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Biological Production of Succinic Acid Using a Cull Peach Medium

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BIOLOGICAL PRODUCTION OF SUCCINIC ACID USING A CULL PEACH MEDIUM

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ABSTRACT

Biological production of succinic acid from a cull peach based medium was studied using *Escherichia coli* AFP 184 in a dual-phase batch process including an aerobic growth phase and an anaerobic production phase. Experiments were performed to study the effects of corn steep liquor supplement and exogenous hydrogen supply. About one half of the sucrose in the media was converted to fermentable sugars glucose and fructose during sterilization. The remaining sucrose was hydrolyzed with invertase. The viscosity of the media was reduced from 350 cP to 50 cP to allow efficient oxygen transfer by a commercial enzyme product. In the media without corn steep liquor supplement, fructose consumption was incomplete and the final succinic acid concentration of 38. 8 g/L was achieved at a yield of 0.77 g/g sugars consumed and a succinate:acetate molar ratio of 5.1:1. When corn steep liquor was supplemented to the media the final succinic acid concentration increased to 47.0 g/L and the yield to 0.87 g/g sugars consumed but the succinate:acetate molar ratio remained at 5.6:1. With hydrogen sparging into the unsupplemented media the final succinic acid concentration increased to 45.5 g/L and the yield to 0.84 g/g sugars consumed. In addition, the succinate:acetate molar ratio increased to 7.7:1. The potential use of cull peach as feedstock for succinic acid production and the possibility of its integration with other biological processes such as butanol and hydrogen productions in a biorefinery are discussed.

DEDICATION

This thesis is dedicated to my loved ones – Priya Krishnakumar, Thangam, Venkatachalam, and Venkatraman. Thank you for your constant love and support.

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CHAPTER ONE

INTRODUCTION

"*Although aromatics are currently synthesized from benzene, toluene, and xylene derived from fossil feedstocks, environmental considerations and the scarcity of petroleum will necessitate development of other industrial routes to these molecules in nations such as the United States"*

-Frost and Lievense, 1994

Succinic Acid – Market & Technology

In 2004 the US Department of Energy (US DOE) released a list identifying 12 most promising building-block or platform chemicals from a pool of 300 candidates, based on their potential market value and possible derivatives, recommending a healthy shift in their production strategies from energy-intensive processes using petroleum-based feedstocks to more environmentally friendly and economically competitive microbial fermentation processes using renewable feedstocks having high carbohydrate contents (Werpy & Petersen, 2004). At the top of the list, succinic acid currently holds the title of being the only niche product with an annual market demand of approximately 30,000 tonnes and a net value of $225 million, which have been projected to increase by six folds in the next three years (Royal Society of Chemistry, 2012).

Succinic acid (HOOC-CH_2-CH_2-COOH), which is also known as amber acid or butanedioic acid, is aptly referred to as a platform or building-block chemical with reference to its potential use as a precursor in the production of several industrially important commodity and specialty chemicals. Some of these chemicals that can be obtained via chemical conversion of succinic acid include 1,4-butanediol (BDO), polybutyrate succinate (PBS), tertahydrofuran (THF), γ-butyrolactone (GBL),

polyamides (PA), polyesters (PE), and biodegradable deicers. Succinic acid along with its derivative products thus find applications in a wide range of industries, from the manufacture of green solvents, which have lesser or no adverse environmental impacts than conventional solvents, and biodegradable plastics to being used as alternatives to some of the currently popular food additives and flavor enhancers such as monosodium glutamate (Zeikus et al, 1999). These derivatives have an annual estimated market value of $4.8 billion and when combined with their demand the potential market of succinic acid soars up to a 1.6 million tonnes per year (Orjuela et al, 2013).

In the traditional petrochemical process, succinic acid is produced from maleic anhydride in the presence of a heterogeneous catalyst such as nickel or palladium. In general, there are two possible routes of this process. Maleic anhydride is either first hydrogenated to succinic anhydride and then hydrated to succinic acid, or hydrated to maleic acid, which is then hydrogenated to succinic acid (Zhou & Zheng, 2013). Maleic anhydride itself is obtained from butane or benzene.

Succinic acid, however, is also commonly found in nature as a metabolite produced by plants, animals, and microorganisms. It is an intermediate of the tricarboxylic acid (TCA) cycle, and also one of the fermentative end products of anaerobic metabolism.

In spite of such economic potential, succinic acid has not been the favorite of many manufacturers of the chemical industry, mainly because of its high production cost. The price of the starting material – maleic anhydride – is considered as major contribution to this effect. Song and Lee (2006) estimated the raw material cost for

succinic acid at $1.207/kg, based on a selling price of maleic anhydride at $ 0.977/kg and a conversion efficiency of 95% by weight. However, a shift in technology towards fermentative strategies for production as suggested by the US DOE would considerably reduce the starting material cost. Fermentations with glucose as the carbon source will have a raw material cost of $0.428/kg of SA, assuming a selling price of glucose at approximately $0.39/kg and 91% yield (Song & Lee, 2006). In addition to this drop in raw material cost, fermentative production steers towards a green technology as it uses renewable feedstocks. Metabolic pathways involved in succinic acid synthesis consume CO_2, which is one of the greenhouse gases, making this strategy an environmentally friendly approach. In other words, replacing the starting material from rapidly depleting hydrocarbon resources with renewable carbohydrate feedstocks, and switching the production process that emits CO_2 with a fermentative strategy that consumes CO_2, succinic acid production can be made greener and cheaper. Biological production of succinic acid thus provides opportunity for development of biorefineries integrating succinic acid production with CO_2-generating processes such as ethanol, butanol, and biohydrogen fermentation.

Microbial Production

Succinic acid is produced by aerobic, facultative, and obligative anaerobic microorganisms. C6 sugars such as glucose and fructose are metabolized by the Embden-Meyerhof-Parnas pathway, which produces two moles of pyruvate, two moles of ATP (energy), and two moles of NADH (reducing power) per mole of sugar. Subsequent

pyruvate metabolism is controlled by the environment, which can be either aerobic or anaerobic.

In an aerobic environment, pyruvate is oxidatively decarboxylated by pyruvate dehydrogenase (PDH) to form acetyl-Coenzyme A (CoA) with NAD^+ acting as the internal electron acceptor. Under anaerobic conditions, pyruvate undergoes the same oxidative decarboxylation reaction to produce acetyl-coA but through the action of a different set of enzymes, which is referred to as pyruvate-ferredoxin oxidoreductase (PFO) or pyruvate-formate lyase (PFL). Ferredoxin is the electron carrier in the case of PFO, while the electrons are internally balanced within the carboxyl group, which is released as formate when the organism uses PFL. PFO has been found in several archaea, while PDH and PFL have not been seen in any. PFO is used by sulfate-reducing bacteria and clostridia, and the PFL is found in enteric bacteria and some lactic bacteria. The acetyl-coA produced using either PFO or PFL is then converted to acetyl-phosphate (acetyl-P) in a reaction catalyzed by phosphotransacetylase (PTA). Acetyl-P then donates the phosphoryl group to ADP to form ATP and acetate in a substrate-level phosphorylation catalyzed by acetate kinase (ACK).

PDH reaction : Pyruvate + NAD^+ + CoA→ acetyl-coA + NaDH + CO_2 (1)

PFO reaction : Pyruvate + Ferredoxin (ox) + CoA → acetyl-coA + Ferredoxin (red) + CO_2 (2)

PFL reaction : Pyruvate + CoA → acetyl-coA + Formate (3)

PTA reaction : acetyl-coA + P → acetyl-P + CoASH (4)

ACK reaction : acetyl-P + ADP → acetate + ATP (5)

Under aerobic conditions, the acetyl-coA then enters the TCA cycle. Through this cycle every mole of acetyl-coA (or pyruvate) is completely oxidized to three moles of CO_2, with the generation of one mole ATP, four moles NADH, and one mole FADH. Succinic acid is one of the intermediates in the TCA cycle (White, 2000).

Though fermenting microorganisms cannot use the TCA cycle to oxidize acetyl-coA to CO_2 at the expense of generating NADH and $FADH_2$, they still need molecules such as oxaloacetate, succinyl-coA, and α-ketoglutarate, which are important precursors in the biosynthesis of cell components. This is achieved by converting the TCA cycle into an incomplete cycle or a reductive pathway with an inactive α-ketoglutarate dehydrogenase. Succinic acid is produced by fumarate reductase, which replaces succinate dehydrogenase found in the complete TCA cycle. Phospohoenol pyruvate carboxylase catalyzes the conversion of phosphoenol pyruvate to oxaloacetate, which is used in the reductive reactions to produce succinate. These reactions are not only seen in fermenting organisms, but also other bacteria that carry out anaerobic respiration.

When acetate and fatty acids are the only carbon sources, aerobic organisms utilize the glyoxylate cycle. This cycle is similar to the TCA cycle but lacks isocitrate dehydrogenase, and instead uses isocitrate lyase. This enzyme cleaves isocitrate into succinate and glyoxylate. The latter is combined with acetyl-CoA to form malate with the help of malate synthase, which regenerates oxaloacetate. Thus the final products of glyoxylate cycle are succinate and one mole of NADH (White, 2000).

Both natural and engineered microorganisms have been studied extensively for the purpose of succinic acid production. Many natural producers are found in the rumen

of monogastric animals, which is explained by the anaerobic environment rich in CO_2 enabling the reductive arm of the TCA reactions resulting in succinic acid production. *Anaerobiospirillum succiniciproducens*, *Mannheimia succiniciproducens*, and *Actinobacillus succinogenes* have extensively been studied as natural overproducers of succinic acid which are found in ruminants (Beauprez et al, 2010). Fungal succinic acid production systems are not very common, with a few known exceptions such as *Fusarium* spp and *Aspergillus* spp. Yeast and fungal strains that produce succinic acid are known to tolerate lower acidic pH than bacterial strains and their use can potentially make recovery and purification of the organic acid easier (Beauprez et al, 2010). *A. succinogenes* is a facultative anaerobe, which is mesophilic, pleomorphic, and capnophilic. Studies have established that this organism could grow on renewable carbon sources such as whey, cane molasses, and wheat hydrolysates to produce succinic acid under anaerobic conditions (Beauprez et al, 2010). *M. succiniciproducenes* is also a facultative anaerobe which is mesophilic and capnophilic. Studies conducted with the strain *M. succiniciproducenes* MBEL55E isolated from the bovine rumen have established the ability of the organism to grow on NaOH-treated wood hydrolysates and whey-based fermentation medium supplemented with corn steep liquor (CSL) (Kim et al, 2004; Lee et al, 2003). Batch experiments resulted in yields of 0.56 g succinic acid/g of glucose and xylose and 0.71g of succinic acid/g of lactose from wood hydrolysates and whey/CSL based medium, respectively (Kim et al, 2004; Lee et al, 2003). *A. succiniciproducens* on the other hand is an obligate capnophilic anaerobe and opportunistic pathogen with requirements for specific amino acids and vitamins as well

as high concentrations of CO_2 for their growth and succinic acid production. These features make this strain unsuitable for commercial production of succinic acid despite its capability of utilizing a wide range of sugars including glucose, fructose, lactose, maltose, and sucrose (Lee et al, 1999). *A. succiniciproducens*, however, is the strain that has been studied most extensively because it was the first organism that was found to be capable of producing succinic acid to over 30 g/L. Recent studies using glucose as carbon source tested the possibility of replacing yeast extract and peptone in the fermentation medium with much cheaper CSL as a source of vitamins and complex nutrients, and found comparable succinic acid yields (Lee et al, 2000b). Research was further extended to determine the feasibility of using non-refined sugar sources such as whey and glycerol, the major byproduct of the biodiesel industry, to grow *A. succiniciproducene* for succinic acid production to reduce raw material costs (Lee et al, 2000a; Lee et al, 2001). Fermentations with these raw materials were supplemented with yeast extract and peptone for high yields and productivity. Interestingly, promising results were obtained with the glycerol medium. The succinic acid yield was 1.33 g/g glycerol consumed and the weight ratio of succinic acid to acetic acid was 25.8 to one, which is 6.5 times that obtained by fermenting glucose.

Recovery & Purification

As in many fermentation processes, product recovery and purification attribute to around 60-70% of the total production cost of biologically produced succinic acid (Bechthold et al, 2008). The organisms used for fermenting carbon sources to succinic

acid mostly operate near neutral pH. Fermentation broth is thus maintained at a constant pH using bases to enable the microorganism to continue succinic acid production. As a result, salts of succinic acid are obtained as the final products. However, industrial applications need succinic acid in its pure form rather than as a salt (Zeikus et al, 1999). To satisfy this requirement elaborate recovery and purification steps are needed, which add to the cost of production. Recovery of succinic acid has been studied extensively by three techniques, which included precipitation, electrodialysis, and liquid-liquid extraction (Kurzrock & Weuster-Boltz, 2010; Bechthold et al, 2008).

Recovery of organic acids by precipitation is a widely studied and practiced technique. In this process $Ca(OH)_2$ is used to precipitate calcium succinate from the fermentation broth. These crystals are separated by filtration and washed to remove cell debris. They are then treated with concentrated sulfuric acid, which forms $CaSO_4$ and succinic acid. The free acid is then purified using activated carbon and recovered by crystallization. Datta et al (1992), in their patent on succinic acid production using *A. succiniciproducens*, obtained a succinic acid yield of 1.2 mole/mole of glucose after precipitation indicating a 15% loss after the recovery process as 1.4 mole succinic acid/mole glucose was obtained through fermentation. In addition to the significant loss in the precipitation process the equimolar generation of $CaSO_4$, which is an unusable byproduct, is the main drawback of this approach (Kurzrock and Weuster-Boltz, 2010; Bechthold et al, 2008). An improvement on this technique was achieved by using ammonium ion base for pH control during fermentation or by substituting the cation of succinate salt created with ammonium cation to produce diammonium succinate. This

soluble salt is then treated with a sulfate ion, in the form of either H_2SO_4 or ammonium bisulfate (NH_4HSO_4), at a pH between 1.5 and 1.8 to produce succinic acid crystals that precipitate due to insolubility at low pH and ammonium sulfate as the only byproduct. The succinate crystals are purified by dissolving in methanol, followed by evaporation. The byproduct is thermally cracked into ammonia and ammonium bisulfate at a temperature of 300°C. This technique produces no waste byproducts and allows for recycle of the acid and base materials used in this process (Berglund et al, 1999).

Electrodialysis for succinic acid recovery is the other extensively researched approach. Glassner et al (1992) patented a technique to obtain high purity succinic acid, which included a desalting step followed by water splitting electrodialysis. Desalting electrodialysis uses positively charged membranes to separate ionic components (salts of succinic acid) and non-ionic components (sugars) in the fermentation broth. The ionic stream is then passed through chelating ion exchange columns where the divalent cation of succinic acid salt is replaced by sodium anion. Sodium succinate and the other ions then are taken through water splitting electrodialysis where water is split to form H^+ and OH^- ions. The positively charged sodium ions, from sodium succinate, move towards the cathode and form NaOH, while negatively charged succinate ions move towards the anode and form succinic acid. A purification yield of 60% was reported by this study. The ion exchange membranes used in this highly energy intensive process are very expensive, and their fouling with time further adds to the total production cost.

Both precipitation and extraction methods lack the required selectivity for succinic acid and the presence of other organic acid byproducts in the fermentation broth make this even more difficult and expensive to achieve.

Liquid-liquid extraction is another technique which is currently under investigation at laboratory scale. Improvement in the distribution coefficient is required to employ this method in larger scales. Tertiary amines are the preferred agent for extraction as its elevated basicity improves reaction with the negatively charged succinate. These tertiary amines are used in water immiscible organic solvents, and form amine-acid complexes in the organic phase, which are then purified through vacuum distillation and crystallization. An extraction efficiency of 99% has been reported (Song et al, 2007). This process depends on several factors such as pH, temperature, nature of the aqueous and organic phases, and the numerical value of the distribution coefficient which is benefits from the highly positive tertiary amine substitution.

Commercial Production

Many rapidly growing companies have started extensive research in the field of biological succinic acid production. Myriant Technologies is a private US company in the renewables and biotechnology sector. This company received a $50 million grant from the US DOE for the construction of America's first commercial succinic acid fermentation plant with capacity of 13,600 tonnes/year in Louisiana. The succinic acid produced by their technology will be a drop-in replacement for the petroleum-based succinic acid. Myriant uses an engineered strain of *E.coli* to produce succinic acid from

corn-derived and unrefined glucose. BioAmber, originally started as Applied CarboChemicals then Diversified Natural Products (DNP) Green, uses an engineered strain of *E.coli* developed by the scientists at the US DOE's National Laboratories through the Alternative Feedstocks Program. BioAmber's semi-commercial plant operating in Pomacle, France since 2010 is the world's first bio-based succinic acid plant and has one of the world's largest bio-based manufacturing fermentors (350,000 L). BASF/Purac, a German and Dutch collaboration, is set to operate their semi-commercial succinic acid fermentation plant in Spain. This plant is designed to produce an estimated 25,000 tonnes of succinic acid/year. Their technology uses a mutant *Basfia succiniciproducens* strain that ferments glycerol to succinic acid. Mitsubishi/Ajinomoto and Reverdia are other companies working in this area, developing technologies using strains of yeast for succinic acid production.

Development of *Escherichia coli* AFP 184

Escherichia coli are facultative anaerobic, Gram-negative, enteric bacteria and the most preferred organism for genetic and metabolic engineering research. Vast data on its genetic make-up and biochemical pathways, ease of handling, and ability to grow under relatively less stringent conditions enable researchers to engineer this organism into over-producers of desired products. In the absence of oxygen, the TCA cycle is modified to become a reductive pathway producing small quantities of succinate. The organism replaces PDH with PFL, and carries out a mixed acid fermentation where the main products are formate, ethanol and acetate, and trace amounts of lactate (Fig 1.1).

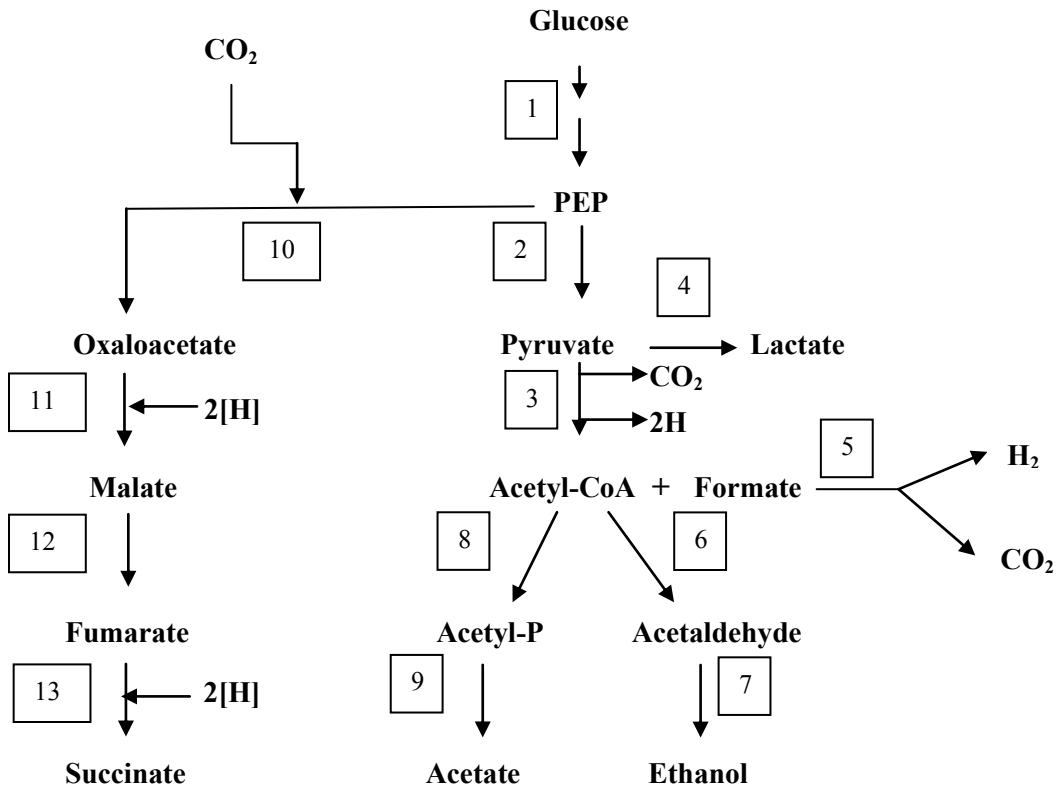

Figure 1.1 Mixed acid fermentation in enteric bacteria like *E. coli*. Enzymes 1:glycolytic enzymes, 2:pyruvate kinase, 3:pyruvate-formate lyase, 4:lactate dehydrogenase, 5:formate-hydrogen lyase, 6:acetaldehyde dehydrogenase, 7:alcohol dehydrogenase, 8:phosphotransacetylase, 9:acetate kinase, 10:PEP carboxylase, 11:malate dehydrogenase, 12:fumarase, 13:fumarate reductase

In order to construct an *E. coli*-based production system for succinic acid fermentation, several mutations have been studied. The pioneering work in this regard was initiated by the US DOE under the Alternative Feedstocks Program (AFP). A team from the Argonne National Laboratory developed several mutant strains for this purpose. The strain development work started with *E. coli* NZN111, which was derived from the wild-type *E. coli* W1485 and lacked the functional genes coding for the enzymes PFL

and lactate dehydrogenase (LDH). With these defectives *E. coli* NZN111 cannot grow fermentatively, and accumulates pyruvate. However, additional chromosomal mutations that spontaneously occurred during culturing of this strain produced another variant that formed succinic acid as the major product of fermentation. This variant was named *E. coli* AFP 111. The additional mutation was identified as a lesion in the gene *ptsG*, coding for the phosphotransferase system (PTS), which is a group of transport proteins participating in glucose uptake by *E. coli*. The absence of functional PFL and LDH altered the mixed acid fermentation reactions restricting conversion of pyruvate into lactate and formate, thereby channeling more carbon into the reductive arm of the TCA cycle to produce succinic acid as the major fermentation product. *E. coli* AFP 111 yielded one mole of succinic acid from one mole of glucose, as opposed to the usual 0.3-0.4 mole (Donnelly et al, 1998). When these mutations were deliberately inserted in a nonproprietary strain C600 (ATCC23724), the engineered strain also produced succinic acid as the major product and had features very similar to AFP 111. This strain with a *pf*l deletion, *ldh* knockout, and inactive *ptsG* from a different mutant is referred to as AFP 184 (Donnelley et al, 2004). *E. coli* AFP 184 gave higher succinic acid yield on glucose than strain AFP 111 and also was much more efficient in simultaneous utilization of glucose and xylose, which are the two major fermentable sugars derived from lignocellulosic feedstocks.

Sugar metabolism by *E. coli* AFP 184

With inoperative genes for PFL and LDH, mixed acid fermentation reactions are altered, blocking reactions that produce acetate, formate, and lactate, funneling carbon into the reductive arm of TCA to produce succinate as the major product. The non-reductive reactions of TCA and glyoxylate shunt are also active, producing succinic acid. Acetate is now the product of an overflow metabolism of pyruvate (see section Microbial Production) (Vemuri et al, 2002b).

The *pts* codes for a group of transport proteins that make up the PTS that regulates uptake of certain sugars by the organism. Key enzyme complexes in this system that are responsible for transporting glucose from the periplasmic space into the cytoplasm are Enzyme I (EI) and Enzyme II (EII). EI is non-sugar specific, facilitating uptake of not only glucose but also fructose and mannose. EII, unlike EI, is sugar specific. The enzymes in the EII complex participating in glucose transportation are II^{glu} and II^{man}. These enzymes in the EII complex contain a soluble protein ($IIA^{glu/man}$) and an integral membrane protein permease ($IIBC^{glu/man}$). The gene *ptsG* codes for $IIBC^{glu}$ and also represses the gene coding for II^{man}. However, in *E.coli* AFP 184 this gene is inoperative and as a result the PTS has no glucose-specific permease protein ($IIBC^{glu}$) and glucose uptake occurs only through the unrepressed II^{man} enzyme complex. It should be noted that the affinity of II^{man} for glucose is lesser than that of II^{glu} (Gosset, 2005; Tchieu et al, 2001). Two other mechanisms that import glucose are the GalP and Mgl systems. These systems transport galactose and glucose, and are ATP dependent processes. As a result, glucose uptake by these is also slower than by the regular PTS

mechanism (Curtis & Epstein, 1975). These features reduce catabolite repression that will be usually caused by the presence of glucose in media, slowing down consumption of other sugars. This imparts the organism an ability to use a myriad of sugars along with glucose. Fructose on the other hand is imported in by the PTS because the genes coding for the enzymes in the PTS responsible for fructose transport are intact (Kornberg et al, 2000).

Based on a redox balance on the anaerobic phase reactions, theoretical yields of succinate from glucose and fructose have been calculated as 1.71 mole/mole (1.12 g/g) and 1.20 mole/mole (0.79 g/g), respectively (Andersson et al, 2007). These differences in theoretical yields can be attributed to the different mechanisms used for glucose and fructose transport into the cell. The overall mechanisms of glucose and fructose metabolisms are illustrated in Figure 1.2.

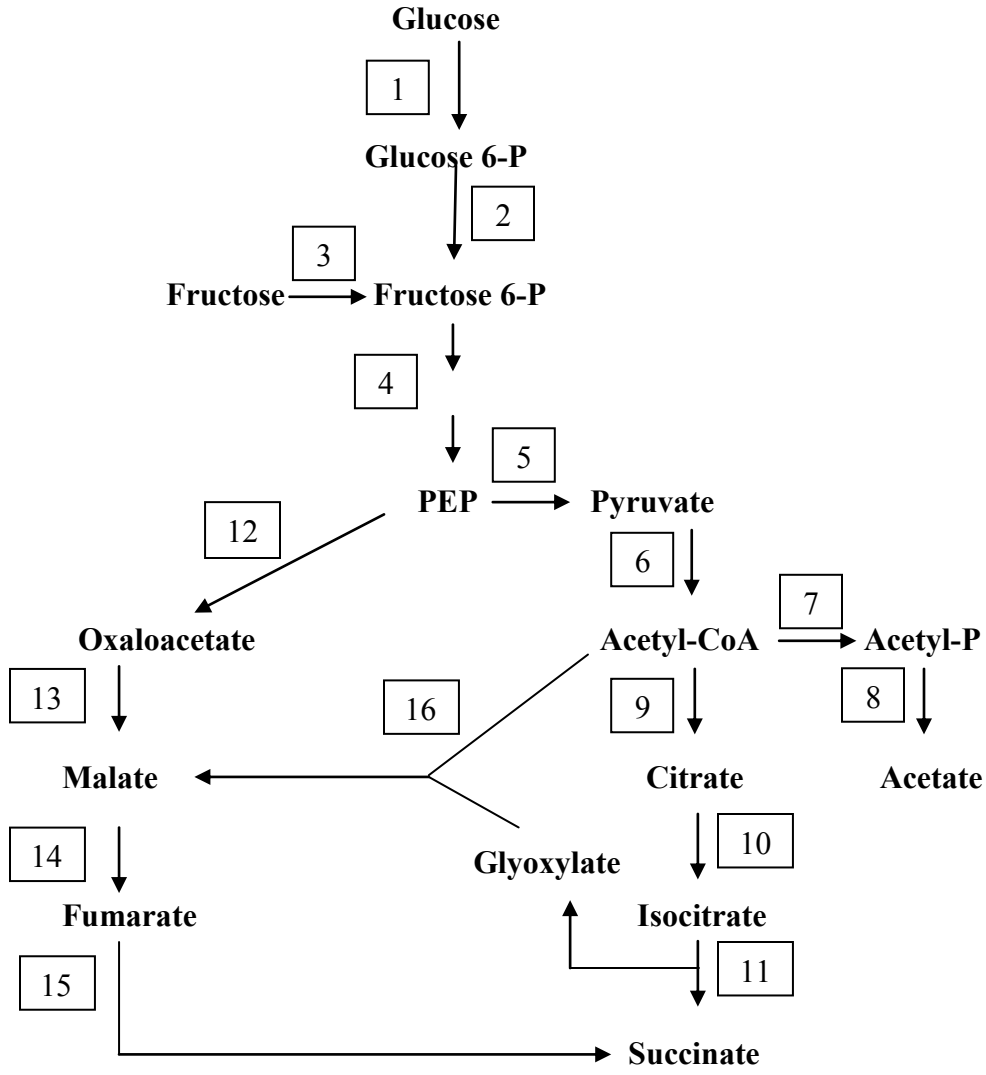

Figure 1.2 *E. coli* AFP 184 metabolism. Key enzymes, 1:glucokinase, 2:glucose-6-phosphate isomerase, 3:phosphotransferase system, 4:glycolytic enzymes, 5:pyruvate kinase, 6:pyruvate dehydrogenase, 7: phosphotransacetylase, 8:acetate kinase, 9:citrate synthase, 10:aconitase, 11:isocitrate lyase, 12:PEP carboxylase, 13:malate dehydrogenase, 14:fumarase, 15:fumarate reductase, 16:malate synthase

Succinic Acid Production Using *E. coli* AFP 184

Another effort of the AFP initiated by the US DOE was the development of a fermentation process for producing succinic acid using the engineered *E. coli* strains. The Oak Ridge National Laboratory focused on this effort. A dual-phase process was developed for succinic acid production using *E. coli* AFP 111 (Nghiem et al, 1999). In the first phase, the microorganism is grown under aerobic conditions to achieve a critical mass. Once this is reached, air supply is cut off to create an anaerobic environment, which is needed for succinic acid production via fermentation. Thus, there is first a growth phase followed by a production phase. The optimal temperature and pH range were determined to be 37°C and 6.5-7.0. This dual phase fermentation process constructively uncouples growth and production phases, and enzymes of the production phase are expressed during aerobic growth phase and remain active throughout the process (Vemuri et al, 2002a). The structural and functional similarities between *E. coli* AFP 111 and 184 allow for successful extension of this procedure and process conditions, developed with *E. coli* AFP 111, to AFP 184 as well (Donnelley et al, 2004).

In addition to glucose, several sugars have been experimented for growing *E. coli* AFP 184. The inability of the organism to metabolize sucrose has been established (Andersson et al, 2007). The organism however could consume glucose, fructose, xylose, and mixtures of glucose and fructose, or glucose and xylose efficiently (Andersson et al, 2007).

Originally yeast extract and tryptone were used as a source of complex nutrients and nitrogen for growing *E. coli* AFP 111. In an effort to develop inexpensive and

commercially suitable growth medium, these components were successfully replaced by CSL (Nghiem et al, 1999; Andersson et al, 2007). CSL is a byproduct of the corn wet-milling industry. It is an inexpensive source of vitamins and trace elements, and other complex nutrients which have to be supplemented for the growth and functioning of the organism. It has approximately 50% solids, and contains many amino acids and small quantities of sugars. Though the exact composition of this component might vary, estimates are found in literature (Table 1.1).

Table 1.1 Corn steep liquor average composition (White & Johnson, 2003)

MAJOR COMPONENT	PERCENT COMPOSITION
Ash	17
Crude protein	47
Fat	0.4
Lactic acid	26
Nitrogen	7.5
Phytic acid	7.8
Reducing sugars	2.5
Water	46

As *E. coli* AFP 184 can metabolize a wide range of sugars, except sucrose, studies aimed at reducing the cost of fermentation medium for succinic acid production by replacing refined sugars with lignocellulosic hydrolysates. Donnelley et al (2004) used hydrolysates obtained by hydrolysis of rice straw using concentrated sulfuric acid to grow *E. coli* AFP 184 and reported succinic acid yields equivalent to those obtained with synthetic solutions prepared with reagent grade glucose and xylose. In the study by Hodge et al (2009), detoxified dilute acid hydrolysate of softwood was successfully used for succinic acid production with *E. coli* AFP 184. These results provide the opportunity

to develop biorefineries using lignocellulosic biomass feedstocks, which are not only inexpensive but are also renewable, and most importantly, environmentally benign.

As illustrated in Figure 1.2, there are two pathways that lead to succinic acid as the final product in *E. coli* AFP 184. They are the reductive and nonreductive reactions of the broken TCA cycle under anaerobic conditions. The former starts with the conversion of PEP to oxaloacetic acid (OAA), which is then ultimately reduced to give succinic acid. The latter starts with the conversion of pyruvate to acetyl-CoA, which then proceeds through incomplete oxidation of citrate to form isocitrate and finally succinate. While the reductive pathway assimilates carbon dioxide into pyruvate to produce OAA, the nonreductive pathway does not need carbon dioxide to carry out its reactions. However, Nghiem et al (2010) showed the preference of the organism for the reductive reactions over the nonreductive reactions under anaerobic conditions to produce succinic acid by demonstrating substantial improvements in succinate yield using external supply of carbon dioxide in the form of sodium carbonate (Na_2CO_3) used for pH control and crude effluent CO_2 from an adjacent ethanol fermentor.

Experiments conducted by Donnelly et al (1998) with *E. coli* AFP 111, reported succinate yield of 0.99 g/g glucose consumed and volumetric productivity of 1.6 g/L-h, with a final concentration of 45 g/L and succinate:acetate mass ratio of 6:1. The organism was shown to be osmotolerant with the ability to produce more than 50 g/L of organic acid salts before product inhibition set in. These results were obtained when the organism was grown anaerobically in a 100% CO_2 atmosphere. When this atmosphere was replaced with CO_2: H_2 mixtures, where the H_2 composition varied from 25% to 100%,

further improvement in succinate:acetate ratio was observed. The mass ratio increased from the aforementioned 6:1 to almost 9:1. This increase in succinate:acetate ratio with exogenous supply of hydrogen, further proves the importance of the oxaloacetate to succinate route over the citrate to succinate route. As illustrated in Figures 1.1 and 1.2, oxaloacetate is reduced to succinate via malate and fumarate, and exogenous hydrogen supply promotes these reactions by providing electrons and hence altering the redox balance, validating the improvement observed in succinate:acetate ratio. This reduction in byproduct formation could potentially lower recovery and purification costs, which attribute to 60-70% of total production cost.

Cull Peaches as Viable Feedstock

As mentioned in the previous section, many agricultural feedstocks have been successfully used as a source of unrefined sugars for succinic acid production with *E. coli* AFP 184. In the US, a large portion of agricultural waste is represented by cull fruits and fruit processing wastes. Cull fruits are those that are deemed unsuitable for fresh market or processing due to over ripening or surface imperfections. Though unfit for human consumption, they are still viable storage of fermentable sugars that can be used in the bioprocessing industry.

In the US, peaches are produced commercially in 28 states. In 2011, a total of 1.2 x 10^9 kg of peaches were produced across the country. South Carolina is the second largest producer of peaches, following California, with an annual production of 2 x 10^8 lbs, out of which an approximate 10% is culled (SCDA, 2012). Peaches are about 90 wt

% water, 8.4 wt % sugars, 0.91 wt % proteins, and 0.25 wt % fat. It also contains minerals like potassium, calcium, magnesium, and iron (225 mg/100 g fruit), and vitamins (9 mg/100 g fruit) (USDA, NAL).

Peach contains a mixture of sugars in which sucrose is the predominant component, followed by fructose and glucose (Genard & Souty, 1996). Colaric et al (2004) reported sugar contents across 19 different peach cultivars and reported fructose and glucose concentrations to be ranging from 7 to 13 g/kg fruit and 5 to 12 g/kg (wet weight basis), respectively. Sucrose concentration was found to be between 46 and 70 g/kg fruit. Although *E. coli* AFP 184 cannot metabolize sucrose (Andersson et al, 2007) this sugar can easily be hydrolyzed with dilute acid or invertase to produce glucose and fructose (Bower 2008; Combes & Monsan, 1983), which then can be efficiently fermented to succinic acid. Thus, peach wastes are a readily available and inexpensive feedstock for commercial succinic acid production.

Research Objectives

The objectives of this study are:

1. Develop a fermentation process for succinic acid production by *E. coli* AFP184 using peach wastes as feedstocks.

2. Investigate and compare the effects of (a) supplementing this medium with corn steep liquor and (b) exogenously sparging this medium with hydrogen on succinic acid production in the developed process.

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CHAPTER TWO

BIOLOGICAL PRODUCTION OF SUCCINIC ACID USING A CULL PEACH MEDIUM

Introduction

Succinic acid is one of the most sought after platform chemicals and is the top candidate selected by the US Department of Energy for production from lignocellulosic feedstocks (Werpy & Petersen, 2004). It has been used in a wide range of industrial applications as a surfactant, ion chelator, food additive, and in the production of pharmaceuticals (Zeikus et al, 1999). Recently, significant efforts have been made to develop catalysts for conversion of succinic acid to important industrial chemicals such as 1,4-butanediol, polybutyrate succinate, tertahydrofuran, γ-butyrolactone, polyamides and polyesters (Nghiem et al, 2000). Succinate-based biodegradable deicers also have recently been developed (Berglund et al, 2003).The total market value of succinic acid and products made from it was estimated at $4.8 billion per year with potential for further expansion (Orjuela et al, 2013).

Succinic acid is traditionally produced from petroleum-based maleic anhydride in the presence of a heterogeneous catalyst such as nickel or palladium. The recent recognition of succinic acid as a commercially important building-block chemical has attracted strong interest in the development of economically viable biological processes using renewable feedstocks for its production on an industrial scale. Many attempts have been made to develop microbial strains suitable for commercial production of succinic

acid. The efforts at the US Department of Energy's National Laboratories resulted in the development of several *Escherichia coli* strains (Donnelly et al, 1998; Nghiem et al, 1999; Donnelly et al, 2004). Among these, strains AFP111 and AFP184 were the two most promising. Both *E. coli* AFP111 and AFP184 are engineered strains with mutations in the following genes: glucose-specific phosphotransferase transport (*ptsG*), lactate dehydrogenase (*ldh*), and pyruvate-formate lyase (*pfl*). These mutations alter the organism's native mixed acid fermentation reactions, thus restricting the formation of lactate, formate, and acetate, and funneling more carbon into synthesis of succinic acid as the major fermentation product. Acetic acid is the only important by-product, which is produced as an overflow of pyruvate metabolism (Donnelley et al, 1998; Donnelley et al 2004). Succinic acid production was designed to be a dual-phase fermentation process, with an initial aerobic phase to achieve a critical cell mass, followed by an anaerobic phase to enable succinic acid production (Nghiem et al, 1999). Under anaerobic conditions, succinic acid production begins with the assimilation of CO_2 into pyruvate by the enzyme phosphoenolpyruvate carboxylase to form oxaloacetic acid, which is then ultimately reduced to give succinic acid, via malic and fumaric acid. The CO_2 requirement for efficient succinic acid production can be provided by using solutions of carbonate salts such as Na_2CO_3, K_2CO_3, and $(NH_4)_2CO_3$ for pH control, or by sparging the fermentation medium with a stream of CO_2 gas (Nghiem et al, 1999, Nghiem et al, 2010). In the presence of exogenous hydrogen, more succinate and less acetate was produced by strain AFP111, presumably due to the altering of the redox balance. The effect of exogenous hydrogen on succinic acid production by strain AFP111 was

observed both in serum bottles and fermentors (Nghiem et al, 2000). This interesting aspect of succinic acid fermentation has not been studied with strain AFP184. Between the two strains *E. coli* AFP184 is more suitable for commercial application than the other strain as it gave higher succinic acid yield on glucose and also is capable of simultaneously utilizing both glucose and xylose, which are the two sugars that can be obtained from lignocellulosic feedstocks (Donnelly et al, 2004).

The ability of *E. coli* AFP184 to produce succinic acid from sugar solutions obtained by acid hydrolysis of lignocellulosic feedstocks has been demonstrated (Donnelly et al, 2004; Hodge et al, 2009). It is of interest to study the possibility of producing succinic acid from other and less expensive feedstocks. In the US, a large portion of agricultural wastes is represented by cull fruits and fruit processing wastes. Though unfit for human consumption, they contain fermentable sugars that can be used in the bioprocess industry. South Carolina is the second largest producer of peaches after California with an annual production of 91,000 metric tons (MT), of which an approximate 10% is culled (South Carolina Department of Agriculture). Peaches contain about 90 wt % water, 8.4 wt % sugars, 0.91 wt % proteins, and 0.25 wt % fat. Other constituents include minerals such as potassium, calcium, magnesium, and iron at 225 mg/100 g fruit and vitamins at 9 mg/100 g fruit (USDA, NAL). Peaches contain several sugars with sucrose being the predominant, followed by fructose and glucose (Genard & Souty, 1996). Colaric et al (2004) reported sugar contents for 19 different peach cultivars with sucrose, fructose, and glucose ranging from 46 to 70 g/kg fruit, 7 to 13 g/kg, and 5 to 12 g/kg, respectively. Glucose and fructose have been shown to be good substrates for

succinic acid production by *E. coli* AFP184 (Anderson et al, 2007). Although sucrose is not metabolizable by this strain it can be easily hydrolyzed by either invertase or dilute acid to glucose and fructose (Combes & Monsan, 1983; Bower et al, 2008), which then can be used for succinic acid production.

The objectives of this study are: 1. Develop a fermentation process for succinic acid production by *E. coli* AFP184 using peach wastes as feedstock, and 2. Investigate the effect of exogenous supply of hydrogen on succinic acid production using the peach-based fermentation media.

Materials and Methods

Materials

E.coli AFP 184 was obtained from the American Type Culture Collection (Manassas, VA). The lyophilized culture was reconstituted in 50 mL sterile LB medium in a 250-mL flask. The flask was incubated in an orbital shaker at 37°C and 250 rpm for 18 hours. The culture was then aseptically mixed with sterile glycerol in 2:1volume ratio. One milliliter of the mixture was dispensed into cryogenic vials and stored at -70°C.

Peaches (*Prunus persica*; type: Zee Lady) were purchased from a local retail store in Wyndmoor, PA. The peaches were allowed to ripen at room temperature for one week. The ripe peaches were then depitted and blended in a kitchen blender for 60 s. The concentrations of the three main sugars in the liquid were 44.1 g/L of sucrose, 18.3 g/L of glucose, and 21.3 g/L of fructose. The mash was transferred to plastic containers (3.5 L each) and kept frozen at -20°C over the course of this study.

Corn steep liquor (CSL) and invertase were purchased from Sigma-Aldrich (St. Louis, MO). The specific activity of the purchased invertase at 55^{o}C and pH 4.5 was listed as146 units/mg. One enzyme unit hydrolyzed 1 μmol of sucrose to glucose and fructose in 1 minute under the conditions of the assay. The enzyme product GC 220 was provided by DuPont Industrial Biosciences (Wilmington, DE). The specific activity of GC 220 was given by the manufacturer as 6200 carboxymethylcellulose units per gram. One enzyme unit liberated 1 μmol of reducing sugars expressed as glucose equivalents from carboxymethylcellulose substrate in 1 minute under specific assay conditions (50^{o}C and pH 4.8).

All chemicals were of reagent grade and purchased from various suppliers.

Methods

Determination of invertase dosage

As discussed previously, sucrose in the peach media must be hydrolyzed to glucose and fructose to maximize the amount of fermentable sugars available for cell growth and succinic acid production. Preliminary experiments showed that 54% of the sucrose in the peach media was converted to glucose and fructose when the fermentor was autoclaved at 121°C for 30 minutes (see below). The remaining sucrose needed to be enzymatically hydrolyzed using invertase.

To determine the required dosage of invertase for use in the succinic acid fermentation experiments, the activity of the enzyme was determined under the fermentation conditions, which were pH 6.5 and 37^{o}C (see below). The glucose and fructose produced in the hydrolysis of sucrose were quantified collectively as glucose

equivalents by the DNS assay (Combes & Monsan, 1983). Stock invertase (0.1 g/L), sucrose (0.3 M), and phosphate buffer (0.5 M, pH 6.5) solutions were prepared in deionized (DI) water. The amounts of each reaction component used in the invertase activity study are shown in Table 2.1. In each experiment, stock sucrose and capped reaction tubes with the enzyme, buffer, and water were allowed to equilibrate in a water bath maintained at 37°C for 2 minutes. The reaction was initiated by adding the stock sucrose solution to the reaction tubes which were then recapped and incubated for exactly 5 minutes in the 37^{o}C water bath. The reaction was stopped by transferring 1-mL aliquot from each tube into to another tube containing 1 mL DNS reagent (Adney & Baker, 1996). The tubes were capped and placed in a boiling water bath for 5 minutes to allow the color to fully develop. DI water (5 mL) then was added to each tube and the absorbance at 540 nm (A_{540}) of the diluted contents was measured on a Spectronic 20D^{+} spectrophotometer (Thermo Fisher Scientific, Madison, WI). A standard curve prepared with glucose was used to determine the amounts of total invert sugars (expressed as glucose equivalents) in the reaction mixtures (Fig 2.1). All experiments were performed in duplicate and the average results are reported. Using the standard curve, the amounts of glucose in test samples were calculated according to the following equation:

Amount of glucose in sample (μmoles) = 3.742 x A_{540}

Table 2.1 Reaction components and their quantities in the invertase activity study

Experiment	1	2	3	4	5
Enzyme (mL)	0	0.3 (0.03)*	0.6 (0.06)*	0.9 (0.09)*	1.2 (0.12)*
Buffer (mL)	0.5	0.5	0.5	0.5	0.5
DI H_2O (mL)	1.5	1.2	0.9	0.6	0.3
Sucrose (mL)	1	1	1	1	1

*Amount of invertase in mg

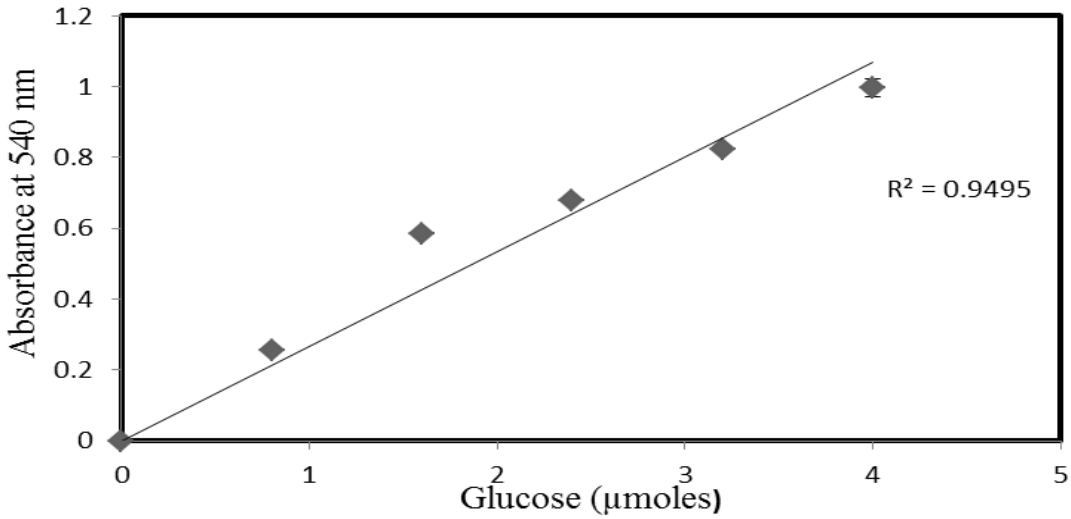


Figure 2.1 The standard curve for invertase assay obtained using DNS method

The rates of sucrose hydrolysis by various amounts of invertase at 37^{o}C and pH 6.5 are shown in Figure 2.2. The specific enzyme activity at these conditions was calculated to be 8.2 units (µmoles sucrose converted per minute) per mg of enzyme. This activity was used to calculate the enzyme dosage required for complete hydrolysis of sucrose. The calculation is shown below.

Invertase had to be added to the fermentor as a filter-sterilized solution. To avoid excess dilution of the substrates and nutrients in the fermentation media it was decided to use an enzyme volume of 1 % of the total mash volume (3 L). Thus, 30 mL of invertase

solution was added to the fermentor. In previous studies, for example by Nghiem et al (1999), it was observed that the rate of succinic acid production started to decrease after about 24 hours due to product inhibition. Thus, it was decided to use quantities of invertase sufficient to completely convert all of the sucrose present in the media to glucose and fructose within the first 24 hours when succinic acid production would still be proceeding at high rates.

The sucrose concentration in the media at the beginning of the fermentation experiments was determined by HPLC to be about 20 g/L. Thus, the desired rate of the hydrolysis was

$$\frac{20\left(\frac{\mathbf{g}}{\mathbf{L}}\right)\times 3(\mathbf{L})}{342.3\left(\frac{\mathbf{g}}{\mathbf{mole}}\right)\times 10^{-6}\left(\frac{\mathbf{mol}}{\mu\mathbf{mol}}\right)\times 60\left(\frac{\mathrm{min}}{\mathbf{h}}\right)\times 24(\mathbf{h})}=121.74\left(\frac{\mu\mathbf{mol}}{\mathbf{min}}\right)$$

The amount of invertase needed to catalyze the hydrolysis of sucrose at the above rate was

$$\frac{121.74\ \left(\frac{\mu\mathbf{mol}}{\mathrm{min}}\right)}{8.2\ \left(\frac{\mu\mathbf{mole}}{\mathbf{mg}.\mathrm{min}}\right)}=14.85\ \mathbf{mg}\sim 15\ \mathbf{mg}$$

The concentration of invertase in the solution added to the fermentor therefore was

$$\frac{15\ \mathbf{mg}}{30\ \mathbf{mL}}=0.5\,\frac{\mathbf{mg}}{\mathbf{ml}}\ \mathbf{or}\ 0.5\,\frac{\mathbf{g}}{\mathbf{L}}$$

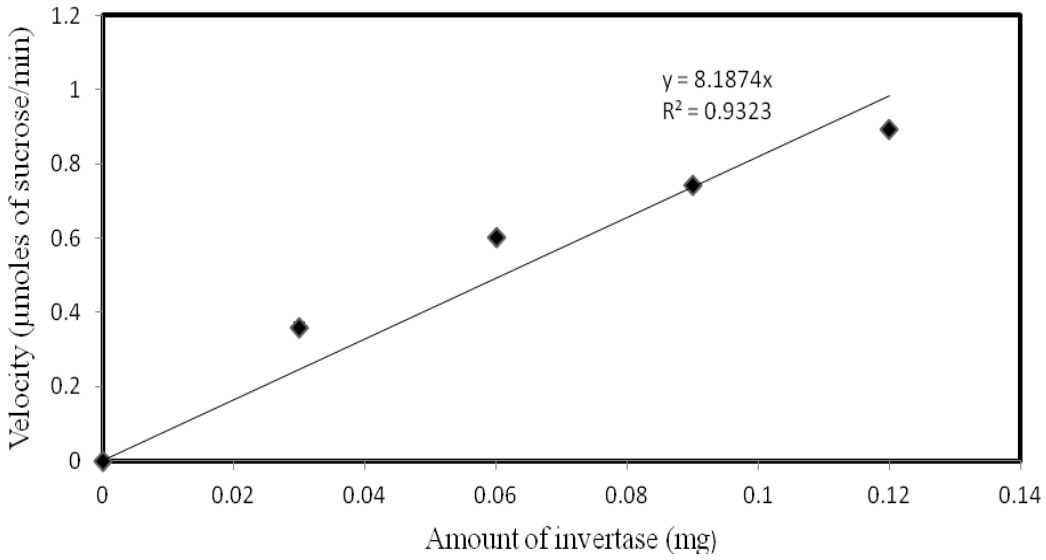


Figure 2.2 Hydrolysis of sucrose by invertase under fermentation conditions

Reduction of viscosity of peach mash

In preliminary experiments it was observed that the viscosity of the peach mash was too high to allow efficient mixing and aeration in the fermentor. Thus it was treated with GC 220, which is a commercial cellulase product formulated for viscosity reduction in ethanol fermentation, prior to being used as the fermentation medium for succinic acid production. To determine the required enzyme dosages and treatment time, 30-mL samples of peach mash were treated with 30 µL and 60 µL of GC 220 at 50^{o}C. The decrease in viscosity with time was followed using a Rapid Visco Analyzer (Newport Scientific, Warriewood, NSW, Australia). The experiments were performed in duplicate and the average results are reported.

Succinic acid fermentation

Batch fermentations were performed in duplicate in 7.5-L Bioflow 110 fermentors (New Brunswick Scientific, Edison, NJ). For each experiment two fermentors were operated simultaneously under the exact same conditions. To prepare the fermentation medium two peach mash containers were removed from the freezer and allowed to thaw at the laboratory temperature (about 25^{o}C). Three liters of the thawed mash then was placed in each of the two fermentors and the following were added (in g/L): K_2HPO_4 1.4, KH_2PO_4 0.6, $(NH_4)_2SO_4$ 3.3, $MgSO_4.7H_2O$ 0.4 (Nghiem et al, 2010). In the experiment performed to study the effect of CSL, this complex nutrient source was also added at 15 g/L. The temperature of the medium in the fermentors was raised to 50°C and 3 mL GC 220 was added. The medium was agitated at 300 rpm for 1 hour. Antifoam A-204 (Sigma-Aldrich, St. Louis, MO) was then added at 1 mL/L. After antifoam addition, the fermentors were sterilized by autoclaving at 121°C for 30 min.

For all experiments, the inoculum medium contained glucose at 5 g/L plus the other components, including CSL, at the same concentrations as in the fermentor medium. The constituents were dissolved in DI water and the pH was adjusted to 6.5 with 10% (w/v) NaOH. Eight 250-mL flasks (four for each fermentor) with 50 mL medium were autoclaved at 121°C for 20 minutes. The flasks were then inoculated with 0.1 mL of the thawed *E. coli* AFP 184 stock culture, and were incubated in an orbital shaker at 37°C and 250 rpm for 18 hours. At the end of this incubation period, contents from four flasks (total 200 mL) were pooled together and used to inoculate one fermentor. Prior to inoculation 30 mL of invertase (0.5 g/L) was syringe-filtered and

added to each fermentor. This concentration of the enzyme solution was determined as shown previously.

After inoculation the aerobic phase (growth phase) was maintained for the first 6.5 hours during which air, filtered through a 0.2 μm sterile filter, was sparged at 1 vvm and the dissolved oxygen was maintained at 20% air saturation via automatic adjustment of the impeller speed. Throughout the experiment the temperature was maintained at 37°C and the pH was maintained at 6.5 using a 2.5 M Na_2CO_3 solution. At the end of the growth phase, air supply was cut off to start the anaerobic phase and initiate succinic acid production. The entire experiment lasted for 72 hours. In the experiment that was performed to study the effect of exogenous hydrogen, this gas was filtered through a 0.2 μm sterile filter and sparged into the fermentation medium immediately after the air supply was suspended and maintained at a flow rate of 5 mL/min throughout the anaerobic phase.

Samples were taken during both aerobic phase and anaerobic phase for analysis. Immediately after collection the samples were centrifuged in a microcentrifuge at 10,000 rpm for 5 minutes. The supernatant was filtered through a 0.2 μm syringe filter and stored in a freezer. At the end of the experiment the final volume of the fermentation broth was measured.

Analytical methods

Fermentation samples were analyzed for sugars (sucrose, glucose, and fructose) and organic acids (succinic and acetic acid) by high-performance liquid chromatography

(HPLC) using an Agilent Technologies system (series 1200) (Santa Clara, CA) equipped with a refractive index detector. The sugar concentrations were determined using an Aminex HPX-87P column (BioRad, Hercules, CA) maintained at 80°C with DI water as the mobile phase at a flow rate of 0.6 mL/min. For organic acid analysis, an Aminex HPX-87H column (BioRad, Hercules, CA) maintained at 65°C was used with 5 mM H_2SO_4 as the mobile phase at a flow rate of 0.6 mL/min.

Statistical analysis

The experimental data were analyzed using Statistical Analysis System (SAS v 9.2, SAS Institute, Cary, NC). The dependent variables used were final succinic acid concentration at 72 h, succinic acid:acetic acid molar ratio, and the final succinic acid yield expressed in g succinic acid/g sugars consumed. Data from the three experiments were compared using Tukey's Studentized range test at a 5% significance level.

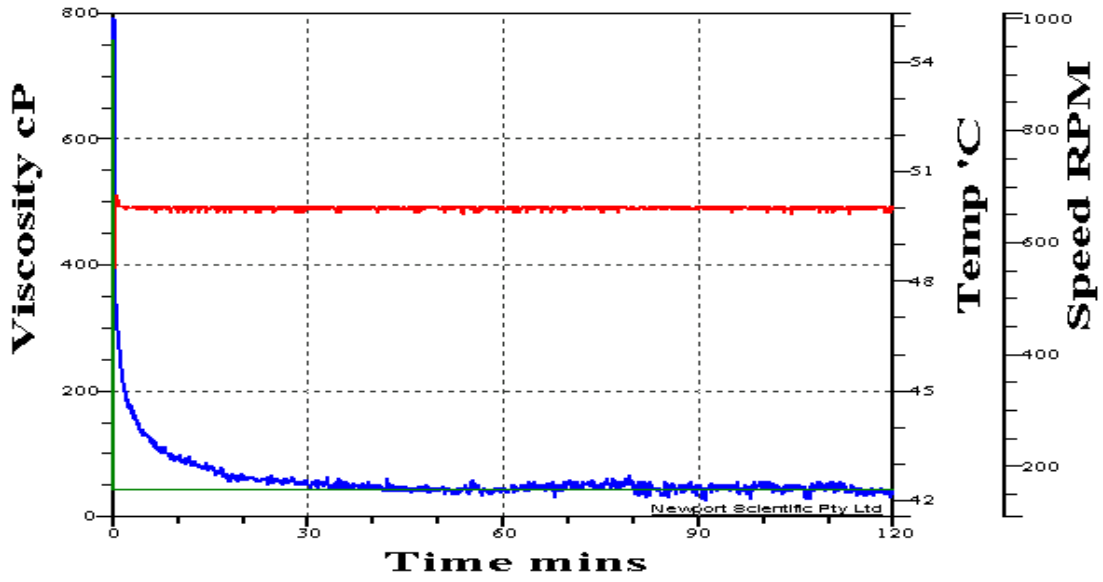
Results and Discussion

Viscosity reduction of peach mash with GC 220

During the first phase of the fermentation process the cells were allowed to grow aerobically until critical cell mass was achieved before aeration was stopped to initiate succinic acid production under anaerobic conditions (Nghiem et al, 1999). Thus, high oxygen transfer rates were required for cell growth during the first phase of the process. During the treatment of the peach medium with both dosages of GC 220 the viscosity decreased from 350 cP to 50 cP after one hour (Figure 2.3). The inverse relationship

between medium viscosity and the oxygen transfer coefficient k_La has been observed in several studies (Ozbek & Gayik, 2001; Kim et al, 2012). Konig et al (1982) reported an exponential increase of k_La as the viscosity of the fermentation medium was decreased. In the present study the k_La value was not determined. However, the steady increase of the impeller speed to maintain dissolved oxygen at 20% of saturation with fixed air flow rate was a clear indication of increasing oxygen demand due to efficient oxygen transfer and high cell growth rates. In fact, microbial growth and active cell metabolism at a medium viscosity of 50 cP have been reported for another organism of the Enterobacteriaceae family (Bandaiphet & Prasertsan, 2006).

(a)



(b)

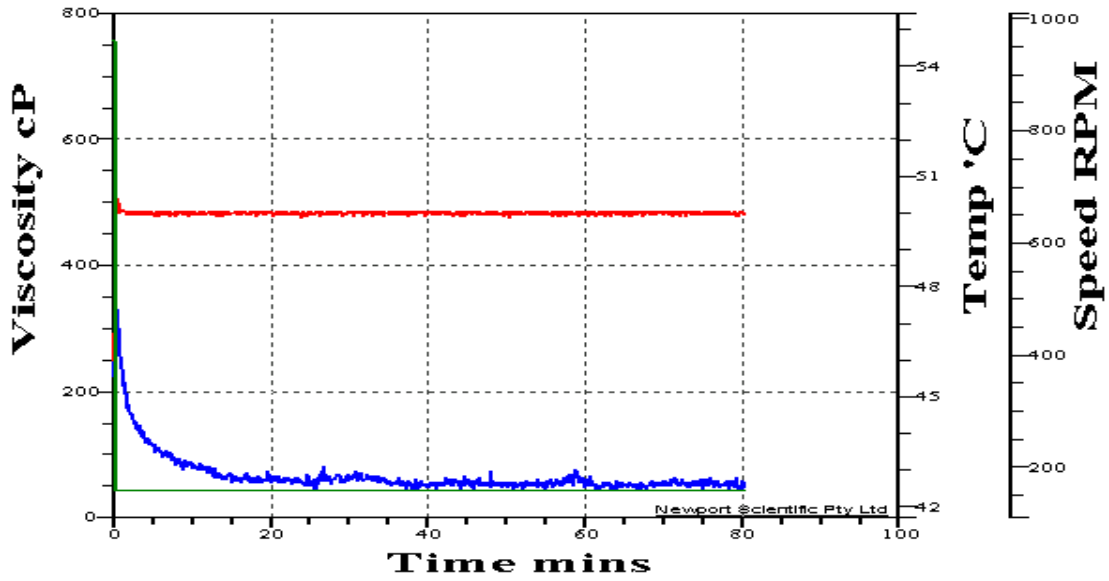


Figure 2.3 Viscosity reduction profile as recorded by the RVA (a) 30 mL peach + 30 µL GC 220 (b) 30 mL peach + 60 µL GC 220. Red: Temperature, Blue: Viscosity, Green: Speed of mixing

Succinic acid fermentation

The concentration profiles of the substrates (sucrose, glucose, fructose) and main products (succinic acid, acetic acid) for the three experiments using the peach media, the peach media supplemented with CSL, and the peach media with hydrogen sparging are shown in Figures 2.4, 2.5, and 2.6, respectively. The final results are summarized in Table 2.2.

The average sucrose, fructose, and glucose concentrations in peach media prior to sterilization were 44.1g/L, 21.3 g/L, and 18.3 g/L, respectively. Sterilization of the fermentors by autoclaving resulted in hydrolysis of 54% of sucrose. Sucrose hydrolysis during autoclaving could be attributed to the low pH of the peach media (3.8-4.0), high

H^+ ion concentration, which at an elevated temperature (121°C), accelerated sucrose conversion to glucose and fructose mimicking the mechanism of acid hydrolysis. The remaining sucrose in the media was then enzymatically hydrolyzed by invertase, which was added to the fermentors at the time of inoculation. As discussed previously, the amount of invertase added was determined to give complete hydrolysis of sucrose by 24 hours. The results showed that this attempt was successful, i.e. there was no sucrose left in the media at the end of that time period.

During the anaerobic production phase, glucose and fructose were metabolized simultaneously and at comparable rates in all three experiments, indicating absence of catabolite repression, similar to the observation reported by Andersson et al (2007). This can be credited to the mutation in the glucose-specific region of the *pts* gene in the strain. The *pts* codes for a group of transport proteins called the phosphotransferase system (PTS) that governs uptake of various sugars. With a mutated glucose-specific *ptsG*, glucose was instead transported by a much slower alternative mechanism involving galactose permease and glucokinase (Curtis & Epstein, 1975). With regions on *pts* that govern fructose uptake still intact, fructose continued to be transported by the PTS. These changes eliminated catabolite repression, allowing fructose consumption even in the presence of glucose.

As succinic acid is an intermediate of the TCA cycle small amounts (less than 1 g/L) were produced during the aerobic phase. However, high rates of succinic acid production did not actually occur until the environment became anaerobic. As peach contains trace amounts of proteins, vitamins, and minerals (USDA, NAL), the organism

was able to grow and use the peach sugars to produce succinic acid even when the fermentation medium was not supplemented with a source of complex nutrients. Final succinic acid concentration of 38.8 g/L with yield of 0.77 g succinic acid/g total sugars (glucose + fructose) consumed and succinate:acetate molar ratio of 5.1:1 were obtained. Though glucose was completely consumed, residual fructose was observed in this case. The addition of CSL resulted in significant improvement of both fructose consumption and succinic acid production. At the end of the fermentation, fructose was completely consumed. The yield was increased to 0.87 g succinic acid/g total sugars consumed and the final succinic acid concentration reached 47.0 g/L. Succinic acid productivity also was improved. The average productivity between 6.5 h (i.e. the start of the anaerobic phase) and 30 h with CSL supplement was 1. 85 g/L-h compared to 1.37 g/L-h observed without CSL. The succinicate:acetate molar ratios in two cases, however, were almost equal (5.6:1 with CSL compared to 5.1:1 without CSL). As shown in Figure 2.7, the two pathways leading to succinic acid in *E. coli* AFP184 branch at phosphoenolpyruvate (PEP). These two pathways are the reductive TCA reactions and the nonreductive TCA reactions that proceed via a glyoxylate shunt. The reductive reactions require bicarbonate to facilitate the PEP carboxylase (PEPC) mediated conversion of PEP (a three-carbon compound) to oxaloacetate (a four-carbon compound), which is then reduced to succinic acid via malic and fumaric acid. The portion of PEP that does not flow into oxaloacetate is converted to pyruvate by the enzyme pyruvate kinase. Pyruvate is then oxidatively decarboxylated into acetyl-CoA by the enzyme pyruvate dehydrogenase, which then enters the nonreductive reactions of TCA and proceeds via the glyoxylate shunt to

produce succinic acid with the help of the enzyme isocitrate lyase. Unlike the reductive TCA reactions, this route does not require bicarbonate or electron acceptors. Acetic acid, which is the only byproduct of this process is the result of an overflow mechanism of pyruvate and is produced via acetyl-CoA. The results indicated that the influence of CSL on the succinic acid biosynthetic pathway stopped at its branch point. In other words, the addition of CSL resulted in improvement of fructose metabolism up to the formation of PEP but did not affect carbon partition between the pathway leading to succinic acid and the one leading to acetic acid. Improvement of succinic acid production observed when CSL was added to the media could also be attributed to improved cell growth, which benefited from the availability of extra nutrients in the CSL. Unfortunately, because of the extremely high insoluble solid contents of the media, it was not possible to accurately measure the cell mass either by optical density or plate count method.

When hydrogen was sparged into the media without CSL supplement significant improvements of succinic acid production was observed for both rate and yield (final concentration of 45.5 vs. 38.8 g/L; yield of 0.84 vs. 0.77 g/g total sugars consumed; average productivity between 6.5 and 30 h of 1.70 vs. 1.37 g/L-h). These improvements were equal to those observed in the experiments where the media were supplemented with CSL but without hydrogen sparging. Complete fructose consumption also was observed. Most interestingly, the molar ratio of succinate:acetate was increased from 5.1:1 to 7.7:1. With exogenous supply of hydrogen additional reducing power in the form of H^+ ions was available, which resulted in larger flow of carbon into the reductive pathway toward succinic acid synthesis (see Figure 2.7). The importance of reducing

power in succinic acid formation in *E. coli* has been demonstrated by others (Nghiem et al, 2000; Hong & Lee, 2002; Vemuri et al, 2002). Nghiem et al (2000) also observed an increase of succinate:acetate molar ratio when hydrogen was sparged into a fermentor where *E. coli* AFP111 was used for succinic acid production in a two-stage process similar to the one used in the present study. These investigators, however, observed a larger increase of the succincate:acetate ratio, from 5.4:1 to 10.3:1. The observation that succinic acid production by *E. coli* AFP184 in peach media could be improved with hydrogen sparging would allow integration of this fermentation with other biochemical processes in a biorefinery where a gaseous byproduct containing carbon dioxide and hydrogen is produced. Examples of such processes include butanol and hydrogen fermentations (Jones and Woods, 1986; Yu & Drapcho, 2011). The off-gas from these fermentors could be sparged into an adjacent succinic acid fermentor to provide both the carbonate and reducing power requirements for succinic acid production at high rates and yields.

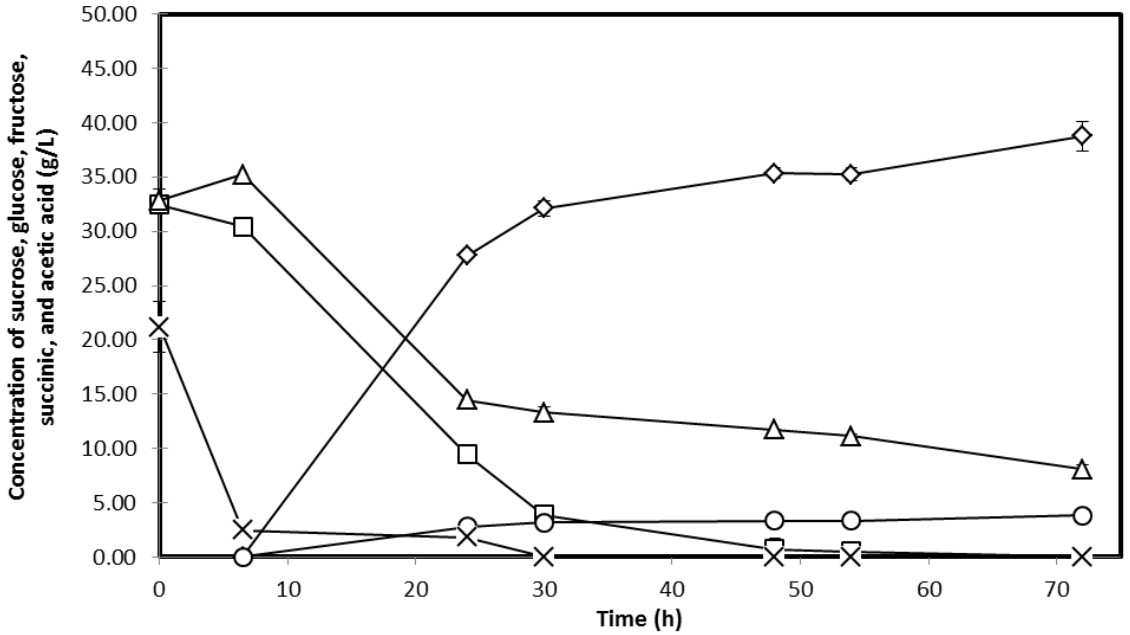


Figure 2.4: Sugar and organic acid concentration profiles from experiment using cull peach medium. Sucrose, Glucose; Fructose; Succinic acid; Acetic acid

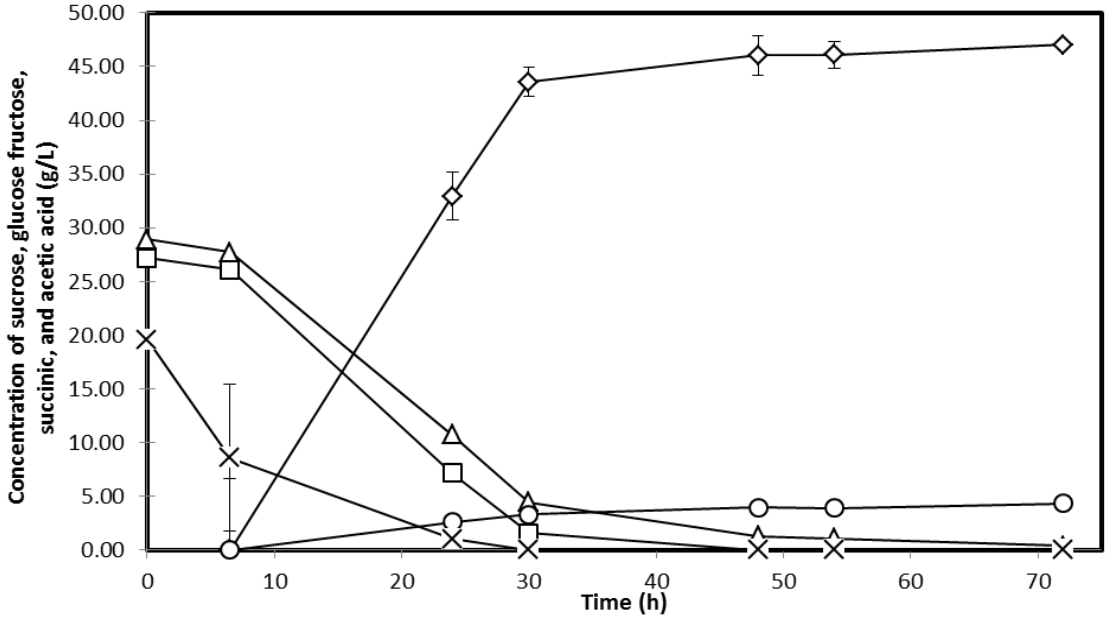


Figure 2.5: Sugar and organic acid concentration profiles from experiment using cull peach medium supplemented with corn steep liquor. Sucrose, Glucose; Fructose; Succinic acid; Acetic acid

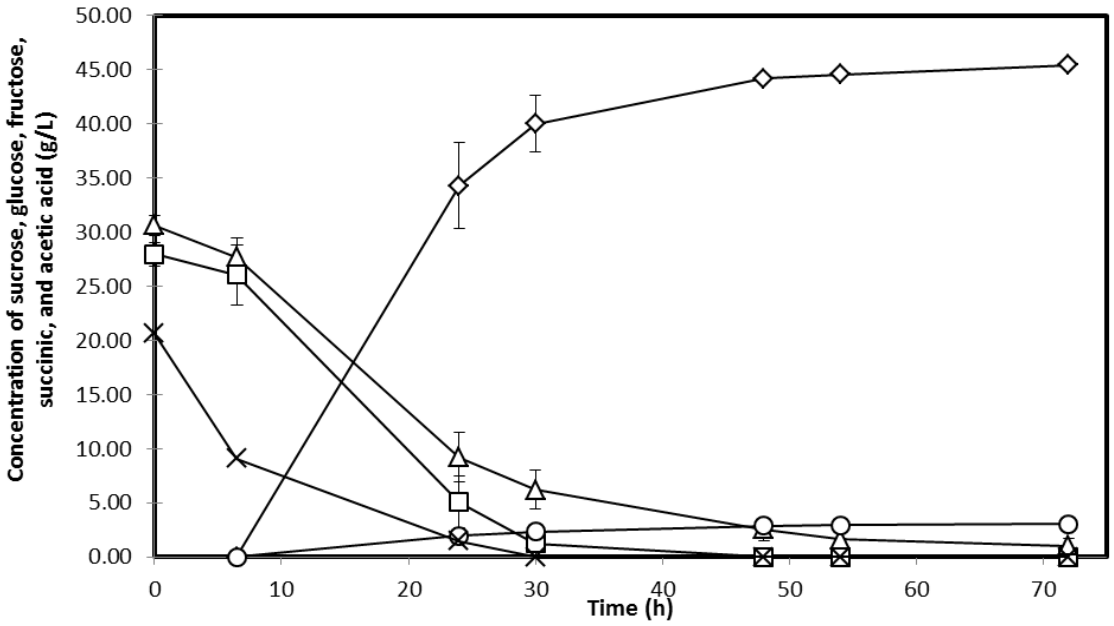


Figure 2.6: Sugar and organic acid concentration profiles from experiment using cull peach medium sparged with hydrogen during anaerobic phase. Sucrose, Glucose; Fructose; Succinic acid; Acetic acid

Table 2.2: Summary of final results[1]

Experiment	Cull peach medium	Cull peach medium supplemented with CSL	Cull peach medium sparged with H_2 during anaerobic phase
Final SA concentration (g/L)	38.75a (1.36)	47.01b (1.27)	45.45b (0.23)
Final AA concentration (g/L)	3.85 (0.21)	4.34 (0.60)	3.03 (0.15)
SA:AA (mole:mole)	5.1c (0.09)	5.6c (0.64)	7.7d (0.42)
Total SA produced (g)	139.80 (4.76)	167.61 (7.68)	162.13 (3.69)
Total AA produced (g)	13.85 (0.72)	15.43 (2.46)	10.76 (0.34)
Total glucose consumed (g)	96.54 (1.81)	94.81 (0.45)	94.73 (0.93)
Total fructose consumed (g)	84.36 (1.43)	98.60 (0.79)	97.65 (3.10)
SA yield (g/g glu+fruc consumed)	0.77e (0.03)	0.87f (0.05)	0.84f (0.04)

[1]Average values of results from duplicate experiments reported with standard errors given in bracket

Different letters along the rows denote significant difference in the data points at a 5% level of significance

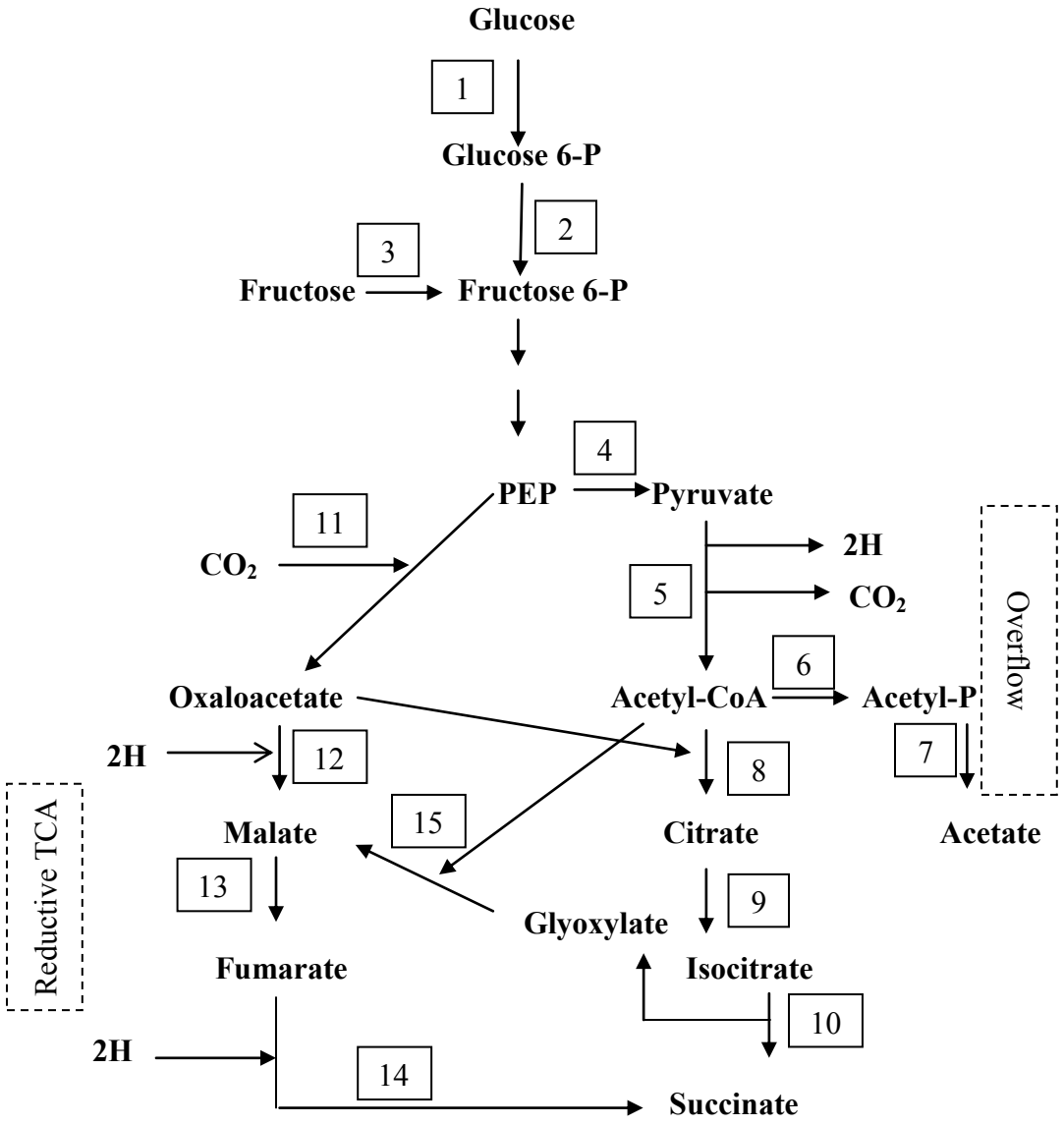


Figure 2.7: *E. coli* AFP 184 glucose and fructose metabolism. Key enzymes, 1:glucokinase, 2:glucose-6-phosphate isomerase, 3:phosphotransferase system, 4:pyruvate kinase, 5:pyruvate dehydrogenase, 6: phosphotransacetylase, 7:acetate kinase, 8:citrate synthase, 9:aconitase, 10:isocitrate lyase, 11:PEP carboxylase, 12:malate dehydrogenase, 13:fumarase, 14:fumarate reductase, 15:malate synthase

Conclusions

This is the first study to report on biological production of succinic acid from a cull peach based medium using *E. coli* AFP 184. Improvement of succinic acid production together with complete consumption of all available sugars were achieved by supplementing the media with CSL and by sparging hydrogen into the media during the production phase. Sparging of hydrogen did not only improve succinic acid production rate and yield but also increase the succinate:acetate ratio. Lower concentrations of the by-product, acetic acid, in the final broth will lower succinic acid recovery cost in an industrial process. Further study is recommended, which should include the following: 1. Determination of the optimal amount of CSL to add to the cull peach medium; 2. Investigation of the effect of hydrogen sparging rate in media supplemented with CSL; and 3. Investigation of the feasibility of using an off-gas containing carbon dioxide and hydrogen from a biochemical process such as butanol and hydrogen fermentations to provide both the carbonate and reducing power requirements for high efficiency succinic acid production in an integrated biorefinery process.

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CHAPTER THREE

CONCLUDING REMARKS

This is the first study on biological production of succinic acid from cull peach based medium using *E. coli* AFP 184. Experiments were performed in laboratory scale fermentors using a dual-phase process including an aerobic growth phase followed by an anaerobic production phase. The organism's inability to metabolize sucrose, the predominant type of sugar found in the peach medium, was circumvented by enzymatically hydrolyzing sucrose into glucose and fructose, which were efficiently consumed by *E. coli* AFP 184 for succinic acid production. The organism's mutation in the glucose-specific *ptsG* gene eliminated catabolite repression, thus allowing for simultaneous consumption of fructose and glucose. A final succinic acid concentration of 38.75 g/L at a yield of 0.77 g succinic acid/g glucose and fructose consumed was obtained when cull peach medium was used. One mole of acetic acid, which was the only major byproduct, was produced for every 5.1 moles of succinic acid. Further improvements in succinic acid yield (0.87 g/g glucose and fructose consumed), final titer (47.01 g/L), and sugar consumption (complete consumption of fructose) were obtained by supplementing the medium with CSL, which is an inexpensive source of nitrogen and complex nutrients. The improved results showed enhanced microbial growth benefited by the addition of CSL. Interestingly, a redox imbalance caused by sparging H_2 during the anaerobic phase of the process, favored production of succinic acid over acetic acid by *E. coli* AFP 184. In this case, the yield (0.84 g/g glucose and fructose) and final titer (45.45 g/L) were similar to those obtained by supplementing the medium with CSL. The

succinic acid to acetic acid ratio however was significantly higher in this case, improving from 5.1:1 to 7.7:1 (mol/mol). This increase in product to byproduct ratio is of significant importance in lowering recovery and purification costs. The sparging of hydrogen into the media to provide additional reducing power was technically similar to the previous study by Nghiem et al (2010) where the CO2 co-product from an ethanol fermentor was sparged into an adjacent succinic acid fermentor to provide the required carbonate for the CO2-fixing step of the pathway. The beneficial effects demonstrated in the two studies can be combined to integrate succinic acid fermentation with other biochemical processes where a by-product gas mixture containing both CO2 and hydrogen is produced such as butanol and hydrogen fermentations. The off-gas from these fermentors can be sparged into an adjacent succinic acid fermentor to satisfy both carbonate and additional reducing power requirements. This practice is highly feasible for a biorefinery, where succinic acid production from inexpensive sources can be sustainably coupled with other processes where CO_2 and H_2 are produced as byproducts. In this study pure H_2 was used. Future research with H_2 and CO_2 mixtures from an adjacent butanol or biohydrogen fermentor could be studied to shed more light on the feasibility and practicality of such integration of bioproducts. The data obtained can also be used for integrated process development. Using agricultural waste products like culled peaches that are not only good sources of fermentable sugars but also contain trace amounts of minerals, vitamins, and proteins, could potentially eliminate the need for exogenous addition of complex nutrient and nitrogen sources. This study shows potential for lowering the cost of sustainably produced succinic acid by decreasing the cost of fermentation medium, and increasing

product:byproduct ratio which can significantly reduce recovery and purification costs. By making use of effluent gases like CO_2 and H_2 from other bioprocesses and agricultural waste products to develop growth medium, this process is also very green and environmentally sound and sustainable.

APPENDIX A

SAMPLE CALCULATIONS

Samples that were taken from the duplicate reactors were analyzed by HPLC. Each sample was injected twice for HPLC analysis and the concentrations were averaged for calculations.

Example:

This data corresponds to the experiment where cull peach medium was used for succinic acid production, without the addition of corn steep liquor or hydrogen.

	unit 1 (g/L)					unit 3 (g/L)				
time (h)	sucrose	glucose	fructose	SA	AA	sucrose	glucose	fructose	SA	AA
before auto	43.52	19.87	22.78	0.00	0.00	43.18	20.29	23.27	0.00	0.00
0	22.82	31.84	32.05	0.00	0.00	19.54	33.01	33.55	0.00	0.00
2.5	13.98	30.93	34.97	0.00	0.00	11.19	32.11	36.71	0.00	0.00
6.5	2.64	30.82	35.22	0.00	0.00	2.28	29.99	35.23	0.00	0.00
24	1.88	9.56	14.49	27.65	2.74	1.69	9.33	14.44	27.92	2.88
30	1.87	3.63	12.99	32.57	3.15	1.63	4.09	13.67	31.63	3.15
48	1.81	0.00	11.70	35.67	3.40	1.64	1.39	11.73	35.02	3.31
54	1.83	0.00	10.99	35.64	3.44	1.57	0.96	11.25	34.78	3.27
72	1.77	0.00	8.37	37.79	3.70	1.48	0.00	7.80	39.71	3.99

	volume (L)	
time (h)	unit 1	unit 3
0	3	3
6.5	3.104	3.105
72	3.5	3.5

waste and sample (mL)	
unit 1	unit 3
20	20
25	25
26	26
25	25
25	25
25	25
24	24
24	25
25	25

sugars and acids removed (g)									
unit 1					unit 3				
sucrose	glucose	fructose	SA	AA	sucrose	glucose	fructose	SA	AA
0.870	0.397	0.456	0.000	0.000	0.864	0.406	0.465	0.000	0.000
0.570	0.796	0.801	0.000	0.000	0.489	0.825	0.839	0.000	0.000
0.363	0.804	0.909	0.000	0.000	0.291	0.835	0.954	0.000	0.000
0.066	0.771	0.881	0.000	0.000	0.057	0.750	0.881	0.000	0.000
0.047	0.239	0.362	0.691	0.069	0.042	0.233	0.361	0.698	0.072
0.047	0.091	0.325	0.814	0.079	0.041	0.102	0.342	0.791	0.079
0.043	0.000	0.281	0.856	0.082	0.039	0.033	0.282	0.841	0.079
0.044	0.000	0.264	0.855	0.083	0.039	0.024	0.281	0.870	0.082
0.044	0.000	0.209	0.945	0.092	0.037	0.000	0.195	0.993	0.100

Amount of glucose consumed during anaerobic phase (6.5-72 h) = [(concentration of glucose at 6.5 h) x (volume at 6.5 h)] – [(concentration of glucose at 72 h) x (volume at 72 h)] + [amount of glucose removed during sampling]

Amount of fructose consumed during anaerobic phase (6.5-72 h) = [(concentration of fructose at 6.5 h) x (volume at 6.5 h)] – [(concentration of fructosee at 72 h) x (volume at 72 h)] + [amount of fructose removed during sampling]

Amount of succinic acid produced during anaerobic phase (6.5-72 h) = [(concentration of succinic acid at 72 h) x (volume at72 h)] – [(concentration of succinic acid at 6.5 h) x (volume at 6.5 h)] + [amount of succinic acid removed during sampling]

Amount of acetic acid produced during anaerobic phase (6.5-72 h) = [(concentration of acetic acid at 72 h) x (volume at72 h)] – [(concentration of acetic acid at 6.5 h) x (volume at 6.5 h)] + [amount of acetic acid removed during sampling]