjusted to 6.5 using NaOH.

Batch growth

ATCC 17753 was initially grown in a 150 mL serum bottle containing 100 mL complex medium for about 10 h, following which 50 mL was transferred to 950 mL of the complex medium in a 2.5 L bioreactor (Bioflo III New Brunswick Scientific Co. Edison, NJ, USA). Nitrogen was sparged throughout the process, and the agitation was 150 rpm. The temperature was maintained at 37°C, and the pH was not controlled.

Steady-state growth

Continuous, steady-state fermentations of 1000 mL volume operated at a dilution rate of 0.125 h^{-1} as carbon-limited chemostats and were initiated in the batch mode in a 2.5 L bioreactor

(Bioflo 310). The influent medium contained the chemostat medium, and additionally either no other components or acrylate (10 or 20 mM) or 2-methyl propenoate (20 mM). A steady state condition was assumed after four residence times at which time the CO_2 concentration in the effluent gas remained unchanged. The temperature was maintained at 37°C, and the pH was controlled at 6.5 using HCl.

Figure 3.1: Set-up of continuous process (chemostat)

Analytical Methods

The optical density at 600 nm (OD) (UV-650 spectrophotometer, Beckman Instruments, San Jose, CA, USA) was used to monitor cell growth. The concentration of carbon dioxide in the off-gas was measured using a gas analyzer (Innova 1313 gas monitor, Lumasense Technologies, Ballerup, Denmark). Concentrations of soluble organic compounds were determined by high performance liquid chromatography as previously described (Eiteman & Chastain, 1997). The

hydrogen was quantified from the headspace of the bioreactor by using gas chromatography as previously described (6850 Network GC system, Agilient Technologies, USA) (Schut *et al.*, 2007).

Enzyme assays

Propionyl-CoA transferase

Propionyl-CoA transferase was measured in a cuvette containing a total volume of 1.0 mL at 25°C (Buckel *et al.*, 1981). The components were (final concentrations): 100 mM potassium phosphate pH 7.0, 200 mM sodium acetate, 1.0 mM oxaloacetate, 1.0 mM 5,3'- dithiobis(2-nitrobenzoate), 20 µg citrate synthase (Boehringer) and 0.1 mM propionyl-CoA. The reaction was initiated by addition of the enzyme (cell extract). The increase of absorbance was followed at 405 nm, $\Delta\square = 13.6\ mM^{-1}\ cm^{-1}$.

Acetate kinase

Acetate kinase activity was measured colorimetrically by its reaction with ferric iron (Rose, 1955). The components were (final concentrations): 100 mM Tris·HCl buffer (pH 7.4), 10 mM ATP, 10 mM $MgCl_2$, 700 mM hydroxylamine, 800 mM potassium acetate. The reaction mixture was maintained at 37°C for 5 minutes, then cell extract was added to initiate the reaction. 10% Trichloroacetic acid was added to stop the reaction. Subsequently 2 mL of ferric chloride solution was added, and the color measured by reading the absorbance at 540 nm.

Results

Batch growth of M. elsdenii ATCC 17753 on lactate

M. elsdenii ATCC 17753 is a rumen bacterium capable of growing on lactate accumulated in the rumen as a result of high fermentable sugar diet (Counotte et al., 1981). We first sought to quantify the growth of ATCC 17753 in an anaerobic batch culture. Figure 3.2

shows the growth on complex medium with 35 mM lactate. The organism attained a specific growth rate of 0.59 h^{-1}.

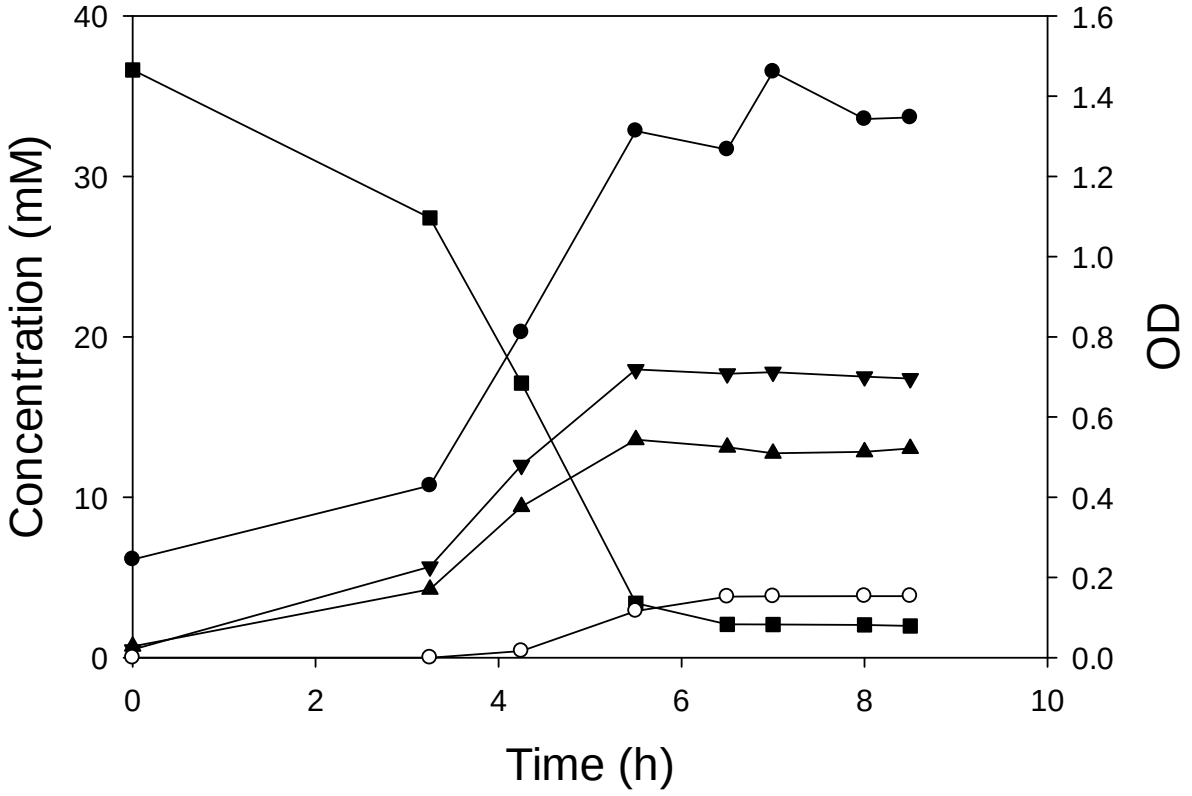

Figure 3.2: Batch culture of *M. elsdenii* ATCC 17753 grown on complex medium with 35 mM lactate. The OD (●) and concentrations of lactate (■), acetate (▲), propionate (▼) and butyrate (○) were measured.

Within 6 h, ATCC 17753 consumed lactate and generated about 13 mM acetate and 17 mM propionate. The concentration of butyrate increased after the concentration of acetate and propionate had increased. Although *M. elsdenii* synthesized acetate and propionate from the onset of the fermentation (Figure 3.2), butyrate was generated increasingly as the fermentation progressed. For example, the acetate and propionate concentrations decreased slightly after 5.5 h

while butyrate increased from 2.9 mM to 3.8 mM. This (late) increase in butyrate was not captured in the flux measurement reported in chapter 2, which was measured early in the fermentation process when the OD was 0.2.

Steady-state growth

Our next goal was to perform carbon-limited chemostats at a growth rate of 0.125 h^{-1} to compare steady state metabolism in the presence of increasing acrylate concentrations (0, 10 and 20 mM). At steady state conditions *M. elsdenii* metabolizes lactate into butyrate, acetate and carbon dioxide as main products of fermentation in contrast to propionate being the main product under batch conditions (Figures 3.2 and 3.4). However, at steady state conditions in the presence of acrylate, *M. elsdenii* generates propionate in addition to acetate, butyrate and carbon dioxide as products (Figure 3.4). Propionate production was observed under steady-state conditions only in the presence of acrylate (Figure 3.4).

The specific consumption rates of the substrates lactate (q_{Lac}) and acrylate (q_{Acr}), and the specific formation rates of the products acetate (q_{Ace}), propionate (q_{Pro}), butyrate (q_{But}), carbon dioxide (q_{CO2}) and (q_{H2}) were calculated and plotted (Figure 3.4). The specific rate of lactate consumption (q_{Lac}) decreased with increasing acrylate consumption (Figure 3.3). With increasing acrylate concentration in the feed (thus increasing q_{Acr}), an increase in q_{Pro} was observed, while there was a simultaneous decrease in q_{But} and q_{CO2} (Figure 3.4). The q_{Ace} did not show a particular trend with increasing acrylate consumption.

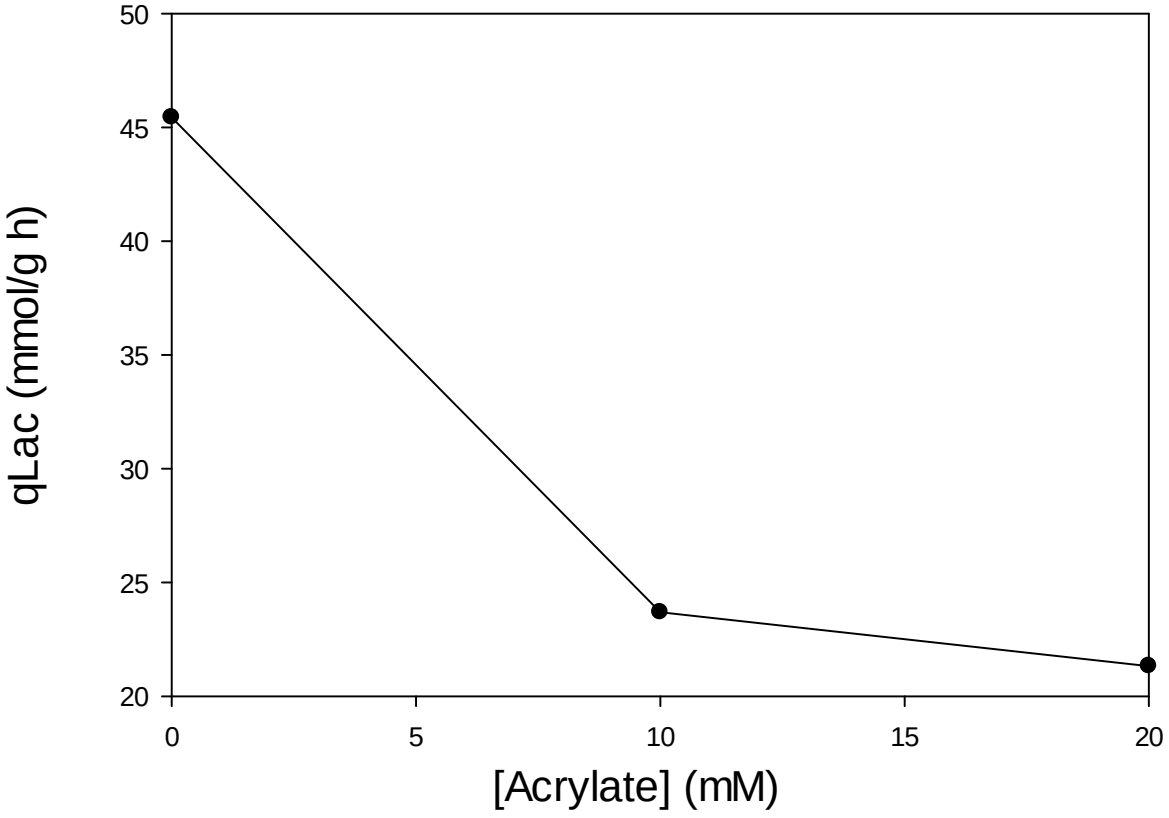

Figure 3.3: Specific lactate consumption rate (q_{Lac}) at varying acrylate concentrations for ATCC 17753 in minimal medium.

The specific rate of propionate production increased with increasing acrylate concentration with a simultaneous decrease in q_{But} and q_{CO2}. (Figure 3.4) further suggesting that the acrylate added externally is being converted to propionate. A decrease in the amount of butyrate and carbon dioxide is observed to compensate for the propionate produced in the presence of increasing acrylate. The data shown in Figures 3.3 and 3.4 represent the separate chemostat runs at increasing acrylate concentration. The carbon balances were calculated to be 81-99%. The low carbon balances might be attributed to the underestimation of CO_2 as it is difficult to recover all the CO_2 formed from the growth medium (Duncan *et al.*, 2002).

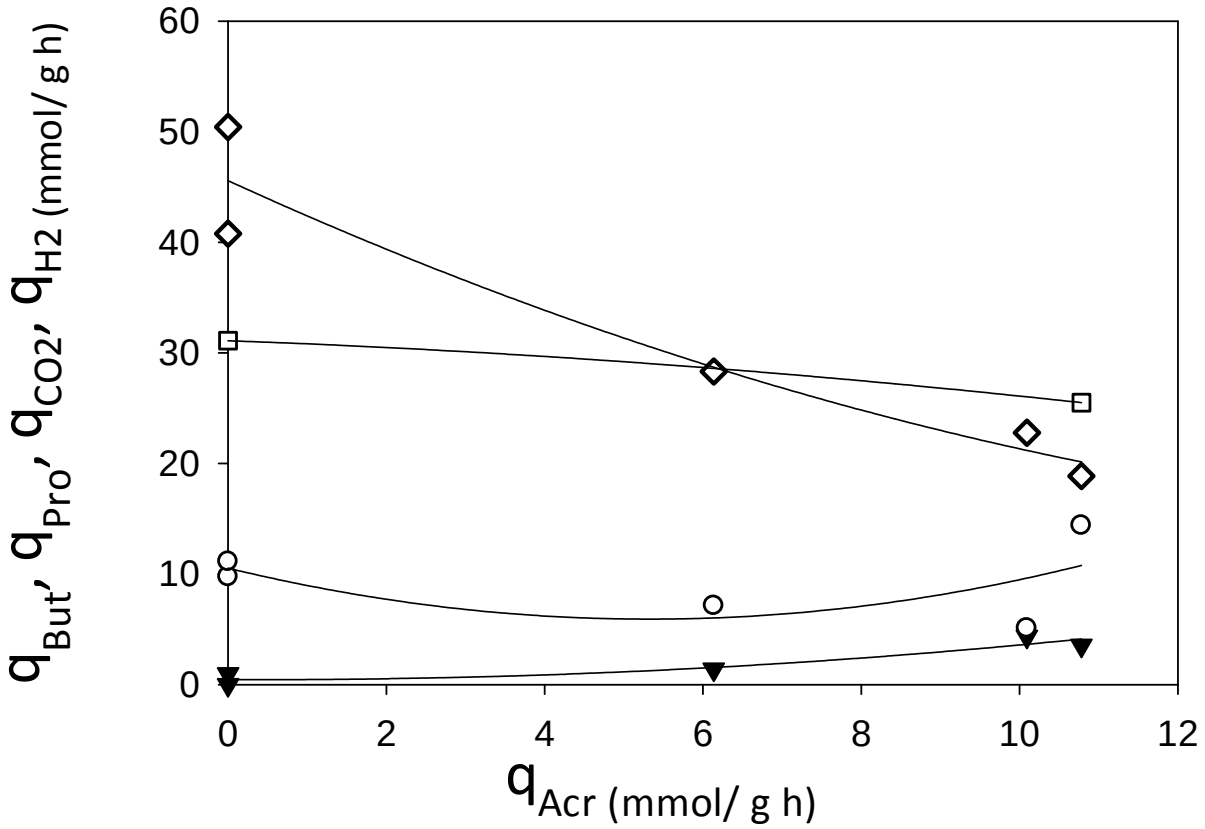

Figure 3.4: Specific production rate of butyrate (○), propionate (▼), CO_2 (◊) or H_2 (□) versus specific rate of acrylate consumption at steady-state for ATCC 17753.

We also calculated q_{H2} at steady-state conditions at increasing acrylate concentrations (0 and 10 mM). The q_{H2} decreases with increase in the acrylate concentration (Figure 3.4). The redox balances and q_{ATP} were also calculated for the various chemostat experiments. The q_{ATP} remained constant at different acrylate concentrations.

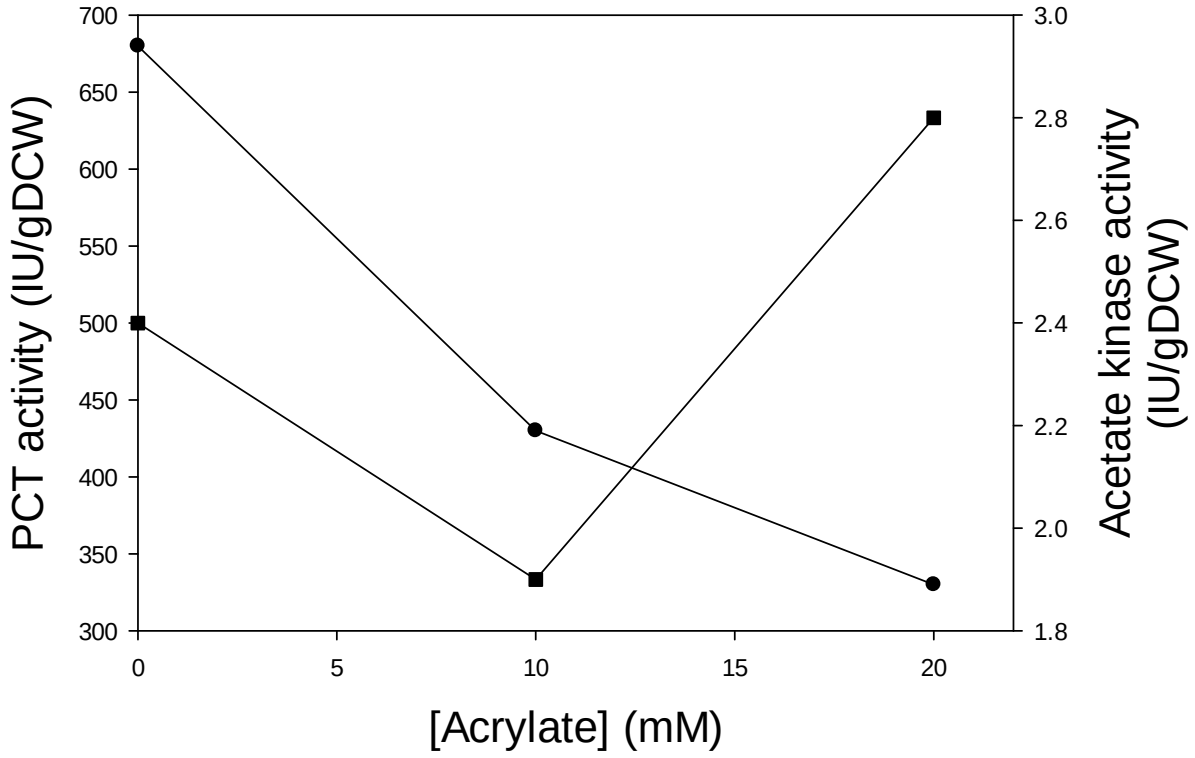

Figure 3.5: Propionyl-CoA transferase (PCT) (●) and acetate kinase (■) enzyme activity calculated for strain ATCC 17753 at increasing acrylate concentration.

We also wanted to quantitate the enzyme activities at increasing acrylate concentrations and test if increasing acrylate concentrations affect the enzyme activity. Propionyl-CoA transferase (PCT) is an important enzyme in the metabolism of lactate in *M. elsdenii* (Tung & Wood, 1975). PCT catalyzes the transfer of a CoA from propionyl-CoA to lactate, resulting in the formation of lactyl-CoA and the terminal product propionate (Selmer *et al.*, 2002). PCT is capable of transferring the CoA moiety to acrylate yielding acrylyl-CoA (Tung & Wood, 1975). Thus, in the presence of increasing acrylate and lactate consumption, PCT should mediate a larger flux. The PCT activity was determined as a function of increasing acrylate concentration in the feed (Figure 3.5). Surprisingly, the PCT activity decreased from 680 IU/g DCW to 330 IU/g DCW for an acrylate concentration of 0 mM to 20 mM respectively (Figure 3.5).

The enzyme acetate kinase physiologically catalyzes the conversion of acetyl phosphate to acetate liberating an ATP molecule. This step appears to be the only energy yielding step during lactate metabolism by ATCC 17753. No butyrate kinase activity was detected (data not shown), supporting the hypothesis that acetate kinase is the sole energy yielding enzyme for the organism. Acetate kinase activity was determined with increasing acrylate concentration in feed (Figure 3.5). No trend was observed in the activity of acetate kinase.

Discussion

M. elsdenii is naturally found in the rumen of cattle and sheep and is considered to be one of the main organism responsible for lactic acid catabolism in the rumen (Marounek & Bartos, 1987). *M. elsdenii* utilizes the acrylate pathway to metabolize lactate into acetate, propionate, and carbon dioxide as major products and butyrate as a minor product (Marounek *et al.*, 1989). However, its fermentation products vary with different substrates due to the extent to which various metabolic pathways are activated in the presence of various substrates (Marounek et al., 1989). Our objective was to grow *M. elsdenii* in carbon-limited steady-state conditions at varying acrylate concentrations and study the effect acrylate concentration had on the organism's metabolism.

M. elsdenii showed a markedly different behavior with respect to product formation under batch conditions as opposed to carbon-limited steady-state conditions (Figures 3.2 and 3.4). *M. elsdenii* grown under batch fermentations with lactate as a carbon source accumulates propionate as the major product (Figure 3.2). On the contrary, continuous cultures of *M. elsdenii* with lactate as the carbon source yielded butyrate as the main product of fermentation with decrease in the proportions of propionate and acetate (Figure 3.4). The presence of acetate in the surrounding medium plays an important role in butyrate synthesis as a molecule of butyrate is

formed from the condensation of two molecules of acetyl-CoA (Whanger & Matrone, 1967). *M. elsdenii* is capable of producing butyrate even under batch conditions when provided with acetate in the growth medium (Hino et al., 1991). Acetate formation thus acts as a prerequisite for butyrate formation, thus suggesting that *M. elsdenii* synthesizes propionate and acetate early on in a batch process until there is sufficient accumulation of acetate in the surrounding medium, after which it switches to synthesizing acetate and butyrate as the main product of fermentation while synthesizing very little propionate at this stage. Propionate being thought of as the major product of lactate fermentation by *M. elsdenii* can be attributed to the dearth of continuous culture studies performed with this organism.

We hypothesized that the production of butyrate under carbon-limited steady-state conditions was an energy favoring mechanism for *M. elsdenii.* Since *M. elsdenii* produces butyrate under carbon limited steady state conditions it could be energetically advantageous to the organism. Had *M. elsdenii* possessed the enzyme butyrate kinase, it would yield an additional molecule of ATP during conversion the of butyryl phosphate to butyrate thus becoming a major component of the energy metabolism of the organism (Valentine & Wolfe, 1960). However, the lack of butyrate kinase activity in *M. elsdenii* suggested that butyrate formation was not related to additional ATP formation in the cell (data not shown). In some rumen bacteria, butyrate production has also been reported by the transfer of a CoA from butyryl-CoA to acetate releasing butyrate and forming acetyl-CoA. For this reaction to occur, butyrate production is dependent on acetate in the surrounding medium or in other words acetate acts as a CoA acceptor for butyrate production (Diez-Gonzalez *et al.*, 1999). Our batch experiment demonstrates the lack of butyrate early on in the process and this can be attributed to the low amount of acetate in the surrounding medium (Figure 3.2).

Stoichiometric reactions in *M. elsdenii* from (Figure 3.6)

1) Lactate to propionate

lactate + propionyl-CoA → lactyl-CoA + propionate

lactyl-CoA → acrylyl-CoA + H_2O

acrylyl-CoA+ NADH →propionate + H_2O + NAD

Summing up: lactate + NADH → propionate + H_2O + NAD

2) Acrylate to propionate

acrylate + NADH → propionate + NAD

3) Lactate to acetate

lactate + NAD → pyruvate + NADH

pyruvate + Fd + CoA → acetyl-CoA + FdH_2 + CO_2

acetyl-CoA + Pi + ADP → acetate + CoA + ATP

Summing up: lactate+ Pi + ADP + NAD + Fd →acetate + NADH + FdH_2 + CO_2 + ATP

4) Lactate to butyrate

2 lactate + Pi + ADP + 2Fd → butyrate + ATP + H_2O + $2CO_2$ + 2 FdH_2

2 FdH_2 → 2 Fd + $2H_2$

(Formation of propionate + acetate from lactate) Summing (1) and (3)

2 lactate + Pi + ADP → propionate + acetate+ $2H_2O$ + FdH_2 + CO_2 + ATP

(Formation of butyrate from lactate)

2 lactate + Pi + ADP + 2Fd → butyrate + ATP + H_2O + $2CO_2$ + 2 FdH_2

From the above equations it is observed that irrespective of the organism synthesizing acetate + propionate or butyrate, one molecule of lactate yields 0.5 molecule of ATP. The organism is able

to completely balance its redox by the synthesis of butyrate by evolving hydrogen gas as a means to oxidize the reduced ferrodoxin.

Another benefit of butyrate synthesis to the organism is that the 2,3-dehydrogenation of propionyl-CoA to acrylyl-CoA is more unfavorable than that of longer acyl-CoAs (Sato *et al.*, 1999). The *M. elsdenii* acyl CoA dehydrogenase exhibits a higher redox potential for the conversion of acrylyl-CoA to propionyl-CoA than for the conversion of crotonyl-CoA to butyryl-CoA which means that the former reaction is thermodynamically more unfavorable in the cell (Reactions in Figure 3.6). The acyl-CoA dehydrogenase isolated from *M. elsdenii* shows higher substrate specificity for butyrate as compared to propionate (Sato et al., 1999). However, butyrate synthesis can take place only after acetate is present in the surrounding medium (Hino et al., 1991).

The consumption of acrylate affects lactate consumption, as well as propionate and butyrate formation (Figure 3.4). With increasing acrylate concentration *M. elsdenii* increasingly generates propionate while consuming less lactate and simultaneously generating lesser butyrate (Figures 3.3 and 3.4). Previous studies have demonstrated that bacteria (*E. coli*, *Corynebacterium* sp., *Eubacterium limosum*) are capable of simultaneously utilizing more than one carbon source under steady state conditions (Lendenmann *et al.*, 1996, Law & Button, 1977); (Loubiere *et al.*, 1992). Washed cell suspensions of *M. elsdenii* are able to ferment lactate and acrylate to generate pyruvate and propionate, and in the presence of phosphate molecules, the pyruvate generated is further converted to acetate (Ladd & Walker, 1959). Carbon labeling studies have showed that all the pyruvate and acetate generated comes from lactate while all the propionate is obtained from acrylate (Ladd & Walker, 1959). This explains our results where we obtain an increase in qPro with increasing qAcr (Figure 3.4). The acrylate added to *M. elsdenii*

cultures is directly reduced to propionate. Thus in the presence of increasing acrylate, the flux of acrylate to propionate increases causing the flux of lactate to butyrate to decrease in order to balance stoichiometry of the cell (Figure 3.6). The acrylate pathway under steady state conditions is as proposed in Figure 3.6. In the absence of acrylate, lactate is metabolized only to acetate and butyrate, while in the presence of acrylate; acrylate is metabolized to propionate and lactate to acetate and butyrate.

Acrylate provides *M. elsdenii* with flexibility in maintaining a redox balance. *M. elsdenii* produces reducing equivalents during the conversion of lactate to acetate and simultaneously consumes these reducing equivalents during the formation of propionate and butyrate (Figure 3.6). *M. elsdenii* has the additional capability of evolving hydrogen gas in order to consume any excessive reducing equivalents present in the cell (Figure 3.6) (Adams, 1990). *C. propionicum* also utilizes the acrylate pathway but lacks a hydrogenase, leading it to balance its redox by the genereation of volatile fatty acids such as propionate and butyrate (Adams, 1990, Ladd & Walker, 1959). As depicted in Figure 3.6, reducing equivalents are produced from the oxidation of lactate to acetate while reducing equivalents are consumed in the reduction of acrylate to propionate and during the formation of butyrate and hydrogen in the cell. Blocking hydrogen formation in *M. elsdenii* results in a build-up of reducing equivalents in the cell, stimulating the cell to further increase butyrate production (Hino et al., 1991). This observation further explains our results that with increasing acrylate concentrations, lesser reducing equivalent is available in the cell, thus decreasing the q_{But} and q_{H2} (Figures 3.4 and 3.6).

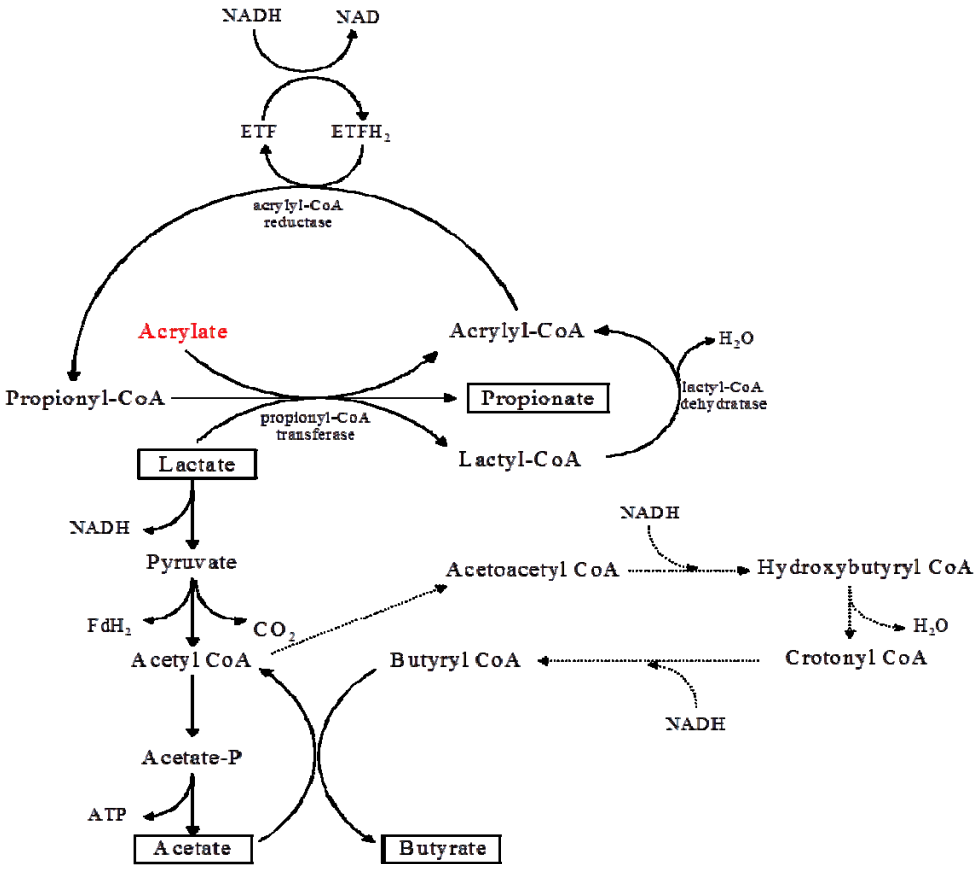

Figure 3.6: Hypothesized acrylate pathway in *M. elsdenii* under carbon limited steady state condition

Propionyl-CoA transferase (PCT) isolated from *M. elsdenii* can catalyze the conversion of acrylate to acrylyl-CoA (Tung & Wood, 1975). The enzyme is also active with various other CoA esters such as butyryl, isobutyryl CoAs but inactive against keto and dicarboxylic acids (Schulman & Valentino, 1976). We proposed that the propionyl-CoA transferase would exhibit a higher activity in the presence of increasing acrylate concentrations. This argument is based on

the assumption that PCT transfers the CoA onto acrylate in *M. elsdenii* cultures therefore having to transfer the CoA onto lactate as well as acrylate. Thus we expected the PCT activity to be higher with increasing acrylate concentration but surprisingly we observed just the opposite (Figure 3.5). Since the PCT activity calculated in our results decreases, it suggests that acrylate is making use of another transferase in the cell in order to form acrylyl-CoA. As mentioned earlier, the PCT transfers CoA onto a molecule of lactate as well, and with increasing acrylate concentrations lactate consumption decreases thus decreasing PCT activity to generate lactyl-CoA.

In summary, this study demonstrated that butyrate is the main product formed from steady state growth of *M. elsdenii* but in the presence of acrylate, propionate is readily generated.

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CHAPTER 4

Conclusions

The long term goal of this project is to synthesize acrylate from renewable sources using fermentation technology. The specific short term goal of this project however has been characterizing bacteria and studying their metabolism in the presence of acrylate. In particular, a major part of this research focused on different strains of *M. elsdenii* which were able to survive in the presence of acrylate and metabolize it. Our first goal was to grow bacteria at increasing acrylate concentrations and select one that grows at highest acrylate concentrations to perform unsteady and steady state flux studies. Ten different *Clostridium* and *Megasphaera elsdenii* strains known to be associated with the acrylate pathway were chosen to test growth characteristics in the presence of lactate supplemented with acrylate. *M. elsdenii* strains (ATCC 17753, ATCC 17752 and ATCC 25940) had the best growth rates at increasing acrylate concentrations and were able to metabolize acrylate into propionate under batch conditions with a simultaneous decrease in consumption of the primary substrate (lactate). ATCC 17753 gave the most consistent results with unsteady state metabolic flux and thus was chosen for the steady state experiments at increasing acrylate concentrations. Under steady state conditions, *M. elsdenii* metabolizes lactate (primary substrate) into butyrate as the main product of fermentation as opposed to propionate under batch conditions. Generating butyrate is energetically favorable to the organism however when acrylate is added to steady state cultures, propionate is generated from the added acrylate. The formation of propionate from acrylate even under steady state conditions further suggests that the bacterium has a active very enzyme to bring about this

conversion. Further knowledge about the enzymes active in the presence of acrylate, enzyme kinetics and metabolic flux calculations might be important information about this organism's capability to synthesize acrylate. Some enzymes in the acrylate pathway are reversible in nature and could be further characterized and engineered into other host organisms to generate acrylate.

Appendix

Steady-state experimental conditions

The data collected for the fermentation experiments are provided in the following pages. Steady-state fermentations were carried out under the following experimental conditions.
Agitation: 150 rpm
Acid: 15% Hydrochloric acid
Gas: Nitrogen in headspace
Gas Flow Rate: 250 mL/m
Initial Fermenter Volume: 900 mL
Inoculum Volume: 50 mL
pH: 6.5
Temperature: 37°C
Dilution rate: 0.125 h^{-1}

Date	4/30/2010
Organism	*M. elsdenii*
Strain	17753
Medium	30 mM Lactate (0 mM Acrylate)
Experiment	Unsteady state metabolic flux in the absence of acrylate for ATCC 17753

OD	ln(OD)	Time (h)	Lactate (mM)	Acetate (mM)	Propionate (mM)	Acrylate (mM)	Butyrate (mM)
0.09	-2.36	0.00	23.93	1.69	0.00	1.79	0.41
0.13	-2.02	0.50	21.76	2.03	0.00	1.86	0.40
0.20	-1.61	1.00	17.92	2.85	0.00	1.68	0.50
0.26	-1.36	1.50	16.67	4.32	0.00	1.93	0.46
0.30	-1.20	1.75	14.44	5.61	0.00	1.71	0.60
0.35	-1.06	2.00	11.59	6.38	0.00	1.59	0.55
0.40	-0.91	2.25	9.90	7.63	0.00	1.65	0.71
0.44	-0.83	2.50	7.82	8.08	0.00	1.75	0.88
0.49	-0.72	2.75	5.96	7.99	0.00	1.59	1.22

Date	4/30/2010
Organism	*M. elsdenii*
Strain	17753
Medium	30 mM Lactate
Experiment	Unsteady state metabolic flux in the presence of 5 mM acrylate for ATCC 17753

OD	ln(OD)	Time (h)	Lactate (mM)	Acetate (mM)	Propionate (mM)	Acrylate (mM)	Butyrate (mM)
0.13	-2.06	0.00	22.96	3.02	2.82	6.72	0.48
0.14	-1.97	0.50	21.79	2.68	3.52	5.79	0.57
0.21	-1.57	1.00	21.69	6.13	5.06	4.26	0.50
0.28	-1.26	1.50	18.76	5.25	6.62	2.86	0.57
0.31	-1.17	1.75	16.25	8.70	7.54	3.07	0.53
0.38	-0.96	2.00	15.85	7.11	9.72	2.42	0.61
0.40	-0.91	2.25	13.07	7.85	10.43	2.17	0.76
0.44	-0.83	2.50	10.54	9.06	11.56	1.93	0.91
0.49	-0.72	2.75	7.77	9.88	12.32	1.79	1.36

Date	4/30/2010
Organism	*M. elsdenii*
Strain	25940
Medium	30 mM Lactate
Experiment	Unsteady state metabolic flux in the presence of 0 mM acrylate for ATCC 25940

OD	ln(OD)	Time (h)	Lactate (mM)	Acetate (mM)	Propionate (mM)	Acrylate (mM)	Butyrate (mM)
0.12	-2.14	0.00	20.96	2.78	3.30	1.75	0.41
0.18	-1.71	0.50	17.82	4.17	4.77	1.72	0.45
0.23	-1.47	0.75	15.50	4.44	4.94	1.68	0.54
0.27	-1.30	1.00	13.84	5.32	4.19	1.31	0.49
0.29	-1.26	1.25	12.57	5.32	5.76	1.50	0.59
0.31	-1.18	1.50	12.17	6.26	6.65	1.56	0.65
0.34	-1.07	1.75	10.34	6.52	5.74	1.33	0.67
0.36	-1.03	2.00	9.89	7.52	7.62	1.58	0.74
0.37	-0.99	2.25	8.16	8.09	8.01	1.61	0.80

Date	4/30/2010
Organism	*M. elsdenii*
Strain	25940
Medium	30 mM Lactate
Experiment	Unsteady state metabolic flux in the presence of 5 mM acrylate for ATCC 25940

OD	ln(OD)	Time (h)	Lactate (mM)	Acetate (mM)	Propionate (mM)	Acrylate (mM)	Butyrate (mM)
0.16	-1.82	0.00	21.08	2.04	1.55	4.37	0.34
0.25	-1.39	0.50	20.33	2.57	3.09	3.77	0.34
0.31	-1.18	0.75	18.20	3.57	4.00	2.91	
0.33	-1.11	1.00	17.43	4.32	4.79	2.68	0.39
0.36	-1.02	1.25	16.52	4.95	4.35	2.32	0.41
0.38	-0.96	1.50	15.31	5.64	5.94	2.38	0.47
0.41	-0.89	1.75	13.34	7.87	7.52	2.07	0.57
0.45	-0.81	2.00	14.67	6.43	5.75	2.11	0.54
0.47	-0.75	2.25	12.12	8.27	8.67	2.29	0.65

Date	4/30/2010
Organism	*M. elsdenii*
Strain	17752
Medium	30 mM Lactate
Experiment	Unsteady state metabolic flux in the presence of 0 mM acrylate for ATCC 17752

OD	ln(OD)	Time (h)	Lactate (mM)	Acetate (mM)	Propionate (mM)	Acrylate (mM)	Butyrate (mM)
0.06	-2.88	0.00	21.28	2.11	2.77	1.78	0.40
0.11	-2.23	0.50	19.43	2.59	2.04	1.50	0.36
0.15	-1.92	1.00	17.52	3.64	2.91	1.44	0.46
0.20	-1.60	1.50	16.51	4.62	4.59	1.73	0.49
0.24	-1.41	1.75	15.25	4.77	4.78	1.60	0.47
0.29	-1.25	2.00	13.94	5.27	5.59	1.49	0.50
0.33	-1.11	2.25	13.80	6.32	5.19	1.46	0.59
0.36	-1.03	2.50	11.94	7.06	6.96	1.63	0.65
0.41	-0.90	2.75	10.59	7.64	7.38	1.55	0.80

Date	4/30/2010
Organism	*M. elsdenii*
Strain	17752
Medium	30 mM Lactate
Experiment	Unsteady state metabolic flux in the presence of 5 mM acrylate for ATCC 17752

OD	ln(OD)	Time (h)	Lactate (mM)	Acetate (mM)	Propionate (mM)	Acrylate (mM)	Butyrate (mM)
0.06	-2.88	0.00	19.59	3.56	4.20	3.79	0.55
0.10	-2.34	0.50	16.13	5.19	6.04	2.91	0.54
0.14	-1.95	1.00	13.42	5.69	6.18	2.14	0.64
0.20	-1.62	1.50	13.48	6.99	7.53	2.21	0.68
0.23	-1.47	1.75	11.32	7.43	8.14	1.96	0.71
0.27	-1.32	2.00	8.58	7.49	8.14	1.71	0.76
0.28	-1.27	2.25	7.86	8.85	7.97	1.46	0.92
0.32	-1.15	2.50	6.25	8.89	8.85	1.51	0.99
0.37	-1.00	2.75	5.42	10.52	10.22	1.66	1.43

Experiment: Summary of results obtained from the various steady state runs at different acrylate concentrations
Strain: ATCC17753
Media: 35 mM defined medium with 0, 10 and 20 mM acrylate

		Acr = 0	Acr = 0	Acr = 10	Acr = 10	Acr = 20
Dilution rate	(h-1)	0.13	0.13	0.13	0.13	0.13
DCW	g/L	0.10	0.07	0.18	0.10	0.21
$Y_{X/L}$	g/g	0.04	0.03	0.06	0.03	0.07
$Y_{X/C}$	g/mmol	1.26	0.92	1.40	0.79	1.33
$Y_{ACE/S}$	mol/mol	0.10	0.13	0.10	0.12	0.18
$Y_{P/S}$	mol/mol	0.00	0.02	0.05	0.07	0.14
$Y_{B/S}$	mol/mol	0.30	0.24	0.24	0.27	0.16
Q_L	mol/mol	3.21	3.07	4.15	4.20	4.40
q_L	mmol/gh	32.96	45.44	23.69	42.01	21.33
Q_{ACR}	mmol/Lh	0.00	0.00	1.07	1.08	2.08
q_{ACR}	mmol/gh	0.00	0.00	6.14	10.78	10.09
Q_{ACE}	mmol/Lh	0.32	0.40	0.51	0.64	1.15
q_{ACE}	mmol/gh	3.25	5.89	2.93	6.42	5.56
Q_P	mmol/Lh	0.00	0.06	0.24	0.36	0.90
q_P	mmol/gh	0.00	0.96	1.38	3.55	4.36
Q_B	mmol/Lh	0.95	0.75	1.25	1.44	1.05
q_B	mmol/gh	9.76	11.13	7.14	14.39	5.10
q_{CO2}	mmol CO_2/g DCW h	40.78	50.42	28.31	18.87	22.78

Experiment: Summary of carbon balance results obtained from the various steady state runs at different acrylate concentrations
Strain: ATCC17753
Media: 35 mM defined medium with 0, 10 and 20 mM acrylate

		Acr = 0	Acr = 0	Acr = 10	Acr = 10	Acr = 20
C-produced: Acetate	mmol C/h	0.6	0.8	1.0	1.3	2.3
C-produced: Propionate	mmol C/h	0.0	0.2	0.7	1.1	2.7
C-produced: Butyrate	mmol C/h	3.8	3.0	5.0	5.8	4.2
C-produced: CO_2	mmol C/h	4.0	3.4	5.0	1.9	4.7
C-produced: Biomass	mmol C/h	0.5	0.3	0.9	0.5	1.0
total C-produced	mmol C/h	8.9	7.7	12.6	10.5	14.9
C-consumed: Lactate	mmol C/h	9.6	9.2	12.4	12.6	13.2
C-consumed: Acrylate	mmol C/h	0.0	0.0	3.2	3.2	6.2
total C-consumed	mmol C/h	9.6	9.2	15.7	15.8	19.4
Carbon Balance	%	92.3	84.1	80.3	66.3	76.8