Analysis of the Enzyme System Responsible for Fermentation Product Formation by
Bacillus licheniformis

by

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Abstract

The enzyme system belonging to *Bacillus licheniformis* involved in the conversion from pyruvate to products was studied and its medium for activity optimized. Various lysis techniques including cell disruption by Misonix sonication and lysis by use of a Parr Cell Disruption Vessel were utilized and optimized for extraction of the enzymes from the microbial cell. Production of intermediates and byproducts such as 16mM acetoin and 13mM succinic acid from 87mM pyruvate by the cell-free enzyme system aided in determining the activity of the enzymes extracted, as did optical density measurements. Analysis of products was determined using HPLC with dilute sulfuric acid as the mobile phase.

The results of the body of work conclude that the Misonix Sonicator was better suited for sonication of cells and retention of enzyme activity than was the Parr Cell Disruption Vessel. The retention of ADH activity obtained from lysis by the Misonix Sonicator (62%) was twice that of the retention of ADH activity obtained from the Parr Cell Disruption Vessel (30%). Other results include the observation that the cell-free enzymes reacted to form product. This answers the question as to whether or not the enzymes would complete fermentation outside of the cells, as they do inside the cells. The cell-free enzymes formed succinic acid and no 2,3-butanediol. Interestingly, the whole cell fermentation of *B. licheniformis* produced 2,3-butanediol and no succinic acid.

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I. Introduction

Fermentations to produce chemicals such as 2,3-butanediol and succinic acid have been completed numerous times with whole cells of bacteria or yeast. Cell-free fermentations, in which the enzymes of these cells are removed and used to complete fermentation outside of the whole cells, have not been done. No literature was found on this subject. Multi-enzyme work, in which purified enzymes are purchased and used to produce specialty chemicals, is used in the pharmaceutical industry. The cost of purified enzymes is very expensive as compared to the cost of lysis and separation of enzymes from the whole cells. Lysis, as used in this thesis, includes all methods to open the cells and release the enzymes, including biological, chemical, and mechanical means. Cell-free fermentations were completed in an attempt to gain knowledge and understanding of the lysis of bacteria, removal of enzymes from the cells, retention of activity of these enzymes after lysis of the whole cells, and production of product, if any, by these cell-free enzymes.

2,3-Butanediol, also known as butanediol, 2,3-butylene glycol, and butane-2,3-diol, is a colorless, odorless liquid with a high boiling point, 177-184°C, depending on the stereoisomer, and a low freezing point of -60°C. Common producers and yields of 2,3-butanediol, batch fermentations, include *Bacillus polymyxa,* 0.87 mol mol^{-1} (Nakashimada, 2000), *Klebsiella pneumoniae*, 0.776 mol mol^{-1} (Jiayang, 2006) and *Bacillus licheniformis*, 0.519 mol mol^{-1} (Perego, 2003). This alcohol has been used extensively as antifreeze, converted to diacetyl for use as a food additive (Perego, 2003) and due to its comparable heating value of 27,198 J g^{-1} to

ethanol's 29,055 J g^{-1}, consideration as a fuel additive has become increasingly popular (Flickinger, 1980).

Succinic acid is used in the agricultural, food, and pharmaceutical industries. It is also used in the synthesis of biodegradable polymers such as polybutyrate succinate (PBS) and polyamides, as well as many important chemicals such as 1,4-butanediol, tetrahydrofuran, N-methyl pyrrolidione, 2-pyrrolidione, succinate salts, and gamma-butyrolactone (Song, 2006).

Succinic acid is mainly produced by petroleum or chemical routes. Liquefied petroleum gas (LPG) or petroleum is used as starting materials for the petroleum process. Producing succinic acid by fermentation would eliminate the need to use LPG and petroleum as starting materials for this chemical and cut down on the use of these fossil fuels. The fermentation process using glucose as substrate has also shown to be less expensive than the petroleum process (Song, 2006).

Producing succinic acid by chemical route involves the conversion of n-butane to maleic anhydride, and finally to succinic acid. The cost of maleic anhydride is the most expensive step in the chemical process to produce succinic acid. The fermentative route eliminates this step as well as its cost, which makes the fermentative route less expensive than the chemical route (Song, 2006). Fermentation to produce succinic acid fixes CO_2 (Song, 2006). Using this process rather than the chemical process reduces the levels of CO_2 escaping into the atmosphere which has negative effects upon the environment.

A variety of cell lysis techniques have been used historically to disrupt bacterial cells and release intracellular content. These techniques include osmotic lysis, chemical lysis, mechanical lysis, as well as many others. Chemical and enzymatic lysis are highly effective methods but also introduce chemicals or materials into the system that must be separated from product (Kido,

2007) as well as the potential of the proteases to deactivate the desired enzymes. Weibull et al., while also working with a *Bacillus* species, chose an enzymatic method of lysis. *Bacillus* cells were lysed enzymatically by destruction of cell walls when dissolved in 0.5mg mL^{-1} lysozyme prepared from egg white (Weibull, 1953). The lysozyme enzyme was not selected for this work due to possible deactivation of the desired enzymes. Mechanical lysis has been more successful when more gentle techniques have failed. Mechanical techniques include, among others, homogenization, pressure bomb, bead-beating, and sonication. Due to the coarse method of breakage, techniques employed by mechanical lysis, especially tissue homogenization, must optimize lysis of cell wall material while also minimizing breakage of any intracellular components (Kido, 2007).

Lysis by use of a pressure bomb takes advantage of pressure changes within the cell. High pressure is applied to the cell and when rapidly decreased the cell walls are burst open, releasing the intracellular content. Foster, Cowan, and Maag lysed bacterial cells using N_2 at 1740 psi over a period of 75 minutes and observed 31.9 to 58.9% rupture (Foster, 1961.) Cell wall destruction by bead-beating was completed by Zoetendal et al. (2001). Their method included addition of prepared cells to a mini bead beater for 3 minutes at 5,000 rpm in 150 µL phenol (Zoetendal, 2001).

Because of the popular uses and interest in 2,3-butanediol, the enzyme system belonging to *B. licheniformis* involved in the conversion from pyruvate to 2,3-butanediol was studied and its medium for activity optimized. The conditions for cell lysis and extraction of the enzymes from the microbial cell were optimized. Two mechanical methods of lysis were chosen for investigation upon *Bacillus licheniformis* because it is a gram positive bacterium and therefore

has a thicker cell wall consisting of intertwined layers of peptidoglycan. Two lysis techniques,

pressure bomb disruption and sonication were used to lyse the bacteria.

II. Literature Review

2,3-Butanediol: Uses and Chemistry

2,3-Butanediol has a molecular weight of 90.121 g mol^{-1}. Its molecular formula is

$C_4H_{10}O_2$. It exists in 3 isomeric forms: D(-), L(+), and meso. Early interest in 2,3-butanediol

dates back to World War II. In the 1930's and 1940's production of 2,3,-butanediol was of

importance because of its ability to be converted to 1,3-butadiene, an organic intermediate in the

production of synthetic rubber (Celinska, 2009). The freezing point of 2,3-butanediol, -60°C,

also lends the compound to be of great interest as an antifreeze agent. The dehydrogenation of

2,3-butanediol results in the compound diacetyl which is a favorable food additive or flavoring

agent (Perego, 2003). Dehydration of 2,3-butanediol results in methyl ethyl ketone (MEK)

which is a favorable fuel additive with a higher heat of combustion than that of ethanol

(Celinska, 2009). The heating value of 2,3-butanediol itself compares highly with ethanol's so

the compound can be used as a fuel additive without the need to dehydrate it to produce MEK

(Flickinger, 1980). MEK is also used as a solvent for resins and laquers. Esterification of 2,3-

butanediol with maleic acid results in polyurethane-melamides (PUMAs) which have been found

to be useful in cardiovascular applications. Additional uses of 2,3-butanediol and its derivatives

are numerous including: production of printing inks, perfumes, fumigants, spandex, moistening

and softening agents, plasticizers, and as a carrier for pharmaceuticals (Celinska, 2009).

Bacterial fermentation of 2,3-butanediol is accomplished by a mixed-acid pathway.

Other products include acetate, lactate, formate, succinate, and ethanol. The precursor to 2,3-

butanediol is acetoin. The conversion of acetoin to 2,3-butanediol is completed by an enzyme, butanediol dehydrogenase, and is a reversible reaction.

To produce 2,3-butanediol bacteria must first complete glycolysis. During glycolysis glucose, with the addition of 2 moles NAD^+, 2 moles ADP and 2 moles inorganic phosphate, is converted to 2 moles pyruvate, 2 moles NADH and 2 moles ATP. Many species of bacteria do not directly use glucose as its substrate, though. Xylose and other sugars can be converted to glucose by the bacteria in order to commence glycolysis.

Once glycolysis is complete the route in which pyruvate is degraded is dependent upon the enzyme system and specific enzymes each bacterial species contains as well as the presence or absence of oxygen. As Figure 1 shows, pyruvate may be converted to α-acetolactate, acetyl-coA, or lactate. One such system converts 1 mole pyruvate to 1 mole α-acetolactate with the release of 1 mole CO_2 by the enzyme acetolactate synthase. This reaction can be coupled to a second enzymatic reaction catalyzed by pyruvate dehydrogenase in which pyruvate along with thiamin diphosphate is converted to 2-(α-hydroxyethyl) thiamine diphosphate and CO_2 (KEGG). The conversion of pyruvate to α-acetolactate is accomplished under low levels of NADH availability. On the other hand, with high levels of NADH 2 moles pyruvate is converted to 2 moles lactate by lactate dehydrogenase (Celinska, 2009). Acetolactate decarboxylase further reduces 1mole α-acetolactate to 1 mole acetoin with the release of another mole of CO_2, under anaerobic conditions (Celinska, 2009). As previously mentioned acetoin, NADH, and H^+ are converted to 2,3-butanediol, and NAD^+ by butanediol dehydrogenase. Acetoin and NAD^+ may also be converted to diacetyl, NADH, and H^+ by diacetyl reductase (KEGG). This reaction takes place if oxygen is present (Celinska, 2009).
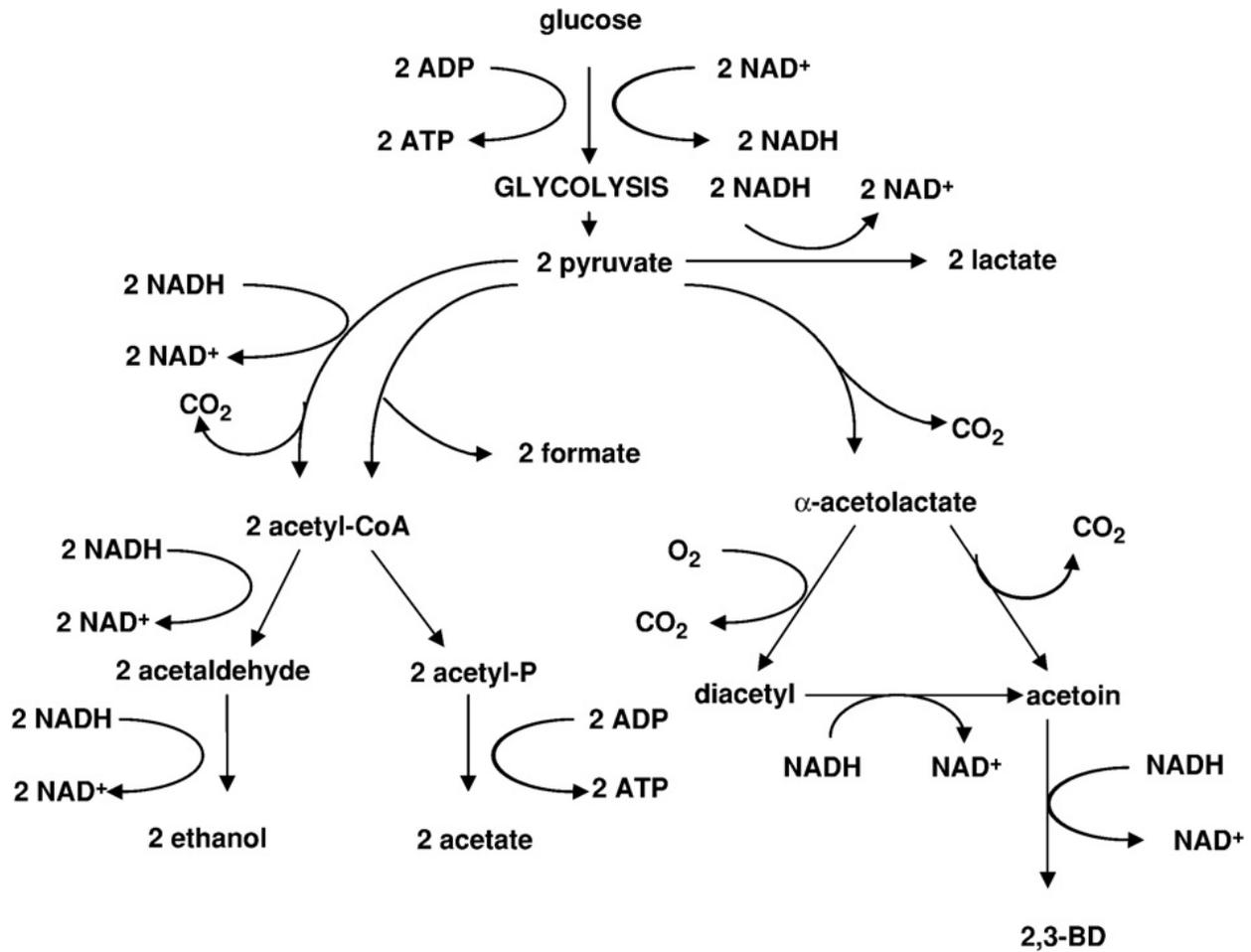
Figure 1. Enzymatic pathways from pyruvate to products (Celinska, 2009).

Due to the branches within the enzyme system, such as pyruvate being converted to either lactate or α-acetolactate, one may attempt to manipulate the system to coerce it to produce one product over the other. One such way to sway the system towards one branch is by use of enzymatic inhibitors. Pyruvate, when converted to lactate, remains lactate. Pyruvate, when converted to α-acetolactate, may continue along its path towards the desirable product, 2,3-butanediol. Akira Yoshida studied and characterized lactate dehydrogenase, the enzyme responsible for the conversion of pyruvate to lactate, isolated from *Bacillus subtilis*. He discovered that the enzyme was partially inactivated when diluted and at a neutral pH. He also

found oxaloacetate, mesoxalate, isocitrate, and D,L,-α-hydroxybutyrate to be competitive inhibitors of lactate dehydrogenase for pyruvate (Yoshida, 1964).

Microbial Producers of 2,3-butanediol

During the early 1900's investigative studies into microbial production of 2,3-butanediol were underway. The best producers of 2,3-butanediol were found to be *Klebsiella pneumonia*, *Klebsiella oxytoca*, *Bacillus polymyxa*, and *Bacillus licheniformis* (Perego, 2003).

K. pneumonia has been found to convert glucose to 2,3-butanediol, in the presence of $(NH_4)HPO_4$, at a yield of $0.763 mol\ mol^{-1}$ in shaking flasks. By maintaining the pH at 6.0 and switching to a fed-batch fermentation the yield rose to $0.858\ mol\ mol^{-1}$ with a productivity of 1-$1.5g\ L^{-1}\ h^{-1}$ (Jiayang, 2006).

D-xylose as well as D-glucose can be utilized as substrate for *K. pneumonia* as was shown by Yu and Saddler. They also showed that low levels of initial substrate, 1-2%, were more efficiently utilized by the organism than were higher substrate concentrations. Prolonged incubation time resulted in decreasing levels of 2.3-butanediol, as the organism may have begun to utilize the alcohol. Yu and Saddler also discovered that 2,3-butanediol yields increased in the presence of acetic acid when working with *K. pneumonia* (Yu, 1982).

The *Klebsiella* species are Biosafety Level 2 organisms which require proper laboratory and safety equipment. Appropriate skill, attention, and specific practices must be applied when working with these pathogenic organisms to prevent transmission of disease from the organism to the workers. Due to the pathogenic nature of this genus, despite its excellent efficiency for producing 2,3-butandiol, large-scale production of product by microbial fermentation of *K.*

pneumonia and *K. oxytoca* is avoided. Biosafety Level 1 microorganisms such as *Bacillus polymyxa* and *B. licheniformis* are much more desirable.

B. polymyxa is a strong producer of 2,3-butanediol and has been seen to produce 0.87mol mol^{-1} (Nakashimada, 2000). Upon examination of the culture medium used by *B. polymyxa* Laube discovered that with increased concentration of yeast extract glucose utilization and 2,3-butanediol production increased. He then went on to determine the key minerals present in yeast extract responsible for the positive effect. Manganese, magnesium, and iron were found to be the important elements within yeast extract. Manganese was determined to be the most effective at an optimal concentration of 1.7μM, which could be due to its requirement by the enzyme acetolactate decarboxylase.

As with *K. pneumonia*, *B. polymyxa* is able to metabolize sugars other than glucose. It was seen to use as substrate mannose, galactose, L-arabinose, cellobiose, starch, and glucose and able to produce significant amounts of 2,3-butanediol (Laube, 1984).

Other fermentative products by *B. polymyxa* include ethanol, acetoin, lactic acid, and acetic acid. *B. polymyxa* also produces large amounts of hydrogen. Production of these side-products reduces the production of 2,3-butanediol by the bacteria.

Fermentation by *B. licheniformis* yields smaller amounts of these by-products but fewer studies have been performed on this microorganism (Perego, 2003). In an attempt to gain insight and gather information about the *B. licheniformis* fermentation Nilegaonkar observed a yield of 2,3-butanediol from glucose of 0.934mol mol^{-1} at pH 6.0. He discovered *B. licheniformis* production of 2,3-butanediol to be highly dependent upon pH with the greatest yield found at pH 6.0 (Nilegaonkar, 1992).

To further broaden the knowledge of *B. licheniformis* 2,3-butanediol production, Perego conducted a study in which the starting substrate concentration, temperature, innoculum size, and carbon source were varied to find the optimal conditions for fermentation. The optimal conditions for the highest yield of 2,3-butanediol were found to be: 20g L^{-1} starting substrate concentration, 37°C, an increase in innoculum size increased the product formation, and starch hydrolyzate as a better carbon source than glucose or sucrose with a 2,3-butandiol yield of 0.628mol mol^{-1} compared to 0.519 and 0.376, respectfully (Perego, 2003).

Cell Lysis

There are a variety of cell lysis techniques including osmotic lysis, enzymatic lysis, mechanical lysis and many others. Each technique works well with a certain cell type and may not work as well for others. Osmotic lysis is a process by which such a large volume of water or solution moves into a cell that the cell walls cannot contain the volume and burst open. Chemical or enzymatic lysis occurs when a chemical or enzyme such as lysozyme is in the presence of a cell. The lysozyme hydrolyzes glycosidic bonds in the bacterial cell wall degrading the linkages in the peptidoglycan. Lysozyme works well against gram positive bacteria because of the large amount of peptidoglycan in its cell walls as opposed to gram negative bacteria which have a lipopolysaccharide outer membrane outside its thin layer of peptidoglycan. Mechanical lysis methods include homogenization, pressure bomb, bead-beating, and sonication. These techniques are used for cells that are hardier and require more energy to break open. Cells which do not have cell walls, such as mammalian cells, are easier to lyse. Osmotic lysis would be sufficient. Prokaryotic cells, such as bacteria and yeast, which contain cell walls, may not be

effectively lysed by osmotic or even enzymatic lysis alone. Mechanical or a combination of

mechanical and enzymatic lysis may be required to disrupt the cell walls of these cells.

One such mechanical lysis technique was proposed by Foster in 1961 as a method of

explosive decompression to rupture bacterial cell walls. He designed an apparatus that

resembled a modern Parr Cell Disruption Vessel in which nitrogen could be introduced, the cells

saturated with the gas, decompression and capture of ruptured cells, as well as sterilization of the

equipment could all be accomplished. Working with a variety of bacteria and a pressure of 1740

psi he observed 31.9 to 58.9% rupture. Pressures lower than 1740 psi showed much less rupture

and he postulated that pressures above 1740 psi would be even more efficient. The concentration

(mg dry weight cells mL^{-1}) seemed to have no effect on rupture efficiency. A volume of 125mL

cell suspension gave 31.9% ruptured cells while a volume of 50mL gave 31.4% (Foster, 1961).

Kurtzman performed mechanical and chemical lysis on *Bacillus cereus* spores in which

glass beads, sodium chloride, and ammonium bicarbonate were used. Rupture was accomplished

with 10mg spores, 100mg salt or glass beads, and a 250mg steel ball in a small steel capsule of

dimension 9.5 x 31.75mm. Results showed glass beads were least effective in rupturing the

spores and retaining glucose dehydrogenase activity and also contaminated the product with

metal which may have damaged the enzymes. Rupture with NaCl proved more effective (higher

glucose dehydrogenase activity) than with glass beads but less effective than with NH_4HCO_3.

Glucose dehydrogenase activity was determined by assay by the method of Bach and Sadoff

(1962) and followed by NADH appearance measured at 340 nm. Results also showed that

removal of NH4HCO3 by sublimation was necessary to avoid reduction in glucose

dehydrogenase activity (Kurtzman 1987).

Sonication experimentation was performed by Feliu, et al. Wild type *E. coli* strains with recombinant proteins joined with ß-galactosidase were lysed in a Braun Labsonic U with a needle titanium probe. Activity of enzymes after lysis was modeled by activity of ß-galactosidase since this enzyme's activity is easily measurable by Miller's method (Miller, 1972). Results indicated that cell concentration and ionic strength did not affect protein release by ultrasonication. Acoustic power and sample volume were found to be the important factors. A decline in probe performance due to aging caused by working time, chemical composition of the samples and acoustic power were also found to negatively influence protein release (Feliu, 1997).

III. Selection of Species

Two species of *Bacillus* were obtained from Northern Regional Research Laboratories, NRRL B-369 *B. polymyxa* and NRRL B-642 *B. licheniformis*. Both organisms were maintained on agar plates stored at 4°C and subcultured twice a month.

Growth culture medium for NRRL B-642 *B. licheniformis* was that of Perego (2003) and sterilized at 121°C, 15 minutes, before the addition of glucose. Growth culture medium for NRRL B-369 *B. polymyxa* was that of Stanier (1944), also sterilized at 121°C, 15 minutes, before the addition of glucose.

Both organisms were grown for a period of 24 hours in 125mL flasks each covered with either a sponge plug or a rubber stopper in a heated shaker at 37°C. Two flasks of *B. licheniformis* were grown for 48 hours. Following completion of the growth cycle a fermentation of 10% and 30% innoculum was prepared for each flask. The fermentation media were those of Perego (2003) and Stanier (1944), respectfully. Fermentation was carried out for 72, or 96 hours and the concentration of 2,3-butanediol produced during this time period by both *Bacillus* species was monitored.

This experiment provided preliminary data and was only intended as a means of choosing one species over the other. Figure 2 revealed that "3", *B. licheniformis*, achieved the highest concentration of 2,3-butanediol during the fermentation. Table 1 shows the slope (mM/hour) for each time period, 0-24 hours, 24-48 hours, 48-72 hours, and 72-96 hours. Samples 3 and 4, (all *B. licheniformis*) have the highest slopes, all during the 0-24 hour time period, which indicate

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high enzymatic activity, as higher slopes are a reflection of higher rates of butanediol production. *B. licheniformis* produced more consistent data, as compared to the inconsistent data and poor performance by *B. polymyxa*. For this reason *B. licheniformis* was chosen as the organism of interest with which to pursue further studies.
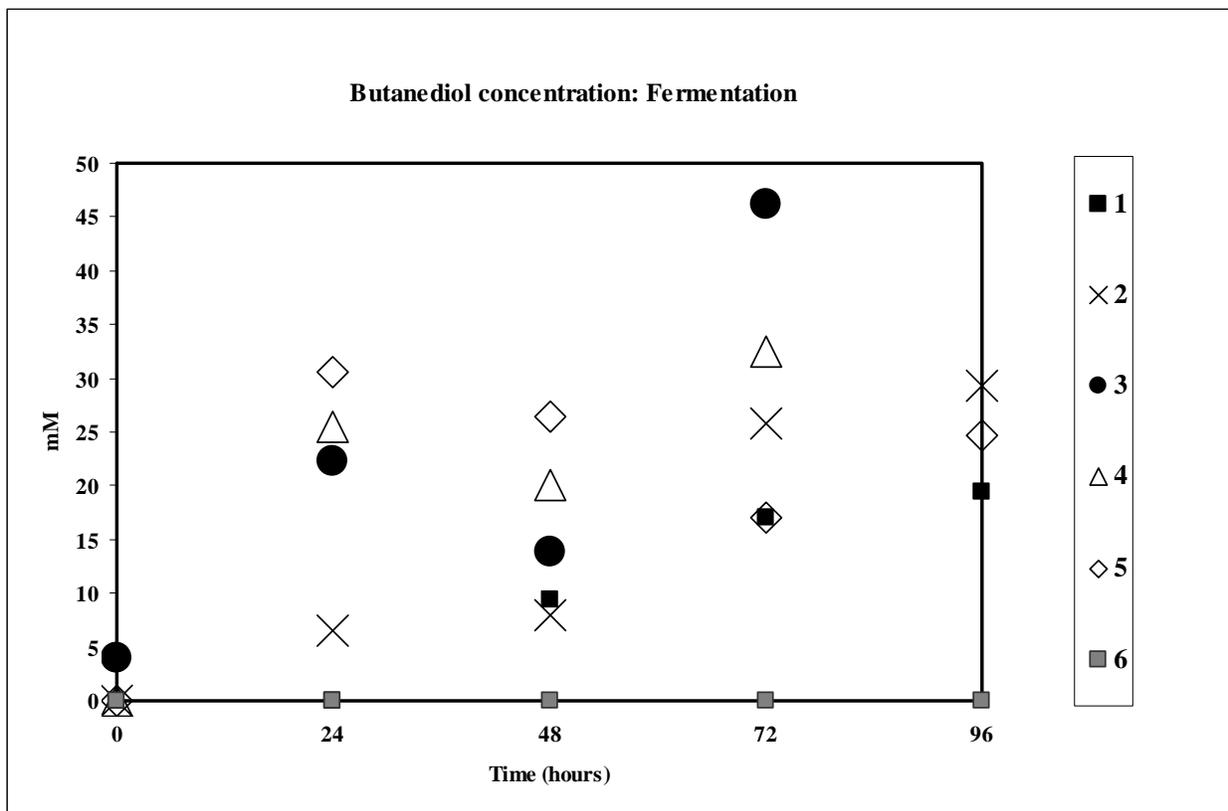
Figure 2. 2,3-Butanediol concentration (mM) during a fermentation over a period of 96 hours. Samples 1,2,3,4 are B. *licheniformis* and samples 5, and 6 are *B. polymyxa*.

Table 1. Samples 1, 2, 3, and 4 are *B. licheniformis*. Samples 5, and 6 are *B. polymyxa*.

Comparison of slopes of change in 2,3-butanediol concentration during each 24 hour time period

from Figure 1.

organism	Slopes (mM/hour)			
	0-24 hr	24-48 hr	48-72 hr	72-96 hr
1	0	0.03	0.03	0.01
2	0.02	0.01	0.07	0.01
3	**0.07**	0.03	0.12	
4	**0.10**	0.02	0.05	
5	0.11	0.02	0.04	0.03
6	0	0	0	0

IV. Cell Growth and Lysis

Materials and Methods

Bacillus licheniformis, NRRL B-642, was obtained from Northern Regional Research Laboratories. B-642 was propagated every 2 weeks on Tryptose agar using a streak plate method. Plates were stored at 4°C. Stock culture was stored at -80°C. The organism was grown microaerobically (Nakashimada, 2000) in 125mL Erlenmeyer flasks in a pre-culture medium of Perego (2003) with 111mM glucose as substrate at 37°C, shaken at 150 rpm, for 24 hours. Afterwards, the organism was aseptically transferred and fermented in a defined medium of Perego (2003) with the same conditions as the growth period. The concentrations of glucose and 2,3-butanediol were determined by a BioRad-HPX-87H column used for measurement of sugar, organic acid and ethanol. A refractive index detector was used with the HPLC with dilute sulfuric acid as the mobile phase at a flow of 0.55mL min^{-1}. All glassware was sterilized at 121°C for 15 minutes.

Cells were lysed using a Parr Cell Disruption Vessel at 2250 psi N_2 for 5 minutes. Cells were also lysed using a Misonix S-4000 Sonicator with a #418 or a #419 A microtip. During lysis by sonication cells were contained in a modified glass graduated cylinder, a conical 15mL polypropylene centrifuge tube, or a round-bottom centrifuge tube in order to position the microtip to optimize circulation and lysis of cell material. Cell lysis during sonication was cycled to reduce frothing with 5 seconds on and 10 seconds off and in an ice bath. Total sonication times refer to the total on time of the sonicator. Cell debris was separated from enzyme solution by centrifugation at 14,000 rpm for 5 minutes. Optical densities of whole cells

and lysed cells were compared using a Varian Cary 3e UV-visible spectrophotometer at 600nm and 280nm.

Because there is no enzyme assay for the enzymes involved in the conversion of pyruvate to 2,3-butanediol the enzyme alcohol dehydrogenase (ADH) served as a model in which any change in its activity would be expected to also occur in the change in activity of the enzymes of interest. The ADH enzyme in bacteria serves a similar function to the enzymes involved in the conversion of pyruvate to 2,3-butanediol and other products. The ADH catalyzes reactions that regenerate NAD^+ for the cells, as do the enzymes of interest. The ADH is a multi-enzyme subunit, as are most dehydrogenases, such as those in the enzyme system within *B. licheniformis*.

The enzymatic activity of alcohol dehydrogenase (ADH) was found by an ADH assay which was completed with the UV-visible spectrophotometer set at 340nm. The assay was conducted at pH 7.8, in an ice bath. Components of the assay include: alcohol dehydrogenase from baker's yeast (ADH) 340units mg solid^{-1}, 200mM disodium phosphate pH 8.8, 95% ethanol, Β-nicotinamide adenine dinucleotide hydrate (NAD^+), and 10mM sodium phosphate buffer pH 7.5. All chemicals were purchased from Sigma Aldrich. To run the assay, 1mg mL^{-1} ADH in 10mM sodium phosphate buffer pH 7.5 was placed in 22.5mL of the same buffer. After swirling, 1mL of this solution was placed in 5mL of 200mM disodium phosphate pH 8.8 and sonicated. After sonication, 1.5mL of 149.2mg NAD^+ in 15mL 200mM disodium phosphate pH 8.8, along with 0.1mL 95% ethanol, and 0.8mL 200mM disodium phosphate pH 8.8, 0.6mL of the sonicated ADH solution were added to a cuvette for a total volume of 3mL. The cuvette was placed in the UV set at 340nm, and the activity of the ADH enzyme was seen as the increase in absorbance of NADH, converted from NAD^+ over a period of six minutes.

Results

      B. licheniformis whole cells were grown for a period of 48 hours during which samples were taken for HPLC measurements in order to follow the concentrations of glucose and 2,3-butanediol over time. The whole cells were also fermented for 48 hours in order to compare the degree of glucose utilization and 2,3-butanediol production during the growth and fermentation phases. Figure 3 shows a comparison of glucose utilization during the growth and fermentation phases. Figure 4 is a comparison of the concentrations of 2,3-butanediol produced during the growth and fermentation phases. From Figure 3 it was found that the concentration of glucose dropped most rapidly during the growth period at the 21-24 hour mark. This was determined to be the time of highest enzymatic activity for the organism to utilize its substrate. For this reason, further growth periods were conducted for 24 hours, at which time the reaction was stopped by centrifugation and freezing of the pellet. This was done in order to collect the bacterial enzymes at their peak activity. Likewise, it is seen from Figure 4 that the highest rate of 2,3-butanediol production occurred at 12 hours into the fermentation phase.

Figure 3. *B. licheniformis* glucose concentrations over 48 hour growth and fermentation periods conducted at 37°C, shaken at 150rpm, pH 6.9. Growth and fermentation broths are those of Perego (2003). All data points represent the average of the results of four experiments. Samples for HPLC were 1mL every 3 hours.
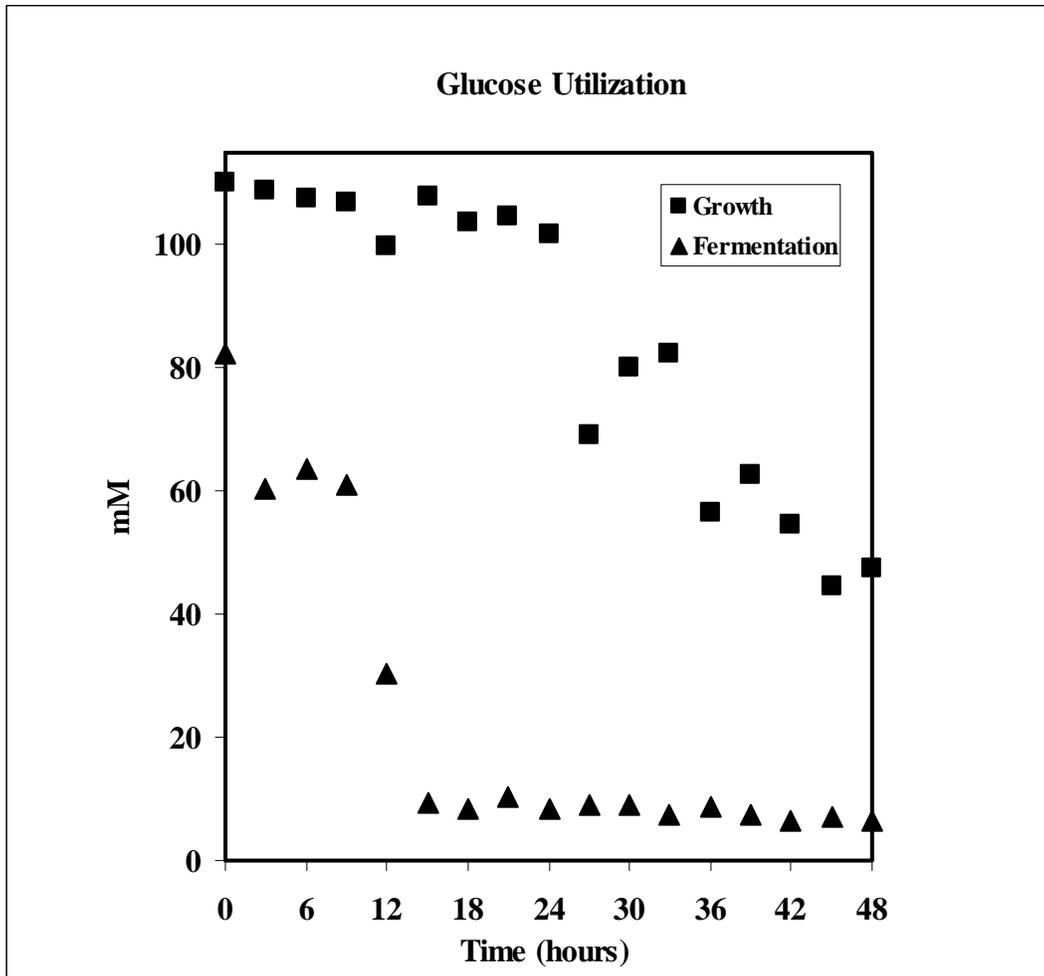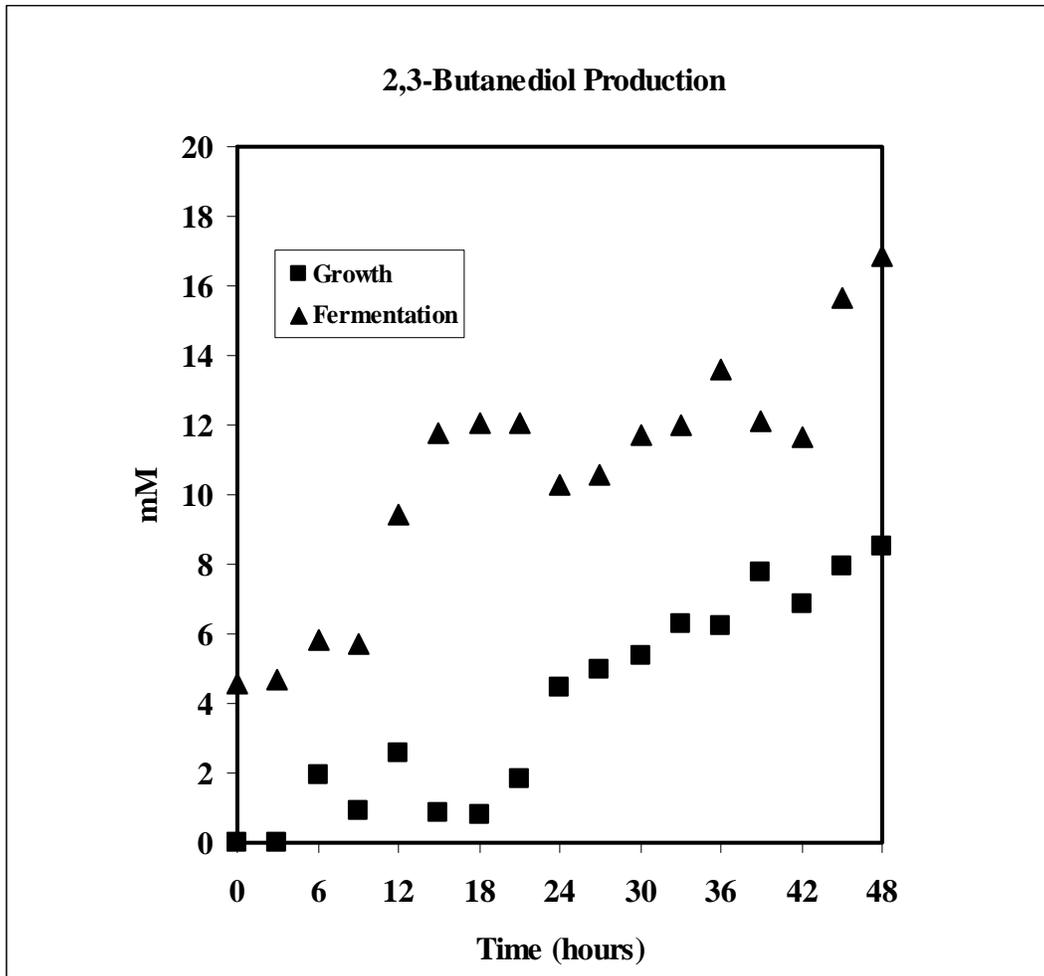
Figure 4. *B. licheniformis* 2,3-butanediol concentrations over 48 hour growth and fermentation periods conducted at 37°C, shaken at 150rpm, pH 6.9. Growth and fermentation broths are those of Perego (2003). All data points represent the average of the results of four experiments. Samples for HPLC were 1mL every 3 hours.

Preliminary experiments of cell lysis were conducted with both the Parr Cell Disruption Vessel and the Misonix Sonicator. Data was collected as a means of choosing one device over the other with which to pursue further experimentation. As Table 2 and Table 3 show, the Parr Cell Disruption Vessel was outperformed by the Misonix Sonicator with regard to the retention of ADH activity after cell lysis. Thus, cell lysis was then continued with the Misonix Sonicator.

Cell lysis was first attempted by a Parr Cell Disruption Vessel. Frozen bacterial pellets were resuspended in 5mL of 10mM sodium phosphate buffer solution at pH 7.5 with the addition of the enzyme alcohol dehydrogenase in order to track its activity after lysis conditions. Analysis of lysis parameters was accomplished by an ADH enzyme assay in which the production of NADH and therefore the activity of the enzyme was found by UV measurements at 340nm. Completing the ADH assay after lysis conditions with the enzyme inside the Parr Cell Disruption Vessel was a method in which to determine the effect cell lysis would have upon the activity of the enzyme. Three runs were conducted at 2250 psi N_2 for 5 minutes. Figure 5 compares the three runs to a run in which the ADH did not experience lysis conditions. As Figure 5 shows, the ADH assay was conducted for a period of 6 minutes, during which time the ADH converted NAD^+ to NADH. The NADH produced is shown as an absorbance at 340nm. The increase in absorbance is a measure of the production of NADH and therefore the activity of the ADH. The higher the slope of absorbance over time the more active the ADH.
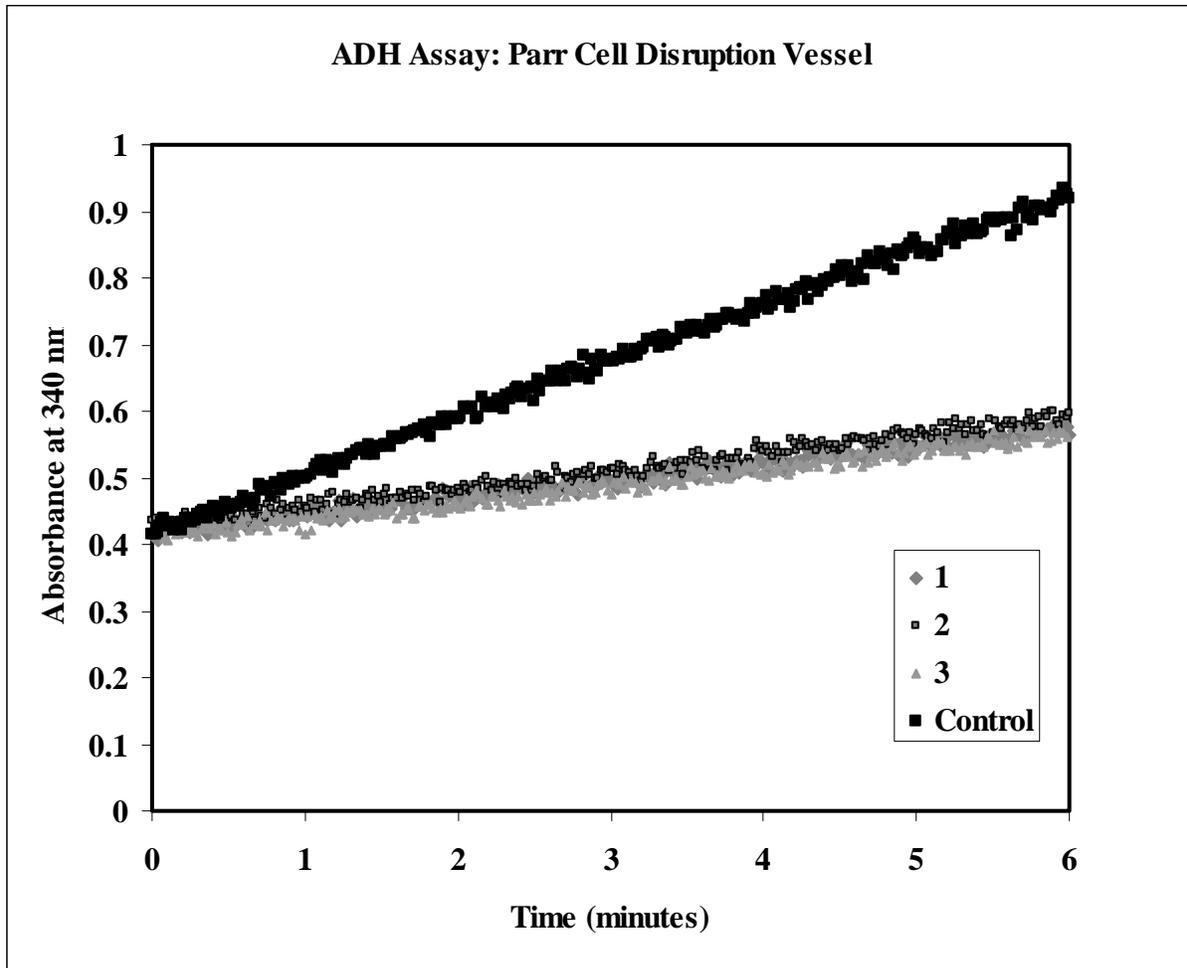
ADH Assay: Parr Cell Disruption Vessel

Figure 5. "1, 2, 3" represent the three runs in the Parr Cell Disruption Vessel with 5mL of 10mM sodium phosphate buffer solution at pH 7.5, ambient temperature. "Control" represents the base run of ADH in which it did not experience lysis conditions.

Table 2. Comparison of slopes of each run from Figure 4 to the slope of the Control. Retention of ADH activity represents the degree of activity ADH retained after lysis conditions.

	Slope (min⁻¹)	Retention of ADH activity
Control	0.084	100
1	0.025	29
2	0.027	31
3	0.025	29

Optical density measurements were taken before and after lysis with the Parr Cell Disruption Vessel. Absorbance at 600nm before lysis was 1.455 and after lysis and centrifugation at 6000 rpm for 10 minutes was 0.070 indicating the cells walls were successfully lysed. The lower optical density after lysis was due to intracellular fluid, cellular organelles, or cell debris. The cell wall and cell debris were separated from cell-free enzyme, intracellular fluid, and remaining cellular organelles by centrifugation.

Cell lysis was also attempted by sonication using a Misonix S-4000 sonicator with #418 and #419 A microtips. The assay was first completed using the #418 microtip at 100, 50, and 10% amplitude sonication for 1 minute, with and without dithiothreitol (DTT) addition, and varying pulse time, in a modified glass graduated cylinder, ambient temperature. DTT was added as an antioxidant to prevent disulfide bonds from forming in the enzymes of interest and rendering them inactive. Figure 6 shows each run as compared to a base run in which the ADH

was not sonicated and no DTT was added. Results from Figure 6 are shown in Table 3 as a

percent of nonsonicated ADH for each condition. The optimal conditions for sonication with the

#418 microtip were found to be 'Run 7'. These conditions include 1mM DTT added to

sonication liquid before sonication, 1 minute of total sonication, pulsing on for 5 seconds and off

for 10 seconds, at 10% amplitude.
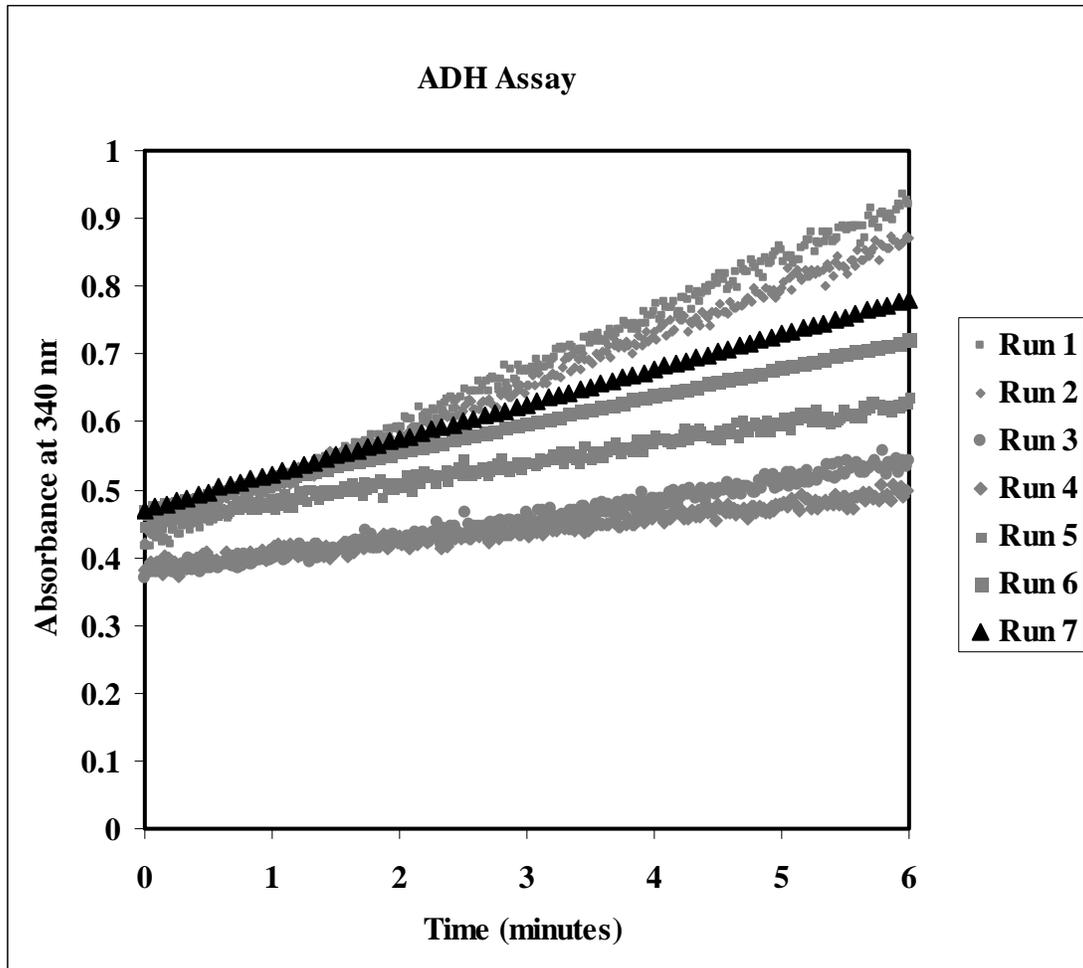
Figure 6. Run 1: Nothing added to ADH, no sonication. Run 2: 1mM DTT added, no sonication.

Run 3: 1mM DTT added, 1 minute sonication, 100% amplitude, 5 sec on, 10 sec off. Run 4:

Nothing added to ADH, 1minute sonication, 100% amplitude, 5 sec on, 5 sec off. Run 5: 1mM

DTT added, 1 minute sonication, 50% amplitude, 5 sec on, 10 sec off. Run 6: Nothing added to

ADH, 1 minute sonication, 10% amplitude, 5 sec on, 10 sec off. Run 7: 1mM DTT added, 1

minute sonication, 10% amplitude, 5 sec on, 10 sec off. The sonicator tip (#418) was positioned

6mm below the surface of the liquid for all runs. All runs were completed in 200mM disodium

phosphate and the pH of the assay was 7.9.

Table 3. Comparisons of each run to a run in which the ADH was not sonicated to determine which set of conditions least affects the activity of the enzymes.

Run	Retention of ADH activity
1	100
2	89
3	34
4	23
5	37
6	49
7	62

A comparison of Table 2 and Table 3 led to the decision that the Misonix was a better choice for cell lysis than was the Parr Cell Disruption Vessel. The retention of ADH activity calculated for the three runs in the Parr Cell Disruption Vessel were all about 30%. Table 3 shows runs 6 and 7 have much higher activity than 30%. For this reason further cell lysis experiments were conducted using the Misonix S-4000.

Frozen bacterial pellets were resuspended in 5mL of 0.15M sodium phosphate buffer at pH 7.5 by brief mixing on a vortex mixer. 1mM DTT was added to the suspension and cell lysis was attempted following the method of 'Run 7' from Figure 6. Optical density measurements at 600nm were taken before and after sonication of cells. Before sonication the optical density of whole cells was found to be 1.445. After sonication the optical density was reduced to 0.084. This reduction in optical density after sonication is an indication that the cell walls were penetrated and protein was released. Absorbance at 280nm is used to determine the concentration of protein in a solution. UV measurements at 280nm of cell-free enzymes produced an average absorbance of 0.931 indicating that protein was free in solution.

Cells were then lysed in a 15mL conical polypropylene centrifuge tube using the #418 and #419A microtips at three different distances for a comparison of conditions. The #418 microtip had a tip diameter of 1.6mm, its specified processing volume was 0.2-5mL, and its maximum amplitude output was 320µm. The #419 A microtip had a tip diameter of 3.2mm, its specified processing volume was 0.5-5mL, and its maximum amplitude output was 240 µm. The tips were placed at distances of 6mm, 16mm, and 30mm below the surface of the liquid. The sonicator was set at the conditions found to be optimal from Figure 6: 1 minute sonication at 10% amplitude, pulsing on for 5 seconds and off for 10 seconds with the addition of 1mM DTT. Again, ADH was added to determine the optimal conditions for retention of enzyme activity. Figure 7 and Figure 8 include "Run 1" and "Run 7" from Figure 6, which are nonsonicated ADH and the optimal conditions for sonication, respectfully.

ADH Assay: Misonix #418 Microtip

Figure 7. Cell lysis by sonication using the #418 microtip at varying distances from the liquid surface and in a 15mL conical polypropylene centrifuge tube. Sonication solution was 200mM disodium phosphate at ambient temperature. The assay was run at pH 7.9. Run 1 and Run 7 were conducted at 6mm below the surface of the liquid.

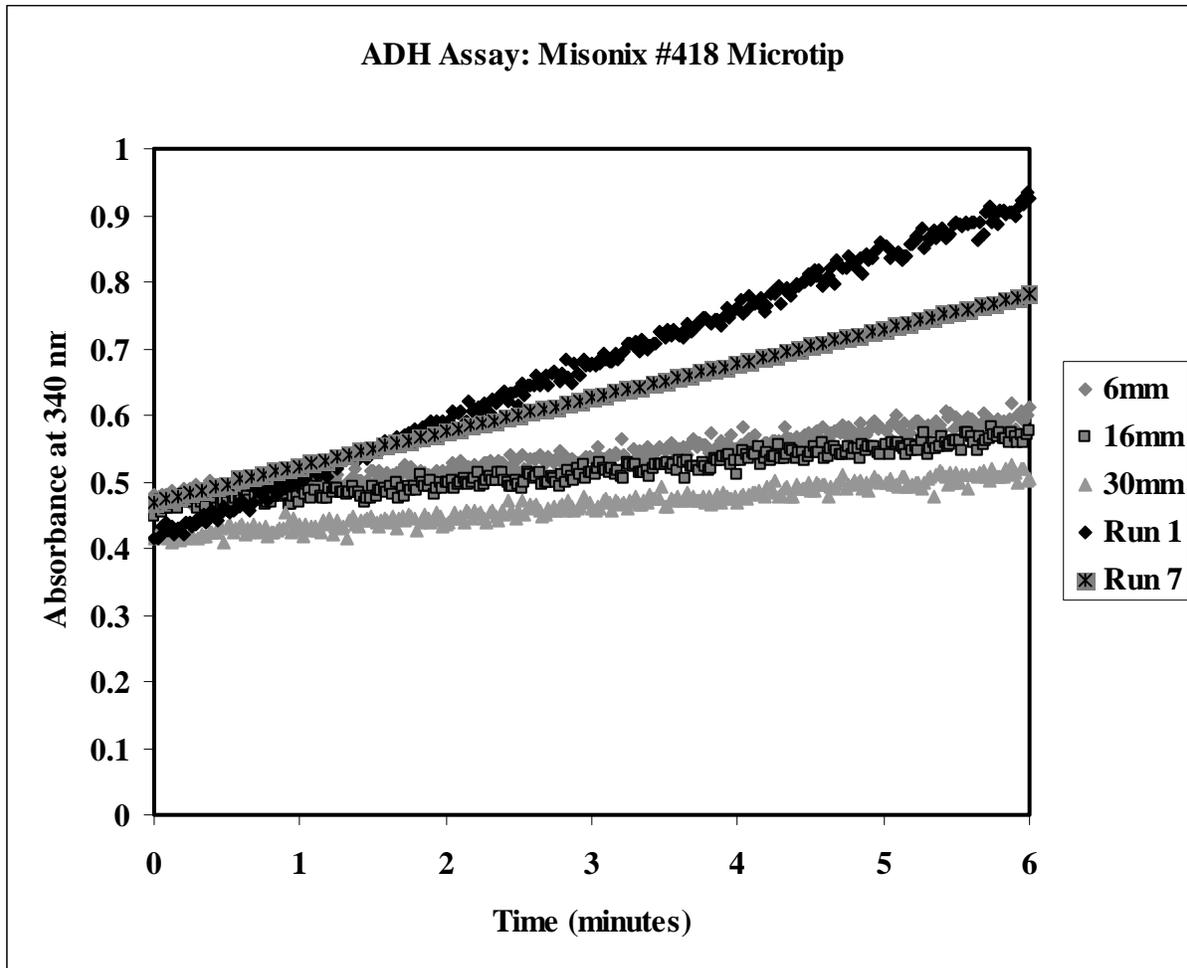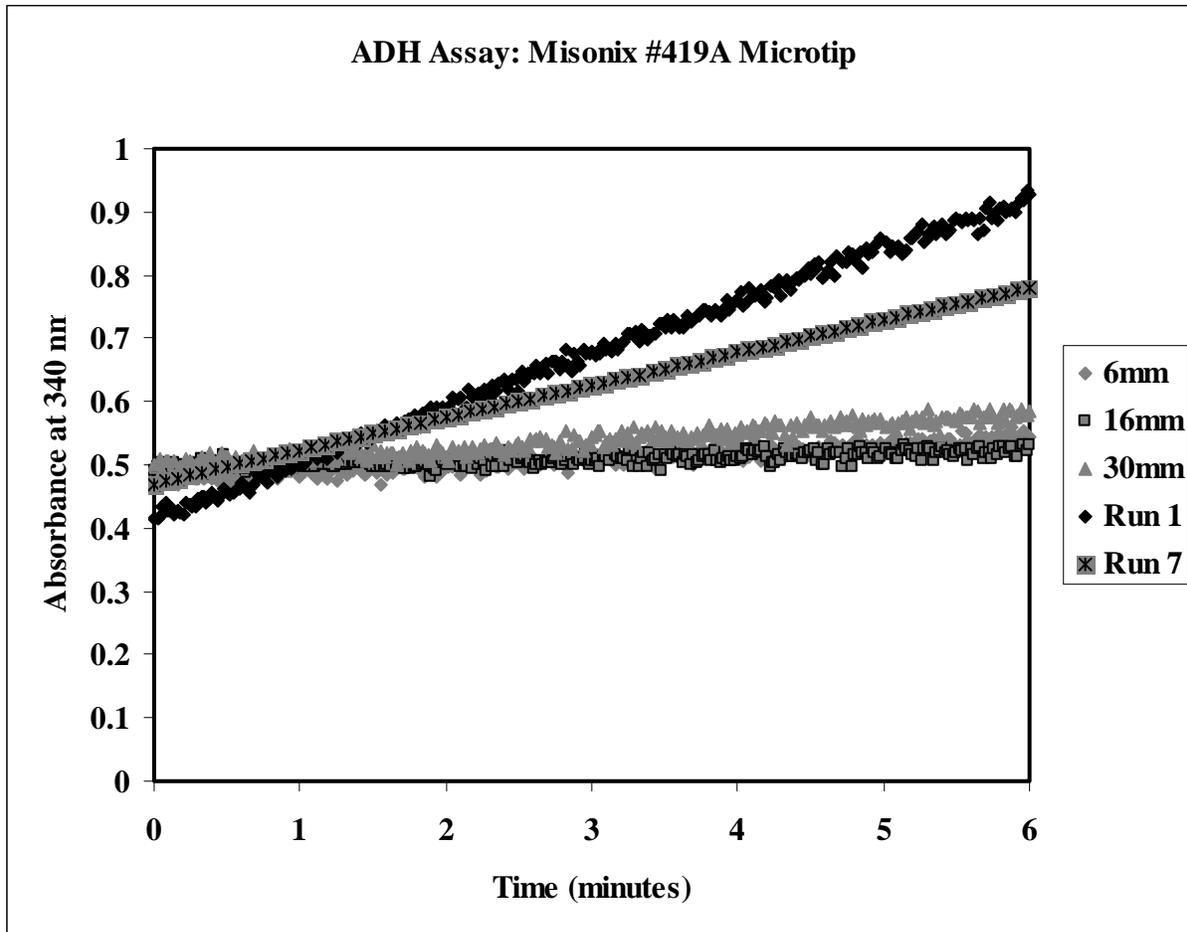**ADH Assay: Misonix #419A Microtip**

Figure 8. Cell lysis by sonication using the #419 A microtip at varying distances from the liquid surface and in a 15mL conical polypropylene centrifuge tube. Sonication solution was 200mM disodium phosphate at ambient temperature. The assay was run at pH 7.9. Run 1 and Run 7 were conducted at 6mm below the surface of the liquid.

Table 4. The retention of enzymatic activity after cell lysis by sonication in a 15mL conical

polypropylene centrifuge tube using either #418 or #419 A microtip.

Run	Retention of ADH activity
"Run 1"	100
#418-6mm	25
#418-16mm	25
#418-30mm	16
#419A-6mm	14
#419A-16mm	8
#419A-30mm	17
"Run 7"	62

The activities of the enzymes shown in Table 4 are much less than that for Run 7, in

which the ADH was sonicated in the flat-bottom glass graduated cylinder. For this reason it was

determined that the geometry of the modified glass graduated cylinder was a more successful

apparatus for sonication than the geometry of the cylindrical centrifuge tube. The geometry of

the apparatus in which the sonication solution was held would affect the way in which the

solution was mixed during lysis.

The glass and polypropylene materials would also have differing affects upon sonication.

Heat transfer across the glass surface could affect sonication in a different way than would the

polypropylene material. The surfaces of each material could also affect sonication as one surface

may allow cells, cell debris, enzyme, etc., to become attached and therefore disrupt the mixing of solution during sonication. Each surface would also alter the cell frothing and enzyme denaturation differently due to the hydrophilic nature of glass and hydrophobic nature of polypropylene.

From Table 4 it was determined that the distance of the microtip from the surface of the sonication solution did not have much affect upon the retention of the ADH activity. With the #418 microtip 25% of the enzyme's activity was retained after sonication at 6mm as well as 16mm. At 30mm from the surface of the sonication solution the ADH enzyme retained 64% of the activity observed at the 6mm and 16mm distances. With the #419 A microtip at 6mm and 30mm distances the ADH enzyme retained almost the same amount of activity. At 16mm the retention of enzyme activity was less. For this reason, further sonication experiments were conducted with the microtip positioned 6mm from the surface of the sonication solution.

In order to determine which microtip was more effective for cell lysis two sets of experiments were completed for each. As before, ADH was added to the sonication liquid. Sonication was carried out at 75, 50, and 10% amplitude for each tip and for 5, 4, 3, 2, and 1 minute total sonication on time each. The pH, watts, joules, and final sonication solution temperature were all monitored. The sonication solution was kept in an ice bath, 1mM DTT was added before sonication, as well as 1mg trypsin protease inhibitor from chicken egg white to prevent breakdown of enzymes by any proteases released during lysis. The ADH assay was completed to determine the amount of active enzyme remaining after sonication. Second, the optical density at 280nm was observed for each to determine the amount of free enzyme after sonication.

Table 5. # 418 microtip sonication data. The pH was checked after completion of the ADH assay. The watts and joules were the power and energy absorbed during sonication. The temperature represents the temperature of the sonication solution after sonication. The OD_{280} of the blank was 0.77. All runs were completed on ice, with DTT, and with protease inhibitor.

#418 microtip	pH	Watts absorbed	Joules absorbed	Final Solution T °C	Retention of ADH activity	OD_{280} minus blank
Blank w/ice, DTT, PI	7.8				100	
75% 5 min	7.8		8710		6	0.94
75% 4 min	7.8	32	7644	17	15	0.68
75% 3 min	7.8	32	5884	18	4	0.73
75% 2 min	7.8	30	3579	36	20	0.44
50% 4 min	7.8	15	3726	24	26	0.73
50% 3 min	7.8	17	3035	11	51	0.56
50% 2 min	7.8	14	1734	19	23	0.65
50% 1 min	7.8	13	838	12	48	0.18
10% 5 min	7.8	8	1738	10	41	0.31
10% 4 min	7.8	5	1283	10	69	0.14
10% 2 min	7.8	4	542	8	79	0.17

Table 6. # 419 A microtip sonication data. The pH was checked after completion of the ADH

assay. The watts and joules were the power and energy absorbed during sonication. The

temperature represents the temperature of the sonication solution after sonication. The OD_{280} of

the blank was 0.77. All runs were completed on ice, with DTT, and with protease inhibitor.

# 419 A microtip	pH	Watts absorbed	Joules absorbed	Final Solution T °C	Retention of ADH activity	OD_{280} minus blank
Blank w/ice, DTT, PI	7.9				100	
75% 5 min	7.9	17	7916	29	11	1.72
75% 4 min	7.9	25	5805	15	26	1.25
75% 2 min	7.9	25	3074	30	3	0.61
75% 1min	7.9	25	1534	29	21	0.77
50% 5 min	7.9	15	4557	10	36	0.80
50% 4 min	7.9	15	4044	10	76	0.56
50% 3 min	7.9	15	2901	8	90	0.45
50% 1 min	7.8	17	1058	23	26	0.81
10% 5 min	7.8	5	1803	17	32	0.48
10% 4 min	7.8	8	1707	11	42	1.10
10% 3 min	7.8	7	1326	9	79	0.32
10% 2 min	7.8	5	893	12	82	0.24
10% 1 min	7.8	5	370	13	97	0.28

From the two tables it was determined that the #419 A microtip was the more effective microtip for cell sonication because it gave a higher retention of ADH activity for each sonication run as compared to the #418 microtip. The two parameters observed to have the best data for this microtip concerning ADH activity and retaining the highest amount of enzyme obtained from the OD_{280} readings were 10% amplitude at 2 minutes and 1 minute. For this reason further sonication was carried out with the #419 A microtip for 1 or 2 minutes. Figures 9 and 10 show the ADH assay for 1 and 2 minutes sonication with the #419 A microtip.
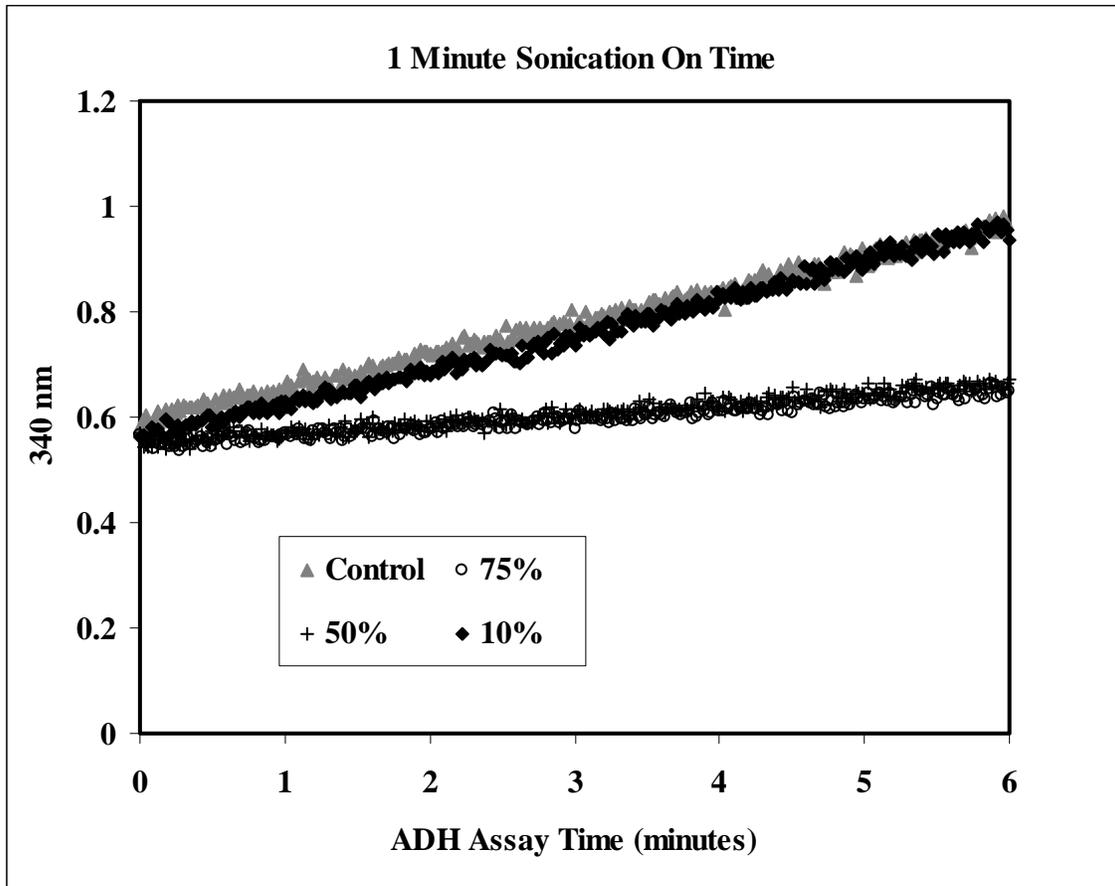
Figure 9. The sonication was attempted with the Misonix S-4000 microptip #419 A for 1 minute total on time at 10, 50, and 75% amplitude. The ADH assay (x axis) was carried out for 6 minutes.
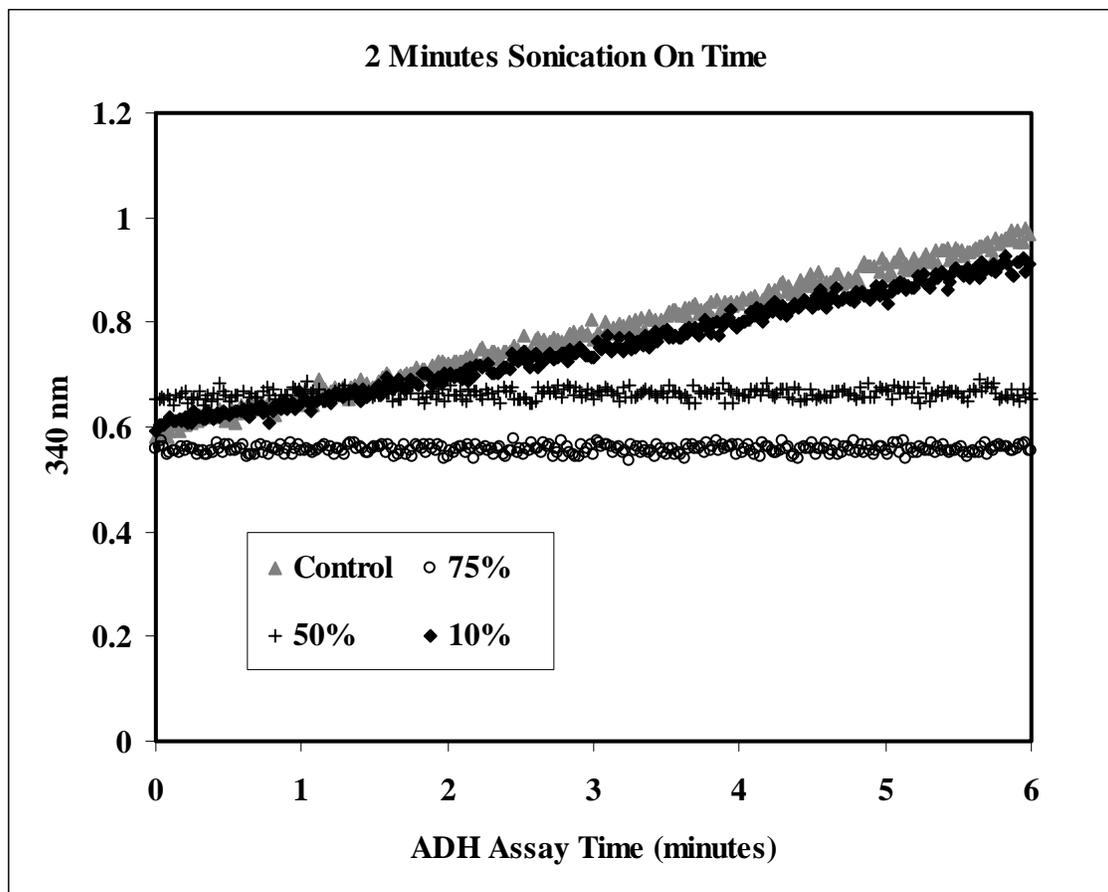
Figure 10. The sonication was attempted with the Misonix S-4000 microptip #419 A for 2 minutes total on time at 10, 50, and 75% amplitude. The ADH assay (x axis) was carried out for 6 minutes.

In order to add to the data previously collected by sonication in a cylindrical centrifuge tube and a flat-bottom graduated cylinder, sonication was completed in a round-bottom centrifuge tube. For comparison, the same set of experiments was conducted for the round-bottom centrifuge tube as were conducted for the choosing of the #418 or #419 A microtips. The data was collected using the #419 A microtip at 10% amplitude as this was chosen as the more efficient tip.

Table 7. The sonication was carried out in a round-bottom centrifuge tube using the #419 A microtip at 10% amplitude in 200mM disodium phosphate. The pH was checked after completion of the ADH assay. The watts and joules were the power and energy absorbed during sonication. The temperature represents the temperature of the sonication solution after sonication.

10% amplitude	pH	Watts absorbed	Joules absorbed	Final Solution T °C	Retention of ADH activity
5 min	7.9	5	1882	10	123
4 min	7.9	5	1526	11	70
3 min	7.9	5	948	10	104
2 min	7.9	9	1639	10	52
1 min	7.9	6	926	12	112

The round-bottom centrifuge tube was determined not to be an appropriate apparatus for sonication because of the variability in data. The position of the microtip within the apparatus was difficult to judge and therefore led to variability among the data and no reproducibility.

Taking into consideration each figure and table it was decided that the best cell lysis was obtained by use of the Misonix S-4000 Sonicator with the #419 A microtip in a glass graduated cylinder. Conditions for sonication were best at 1 minute or 2 minutes sonication pulsing on for

5 seconds and off for 10 seconds at 10% amplitude with the addition of 1mM DTT and 1mg trypsin inhibitor.

Figures 11 and 12 show a relationship between the OD_{280} (indicating protein) resulting from sonication and the Joules absorbed during sonication of ADH. This relationship was seen by Feliu while conducting sonication on ß-galactosidase, as extracellular ß-galactosidase (%) and ß-galactosidase activity (%) were plotted versus sonication time (Feliu, 1997).

The relationship shows that as more energy is put into the cell during sonication, more enzymes are released and the higher the OD_{280} results. The highest OD_{280} readings correlating to the highest Joules are also due to the breakdown of cell walls and cell debris, as well as released enzymes and cellular organelles. Therefore a lower range of OD_{280} is more desirable as this would indicate less damage to the enzymes of interest as well as unnecessary breakdown of the cell wall and creation of cell debris.
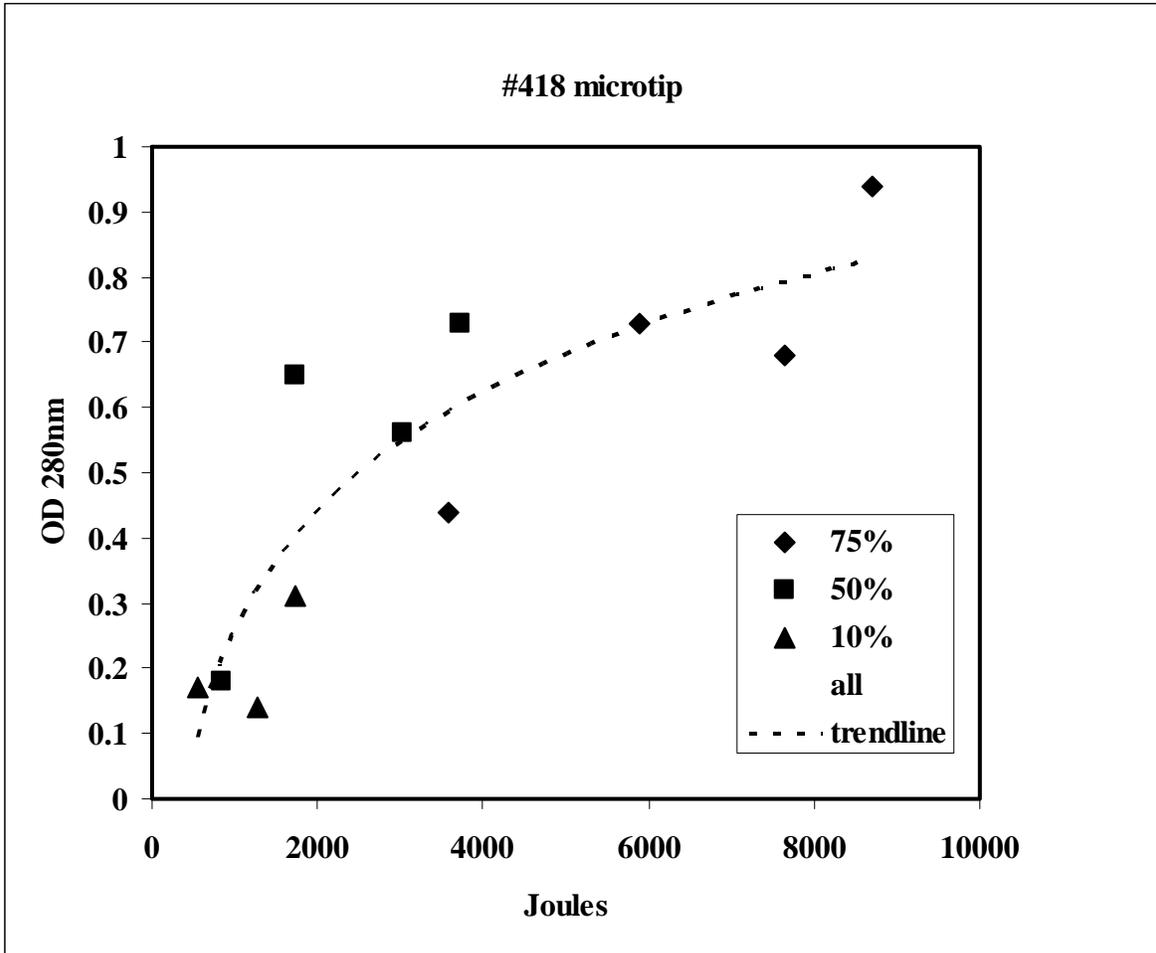
Figure 11. The relationship between amount of enzyme released during sonication and the energy absorbed during sonication Sonication was completed with the #418 microtip at 75, 50, and 10% amplitude in a modified glass graduated cylinder, in an ice bath.
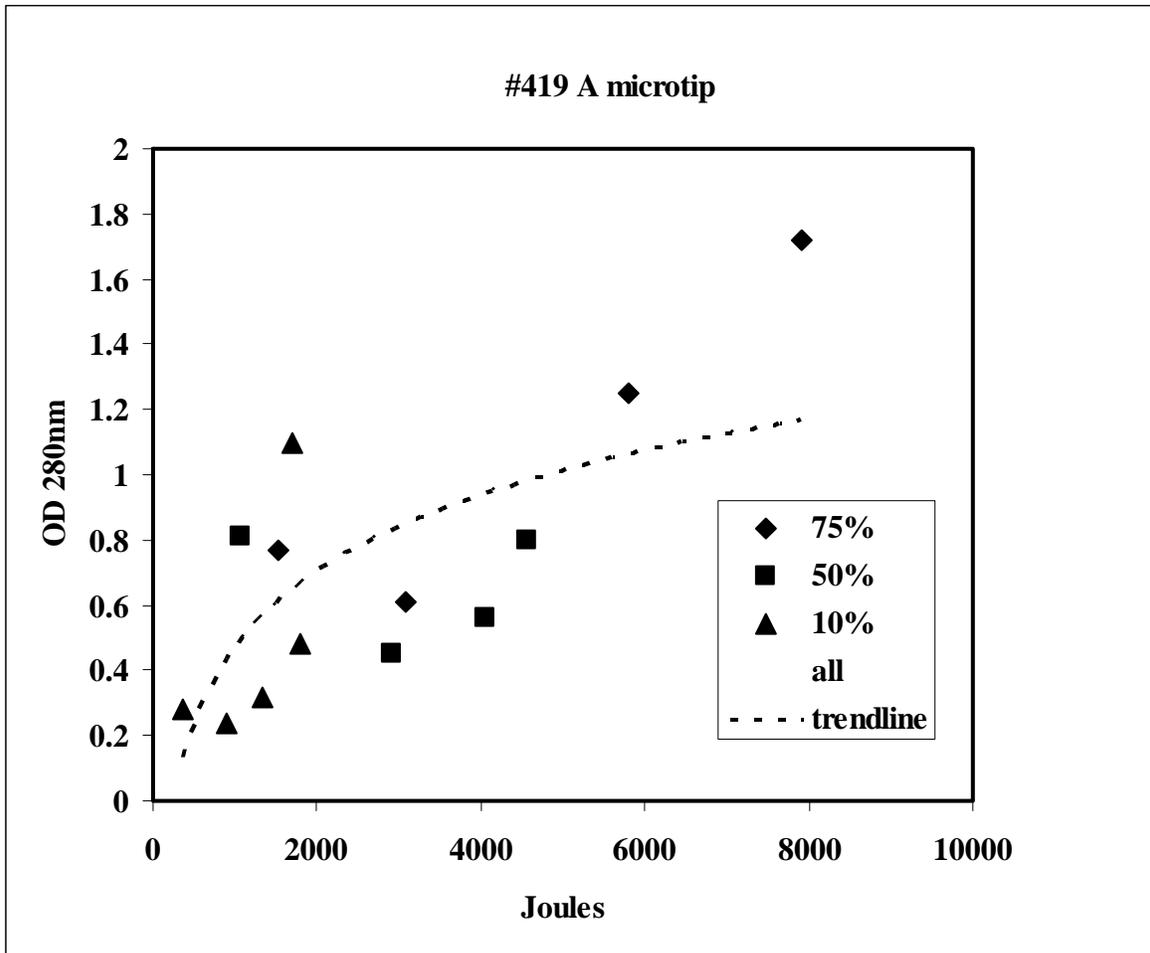
#419 A microtip

Figure 12. The relationship between amount of enzyme released during sonication and the energy absorbed during sonication. Sonication was completed with the #418 microtip at 75, 50, and 10% amplitude in a modified glass graduated cylinder, in an ice bath.

Conclusion

The data collected represent pioneering research in the area of cell-free enzyme systems. Very little literature exists on the fermentation of whole cells of *B. licheniformis*, as well. Cell lysis was attempted on *B. licheniformis*, which is a gram positive bacterium, meaning it has a thick cell wall containing many layers of peptidoglycan. Lysis of gram positive bacteria is more difficult than lysis of gram negative bacteria, such as *Escherichia coli* (*E. coli*). Gram negative bacteria have a much thinner cell wall of peptidoglycan with a polysaccharide membrane surrounding it. The peptidoglycan gives structure and strength to the cell, rendering it more different to penetrate than the polysaccharide membrane. Cell lysis of *E. coli* and analysis of the activity of a recombinant protein, β-galactosidase, was attempted by Feliu (1997). Comparison of this work to the work by Feliu involves the difference in cell wall, gram positive versus gram negative, as well as the enzyme of interest with regard to enzyme assays, alcohol dehydrogenase versus β-galactosidase. Alcohol dehydrogenase is a multi-enzyme subunit with a large molecular weight. Its structure is more fragile than proteases such as β-galactosidase. Cell lysis would tend to have more of an affect on the activity of these enzymes, rather than the proteases. Therefore, a comparison of results of this work to the work by Feliu must be done carefully, as the two cases have significant differences.

The results of the body of work conclude that the Misonix Sonicator was better suited for sonication of cells and retention of enzyme activity from *B. licheniformis* than was the Parr Cell Disruption Vessel. The retention of ADH activity obtained from lysis by the Misonix Sonicator (62%) was twice that of the retention of ADH activity obtained from the Parr Cell Disruption Vessel (30%). The temperature of the sonication solution during sonication was very important as high temperatures resulted in very low enzyme activity. Sonication for longer periods of time,

3-5 minutes, and higher amplitudes, 50-100%, correlated with higher energy absorbed by the

solution during sonication. These factors also increased the temperature during sonication.

Sonication for 1-2 minutes and at 10% amplitude did not raise the sonication solution

temperature above the level for which sufficient enzyme activity remained.

V. Cell-Free Enzyme Reactions and Production of Products

Materials and Methods

Enzyme reaction media was derived from the whole cell fermentation media of Perego (2003) with the addition of sodium pyruvate, trypsin inhibitor from chicken egg white, ß-nicotinamide adenine dinucleotide hydrate (NAD$^+$), ß-nicotinamide adenine dinucleotide reduced disodium salt hydrate (NADH), DL-dithiothreitol (DTT), and thiamine pyrophosphate (TPP). All chemical were purchased form Sigma-Aldrich. Sodium pyruvate was used as substrate. Trypsin inhibitor from chicken egg white was incorporated to protect against proteases in the system and those that may have been released during lysis which would break down the enzymes of interest. NAD$^+$ and NADH were added as necessary cofactors for the activity of the enzymes. DTT acted as an antioxidant to prevent disulfide bonds from forming in the enzymes and therefore inactivating them. TPP was also added as a necessary cofactor for the activity of the enzymes. Frozen whole cell pellets were resuspended with 5mL 0.05M sodium phosphate buffer, pH 7.0, and shaking on a vortex. Enzymes were collected by use of a Parr Cell Disruption Vessel or Misonix Sonicator and followed by centrifugation at 14,000 rpm for 5 minutes. Identification of products was completed by HPLC with dilute sulfuric acid as the mobile phase at 0.55mL min^{-1}.

Results

       The effect of cell harvest time in the whole cell fermentation was investigated. Whole cells of *Bacillus licheniformis* NRRL B-642 were grown for 48 hours and fermented for 48 hours. Amounts of glucose and 2,3-butanediol were tracked over time. From Figures 13-16 it is seen that the microorganism's rate of utilization of glucose and production of 2,3-butanediol shifted over time as compared to Figures 3 and 4. For this reason the harvest time was adjusted in order to capture the enzymes of interest at their peak activity. As evidenced by Figure 14 and Figure 16, the greatest change in concentration of both glucose and 2,3-butanediol occurred between 3-9 hours into the whole cell fermentation period. Hence, cells were harvested at time periods 3, 6, and 9 hours into the whole cell fermentation for further experimentation.
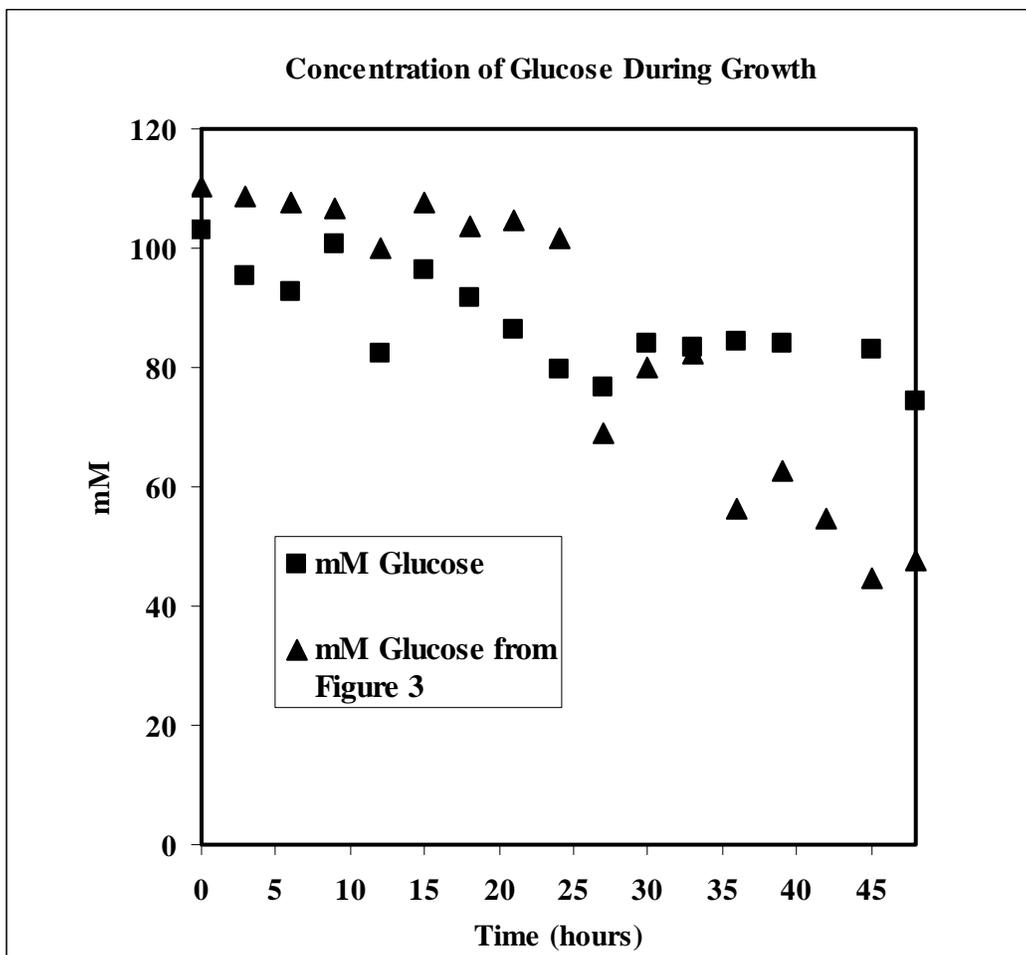
Figure 13. Comparison of glucose concentrations during growth for the current harvest experiment to those of Figure 3. Data points represent the average of the results of four experiments for "mM Glucose from Figure 3" and the average of the results of three experiments for "mM Glucose". Samples for HPLC were 1mL every 3 hours. Growth medium consisted of: 2.0g L^{-1} yeast extract, 5.0g L^{-1} peptone, 5.0g L^{-1} sodium chloride; and 1.0g L^{-1} beef extract powder, with the addition of 20.0g L^{-1} glucose (Perego, 2003). Growth temperature was 37°C and pH was 6.0.
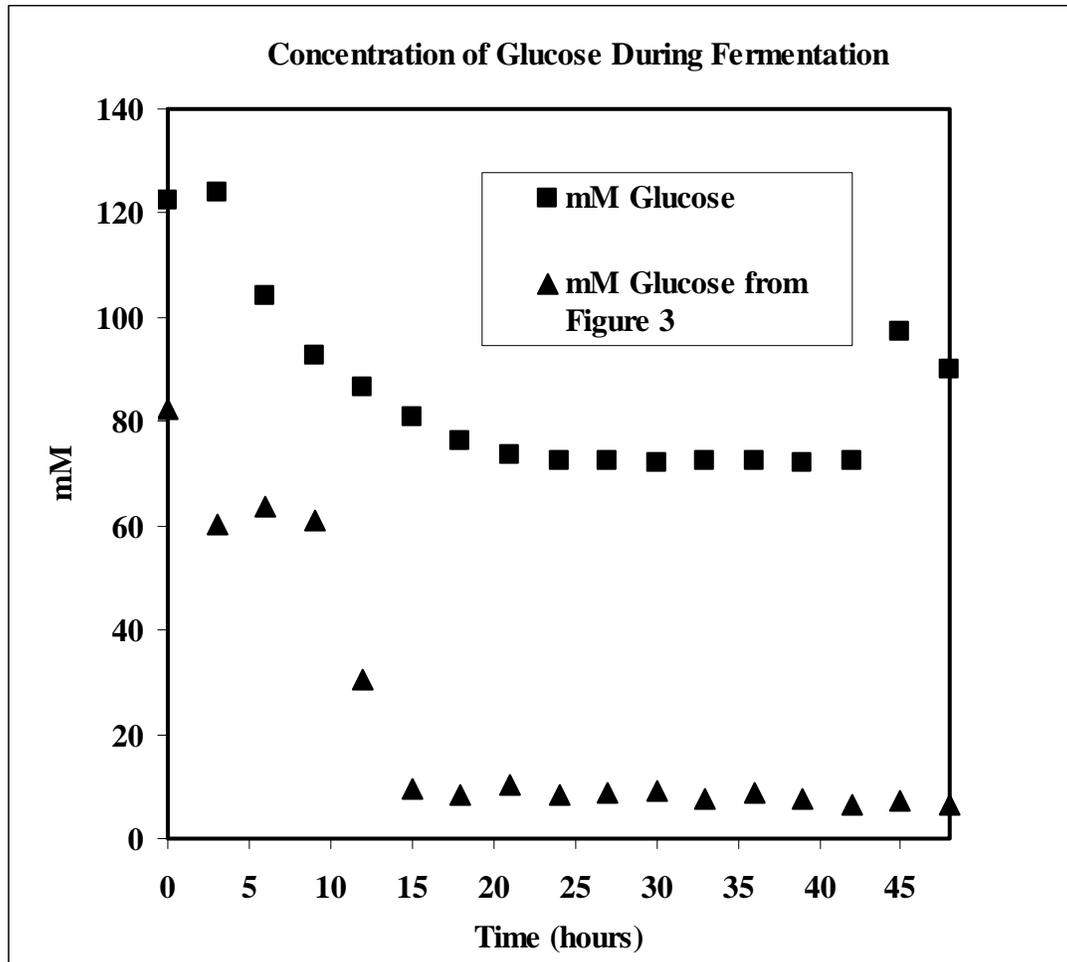
Figure 14. Comparison of glucose concentration during fermentation for the current harvest experiment to those of Figure 3. Data points represent the average of the results of four experiments for "mM Glucose from Figure 3" and the average of the results of three experiments for "mM Glucose". Samples for HPLC were 1mL every 3 hours. Fermentation medium for whole cells consisted of: 5.0g L^{-1} yeast extract, 6.0g L^{-1} potassium phosphate monobasic, 14.0g L^{-1} potassium phosphate dibasic, 2.0g L^{-1} ammonium sulfate, 1.0g L^{-1} sodium citrate monobasic, and 0.20g L^{-1} magnesium sulfate heptahydrate, supplemented with 20.0g L^{-1} glucose (Perego, 2003). Fermentation temperature was 37°C and pH was 6.0.
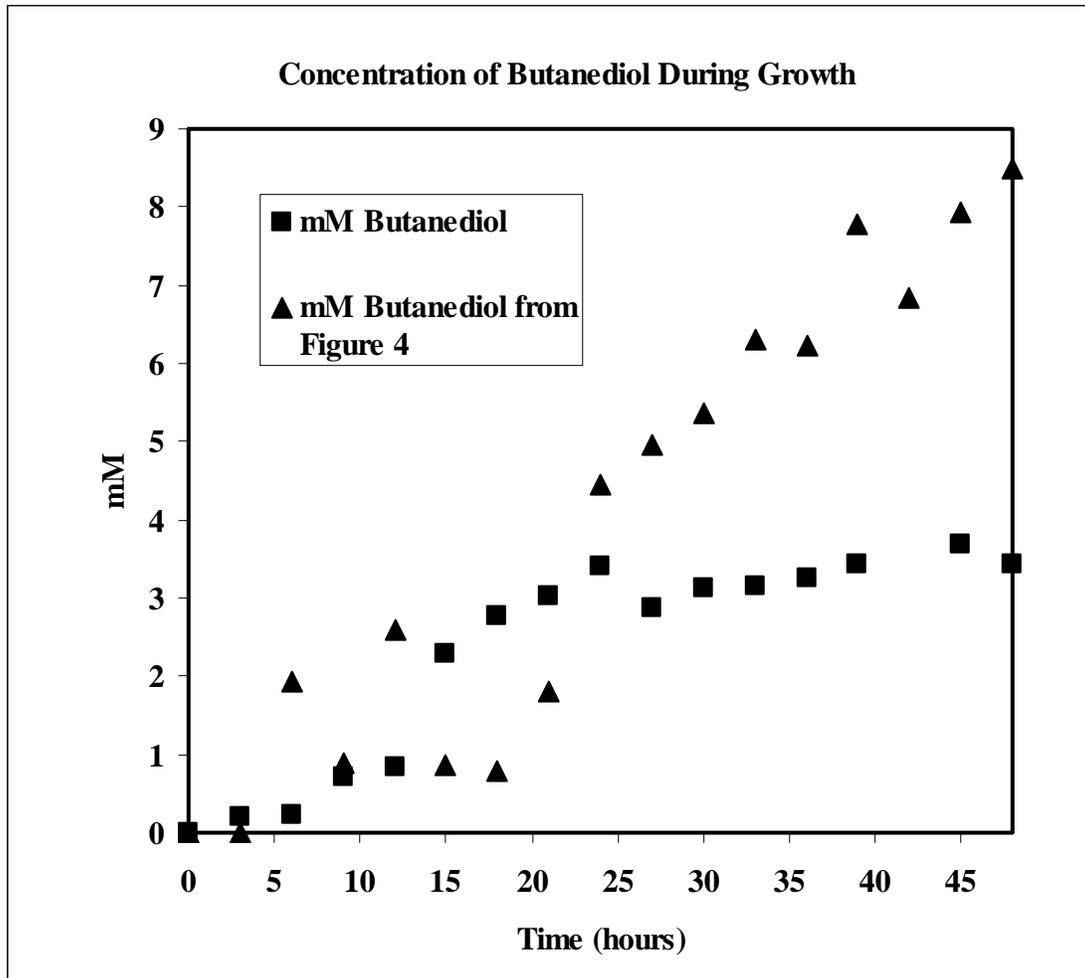
Figure 15. Comparison of 2,3-butanediol concentration during growth for the current harvest experiment to those of Figure 4. Data points represent the average of the results of four experiments for "mM Butanediol from Figure 4" and the average of the results of three experiments for "mM Butanediol". Samples for HPLC were 1mL every 3 hours. Growth medium consisted of: 2.0g L^{-1} yeast extract, 5.0g L^{-1} peptone, 5.0g L^{-1} sodium chloride; and 1.0g L^{-1} beef extract powder, with the addition of 20.0g L^{-1} glucose (Perego, 2003). Growth temperature was 37°C and pH was 6.0.
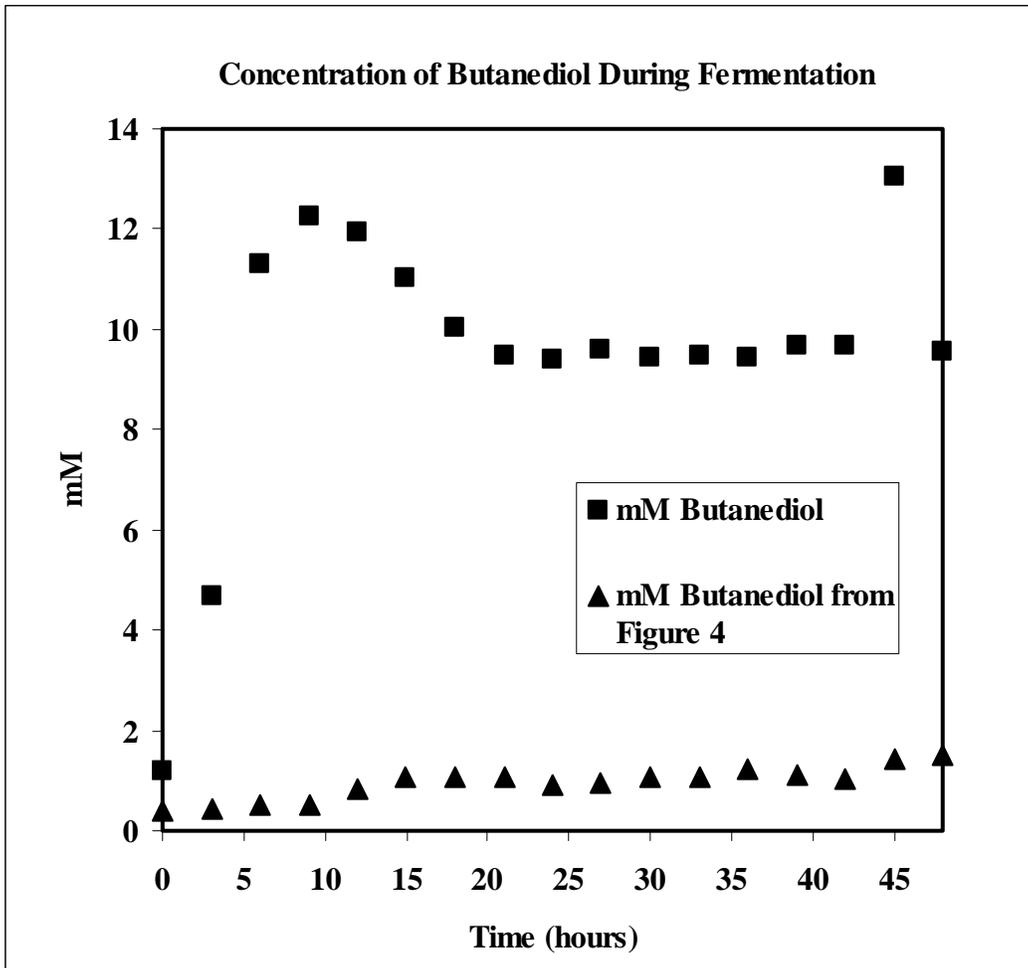
Figure 16.   Comparison of 2,3-butanediol concentration during fermentation for the current harvest experiment to those of Figure 4. Data points represent the average of the results of four experiments for "mM Butanediol from Figure 4" and the average of the results of three experiments for "mM Butanediol". Samples for HPLC were 1mL every 3 hours. Fermentation medium for whole cells consisted of: 5.0g L^{-1} yeast extract, 6.0g L^{-1} potassium phosphate monobasic, 14.0g L^{-1} potassium phosphate dibasic, 2.0g L^{-1} ammonium sulfate, 1.0g L^{-1} sodium citrate monobasic, and 0.20g L^{-1} magnesium sulfate heptahydrate, supplemented with 20.0g L^{-1} glucose (Perego, 2003). Fermentation temperature was 37°C and pH was 6.0.

Growth and fermentation media for whole cells were both from Perego (2003). Growth medium consisted of: 2.0g L^{-1} yeast extract, plant cell culture tested; 5.0g L^{-1} peptone, vegetable No.1 for microbiology; 5.0g L^{-1} sodium chloride; and 1.0g L^{-1} beef extract powder, with the addition of 20.0g L^{-1} glucose after autoclaving at 121°C for 15 minutes. Fermentation medium for whole cells consisted of: 5.0g L^{-1} yeast extract, 6.0g L^{-1} potassium phosphate monobasic, 14.0g L^{-1} potassium phosphate dibasic, 2.0g L^{-1} ammonium sulfate, 1.0g L^{-1} sodium citrate monobasic, and 0.20g L^{-1} magnesium sulfate heptahydrate, supplemented with 20.0g L^{-1} glucose after autoclaving at 121°C for 15 minutes.

Cell lysis solution consisted of the whole cell fermentation medium with the addition of 150mM sodium acetate, 1mM DTT, and 1mg trypsin inhibitor from chicken egg white.

Cell-free enzyme reaction medium consisted of the whole cell fermentation medium with the addition of 150mM sodium acetate, 87mM sodium pyruvate, 10mM NADH, 1mM NAD^+, 10mM dithiothreitol, 1mM thiamine pyrophosphate, and 1.11mg mL^{-1} trypsin protease inhibitor.

Cell lysis was performed by Misonix sonication with #418 and #419 A microtips (for comparison) at 10% amplitude for 1 or 2 minutes total on time, pulsing on for 5 seconds, off for 10 seconds, with the addition of 1mM DTT and 1mg trypsin inhibitor, in an ice bath. In addition, 150mM acetate was added to reaction medium, as well as fermentation medium, to stimulate production of product (Nakashimada, 2000). Cells were harvested at 3, 6, and 9 hours into fermentation and frozen in 15% v/v glycerol. Enzyme reactions took place at pH 6.6, which was found to be optimal for whole cell fermentations in the literature (Nakashimada, 2000).

Samples were taken at 3, 6, and 9 hours whole cell fermentation for comparison with cell-free enzyme reaction products. HPLC analysis of whole cell fermentation products are shown in Table 8. Glucose was substrate and therefore pyruvate amounts are zero. Glycerol

amounts are also zero because it was added to harvested cells after these samples were taken. 150mM sodium acetate was added to fermentation media and therefore acetic acid concentrations remain high compared to other products.

Table 8. Products of 3, 6, and 9 hour whole cell fermentations. Concentrations of all products are mM amounts. Fermentation medium for whole cells consisted of: 5.0g L^{-1} yeast extract, 6.0g L^{-1} potassium phosphate monobasic, 14.0g L^{-1} potassium phosphate dibasic, 2.0g L^{-1} ammonium sulfate, 1.0g L^{-1} sodium citrate monobasic, and 0.20g L^{-1} magnesium sulfate heptahydrate, supplemented with 20.0g L^{-1} glucose (Perego, 2003). Fermentation temperature was 37°C and pH was 6.0.

	3 hour	6 hour	9 hour
Acetic Acid	119mM	119mM	116mM
Acetoin	5	4	5
2,3-Butanediol	1	5	8
Ethanol	0	0	0
Lactic Acid	0	4	2
Pyruvate	0	0	0
Glucose	162	164	149
Glycerol	0	0	0
Succinic Acid	0	0	0

At 3, 6, and 9 hours into the whole cell fermentation, reactions were stopped by addition of 15%v/v glycerol and centrifugation at 6000 rpm for 10 minutes. Supernatant was pippetted off and the pellet was frozen for later use.

Before cell lysis frozen pellets were resuspended in 0.05M sodium phosphate buffer pH 6.5. Cells were not washed. A small amount of the growth and fermentation media remained within the pellet. Cells were lysed in the cell lysis solution and per the parameters previously mentioned. After lysis, cell-free enzymes were reacted for 4 hours in the cell-free enzyme reaction media. HPLC analysis revealed the primary products to be succinic acid and acetoin by the cell-free enzyme system. Figures 17-22 show each set of reactions produced an increase of about 10mM succinic acid and 15mM acetoin over the 4 hour cell-free enzyme reaction time period for the 3 and 6 hour harvested enzymes. Interestingly, no succinic acid was produced during the whole-cell fermentation. Also, the 9 hour harvest cell-free enzyme mixture contained about 6-8mM succinic acid and 15-18mM acetoin at time zero reaction.

As well as an increase in succinic acid concentration an increase in concentration of acetoin was seen over the four hour cell-free reaction time period. The enzymes harvested at 3 and 6 hours whole cell fermentation show a clear increase in acetoin most markedly between hours 2 and 3 cell-free enzyme reaction (Figures 20 and 21). This was the same time period when the succinic acid production began (Figures 17 and 18). On the other hand, Figure 22 shows the enzymes harvested at 9 hours whole cell fermentation did not clearly produce acetoin as the concentration remained fairly constant and slightly decreased

3 hour harvest- Succinic Acid Concentrations

Legend:
- ◆ #418 1 minute
- ▲ #418 2 minutes
- ✳ #419A 1 minute
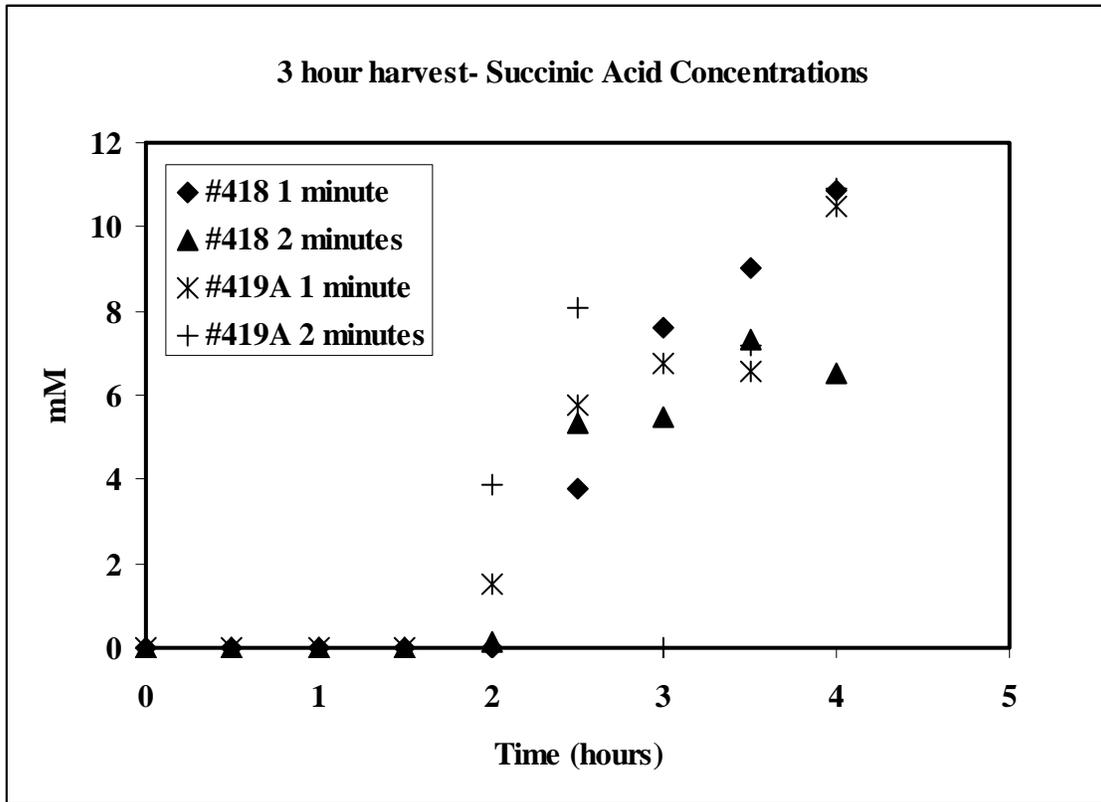- ＋ #419A 2 minutes

Y-axis: mM
X-axis: Time (hours)

Figure 17. Production of succinic acid by cell-free enzymes harvested after 3 hours fermentation. #418 and #419 A refer to the microtip used for sonication for 1 or 2 minutes. Cell-free enzyme reaction medium consisted of 5.0g L^{-1} yeast extract, 6.0g L^{-1} potassium phosphate monobasic,14.0g L^{-1} potassium phosphate dibasic, 2.0g L^{-1} ammonium sulfate, 1.0g L^{-1} sodium citrate monobasic, and 0.20g L^{-1} magnesium sulfate heptahydrate, with the addition of 150mM sodium acetate, 87mM sodium pyruvate, 10mM NADH, 1mM NAD^+, 10mM dithiothreitol, 1mM thiamine pyrophosphate, and 1.11mg mL^{-1} trypsin protease inhibitor. Cell-free enzyme reaction took place at ambient temperature and pH 6.6.
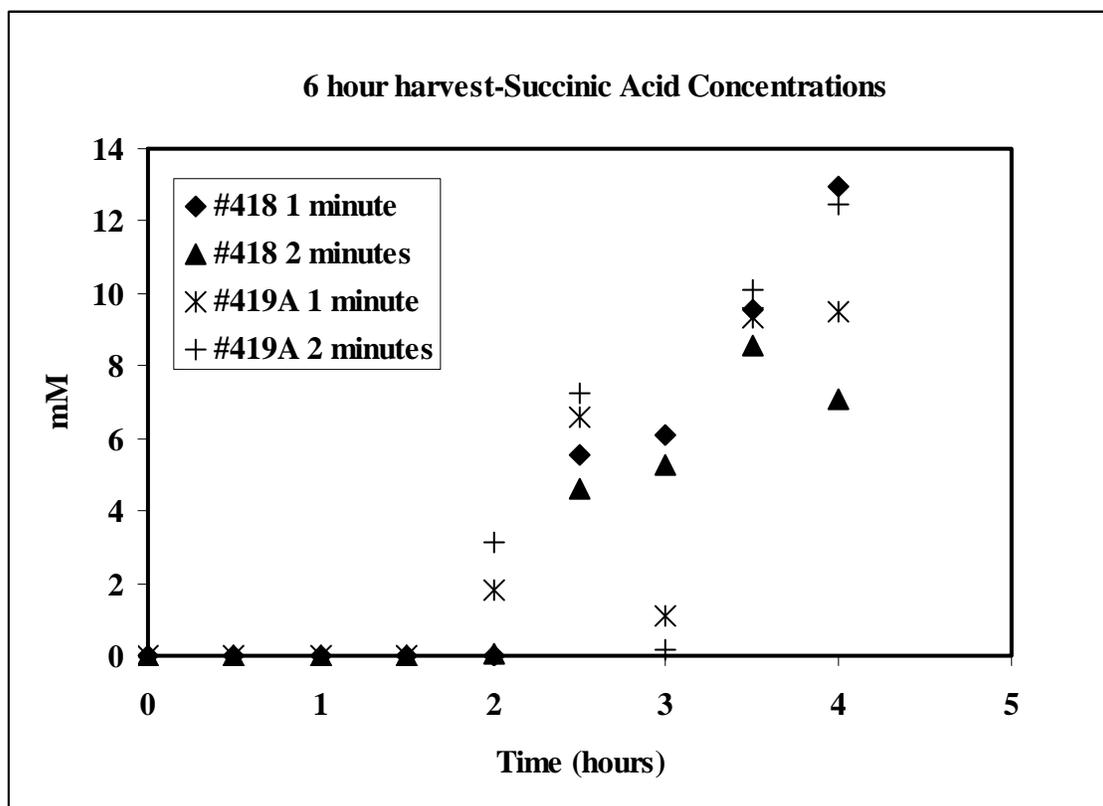
Figure 18. Production of succinic acid by cell-free enzymes harvested after 6 hours fermentation. #418 and #419 A refer to the microtip used for sonication for 1 or 2 minutes. . Cell-free enzyme reaction medium consisted of 5.0g L^{-1} yeast extract, 6.0g L^{-1} potassium phosphate monobasic,14.0g L^{-1} potassium phosphate dibasic, 2.0g L^{-1} ammonium sulfate, 1.0g L^{-1} sodium citrate monobasic, and 0.20g L^{-1} magnesium sulfate heptahydrate, with the addition of 150mM sodium acetate, 87mM sodium pyruvate, 10mM NADH, 1mM NAD^{+}, 10mM dithiothreitol, 1mM thiamine pyrophosphate, and 1.11mg mL^{-1} trypsin protease inhibitor. Cell-free enzyme reaction took place at ambient temperature and pH 6.6.
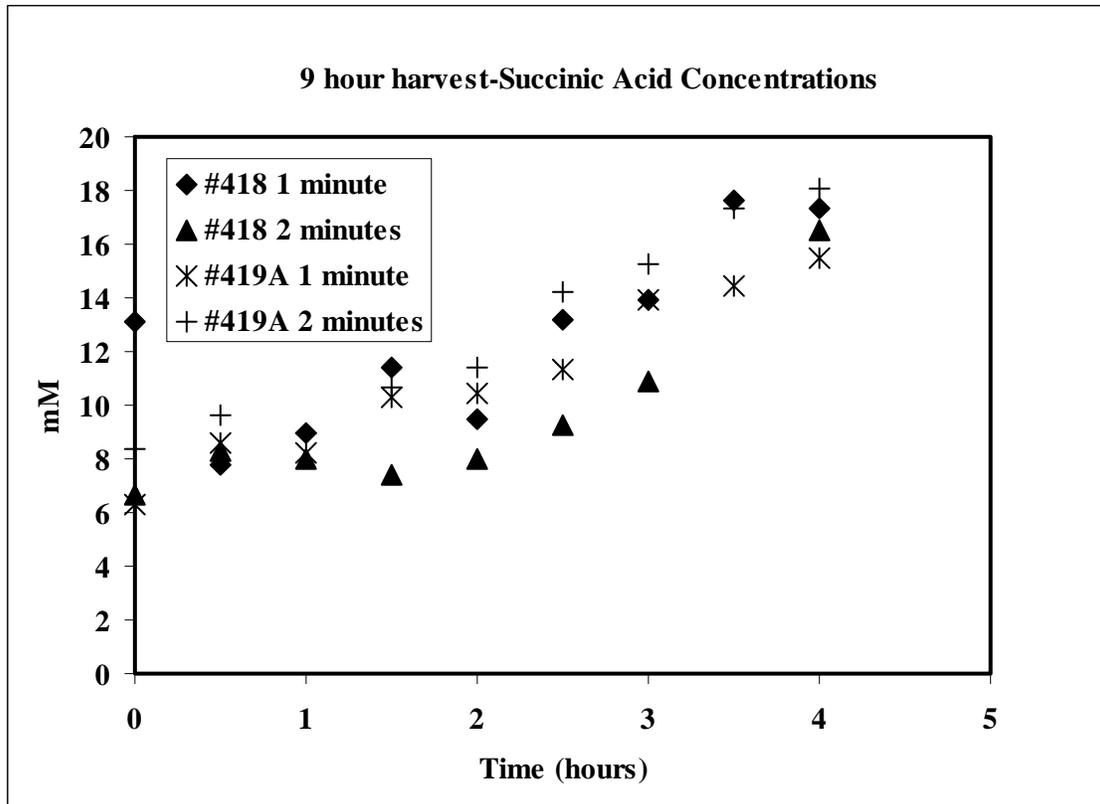
9 hour harvest-Succinic Acid Concentrations

Figure 19. Production of succinic acid by cell-free enzymes harvested after 9 hours

fermentation. #418 and #419 A refer to the microtip used for sonication for 1 or 2 minutes. At

reaction time zero 6-8mM succinic acid was present. Cell-free enzyme reaction medium

consisted of 5.0g L^{-1} yeast extract, 6.0g L^{-1} potassium phosphate monobasic,14.0g L^{-1} potassium

phosphate dibasic, 2.0g L^{-1} ammonium sulfate, 1.0g L^{-1} sodium citrate monobasic, and 0.20g L^{-1}

magnesium sulfate heptahydrate, with the addition of 150mM sodium acetate, 87mM sodium

pyruvate, 10mM NADH, 1mM NAD^+, 10mM dithiothreitol, 1mM thiamine pyrophosphate, and

1.11mg mL^{-1} trypsin protease inhibitor. Cell-free enzyme reaction took place at ambient

temperature and pH 6.6.

3 hour harvest-Acetoin Concentrations

Legend:
- ◆ #418 1 minute
- ▲ #418 2 minutes
- ✳ #419 A 1 minute
- + #419 A 2 minutes

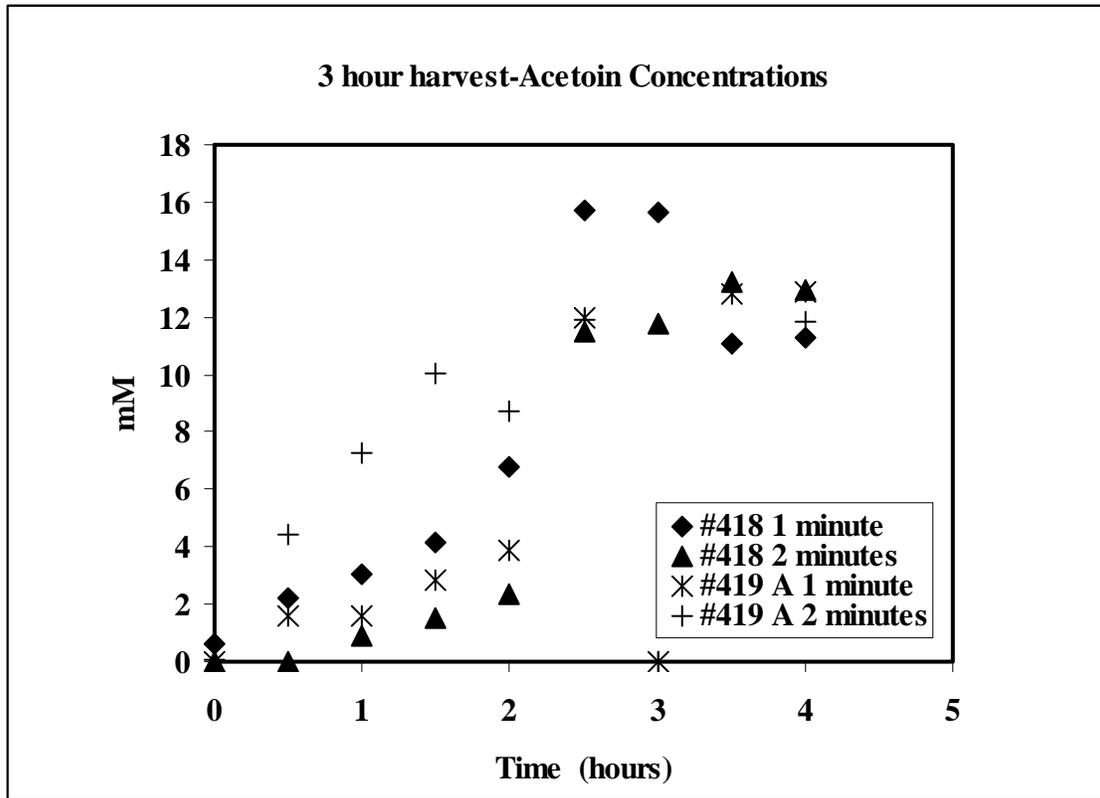Axis labels: mM (y-axis), Time (hours) (x-axis)

Figure 20. Production of acetoin by cell-free enzymes harvested after 3 hours fermentation. #418 and #419 A refer to the microtip used for sonication for 1 or 2 minutes. Cell-free enzyme reaction medium consisted of 5.0g L^{-1} yeast extract, 6.0g L^{-1} potassium phosphate monobasic,14.0g L^{-1} potassium phosphate dibasic, 2.0g L^{-1} ammonium sulfate, 1.0g L^{-1} sodium citrate monobasic, and 0.20g L^{-1} magnesium sulfate heptahydrate, with the addition of 150mM sodium acetate, 87mM sodium pyruvate, 10mM NADH, 1mM NAD^{+}, 10mM dithiothreitol, 1mM thiamine pyrophosphate, and 1.11mg mL^{-1} trypsin protease inhibitor. Cell-free enzyme reaction took place at ambient temperature and pH 6.6.
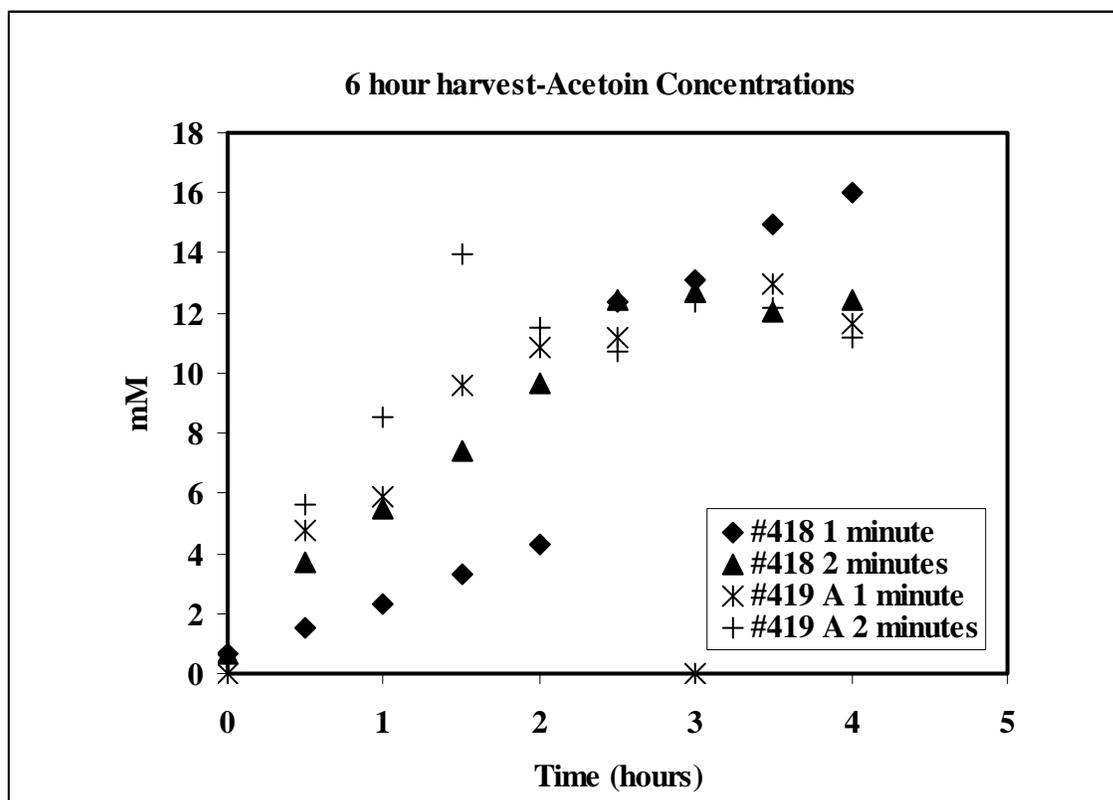
Figure 21. Production of acetoin by cell-free enzymes harvested after 6 hours fermentation.

#418 and #419 A refer to the microtip used for sonication for 1 or 2 minutes. Cell-free enzyme

reaction medium consisted of 5.0g L^{-1} yeast extract, 6.0g L^{-1} potassium phosphate

monobasic,14.0g L^{-1} potassium phosphate dibasic, 2.0g L^{-1} ammonium sulfate, 1.0g L^{-1} sodium

citrate monobasic, and 0.20g L^{-1} magnesium sulfate heptahydrate, with the addition of 150mM

sodium acetate, 87mM sodium pyruvate, 10mM NADH, 1mM NAD^{+}, 10mM dithiothreitol,

1mM thiamine pyrophosphate, and 1.11mg mL^{-1} trypsin protease inhibitor. Cell-free enzyme

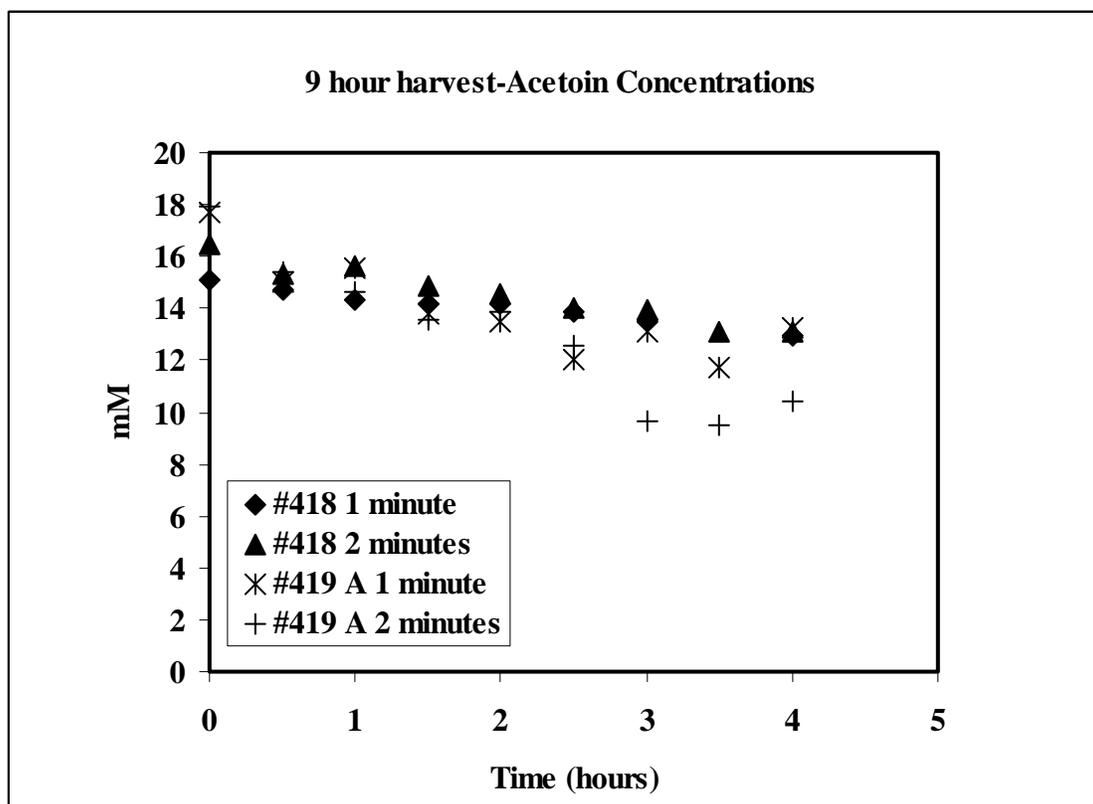reaction took place at ambient temperature and pH 6.6.

Figure 22. Concentrations of acetoin by cell-free enzymes harvested after 9 hours fermentation.

#418 and #419 A refer to the microtip used for sonication for 1 or 2 minutes. At reaction time

zero 15-18mM acetoin was present. Cell-free enzyme reaction medium consisted of 5.0g L^{-1}

yeast extract, 6.0g L^{-1} potassium phosphate monobasic,14.0g L^{-1} potassium phosphate dibasic,

2.0g L^{-1} ammonium sulfate, 1.0g L^{-1} sodium citrate monobasic, and 0.20g L^{-1} magnesium sulfate

heptahydrate, with the addition of 150mM sodium acetate, 87mM sodium pyruvate, 10mM

NADH, 1mM NAD^{+}, 10mM dithiothreitol, 1mM thiamine pyrophosphate, and 1.11mg mL^{-1}

trypsin protease inhibitor. Cell-free enzyme reaction took place at ambient temperature and pH

6.6.

Figure 23 shows metabolic pathways in which acetoin is formed. Also shown are pathways for lactate, ethanol, acetate, formate, and 2,3-butanediol (Celinska, 2009).
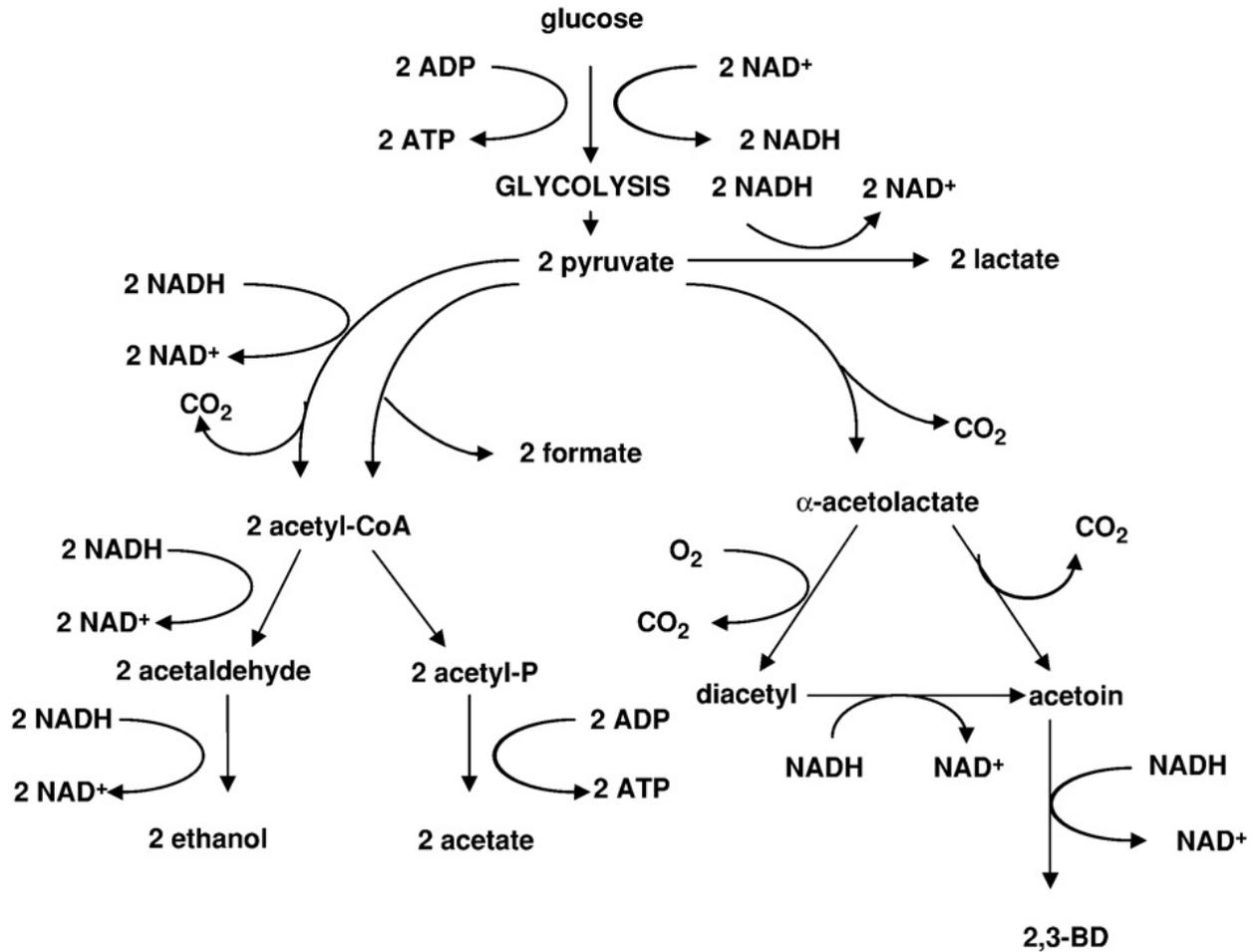

Figure 23. Metabolic pathways to produce acetoin (Celinska, 2009).

Figures 24-34 show concentrations of ethanol, lactic acid, glucose and pyruvic acid in the cell-free enzyme reaction system. No 2,3-butanediol was produced and pyruvate was assumed to be consumed within the first half hour of reaction.

Figure 24. Concentrations of ethanol by cell-free enzymes harvested after 3 hours fermentation. #418 and #419 A refer to the microtip used for sonication for 1 or 2 minutes. Cell-free enzyme reaction medium consisted of 5.0g L^{-1} yeast extract, 6.0g L^{-1} potassium phosphate monobasic,14.0g L^{-1} potassium phosphate dibasic, 2.0g L^{-1} ammonium sulfate, 1.0g L^{-1} sodium citrate monobasic, and 0.20g L^{-1} magnesium sulfate heptahydrate, with the addition of 150mM sodium acetate, 87mM sodium pyruvate, 10mM NADH, 1mM NAD^+, 10mM dithiothreitol, 1mM thiamine pyrophosphate, and 1.11mg mL^{-1} trypsin protease inhibitor. Cell-free enzyme reaction took place at ambient temperature and pH 6.6.
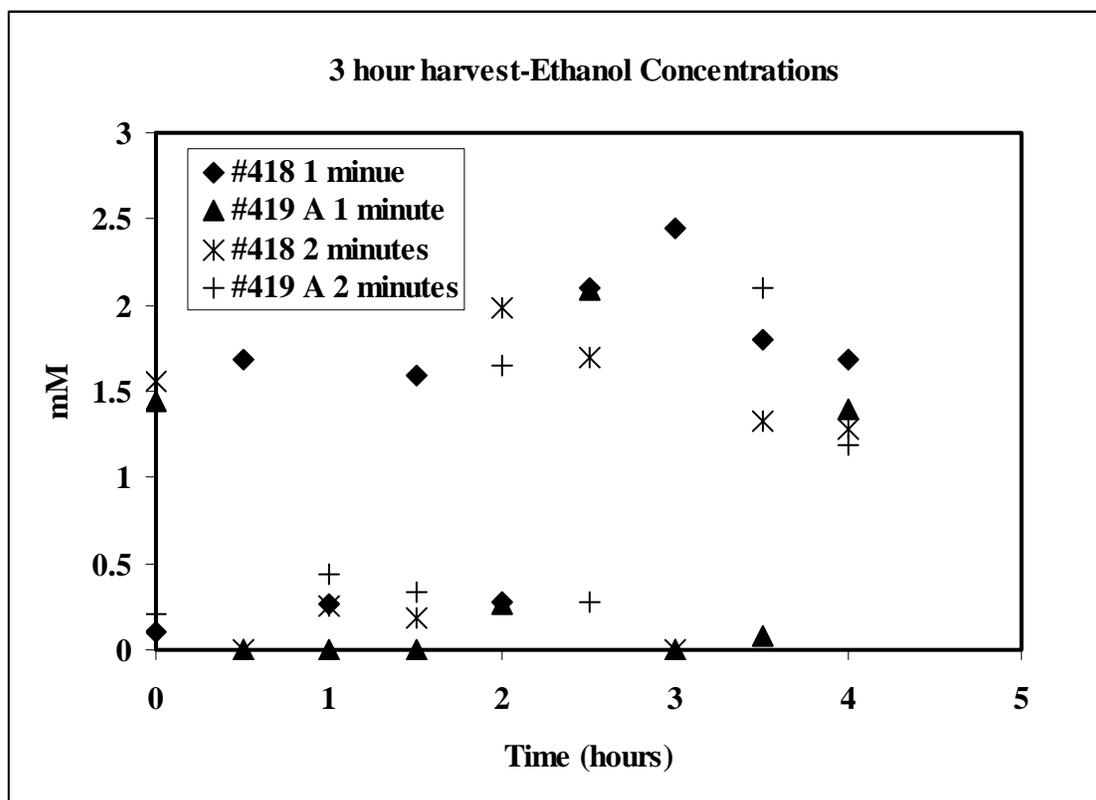
Figure 25. Concentrations of ethanol by cell-free enzymes harvested after 6 hours fermentation. #418 and #419 A refer to the microtip used for sonication for 1 or 2 minutes. Cell-free enzyme reaction medium consisted of 5.0g L^{-1} yeast extract, 6.0g L^{-1} potassium phosphate monobasic,14.0g L^{-1} potassium phosphate dibasic, 2.0g L^{-1} ammonium sulfate, 1.0g L^{-1} sodium citrate monobasic, and 0.20g L^{-1} magnesium sulfate heptahydrate, with the addition of 150mM sodium acetate, 87mM sodium pyruvate, 10mM NADH, 1mM NAD^+, 10mM dithiothreitol, 1mM thiamine pyrophosphate, and 1.11mg mL^{-1} trypsin protease inhibitor. Cell-free enzyme reaction took place at ambient temperature and pH 6.6.
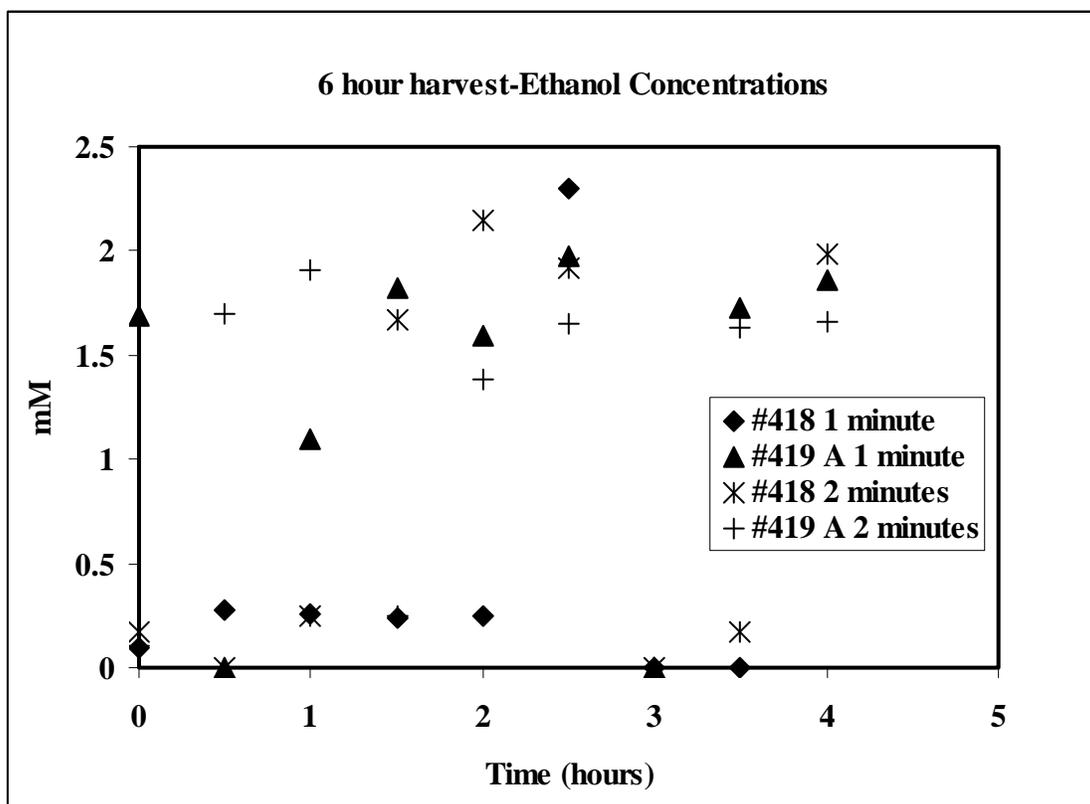
Figure 26. Concentrations of ethanol by cell-free enzymes harvested after 9 hours fermentation. #418 and #419 A refer to the microtip used for sonication for 1 or 2 minutes. Cell-free enzyme reaction medium consisted of 5.0g L^{-1} yeast extract, 6.0g L^{-1} potassium phosphate monobasic, 14.0g L^{-1} potassium phosphate dibasic, 2.0g L^{-1} ammonium sulfate, 1.0g L^{-1} sodium citrate monobasic, and 0.20g L^{-1} magnesium sulfate heptahydrate, with the addition of 150mM sodium acetate, 87mM sodium pyruvate, 10mM NADH, 1mM NAD^+, 10mM dithiothreitol, 1mM thiamine pyrophosphate, and 1.11mg mL^{-1} trypsin protease inhibitor. Cell-free enzyme reaction took place at ambient temperature and pH 6.6.

Figure 27. Concentrations of lactic acid by cell-free enzymes harvested after 3 hours

fermentation. #418 and #419 A refer to the microtip used for sonication for 1 or 2 minutes.

Cell-free enzyme reaction medium consisted of 5.0g L^{-1} yeast extract, 6.0g L^{-1} potassium

phosphate monobasic,14.0g L^{-1} potassium phosphate dibasic, 2.0g L^{-1} ammonium sulfate,   1.0g

L^{-1} sodium citrate monobasic, and 0.20g L^{-1} magnesium sulfate heptahydrate, with the addition

of 150mM sodium acetate, 87mM sodium pyruvate, 10mM NADH, 1mM NAD^{+}, 10mM

dithiothreitol, 1mM thiamine pyrophosphate, and 1.11mg mL^{-1} trypsin protease inhibitor. Cell-

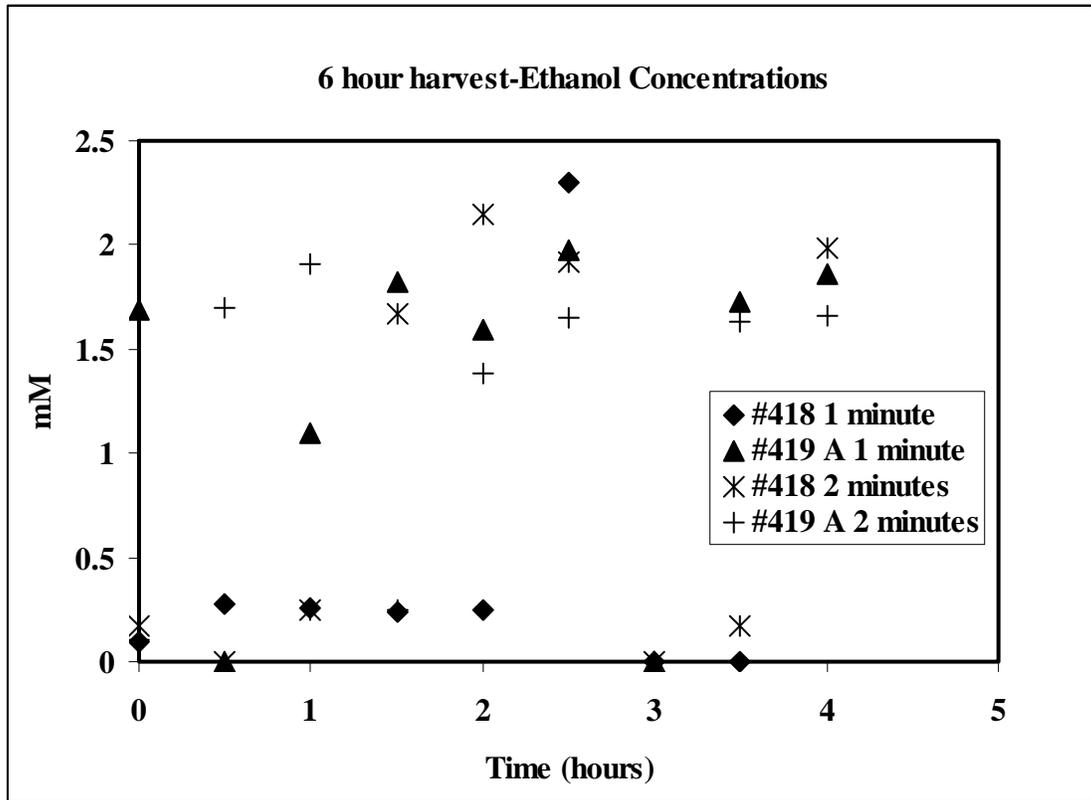free enzyme reaction took place at ambient temperature and pH 6.6.

Figure 28. Concentrations of lactic acid by cell-free enzymes harvested after 6 hours fermentation. #418 and #419 A refer to the microtip used for sonication for 1 or 2 minutes. Cell-free enzyme reaction medium consisted of 5.0g L^{-1} yeast extract, 6.0g L^{-1} potassium phosphate monobasic,14.0g L^{-1} potassium phosphate dibasic, 2.0g L^{-1} ammonium sulfate, 1.0g L^{-1} sodium citrate monobasic, and 0.20g L^{-1} magnesium sulfate heptahydrate, with the addition of 150mM sodium acetate, 87mM sodium pyruvate, 10mM NADH, 1mM NAD^{+}, 10mM dithiothreitol, 1mM thiamine pyrophosphate, and 1.11mg mL^{-1} trypsin protease inhibitor. Cell-free enzyme reaction took place at ambient temperature and pH 6.6.

9 hour harvest-Lactic Acid Concentrations

Legend:
- ◆ #418 1 minute
- ▲ #419 A 1 minute
- ✳ #418 2 minutes
- + #419 A 2 minutes

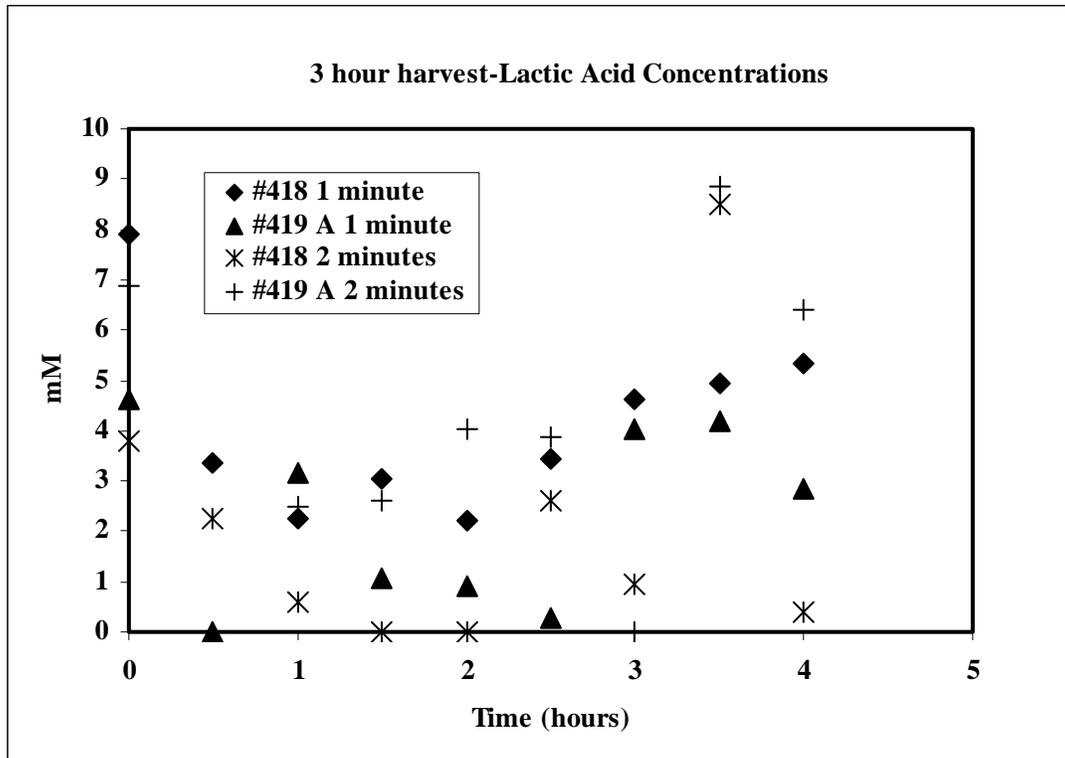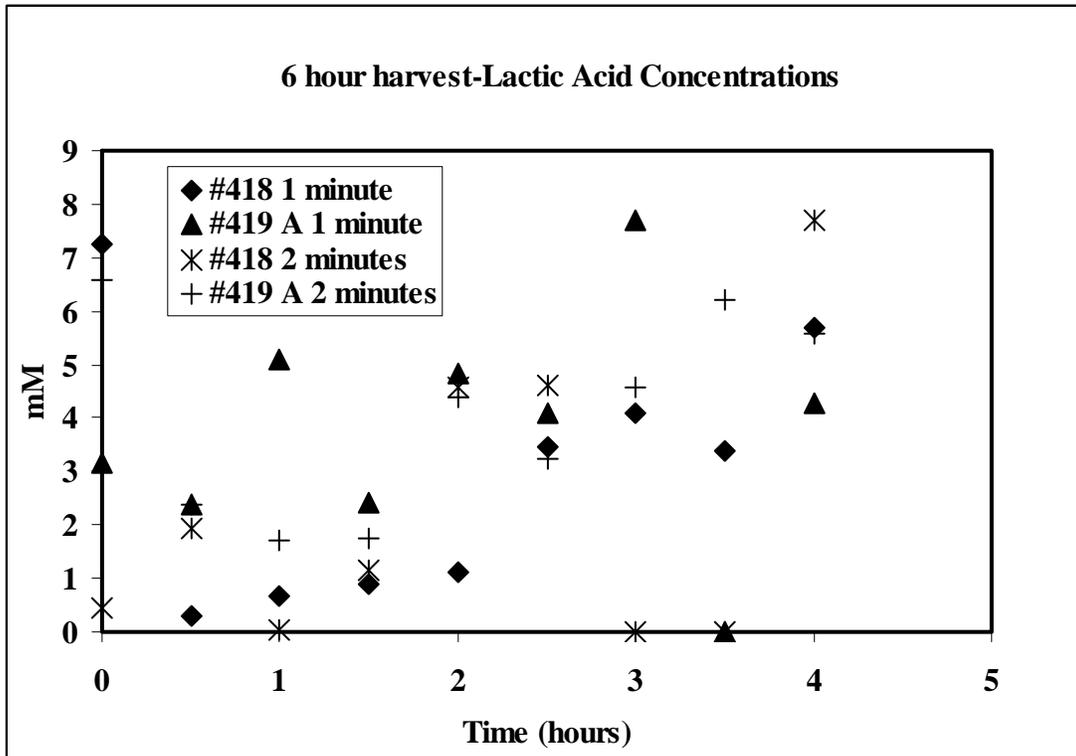Y-axis: mM
X-axis: Time (hours)

Figure 29. Concentrations of lactic acid by cell-free enzymes harvested after 9 hours fermentation. #418 and #419 A refer to the microtip used for sonication for 1 or 2 minutes. Cell-free enzyme reaction medium consisted of 5.0g L^{-1} yeast extract, 6.0g L^{-1} potassium phosphate monobasic,14.0g L^{-1} potassium phosphate dibasic, 2.0g L^{-1} ammonium sulfate, 1.0g L^{-1} sodium citrate monobasic, and 0.20g L^{-1} magnesium sulfate heptahydrate, with the addition of 150mM sodium acetate, 87mM sodium pyruvate, 10mM NADH, 1mM NAD^{+}, 10mM dithiothreitol, 1mM thiamine pyrophosphate, and 1.11mg mL^{-1} trypsin protease inhibitor. Cell-free enzyme reaction took place at ambient temperature and pH 6.6.

3 hour harvest-Glucose Concentrtations

Figure 30. Concentrations of glucose in cell-free enzymes harvested after 3 hours fermentation. #418 and #419 A refer to the microtip used for sonication for 1 or 2 minutes. Sample peaks for times 0-2 hours were not differentiable. The glucose peak occurred directly beside a peak for the media and was engulfed. Cell-free enzyme reaction medium consisted of 5.0g L^{-1} yeast extract, 6.0g L^{-1} potassium phosphate monobasic,14.0g L^{-1} potassium phosphate dibasic, 2.0g L^{-1} ammonium sulfate, 1.0g L^{-1} sodium citrate monobasic, and 0.20g L^{-1} magnesium sulfate heptahydrate, with the addition of 150mM sodium acetate, 87mM sodium pyruvate, 10mM NADH, 1mM NAD^+, 10mM dithiothreitol, 1mM thiamine pyrophosphate, and 1.11mg mL^{-1} trypsin protease inhibitor. Cell-free enzyme reaction took place at ambient temperature and pH 6.6.
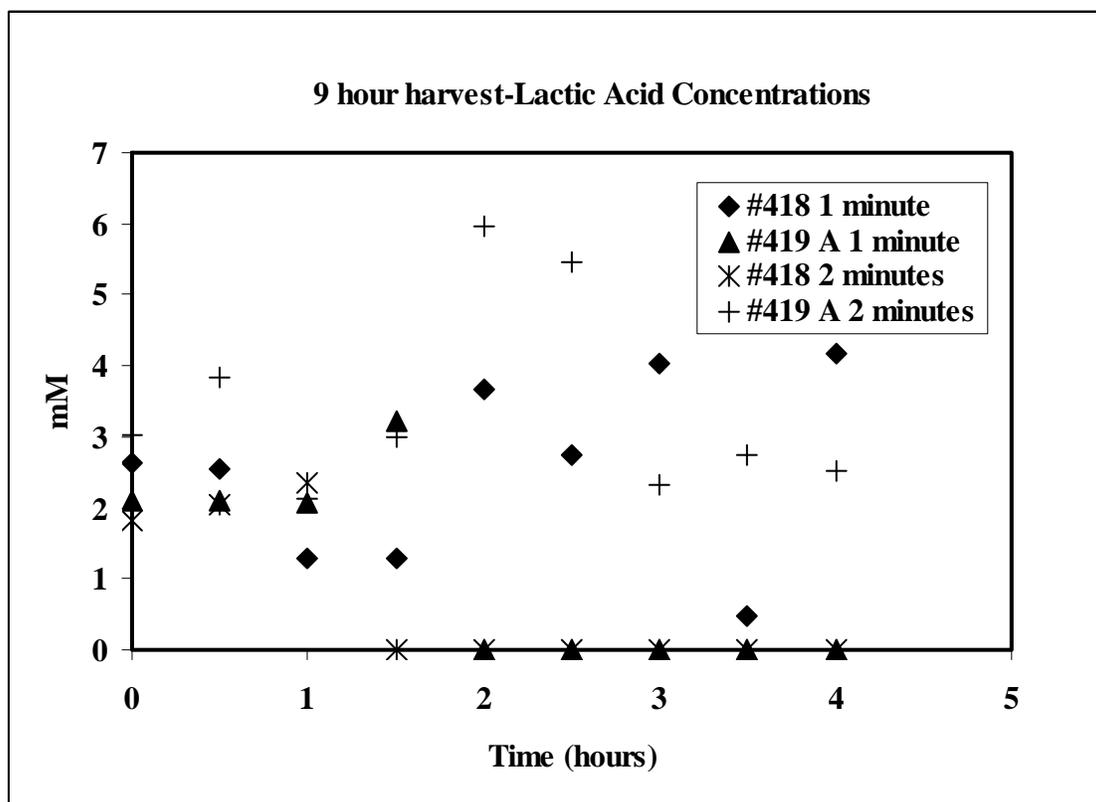
6 hour harvest-Glucose Conentrations

Figure 31. Concentrations of glucose in cell-free enzymes harvested after 6 hours fermentation. #418 and #419 A refer to the microtip used for sonication for 1 or 2 minutes. Sample peaks for times 0-2 hours were not differentiable. The glucose peak occurred directly beside a peak for the media and was engulfed. Cell-free enzyme reaction medium consisted of 5.0g L^{-1} yeast extract, 6.0g L^{-1} potassium phosphate monobasic,14.0g L^{-1} potassium phosphate dibasic, 2.0g L^{-1} ammonium sulfate, 1.0g L^{-1} sodium citrate monobasic, and 0.20g L^{-1} magnesium sulfate heptahydrate, with the addition of 150mM sodium acetate, 87mM sodium pyruvate, 10mM NADH, 1mM NAD^{+}, 10mM dithiothreitol, 1mM thiamine pyrophosphate, and 1.11mg mL^{-1} trypsin protease inhibitor. Cell-free enzyme reaction took place at ambient temperature and pH 6.6.
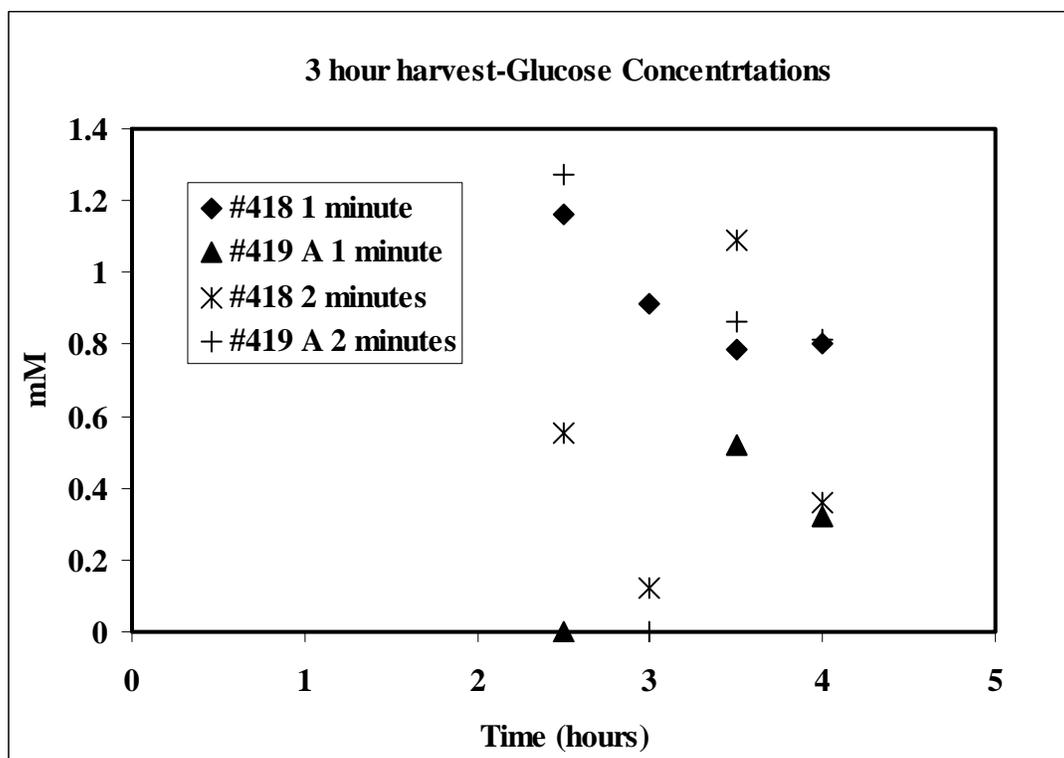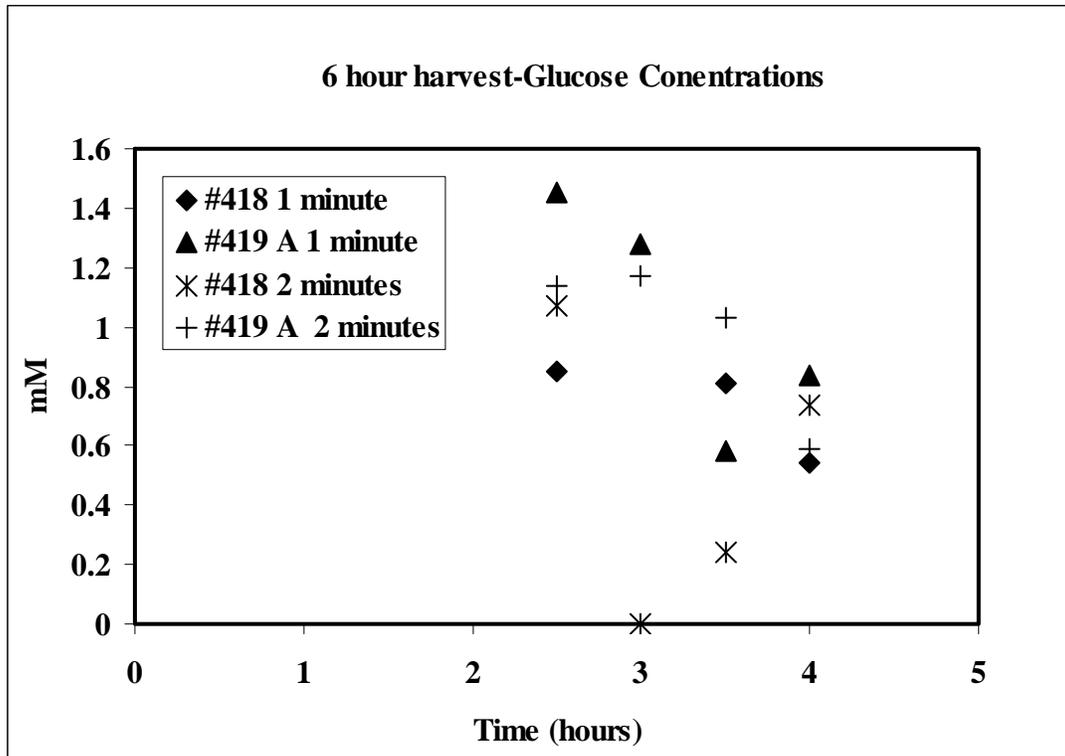
Figure 32. Concentrations of glucose in cell-free enzymes harvested after 9 hours fermentation. #418 and #419 A refer to the microtip used for sonication for 1 or 2 minutes. At reaction time zero 0.4-0.8mM glucose was present. Cell-free enzyme reaction medium consisted of 5.0g L^{-1} yeast extract, 6.0g L^{-1} potassium phosphate monobasic,14.0g L^{-1} potassium phosphate dibasic, 2.0g L^{-1} ammonium sulfate, 1.0g L^{-1} sodium citrate monobasic, and 0.20g L^{-1} magnesium sulfate heptahydrate, with the addition of 150mM sodium acetate, 87mM sodium pyruvate, 10mM NADH, 1mM NAD^{+}, 10mM dithiothreitol, 1mM thiamine pyrophosphate, and 1.11mg mL^{-1} trypsin protease inhibitor. Cell-free enzyme reaction took place at ambient temperature and pH 6.6.

3 hour harvest-Pyruvic Acid Concentrations

Legend:
- ◆ #418 1 minute
- ▲ #418 2 minutes
- ✳ #419 A 1 minute
- + #419 A 2 minutes

Y-axis: mM (0 to 100)
X-axis: Time (hours) (0 to 1)

Figure 33. Concentrations of pyruvic acid in cell-free enzymes harvested after 3 hours

fermentation. #418 and #419 A refer to the microtip used for sonication for 1 or 2 minutes.

Sample peaks for times 1-4 hours were not differentiable. The pyruvic acid peak occurred

directly beside a peak for the media and glucose and was engulfed. The pyruvate was probably

consumed within the first hour of reaction. Cell-free enzyme reaction medium consisted of 5.0g

L^{-1} yeast extract, 6.0g L^{-1} potassium phosphate monobasic,14.0g L^{-1} potassium phosphate

dibasic, 2.0g L^{-1} ammonium sulfate, 1.0g L^{-1} sodium citrate monobasic, and 0.20g L^{-1}

magnesium sulfate heptahydrate, with the addition of 150mM sodium acetate, 87mM sodium

pyruvate, 10mM NADH, 1mM NAD^{+}, 10mM dithiothreitol, 1mM thiamine pyrophosphate, and

1.11mg mL^{-1} trypsin protease inhibitor. Cell-free enzyme reaction took place at ambient
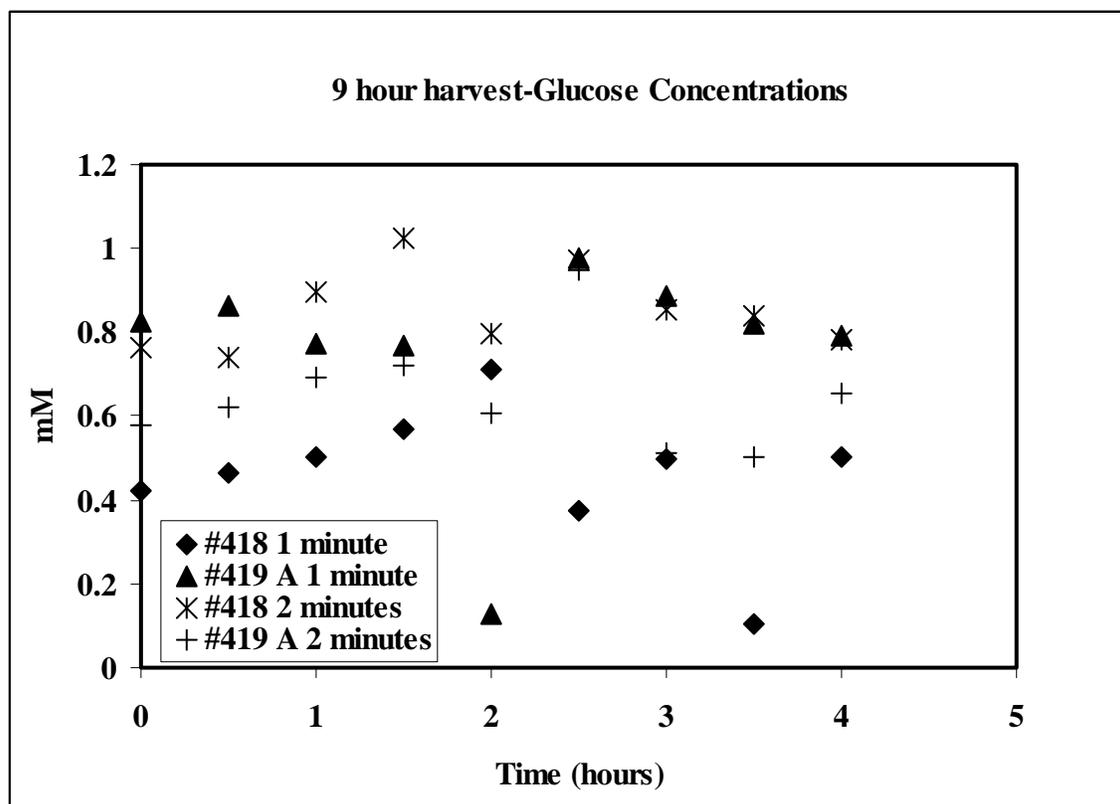
temperature and pH 6.6.

70

6 hour harvest-Pyruvic Acid Concentrations

Legend:
- ◆ #418 1 minute
- ▲ #418 2 minutes
- ✳ #419 A 1 minute
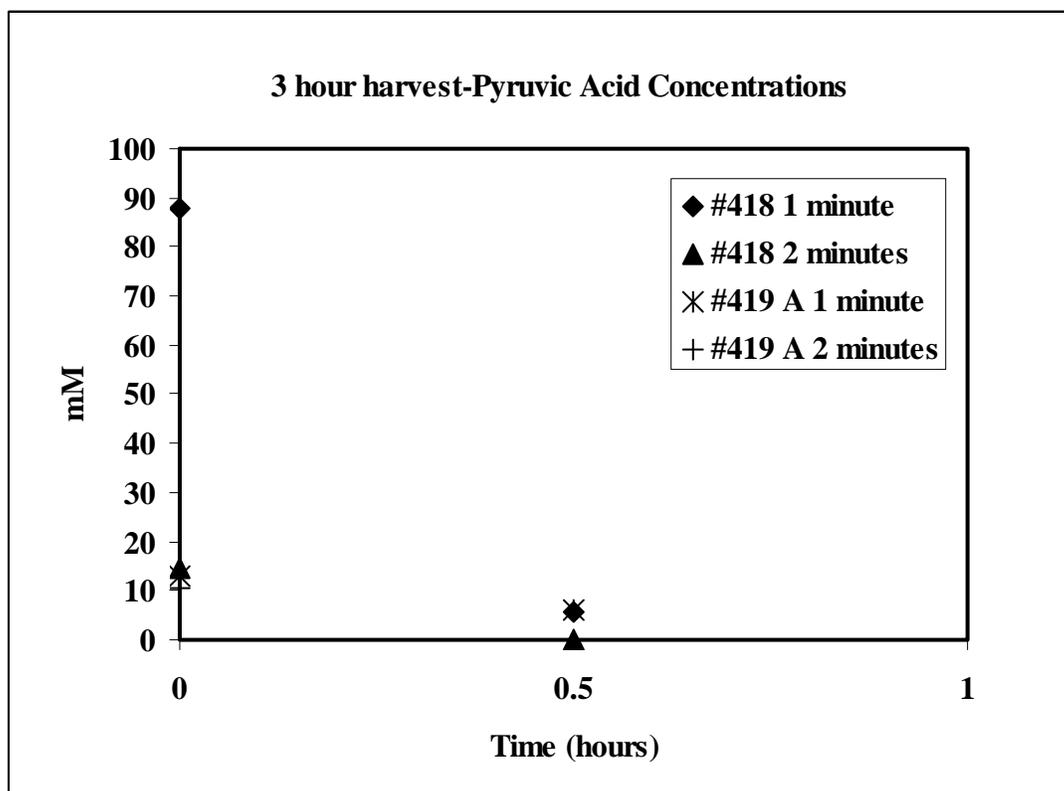- + #$19 A 2 minutes

Y-axis: mM
X-axis: Time (hours)

Figure 34. Concentrations of pyruvic acid in cell-free enzymes harvested after 3 hours fermentation. #418 and #419 A refer to the microtip used for sonication for 1 or 2 minutes. The pyruvic acid peak occurred directly beside a peak for the media and glucose and was engulfed. The pyruvate was probably consumed within the first hour of reaction. Cell-free enzyme reaction medium consisted of 5.0g L^{-1} yeast extract, 6.0g L^{-1} potassium phosphate monobasic,14.0g L^{-1} potassium phosphate dibasic, 2.0g L^{-1} ammonium sulfate, 1.0g L^{-1} sodium citrate monobasic, and 0.20g L^{-1} magnesium sulfate heptahydrate, with the addition of 150mM sodium acetate, 87mM sodium pyruvate, 10mM NADH, 1mM NAD^{+}, 10mM dithiothreitol, 1mM thiamine pyrophosphate, and 1.11mg mL^{-1} trypsin protease inhibitor. Cell-free enzyme reaction took place at ambient temperature and pH 6.6.

The pyruvic acid concentrations for the 9 hour harvest reaction were generally not detectable. The pyruvic acid peak occurred directly beside a peak for the media and glucose and was engulfed.

Figures 35-37 show the concentrations of glycerol over the 4 hour cell-free enzyme reaction time. As compared to the increase in concentrations of succinic acid and acetoin over the same time period, the concentrations of glycerol remain fairly constant.

3 hour harvest-Glycerol Concentrations

Legend:
- ◆ #418 1 minute
- ▲ #418 2 minutes
- ✳ #419A 1 minute
- + #419A 2 minutes

Y-axis: mM

X-axis: Time (hours)

Figure 35. Concentrations of glycerol contained in media for cell-free enzyme reaction. Enzymes were harvested after 3 hours fermentation. #418 and #419 A refer to the microtip used for sonication for 1 or 2 minutes. Concentrations are high for series #418 2 minutes and #419 A 1 minute due to an inadvertent addition of excess glycerol to the system. However, these samples show very little change in glycerol concentration over time and no unusual products nor a significant increase in any of the current products were formed as a result. Cell-free enzyme reaction medium consisted of 5.0g L^{-1} yeast extract, 6.0g L^{-1} potassium phosphate monobasic,14.0g L^{-1} potassium phosphate dibasic, 2.0g L^{-1} ammonium sulfate, 1.0g L^{-1} sodium citrate monobasic, and 0.20g L^{-1} magnesium sulfate heptahydrate, with the addition of 150mM sodium acetate, 87mM sodium pyruvate, 10mM NADH, 1mM NAD^{+}, 10mM dithiothreitol, 1mM thiamine pyrophosphate, and 1.11mg mL^{-1} trypsin protease inhibitor. Cell-free enzyme reaction took place at ambient temperature and pH 6.6.
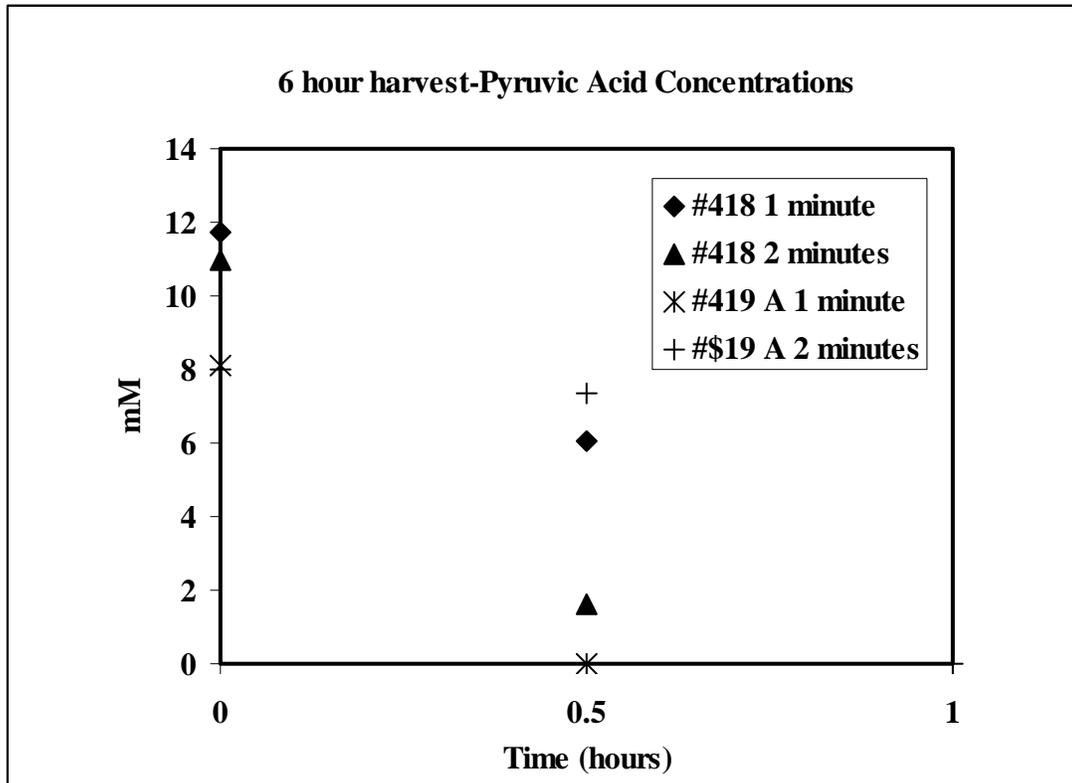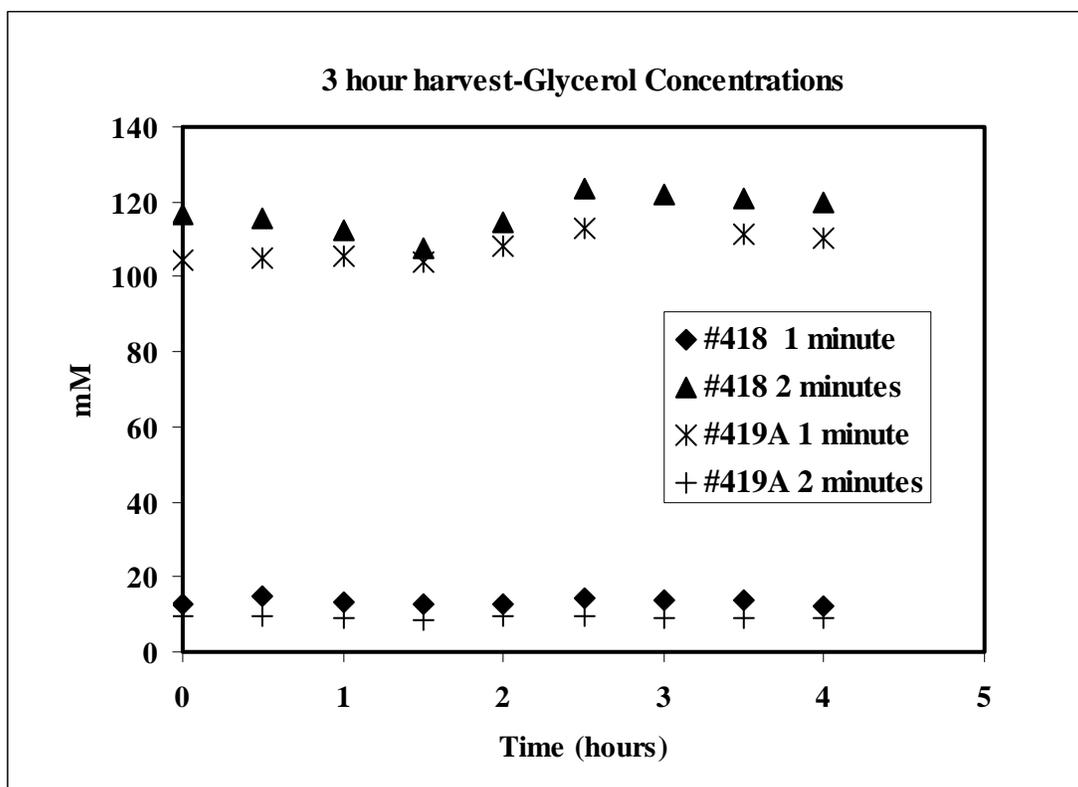
Figure 36. Concentrations of glycerol contained in media for cell-free enzyme reaction.

Enzymes were harvested after 6 hours fermentation. #418 and #419 A refer to the microtip used

for sonication for 1 or 2 minutes. Cell-free enzyme reaction medium consisted of 5.0g L^{-1} yeast

extract, 6.0g L^{-1} potassium phosphate monobasic, 14.0g L^{-1} potassium phosphate dibasic, 2.0g L^{-1}

ammonium sulfate, 1.0g L^{-1} sodium citrate monobasic, and 0.20g L^{-1} magnesium sulfate

heptahydrate, with the addition of 150mM sodium acetate, 87mM sodium pyruvate, 10mM

NADH, 1mM NAD^{+}, 10mM dithiothreitol, 1mM thiamine pyrophosphate, and 1.11mg mL^{-1}

trypsin protease inhibitor. Cell-free enzyme reaction took place at ambient temperature and pH
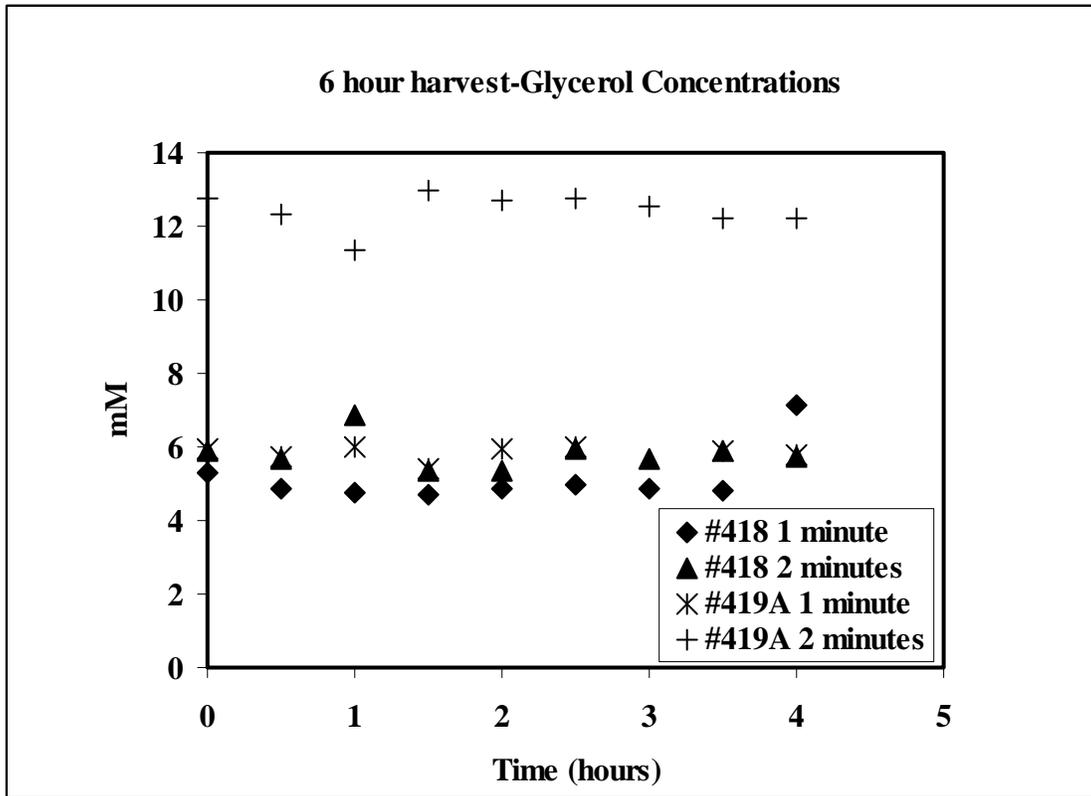
6.6.

Figure 37. Concentrations of glycerol contained in media for cell-free enzyme reaction. Enzymes were harvested after 9 hours fermentation. #418 and #419A refer to the microtip used for sonication for 1 or 2 minutes. Cell-free enzyme reaction medium consisted of 5.0g L^{-1} yeast extract, 6.0g L^{-1} potassium phosphate monobasic,14.0g L^{-1} potassium phosphate dibasic, 2.0g L^{-1} ammonium sulfate, 1.0g L^{-1} sodium citrate monobasic, and 0.20g L^{-1} magnesium sulfate heptahydrate, with the addition of 150mM sodium acetate, 87mM sodium pyruvate, 10mM NADH, 1mM NAD^{+}, 10mM dithiothreitol, 1mM thiamine pyrophosphate, and 1.11mg mL^{-1} trypsin protease inhibitor. Cell-free enzyme reaction took place at ambient temperature and pH 6.6.

Although glycerol can be used as a carbon source to produce 2,3-butanediol (Blankschien, 2010) it does not appear to be the case for this reaction. Pyruvate, with the addition of NAD(P)H, can react to form malate. Or, with the addition of ATP, pyruvate can form oxaloacetate and then malate with the addition of NADH. Malate can then form fumarate, and finally succinate (Blankschien, 2010). Because only NAD^+ and NADH were added as a cofactor in these experiments it is not clear how the succinate was formed and which enzymes were responsible. There are many enzyme pathways that lead to succinate and no literature was found for succinate providing enzyme pathways in this particular bacterium.
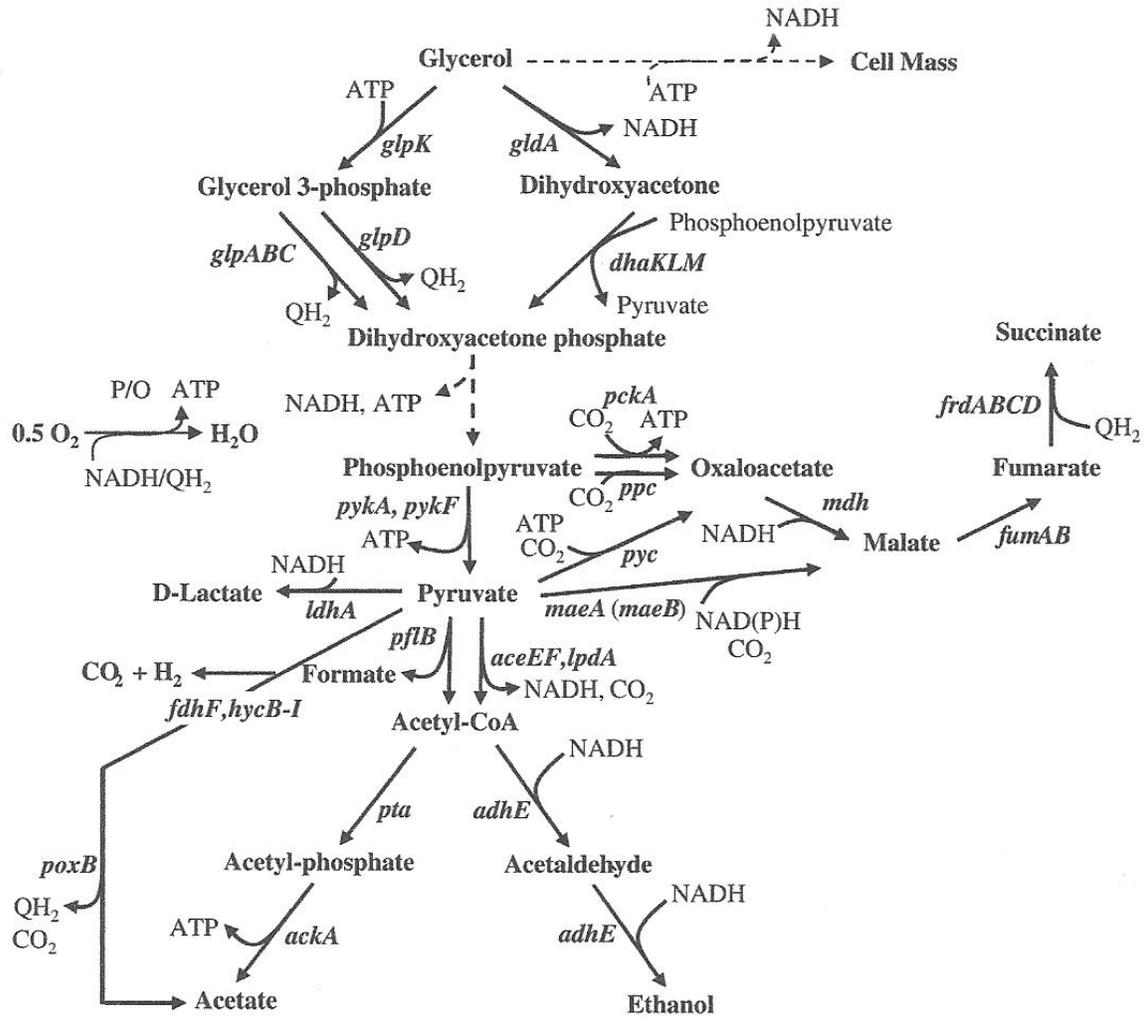
Figure 38. Enzymatic pathways in the conversion of pyruvate to products (Blankschien, 2010)

In order to determine the cause of the production of succinic acid a new set of experiments was performed. The previous procedure was repeated. Sonication was completed by #419A microtip for 2 minutes. Samples for HPLC were taking at 9 hours fermentation harvest, after addition to sonication solution but before sonication, directly after sonication, and after sonication and addition to cell-free enzyme reaction medium.

Table 9. Concentrations of succinic acid at various time periods.

Sample	mM Succinic Acid
9 hour whole cell fermentation	0.4
Cell-free enzyme medium	0
Before sonication	0
After sonication	0
After addition to medium	12.0

As Table 9 shows, succinic acid was not produced during sonication. It was seen in very small concentrations during the whole cell fermentation and in significantly larger concentrations after the enzymes were added to the reaction medium. The assumption is that the peak seen on the HPLC analysis was succinic acid, and not some other compound with the same retention time as succinic acid. Therefore, the succinic acid has to be formed in the cell-free enzyme reactions.

Conclusion

It is postulated, along with the data from Figures 17-22 that the cell-free enzymes produced 13mM succinic acid as well as 16mM acetoin from 87mM pyruvate. If all of the succinic acid was produced from the pyruvate then the theoretical yield of the combination of these was 52%. Comparatively, Perego observed a yield of 51.9mM 2,3-butanediol and 4.5mM acetoin by *B. licheniformis* whole cells. When considering 2,3-butanediol and acetoin together and glucose as substrate, 56.4% of the theoretical yield was achieved (Perego, 2003). Note that these results are based on glucose as substrate. No literature was found with pyruvate as substrate for *Bacillus*.

Conventional whole cell fermentation or even engineered bacterial fermentation to produce succinic acid also produces byproducts such as acetic acid, formic acid, and lactic acid. Formation of these byproducts reduces the yield of succinic acid formed (Song, 2006). The proposed cell-free enzyme reaction produced mostly acetoin as a side product, and very small amounts of anything else. This process would therefore increase the yield of succinic acid produced by decreasing production of side products as compared to conventional fermentation. Separation of succinic acid from byproducts would also be less expensive and more profitable.

Other results include the observation that the cell-free enzymes reacted to form product. This answers the question as to whether or not the enzymes would complete fermentation outside of the cells, as they do inside the cells. The cell-free enzymes formed succinic acid and acetoin and no 2,3-butanediol. Interestingly, the whole cell fermentation of *B. licheniformis* produced 2,3-butanediol and no succinic acid.

References

Bach J. A., Sadoff H. L., "Aerobic sporulating bacteria. I. Glucose dehydrogenation of *Bacillus cereus*." <u>Journal of Bacteriology</u> 83, (1962): 699-707.

Blankschien Matthew D., Clomburg James M., Gonzalez Ramon "Metabolic Engineering of Escherichia coli for the production of succinate from glycerol." <u>Metabolic Engineering</u> 12, (2010): 409-419.

"Butanoate Metabolism." <u>KEGG Pathway Database</u>. 2009. May 2008 <http://www.genome.jp/kegg/metabolism.html>.

Celinska E., Grajek W. "Biotechnological Production of 2,3-butanediol-Current state and prospects." <u>Biotechnology Advances</u> 27, (2009): 715-725.

Feeney Robert E., Stevens Frits C., and Osuga David T. "The Specificities of Chicken Ovomucoid and Ovoinhibitor." <u>The Journal of Biological Chemistry</u> 238, 4 (1963): 1415-1418.

Feliu J. X., Cubarsi R., Villaverde A. "Optimized Release of Recombinant Proteins by Ultransonication of *E. Coli* Cells." <u>Biotechnology and Bioengineering</u> 58, 5 (1997): 536-540.

Flickinger, M.C. 1980 "Current biological research in conversion of cellulosic carbohydrates into liquid fuels: how far have we come?" <u>Biotechnology and Bioengineering</u> 22, (1980): 27-48.

Foster John W., Cowan Robert M., and Maag Ted A. "Rupture of Bacteria by Explosive Decompression." <u>Journal of Bacteriology</u> 83,2 (1962): 330-334.

Jiayang Qin, Zijun Xiao, Cuiqing Ma, Nengzhong Xie, Peihai Liu, and Ping Xu "Production of 2,3-Butanediol by *Klebsiella pneumoniae* Using Glucose and Ammonium Phosphate." <u>Chinese Journal of Chemical Engineering</u> 14, 1 (2006): 132-136.

Kido Horacio, Micic Miodrag, Smith David, Zoval Jim, Norton Jim, and Madou Marc "A novel, compact disk-like centrifugal microfluidics system for cell lysis and sample homogenization." <u>Colloids and Surfaces B: Biointerfaces</u> 58 (2007): 44-51.

Kurtzman R. H. "Rupture of bacterial spores with ammonium bicarbonate subsequently removed by sublimation." <u>Letters in Applied Microbiology</u> 5, 6 (1987): 111-113.

Laube V. M., Groleau D,. Martin S. M. "The Effect of Yeast Extract on the Fermentation of Glucose to 2,3-Butanediol by *Bacillus polymyxa*." Biotechnology Letters 6, 8 (1984): 535-540.

Miller J. H. Experiments in Molecular Genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 1972.

Nakashimada Yutaka, Marwoto Bambang, Kashiwarmura Takashi, Kakizono Toshihide, and Nishio Naomichi "Enhanced 2,3-Butanediol Production by Addition of Acetic Acid in *Paenibacillus polymyxa*." Journal of Bioscience and Bioengineering 90, 6 (2000): 661-664.

Nilegaonkar S., Bhosale S. B., Kshirsagar D. C., Kapadi A. H., "Production of 2,3-butanediol from glucose by *Bacillus licheniformis*." World Journal of Microbiology and Biotechnology 8 (1992): 378-381.

Perego P., Converti A., and Del Borghi M. "Effects of temperature, inoculum size and starch hydrolyzate concentration on butanediol production." Bioresource Technology 89 (2003): 125-131.

Poulsen C. and Stougaard P. "Purification and properties of *Saccharomyces cerevisiae* acetolactate synthase from recombinant *Escherichia coli*." European Journal of Biochemistry 185 (1989): 433-43Song Hyohak, Sang Yup Lee "Production of succinic acid by bacterial fermentation." Enzyme and Microbial Technology 39 (2006): 352-361.

Stanier R. Y., and Adams G. A. "The Nature of the *Aeromonas* Fermentation." Biochemical Journal 38 (1944): 168-171.

Weibull, Claes "The Isolation of Protoplasts From *Bacillus megaterium* by Controlled Treatement with Lysozyme." Journal of Bacteriology 66, 6 (1953): 688-695.

Yoshida Akira "Enzymatic Properties of Lactate Dehydrogenase of *Bacillus subtilis*." Biochemica et Biophysica acta 99 (1965): 66-77.

Yu Ernest K. C,. Saddler John N. "Enhanced Production of 2,3-Butanediol by *Klebsiella pneumoniae* Grown on High Sugar Concentrations in the Presence of Acetic Acid." Applied and Environmental Microgiology 44, 4 (1982): 777-784.

Zoetendal Erwin G., Ben-Amore Kaouther,. Akkermans Antoon D. L, Abee Tjakko, and DE Vos Willem M. "DNA Isolation Protocols Affect the Detection Limit of PCR Approaches of Bacteria in Samples from the Human Gastrointestinal Tract." Systematic and Applied Microbiology 24 (2001): 405-410.

Appendix

Experiment Protocols

Bacterial Cell Propagation and Aseptic Technique

Maintain live bacterial cells on agar plates. Every two weeks streak new plates using the most recent plates stored in the refrigerator. Place both sets of plates into the biological safety hood. Ensure the hood is used properly. Do not open the hood above the indicated height. Wear a laboratory coat that is only used when working directly with the bacteria. Wear gloves. Turn on the butane torch and set to the hands free setting. Sterilize a loop by holding it in the butane flame until the loop turns bright orange. Run the length of the loop through the flame so that each portion of the length of loop turns orange while in the flame. Let the loop cool for a few seconds and then gently scrape the loop across the plate to grab bacterial cells. Gently spread the loop with cells onto a fresh plate applying a small amount of pressure to ensure the loop does not puncture the agar. Do not repeat the procedure for a singular plate. Only streak a plate one time. Reflame the loop after spreading the cells onto the first plate to reduce the chance of spreading any contamination from one fresh plate to the next. Repeat the procedure for a second plate. Label each plate with the name of the species, the date, and initials. Place each plate in the incubator and ensure the temperature is correct. Allow adequate time for the cells to grow. This time will be dependent upon the species. The cells will have grown sufficiently once they can be seen easily with the naked eye and are of a decent size. Remove the cells from the incubator and store in the refrigerator.

Sterilization

Sterilize all glassware before and after each growth and fermentation or enzyme reaction by autoclaving at 121°C for 15 minutes. All glassware must be sterile to avoid contamination of each experiment. All glassware must be sterilized after it has come into contact with bacteria to ensure the bacteria is killed and does not contaminate the water supply or any surfaces in the building.

Everything that has had any contact with the bacteria must be autoclaved. Wrap old agar plates with bacteria on them in aluminum foil and autoclave at 121°C for 15 minutes. The plates will partially melt. After autoclaving the plates may be trashed. Repeat the same procedure for any centrifuge tubes that have contained either bacterial cell pellets or enzyme. Once the centrifuge tubes have been sterilized they may be thrown away. Do not place anything directly into the trash that has been in contact with the bacteria. It <u>must</u> be autoclaved first. Keep all bacteria within the biological laboratory. Do not transport outside the biological laboratory unless centrifugation and cell lysis is not possible within this lab.

Bacterial Growth/Fermentation and use of Heated Shaker

Prepare growth medium and autoclave at 121°C for 15 minutes. <u>Do not store medium; use it immediately</u>. Fill 10 flasks with 50mL each of growth medium and 1 gram of glucose (or 20g/L). Aseptically inoculate (with butane torch) a loop of bacterial cells into each flask. Cover each flask with a rubber stopper for anaerobic growth. Place each flask securely in the heated shaker. Close the hood and adjust the rpm to 150. Set the temperature to 37°C. Monitor the temperature periodically because it will fluctuate. Be sure the temperature does not exceed or

decrease more than a few degrees beyond or below 37. Adjust the safety knob if needed to allow for the correct temperature setting.

Prepare fermentation medium and autoclave at 121°C for 15 minutes. <u>Do not store</u> <u>medium</u>. Have the medium prepared so that its sterilization will be complete at the same time the growth cycle will be complete. Fill 10 flasks each with 1g glucose and 35mL of fermentation medium. Pipette 15mL from each of the ten growth flasks into each of the ten fermentation flasks for a total of 50mL per flask and 30% innoculum. Cover each flask with a rubber stopper for anaerobic fermentation. Place each fermentation flask securely into the heated shaker. Continue with the same speed and temperature settings as for the growth for the allotted fermentation time period.

Cell Harvesting

Follow the procedure for the growth and fermentation of the bacterial cells. Once the proper growth or fermentation end time has been reached harvest the cells by turning off the heated shaker and pouring the 50mL of growth or fermentation liquid with bacteria into a 50mL centrifuge tube with the addition of 15% v/v glycerol. Centrifuge at 6000 rpm for 10 minutes. Pipette off the supernatant. Keep the pellet in the centrifuge tube and store in the freezer until ready to use. Autoclave the supernatant to insure no live bacterial cells will persist.

Cell Lysis by use of Parr Cell Disruption Vessel

Follow growth, fermentation, and cell harvesting procedures prior to cell lysis. Remove frozen cell pellet from the freezer. Resuspend the pellet by adding 5mL of 0.05M sodium phosphate buffer at a neutral pH and placing on the vortex briefly. The pellet will be suspended

once no part of it remains on the bottom of the centrifuge tube. Unscrew the chamber of the Parr Cell Disruption Vessel and pour in the cell suspension. To close, place a drop of water on the O-ring, set the head of the vessel in place and push it down until it rests firmly in the cylinder. Turn the screw cap down by hand. Secure the neck and chamber of the vessel on the table with test tube clamps. Connect the nitrogen filling connection by inserting the hose fitting into the adapter on the inlet valve and tighten. Open the nitrogen tank valve no more than one quarter turn. Open the bomb inlet valve one or two turns. Open the filling connection control valve slowly and watch the bomb gage as the pressure rises to the desired level. Close the control valve momentarily and watch the pressure gage to see if the pressure drops significantly. If the pressure drops significantly, add nitrogen to maintain the desired pressure level. Close all valves and open the dump valve on the underside of the filling connection to release the residual pressure in the filling hose. Disconnect the hose from the inlet valve. Close the dump valve. Allow significant time for the nitrogen to dissolve and come to equilibrium with the cells. (This time is dependent upon bacterial species.) Open the discharge valve slowly and slowly release the sample through the discharge tube into a test tube. (To prevent splattering, close the discharge valve after the bulk of the sample has been recovered and release the remaining nitrogen through the inlet valve.) After the sample has been recovered, release the residual nitrogen through the inlet valve. Remove the clamps from the vessel and unscrew the head. Thoroughly rinse the head, chamber, and discharge tube with distilled water. Allow the assembly to dry. Replace the head onto the chamber vessel and store the vessel appropriately. (The vessel assembly may be kept in the refrigerator so that it is cooled for heat sensitive samples).

Cell Lysis by use of Misonix Sonicator

Follow growth, fermentation, and cell harvesting procedures prior to cell lysis. Remove frozen cell pellet from the freezer. Resuspend the pellet by adding 5mL of 0.05M sodium phosphate buffer at a neutral pH and placing on the vortex briefly. Add 1mM dithiothreitol to the cell suspension. Pour the suspension into the modified graduated cylinder. Place the graduated cylinder in the hood and secure with test tube clamps. Lower the sonicator tip into the cell suspension so that it is 6mm below the surface of the liquid being careful to ensure no part of the tip comes in contact with the graduate cylinder. Power on the Misonix and follow the on-screen instructions to set the amplitude, total time, and pulse time for the sonication. Close the hood doors. Monitor the Misonix while in use. Become familiar with the normal sound the tip makes when sonicating as a change in sound could indicate a problem that will need to be resolved. After the allotted sonication time raise the tip, remove the graduated cylinder from the hood and power off the Misonix. Autoclave the sonicator tip so it is ready for the next experiment.

Enzyme Reactions and use of Ambient Shaker

Follow growth, fermentation, cell harvesting, and cell lysis by use of Parr Cell Disruption Vessel or Misonix procedures prior to commencing enzyme reactions. Prepare enzyme reaction medium before beginning the lysis process. <u>Do not store the enzyme reaction medium</u>. <u>Prepare fresh before each enzyme reaction</u>. Immediately following cell lysis by Parr Cell Disruption Vessel or Misonix pipette the lysed cells into 1.5mL centrifuge tubes. (<u>Do not store lysed cells. Use them immediately</u>.) Centrifuge at 14,000 rpm for 5 minutes. After centrifugation pipette the enzyme (supernatant) into the enzyme reaction medium, a total of 10mL in a 125mL flask. Recheck the pH of the medium and adjust if necessary. Cover each flask with a rubber stopper.

Place each flask in the uncovered, ambient temperature shaker. Power on the shaker and set the

speed to 150 rpm and set the time. Be sure not to use the heated shaker as heat will denature the

enzyme and no reaction will occur.

ADH Assay

Reagents: A. 200mM Disoodium Phosphate pH 8.8

B. 95% Ethanol

C. 15mM B-Nicotinamide Adenine Dinucleotide Solution

D. 10mM Sodium Phosphate buffer pH 7.5

E. 1mg mL^{-1} solution ADH in reagent D

F. Dilute reagent 'E' to 0.75units mL^{-1}

Reaction: Ethanol + B-NAD$^+$ <u>Alcohol Dehydrogenase</u>-> Acetaldehyde + B-NADH

Conditions: Temperature = 25°C, pH = 8.8, A$_{340nm}$, Light path = 1 cm, Varian Cary 3e UV-

visible Spectrophotometer

Principle: Alcohol dehydrogenase is an enzyme that reduces ethanol and NAD$^+$ to

acetaldehyde and NADH. The production of NADH is followed by reading the increase in

absorbance at 340nm. Thus, the activity of the enzyme is determined by reading the absorbance

of NADH produced. Sonication of the ADH enzyme followed by the ADH assay allows one to

collect data concerning the effect of sonication on the activity of the enzyme.

Procedure: 1. Pipette 0.8mL reagent A into a cuvette.

2. Add 0.1mL reagent B to cuvette.

3. Add 1.50mL reagent C to cuvette.

4. Mix by inversion and find the 'blank' reading on the UV at 340nm.

5. After the 'blank' reading has equilibrated, add 0.6mL reagent F, mix by
   inversion and record the A_{340nm} over a period of 6 minutes.

Calculations: Graph the A_{340nm} minute^{-1}. Compare the graphs of nonsonicated ADH A_{340nm} minute^{-1} to those of varying sonicated ADH. The absorbance, and therefore production of NADH, will be highest in nonsonicated ADH assays. Once can take the slope of each A_{340nm} minute^{-1} graph and determine the percentage of nonsonicated ADH activity each sonicated enzyme retains. This will allow one to determine the optimum conditions for sonication with the least effect upon the enzyme of interest.

Protein Assay

To check for the presence of free protein (enzyme) within a solution set the UV spectrophotometer to 280 nm. Place the cuvette with the protein solution within the spectrophotometer and read the absorbance.

Cell Mass Assay

To check for the cell mass of whole cells set the visible spectrophotometer to 600 nm. Place the cuvette with the solution containing whole cells within the spectrophotometer and read the absorbance.