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Lactic Acid Production Using Sweet Potato Processing Waste

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By

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ABSTRACT

Organic waste generated from industrial sweet potato canning is estimated to be 30% of incoming raw material. This waste contains carbohydrates (sugars and starch) that could be used as substrates for the production of useful compounds via fermentation (e.g. lactic acid), resulting in the production of value-added products. The goal of this research project is to produce a substrate from the sweet potato processing waste material that supports the growth of lactic acid bacteria, which results in the production of lactic acid. The sweet potato waste product was characterized, and found to contain 16.5% solids. The solids components were 18.5% ash, 4.4% protein, and the rest assumed to be carbohydrates. The carbohydrate component was found to contain 20.5% sugars, mostly in the form of sucrose, 19% soluble starch, and the rest assumed to be fiber. Conditions for enzymatic starch hydrolysis were explored, and using 80U glucoamylase/100 gram waste material for a 24 hour treatment at 35°C and pH 4 yielded a greater than 95% conversion efficiency to glucose while minimizing total enzyme required. Screening of 3 lactic acid bacteria strains in a control medium (YM Broth) yielded highest lactic acid production by *Lactobacillus rhamnosus*. Different dilutions of the hydrolyzed sweet potato waste, with and without pH control, were used as a fermentation substrate for *L. rhamnosus,* and lactic acid production was highest in the undiluted hydrolyzed waste at pH set point 5.0, yielding 10g/L in 72 hours. Lactic acid production from sweet potato waste will provide a valuable product from a waste stream for local processing facilities.

This thesis is approved for recommendation
to the Graduate Council.

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INTRODUCTION

The transformation of fruit and vegetable wastes from the food industry into value-added products is desirable economically as well as environmentally. Plant materials that are either composted or used as animal feed contain nutrients and carbon, which can be transformed into a new product with unique or enhanced functionality. The canning industry estimates that up to 30% of the incoming raw material is designated as waste and not used for human consumption (Schaub et al. 1996). Byproducts of the canning industry are usually discarded, composted, or fed to ruminants. Other possible uses include extraction of bioactive compounds (Eguees et al. 2012, Babbar et al. 2011) and fermentation to produce a variety of value-added chemicals (Kroyer 1991, Haddadin et al. 2001).

Previous research has shown that it is possible to use fruit, vegetable, and grain byproducts as a base for fermentation media (Huang et al. 2005, John et al. 2007, Nakanishi et al. 2010, Shindo et al. 2004). Examples of waste streams previously evaluated include different fruit pomaces, corn husks, sugarcane bagasse, spent grain, trimming vine shoots, and potato processing wastewater. Products resulting from fermentation of canning wastes, such as ethanol, methane, microbial protein, and lactic acid, have been researched and show potential for value addition (Nigam 1999, Hills et al. 1982, El-Masry et al. 1991, John et al. 2005).

Production of lactic acid is desirable because it is prevalent in our food system and has many uses, including flavor enhancement, preservation, and pH control. Not only is the use of lactic acid well-documented and widely used as a food additive, it is also utilized in other industries including bioplastics, chemicals, and cosmetics. Currently, lactic acid is being

produced by lactic acid bacteria (LAB) on a commercial scale with raw materials such as corn starch (in the northern hemisphere) and cane sugar (in tropical and semitropical countries) (Calabia et al. 2007).

LAB have been utilized for food preservation and flavor development for hundreds of years and, over the years, the widespread use of LAB has resulted in the availability of a wide variety of strains. Fermentations using certain LAB produce almost exclusively D (-) or L (+) lactic acid, of which L (+) is the preferred form for the bioplastics industry (Datta et al. 1995). Each strain has a different growth curve as well as different environmental and medium requirements for acceptable yields. Conditions for optimal growth and lactic acid production must be evaluated and optimized for each strain. The parameters requiring optimization include, but are not limited to, the following: nutrients for growth and production, temperature, pH, time, vessel size, and oxygen.

The processing of sweet potatoes generates a waste byproduct rich in minerals, polysaccharides, and free sugars. With enzyme treatments, the starch can be broken down into glucose, yielding a greater concentration of free sugars that may be a rich substrate for fermentation. Production of lactic acid via fermentation of raw starch derived from sweet potato has been successfully tested (Wongkhalaung 1995), but not waste material from sweet potato processing.

The first goal of this project was to produce a fermentation substrate from the waste stream of sweet potato processing that has the potential to support the growth of lactic acid bacteria and the production of lactic acid. The second goal of this project was to find a LAB strain that can grow and produce lactic acid with minimal supplementation of the sweet potato

substrate. The overall project goal was to put together a system that allows for an efficient use and economical transformation of the sweet potato waste material generated by the canning and processing industry.

OBJECTIVES

Objective 1: Determine suitable conditions for the conversion of sweet potato byproduct from the food processing industry into a substrate suitable for subsequent fermentation into lactic acid.

Objective 2: Screen lactic acid bacteria strains to maximize the production of lactic acid using substrate obtained in objective 1.

LITERATURE REVIEW

Sweet potato processing and waste material production

Sweet potato is a worldwide crop with production of over 100 million metric tons in 2009 (USDA Economics, Statistics, and Market Information website, 2012). The USDA estimates that approximately 5% of total sweet potato production is lost during processing, generating 5 million metric tons of waste annually (USDA Economics, Statistics, and Market Information website, 2012). Approximately 25% of all sweet potatoes produced in the US are canned, while the remainder are purchased fresh or processed into frozen and dried products (Economic Research Service, 2002). The canning industry generates up to 50% waste from the incoming raw material (BOA, 1983).

Much of the usable waste from sweet potato canning processes is generated during the peeling process. The peeling operation can be accomplished by several methods, including mechanical abrasion, sodium hydroxide (lye), and high pressure steam. Mechanical abrasion is

seldomly used commercially due to excessive yield losses (Edmond, 1971), and lye peeling, although efficient, results in the generation of large amounts of caustic waste and can discolor the flesh (Walter and Schadel, 1982). Steam peeling, consisting of a short duration of exposure to super heated steam and subsequent water washing, is an efficient and less wasteful peeling process (Smith et al. 1983). Washings are high in nutrients including starch and free sugars, as well as minerals and other trace elements (Colston and Smallwood Jr, 1973, Olaoye and Sanni, 1988).

Fermentation of plant waste materials

Since plant waste products from the canning industry can be high in nutrients, interest in the use of these products for other than composting or animal feed has been growing. Knol et al. (1978) proved the feasibility of producing biogas from fruit and vegetable canning wastes. Paige and Boulton (1978) concluded that production of ethanol from canning and agricultural wastes was economically viable. More recently, Del Campo et al. (2006) explored the production of bioethanol from tomato, roasted red pepper, and artichoke wastes. Production of these value added products is generally accomplished via fermentation with microorganisms, since a range of products from biofuels to drugs to chemicals can be generated (Du et al. 2011).

Fermentation using microorganisms requires the generation of a usable energy source from these plant waste materials, mainly carbohydrates. These can be in the form of refined (ex. glucose, sucrose, and starch) or complex (cellulose, whole cereal grains, and waste materials). However, the fermentation organism does not have the ability to convert these carbohydrates into glucose for energy of growth and maintenance, so pretreatment of the plant waste material will be necessary (Litchfield, 1996). Efficient hydrolysis of gelatinized starch generally requires

multiple enzymes or acid hydrolysis. Enzymatic hydrolysis is preferable to the use of acid due to the toxic compounds formed during acid treatment (Gurgel et al. 1998). The enzymes previously used for sweet potato starch hydrolysis include α-amylase, β-amylase, and glucoamylase (Baba and Kainuma, 1987, Chang Rupp and Schwartz, 1988, Noda et al. 1992). Alpha-amylase causes endohydrolysis of α-1,4 linkages and works quickly to reduce molecular weight and decrease viscocity. Beta-amylase causes exohydrolysis from α-1,4 linkages, releasing individual maltose units. Glucoamylase causes successive exohydrolysis of α-1,4 linkages, as well as α-1,6 linkages to a slower extent, and results in glucose formation from starch. Alpha-amylase is commonly used in laundry detergents to enhance stain removal, so large quantities are available at a low cost, and the combination of α-amylase and glucoamylase is often used as a cost-effective, enzymatic method to degrade starch into glucose (Kirk-Othmer, 2004). The energy source generated by starch hydrolysis, namely glucose, is required by most microorganisms during fermentation in order to manufacture the chemicals that are value-added products. One of the chemicals of interest produced via fermentation is lactic acid, mainly produced by lactic acid bacteria.

Importance of lactic acid

Lactic acid and LAB have been part of our food system for thousands of years; however, the mechanism for preservation and sensory qualities bestowed by these bacteria was not understood until the 19th century with technological advances in microbiology. Since then, studies have demonstrated that LAB not only produce compounds that decrease pH but also a variety of antimicrobial agents that discourage the growth of other microbes (Vandenbergh 1993). In the food industry, lactic acid is used as a preservative, flavouring agent, pH buffer, and acidulant (Narayanan et al. 2004). It is estimated that global demand will be 259,000 metric tons

by 2012 and 328,900 metric tons by 2015 (Global Industry Analysts 2011). NatureWorks LLC (Blaine, Nebraska, USA) currently produces 140,000 metric tons (400 million pounds) of lactic acid annually, and is currently looking for a site to build a second lactic acid facility (Vink et al. 2007). Lactic acid is one of the most widely used chemicals, not only in food, but also in the medical, pharmaceutical, plastics, and cosmetic industries.

Uses of lactic acid

Lactic acid, 2-hydroxy-propanoic acid, is widely used because the structure lends itself to a variety of chemical reactions—it consists of both a hydroxyl group and a carboxylic acid group, as shown in Figure 1.1.

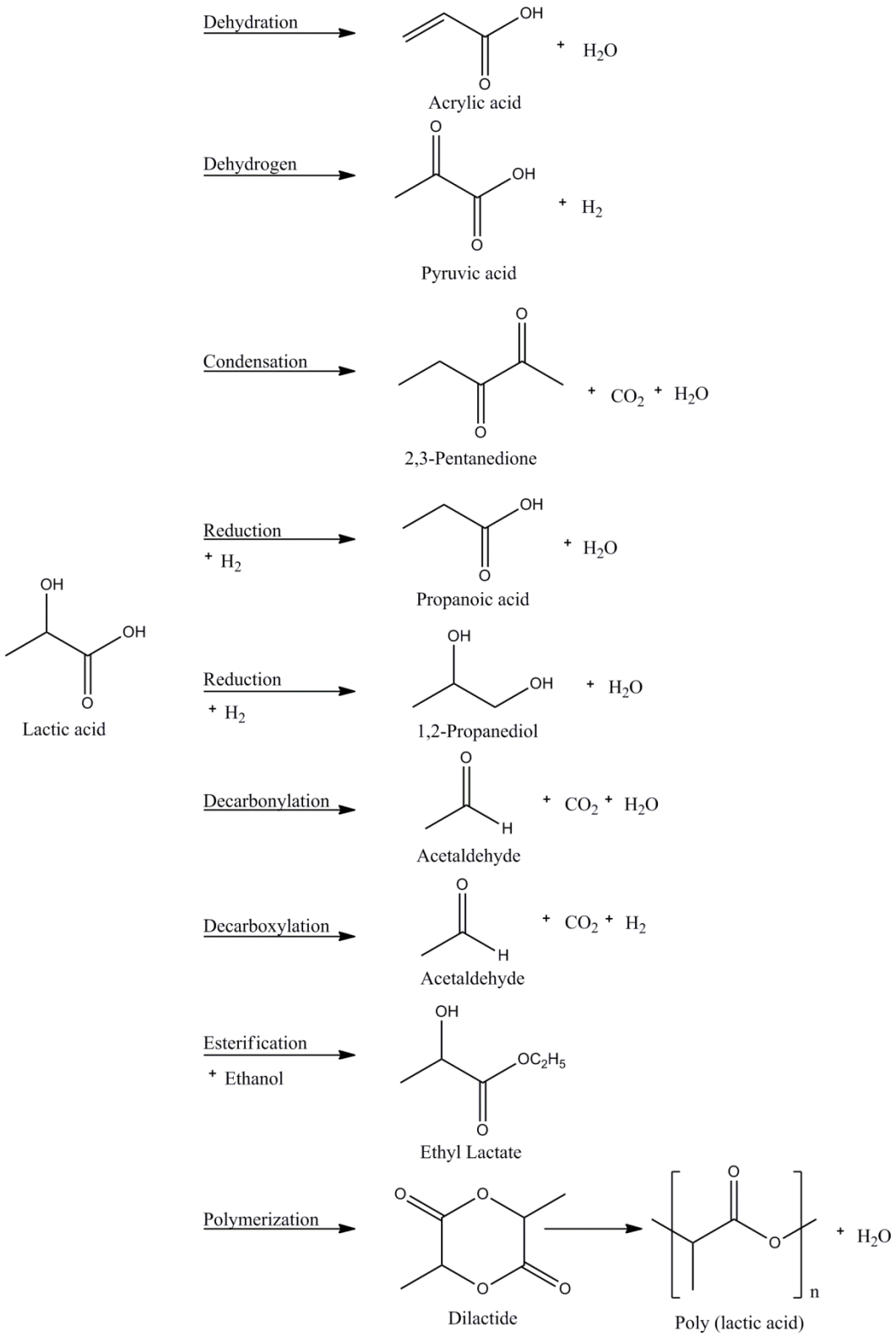
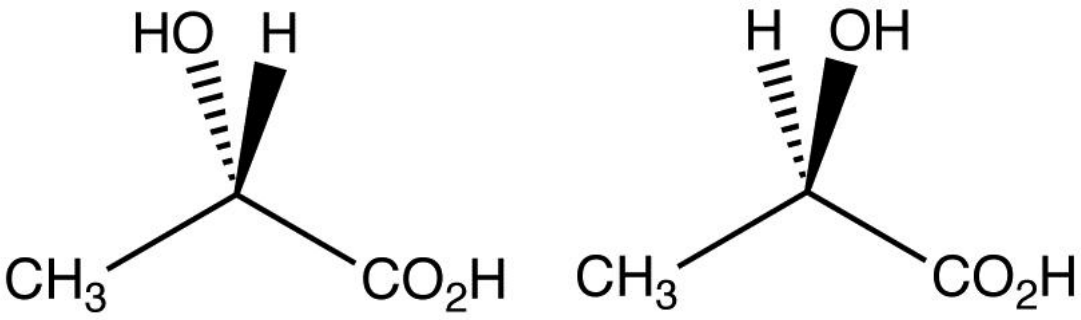


Figure 1.1. Common chemical reaction pathways of lactic acid (Fan et al. 2009)

Polymerization of lactic acid, with generation of dilactide as an intermediate, generates poly lactic acid (PLA) (Figure 1.1). Commercially used as a bioplastic, this polymer can be employed in a variety of applications ranging from clothing fibers to films to paper coatings (Pang et al. 2010). Several research groups have been developing processes to produce PLA materials, resulting in products that have similar crystallinity, glass transition temperatures, melting temperatures, and flexibility as petroleum based plastic products (Nijenhuis et al. 1996, Ouchi et al. 2003, Sarasua et al. 1998, Tsuji 2002, Wang et al. 2006). These groups have found that a pure form of lactic acid allows for greater control of the final PLA product during manufacturing. Two stereoisomers of lactic acid exist, L (+) and D (-) (Figure 1.2), and the physical properties of PLA are directly influenced by the isomer used to manufacture this material. Stereospecificity of the precursor for the production of PLA determines the clarity, melting point, and strength of the bioplastic. Racemic lactic acid mixtures produce non-crystalline PLA polymers with very low glass-transition temperatures and low melting points (Pang et al. 2010). When first produced, PLA production was an expensive and labor intensive process to go from fermentation substrate input to plastic output. Advances in technology, as well as the opening of the first large scale polylactide (PLA) plant in 2002, have reduced the production costs and enhanced the quality of PLA, making it more competitive with petroleum-based plastics (Carole et al. 2004).



L (+) Lactic Acid D (-) Lactic Acid

Figure 1.2. Stereoisomeric forms of lactic acid

Recent studies predict a 37% increase in the consumption of bio-based plastics from 2007 to 2013 (Shen et al. 2009). Based on a report by the U.S. Department of Energy, PLA is one of the top three bio-based plastics, and lactic acid is in the top 30 for value-added chemicals from biomass (Energy Efficiency and Renewable Energy, 2004). Lactic acid can be produced via chemical synthesis from fossil fuel feedstocks such as petroleum and natural gas. However, the input is derived from a non-renewable source and the process generates a racemic mixture of isomers, which is undesirable for the production of PLA, but microbiological fermentation can yield either a mixture or a pure form of L (+) or D (-) depending on the LAB strain used (Caplice et al. 1999).

Lactic acid producing microorganisms

The physical properties and characteristics of PLA products depend on the form of lactic acid isomer used, which in turn depends on the organism used to produce it. Table 1.1 lists the fermentation pattern as well as the isomer produced by selected lactic acid-producing bacteria and fungi. LAB are gram-positive, facultative anaerobes or microaerophilic, and are classified as either homofermentative or heterofermentative. Homofermentative LAB produce lactic acid without other metabolic byproducts and a theoretical conversion of 1 mole glucose to 2 moles lactic acid. Heterofermentative LAB produce lactic acid as well as other byproducts including ethanol, diacetyl, and carbon dioxide; thus, conversion of 1 mol glucose results in less than 2 mol lactic acid. For this reason, homofermentative LAB strains under ideal conditions have the ability to produce higher lactic acid yields than heterofermentative strains (Litchfield 1996).

Table 1.1. Fermentation patterns and isomeric forms of lactic acid generated by various microorganisms (Litchfield 1996)

Organism	Fermentation Pattern	Lactic Acid Isomer
Bacteria		
Bacillus coagulans	Heterofermentative	L (+)
Bacillus laevolacticus	Heterofermentative	D (-)
Lactobacillus amylophilus	Homofermentative	L (+)
Lactobacillus amylovorus	Homofermentative	D/L
Lactobacillus casei subsp. casei	Heterofermentative	L (+)
Lactobacillus delbruekii subsp. bulgaricus	Homofermentative	D (-)
Lactobacillus helveticus	Homofermentative	D/L
Lactobacillus rhamnosus	Heterofermentative	L (+)
Lactococcus lactis subsp. lactis subsp. cremoris	Homofermentative	L (+)
Streptococcus faecalis	Homofermentative	L (+)
Streptococcus thermophilus	Homofermentative	L (+)
Sporolactobacillus inulinis	Homofermentative	D (-)
Fungi		
Rhizopus arrhizus	Heterofermentative	L (+)
Rhizopus delmar	Heterofermentative	L (+)
Rhizopus oryzae	Heterofermentative	L (+)
Rhizopus stolonifer	Heterofermentative	L (+)
Rhizopus sp. G-36	Heterofermentative	L (+)

Fungi are also used for lactic acid production, most commonly *Rhizopus arrhizus and Rhizopus oryzae*. Yields from direct fermentation of potato starch wastewater with *Rhizopus arrhizus* and *Rhizopus oryzae* were 1.02 g lactic acid/g starch and 0.78 g lactic acid/g starch, respectively, and the maximum productivities (per liter of wastewater) were 0.67 g/L/h and 0.40 g/L/h, respectively (Huang et al. 2005). Furthermore, using *Rhizopus arrhizus* to ferment waste potato starch, Zhang et al. (2010) reported a lactic acid yield of 0.865 g lactic acid/g glucose with a maximum productivity of 2.2 g/L/h. Both studies produced similar values for conversion of glucose to lactic acid, but Zhang et al. (2010) reported a volumetric productivity of 3-5 times higher than Huang et al. (2005).

Although the advantages of using fungi include a simpler nutrient requirement, the capability to hydrolyze starch, and the means to metabolize high concentrations of glucose, the main disadvantage is the production of high levels of byproduct, including ethanol and fumaric acid (John et al. 2007). Even though recent yields from fungi-based systems indicate that this might be a viable production system, the predominant lactic acid production systems continue to be based on LAB (John et al. 2007).

According to Litchfield (1996), the best bacterial strain for lactic acid production depends on the desired isomer, input substrate, production process temperature and pH, and productivity. One common bacterial strain used for lactic acid production is *Lactobacillus delbrueckii*. Ray et al. (1991) reported that the strain was capable of a lactic acid yield of 69% on the basis of glucose weight (0.69 g lactic acid/g glucose). More recently, John et al. (2005) reported that *L. delbrueckii* produced 0.249 g lactic acid/g starch under nutrient limited conditions and without pH control. Another study utilizing *L. delbrueckii* was published recently by Lu et al. (2009) in which unpolished rice saccharificate, wheat bran powder, and yeast extract were used as the

culture medium. The lactic acid yield was 0.73 g/g starch and the productivity rate was 1.5 g/L/h. Fukushima et al. (2004) reported large-scale lactic acid yields of 0.66 g/g sugar. The main carbon source in the medium was hydrolyzed rice starch, and the strains screened in the study were: (1) seven different *lactobacilli* strains, including *L. casei*, *L. bulgaricus*, *L. delbrueckii*, *L. helveticus*; (2) five different *lactococci* strains; and (3) two *sporolactobacilli* strains. Overall, the *L. delbrueckii* strains produced the most lactic acid.

Other strains that have been used for the production of lactic acid from a variety of substrates are *Lactobacillus rhamnosus*, *Lactobacillus lactis*, *Lactobacillus paracasei*, *Lactobacillus pentosus*, and *Lactobacillus amylophilus* (Table 1.2). Shindo et al. (2004) fermented spent grain, a by-product of beer production, with *L. rhamnosus*; the fermentation yielded 0.475 g/g sugar. *L. lactic* is reported to utilize cellobiose efficiently with yields as high as 0.8 g lactic acid/g cellobiose with a maximum productivity rate of 1.66 g/L/h (Singhvi et al. 2010). Nakanishi et al. (2010) used *L. paracasei* to ferment sugars from canned pineapple syrup and were able to generate 0.93 g/g sugar in a pH controlled process. Additionally, Bustos et al. (2005) used *L. pentosus* to produce lactic acid at a rate of 0.36 g/L/h from trimming waste of vine shoots while Altaf et al. (2007) used *L. amylophilus* to produce 0.78 g lactic acid/g starch from corn flour supplemented with red lentil flour and baker's yeast.

A comparison of these studies (Table 1.2) reveals there is not one "perfect" microorganism for the production of lactic acid, but the best candidate depends on available nutrients, duration of production process, and method of production. Since production of lactic acid from canning byproducts must be economically viable, using the best possible microorganism for fermentation is crucial to optimizing production and maximizing product output.

Table 1.2. Lactic acid production by various lactic acid-producing bacteria using a variety of biomass substrates (Wang et al. 2010)

		Lactic Acid		
Substrate	Organism	Concentration (g/L)	Productivity (g/L/h)	Yield (g/g substrate)
Alfalfa Fibers	*L. delbrueckii*	35.4	0.75	0.35
	L. plantarum	46.4	0.64	0.46
Apple Pomace	*L. rhamnosus*	32.5	5.4	0.88
Barley Bran Hydrolysates	*L. pentosus*	33.0	0.60	0.57
Cellobiose and Cellotriose	*L. delbrueckii*	90.0	2.3	0.90
Cellulose	*L. delbrueckii*	65.0	0.18	-
Corncob	*L. pentosus*	26.0	0.34	0.53
Lignocellulosic Hydrolysates	*L. sp RKY2*	27.0	6.7	0.90
Molasses	*L. delbrueckii*	166	4.2	0.87
	L. rhamnosus	73.0	2.9	0.97
Paper Sludge	*B. coagulan 36D1*	92.0	0.96	0.77
	B. coagulan P4-102B	91.7	0.82	0.78
Rice Bran	*L. delbrueckii*	28.0	0.78	0.28
Sugarcane Bagasse	*Bacillus sp. strain*	55.5	0.39	0.77
	Lactococcus lactis	10.9	0.17	0.36
Trimming Vine Shoots	*L. pentosus*	24.0	0.51	0.76
Wastepaper	*L. delbrueckii*	31.0	-	-
Wheat Bran Hydrolysate	*L. bifermentans*	62.8	1.2	0.83
Wood Hydrolysate	*Enterococcus faecalis*	93.0	1.7	0.93
Xylose	*L. plantarum*	41.2	-	0.82
Corncob Molasses	*Bacillus* sp. strain	74.7	0.38	0.50

Lactic acid production processes

Even though lactic acid production capability is dictated, to a large extent, by the microorganism used for fermentation, the media and process are also significant factors in determining the success of a production process, with growth medium being a key factor. In testing various carbon sources, Olaoye et al. (2008) found that glucose and galactose were the preferred carbon sources for various strains of *Lactobacillus pediococcus*, while Calabia et al. (2007) found that sucrose from sugar cane and sugar beet juice was an acceptable carbon source, without modification, for *L. delbruekii*. In contrast, Chervaux et al. (2000) found the fastest growth in 22 different strains occurred with media comprised of lactose as opposed to glucose, mannose, or fructose. Nitrogen sources are also an important factor in microbial growth media, and commercial formulations for LAB, such as LB Broth and MRS Broth, contain protein hydrolysates from soy, casein, meat, yeast, or other sources. As sweet potatoes are low in overall protein content (Purcell et al. 1978), choosing the appropriate LAB strain will be critical because the addition of supplements would decrease the economic benefit of transforming this waste into a value-added product.

Although growth medium is a key factor in determining success of a fermentation process, mode of production is also important. The three main modes of production used for fermentation are batch, fed-batch, and continuous. Continuous production, also called perfusion, is an effective method to achieve high cell densities and produce large quantities of product (Ohashi et al. 1999). Unfortunately, bench-top versions of this production mode require specialized, expensive equipment and extensive optimization. The time and money investment for this technology renders it impractical for most applications. Alternatively, current batch and fed-batch technologies are simple, inexpensive, and high throughput compared with perfusion

(Huber et al. 2009). Shake flasks are a reasonable representative of the bioreactor batch mode, and are a commonly used method for small scale development and optimization of lactic acid production using LAB (Mel et al. 2008, Yu et al. 2008, De Lima et al. 2010).

Utilization of sweet potato waste product

Ray and Ward (2006) specifically mention sweet potato waste may be a good candidate for microbial fermentation due to the abundant supply in several Asian countries. Furthermore, it is currently only being used as animal feed. Sistrunk and Karim (1977) showed that fermentation of lye-peelings from sweet potatoes extends storage life for livestock feed. In addition, sweet potato residue has been fermented to generate tetracycline (Yang et al. 1989, Yang et al. 1990), microbial proteins (Yang 1993, Aziz et al. 2002, Wang et al. 2008), and ethanol (Moore et al. 2008). However, in each study, the sweet potato residue is generated in the lab by peeling the raw potatoes, steaming the flesh, and sieving to break up large clumps. Several studies examine fermentation of sweet potato with lactic acid bacteria (Ray et al. 1991, Jiang et al. 1993, Wongkhalaung 1995), but the fermentation substrate is always the flesh of the sweet potato–raw, cooked, or dried. Sweet potato flesh has been used to make several valuable products, including ethanol, citric and lactic acid, and sugar syrups (Palaniswami et al. 2008).

Studies on the nutritional properties of sweet potatoes indicate the presence of several essential amino acids (Purcell et al. 1978), high levels of starch and free sugars (Ravindran et al. 1995), and a peel composition of cellulose, pectic substances, and hemicellulose (Noda et al. 1994). However, it is noted by Noda et al. (1992) that starch properties and chemical composition is subject to cultivar and tuber developmental stage. Type of sweet potato processing, whether they are peeled via mechanical methods, with lye, or by steam, also affects

the chemical composition of the processing waste. Previous research involves the use of sugar generated from sweet potato starch, so it is very likely that the residual starch in sweet potato processing waste could be converted to glucose and used for the same purpose. No research exists on the fermentation of sweet potato processing waste from a canning facility for production of a value added product.

Chapter 2: TRANSFORMATION OF SWEET POTATO PROCESSING WASTE INTO A FERMENTABLE SUBSTRATE

INTRODUCTION

Waste from the sweet potato canning process is a potential source of nutrients that can be turned into useful products via fermentation. Fermentation of several different plant waste materials has already been proven possible, and in some cases, economically viable. Sweet potatoes are produced globally, potentially providing an endless stream of substrate for transformation. Fermentation requires a usable carbon source, which is generally glucose. This is obtained through hydrolysis of starch, and several enzymes specifically and efficiently complete this task. Enzymes can be a costly reagent in this type of process, and it is important to minimize their cost as much as possible, since this is a process for turning waste material into a value added product.

Objective 1: Determine suitable conditions for the conversion of sweet potato byproduct from the food processing industry into a substrate suitable for subsequent fermentation into lactic acid.

MATERIALS AND METHODS

A 20-gallon bucket of sweet potato waste material harvested at the steam peeler discharge—primarily comprised of peel and water with residual flesh—was provided by Allen Canning, Inc. (Siloam Springs, AR, USA) in September 2010.

Characterization of sweet potato processing waste

The sweet potato waste was divided into gallon-sized Ziploc© bags with each bag containing 480-500g. Bags were stored at -20°C until use. To thaw waste material, bags were held at 4°C for 48-72 h, or until the waste material was free of ice crystals.

Solids content determination

Solids content was determined by oven drying (VWR model #1326) quadruplicate weighed samples of sweet potato waste in 1 g aluminum tins at 60°C for 18 h. Tins were then allowed to equilibrate in a dessicator for 1 h before final weight was obtained. Equation for calculating solids content was:

$$\%\, Total\, Solids = \frac{dried\ sample\ weight\ (g)}{sample\ weight\ (g)} \times 100 \qquad \text{[Equation 2.1]}$$

Preparation of dried material for analyses

A large batch of dried ground powder was generated from the waste. One bag of sweet potato waste material was mixed 1:1 with deionized water, and blended in a Waring commercial laboratory blender (East Windsor, NJ, USA) at speed 6 for 3 min. This mixture was then dried in an oven at 50°C for 24 h. The resulting material was pulse ground in a coffee grinder (Mr. Coffee, Rye, NY, USA) and sieved through a #40 (0.425mm) mesh screen. The powder was stored in an air-tight container at room temperature until use.

Sugar and starch determination

Total sugar content was determined by a modified phenol sulfuric acid method (Dubois et al. 1956). A 0.5 mL sample of ethanol extracted sweet potato powder was mixed with 0.5 mL of 5% phenol reagent and 2.5 mL of concentrated sulfuric acid. After a 30 min incubation at room temperature (25^{o}C), the absorbance at 490 nm was read with a UV-1700 PharmaSpec spectrophotometer (Shimadzu, Columbia, MD, USA) and compared to a standard curve established by glucose.

Total starch was determined using Megazyme's Total Starch assay kit (Dublin, Ireland) after an ethanol pre-wash step to remove free sugars. The ethanol pre-wash procedure was the addition of 5.0 mL of 80% (v/v) aqueous ethanol to 100 mg sample in a 15 mL centrifuge tube, with incubation at 85°C for 5 min. Then an additional 5 mL of ethanol was added, and the contents were mixed on a vortex mixer. The tube was centrifuged for 10 min at 1,800 x g in a Beckman Coulter Allegra X-22R with SX4250 rotor, and the supernatant was discarded. The pellet was resuspended in 10 mL of 80% aqueous ethanol, and mixed on a vortex mixer. The tube was centrifuged at 1,800 x g for 10 min, and the supernatant was carefully removed. The resulting pellet was used for total starch determination by addition 3 mL of thermostable α-amylase (100 U/mL in 100 mM sodium acetate buffer, pH 5.0) and incubation in a boiling water bath for 12 min, with vortex mixing at 4, 8, and 12 min. The tube was then placed in a 50°C water bath for 5 min, and 0.1 mL of amyloglucosidase (3300 U/mL) was added, mixed by vortexing, and incubated at 50°C for 30 min. The volume of tube was then adjusted to 10 mL with deionized water, and centrifuged at 1,800 x g for 10 min. 1.0 mL of supernatant was diluted with 9.0 mL deionized water, and 0.1 mL of this solution was transferred to the bottom of a glass test tube (16x100 mm). 3.0 mL of Glucose Determining Reagent (glucose oxidase plus peroxidase plus 4-aminoantipyrine in 1.0 M potassium phosphate buffer with 0.22 M p-hydrobenzoic acid and 0.4% sodium azide) was added, and the tube was incubated at 50°C for 20 min. Absorbance at 510 nm was read and compared with the D-glucose control. Calculation of total starch was as follows:

$$\%Starch = \Delta A \times \frac{9}{\Delta Abs} \qquad \text{[Equation 2.2]}$$

Where:

ΔA = Absorbance of sample at 510 nm read against a reagent blank
ΔAbs = Absorbance of glucose standard at 510 nm read against a reagent blank

Sugars analysis

The identity of sugars in the sweet potato processing waste was determined using high-performance size exclusion chromatography with refractive index detection (HPSEC-RI) (Waters, Milford, MA, USA). The system consisted of a 1515 HPLC pump with a manual injector valve, a 50-µL sample loop, and a 2410 refractive index detector maintained at 40 ºC. Sugars were separated by two Shodex columns, an OH Pack SB-802 HQ (300 x 8 mm), and an OH Pack SB-804 HQ (300 x 8 mm) connected in series and maintained at 55ºC by a column heater, preceded by a Shodex OH pack SB-G (50 x 6 mm) guard column. The mobile phase was 0.1 M $NaNO_3$ with 0.2% NaN_3 ($NaNO_3$ 8.499 g + NaN_3 0.2 g in 1 L distilled water) at a flow rate of 0.4 mL/min. Samples for HPSEC-RI were prepared by adding 5 g of sweet potato waste powder to 25 g HPLC grade water, and mixing at 50°C for 15 min. Samples were then centrifuged at 1,800 x g for 10 min and the resulting supernatant was filtered through a 0.45-µm nylon membrane before injection.

Ash content

Ash content of the sweet potato waste was determined using a modified AACC method 08-17, Ash in Starch. Triplicate crucibles were first placed in a muffle furnace and held at 600°C for one h, and then allowed to equilibrate to room temperature in a dessicator overnight. Three g of sweet potato waste powder were added to each crucible, heated on a hot plate until fully carbonized, placed in a muffle furnace, and burned at 600°C for 16 h. Crucibles containing the burned sample were then placed in a dessicator and cooled to room temperature for one h before weighing. Calculation of ash content was as follows:

$$\%\ Ash\ \ (as-is) = \frac{Ash\ \ weight\ \ (g)}{Sample\ weight\ \ (g)} \times 100 \qquad \text{[Equation 2.3]}$$

Protein, total nitrogen, and mineral analysis

Protein and mineral analysis were conducted by a contract lab (Agricultural Diagnostic Laboratory, Fayetteville, AR, USA). Total nitrogen was determined using combustion with LECO FP428 nitrogen analyzer, and mineral elements were determined using a concentrated nitric acid/hydrogen peroxide digest on a heated block and analysis by SPECTRO ICP.

Thermal properties

Thermal properties were assessed by a differential scanning calorimeter (DSC, Perkin-Elmer Co., Norwalk, Conn., USA). Approximately 10 mg of sweet potato waste powder was weighed into a steel DSC pan, and 20 µL of deionized water was added with a microsyringe. The mixture was hermetically sealed and equilibrated at room temperature for 24 h prior to heating from 25°C to 120°C at 10°C/min. An empty pan was used as reference.

Hydrolysis of sweet potato processing waste

Pre-hydrolysis treatment

Thawed sweet potato waste was mixed 1:1 with deionized water, and boiled in a covered glass container for 5 min to decrease microbial load. Mixture was cooled to 35°C, and half of the mixture was removed and homogenized with a Waring commercial laboratory blender at speed 6 for 3 min. The portion of waste remaining was further split and one-half was centrifuged at 3,900 x g for 30 min while the remainder was untreated. Following centrifugation, the liquid fraction was isolated and the solids were discarded. The homogenized mixture was also split into two portions—one centrifuged and one untreated (Figure 2.1).

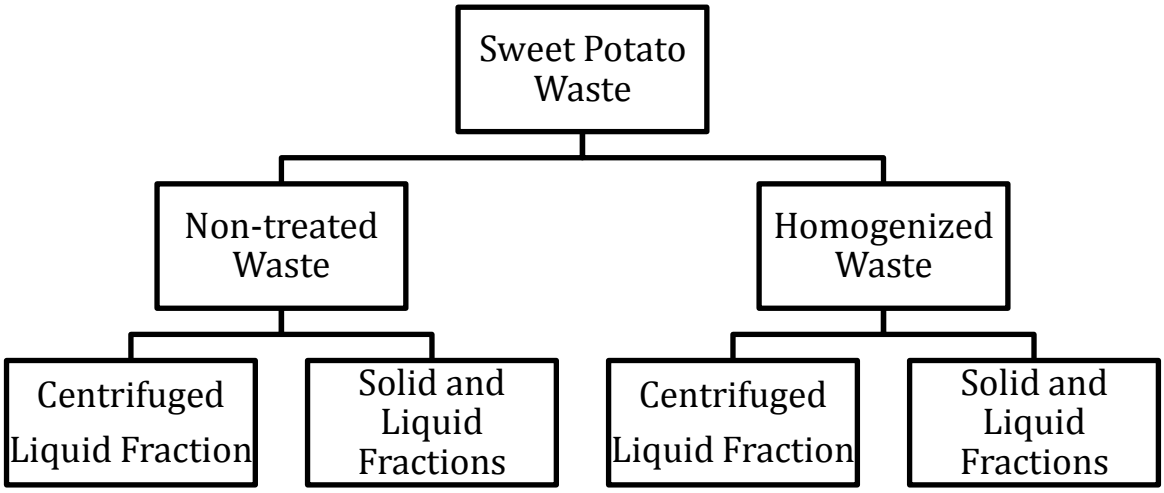


Figure 2.1 Schematic representation of pre-hydrolysis treatment of sweet potato waste

Enzyme hydrolysis

Effectiveness of pre-hydrolysis treatments was tested by addition of 100 U of glucoamylase (MP Biomedicals LLC, Solon, OH, USA) to 50 g sample in duplicate for each of the 4 pretreatment conditions, which were then incubated at 37°C for 24 h. One mL samples were extracted at various time points, placed in a boiling water bath for 5 min to inactivate the enzyme, and centrifuged at 3,900 x g for 10 min. The resulting supernatant was analyzed for glucose concentration using Megazyme's GOPOD assay kit (glucose oxidase/peroxidase). The assay kit procedure was addition of 1.5 mL of Glucose Determining Reagent to 0.05 mL sample and incubated at 45°C for 20 min. Absorbance at 510 nm was read and compared with the D-glucose control. Calculation of glucose concentration was as follows:

$$D-Glucose\,(mg/mL)=\frac{\Delta A_{sample}}{\Delta A_{standard}}\times dilution\,factor \qquad \text{[Equation 2.4]}$$

Where:

ΔA_{sample} = Absorbance of sample at 510 nm read against a reagent blank
$\Delta A_{standard}$ = Absorbance of glucose standard at 510 nm read against a reagent blank

Effectiveness of sweet potato processing waste starch hydrolysis by α-amylase (MP Biomedicals LLC, Solon, OH, USA) was measured using a modified Park-Johnson assay for reducing sugars. One gram of sweet potato waste powder was mixed with 30 mL deionized water, the pH adjusted to 6.5 with 1 M KOH, and heated to 35°C on a magnetic stirring platform. Eighteen mg of α-amylase was added, and samples were taken at time 0, 1, 2, and 3 h. Samples were then placed in a boiling water bath for 5 min to inactivate the enzyme, and centrifuged at 3,900 x g for 10 min. Liquid was retained for analysis, and solids pellet was discarded. One mL of the liquid sample, diluted to contain approximately 5 μg of reducing sugars, was mixed with 1 mL of reagent A (0.5 g $K_3Fe(CN)_6$ dissolved in 1 L deionized water) and 1 mL of reagent B (4.8

g Na_2CO_3 + 9.2 g $NaHCO_3$ + 0.65 g KCN dissolved in 1 L deionized water) in a ground glass stoppered test tube (1.5 cm x 20 cm) and heated in a boiling water bath for 20 min. After cooling for 5 min in running tap water, 2.5 mL of reagent C (3 g $NH_4Fe(SO_4)_2$ dissolved in 1 L of 0.05 M H_2SO_4) was added and allowed to stand at room temperature for 20 min. Absorbance was determined at 715 nm, and reducing sugars were calculated using a standard curve generated by maltose.

Enzyme hydrolysis of sweet potato waste by α-amylase and glucoamylase was completed by adding 35 g of sweet potato waste material (as is) and various enzyme concentrations to 250 mL Erlenmeyer flasks on a Barnstead/Thermolyne (Dubuque, IA, USA) heat controlled stirring platform and incubated at 37°C for 3 h. Experimental conditions are shown in Table 2.1. Alpha-Amylase concentrations were: low = 0.001 g of enzyme per gram of waste material, medium = 0.01 g of enzyme per gram of waste material, and high = 0.1 g of enzyme per gram of waste material. Glucoamylase concentrations were: low = 10 units of enzyme per gram of waste material, medium = 50 units of enzyme per gram of waste material, and high = 200 units of enzyme per gram of waste material.

Table 2.1 Enzyme concentrations used for each experimental condition

Condition	α-amylase	glucoamylase
1	0	0
2	low	0
3	medium	0
4	high	0
5	0	low
6	low	low
7	medium	low
8	high	low
9	0	medium
10	low	medium
11	medium	medium
12	high	medium
13	0	high
14	low	high
15	medium	high
16	high	high

Each condition was run in duplicate and sampled at time 0, 1 h, and 3 h, and enzyme effectiveness was measured using GOPOD assay kit. Conditions 1-16 were randomized and, since a maximum of 4 conditions could be tested per run due to equipment limitations, blocked for run using JMP 9.0 software (Cary, NC, USA). Blocks were grouped as shown in Table 2.2.

Enzyme hydrolysis of sweet potato waste by glucoamylase was completed by adding 35 g of waste material (as is), which was at pH 5.0, and enzyme concentrations of 0.4 U, 0.8 U, 2.0 U, or 4.0 U (U=units enzyme per gram waste material) to 250 mL Erlenmeyer flasks on a heat controlled stirring platform and incubated at 37°C for 24 h. Triplicate conditions were randomized (Table 2.3) but not blocked, since all conditions were in the same run. Enzyme effectiveness was measured using GOPOD assay kit.

Statistical analysis

Statistical analysis was performed using JMP 9.0 software (Cary, NC, USA). Experiments were designed as a full factorial and completely randomized (with or without block), and analysis was ANOVA ($p < 0.05$), followed by least square means comparison ($\alpha=0.05$) using Tukey HD. All statistical models were set up to test for main effects as well as effect interactions.

Table 2.2 Randomized blocks for enzyme hydrolysis

Run 1	Condition
1	4,13,15,1
2	6,5,10
3	16,11,14
4	1,11,2
5	13,7,8
6	3,2,7
7	9,15,4
8	6,12,10
9	8,5,9
10	14,16,12,3

Table 2.3 Glucoamylase concentrations in randomized flask conditions

Condition	Glucoamylase
1	0.4 U
2	2.0 U
3	0.4 U
4	2.0 U
5	0.8 U
6	0.8 U
7	4.0 U
8	4.0 U
9	0.8 U
10	2.0 U
11	0.4 U
12	4.0 U

RESULTS AND DISCUSSION

Characterization of sweet potato processing waste

Solids content of the sweet potato processing waste material from Allen canning, Inc. averaged 16.5%. Carbohydrates were composed of 19.0% starch, 20.5% sugars, and the rest assumed to be fibrous material. Sugars were almost entirely sucrose, which was confirmed by comparison with known monosaccharide standards using HPSEC-RI.

The dried sweet potato powder had a fairly high ash content of 18.5%. Total nitrogen was 0.70%, or 4.4% total protein. Minerals present include 0.25% phosphate, 2.98% potassium, 0.62% calcium, 0.11% magnesium, 0.08% sulfur, 498 mg sodium /kg, 919 mg iron/kg, 161 mg manganese/kg, 15.7 mg zinc/kg, 10.7 mg copper/kg, and 9.4 mg boron/kg. The minerals present are similar to various chemically defined media formulations for acidic microorganisms, and the starting pH of the waste is 5.0. Many of the essential nutrients for growth, namely a carbon source, essential amino acids, and various minerals, are present in sweet potatoes and were found to be present in the waste material. The comparison of media formulations in Table 2.4 verifies the potential for this waste material to be a good substrate for lactic acid fermentation.

Table 2.4 Comparison of defined media formulations to whole sweet potato and the sweet potato waste material

	Component	MPL[a]	CDM1[b]	Wine CDM[c]	Sweet Potato[d,e]	Sweet Potato Processing Waste
Carbon Sources	Lactose	✓				
	Glucose		✓			✓
	Fructose		✓			✓
	Sucrose					✓
	D-Ribose			✓		
Nucleic and Amino Acids	Adenine	✓	✓	✓		
	Cytosine			✓		
	Guanine	✓	✓	✓		
	Thymine			✓		
	Xanthine	✓	✓	✓		
	Uracil	✓	✓	✓		
	L-Glutamine	✓	✓	✓		
	Essential Amino Acids	✓	✓	✓	✓	
Salts, Trace Elements, Minerals, Selective Agents	Sodium thioglycolate	✓				
	Tween 80	✓	✓			
	Na_2HPO_4	✓				
	$NaH_2PO_4.H_2O$	✓				
	KH_4PO_4		✓			✓
	K_2HPO_4		✓	✓		
	Potassium acetate	✓				
	Sodium acetate		✓			
	NaCl		✓			✓
	$CaCl_2$	✓		✓		✓
	$MgSO_4.7H_2O$	✓	✓	✓		✓
	$MnSO_4.H_2O$	✓	✓	✓		✓
	NH_4Cl		✓			
	$FeSO_4.7H_2O$	✓	✓	✓		✓
	$(NH_4)_6Mo_7O_{24}.4H_2O$	✓				
	$CoCl_2.6H_2O$	✓				
	H_3Bo_3	✓				✓
	$CuSO_4$	✓		✓		✓
	$ZnSO_4.7H_2O$	✓		✓		✓
	Cobalamin (B12)	✓	✓	✓		
	Riboflavin	✓	✓	✓	✓	
	Ca pantothenate	✓	✓	✓		
	Niacin	✓				
	Nicotinic Acid		✓	✓	✓	
	Folic Acid	✓	✓	✓		
	Pyridoxal	✓				
	Pyridoxine		✓	✓		
	Thiamin		✓	✓	✓	
	p-Aminobenzoate		✓	✓		
	D-Biotin		✓	✓		
	Myo-inositol		✓	✓		
	Choline Chloride			✓		

[a] Chervaux et al. (2000)
[b] Savijoki et al. (2006)
[c] Terrade et al. (2009)
[d,e] Kotecha and Kadam (1998) and Purcell et al. (1978)

DSC analysis proved the state of starch in the waste material was already gelatinized, because replicates showed no endotherms at typical starch gelatinization temperatures of 50-80°C. Endotherms are a graphical representation of thermal events, and since gelatinization is defined as a melting of starch crystalline structure (Zobel et al. 1988), presence of an endotherm denotes a change in starch structure–there is a change in phase from crystalline to non-crystalline–which requires additional energy/heat. The state of starch in the sweet potato processing waste is important because it dictates the treatment required for enzymatic hydrolysis. A starch granule in its raw/native state is not easily digestible by enzymes, whereas gelatinized starch has lost its crystalline structure and is easily accessible to enzyme action.

Hydrolysis of sweet potato processing waste

Pre-hydrolysis treatment

Pretreatment was applied in an attempt to release additional hydrolysable starch from the solids fraction of the sweet potato waste. Comparison of glucose concentration after enzyme treatment (Table 2.5) yielded no significant difference between the non-treated sweet potato waste and pre-treatment homogenization. Removal of solids before enzyme treatment resulted in a significantly lower glucose concentration than hydrolysis of the whole material, indicating that there are additional starch particles in the solids fraction that are accessible to enzyme action. It was determined that pretreatment of the waste material by blending or centrifugation was not necessary to obtain maximum hydrolysis yields. This is advantageous due to the minimal processing required to hydrolyze this waste material into a fermentable substrate.

Table 2.5 Glucose concentrations resulting from enzyme hydrolysis of pre-treated sweet potato processing waste. Means sharing the same letter are not significantly different

Treatment	Glucose concentration (g/L)
Non-treated solids and liquids fraction	11.38[a]
Non-treated centrifuged liquid fraction	8.69[b]
Homogenized solids and liquids fraction	11.41[a]
Homogenized centrifuged liquid fracton	11.48[a]

Enzyme hydrolysis

Two enzymes, α-amylase and glucoamylase, were tested separately and in combination, in an attempt to determine the most efficient conditions to release maximum fermentable sugars, namely glucose, using the lowest possible enzyme concentration(s). Alpha-amylase hydrolysis of sweet potato waste was followed by the presence of reducing sugars (Figure 2.2). A reducing sugar, i.e. a sugar containing a free or potentially free aldehyde group, is formed during hydrolysis of starch, and an increase in reducing sugars is directly related to enzyme degradation (Kruger and Marchylo, 1972, Chang Rupp and Schwartz, 1988). Figure 2.2 shows that there was close to a 3-fold increase in reducing sugars of the sweet potato waste after 3 h of enzymatic treatment.

Results from hydrolysis of the waste material using both α-amylase and glucoamylase at three h can be seen in Figure 2.3. Only the highest concentration of α-amylase was significantly better than the other concentrations, and there was no significant difference at the one h time point. As can be expected in the test conditions containing only α-amylase, there is very little glucose produced in three h because α-amylase works to reduce molecular weight of starch molecules, not produce glucose. Conversely, increasing concentrations of glucoamylase produce increasing concentrations of glucose, and at each concentration, the enzyme produced more glucose with more time. Each set of glucoamylase concentrations should have increased levels of glucose as there is an increase in α-amylase concentrations. There is only a small effect of α-amylase on the efficiency of glucose production from the starch in this waste material, and brings into question the necessity of its addition.

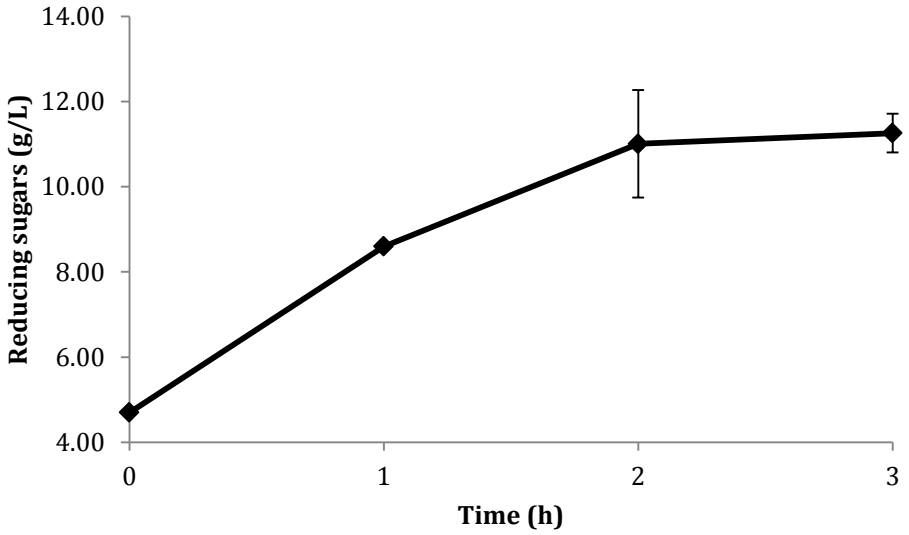


Figure 2.2 Changes in reducing sugars on 1 g of sweet potato waste by the action of 18 mg α-amylase

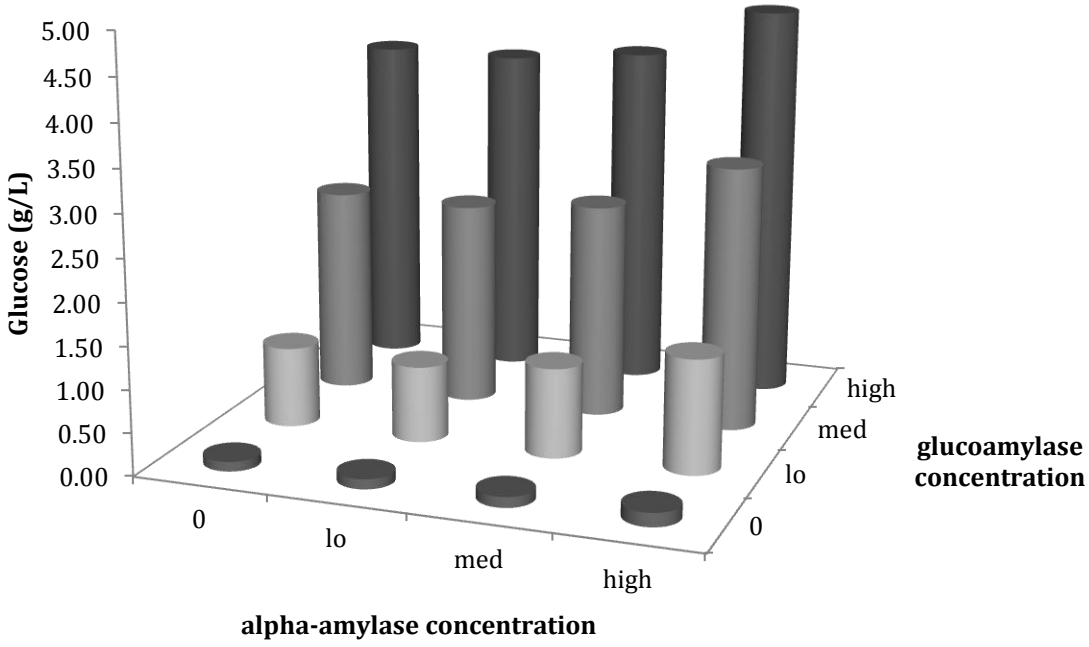


Figure 2.3 Sweet potato processing waste hydrolysis at 3 h with α-amylase and glucoamylase

Figure 2.4A represents the sweet potato waste material with no enzyme treatment. These peaks are starch fragments, and are likely present due to the processing conditions used at the canning facility where this waste material is produced. The steam peeling process results in destruction of the starch granules and fragmentation of the amylose and amylopectin. The heat and pressure have broken apart the starch molecules, and consequently reduced the components to dextrins easily hydrolyzed by glucoamylase alone. This is also a likely explanation for why there is no gelatinization endotherm during DSC, since there are no intact crystalline structures.

Figure 2.4B, C, and D represent the samples taken from the enzyme treated sweet potato waste after three h. A comparison of plots A and B indicate almost no effect of α-amylase on size decrease of the starch fragments. A comparison of plots A and C indicate the action of glucoamylase is effective in hydrolysis of the starch fragments to glucose (peak present at 45.3 min), and a comparison of plots C and D indicate a very slight synergistic effect of the two enzymes in producing glucose.

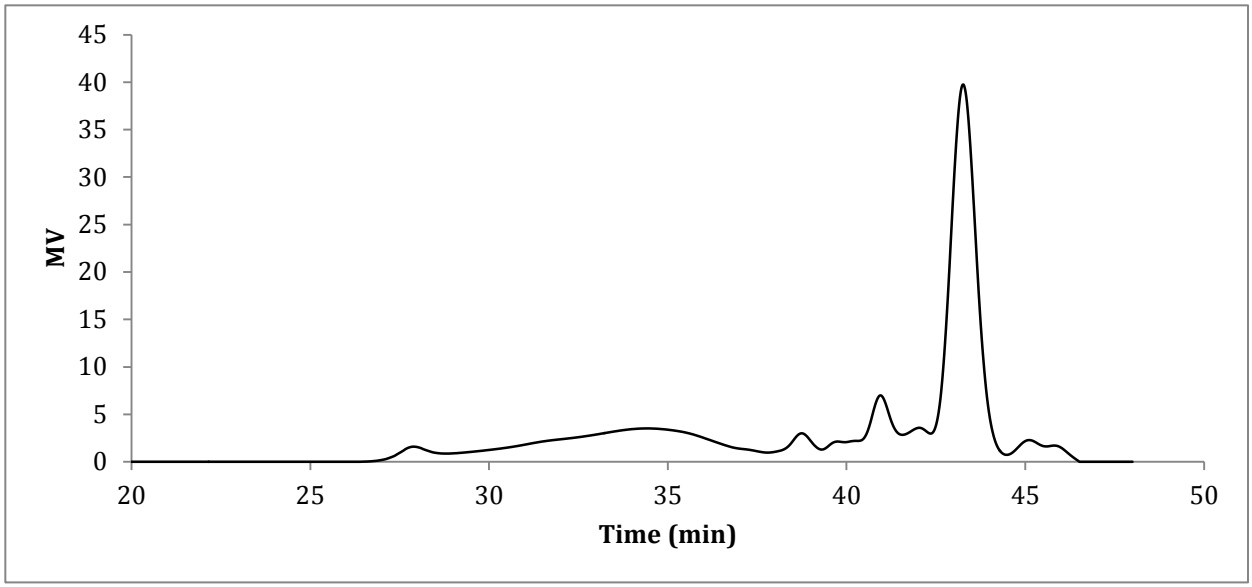


Figure 2.4A HPSEC-RI profile of sweet potato waste samples after three h with no α-amylase and no glucoamylase

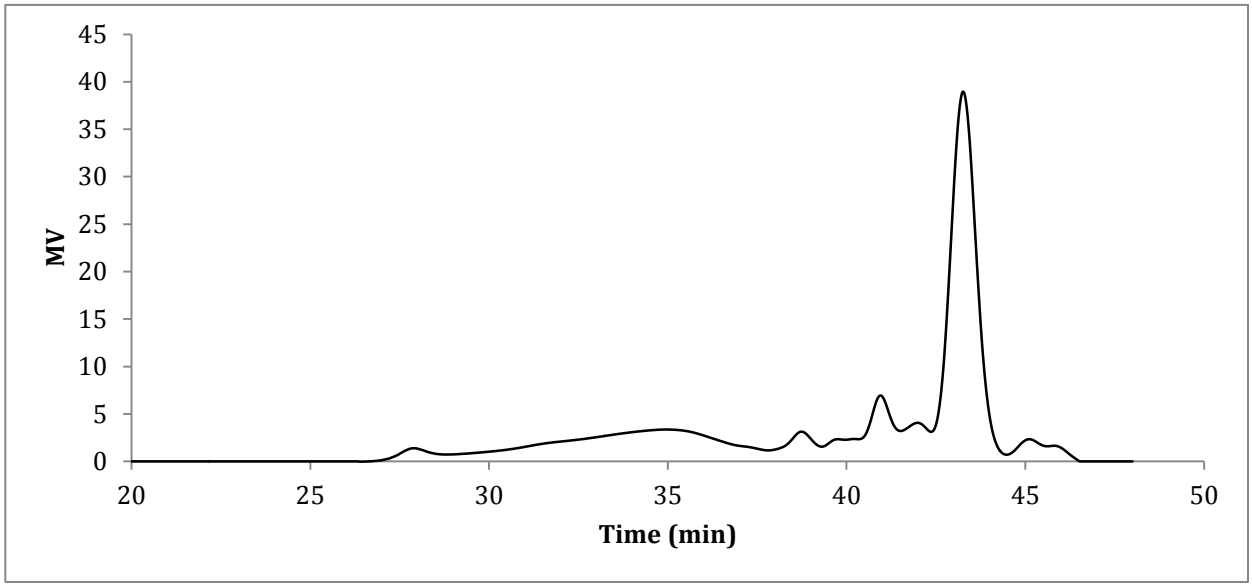


Figure 2.4B HPSEC-RI profile of sweet potato waste samples after three h with a high concentration of α-amylase and no glucoamylase

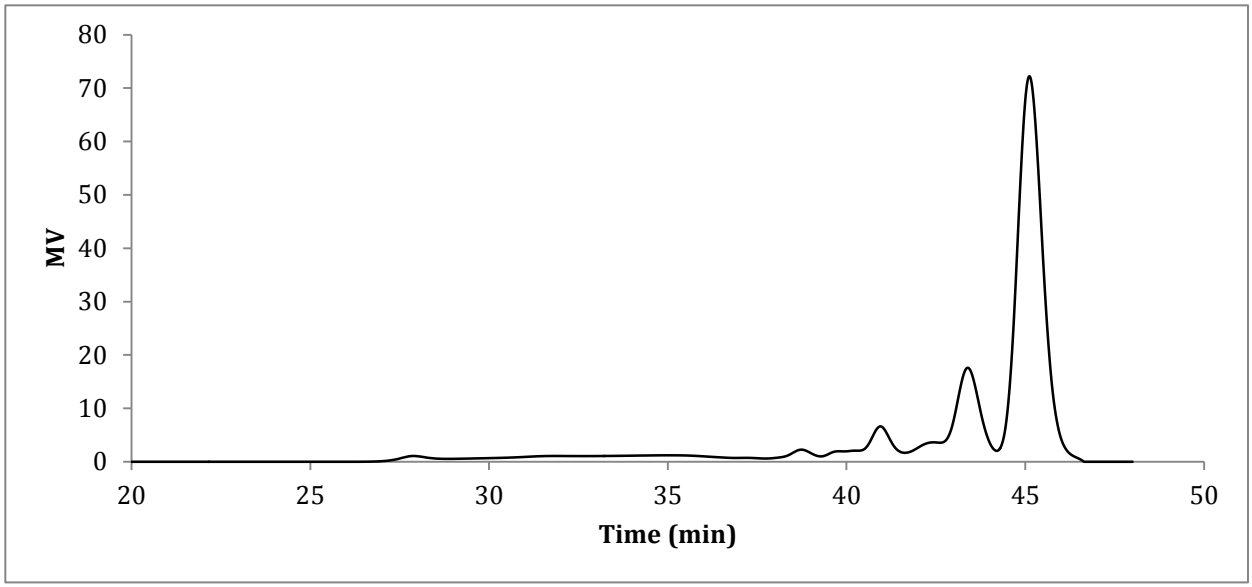


Figure 2.4C HPSEC-RI profile of sweet potato waste samples after three h with no α-amylase and a high concentration of glucoamylase

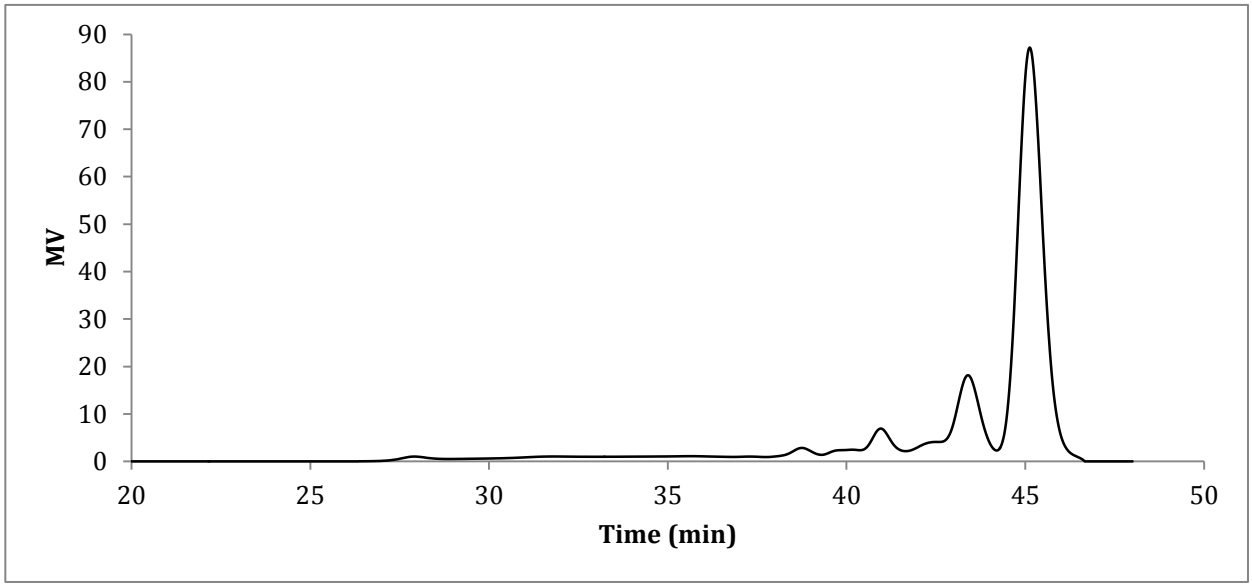


Figure 2.4D HPSEC-RI profile of sweet potato waste samples after three h with a high concentration of α-amylase and a high concentration of glucoamylase

Due to the minimal effects of α-amylase and the likely presence of starch fragments seen in the HPSEC-RI profiles, it was determined that the only enzyme necessary for hydrolysis of the sweet potato waste was glucoamylase. This enzyme was tested on the sweet potato processing waste for the lowest concentration that would yield maximum glucose in 24 h. Figure 2.5 indicates that the enzyme activity continues through 24 h, since the lowest enzyme concentration is still increasing in glucose at the last tested time point. The use of a higher concentration of glucoamylase results in a faster conversion of starch to glucose, with the highest concentration completing hydrolysis within six h. Theoretical hydrolysis of 100 g sweet potato waste, with a solids content of 17% and soluble starch content within the solids of 19%, is 3.2 g of glucose. At 24 h, all levels of glucoamylase concentrations tested, with the exception of the lowest, result in very close to maximum theoretical yield. It is quite possible that the lowest concentration of enzyme would eventually reach this level of hydrolysis given more time. As a result, it is possible to use lower enzyme concentrations and still achieve the same level of hydrolysis. For the purpose of this set of experiments, a balance of enzyme concentration and time to full hydrolysis was required. The chosen hydrolysis conditions moving forward will be 80 U/100 gram of sweet potato waste with an incubation time of 24 h.

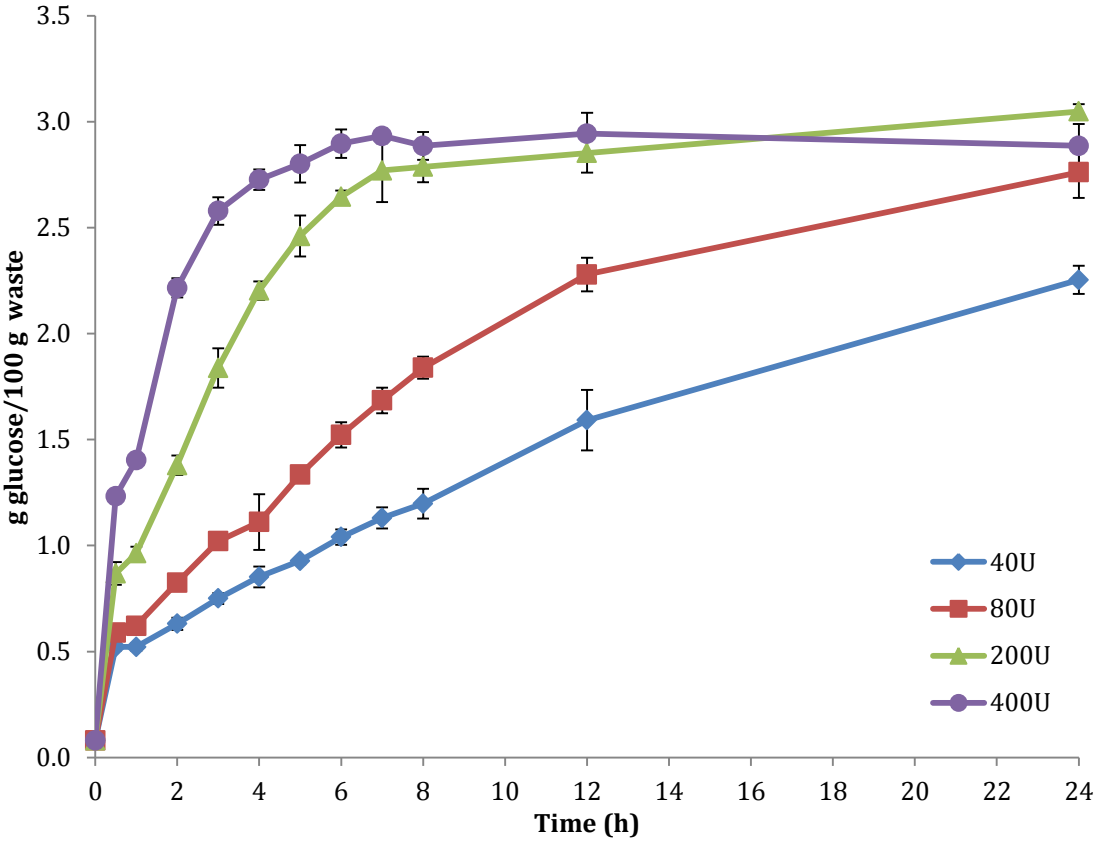


Figure 2.5 Hydrolysis of sweet potato waste by glucoamylase (U/100 g waste)

CONCLUSION

The waste produced from sweet potato canning facilities is a potentially good source for a fermentation substrate. The presence of several essential nutrients indicates the possibility that this substrate could be used for fermentation without supplementation. The steam peeling process results in starch that is fragmented due to the extreme temperature used. This fragmentation results in a simpler hydrolysis process, since no pretreatment of the starch in the waste material is required for efficient enzyme hydrolysis. Glucoamylase action on the available starch fragments in the sweet potato processing waste provided levels of glucose that are comparable to several commercially used fermentation media (Table 2.4). For full hydrolysis of available starch in 24 h, the minimal enzyme concentration was 80 U/100 g of sweet potato waste material.

Chapter 3: FERMENTATION OF HYDROLYZED SWEET POTATO PROCESSING WASTE

INTRODUCTION

Fermentation of hydrolyzed agro-wastes using lactic acid bacteria (LAB) is affected by substrate, LAB strain, and production mode and parameters (John et al. 2007). There are many strains of LAB, and each displays a unique set of nutritional requirements and production kinetics (Litchfield 1996), therefore it is critical to match strain to substrate for optimal production of lactic acid. Due to the recent uses for lactic acid in the bioplastics industry, LAB that produce only one stereoisomer will be evaluated for growth and production in the hydrolyzed sweet potato waste. Some tools used to evaluate and compare strains include: viable cell counts, pH monitoring and controlling, glucose consumption, and lactic acid production. Production of lactic acid from the hydrolyzed sweet potato production waste was the goal of this project, and all of these tools were used to evaluate and optimize production. The objective of this chapter (Objective 2 of the research) was to screen lactic acid bacteria strains that would maximize the production of lactic acid using as a substrate hydrolyzed sweet potato waste obtained in Chapter 2.

MATERIALS AND METHODS

Materials

Nine LAB were received from the USDA Agriculture Research Service (Washington, DC, USA). Each strain was a dried pellet contained in a glass vial, and the general characteristics of each strain are listed in Table 3.1.

Table 3.1 Characteristics of LAB strains

Strain	Lactic Acid Isomer	Metabolism	Ideal Temperature	Reference
L.delbrueckii subsp. *delbrueckii*	D	Homofermentative	37°C	Litchfield (1996)
L.rhamnosus	L	Heterofermentative	37°C	Litchfield (1996)
L.amylophilus	L	Homofermentative	30°C	Litchfield (1996)
Sporolactobacilus inulinus	D	Homofermentative	37°C	Litchfield (1996)
L.pentosus	DL	Heterofermentative	37°C	Zanoni et al (1987)
L.delbrueckii subsp. *bulgaricus*	D	Homofermentative	37°C	Litchfield (1996)
L.sharpeae	L	Homofermentative		Kandler and Weiss (1986)
L.ruminis	L	Homofermentative		Kandler and Weiss (1986)
L.salivarius subsp. *salicinius*	L	Homofermentative	37°C	Kandler and Weiss (1986)

Each glass vial was stored at 4°C until use. All manipulation of live cell cultures was completed in a class II type A2 biosafety cabinet (ESCO, Hatboro, PA, USA) using sterile technique. The growth medium used for strain comparison was Difco YM Broth (Becton Dickinson, Franklin Lakes, NJ, USA), which was solubilized by addition of 21.0 g powder into 1 L MilliQ water and filtered through a sterile 0.22-μm polyethersulfone (PES) membrane from Corning (Corning, NY, USA). To preserve LAB by freezing, dimethylsulfoxide (DMSO) from Sigma Aldrich (St. Louis, MO) was used as a media supplement to YM Broth, and vials, a freezing jar, and freezer storage boxes were all obtained from Nalgene (Rochester, NY, USA). Enumeration of cells was accomplished using a Hausser Scientific brightline hemacytometer (Horsham, PA, USA), and cell viability was assessed using a 0.4% trypan blue liquid solution from Amresco (Solon, OH, USA). Lactic acid standards used for detection and quantification method development were 85% pure L-lactic acid (Purac, Lincolnshire, IL, USA), 95% pure D-lactic acid (Sigma-Aldrich, St Louis, MO, USA), and an 85% pure equal mixture of D (-) and L (+) lactic acid (Sigma-Aldrich, St Louis, MO, USA). Cell culture supernatants for lactic acid analysis were passed through 0.45-μm Nylon syringe filters from Acrodisc (Pall, Port Washington, NY, USA). Hydrolyzed sweet potato waste used for fermentation was diluted using MilliQ filtered water (Millipore, Germany), and any pH adjustment during fermentation was achieved with dropwise addition of 1 M NaOH. After each sampling event, culture vessels were flushed with filtered nitrogen gas for 5 s and then tightly sealed.

Methods

Growth and proliferation

Initial cultures received from the USDA-ARS were reanimated by suspension of the pellet in 20 mL of fresh growth medium in a sterile 250 mL flask. Flasks were incubated at 30-

37°C, depending on strain requirements, on a MaxQ 4450 shaking platform (Thermo Scientific, Waltham, MA, USA) maintained at 95 rpm. After 24-48 h, backup cultures were frozen in growth medium supplemented with DMSO to a final concentration of 10% in sterile, 2 mL freezing vials. These vials were placed in a freezing jar and stored at −80°C for 24 h. Frozen vials were then transferred to a storage box and stored at −80°C until use.

For growth, each vial of a desired LAB strain was quickly thawed by submersion in a water bath set at 37°C. Once there were no longer any visible ice crystals, the contents of the vial were placed in 8 mL of growth medium and centrifuged at 1,900 x g for 5 min. Once the resulting supernatant was discarded, the cell pellet was resuspended in 20 mL growth medium, transferred to a sterile 250 mL flask, flushed with filtered nitrogen gas, and incubated at 30-37°C with shaking at 95 rpm.

Cells were counted using a hemacytometer with counting chambers (as shown in Figures 3.1 and 3.2) and a microscope with a 45x objective (Bausch and Lomb, Rochester, NY, USA). Cell suspension was diluted in YM broth to yield approximately 2-4x10^6 viable cells per mL. The diluted cell suspension was well mixed on a vortex mixer, and a 50 µL aliquot was mixed with 50 µL trypan blue solution in a 1.5 mL Eppendorf tube. For analysis, a 10 µL sample of the trypan blue dyed cell suspension was slowly injected under the cover slip and into the hemacytometer chamber. One square millimeter of grid was counted, and cell concentration calculated (according to Eq. 3.1). Dead cells were differentiated from live cells by color and movement. Dark blue/black cells that were not visibly moving were counted as dead, and light reflective white, light blue, or blue cells showing movement were counted as live.

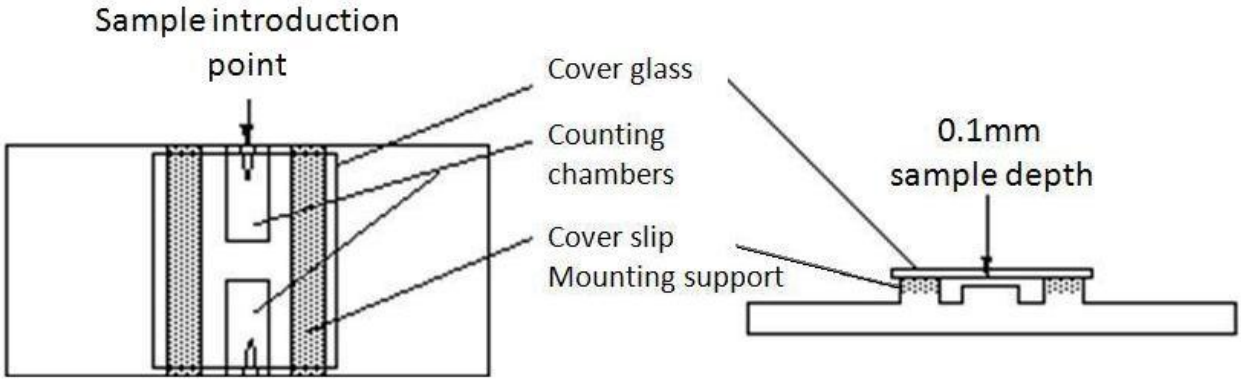


Figure 3.1 Hausser brightline hemacytometer (image from www.dtc.pima.edu)

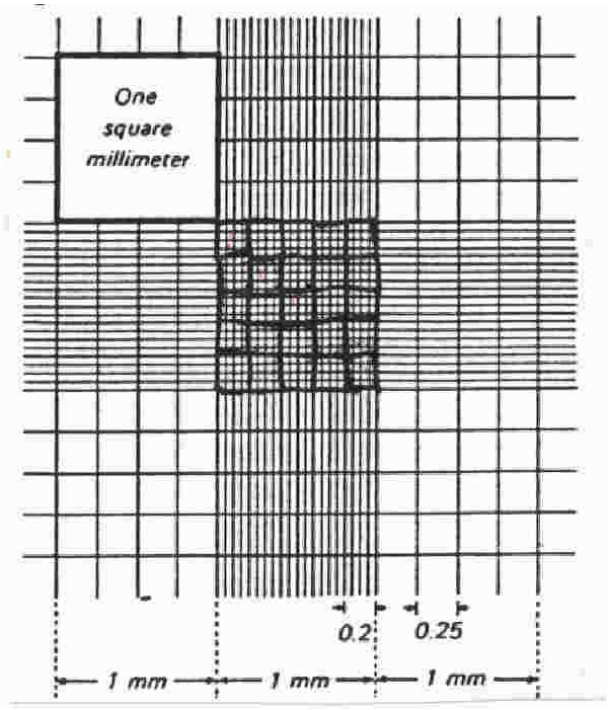


Figure 3.2 Magnified hemacytometer counting chamber grid (image from www.dtc.pima.edu)

$$\frac{Viable Cells}{mL} = number of\ cells\ in\ 1\,mm^2 \times dilution\ factor \times \frac{10^4\,mm^2}{mL} \qquad \text{[Equation 3.1]}$$

Fermentation

Comparison of growth and production kinetics from the viable LAB strains was carried out in duplicate sterile, 500 mL flasks with a fill volume of 200 mL, a starting cell inoculum of 1-2x10^5 viable cells per mL (VC/mL), and incubation on a shaking platform set at 95 rpm and 37°C. All cultures were started at the same time, and samples were taken at set time intervals between 0 and 72 h. After each sample point, flasks were flushed with nitrogen gas, resealed, and returned to the shaker platform. Each culture sampling consisted of a 3-mL extraction of culture liquid with a sterile pipette, placement of this sample into a 15-mL centrifuge tube, and vortex mixing at medium-high speed for 30 s. An aliquot of each sample was diluted for cell count determination with a hemacytometer, and the remainder of the sample was centrifuged at 3,900 x g for 10 min. The pH of the resulting supernatant was determined by submersion of a sympHony SP70P pH probe (VWR, Radnor, PA, USA). Following the pH determination, the supernatant was transferred to 1.5 mL Eppendorf tubes and stored at −20°C for glucose and lactic acid analysis. The cell pellet was discarded.

Fermentation using hydrolyzed sweet potato waste material (hydrolysis was achieved according to the procedure outlined in chapter 3) was carried out in duplicate sterile, 500 mL flasks with a fill volume of 200 mL, a starting cell inoculum of 1-2x10^5 VC/mL, and incubation on a shaking platform set at 95 rpm and 37°C. Sampling of flasks was according to the same procedure as with growth and production kinetics (detailed in previous paragraph). Dilutions of the hydrolyzed sweet potato waste material used for LAB growth and production kinetics are shown in Table 3.2.

Table 3.2 Waste material dilutions and final glucose concentrations

Dilution	Hydrolyzed Sweet Potato Waste	MilliQ Water	Final glucose concentration (g/L)
1:0	200 mL	0 mL	30
1:1	100 mL	100 mL	15
1:3	50 mL	150 mL	7.5

Fermentation using hydrolyzed sweet potato waste material with pH control was carried out in duplicate sterile, 500 mL flasks with a fill volume of 200 mL, a starting cell inoculum of 1-2x10^5 VC/mL, and incubation on a shaking platform set at 95 rpm and 37°C. Sampling of flasks was according to the same procedure as with growth and production kinetics (detailed in previous paragraph). The pH was maintained by the addition of sodium hydroxide each time the flasks were sampled. Control of pH at the 5.0 set point required a total volume of 2.1 mL base addition from 9-24 h, and the 4.0 set point required a total volume of 2.1 mL base addition from 21-48 h.

Glucose and lactic acid analysis

The supernatant taken from each sample during fermentation experiments was analyzed for glucose by Megazyme GOPOD assay kit (Dublin, Ireland) as described in the previous chapter. Lactic acid concentration was determined using high-performance liquid chromatography (HPLC). The equipment was a Shimadzu (Shimadzu, Japan) consisting of two pumps (Model), an autosampler (SIL-10AF) equipped with a 50-µL sample loop, adegasser (DGV-20A3), column oven (CTO-20A) set at 65°C, and SPD-20AV UV-Vis detector set at 210 nm. Supernatant cell culture samples were thawed at 4°C, filtered through a 0.45-µm syringe filter, and 50 µL injected. Separation was conducted on 2 Biorad Aminex HPX-87H organic acid columns in series with aqueous 0.005 N H_2SO_4 at a flow rate of 0.3 mL/min. Concentrations were determined by comparison to a standard curve generated using lactic acid standards.

Statistical analysis

All experimental conditions were completely randomized and run in duplicate. Reported values were means ± the standard error.

RESULTS AND DISCUSSION

Comparison of LAB strains for growth and production

Upon reanimation from shipping vials, only three of the nine strains grew in the culture media-*L. delbrueckii* subsp. *delbrueckii*, *L. rhamnosus*, and *L. delbrueckii* subsp. *bulgaricus*. A probable explanation as to why the other six strains did not grow is their intolerance to oxygen. LAB do not possess the ability to make catalase or superoxide dismutase, so the presence of reactive oxygen species can be toxic (Kandler and Weiss 1986). Although each flask was flushed with nitrogen gas, there may have been a small amount of residual oxygen dissolved in the liquid medium that inhibited growth.

Since the ideal lactic acid production process should be simple, inexpensive, and easy to implement, the three strains chosen for continued study were the most suitable because they were the most tolerant of a microaerobic environment and grew well upon reanimation. A LAB strain that can tolerate some amount of oxygen will be a more flexible candidate to meet production goals than a strict anaerobe.

Hemacytometer counts, pH values, glucose, and lactic acid production of the three strains are shown in Figures 3.3-3.6 respectively. Growth curves in Figure 3.3 show all three strains have similar curve shapes, but *L. bulgaricus* and *L. delbrueckii* grew to a much higher cell density than *L. rhamnosus*. One of the goals of this experiment was to see the three distinct phases of growth-lag, exponential, and stationary. Unfortunately, the lag phase was not readily observable for *L. bulgaricus* or *L. delbrueckii*. It probably occurred within the first nine h, but was not observed because a sample was not taken during that time. Although counting by hemacytometer allowed the differentiation of live and dead cells, there were very few dead cells,

and accurate enumeration was difficult because of the small cell size and tendency of all three strains to grow in clusters of 2-10 cells. This is a common problem, however, since bacterial cells are difficult to evaluate and accurately enumerate without expensive, specialized equipment (Edwards 1996).

Initially, the culture pH of *L. bulgaricus* and *L. delbrueckii* dropped dramatically (Figure 3.4), corresponding with a rapid increase in cell number, but after 15 h, there was no change in pH. This is the same time point when there is a transition from exponential to stationary growth phase (Figure 3.3). Conversely, *L. rhamnosus* continued to produce compounds that deceased pH throughout the exponential and stationary phases of growth. There is a notable decrease in pH between 9 h and 18 h, corresponding almost exactly with the exponential phase of growth (Figure 3.3), and then a continuous, gradual decline throughout the remainder of the experiment. LABs that are more tolerant of lower pH would be advantageous for a lactic acid production system, since they are less inhibited by the product they are producing. Based on tolerance to a lower medium pH, *L. rhamnosus* would be the preferred candidate for lactic acid production.

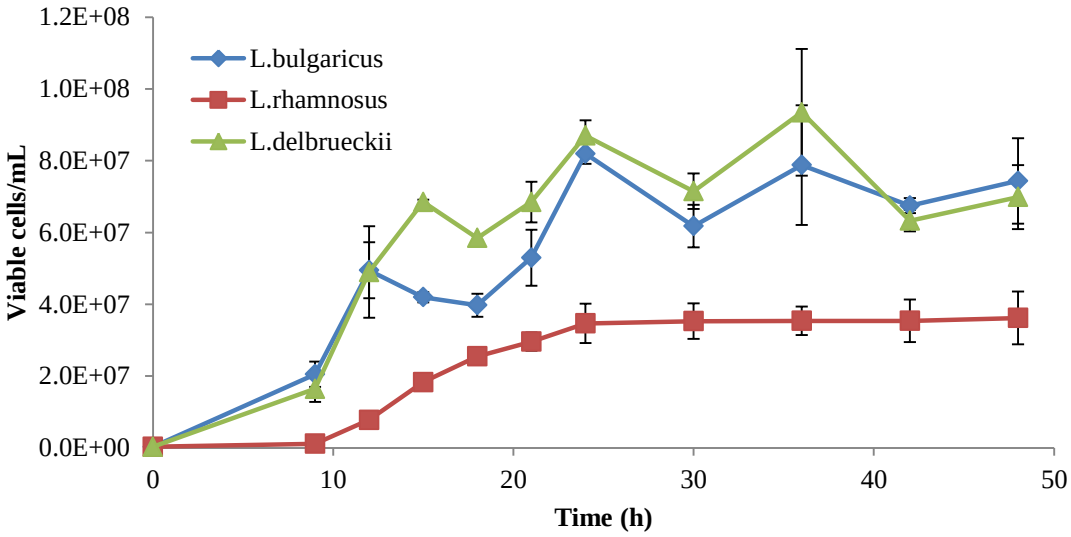


Figure 3.3 Hemacytometer counts (VC/mL) of *L. bulgaricus*, *L. delbrueckii*, and *L. rhamnosus* during growth in traditional media

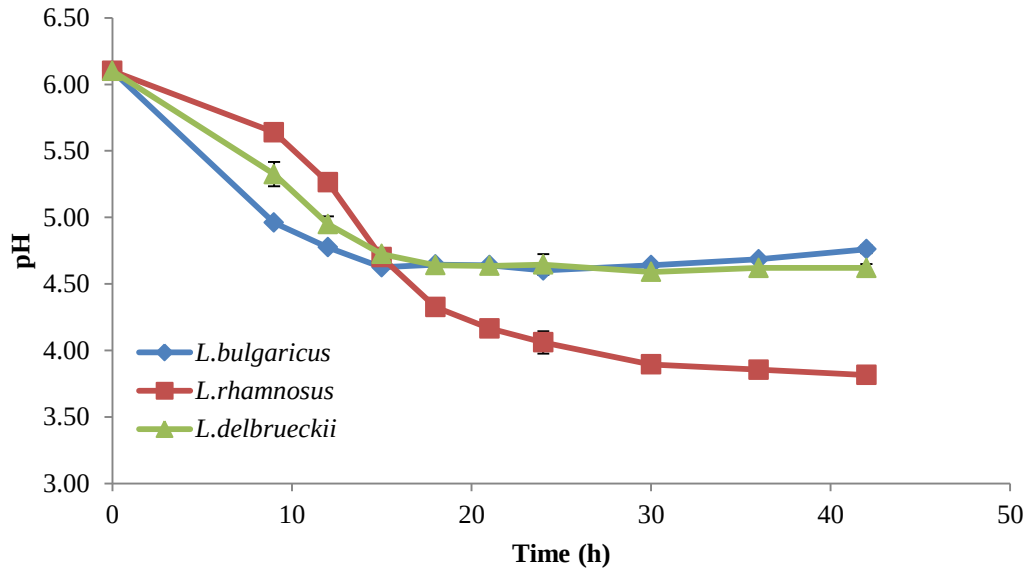


Figure 3.4. The pH measurements of *L. bulgaricus*, *L. delbrueckii*, and *L. rhamnosus* during growth in traditional media

Glucose utilization in Figure 3.5 shows a similar pattern to the pH drop in Figure 3.4. Since *L. bulgaricus* and *L. delbrueckii* are homofermentative, it is assumed that they produce lactic acid from glucose more efficiently than *L. rhamnosus*. It can be observed, however, that *L. rhamnosus* continues to metabolize glucose throughout the duration of the experiment, whereas the other strains stop after the exponential phase of growth. This is probably a function of the sensitivity of these strains to low pH, which is corroborated by the data in Figure 3.4. Overall, *L. rhamnosus* is the most tolerant and continues to metabolize despite a very low pH.

All of these measurements are indicators of the product of interest, specifically lactic acid. A comparison of lactic acid production by each of the LAB strains (Figure 3.6) agrees with the previous indicators of cell count, pH, and glucose consumption. *L. rhamnosus* continues to produce lactic acid throughout the stationary growth phase, while *L. bulgaricus* and *L. delbrueckii* stop after the exponential growth phase, probably due to pH inhibition. None of the strains utilized all of the available glucose, although *L. rhamnosus* was still metabolizing at the last time point sample. Given more time, it is very possible that most or all of the glucose could have been utilized by *L. rhamnosus*.

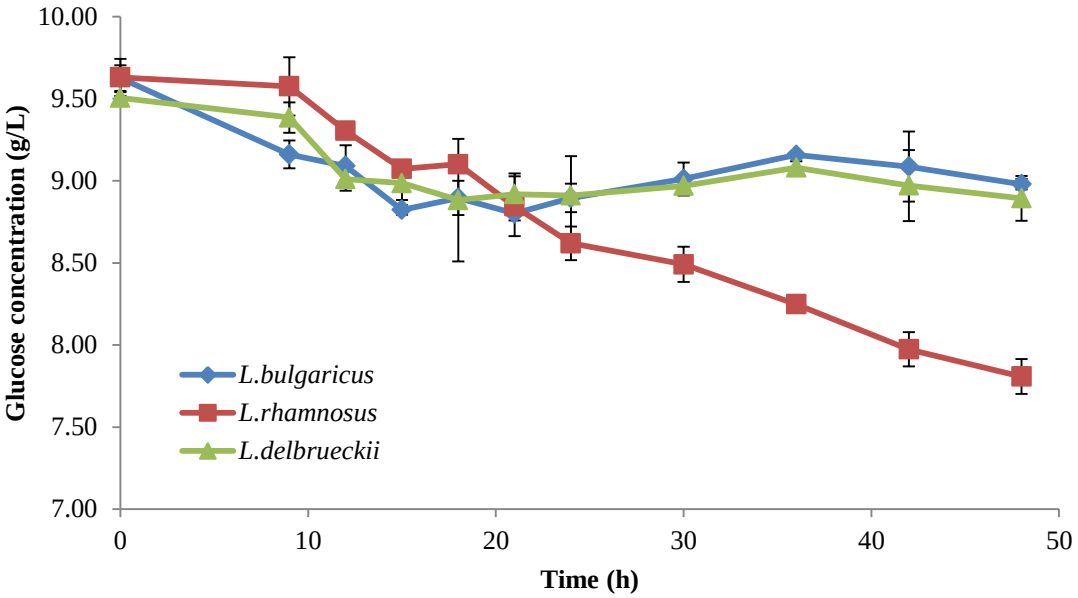


Figure 3.5 Glucose concentration of *L. bulgaricus*, *L. delbrueckii*, and *L. rhamnosus* during growth in traditional media

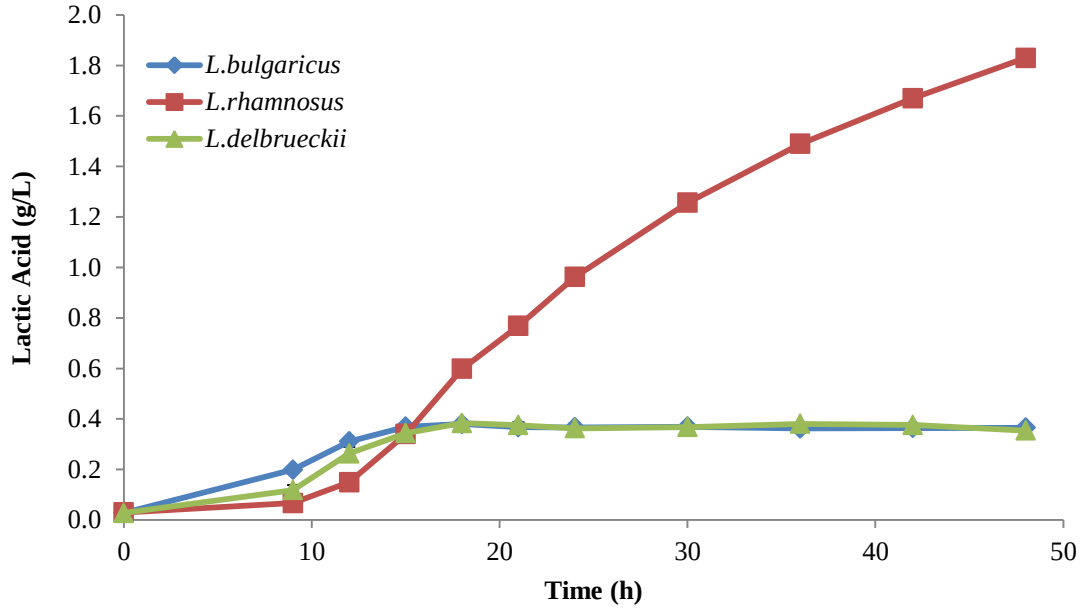


Figure 3.6 Lactic acid production of *L. bulgaricus*, *L. delbrueckii*, and *L. rhamnosus* during growth in traditional media

In order to complete a comparison of these three LAB for the purpose of selecting one for fermentation in the hydrolyzed sweet potato waste material, calculations were made to determine the efficiency of each strain at converting glucose to lactic acid. Production kinetics for each strain are represented by Figures 3.7-3.9. The following equations (3.3 and 3.4) were used to plot points and calculate the slope of a best fit line that represents the amount of lactic acid (product, in grams) produced per gram of glucose (substrate) consumed:

$$Substrate\,consumption_t = S_0 - S_t \qquad \text{[Equation 3.2]}$$

$$Lactic\,acid\,production_t = P_t - P_0 \qquad \text{[Equation 3.3]}$$

S_0 = glucose concentration at time 0
S_t = glucose concentration at time t
P_t = lactic acid concentration at time t
P_0 = lactic acid concentration at time 0

The slopes from Figures 3.7-3.9 represent lactic acid production for *L. bulgaricus, L. delbrueckii,* and *L. rhamnosus*, which were 0.43, 0.53, and 0.96 g lactic acid/g glucose, respectively. Therefore, *L. rhamnosus* produced lactic acid much more efficiently than either of the other strains and was chosen as the one strain to use for fermentation with the hydrolyzed sweet potato waste material.

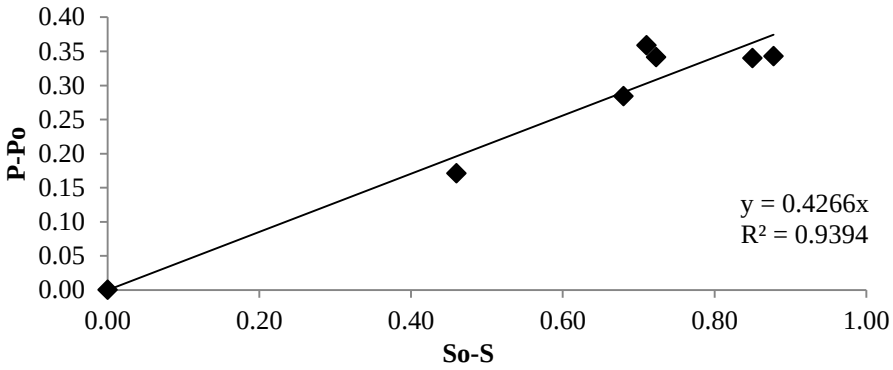


Figure 3.7 *L. bulgaricus* lactic acid production kinetics

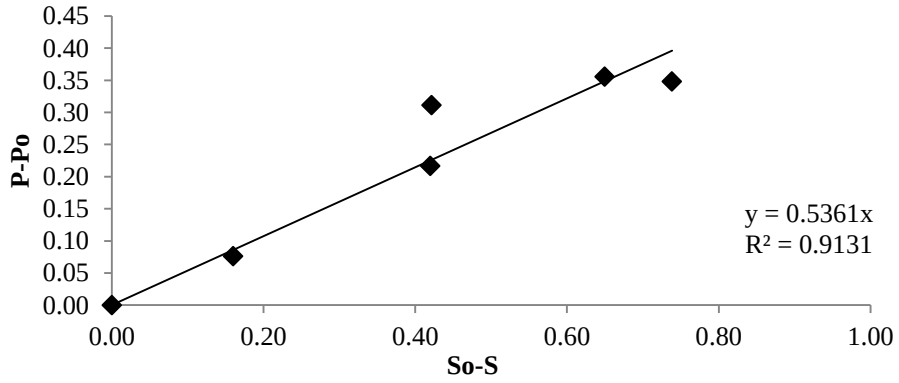


Figure 3.8 *L. delbrueckii* lactic acid production kinetics

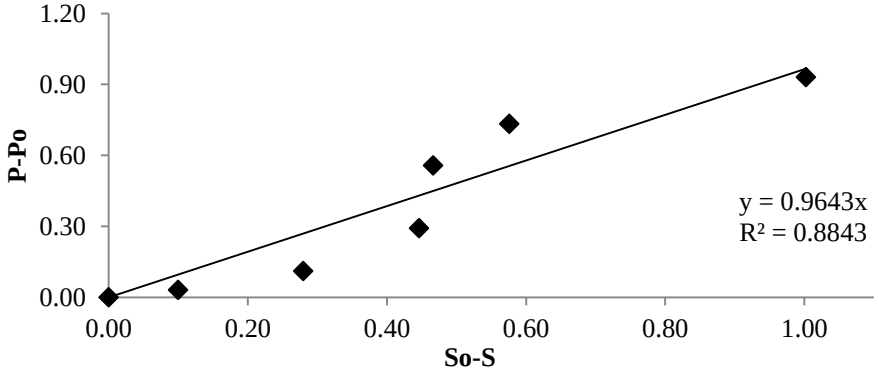


Figure 3.9 *L. rhamnosus* lactic acid production kinetics

Comparison of control medium to hydrolyzed sweet potato waste material

There was a concern that using the hydrolyzed material as is might create a highly unbalanced osmotic environment for *L. rhamnosus*, due to the high concentration of glucose–approximately 30 g/L. This is why 2 different dilutions (1:1 and 1:3), as well as the material as is (1:0) were compared to the control medium (YM Broth) for growth and production. The growth curves in Figure 3.10 show a similar rate of growth in the 1:3 diluted hydrolyzed sweet potato waste material as in the control, but the 1:1 diluted and undiluted material support a faster growth rate and a higher cell density. Due to high flask to flask variability, counting was discontinued after 36 h, although samples for glucose and lactic acid analysis were gathered through 70 h. *L. rhamnosus* grew better in the hydrolyzed sweet potato waste material than the control medium.

The pH measurements (Figure 3.11) were very similar for all conditions throughout the experimental time period. The hydrolyzed sweet potato waste material had a lower starting pH than the control medium, but after 10 h, there was little difference.

Monitoring the factors of cell growth, pH, and substrate consumption is done mainly to get an understanding of why or why not a system is producing a product, in this case, lactic acid. However, maximizing the production of lactic acid is the ultimate objective, so the total lactic acid concentration is the most vital factor in determining whether the ideal growth media for *L. rhamnosus* is the control medium or the hydrolyzed sweet potato material. A comparison of lactic acid concentrations (Figure 3.13) for each test condition reveals what the indicator measurements were hinting at–*L. rhamnosus* in the undiluted hydrolyzed sweet potato waste material produced more lactic acid in a 70-h period than the other conditions.

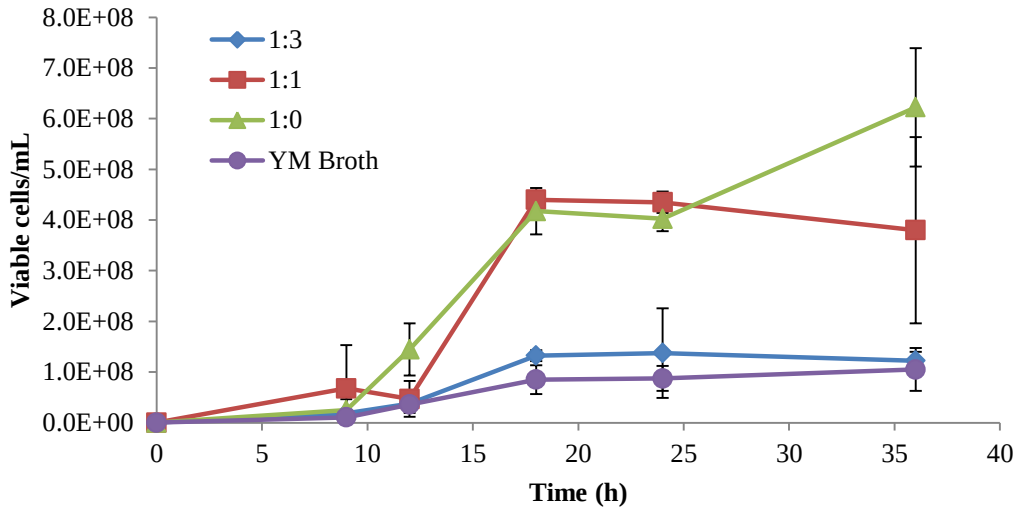


Figure 3.10 Hemacytometer cell counts (VC/mL) of *L. rhamnosus* grown in control medium and hydrolyzed sweet potato waste

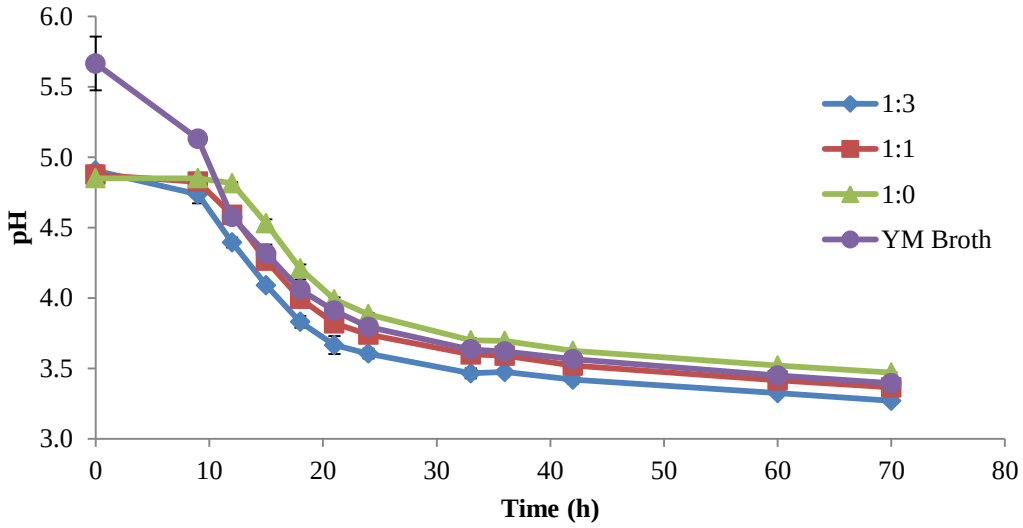


Figure 3.11 The pH values of *L. rhamnosus* grown in control medium and hydrolyzed sweet potato waste

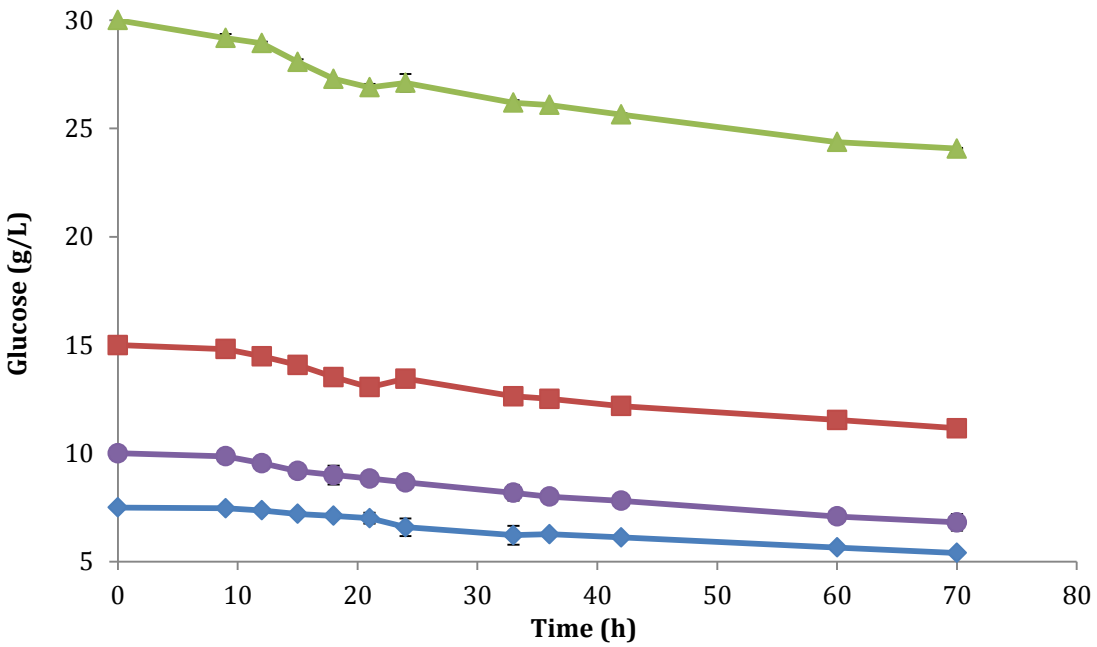


Figure 3.12 The glucose concentration at various time intervals of *L. rhamnosus* in control medium (●), 1:0 hydrolyzed sweet potato waste material (▲), 1:1 diluted hydrolyzed sweet potato waste material (■), and 1:3 diluted hydrolyzed sweet potato waste material (◆)

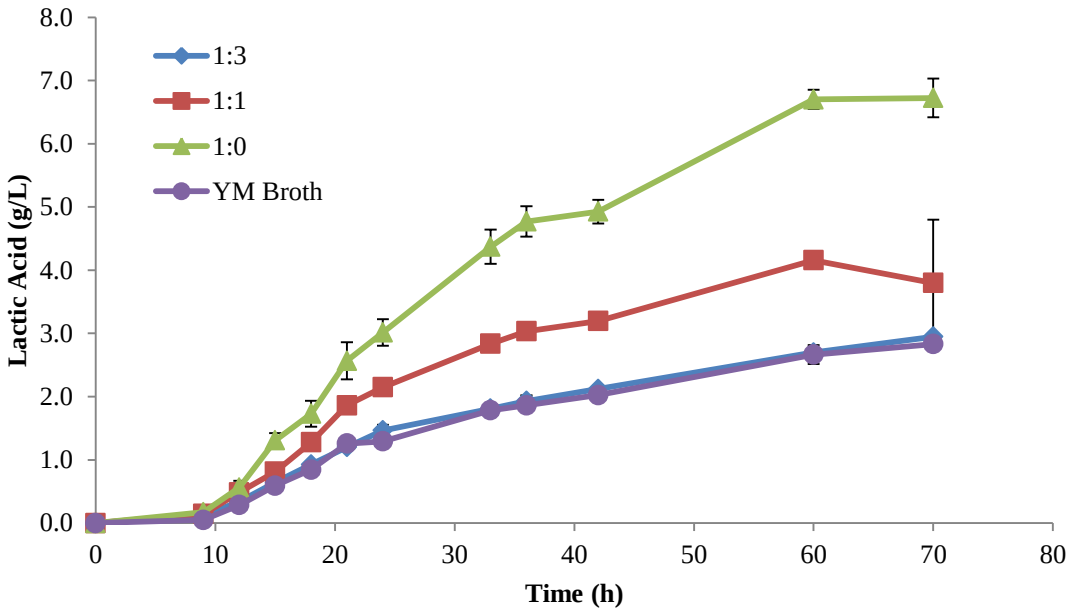


Figure 3.13 *L. rhamnosus* lactic acid production in control medium and hydrolyzed sweet potato waste

It is important to note that the hydrolyzed sweet potato material already contains a small amount of lactic acid, and the time zero points in Figure 3.13 have been adjusted by subtracting out this amount. The enzyme hydrolysis process occurs for 24 h at 37°C, and is not done in completely sterile conditions in order to mimic a potential production process at a canning facility. Before incubation with *L. rhamnosus*, the hydrolyzed material is autoclaved, so there is little chance that contaminating organisms are carried over from the hydrolysis process to the production process. The scaled down production process (shake flask) is also conducted under clean but not completely sterile conditions, in order to mimic a larger scale process. A completely sterile process would be incredibly costly and time-consuming.

An analysis of production kinetics (data not shown) reveals the same conversion of glucose to lactic acid–1.0 g lactic acid/g glucose–in all media formulations as in the previous experiment (see Figure 3.9), as well as similar lactic acid concentrations in the control medium at the same time points, demonstrating the reasonably good reproducibility of the shake flask method.

Production in hydrolyzed sweet potato waste material with pH control

Several studies have confirmed the beneficial effects of pH control during production of lactic acid (Calabia et al. 2007, Nakanishi et al. 2010, Zhang et al. 2010). There are different methods to control pH, but the simplest to employ in a small scale experiment without detrimental effects to the product is addition of base when pH drops below a set value. Figure 3.11 indicates a *L. rhamnosus* working pH range of 3.2-5, so the pH set points designated for this experiment were 4.0, 5.0, and a control, which received no pH adjustment. Figure 3.14 shows

that growth of *L. rhamnosus* was affected by pH adjustment, but only at the 5.0 set point. The cell counts of pH set point 4.0 and the control were within flask to flask variability.

It is possible that too much base addition would increase the medium osmolarity, and in turn cause the bacterial cells to swell and eventually burst. *Lactobacillus* strains are generally tolerant to high salt environments (Litchfield 1996), but can only survive up to a certain point. For this reason, the addition of base to maintain pH set points was discontinued at 24 h for set point 5.0, and 48 h for set point 4.0. Figure 3.15 shows pH values before adjustment, and directly after adjustment, for the duration of the experiment.

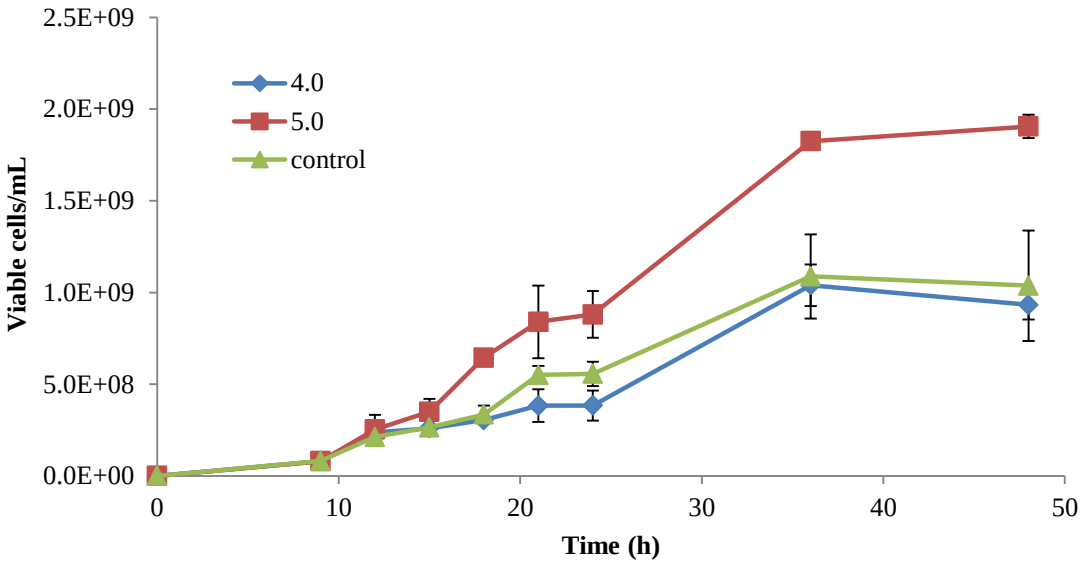


Figure 3.14 Hemacytometer cell counts (VC/mL) of *L. rhamnosus* grown in undiluted hydrolyzed sweet potato waste with various pH set points

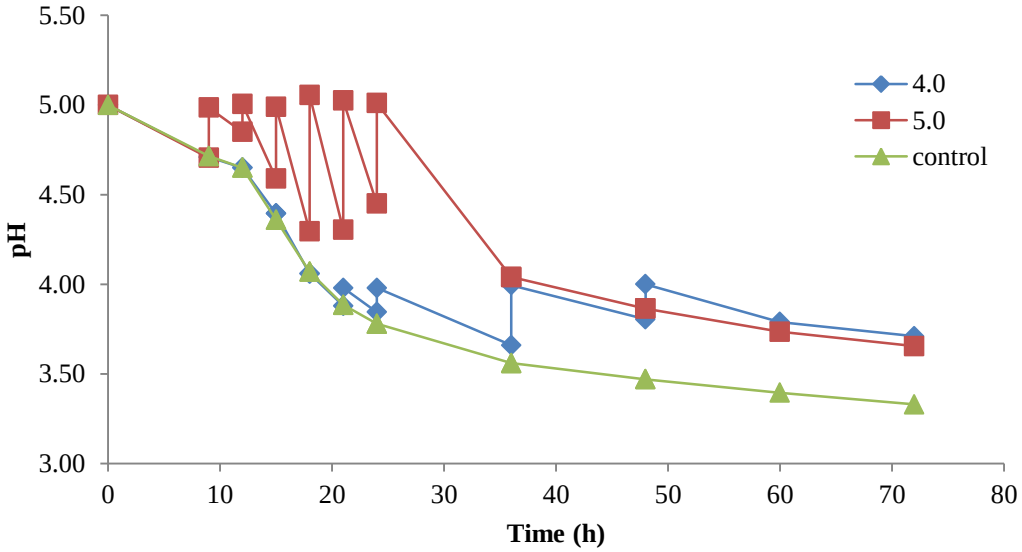


Figure 3.15 The pH during growth of *L. rhamnosus* before and after adjustment in undiluted hydrolyzed sweet potato waste

Figure 3.16 indicates that consumption of glucose by *L. rhamnosus* was generally higher in the pH 5.0 set point than pH 4.0 set point or control. The slope of each line represents the rate of glucose consumption, which is higher (steeper) at pH set point 5.0 than the other 2 treatments. This is probably due to a higher number of cells in the flasks maintained at a pH of 5.0, but could also be attributed to a higher rate of metabolism per cell. This can be calculated given accurate cell counts, but as described earlier in this chapter, getting accurate cell counts was very difficult. Any calculations based on those numbers would be highly suspect.

Not surprisingly, the pH set point of 5.0 had the highest supernatant lactic acid concentration, while no pH control had the lowest (Figure 3.17). Interestingly, although the pH 5.0 set point condition had almost twice as many cells as the pH 4.0 set point and the control, there was not twice the concentration of lactic acid. The rate of lactic acid production is faster (has a steeper slope) in the first 36 h of production than in the last 24 h. This could be affected by the increased osmotic pressure due to the addition of sodium hydroxide. Figure 3.14 indicates that the exponential growth phase of this treatment corresponds with the faster rate of production. Interestingly, the pH 4.0 set point condition did not exhibit the same change in growth rate as the pH 5.0 set point. It is possible that the change in osmotic pressure caused by the additional of excess base affects cells in the exponential growth phase more than it affects cells in the stationary growth phase. Indeed, Piuri et al. (2005) found that modifications in cell wall allow *L. casei* to swell in response to a high salt environment, which slows growth and inhibits cell division when compared to cells grown without excess salt.

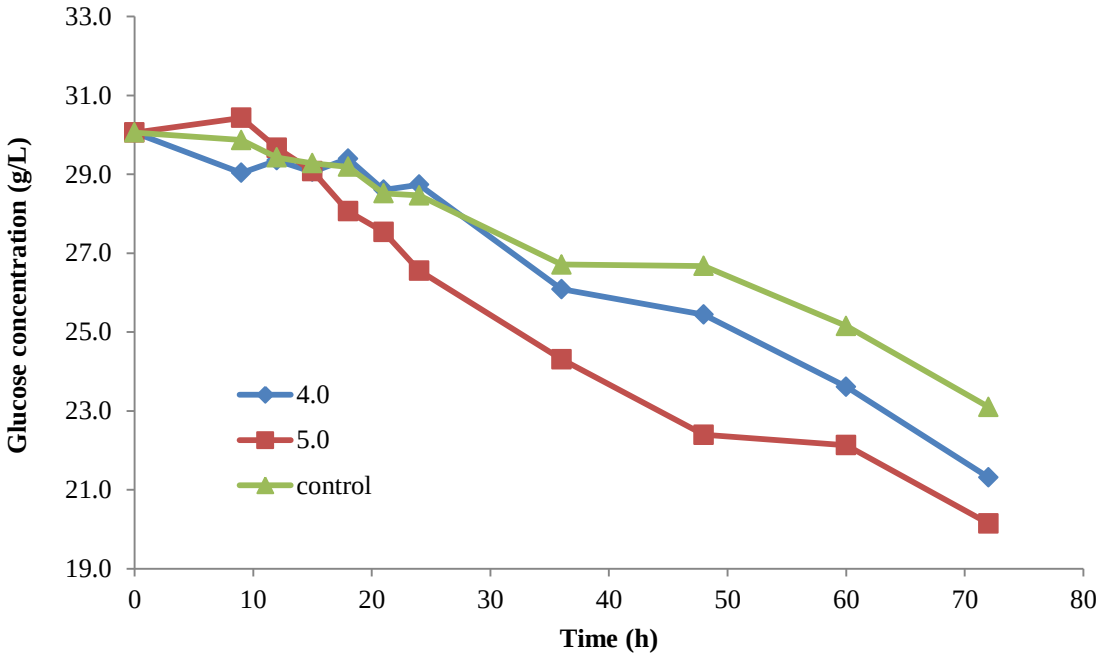


Figure 3.16 Glucose concentrations during growth of *L. rhamnosus* in undiluted hydrolyzed sweet potato waste

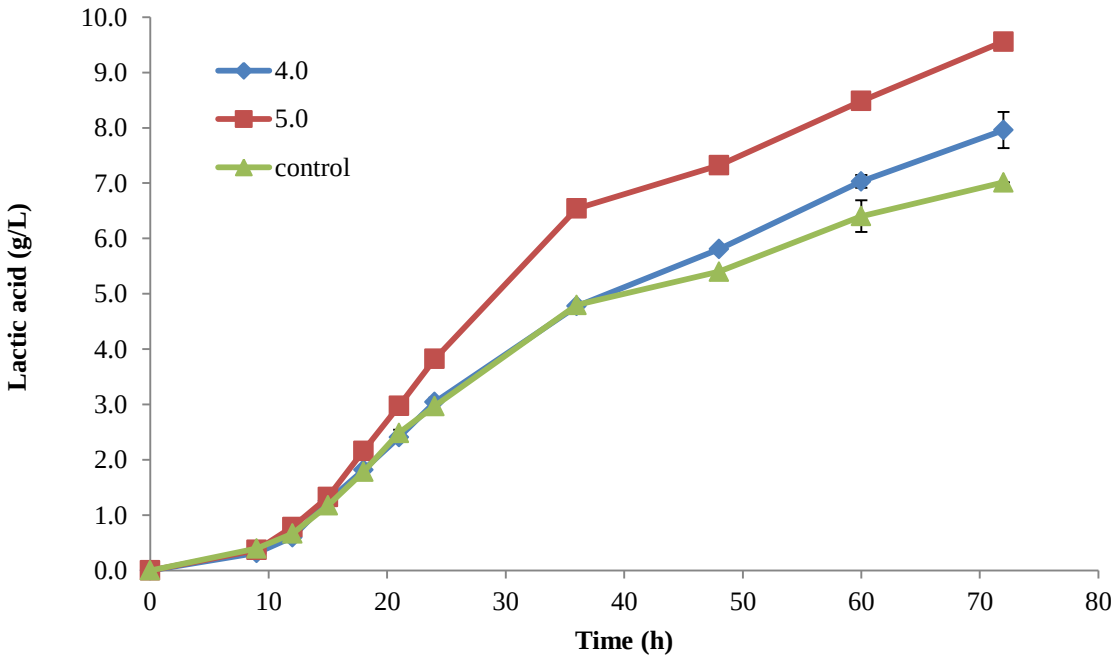


Figure 3.17 Lactic acid production during growth of *L. rhamnosus* in undiluted hydrolyzed sweet potato waste media

CONCLUSION

L. rhamnosus has the ability to effectively grow and produce lactic acid in hydrolyzed sweet potato processing waste without further supplementation. A maximum lactic acid yield of almost 10 g/L in the undiluted hydrolyzed sweet potato waste material shows great promise for economical conversion of this waste material. *L. rhamnosus* can produce 1 g of lactic acid per 1 g glucose consumed, and an optimization of the production system starting with 30 g/L glucose in the sweet potato waste could theoretically yield 30 g/L lactic acid.

Chapter 4: CONCLUSIONS AND FUTURE RESEARCH

Utilization of sweet potato processing waste for production of lactic acid has been proven possible in this research. A simple hydrolysis process involving treatment with 80 U of glucoamylase per 100 g of waste efficiently hydrolyzes the available starch into glucose within 24 h. Fermentation of this hydrolyzed waste using *Lactobacillus rhamnosus* and pH control produced close to 10 g/L lactic acid. Optimization of production processes could result in higher lactic acid yields, since approximately 66% of the available glucose remained unused.

Supplementation with other agricultural wastes rich in protein, as used by Altaf et al. (2007) and Lu et al. (2009), might be a cost effective method to extend the logarithmic growth phase and increase cell number. Simultaneous enzyme hydrolysis and fermentation could result in reduced fermentation time, as would inoculation of higher starting cell densities. Another possible improvement of lactic acid yields might be achieved using a continuous production process. Perfusion technology results in increased cell densities, and the ability of *L. rhamnosus* to produce lactic acid throughout the stationary growth phase makes this cell line a good candidate for this technology. This research proves that sweet potato processing waste can be used to produce lactic acid in batch mode, and the production of other value-added products is possible via fermentation.

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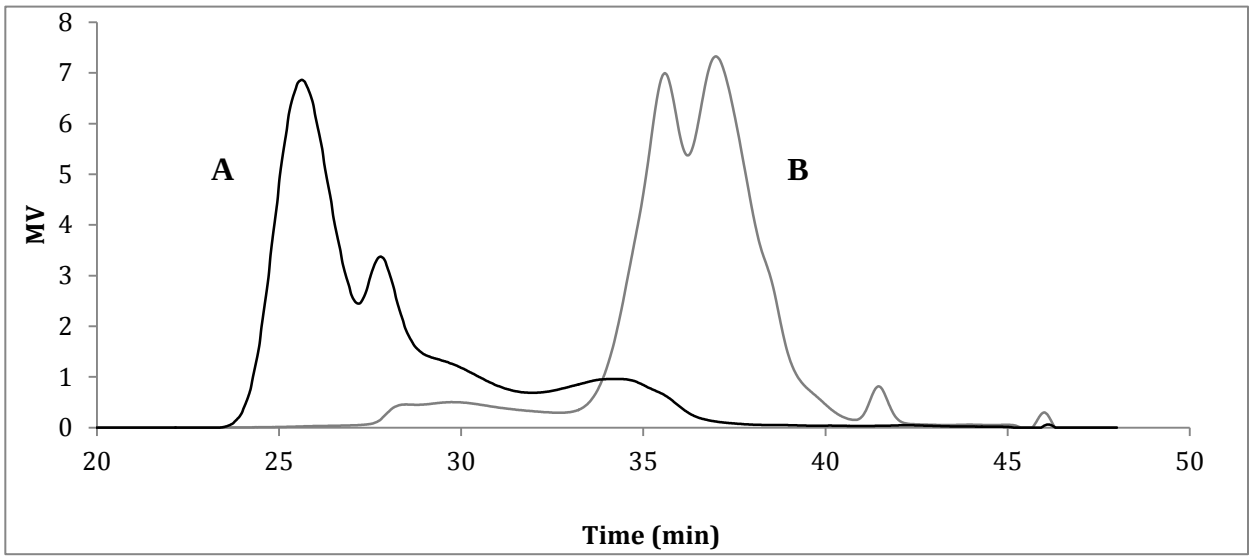
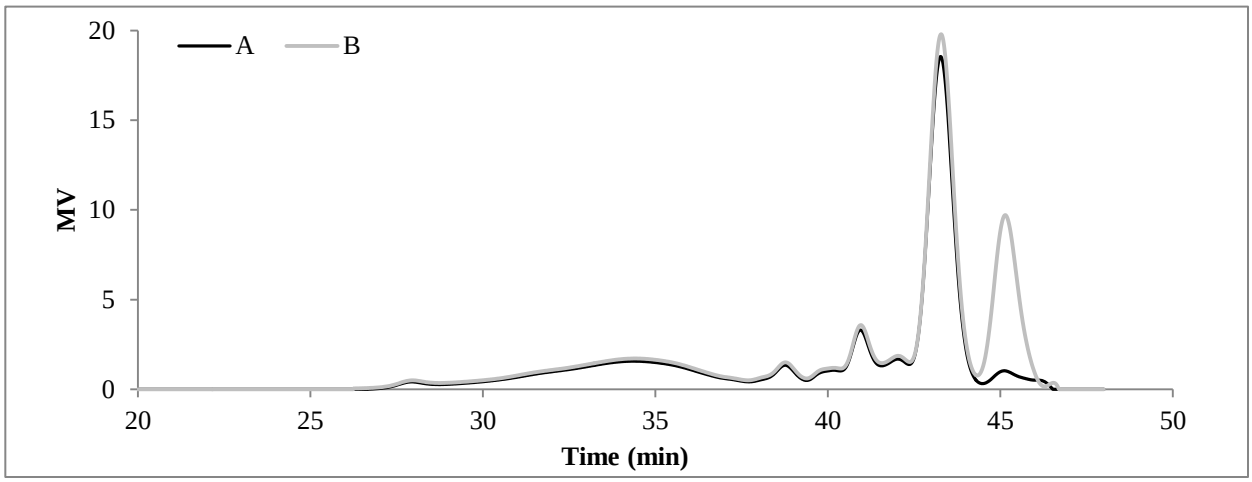
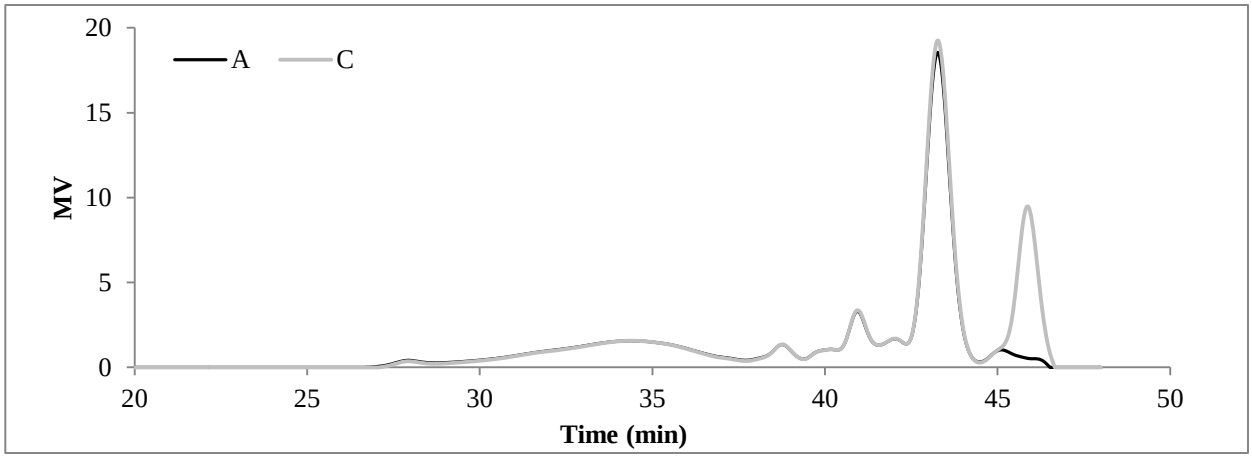
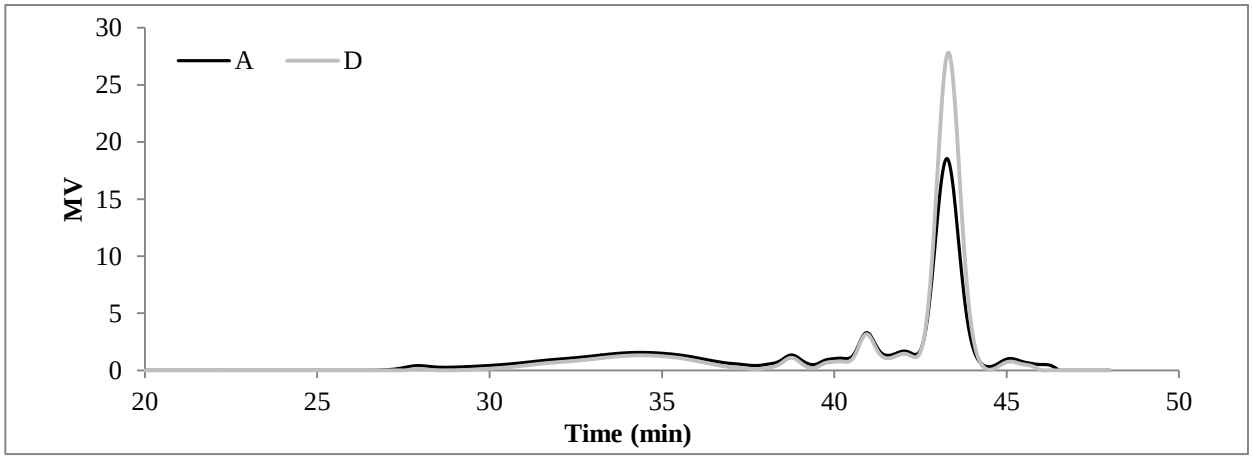
APPENDIX 1: Verification of HPSEC-RI column performance using DMSO solubilized potato starch (A) and isoamylase debranched potato starch (B)



APPENDIX 2: HPSEC-RI profiles of sweet potato processing waste (A) compared to sweet potato processing waste with 1 mg/mL spikes of glucose (B), fructose (C), or sucrose (D)







APPENDIX 3: HPLC profiles of *L.rhamnosus* culture supernatant with a 1 mg/mL L-lactic acid spike (A) and without (B)