BIOCONVERSION OF PAPER MILL LIGNOCELLULOSIC MATERIALS TO LACTIC ACID USING CELLULASE ENZYME COMPLEX AND MICROBIAL CULTURES

by

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Abstract

Paper mill sludge is a solid waste generated from the paper-making industry. Cellulose in the sludge can be hydrolyzed into glucose using a cellulase enzyme complex, which can then be fermented to produce value added chemicals, such as lactic acid. The enzyme requirement for hydrolysis of the cellulose in paper sludge was benchmarked against paper pulp. Enzymatic requirements for complete conversion of cellulose in paper pulp was found to be 12 fpu cellulase, supplemented with 5 egu of beta-glucosidase per gram of cellulose. However, beta-glucosidase supplementation had to be increased to 38 egu to obtain a similar level of hydrolysis in the case of paper sludge indicating a decrease in enzyme activity due to sludge components.

Response Surface Methodology (RSM) was used to study the lactic acid yield from paper sludge using enzyme dosage and temperature as parameters and operating in simultaneous saccharification and fermentation (SSF) mode. Maximum lactic acid yield of 0.75 g/g glucose was obtained within 36 hours using 10 fpu cellulase supplemented with 32 egu beta-glucosidase at a temperature of 39 degree C. Using the optimization function of the software, the optimal operational conditions for paper sludge hydrolysis were found to be 9 fpu cellulase, 12.5 egu beta-glucosidase at 40 degree C which resulted in a lactic acid yield of 0.58 g /g glucose.

Lactic acid producing microbial cultures, *Lactobacillus plantarum* and *Rhizopus oryzae* were evaluated for fermentation of the pulp and sludge hydrolyzate at 125-ml shake flask and 2-L fermenter levels. In paper pulp media, the yields obtained by bacterial and fungal fermentations were 0.89 and 0.36 g/g glucose, respectively. In the case of paper sludge, the yield remained same, but

inhibition of bacterial growth occurred. This resulted in lower substrate uptake and productivity than those obtained in paper pulp. On the other hand, fungal growth rate was enhanced due to the high solids content of paper sludge. The yield of lactic acid from paper sludge using *L. plantarum* and *R. oryzae* was 0.88 and 0.72 g/g glucose, respectively. Microbial cultures native to the sludge were isolated and evaluated for their performance of lactic acid production.

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CHAPTER 1 - Introduction

As fossil fuel reserves deplete steadily, there has been increasing awareness and acceptance of biomass as a potential renewable source of energy and carbon. Production of biofuels and biomaterials has been a subject of extensive debate mainly because they are presently being made from cereal grains, and thus compete with the food chain. As a result, efforts are being made to shift to the next generation of biofuels and biomaterials derived from cellulosic sources (Sun et al. 2005). Making them from cellulose dramatically expands the types and amount of material available for their production. This includes agricultural byproducts (Mohaghegi et al. 1992), energy crops such as fast-growing trees and grasses (Galbe et al. 2002) and many materials now regarded as wastes requiring disposal, such as municipal wastes and paper mill sludge (Lynd et al. 2001). The benefits of biomass conversion technology are: increased national energy security; reduction in greenhouse gas emissions; use of renewable resources; foundation of a carbohydrate-based chemical process industry; and macroeconomic benefits for rural communities and the society at large.

To make the production of biobased fuels and materials technically feasible and thus economically competitive with the available petroleum alternatives, the concept of biorefinery has been put forth by National Renewable Energy Laboratory (NREL). As with the petrochemical refineries, the biorefinery would integrate several conversion processes to produce transportation fuels, chemicals or products, including ones that are presently derived from petroleum. However, instead of crude oil, the starting material would be biomass in case of biorefineries.

Pulp and paper mills are one of the few present industries that incorporate some of the characteristics of such biorefineries in that they produce a combination of food, feed, power and

industrial and consumer products using biomass (Lynd et al. 2005). Primarily producing different types of papers, paper mills often produce different lignin-based by-products and generate a large part of their power requirements by burning some of their energy-rich waste streams. Their product base can be further broadened by utilizing the cellulose present in their principle waste stream, referred to as paper sludge; to produce organic chemicals via the biochemical pathway which comprises enzymatic hydrolysis and fermentation (Schmidt et al. 1997).

Presently, paper sludge is disposed either in landfills or is burnt. If the cellulosic feedstock is utilized for production of useful chemicals, it would not only allow for additional revenues but will also have a positive effect on waste management. Though limited in availability, paper sludge is more promising for such bioconversion processes as compared to other lignocellulosic biomass because it already undergoes processing and thus no pretreatment is required. This makes it an attractive point-of-entry and proving ground for commercial processes featuring enzymatic hydrolysis of cellulose (Fan et al. 2006).

Many studies have focused on production of different types of value-added chemicals from paper sludge. It has been studied as a substrate for production of carboxy methyl cellulose (Barkalow et al. 1985), activated carbon (Khalili et al. 2000) and for cellulase (Maheshwari et al. 1994). It has been widely studied for suitability of conversion to ethanol (Lark et al. 1997; Ballesteros et al. 2002; Kadar et al. 2004; Yamshita et al. 2006: Marques et al. 2008) and lactic acid (Nakasaki et al. 1999; Lee et al. 2005; Marques et al. 2008).

Lactic acid was discovered in 1780 by Carl Wilhelm Scheele, a swedish chemist who isolated the acid from sour milk as an impure brown syrup. The French scientist Frémy produced lactic acid by fermentation and this gave rise to industrial production in 1881. Pure and anhydrous racemic lactic acid is a white crystalline solid with a low melting point. It exists in

two optically isomeric forms, L(+) lactic acid and D(-) lactic acid. L(+) lactic acid is the biological isomer and is ubiqitous in the living kingdom as an important metabolite involved in several biochemical pathways.

Pyruvate is the end product of glycolysis, the first set of steps for metabolic energy generation. Under aerobic conditions, it is completly oxidized to CO_2 and water *via* the citric acid cycle and generates ATP . Under anaerobic conditions, no furthur oxidation and consequently, no ATP production occurs. However, the NAD+ reduced during glycolysis needs to be regenerated for glycolysis to continue. For this, the pyruvate is either converted to ethanol by the action of the enzyme pyruvate decarboxylase or to lactic acid by the action of lactate dehydrogenase, thereby regenerating the NAD+. This is the main route of lactic acid production in biological systems. (Wood BJ, Genera of Lactic Acid Bacteria, Springer.)

Lactic acid is an important organic chemical used in several industries. Predominant among these is the food industry where it is used as

- sodium or potassium lactate in meat, poultry and fish to extend shelf life
- acidity regulator in beverages such as soft drinks and fruit juices.
- preservative of vegetables such as olives, gherkins, etc. preserved in brine.
- additive to hard-boiled candy, fruit gums and other confectionery products for reduced stickiness and a longer shelf life.
- acidification agent for dairy products which also enhances the dairy flavor.

Also, lactic acid is a natural sourdough acid, which gives the bread its characteristic flavor, and therefore it can be used for direct acidification in the production of sourdough. The esters of lactic acid with long chain alcohols are used as emulsifying agents in bakery products.

Besides these uses, lactic acid is also an important platform chemical and serves as the precursor of various other useful organic chemicals. For example, by dehydration it yields acrylic acid, oxidation produces malonic acid, while it is hydrogenated to produce 1-3 propanediol. However, over recent years, the biggest surge in demand has been for the polymerization product, polylactic acid, a biodegradable plastic which has multiple applications in the packaging industry. Two molecules of lactic acid can be dehydrated to lactide, a cyclic lactone, which as biodegradable polyesters is currently used to manufacture tissue engineering materials such as resorbable screws and sutures. It is also increasingly being used as an intermediate in the synthesis of high volume oxygenated chemicals such as propylene glycol, and the esters of lactic acid with low molecular weight alcohols are being used to produce environmental friendly solvents (Dutta et al. 2006).

Industrially, Lactic acid is manufactured either using a chemical or a biochemical route. The chemical route involves the hydrolysis of lactonitrile using strong acids that yields a racemic mixture. The biochemical process involves fermentation of sugars such as glucose and lactose (Vadlani et al. 2008) using appropriate microorganisms.

The objective of the present work is to compare lactic acid production from paper pulp and paper sludge and devise an efficient bioprocess for the conversion of paper sludge into lactic acid using appropriate cellulase enzymes and efficient microbial cultures.

CHAPTER 2 - Hydrolysis of paper mill pulp and sludge to constituent sugars using cellulase enzyme complex

Introduction

Cellulose is the most abundant organic material on earth. It has a huge potential to serve as a renewable source of energy and carbon to meet the burgeoning world demand for fuels and chemicals. However, low-cost technologies to use cellulose as a feedstock to produce chemicals are yet to be developed. One of the chief roadblocks in this regard is the recalcitrance of lignocellulosic biomass against hydrolysis to yield constituent sugars, a primary requirement for processing them further into the desired products (Himmel. 2007). In nature, what is one of the most important processes for carbon recycling, is carried out by some fungi and bacteria. Cellulolytic fungi produce a host of hydrolases, commonly referred to as the cellulase enzyme complex, which acts in tandem to progressively break down cellulose. Bacteria have complexed cellulase systems called polycellulosome organelles that are exposed on bacterial cell surface that aid in more efficient uptake of the released glucose by preventing loss by diffusion (Zhang et al. 2004).

Chemically, cellulose is a linear condensation polymer consisting of D-glucose joined together by β-1,4-glycosidic bonds with a degree of polymerization (DP) ranging from 100 to 20,000 (Lee et al, 2002). Anhydrocellobiose is the repeating unit of cellulose. Coupling of adjacent cellulose chains and sheets of cellulose by hydrogen bonds and van der waal's forces results in a parallel alignment and a crystalline structure with straight, stable supra-molecular

fibers of great tensile strength and low accessibility (Zhang et al. 2004). This makes cellulose breakdown difficult.

The widely accepted mechanism for enzymatic cellulose hydrolysis involves synergistic actions by endoglucanase (EC 3.2.1.4), exoglucanase or cellobiohydrolase (EC 3.2.1.91), and β-glucosidase (EC3.2.1.21). Endoglucanases hydrolyze accessible intramolecular β-1,4-glucosidic bonds of cellulose chains randomly to produce new chain ends; exoglucanases processively cleave cellulose chains at the ends to release soluble cellobiose or glucose; and β-glucosidases hydrolyze cellobiose to glucose. These three steps occur simultaneously to degrade cellulose(Lee et al. 2002). This complex has been found to be remarkably resistant to inhibitors except product and substrate inhibition resulting from cellobiose and cellulose respectively (Howell et al. 1975).

Improving biomass hydrolysis using fungal cellulase has been a very active area of research in recent years. The biotechnology companies, Danisco and Novozymes Inc. have taken the lead in this and have reported significant reduction of enzyme costs for the cellulose-to-ethanol process from US$5.40 per gallon of ethanol to approximately 50 cents per gallon of ethanol (Moreira, 2005). The two main strategies pursued to achieve this goal are (i) an economical improvement in the production of cellulase enzyme complex by process improvisation such as low-cost media components and fungal strain enhancement; (ii) designing of a more efficient cellulase enzyme complex so as to reduce enzyme requirements by designing better cocktails and by individual component improvement by processes such as protein engineering (Moreira, 2005).

However, the enzyme costs for lignocellulosic hydrolysis needs to go down further to make bioprocessing a commercial reality. NREL estimates total value to be around 10 cents to produce a gallon of ethanol (Stephanopolous, 2007).

Different lignocellulosic materials have been studied for their use as substrates in biochemical conversions. Lynd and his coworkers, 1996, were the first to study the suitability of paper sludge for biochemical conversion. They studied thirty six different types of paper sludges for their amenability to hydrolysis and reported that different sludges vary in their level of enzymatic susceptibility. Nakasaki and coworkers (1999) were the first to study lactic acid production from different types of sludge including paper mill sludge. In a sequential process of hydrolysis and fermentation, using carboxy methyl cellulase (EC 3.2.1.4.), they reported a saccharification level of 50% that yielded a solution with 10g/l glucose after 100 hours. Romani and coworkers (2007) evaluated the effect of various operational conditions on the hydrolysis of sludge. These include the effect of surfactant addition, cellulase to solids ratio and liquid to solids ratio. In the most recent study, Marques and coworkers (2008) reported complete saccharification as well as fermentation in a study investigating the suitability of recycled paper sludge for lactic acid production.

All previous studies used one-factor-at-a-time variation which is not appropriate method for use in process optimization. It is time consuming, cannot determine the effect of all possible combinations of factors, and is not very accurate because cellulase has partial beta glucosidase activity and vice versa which means that it is not possible to hold one level constant while changing the other. In this study, the results from these experiments were used to fix the design space, that is, to determine the highest and the lowest levels of factors to generate a model using RSM that can then be used for process optimization.

Additionally, none of these studies tested β-glucosidase as a variable. Minimizing the β-glucosidase requirement is as important as minimizing cellulase requirement to bring down the total enzyme cost.

Different substrates have varying amenability to enzymatic hydrolysis. Consequently, hydrolysis experiments were performed to determine the maximum extent of hydrolysis possible, hydrolysis levels resulting from different levels of enzyme addition as well as the time required for hydrolysis. This set of experiments therefore, is a prerequisite whenever studies requiring lignocellulosic hydrolysis are to be undertaken.

The factors tested in this study are the effect of two enzymes: cellulase, the primary cellulose hydrolyzing enzyme; and β-glucosidase: the enzyme which breaks down cellobiose, a glucose dimer that is generated by the action of cellulases. The cellulase mixtures obtained commercially are often deficient in β-glucosidase and must be supplemented with β-glucosidase. Other accessory enzymes such as xylanase, furfuryl esterase are used while hydrolyzing complex substrates (Margeot et al. 2009). However, they were not required in this case as the cellulose fiber present in paper pulp and sludge has already been stripped of all other components that in native lignocellulosics bind to cellulose and prevent enzyme access. The optimum temperature and pH for the enzymes were specified by the manufacturer and thus their values were maintained as suggested.

Materials and methods

Feedstock

Bleached softwood paper pulp and paper mill primary clarifier sludge (before dewatering) were obtained from Crompton mill, MeadWestvaco. Softwood paper pulp is obtained by kraft pulping of heart-wood obtained from softwood tree species. Lignin is completely stripped of the process of bleaching. The clarifier sludge is the suspended solids in

the wastewater stream of the paper mill that pass through the filter screens and settle to the bottom of the clarifier tank. Sludge is recovered and subsequently dewatered before disposal. The moisture content of pulp and sludge were 83 and 90% respectively on a wet basis. They were stored in plastic buckets at 4°C in the cold room until use.

Enzymes

The enzyme units for cellulase activity is Filter paper unit (fpu), which is that amount of enzyme that causes 1μmol of reducing sugar equivalents release in 1 min from Whatman filter paper at 50°C and pH 4.8. Endoglucanase unit (egu) is defined as the amount of enzyme which releases 1.0 μmol of glucose units from cellobiose per min under the same assay conditions. The enzymes used were NS50013 (cellulase) and NS50010 (β-glucosidase) with activities of 70 fpu/g and 250 egu/g respectively. They were obtained from Novozymes inc. and were a part of their complete biomass hydrolysis kit. The enzymes were stored at 4°C in plastic bottles, away from sunlight.

Compositional analysis

The water content of the sludge was determined by using convection oven drying method. Three gm of wet paper pulp and paper sludge each were weighed and left to dry in a convection oven at 80°C for 24 hours. Analysis by Denver Infrared Moisture Analyzer, Model IR35 (Fisher Scientific, USA), confirmed that the samples were completely dried. The weight of the dried samples were then determined. The difference in weight is the moisture content of the material. The carbohydrate content of the dried paper pulp and paper sludge was determined by a two-step quantitative hydrolysis process according to NREL/TP-510-42618 (www.nrel.gov/biomass/pdfs/42618). The samples were centrifuged and the pellet was air dried

to a constant weight. A 0.3gm air dried sample was weighed in an Erlenmeyer flask and hydrolyzed by adding 3 ml of 72% (w/w) sulphuric acid and agitating the mixture for 1 hour. This was followed by a second hydrolysis at 121° C in 4% (w/w) sulphuric acid (adjusted by diluting the above mixture with 84 ml water) for 1 hour in an autoclave. Each sample was then centrifuged and the supernatant analyzed for pentose and hexose sugars by HPLC using a Shimadzu CBM-20A HPLC system connected to a Shimadzu RID-10A refractive index detector. A monosaccharide column (300 x 7.8 mm; Phenomenex, Calif.) was used and the analysis conditions maintained were 80°C, mobile phase 0.0025M sulphuric acid, retention time 15 minutes and flow rate 0.4 ml/min. Values for weight percent cellulose in the sample were calculated using the following equation,

$$\%\ Cellulose = \left[\left(\frac{g\ glucan}{g\ solid}\right)_{QH1} + \left(\frac{g\ solid\ residue}{g\ solid}\right)_{QH2} \times \left(\frac{g\ glucan}{g\ solid\ residue}\right)_{QH2}\right] \times 100$$

A similar calculation was followed for the hemicellulose content by replacing glucan with xylan.

Enzymatic hydrolysis

Enzymatic hydrolysis was performed in 125 ml Erlenmeyer flasks with a working volume of 20 ml. Cellulose content in the flask was maintained at 2% w/v. The flasks were incubated at a temperature of 45°C in a temperature controlled shaker and at an agitation rate of 120 rpm (Innova 2025, New Brunswick scientific, NJ). Enzymes were added as per the requirement of each individual experiment. All experiments were done in triplicate. Hydrolysis was carried out for 32 hours, after which no further increase in glucose levels were observed.

Pulp hydrolysis was carried out in aqueous media, which had equal volumes of distilled water and 0.01 M citrate buffer at pH 5.2, the optimum conditions for cellulase activity. Paper

sludge obtained was in a liquid form (90% moisture) and had a pH of 7.5. Thus, the required pH could not be obtained using citrate buffer. Instead, concentrated hydrochloric acid was used to adjust the pH of the sludge to 5.0. The sludge was then autoclaved before enzymatic hydrolysis to prevent contamination.

Calculations and statistical analysis

Product yield (Yp/s) is based on the amount of product synthesized (g) divided by the amount of substrate consumed (g). Theoretically, the maximum yield of glucose is 1.1g/g from cellulose and the maximum yield of lactic acid is 1g/g from glucose. Productivity is calculated by final product concentration divided by the time taken for fermentation. All shake flask level experiments were done as triplicates and the average values are reported. The standard deviations are depicted as error bars in the figures.

Results and discussion

Composition

Paper sludge was found to have a moisture content of 90%. Paper sludge consists of an organic fraction which are materials of plant origin such as polysaccharides and lignin and an inorganic ash fraction. The inorganic fractions result from different additives such as silica, lime, clay introduced in the production stream at various stages of paper manufacturing. Table.1-1 summarizes the composition of the paper sludge sample used in this study. Paper pulp was found to be mostly cellulosic with 91% glucan and 6% xylan content, on a dry basis.

Enzymatic hydrolysis

Paper pulp hydrolysis. A two gm sample of pulp was dispersed in 100 ml citrate buffer, and cellulase at three different levels were added, ranging from 5-20% of the pulp weight. The flasks were incubated in a rotary shaker. Samples were collected after 4, 12, 24, 36 hrs and analyzed for glucose and cellobiose concentrations.

Hydrolysis increased progressively from 50% to 82% when the enzyme loading was increased from 5% to 20% respectively (Figure1-1). However, the marginal increase of glucose release with increasing enzyme loading decreased progressively.

The next experiment assessed the effect of β-glucosidase supplementation on hydrolysis. The three cellulase levels used previously were supplemented with 5egu of β-glucosidase. As can be seen from the graph (Figure1-2), addition of β-glucosidase had a significant positive affect on the amount of glucose release at all cellulase loadings in addition to faster reaction kinetics, particularly in the initial stages. Cellulase has partial cellobiose cleaving activity and thus cellobiose accumulation occurs in the initial stages of hydrolysis (Data not shown). This then slowly gets hydrolyzed to glucose. Supplementation with β-glucosidase hastens the breakdown of the cellobiose leading to faster glucose release. If the amount of cellulase is low, there would be insufficient β-glucosidase activity, which would result in a lower glucose release as part of it will remain bound as cellobiose. Addition of β-glucosidase in such a case increases the level of glucose release.

Paper sludge hydrolysis. As was the case for paper pulp, the first set of hydrolysis experiments was done using only cellulase. It was observed that even with progressively higher levels of cellulase, the concurrent increase in glucose was very low. Additionally, a large amount of cellobiose accumulation occurred (data not shown). This suggested β-glucosidase inactivation,

probably due to enzyme immobilization by one or multiple components of sludge. Thus, the next experiments were done by progressively increasing β-glucosidase concentration and keeping the cellulase level constant at 12 fpu. (Figure1-3) Finally, at 38 egu, a similar hydrolysis level was observed as in the case of paper pulp using the same level of cellulase supplemented with 5 egu β-glucosidase. Table 1-2 compares hydroysis pattern of paper pulp and paper sludge.

Conclusion. The cellulose present in both the feedstocks tested were found to be very amenable to hydrolysis under proper conditions. Such a high degree of hydrolysis using similar enzyme levels and within similar residence times has not been reported In the case of other lignocellulosic biomass.

The increase in enzyme requirements observed for sludge is undesirable. There needs to be further studies to investigate if this is due to enzyme adsorption or enzyme inhibition, possibly caused by the presence of heavy metal ions. The enzyme could also become irreversibly adsorbed to other components present in sludge such as lignin, clay or calcium carbonate and thus become unavailable for sludge hydrolysis. This could be tested by techniques such as Fourier Transform Infra Red (FTIR) spectroscopy. Also, it is well known that most enzymes are inhibited in the presence of heavy metal ions. Paper sludge has a considerable heavy metal salt content (Poikio et al. 2007) that could also decrease enzyme activity. One could also dialyze to remove metal ions and repeat hydrolysis to compare hydrolysis activity. This would simulate membrane separation. If the component/components responsible for increased enzyme requirement is recognized, eliminating them from entering the waste stream would effectively decrease the difference in enzyme requirements of paper pulp and sludge and decrease process cost considerably.

Table 2.1 Composition of paper sludge

Components	Concentration (w/w%)
Cellulose	21.1 ± 1.1
Hemicellulose	4.1 ± 0.3
Lignin	13.9 ± 0.6
Ash	46.5 ± 1.4

Table 2.2 Comparison of enzymatic hydrolysis of paper pulp and paper sludge

	Paper pulp	Paper sludge
Maximum glucose release	0.93[a]	0.94[a]
Glucose release at 12fpu cellulase only	0.81[a]	0.23[b]
Required β-glucosidase supplementation for maximum glucose release (in egu)	5	38

For a single parameter, values with same superscript do not vary significantly. Values with different superscripts differ significantly ($P < 0.05$)

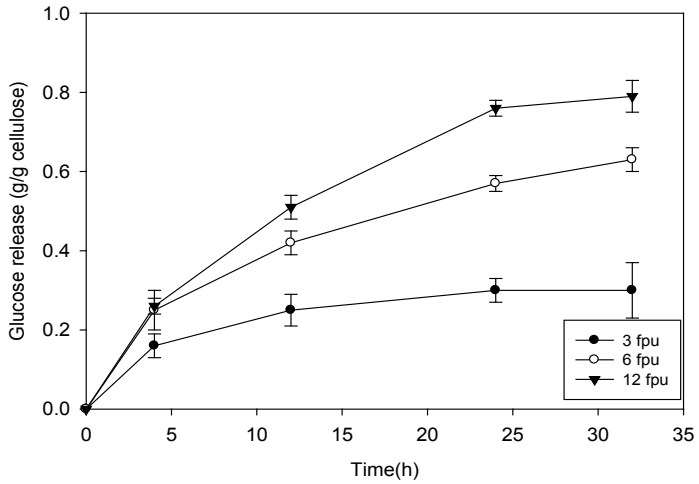

Figure 2.1 Paper pulp hydrolysis at varying cellulase levels

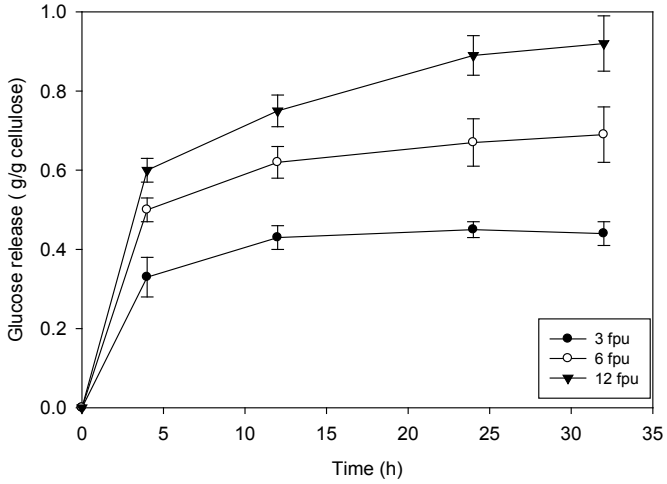

Figure 2.2 Paper pulp hydrolysis at varying cellulase levels and supplemented with 5 egu β-glucosidase

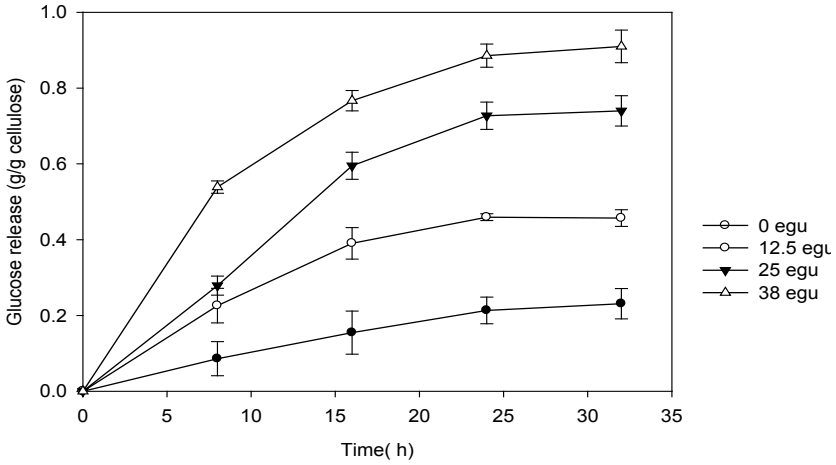

Table 2.3 Hydrolysis of paper sludge with constant cellulase level of 12 fpu and varying β-glucosidase concentrations

CHAPTER 3 - Fermentation of sugars derived from paper pulp and paper sludge to L(+) Lactic acid using *Lactobacillus plantarum* and *Rhizopus oryzae.*

Introduction

After the sugars have been released by the action of enzymes, they can then be converted to a variety of fermentation products using suitable microorganisms. Manipulating the growth conditions suitably leads to increase in the level of the desired product and suppression of by-product formation.

Lactic acid bacteria (LAB)

Lactic acid bacteria are a group of Gram positive bacteria that produce lactic acid as a result of carbohydrate fermentation. These microbes are used in the production of fermented food products. Lactobacilli vary in morphology from long, slender rods to short, round cocci. Some species are aerotolerant and may utilize oxygen through the enzyme flavoprotein oxidase, while others are anaerobic (Codon, 2006). The growth is optimum at pH 5.5-5.8 and temperature of 32-35°C. They are heterotrophic and generally have complex nutritional requirements because they lack many biosynthetic capabilities**.**

Based on the pathway followed for carbohydrate metabolism, LAB can be grouped into two categories (Wood BJ, Genera of Lactic Acid Bacteria, Springer.)

- Homofermentative LAB catabolize glucose through the glycolytic pathway to yield two moles of pyruvate and two moles ATP per mole glucose consumed. Intracellular redox balance is maintained through the oxidation of NADH, concomitant with pyruvate reduction to lactic acid. Thus, two moles of lactic acid are formed for every mole of glucose consumed.
- Heterofermentative LAB use the pentose phosphate pathway. One mole Glucose-6-phosphate is dehydrogenated to 6-phosphogluconate and then decarboxylated which yields a mole each of carbondioxide and pentose-5-phosphate (P5P). P5P is then cleaved into one mole glyceraldehyde phosphate (GAP) and one mole acetyl phosphate. GAP enters the glycolytic pathway, while the acetyl phosphate is reduced to ethanol. Thus, one mole each of lactic acid, ethanol and CO_2 is formed from a mole of glucose.

Different species of homofermentative *Lactobacillus* are used industrially to produce lactic acid from sugar or starch-rich sources. *Lactobacillus plantarum*, the LAB used in this study, is one such species. Lactic acid yields obtained are very high and the fermentation time is lowered. A yield of 98% has been reported (Vadlani et al. 2007) while productivities can go up to 2.1 g/l/h (Reddy et al. 2008). However, the drawbacks associated with using bacterial cultures like *L.plantarum* is that supplementation of the media with organic supplements such as peptone and yeast extract is required (Chopin, 1993). This increases the production cost and also makes downstream processing difficult.

Rhizopus oryzae

Fungi belonging to the genus *Rhizopus* are saprophytic and utilize dead and decaying matter as their source of nutrition. They release large amounts of different types of extracellular

hydrolytic enzymes to be able to effectively metabolize nutrients from their surroundings. This property has been exploited industrially to produce different enzymes, primarily, lipase and cellulase. *Rhizopus* sp. also produce a wide range of low molecular weight organic acids. For example, some species of *Rhizopus* such as *R.oligosporus* and *R. oryzae* are able to produce large quantities of lactic acid. However, different strains differ in their ability to produce this acid. *R. oryzae* strain NRRL 395 has been identified as a very efficient producer of lactic acid (Soccol et al. 1994 ; Oda et al. 2003).

Several studies report the use of *R. oryzae* for the production of lactic acid from starch-based materials (Akerberg et al. 2000: Huang et al. 2003; Linko et al. 2006). However, only a limited number of studies report fermentation using *Rhizopus* sp. on lignocellulosic substrates. Meiura et al report (2004) the production of lactic acid from corncob hydrolyzate using *Rhizopus*. 25 g/l of L-lactic acid was produced from the hydrolyzate containing 55 g/l of pentose and hexose sugars. In another study, Woiciechowski et al (1999) studied wood hydrolyzate as a substrate for the fungal fermentation to produce lactic acid and reported a yield of 0.58 g/g total sugars.

The yield and productivity associated with the fungal fermentations is less than that obtained in bacterial fermentations. The maximum yield reported using *R. oryzae* is 0.85 g lactic acid per gram starch (Huang et al. 2005). This is because only a part of the carbon is used for the formation of the desired product and the rest of the flux is diverted to by-products such as xylitol, glycerol, ethanol, CO_2 and fungal biomass. Another drawback is that most fungal fermentations require aerobic conditions. In oxygen deficient conditions, *R. oryzae* starts to produce more ethanol, and lactic acid production decreases (Skory et al. 1998). Provision of aeration needs additional infrastructure and the energy associated with provision of aeration is significant.

However, in spite of the above constraints, fungal fermentations are often used by industry because of certain distinct advantages. Due to the way they grow, either as mycellial mats or as pellets, downstream processing becomes easier. Nutrient supplementation with inorganic salts is sufficient; an additional organic nitrogen source is not required, thus bringing down the cost of the media (Soccol, 1994). Certain strains of the fungus have amylolytic activity, which decreases the enzyme requirements for starch hydrolysis (Linko et al. 2006). Fungal strains can partially utilize pentose sugars for lactic acid production unlike bacteria which can use them only for growth requirements. This is a significant advantage when the substrate is cellulosic biomass as it has substantial pentose sugar content. Finally, the fungal biomass generated can be used for a variety of purposes such as chitosan isolation (Pochanavanich et al. 2002) and for addition to animal feed (Kusumaningtyas et al. 2006).

Numerous studies have been undertaken in an effort to increase the productivity of fungal cultures. Of these different approaches, the most common is to optimize process parameters such as aeration and pH, modifying morphology and immobilization on solid supports. One of the important factors affecting lactic acid production in *Rhizopus* is morphology. Generally, fungal mycellia tends to grow as clumps and this morphological form is a poor producer of lactic acid. Studies report different methods of making the fungus grow in the form of flocs (Yua et al. 2007) or very small pellets (Bai et al. 2003) which increases the level of mass transfer, and consequently, the yield. Yua et al (2007) observed yields of 0.87 g/g and a productivity of 1.73g/l/h while working with a medium with 12.5% glucose if the fungus was allowed to grow as floc. By immobilizing the fungus on polyurethane foams implanted using ion beams, yields of 0.8g/g glucose was obtained (Fan et al. 2008).

This study, will test a well-known homofermentative lactic acid bacteria, *L. plantarum*, and a fungus, *R. oryzae* for their performance in the production of lactic acid from paper pulp and paper sludge. All studies involving production of lactic acid from paper sludge have utilized different bacterial species belonging to the genus *Lactobacillus*. No studies have been reported that use *R.oryzae* for fermentation of sugars from paper sludge though it has been studied for production of lactic acid using other lignocellulosic substrates (Park et al. 2004).

Simultaneous saccharification and fermentation (SSF)

Biomass conversion is a two step process involving initial hydrolysis and subsequent fermentation. These two steps can be carried out sequentially or simultaneously. SSF is the spatial and temporal integration of the hydrolysis and fermentation steps. In it, the enzymes and the inoculum are added to the bioreactor at the same time at the beginning of the reaction and the process is then allowed to proceed until completion. SSF has been widely reported to result in better yields due to avoidance of product inhibition (Philippidis et al. 1993) as well as decreasing the total process time (Lee et al. 2004). However, the temperature optima for hydrolysis and fermentation are often very different and the temperature for SSF lies somewhere between the two optima. The difference in temperature and initial substrate concentration as compared to the sequential process leads to changes in kinetics and thus yield and productivity values.

Fermentation studies are typically first carried out at shake flask levels to determine the effects of the factors being tested and to identify the optimum conditions. However, to determine the actual yield and productivities obtainable under optimized conditions, fermentation is performed in bioreactors or fermenters. Laboratory scale fermenters typically range from 1-10 liters in capacity and has computer controlled main consoles accurate control of process conditions. For example, it maintains a constant pH by monitoring and adjusting it to the optimum level by the

addition of requisite quantities of acid or base via peristaltic pumps. Fermenters allow for effective agitation and efficient mass and heat transfer. All these factors lead to much higher nutrient uptake by the microbes and thus greater product concentration and yield can be achieved. The results obtained using fermenters can be scaled up to pilot and industrial levels of operation.

Materials and methods

Microbial cultures: *L.plantarum* 35423 and *R. oyzae* strain NRRL-395 were obtained from American type culture collection (ATCC). All dehydrated media were procured from Difco, BBL, USA, and the analytical grade chemicals were procured from Fisher Scientific, USA.

Inoculum preparation

L. plantarum: Glycerol stocks of the culture was prepared and stored at -80° C. The thawed glycerol stock solution was inoculated in deMan Rogosa Sharpe (MRS) broth, specific for LAB and allowed to grow for 15 hours, to be used as inoculum.

R. oryzae: Lyophilized cultures were inoculated on plates containing potato dextrose agar. Spores were collected from the plates by washing them with water and Tween 80. They were then inoculated in a liquid mineral medium for germination**.** The medium was adjusted such that the spore count was maintained at 10^7 spores/ml. The composition of the medium was glucose 20g/l, ammonium sulphate 3g/l, zinc sulphate 0.02g/l, potassium phosphate 0.05g/l magnesium sulphate 0.075 g/l and calcium carbonate 10g/l. Insoluble calcium carbonate was

found to be necessary for the spores to germinate. The spores were allowed to germinate for 24 hours and the solution was used as inoculum.

Fermentation in shake flasks

Experiments were performed in 125 ml Erlenmeyer flasks containing 20ml of the culture medium. Three different carbon sources; synthetic glucose, pulp and sludge hydrolyzate were compared for their performance in fermentation. The starting pH of the media was set at 5 as the hydrolyzates were adjusted to that pH during enzymatic hydrolysis. In case of *L.plantarum*, the pulp and the sludge hydrolyzate were supplemented with 5 g/l peptone, the temperature of fermentation was maintained at 37°C and fermentation duration was 28 hours. For *R.oryzae*, medium supplementation was done with 5g/l ammonium sulphate as the nitrogen source, fermentation temperature was 35°C and fermentation duration was 72 hours. Inoculation was done with 5% v/v of inoculum and fermentation was allowed to proceed in an orbital shaker (Innova 23025, New Brunswick Scientific, NJ) under constant agitation of 150 rpm. Samples were collected at intervals and were centrifuged, filtered and analyzed for lactic acid and residual glucose concentration using HPLC. The conditions maintained for analysis were as reported in chapter I.

Fermenter experiments

After the hydrolysis and fermentation experiments using shake flasks, the study was scaled up to a 2 L fermenter (Biostat B. B. Braun Biotech International GMbH Melsungen, Germany). In bioprocessing applications, it is desirable to have a higher initial substrate concentration so that higher product concentrations are obtained to ensure an economical operation. A product concentration of 40 g/l of product has been identified as an economically

viable point (Fan et al. 2005). Thus, paper pulp and glucose was added in quantities that would make it possible to achieve a glucose concentration of 40g/l in the medium. However, paper sludge hydrolyzate could not be adjusted appropriately as increasing the glucose content would necessitate drying which would adversely affect its amenability to hydrolysis. Thus glucose was maintained at 20g/l in this case.

The synthetic glucose media was directly inoculated while the paper pulp and paper sludge media was first hydrolyzed using cellulase enzyme levels determined by the hydrolysis experiments described in chapter I, and then inoculated. Similar conditions of temperature and starting pH were maintained as in the shake flask experiments. The agitation rate was 300 rpm. pH was maintained using 2N sodium hydroxide solution in case of synthetic glucose media and pulp hydrolyzate. No pH change was observed in sludge hydrolyzate due to the buffering action attributed to the high ash content, and thus no pH control was required. Additionally, air was pumped in during the entire period of fermentation at the rate of 2 volume/ volume/ minute (VVM) during fungal fermentation.

SSF

The enzyme at the required dosages and 5% (v/v) of the *L. plantarum* inoculum, grown in MRS media for 24 hours were added to sludge that had been pH adjusted and autoclaved. Additionally, the sludge was supplemented with 5g/ l of peptone. SSF was allowed to proceed for 36 hours.

Results and discussion

Shake flask experiments

L. plantarum: The lactic acid concentration and residual glucose was determined after fermentation and the results obtained are presented in Table 2-1. As can be observed, glucose utilization as well as yield was high in paper pulp and synthetic media. However, in case of paper sludge, there was a considerable amount of residual glucose which resulted in lower lactic acid concentration. Even when the fermentation was allowed to continue for more time, no significant decrease in glucose concentration was observed (Data not shown).To obtain the cell count, the sludge was dilution plated along with a control; synthetic medium as the control that had the same glucose concentration and was fermented under the same conditions. The colony forming units (CFU) in control and sludge hydrolyzate media were 2.1×10^8 and 9.2×10^7 respectively. This suggested that inhibition of bacterial growth had occurred. When using paper sludge as a medium for microbial growth, some studies report complete conversion of available glucose (Li et al.2004) (Marques et al.2008) while others observed a suppression of bacterial growth and thus incomplete glucose utilization (Yamashita et al. 2008). It may be postulated that paper sludges vary widely in their compositions, particularly with respect to minor additives such as heavy metal salts that act as inhibitors, and thus account for the difference in observations.

R. oryzae: The observed residual glucose and lactic acid concentrations at the end of 72 hour of fermentation are presented in Table 2-2. Contrary to the observation in case of *L. plantarum*, *R. oryzae* performed better in paper sludge media than in paper pulp or synthetic glucose media. This could be due to better growth of the fungus as the media had large amount of suspended material that enhanced fungal mycellial growth (Li et al. 2003). Complete consumption of glucose was observed, even at the shake flask level, unlike in the case of *L.*

plantarum. However, the efficiency of substrate utilization, represented by a yield of 0.37g/g, for synthetic medium was much lower than that obtained by using *L. plantarum* (0.97g/g). Additionally, substantial by-product formation was observed in all the three media. The major by-products were ethanol and malic acid at concentrations 2.3 and 1.7 g/l, respectively.

Fermenter experiments

L .plantarum: The profile of glucose and lactic acid concentration with time for the three fermenter runs are presented in Figure 2-1, 2-2 and 2-3 respectively. A similar trend in glucose consumption was observed as in the shake flask level. However, the lactic acid concentration was higher due to higher glucose consumption in the fermenter than in the respective shake flasks levels. In all the three cases this was presumably due to better process control.

R .oryzae: Only paper sludge as a substrate was evaluated in this experiment. The time course profile of glucose consumption and lactic acid production is shown in Figure 2-4. No byproduct formation was observed. Due to better aeration conditions in the fermenter, the fungus produced only lactic acid while it tends to produce ethanol in oxygen limiting conditions (Skory et al. 1998). Because the carbon flux was not diverted to the production of by-products, the lactic acid yield and concentration increased to 13.6g/l and 0.68g/g glucose respectively**.** However, because fungal fermentation takes so long, the productivity was very low (0.16g/l/h).

The yield, concentration and productivity values obtained in the fermenter runs conducted are presented in the form of a table (Table 2.3).

SSF

SSF was carried out using paper pulp and sludge at the shake flask level. No significant increase in the amount of lactic acid production was observed in either paper pulp or paper

sludge as compared to sequential hydrolysis and fermentation. Though there was no yield increase, a substantial decrease in total process time and thus enhanced productivity was observed. Sequential hydrolysis and fermentation took 18 and 28 hours respectively resulting in 46 hours of total time required while SSF was complete within 36 hours. The same results were obtained when SSF was carried out using paper sludge (Figure 2-5).

Conclusions. In fermentation experiments using *L. plantarum*, it was observed that yield changes were not large when the bacteria were grown on different substrates. This is because, irrespective of the source, the same sugar is metabolized (glucose) and no change in yield occurs. However, the presence of inhibitors in paper sludge slows the growth and product formation rate since lactic acid production is partly growth associated. Slow growth limits sugar uptake, and as a result, the final product accumulation. Slow product formation and low final product concentration decreased the productivity value in case of paper sludge media. Further studies are needed to identify the inhibitors and assess their effect on growth and product formation rates. Eliminating inhibitors from the waste stream could decrease the difference in concentration and productivity values between paper pulp and paper sludge.

To make lactic acid production from *R. oryzae* more competitive, further studies are required for increasing yield and productivity by implementing the strategies discussed earlier in the chapter to evaluate their effectiveness in paper sludge media.

Table 3.1 Lactic acid production in shake flask using different carbon sources and *L. plantarum*

	Initial glucose concentration (g/l)	Residual glucose concentration (g/l)	Final lactic acid concentration (g/l)	Yield (g/g glucose)
Synthetic media	10	3	6.7[a]	0.98[a]
Paper pulp	10	3.2	6.5[a]	0.96[a]
Paper sludge	10	5.7	3.6[b]	0.84[b]

Values with same superscript do not vary significantly. Values with different superscripts differ significantly ($P < 0.05$)

Table 3.2 Lactic acid production in shake flask using different carbon sources and *Rhizopus oryzae*

	Initial glucose concentration (g/l)	Residual glucose concentration (g/l)	Final lactic acid concentration (g/l)	Yield (g/g glucose)
Synthetic media	20	0	7.4[a]	0.37[a]
Paper pulp	20	0	7.8[b]	0.40[b]
Paper sludge	20	0	10[c]	0.50[c]

Values with same superscript do not vary significantly. Values with different superscripts differ significantly ($P < 0.05$)

Table 3.3 Lactic acid production using different carbon sources in a fermenter

	Concentration (g/l)	Yield(g/g glucose)	Productivity (g/l/h)
Paper pulp (L.plantarum)	35.6	0.89	0.82
Paper sludge (L.plantarum)	13.4	0.67	0.32
Paper sludge (R.oryzae)	13.6	0.68	0.16
Paper sludge (SSF)	14.0	0.70	0.52

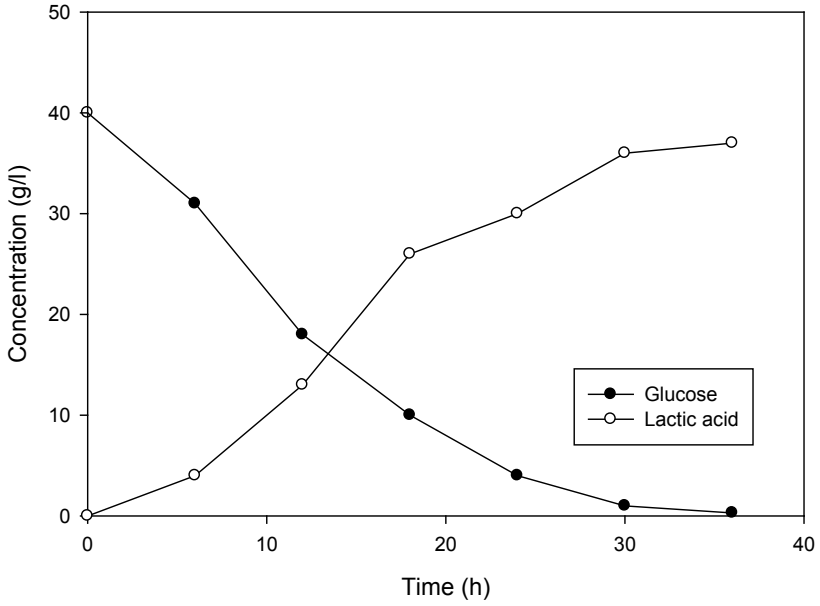

Figure 3.1 Fermentation profile of synthetic glucose media using *L. plantarum*

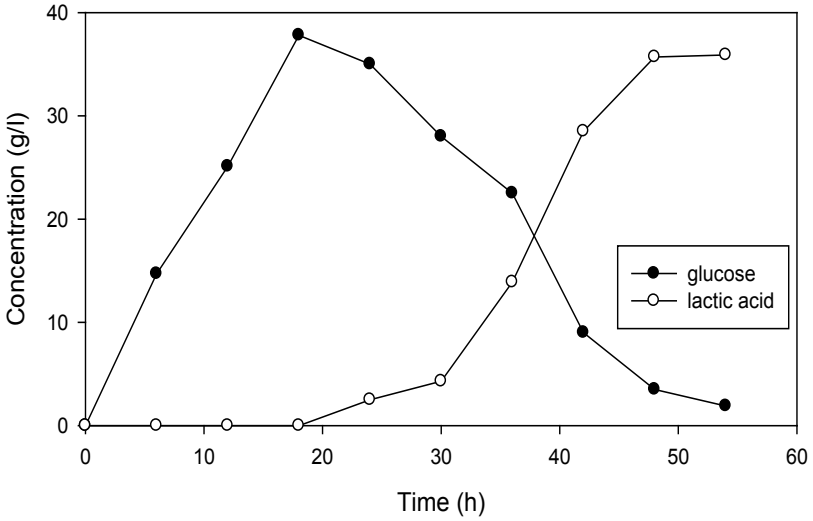

Figure 3.2 Fermentation profile of paper pulp hydrolyzate media using *L. plantarum*

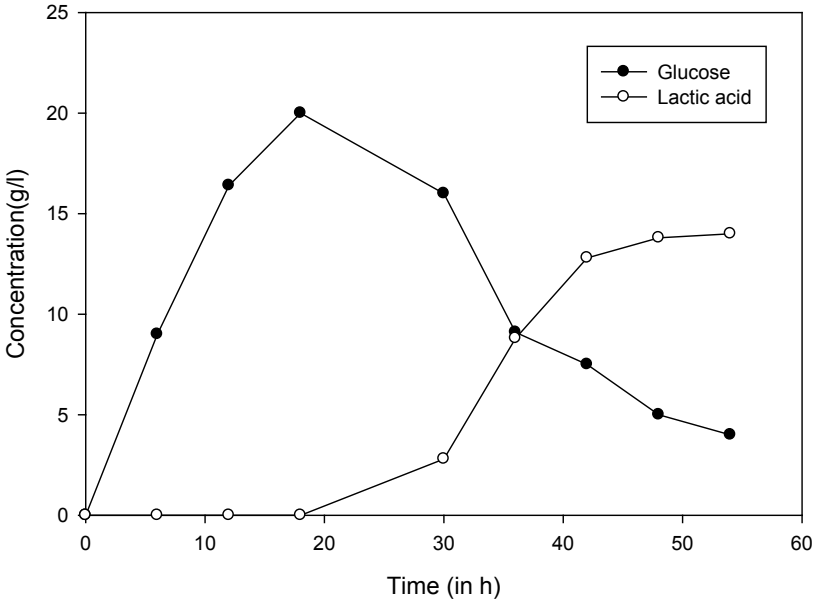

Figure 3.3 Fermentation profile of paper sludge hydrolyzate media using *L. plantarum*

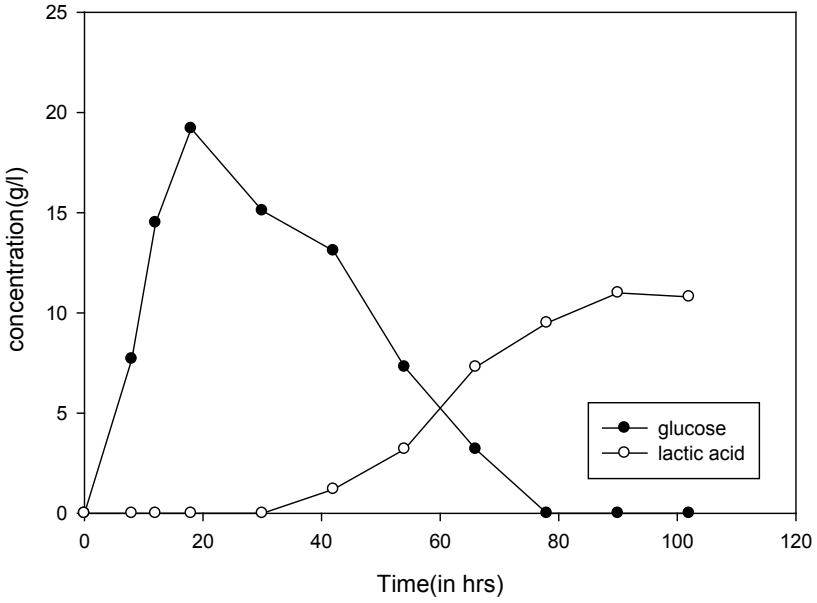

Figure 3.4 Fermentation profile of paper sludge hydrolyzate media using *R. oryzae*

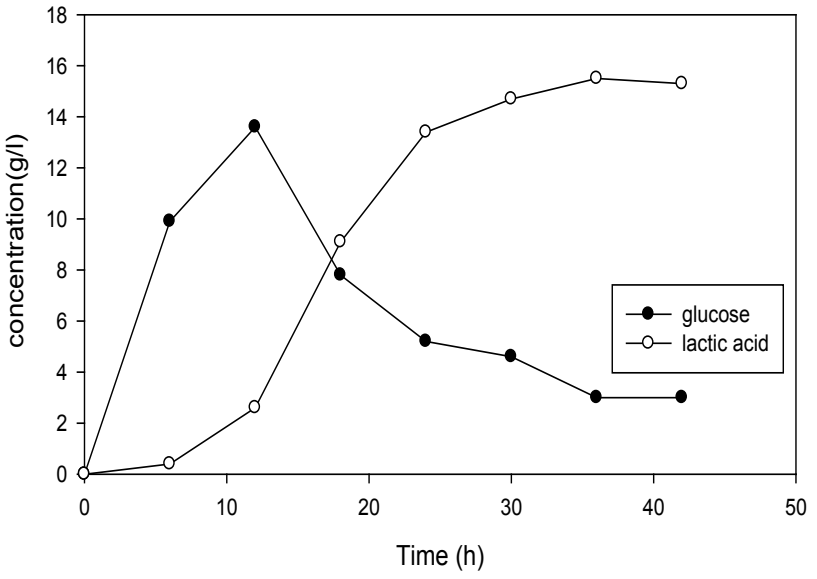

Figure 3.5 Simultaneous saccharification and fermentation of paper sludge using *L. plantarum*

CHAPTER 4 - Determining the optimum enzyme dosage for sludge hydrolysis using response surface methodology (RSM)

Introduction

Response surface methodology (RSM) is a collection of statistical design and numerical optimization techniques often used to optimize industrial processes. RSM includes aspects of experimental design, hypothesis testing, and regression analysis. The experimental design aspect deals with the choice of suitable variables and their levels, and regression analysis enables a mathematical form to be fitted to the observations. Hypothesis testing is relevant to both these processes (Morton H, http://www.stat.auckland.ac.nz/~iase/publications/18/BOOK2/B4-7.pdf.)

In other words, RSM searches for the input combinations, called the independent variables, which optimize the simulation output, referred to as the response, within a predetermined design space. This is done by-

- Fixing the experimental design space.
- Designing appropriate experiments within the design space and performing them.
- Using the quantitative data from these experiments to determine and simultaneously solve multivariate equations.
- Estimating the main effects, interaction effects and quadratic effects of the explanatory variables, and based on this, generating the shape of the response surface under investigation.

- Finally, using this response surface, identifying the optimum point where all specifications can be met.

There are different classes of designs included in RSM that are used in different production situations. A central composite design (CCD) is an experimental design for building a second order (quadratic) model for the response variable without setting up a complete three-level factorial experiment (Anderson, RSM simplified).

The design consists of three distinct sets of experiments:

1. A factorial design in the factors studied, each having two levels.

2. A set of *centre points*, experimental runs whose values of each factor are the medians of the values used in the factorial portion. This point is often replicated in order to improve the precision of the experiment.

3. A set of *axial points*, experimental runs identical to the centre points except for one factor, which will take on values both below and above the median of the two factorial levels, and typically both outside their range. All factors are varied in this way.

RSM has been widely used for process parameter optimizations such as enzymatic hydrolysis for recycled paper (Liu et al. 2009) and for production of lactic acid from apple pomace (Gullon et al. 2007).

Process optimization involves not only finding the best output, but also minimizing the amount of inputs. Because the cost of enzymes is one of the most significant process costs, it would be desirable to operate the process at a point where the maximum sugar release is obtained

per unit amount of enzyme used. Thus, a design that could predict the hydrolysis levels depending on the amount of enzymes would be very useful. Response surface methodology was used to generate such a model. However, since this process is economically operated as SSF (Phillipidis et al. 1993), it would be more meaningful to include temperature as one of the explanatory variables and the yield of the final product as the response variable rather than to consider saccharification in isolation.

Methods

Using RSM the effect of three operational variables: concentrations of the two enzymes (cellulase and β-glucosidase); and temperature was studied on the response variable, lactic acid concentration during SSF. This experimental approach is useful to investigate multiple parameters affecting a reaction simultaneously and also identifies interactions among the parameters when present. An estimate of the enzyme levels corresponding to different hydrolysis levels obtained in the above experiments was used to set the upper and lower limits of the enzyme loadings. Table 3.1 shows the ranges of the operational variables used for this design. The microorganism used is *L .plantarum* as it has higher yield and productivity values than *R .oryzae*.

A 2^3 factorial central composite experimental design (CCD) was set-up with six axial points and six replications at the center point resulting in a total of twenty runs. The experimental design matrix is described in Table 3-2. The runs were conducted, accordingly, for thirty six hours and the lactic acid concentration determined. Experimental data from the CCD was analyzed using RSM algorithm Design Expert 7.1 (Stat-ease, Minn. USA) and fitted according to Eq. (1) as a

second-order polynomial equation including main effects and interaction effects for each variable:

$$y = \beta_o + \sum_{i=1}^{3} \beta_i x_i + \sum_{i=1}^{3} \beta_{ii} x_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \beta_{ij} x_i x_j \quad (1)$$

where, y = predicted response, β_o = constant coefficient, β_i= linear coefficient, β_{ii}= quadratic coefficient, and β_{ij}= interaction coefficient. Analysis of variance (ANOVA) and contour plots were generated using Design Expert 7.1. Optimized values of three independent variables for maximum lactic acid yield were determined using a numerical optimization package of Design Expert 7.1. Numerical optimization searches the design space using a fitted model to find the optimized values of independent variables that maximizes the response variable (Brijwani et al. 2009).

SSF -- To 20 ml of paper sludge that had been autoclaved and supplemented with 5 g/l peptone. Enzymes were added at the levels identified by the software, inoculum was added and were incubated at different temperatures as defined by the software.

Results and discussion

Analysis

SSF experiments were conducted as outlined in the methods section and a polynomial second order equation was generated by the software using in the values obtained in those experiments. The quality of fit of the second order equation was expressed by the coefficient of determination, R^2, and its statistical significance was determined by F-test and the significance of each coefficient was determined using Student's t-test. As shown in table 3-3, the model F value

of 73.9 implies that the model is significant. An R^2 value of 0.985 and a very close adjusted R^2, 0.962, showed that the model can be very precise for predictions. A reasonably low value of coefficient of variation (CV), 7.71 proved that the experimental runs conducted were precise. The significance tests of each of the coefficients (Table 3-3) showed that all the factors tested were significant. However, the factor with the maximum influence was cellulase level followed by β-glucosidase. Both the factors had a uniformly sloped positive effect on the yield all through their variation range, but the slope for cellulase was much steeper than that of β-glucosidase (Figure 3-1). Temperature was found to have a moderate degree of negative correlation to lactic acid production (Figure 3-2). Temperature and β-glucosidase showed a significant interaction effect.

Process optimization

Using Design Expert 7.1, the design space was explored with the fitted quadratic model to arrive at optimum levels of the independent variables. The optimized variables were found using a desirability objective function that assigns relative importance to the variables. To maximize lactic acid yield, the solution with the maximum desirability gave an operation condition of enzyme concentrations of 10 fpu of cellulase and 35 egu β-glucosidase at 38°C to achieve a maximum lactic acid yield of 0.75g/g glucan. However, this point is at the corner of the design space and it is obvious that higher levels of enzyme would lead to higher yields. Beyond this, the yield cannot go up as the maximum lactic acid yield obtainable in case of paper sludge is 0.75 g/g glucose. Thus the yield will plateau beyond this design space. To optimize the process such that higher lactic acid yield can be achieved with the minimum amount of enzymes, the relative importance of minimizing the enzyme requirements was increased to the same level as that of maximizing product yield. In this case, the maximum desirability gave an operation

condition of 8.5 fpu cellulase, 12.5 egu β-glucosidase at 40°C which would result in 0.58 g lactic acid per gram of glucan present in the media.

The data from the initial hydrolysis experiments clearly demonstrates that β-glucosidase has a very significant effect on time of hydrolysis and has some effect on the total amount of glucose released. The correlation of β-glucosidase with lactic acid concentration was found to be smaller than that expected from the one-factor-at-a-time results. This is due to the mode of action of the two enzymes used. β-glucosidase quickly removes the bottleneck caused by cellobiose accumulation by cleaving it into glucose units. Thus, it hastens the reaction. However, instead of 28 hours as in the case of the hydrolysis experiments, SSF was allowed to proceed for 36 hrs. In this additional time, the residual β-glucosidase activity of the cellulase can hydrolyze the cellobiose units thus decreasing the effect of β-glucosidase addition. This result confirms that reported by Marques et al (2008) where they observed the same level of hydrolysis with and without β-glucosidase supplementation when supplemented with high cellulase loadings (25 fpu/g carbohydrates) and a high residence time of 72 hours. Thus, a proper level of β-glucosidase supplementation effectively brings down the cellulase loading as well as hydrolysis time.

The desirable temperature for the operation of SSF was predicted to be around 38°C, confirming that microbial growth is much more susceptible to temperature deviations from the optimum than enzymatic hydrolysis. As can be seen from Figure 3-2, when the temperature is on the lower range, the effect of increasing enzymes is directly translated into increasing yields. But as the temperature starts to increase, even if saccharification increases due to higher enzyme concentrations, the sugar cannot be utilized effectively by the microorganisms to produce the acid.

Using the results from the design experiment, the total enzyme requirement of the process, for hydrolysis levels greater than 90% was estimated to be 280 µl/ gm of carbohydrates. This compared favorably to all earlier studies reporting the enzymatic requirements of sludge hydrolysis. This result could be partly due to the nature of the sludge, but is mostly attributed to the fact that the model could thoroughly search the design space, much more effectively than can be possibly done by actual experimentation to identify points of maximum hydrolysis as well as highest yield per unit of enzyme used.

Conclusion. Optimizing operational conditions to obtain the maximum amount of product with the minimum input costs and time is a primary requirement for designing an economically feasible bioconversion process. In this regard, the model generated in this study is a very powerful tool to determine the point in the design space at which to operate by taking into consideration the cost of the individual enzyme and the product so that highest economic gains can be made. Though different sludge samples may vary slightly in their enzymatic requirements, such models can be prepared by varying the upper and lower limits accordingly to determine their ideal enzyme dosages.

Table 4.1 Variables and experimental design region for response surface

Factor	Name	Units	Type	Low actual	High actual	Low coded	High coded
A	cellulase	fpu/g cellulose	numeric	3.5	10	-1	1
B	β-glucosidase	egu/g cellulose	numeric	12.5	38	-1	1
C	temperature	°C	numeric	35	45	-1	1

Response variable- Lactic acid yield, units-g/l.

Table 4.2 Experimental design with the real values of lactic acid yield

Run	Factor A(fpu/g)	Factor B(egu/g)	Factor C(°C)	Response(g/l)
1	10.50	38.00	35.00	0.72
2	7.00	4.00	40.00	0.43
3	7.00	25.00	40.00	0.53
4	7.00	4.00	40.00	0.74
5	7.00	25.00	40.00	0.55
6	7.00	25.00	40.00	0.55
7	7.00	25.00	40.00	0.53
8	13	25.00	40.00	0.7
9	3.50	12.50	45.00	0.14
10	10.50	12.50	35.00	0.58
11	7.00	25.00	48.41	0.16
12	7.00	25.00	40.00	0.53
13	10.50	25.00	45.00	0.65
14	3.50	37.00	45.00	0.2
15	1.00	25.00	40.00	0.1
16	10.50	12.50	45.00	0.61
17	7.00	25.00	31.59	0.34
18	7.00	25.00	40.00	0.54
19	3.50	37.00	35.00	0.41
20	3.50	12.50	35.00	0.12

Table 4.3 Regression analysis for the second order polynomial model

Factor	Coefficient estimate	Standard error	F value	P value
Intercept	0.54	0.014	73.9	<0.0001
A-A	0.20	9.526e-003	430.45	<0.0001
B-B	0.077	9.526e-003	65.31	<0.0001
C-C	-0.0039	9.526e-003	16.77	0.0022
AB	-0.021	0.012	2.92	0.1186
AC	0.019	0.012	2.27	0.1629
BC	-0.041	0.012	10.98	0.0078
A^2	-0.044	9.273e-003	22.19	0.0008
B^2	0.022	9.273e-003	5.49	0.0411
C^2	-0.097	9.273e-003	108.78	<0.0001

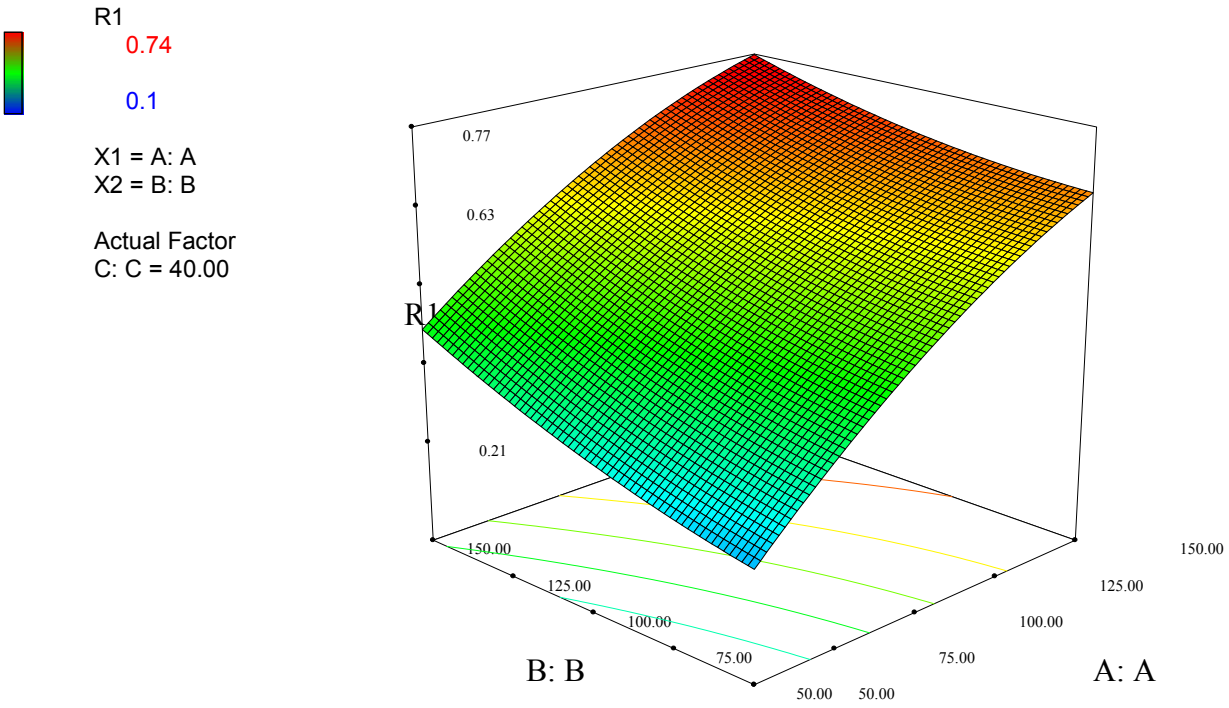

Figure 4.1 Response surface showing the effect concentration of two enzymes on lactic acid yield.

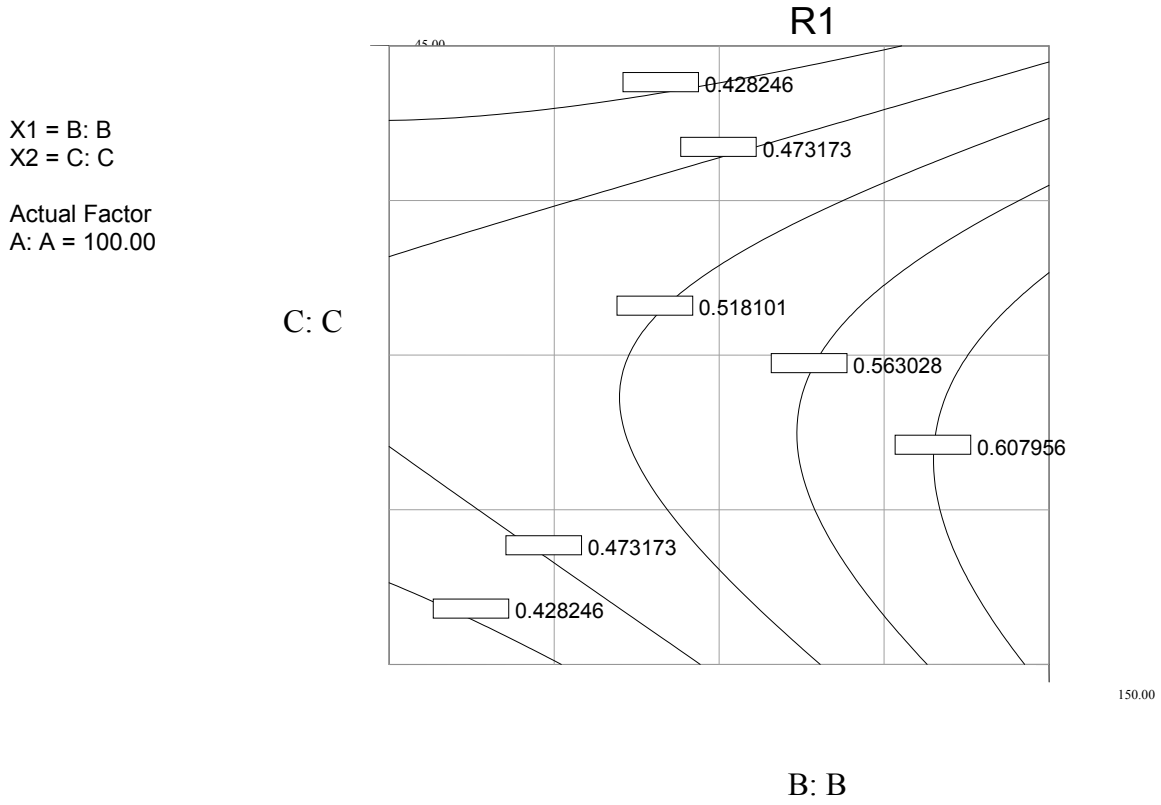

Figure 4.2 Contour plot showing the effect of β-glucosidase concentration and temperature on lactic acid yield.

CHAPTER 5 - Microbial isolates with enhanced lactic acid production from paper sludge

Introduction

Isolation and screening of microorganisms from natural sources is one of the most common methods of obtaining superior performing microbial strains for use in industrial production. Many examples exist in literature in which strains with different desirable characteristics have been isolated from nature. For example, Adnan et al. (2007), isolated naturally occurring strains of *Lactobacillus* from tapai (fermented tapioca), that were resistant to very high lactic acid concentrations.

In this study, a marked decrease in growth of the ATCC cultures was observed when inoculated in paper sludge. This suggested that the presences of some component/ components in the sludge are detrimental to microbial growth. Additionally, it was observed that when the medium was hydrolyzed without autoclaving, at the end of hydrolysis, some lactic acid accumulation occurred. This was possibly due to the action of lactic acid bacteria native to the sludge. The hypothesis here is that the native lactic acid bacterial populations that had been growing in the same medium for generations could have developed some resistance to these inhibitory substances by evolutionary mechanisms and thus were more efficient lactic acid producers.

Method

Preparation of a selective medium

A medium for the selective isolation of acid producing bacteria was prepared. Solid MRS medium was prepared by adding agar to MRS broth. Before autoclaving the medium, citric acid was used to bring down the pH to 5.2. 0.0025g/l of antifungal agent cycloheximide and 0.05 g/l bromocresol green, a pH indicator which is green at pH above 5 and turns yellow at a lower pH was added to MRS agar. The media was autoclaved and used for plating the sludge solution. It was observed that the acid produced by the acid producing colonies diffused into the medium which resulted in fainter, bigger yellow zones, often around a group of colonies. This made it difficult to identify the colonies that were actually producing the acid. To prevent diffusion, calcium carbonate at a concentration of 20g/l added during the preparation of the medium. This effectively prevented acid diffusion and the halos were darker and more distinct around the actual acid producing colonies.

Isolation

The hydrolyzed sludge was enriched with 5g/l peptone and incubated at 37°C for 24 hours. To maintain a low oxygen environment suitable for LAB, 100 µl of this hydrolyzed sludge was pour plated instead of spread plated on the above described agar. The plates were incubated at 37°C for two days. Colonies that grew on the plates were streaked individually on fresh plates to obtain pure cultures.

Each of the isolated cultures was gram stained and the gram positive strains were subjected to the gel plug test (Gibson et al. 1945) to determine if the isolate is a homofermentative or a heterofermentative species. The isolates that tested negatively for the

tested were those that did not produce any CO_2 and were homofermentative LAB. They were grown in MRS media as well as on sludge hydrolyzate and their lactic acid production levels were tested against the ATCC culture.

Results and Discussion

Five colonies were selected from both the glucose and the lactose media that showed the biggest acid zones. By the gel plug test, all the isolates were confirmed to be homofermentaive lactic acid bacteria. In the synthetic medium, all of these isolates had lactic acid yields and productivities similar to the ATCC culture. On inoculating them in sludge media, it was found that even in this medium, the highest concentration of lactic acid that could be achieved were similar to that obtained in the case of ATCC cultures (Table 4-1). A screening technique that has a higher throughput and sensitivity would thus be required for isolating strains that can produce higher lactic acid concentration, provided such species are present in the sludge.

Table 5.1 Lactic acid concentration obtained by fermentation using bacterial isolates on paper sludge hydrolyzate

Culture	Concentration (g/l)
L. plantarum ATCC	0.74[a]
O1	0.73[a]
O2	0.69[b]
O3	0.67[b]
O4	0.71[a]
O5	0.73[a]

Values with same superscript do not vary significantly. Values with different superscripts differ significantly ($P < 0.05$).

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