Lactic Acid Production from Renewable Carbon Sources by Genetic Engineered *Bacillus subtilis* and Mixed Culture with *Lactobacillus rhamnosus*

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Lactic Acid Production from Renewable Carbon Sources by Genetic Engineered *Bacillus subtilis* and Mixed Culture with *Lactobacillus rhamnosus*

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Certificate of originality

I hereby declare that this thesis is my own work and that, to the best of my knowledge and belief, it reproduces no material previously published or written, nor material that has been accepted for the award of any other degree or diploma, except where due acknowledgement has been made in the text.

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Abstract

Lactic acid has been produced commercially by fermentation since 1881. The fermentative production of this product accounts for half of the world production. The remainder is produced synthetically from e.g. lactonitrile. The main users of lactic acid are the food and pharmaceutical industries, but there are technical applications as well, e.g. production of poly lactic acid (PLA). PLA can be used as cell microcarriers, bone tissue engineering etc.

Batch fermentation experiments were conducted to establish optimum operating conditions for the simultaneous saccharification and fermentation (SSF) of potato starch to lactic acid using *Lactobacillus rhamnosus* (ATCC 10863) with immobilized *Aspergillus niger* (ATCC 13496). Starch was first liquefied by bacterial alpha amylase. The alpha amylase solution (59kunit/ml) was obtained from *Bacillus subtilis* Φ105 MU331 with alpha amylase gene inserted, after 6 hours thermoinduction. Immobilized *Aspergillus niger* alginate beads were cultivated in a sucrose medium followed by glucoamylase induction in a starch solution. The beads and *Lactobacillus rhamnosus* inoculum were mixed together with fermentation broth. All SSF were kept at pH 5.0 – 5.5 adjusted by feeding calcium carbonate at 43℃.

The yield of lactate based on total sugar was about 75%. Lactate productivity was 1.354 g/l/h for SSF conducted under optimum conditions with 100 g/l potato starch; 1.389 g/l/h with initial 150 g/l potato starch. The productivities and yields did not change with the addition of yeast extract in SSF.

Bacillus subtilis is an atypical strain for lactic acid production and it is rarely used as a lactic acid producer. In its' metabolism, glucose was metabolized through glycolysis, Krebs cycle and electron receptors yielding ATP. Lactic acid was produced only when there is a lack of oxygen. The aerobic condition showed a positive influence on growth and a negative effect on lactate production. Based on this, a 2-stage fed batch fermentation was developed, the initial aerobic culture with aeration rate of 2l/l/min was changed to a micro-aerobic culture with aeration rate of 0.5 l/l/min. The biomass and lactic acid concentration produced were 6.2 and 10.0 g/l, respectively. To improve the strain, UV mutagenesis was performed. Mutants were isolated by mutagenizing the parent strain *Bacillus subtilis* Φ105 MU331. After fermentation, the concentration of biomass and lactic acid of the mutants were approximately 6.4 and 21 g/l, respectively. The productivity of lactic acid is high (2.625 g/l/h), in comparison with mesophilic lactic acid bacteria. The major metabolites produced was analyzed and identified by HPLC.

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Project Objectives

1. Study the feasibility of using *Bacillus subtilis* MU331 (pSG03 + AMY, genetically engineered to produce high levels of α amylase) for lactic acid production.

2. Use a mixed culture in a single or multiple stage fermentation process to convert starch to lactic acid.

3. Study the feasibility of using industrial waste (malt waste) as a substrate to produce lactic acid by fermentation and develop processes in which *Lactobacillus rhamnosus* can produce lactic acid with industrial wastes containing cellulose, starch as carbon source.

1 Introduction

1.1 Lactic acid

Lactic acid ($C_3H_6O_3$) can be produced by our body and other organisms. In our body, through daily activities, our body constantly produces lactic acid due to anaerobic respiration. It is produced by microorganisms and the simplest production formula is

Sugars + Water + Microorganisms → Lactic Acid

Lactic acid is a versatile chemical, naturally occurring multifunctional organic acid used as an acidulant, flavor and preservative in the food, pharmaceutical, leather and textile industries. It is also used for the production of base chemicals. Nowadays, one of its most promising applications is its use for biodegradable and biocompatible polylactate polymers such as PLA, an environmentally friendly alternative to biodegradable plastics (Datta, 1995; Hofvendahl et al., 1999; Khalaf, 2001). Lactic acid is largely produced for making biodegradable plastics polylactic acid polymer (Vickroy, 1985; Kharas et al., 1994). Owning to the unique property of PLA, lactic acid has the potential to be a substitute for biodegradable plastics manufacture and becomes a very large-volume commodity chemical intermediate. Lactic acid exists as two optical isomers, D- and L-lactic acid. Both isomeric forms of lactic acid can be

polymerized and polymers with different properties can be produced depending on the composition. Since elevated levels of the D-isomer are harmful to humans, L-(+)-lactic acid is the preferred isomer in food and pharmaceutical industries (Akerberg, 1998; Hofvendahl, 2000). Over 80000 tons of lactic acid are produced worldwide every year about 90% are made by lactic acid bacterial fermentation and the rest is produced synthetically by the hydrolysis of lactonitrile (Datta, 1995; Hofvendahl, 2000). Fermentation production has the advantage that by choosing a strain of lactic acid bacteria producing only one of the isomers, an optically pure product can be obtained, whereas synthetic production always results in a racemic mixture of lactic acid. Another significant advantage over the chemical synthesis is that biological production can use cheap raw materials, such as whey, molasses, starch waste, beet- and cane-sugar and other carbohydrate-rich materials. In commercial processes, sugars and starches have been widely used as substrates for biological production of lactic acid (Tsao, 1999; Richter, 1998; Anuradha, 1999; Aristidous, 2000; Huang, 2003). Renewable resources do not give any net contribution of carbon dioxide to the atmosphere, as do the limited oil and fossil fuel based sources. Cellulose, hemicellulose and starch are the most abundant compounds in the world, and when hydrolyzed to mainly glucose they are fermentable by a number of microorganisms. Hemicellulose, in contrast to starch and cellulose,

contains pentoses, which give rise to by products such as acetate and ethanol, decreasing the lactic acid yield. Fermentative lactic acid production from renewable resources comprises the following steps: pretreatment of substrate including hydrolysis to sugars, fermentation of sugars to lactic acid, separation of bacteria and solid particles from the broth, and purification of lactic acid.

Biotechnological production is primarily carried out by bacterial fermentation of simple sugars, and bacterial species *Lactobacillus* and *Lactococcus* have received a worldwide interest in industrial processes because of their high growth rates and product yields (Cheng, 1991; Gonzalez, 2001; Linko, 1996). Lactic acid bacteria are cocci, with the exception of lactobacilli which are rods, they are unable to synthesize ATP by respiration, and lactic acid is produced as the major end product from energy conserving fermentation of sugars. Most lactic acid bacteria are facultatively anaerobic, catalase negative, nonmobile and nonspore forming. They have high acid tolerance and can survive at pH 5 and lower. Their acid tolerance gives them a competitive advantage over other bacteria. The optimal temperature for growth varies between the genera from 20 to 45℃ (Wood, 1995). Most of them are considered as GRAS (generally regarded as safe), but some strains are pathogenic.

Lactic acid bacteria have complex nutrient requirements, due to their limited ability to synthesize B-vitamins and amino acids (Chopin, 1993). Therefore they are naturally found in nutrient rich environments such as in plants, milk, and inside the human and animal bodies. A complex natural environment renders a microorganism able to metabolize many different carbohydrates (Viniegra-Gonzalez, 1984). Lactic acid bacteria have been used by humans for the fermentation of food and feed products since ancient days, and today their major applications are still in the food and feed industry such as production of dairy products, pickles, meat and wine. The present technical applications of lactic acid bacteria include the production of nisin by Lactococcus, and the production of lactic acid for different applications (Dellaglio, 1995). It has also been suggested that lactic acid bacteria could be used as oral vaccine vectors (Wells, 1996).

Lactic acid bacteria ferment sugars via different pathways resulting in homo, hetero or mixed acid fermentation. Homo-fermentation gives only lactic acid as the end product of glucose metabolism, and the Embden Meyerhof Parnas pathway is used (Thomas, 1979; Smith, 1975). In hetero-fermentation lactic acid, carbon dioxide and ethanol or acetate are formed from glucose via the phosphoketolase pathway (Axelsson, 1993; Garvie, 1980). This pathway is used by facultative

heterofermenters, such as lactobacillus casei, for the fermentation of pentoses, and for the fermentation of hexoses and pentoses by obligate heterofermenters (Kandler, 1983). According to Kandler, all lactic acid bacteria except lactobacilli of type I are able to ferment pentoses, i.e. they are facultative heterofermenters (Kandler, 1983). Lactic acid bacteria is recognized for the conversion of glucose to lactic acid. The most common species used in industries was *Lactobacillus casei*. The optimum temperature, sources of nitrogen and growth factors had already been investigated (Hujanen and Linko, 1996).

Various fermentative lactic acid production processes have been patented. These include simultaneous saccharification and fermentation process with mixed culture using industrial starchy waste (Tsai and Millard, 1994), the use of recombinant lactobacillus enabling the fermentation of 5 carbon sugar (xylose) (Picataggio et al., 1997), purification of lactic acid through electrodialysis (Datta, 1989; Miura and Kumagai, 1995), membrane separation (Russo and Kim, 1996), and esterification (Kumagai, 1994).

At the present time, researches are focused on fermentation techniques for higher substrate conversion and production yields (Nancib et al., 2001). Plastic products

made from polylactic acid can bypass recycling efforts and end up directly in a landfill; it is naturally metabolized by microorganisms into CO_2 and water and defined as biodegradable product. In addition, it eliminates emissions of dioxins and other harmful chemicals if incinerated as the material used is natural. Also, this plastic reduces the use of petroleum products from the start. Lastly, the biodegradable plastic takes less energy to produce then the conventional plastic. Therefore, it is already being widely used in textiles, medical and packaging industries, such as plastic bags, vegetables and liquid food packaging, fiber and non-woven. (Balkcom et al., 2002)

To produce lactic acid commercially, glucose can be converted to lactic acid by lactic acid bacteria. Even banana can be the substrate for lactic acid production, as sucrose, glucose and fructose are banana is major sugars in the pulp (Chan-Blanco et al., 2003). However, other than glucose, it is possible to use other starting substrates to lower the production cost. Corn, wheat, sorghum, rice, soybean and cereal contain high percentage of starch, these starchy substrates can be hydrolyzed into glucose; therefore, the substrate cost is lowered (Kwon et al., 2000).

Molasses and whey are typical raw materials for lactic acid production by

fermentation. However, these sources are not in plentiful supply and are quite expensive. Starch as a cheap carbon source has been used in many microbial processes to produce fermentative products. Starch generally exists in two forms, which are amylose and amylopectin; they are linear and branched form respectively. They can both be hydrolyzed by alpha-amylase, beta-amylase, gamma-amylase, alpha-glucosidase and pullulanase (Janse et al., 1993). Glucose can be derived through saccharification of starch by amylases but the product, glucose inhibits the enzyme. A process known as simultaneous saccharification and fermentation (SSF) has shown experimentally that the inhibition can be overcome and starch can be used as a raw carbon source for lactic acid production. SSF couples saccharification by amylases and fermentation at the same time (Anuradha et al., 1999). This process is much more economical not only in terms of shortening the operational time but also in reducing bioreactor volume. Thus the SSF process uses starch as the primary raw material, which is cheap, abundant and present in a variety of agricultural resources, e.g., rice, potato and tapioca. The advantage of the SSF process is to prevent amylase inhibition caused by glucose accumulation thus an enhancement in the lactic acid productivity.

1.2 Multi Stages

In order to convert starch into lactic acid, the process had to go through several steps. These steps are gelatinization, liquefaction, saccharification and lactic acid production. (Starch → liquefied starch → glucose → lactic acid) Conventional biotechnological production of lactic acid from starch materials, for instance, requires pretreatment by gelatinization and liquefaction, which is carried out at high temperatures of 90 - 130 ℃ for 15 min followed by enzymatic saccharification to glucose and subsequent conversion of glucose to lactic acid by fermentation (Lynd, 1991). The second and third steps are carried out by biological catalysts. These biological catalysts are industrial enzymes, they can eliminate the time, cost and waste to produce useful products, and therefore, they are recognized as a trend in biotechnology. Different approaches are used for discovering industrial enzymes, e.g. either by finding new microbes or cloning gene into other microorganisms (Marrs B. et al., 1999). For industrial purposes, thermostable bacterial enzymes are commonly used because the process may need to be maintained at a higher temperature and the enzyme therefore had to withstand the condition. The most widely used thermostable enzymes are the amylases in the starch industry (Poonam and Dalel, 1995; Crab and Mitchinson, 1997; Emmanuel et al., 2000; Sarikaya et al., 2000). Thermostable enzymes are gaining wide industrial and biotechnological interest due to the fact that

these enzymes are better suited for harsh industrial processes. The applications of thermostable enzymes are alpha amylase (bacterial and fungal) in starch hydrolysis, brewing, baking, detergents, production of maltose; pullulanase in the production of glucose syrup; xylanase in the pulp and paper industry; cellulase in cellulose hydrolysis, polymer degradation in detergents. The advantage of conducting such high temperature processes is reducing the risk of contamination by common mesophiles. Other values of elevated process temperatures include higher reaction rates due to a decrease in viscosity and an increase in diffusion coefficient of substrates and higher process yield due to increased solubility of substrates and products. The enzymes are also used as a model for understanding thermostability and thermo activity, which is useful for protein engineering

The starch industry is one of the largest users of enzymes for the hydrolysis and modification of this useful raw material. The starch polymer, like other such polymers, requires a combination of enzymes for its complete hydrolysis. These include alpha amylases, glucoamylases, beta amylases, and isomerases or pullulanases. The enzymes are classified into endo-acting and exo-acting enzymes. Alpha amylase is an endo-acting enzyme and hydrolyses linkages in a random fashion and leads to the formation of linear and branched oligosaccharides, while the

rest are exo-acting enzymes and attack the substrates from the non-reducing end, producing oligo-, di- or mono-saccharides.

The enzymatic conversion of all starch includes gelatinization, which involves the dissolution of starch granules thereby forming a viscous suspension, liquefaction, which involves the partial hydrolysis and loss in viscosity and saccharification, involving the production of glucose and maltose via further hydrolysis. Gelatinization is achieved by heating starch with water, and starch is water soluble only at high temperatures which are dependent on the source. For hydrolysis of the starch to proceed immediately after gelatinization, hence, among other things avoiding a lot of cooling time, the enzyme has to be thermostable. This two step process involving consecutive enzymatic hydrolysis and fermentation makes it economically unattractive. The bioconversion of carbohydrate materials to lactic acid can be made much more effective by coupling the enzymatic hydrolysis of carbohydrate substrates and microbial fermentation of the derived glucose into a single step, known as simultaneous saccharification and fermentation (SSF). SSF has been successfully employed for lactic acid production from raw starch materials and many representative bacteria including *Lactobacillus* and *Lactococcus* species have been used (Zhang, 1994; Vishnu, 2000).

One of the concerns of the starch industry is the calcium requirement of the alpha amylase enzymes and the formation of calcium oxalate, a substance that may block process pipes and heat exchangers. Besides, such accumulation in some products like beer is not acceptable. Its precipitation can be reduced through a decrease in calcium ion requirement of enzymes and lowering of the pH of the production process. Activities of a number of the starch hydrolyzing enzymes, however, are calcium dependent although the degree of utilization varies. Saccharification of liquefied starch is carried out at low pH values. However, currently used thermostable alpha amylases are not stable at such low pH. An economical process could be attained through the use of amylases stable at the saccharification stage. A challenge is placed to scientific and business area in achieving a perfect enzyme system to commit to new conditions and characteristics.

Refined sugars, although expensive are the most commonly used substrate for production of lactic acid by fermentation. Lactic acid is also produced from cheaper substrate like starch in two-step process of saccharification followed by lactobacillus fermentation (Yumoto and Ikeda, 1995). Use of a microorganism which directly ferments starch to L-lactic acid would eliminate saccharification cost and

Lactobacillus amylophilus GV6 is one such organism having high production efficiency in submerged fermentation as reported (Vishnu et al., 1998, 2000).

1.3 Alpha-amylase

There are two main classes of amylase for starch degrading processes: α-amylase and glucoamylase. α-amylase is produced by lots of organisms, including bacteria, fungi, animals and plants. It is commonly produced by *Aspergillus sp.* and *Bacillus sp.* because they produce extracellular enzyme and therefore are easier to collect by centrifugation (Amylase Research Society of Japan, 1988). α-amylase randomly cleaves the 1,4-α-D-glucosidic linkages between the adjacent glucose units of linear amylase chains (Ellaiah et al., 2002). It can be divided as starch liquefying and starch saccharifying type, depending on their protein nature and starch degrading mechanism. However, the saccharifying type is not commonly used in industries as their activity is lower than glucoamylase (Amylase Research Society of Japan, 1988).

Alpha-amylase was investigated under industrial conditions; temperature, pH, rotation speed were subjected to the investigation. Results showed that the highest utilizing rate of dextrose was at 90°C and pH 6. All the above conditions directly affected the glucose production from starch (Marchal et al., 1999). Bacillus species

are considered to be the most important sources of α-amylase and have been used for enzyme production using simultaneous saccharification and fermentation (SSF). SSF is considered as a potential tool for achieving economy in enzyme production and starch hydrolysis. In the SSF process, starch is simultaneously degraded to oligosaccharide and hydrolyzed to glucose by the alpha amylase or glucoamylase, oligosaccharide is converted to monomeric glucose by amylglucosidase, and glucose is catabolized primarily to lactic acid, cell mass and carbon dioxide by a fermentative microorganism. In this process, the saccharification and fermentation steps are integrated, i.e., performed simultaneously. This method eliminates the need for a complete hydrolysis step prior to the fermentation step (Jin, 1999; Jin, 2001; Moldes, 2001). In the SSF process, enzymatic hydrolysis, cell growth and microbial production occur simultaneously. Another direct benefit of the SSF is a decrease in the inhibition caused by glucose accumulation, leading to an increase in the saccharification rate, consequently increasing productivity and reducing reactor volume and capital costs (Takagi, 1977).

The source of α-amylase used in this project was *Bacillus subtilis* MU 331 (pSG03+AMY), the gene for enzyme production was originally from *Bacillus licheniformis*, and it is a thermostable enzyme and is intense in starch liquefaction

(Joel and Ana, 2003).

1.4 Glucoamylase

Glucoamylase is an exosplitting enzyme where it removes a glucose molecule from an oligosaccharide one at a time. It is also produced from a wide variety of organisms, such as yeast, bacteria, fungi, and the common microbes that are used in industries are *Aspergillus sp., Clostridium sp.* and *Saccharomycopsis sp.* (Selvakumar et al., 1996).

It is traditionally produced from submerged fermentation; however, recently the application of solid state fermentation (SS) has become more significant in enzyme production. SS have many advantages over submerged fermentation, especially the continuous enzyme production and the convenience of enzyme collection (Lonsane and Ramesh, 1990). Extensive work has been carried out on the production of glucoamylase in solid state cultures using Aspergillus niger. The studies included screening of a number of agro-industrial residues such as wheat bran (Sandhya, 2005), rice bran (Sandhya, 2005), rice husk (Sandhya, 2005), gram flour (Pandey et al., 1993), wheat flour (Sandhya, 2005), corn flour (Pandey et al., 1993), tea waste (Selvakumar et al., 1998), copra waste (Pandey et al.,1995) etc for the production of

enzyme.

1.5 Fungus glucoamylase

Fungus is one of the two major microbes for producing glucoamylase. In this project, *Aspergillus niger* is chosen as the source. The major advantage of using the fungi over the bacteria is the lower costs, due to (1) use of raw and/or waste materials, (2) no specific nutrients required, (3) little pH maintenance required, as most fungi can tolerate low pH environments, and (4) easy and un-expensive separation of filamentous or pellet biomass from the fermentation broth (Soccol, 1994; Rosenberg, 1995). Many factors, such as pH, temperature, substrates, and product concentration of glucose and lactic acid can affect the SSF mechanisms (Anuradha, 1999; Vishnu, 2000). A disadvantage of SSF is the difference in cultivation conditions, such as pH and temperature, required for saccharification and fermentation. In many cases, the low pH, e.g., lower than 5, and high temperature, e.g., >40 ℃, may be favorable for enzymatic hydrolysis, whereas the low pH can surely inhabit the lactic acid production and the high temperature may affect adversely the bacteria cell growth (Jin, 1999; Jin, 2001). Therefore, to identify the microbial and biochemical kinetics and determine the optimal process conditions which enhance the SSF performance is of importance for an industrial process for the lactic acid production. However, little

information of SSF kinetic using fungal microorganisms for lactic acid production is available in the literature.

Aspergillus sp. has a long history in fermentation for enzyme production. Some of the strains can secret in excess of 20g/L of enzyme, including glucoamylase. In submerged culture, the filamentous fungi will exhibit a range of morphology from mycelial trees to densely interwoven mycelial masses (Papagianni and Moo-Young, 2002). *Aspergillus niger* has been used for a long time in industries, glucoamylase production was studied in carbon limited chemostat (Metwally et al., 1991). Fed-batch cultivation is the most frequently used in industries (Stanbury et al., 1995).

2 Project significance and value

1. A one or two stage mixed culture batch fermentation process which can use starch to overproduce lactic acid is better than a process with 2 steps involving an enzymatic stage and a fermentation stage. The reasons are economics and simpler process operation.

2. Isolation of improved acid tolerant *Lactobacillus rhamnosus* mutants using continuous culture selection methods could result in higher levels of lactic acid production.

3. It would be of interest academically and industrially if a recombinant *Bacillus subtilis* (MU331) which overproduce α amylases can be developed to overproduce lactic acid from starch. The directing of the carbon flux to lactic acid would involve fermentation manipulations and genetic techniques, which if successful, can produce the target metabolite in large amounts. *Bacillus subtilis* can grow in less expensive media than *Lactobacillus*, and it can be developed later to use inexpensive carbon sources such as starch, agricultural, food and industrial waste.

3 Research methodology

A brief introduction is given here. More details will be given in later chapters.

Fermentation

The first part is optimizing the fermentation conditions, using different fermentation conditions and medium development to optimize lactic acid production by *Bacillus subtilis* MU331 and *Lactobacillus rhamnosus* (ATCC 10863).

Alpha amylase

A genetically engineered *Bacillus subtilis* MU331 is used as the source of alpha amylase. The *Bacillus subtilis* MU331 would overexpress the extracellular alpha amylase after heat shock. The enzyme will be used in the starch liquefaction process.

Glucoamylase

Aspergillus niger (ATCC 13496) is one of the well known glucoamylase producers. It produces glucoamylase extracellularly. In this study, immobilized *Aspergillus niger* cultures will be used. Immobilization has several advantages over submerge fermentation such as ease of handling of biomass, enzymes being trapped inside beads and a boost of metabolite conversion rate etc. The immobilized cultures will be

used for the starch saccharification process.

Industrial waste

Malt waste was used as the substrate in solid state fermentation by *Aspergillus niger*. It is to examine the feasibility of lactic acid production by *Lactobacillus rhamnosus* from the fungal hydrolysate and acid hydrolysate of malt waste.

4 Materials and methods

4.1 Microorganisms and culture conditions

4.1.1 *Bacillus subtilis*

Strain

Bacillus subtilis MU 331 (pSG03+AMY) genetically engineered to produce high levels of *α-amylase* was used; the strain was stored in brain heart infusion in a –80°C refrigerator. It is kindly provided by Dr. Leung Yun-chung, Thomas.

Shake flasks

α-amylase

The medium used in the shake flask culture contained 37g/L brain heart infusion and 5g/l yeast extract (BHY). The media were sterilized at 121°C (15 p.s.i.) for 20 minutes before inoculation. The culture was grown in 1L flask, on orbital shaker at 270 rpm, with 100 ml culture medium inoculated with a loop of *Bacillus subtilis* MU 331 from a nutrient agar plate, at 37°C and initial pH 7.4 for 12 hours. Then, the culture was heat shocked for 5 minutes at 50°C in a water bath to induce *α-amylase* production. Samples were then taken out for analysis.

Lactic acid production

The characterization begins with various culture volumes (100ml, 300ml, 500ml and 700ml in 1L flasks) in order to determine the effect of different oxygen transfer rates on lactic acid production by *Bacillus subtilis* MU331. Then, the effect of oxygen transfer was studied by varying the rpm of the incubation shaker. In addition, extra glucose (18g/l) was used to avoid glucose limitation.

Then, 2 stage fed batch fermentations were investigated. The cultures were grown in 1L flasks, in an orbital shaker at 270 rpm, with 100ml BHY inoculated with a loop of *Bacillus subtilis* MU 331, at 37°C and initial pH 7.4. After 12 hours, the culture was transferred to 4 100ml flasks at different volumes (i.e. 10ml, 20ml, 30ml and 40ml). ~15g/l of glucose and/or 10g/l of yeast extract were added at the beginning of the second stage fermentation (120rpm at 37°C) with 5g/l calcium carbonate.

Fermentor fed-batch culture

The fermentor cultures were conducted at 37°C and pH 7.2. A Bioengineering 2000 fermenter with a 3.7 L glass tank was used. All runs begin with 2.0L of PNB medium. It contains 5.0g/l glucose, 3.0 g/l yeast extract, 13.0 g/l nutrient broth, 10.0 g/l

Primatone RL. Sigma antifoam 289 was added to minimize foaming. The pH was controlled with 1.5 M NaOH and 1.5 M HCL. The dissolved oxygen tension was monitored with a METTLER TOLEDO dissolved oxygen sensor. The pH measured by pH/Redox electrodes with refillable liquid electrolyte. The fermenter was interfaced by a Modicon device interface and controlled by Genesis software. Samples were taken out through a sampling tube. At the beginning of the second stage fermentation, glucose was added to a level of around 3g/l. Then, constant feeding of a 20% glucose solution (100g / 500ml) was started. Feeding was continued until the end of the experiment. Glucose solution was fed at a rate of 50 ml/hour. During the fermentation, agitator rotation speed was set at 400 rpm and aerated with 2 l/l/m air, later changing to 0.5 l/l/m.

Strain improvement – UV_{254}

The parent strain *Bacillus subtilis* MU 331 was exposed to UV irradiation at 254 nm for 0–45 min at a distance of 20 cm. The colonies forming units/ml (CFU/ml) in the culture broth were maintained at 47×10^6 cells/ml for UV-irradiation and further studies. Survival curve was prepared and time of exposure giving over 99% kill rate was selected for mutation of the organism. Samples were taken out at specific time points then spread over nutrient agar plates. The plates were placed in a 37°C

incubator for about 12 hours until colonies were formed. The suspected mutants were screened in the 2 stage fed-batch shake flask experiments.

Inoculum and frozen stock

The inoculum was grown in the nutrient agar plate by streaking a small piece of frozen stock and incubating at 37°Cfor 24 hours. The frozen seed culture was preserved in 1.5 ml vials containing 1 ml cell broth with 20% glycerol and stored at –80°C.

4.1.2 Lactic acid bacteria

A lactic acid bacteria *Lactobacillus rhamnosus* (ATCC 10863) was used, the strain was stored in MRS broth in a –80°C refrigerator.

Medium development

YE10 was developed to replace the use of MRS. This is to minimize the use of expensive and complex nitrogen sources to a single nitrogen source, yeast extract. The medium (YE10) in the shake flask culture contained 20g/L glucose, 10 g/L yeast extract, 5 g/L sodium acetate, 2 g/L KH_2PO_4, 0.2 g/L $MgSO_4$, 0.05 g/L $MnSO_4$, Tween 80 1ml/L. The media were sterilized at 121 ºC (15 p.s.i.) for 20 minutes before being inoculated. The culture was grown in 250ml flasks, in an orbital shaker at 250 rpm, with 100 ml culture medium containing 5% inoculum, at 37 ºC and initial pH 6.0 for 24 hours. The effect of corn steep liquor was studied. Various carbon sources including 6 carbons and 5 carbons were also examined.

Fermentor batch culture

The fermentations were conducted at 37°C/ 43°C and pH 6.0. A Bioengineering 2000 fermenter with a 3.7 L glass tank was used. All runs begin with 2.0L of YE10 medium and dissolved oxygen (DO) level below 10%. During the fermentation,

agitator rotation speed was set at 200 rpm without aeration. The pH was controlled with 8.0 M NaOH and 1.5 M HCL. The dissolved oxygen tension was monitored with a METTLER TOLEDO dissolved oxygen sensor. The pH measured by a pH/Redox electrode with refillable liquid electrolyte. The fermenter was interfaced by a Modicon device interface and controlled by Genesis software. Samples were taken out through a sampling tube.

The inoculum was grown in YE10 medium as the fermentation medium. After sterilization of the medium, the *Lactobacillus rhamnosus* was inoculated from a frozen seed (1ml) into a 250ml flask containing 100ml medium. The flask was incubated in a rotary shaker at 37℃ and 250 rpm for 15 hours, the initial pH was 6.0. The frozen seed culture was preserved in 1.5 ml vials containing 1 ml cell broth with 20% glycerol and stored at –80℃.

Strain improvement - Continuous culture

The fermentation parameters for the continuous cultures were similar to the batch fermentations described previously. The working volume was 2.0L with different pH to select for mutants at a dilution rate (D) of 0.03hr^{-1}. YE10 was used for the continuous cultivation. Steady state was reached after approximately 99 hours (3

turnover of the cultures). Isolated colonies were screened with various glucose concentrations in shake flask with respect to volumetric concentration, yield and volumetric productivity of lactic acid.

4.1.3 Fungi

Aspergillus niger (ATCC 13496) was used, the strain was maintained on potato dextrose agar slants at 4°C.

Alginate beads preparation

Aspergillus niger is grown at 25°C on potato dextrose agar plate for 7 days to obtain cultures with dense sporulation. The spores are collected with saline for immobilization. The spores are entrapped in calcium alginate gel beads under aseptic conditions. 100ml of sterilized 6% sodium alginate solution is mixed with 10ml of spore suspension (~10^7 spores/ ml) and extruded through a syringe into 200ml of 0.5 M calcium chloride solution with stirring. The gel beads formed are hardened and stirred for 20 minutes, and finally washed with 0.5% calcium chloride until free spores are not found in the solution. The beads are stored in a 4°C refrigerator.

Beads germination and enzyme induction

The medium in the shake flask culture contained 40g/L sucrose, 10g/L sodium nitrate, 0.5g/L KH_2PO_4, 0.5g/L $MgSO_4$ and 0.01g/L $FeSO_4$ (MYG). The media were sterilized at 121°C (15 p.s.i.) for 20 minutes before inoculation. The culture was grown in 250ml flasks, in an orbital shaker at 250 rpm, with 50 ml culture medium

containing ~3g beads, at 30°C and initial pH 6.0 for 24 hours. The above medium is used for the spores germination and growth of the mycelium within the beads. Then, the sucrose medium is replaced by 20g/L starch solution (enzyme production medium). The culture was grown in 250ml flasks, in an orbital shaker at 250 rpm, with 50 ml 2% starch medium containing 3g beads from the above mycelium growth medium, at 30°C and initial pH 6.0 for 24 hours. The beads are used for the saccharification process.

Liquefaction and saccharification

Starch was liquefied by α-amylase in order to reduce its viscosity. For every 10g of starch, 1ml of α-amylase (59kunit/ml) was added to the gelatinized starch solution. 10ppm of calcium was added in order to enhance the process. Liquefaction was carried out during autoclaving.

The 3g enzyme induced beads were added to the sterilized 100ml of liquefied starch solution. The cultures in 250ml flasks, were incubated in an orbital shaker at 250 rpm, with 100ml liquefied starch medium at 43 ºC and initial pH 6.0 for 24 hours. The media were sterilized at 121 ºC (15 p.s.i.) for 20 minutes before being inoculated.

4.2 *Simultaneous saccharification and fermentation (SSF) of lactic acid*

Batch fermentation was carried out in a Lh (1.0L) fermenter to characterize the SSF process. The initial conditions of all fermentations were pH 5.5, 43°C, dissolved oxygen (DO) level at around 10% without aeration. The medium was continuously stirred at a constant rate of 200rpm during all the runs. The temperature of the fermentor was controlled at 43 ± 0.1°C. A Cole Parmer sealed pH electrode was used to measure the pH.

Four 250ml flasks, each containing 3g of beads were cultivated in 50ml sucrose medium at 30^{o}C, 250rpm for 24 hours. To induce the glucoamylase production, the sucrose medium was replaced by 50ml of 2% liquefied starch and incubated for 24 hours. After 24 hours, 12g of beads and 20ml of *Lactobacillus rhamnosus* inoculum were together inoculated into the fermentor. For the SSF, 400 ml of SSF medium containing either 2, 10, 15% of liquefied starch, 10 g/L yeast extract, 5 g/L sodium acetate, 2 g/L KH_2PO_4, 0.2 g/L $MgSO_4$, 0.05 g/L $MnSO_4$, Tween 80 1ml/L was used. 20ml seed culture of *Lactobacillus rhamnosus* was then inoculated. $CaCO_3$ was used for neutralization of lactic acid. Samples were taken out at different intervals for analysis.

In the fermentor runs, industrial grade starch was used. It contains 20% moisture.

α-amylase activity

Bacillus subtilis MU331 overproduce α-amylase after heat shock at 50℃ for 5 minutes. After the heat shock, 1 ml of the sample is collected and centrifuged at 13000 rpm for 2 minutes, the supernatant is subjected to enzyme assay.

50µl of 0.5M glycine-NaOH buffer at pH 9 and 50µl of sample supernatant which contain α-amylase are added into 100µl of 0.2% starch solution for reaction. The mixture was incubated at 50℃ water bath for 10 minutes. After that, 200µl of 1.5M acetic acid was added to quench the reaction. 200µl of iodine solution was added to the mixture and followed by 3.4ml of distilled water. The absorbance was taken at 690nm. The amount of dark blue complex detected is inversely proportional to the enzyme activity.

The blank was made by using the same amount as above by substituting 0.2% starch solution with distilled water. A negative control was made by using the above components but using distilled water instead of the sample.

Enzyme activity can be calculated by the following equation, α-amylase (units/ml)

$$= \frac{\text{Abs of –ve control} - \text{Abs of sample}}{\text{Abs of –ve control}} \times \frac{100}{2.5} \times \frac{\text{dilution factor}}{\text{vol. of supernatant added}}$$

0.2% starch solution

2g of soluble starch is added into 10ml distilled water in room temperature and stirred. Then it was slowly poured into 90ml boiled distilled water until it dissolved. It was adjusted to 100ml with distilled water. 1ml of 2%starch solution was taken out and made up to 10ml with distilled water.

Iodine solution

A solution containing 0.2% iodine and 2% potassium iodide was freshly prepared from a five times concentration stock solution by dilution with water.

Absorbance measurement at 600nm

Measuring the O.D. of the sample at 600nm with the spectrophotometer was used to detect the growth of cells. Serial dilution of samples were needed when the O.D. was larger than 0.6.

pH

Measured by pH meter

4.3 Fermentation kinetics

Amount of glucose utilized (g/l)	= (Initial – final) glucose concentration
Rate of glucose utilization (g/l/hr)	= Amount of glucose utilized / fermentation time
$Y_{LA/GLU}$ (g/g)	= Final lactic acid concentration (g/L) / Amount of glucose utilized (g/L)
Lactate productivity (g/l/hr)	= Final lactic acid concentration (g/L) / fermentation time (hr)
Theoretical glucose (g/l)	= concentration of starch (g/l) * 1.11 (Roy et al., 2001)
Dried weight (g/l)	= 1ml centrifuged culture sample dried at 95℃ to constant weight * 1000

Specific lactic acid production rate (g/g/l)	= Final lactic acid concentration (g/L) / fermentation time (hr) / cell dried weight (g/l)

Preparation of the acid hydrolysate

For the acid hydrolysis of industrial waste, 0.1 M sulphuric acid and a waste to acid ratio of 1:5 (w/w) was used. The acid with the wastes were heated at 121º C (15 p.s.i.) for 30 minutes in the autoclave. After hydrolysis, the solid waste and the hydrolysate were separated by filtration to obtain a pale brown solution. 10 M NaOH was used to neutralize the filtered hydrolysate to pH 6.0. After neutralization, the hydrolysate contained precipitate, which was removed by centrifuge and filtered by using a 0.45 µm filter membrane. A clear brown hydrolysate was obtained.

Measurement of organic carbon; glucose and lactate

For organic carbon measurement, a TOC Analyzer, Shimadzu TOC-5000A was used. Samples are diluted to the appropriate concentrations so that the value falls within the 100 to 1000 ppm standard curve.

For glucose and lactate measurements, a YSI 2700 SELECT Biochemistry Analyzer was used. The samples were centrifuged at 12,000 rpm for 2 minutes and then the amount of glucose and lactate in the supernatant was measured. For glucose measurements, samples are diluted within 0 to 9 g/l. For lactate measurements, samples are diluted within 0 to 2.67 g/l.

4.4 Solid state fermentation process

Solid state fermentation (SS) was performed in order to obtain a hydrolysate for lactic acid production. For the solid state fermentation of malt waste, 25g of malt waste and a waste to distilled water ratio of 1:5 (w/w) was used. The soaked wastes (supplemented with minerals) were autoclaved at 121º C (15 p.s.i.) for 20 minutes in a 250ml shake flask. Minerals are used in following concentration (per 100g of dried waste) 3.5g of KH_2PO_4, 0.5g of $MgSO_4$, 8.7mg of $FeSO_4$, 3.5mg of $CaCl_2$, 2.8mg of $MnSO_4$ and 2.5mg of $ZnSO_4$. It was inoculated with 1ml *Aspergillus niger* spores suspension (~10^7 spores/ml). The inoculated solid state substrate culture was incubated in a 25°C incubator for 24hours. Then, 25ml of distilled water was added. The flask was incubated in an orbital shaker at 250 rpm, at 60º C for another 24 hours. After hydrolysis, the solid waste and the hydrolysate were separated by filtration, a pale brown solution was obtained.

1M NaOH was used to neutralize the hydrolysate to pH 6.0. The precipitate in the neutralized hydrolysate was removed by centrifuge at 4000rpm. A clear brown hydrolysate was obtained. Then, the fungal hydrolysate was used as medium in lactic acid production.

4.5 High Performance Liquid Chromatography (HPLC)

HPLC was used to study the metabolites produced in the fed batch fermentation by *Bacillus subtilis* MU331. In this study, an alltech IOA-1000 organic acid column was used. 0.005N H_2SO_4 was used as the mobile phase. The wavelength was monitored at 210nm. Flow rate of the pump was set at 0.3 ml/min. The whole system was allowed to equilibrate until a stable baseline was attained in the detector. 20µl of sample was injected. Each sample runs for 180 minutes until all the compounds elute out.

5 Results and discussion

5.1 Lactic acid production by *Bacillus subtilis*

5.1.1 Lactic acid production by *Bacillus subtilis* in shake flasks

Since shake flask experiments are easier to handle than fermenter due to its smaller scale, they had been used to characterize the growth patterns and lactic acid production by *Bacillus subtilis*.

In this study, shake flask experiments had been used to demonstrate the growth of *Bacillus subtilis* using glucose as the carbon source. The cultures were grown in 1L flasks, in an orbital shaker at 270 rpm, with culture medium (BHY) inoculated with a loop of bacteria from nutrient agar plates, at 37 ºC and initial pH 7.4.

Factors being determined using these shake flask experiments:

1) Determine the effect of oxygen transfer rate (using different culture volumes) on the growth and lactic acid production by *Bacillus subtilis.*

2) Determine the effect of shaker speed (rpm) on the growth and lactic acid production by *Bacillus subtilis.*

3) Determine the effect of additional nutrient supplements on the growth and lactic acid production by *Bacillus subtilis.*

4) Determine the effect of a two stage fermentation on the growth and lactic acid production by *Bacillus subtilis*.

Sulfite oxidation method was used to demonstrate the relationship between OTR and different volume/shaking speed (see appendix).

Effect of oxygen transfer rate (different culture volumes in 1 L flasks) at 270rpm

Fig 5.1.1 Glucose concentrations with different culture volumes

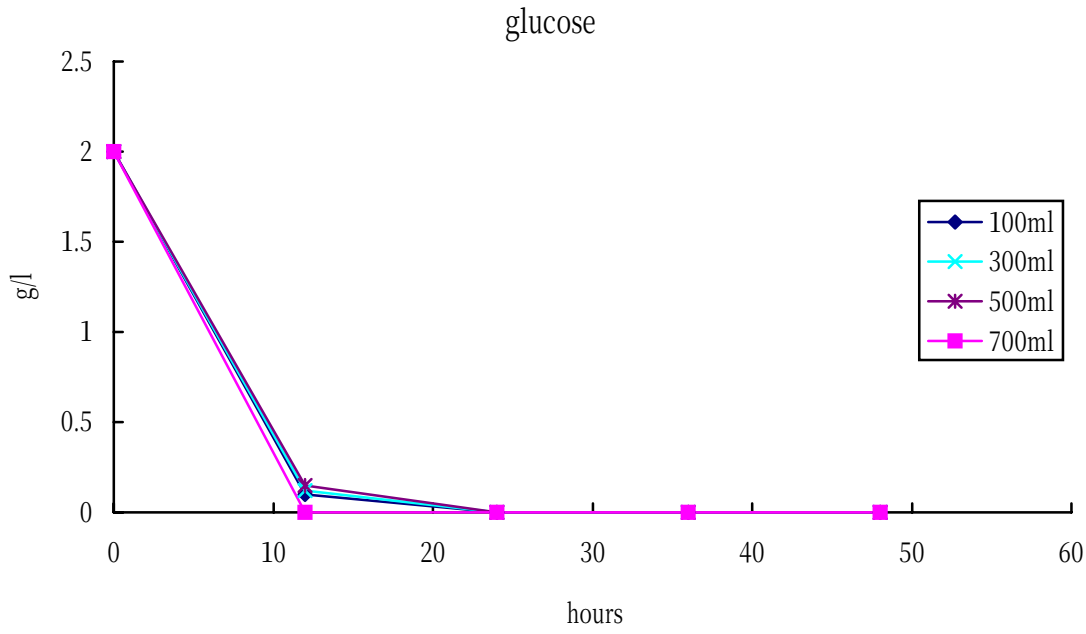


Fig 5.1.2 Lactate production with different culture volumes

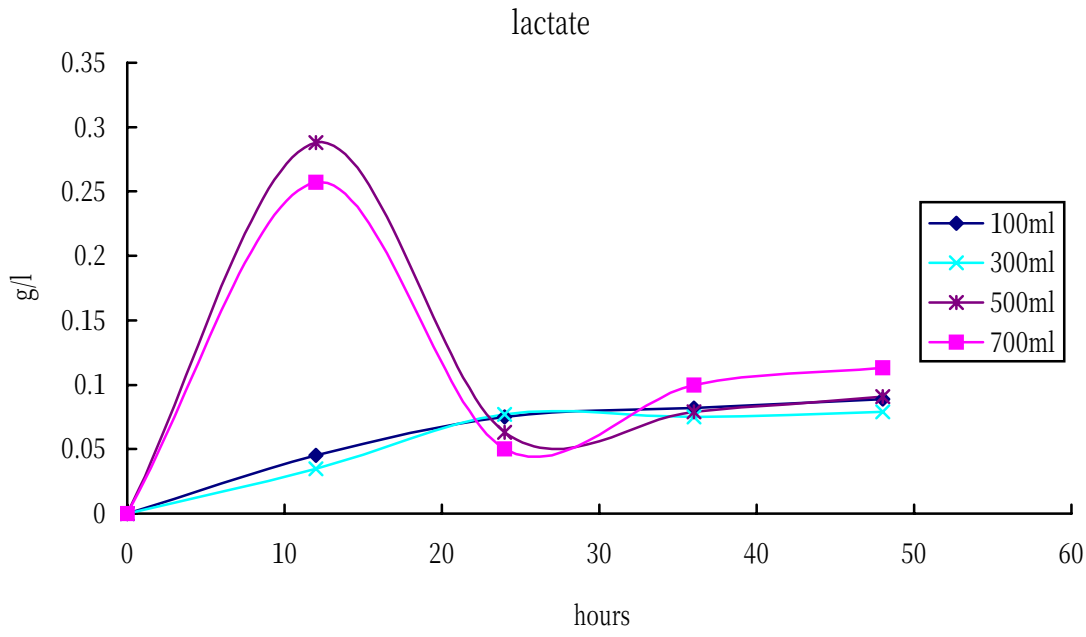


Table 5.1.1 Glucose utilization rate of Bacillus subtilis with different culture volumes in 1L flasks

Culture volume in 1L flask	Initial glucose concentration (g/L)	Residual glucose concentration* (g/L)	Amount of glucose utilized (g/L)	Rate of glucose utilization (g/L/hr) [process hours]
100ml	2	0	2	0.167 [12]
300ml	2	0	2	0.167 [12]
500ml	2	0	2	0.167 [12]
700ml	2	0	2	0.167 [12]

* Glucose concentration at the highest lactic acid concentration

Table 5.1.2 Conversion efficiency of lactic acid with different culture volumes

Culture volume in 1L flask	Amount of glucose utilized (g/L)	Highest lactic acid concentration (g/L)	$Y_{LA/GLU}$ (g/g)	Productivity (g/l/h) [process hours]
100ml	2	0.082	0.045	0.002 [12]
300ml	2	0.077	0.040	0.003 [12]
500ml	2	0.288	0.144	0.024 [12]
700ml	2	0.257	0.129	0.021 [12]

This experiment was designed to find out the relationship between oxygen transfer and lactic acid production by *Bacillus subtilis*. Based on the oxygen transfer rate (OTR) theory, smaller the interfacial area per unit volume is, smaller the OTR value.

Fig. 5.1.2 shows that the maximum lactate concentration was 0.288 g/L from the 500ml culture volume flask at 12 hours and 0.257 g/L from the 700ml culture volume flask at 12 hours. Based on these results, oxygen transfer rate would directly affecting lactic acid production by *Bacillus subtilis*.

To determine the relationship between OTR and lactic acid production, 4 different culture volume in 1L flasks were used. According to the results, the lactic acid production obtained in 500ml and 700ml culture volumes were higher than the others, this indicates that the oxygen limitation or depletion may stimulate lactic acid production. Based on the theory, less the interface for gaseous exchange is, lower oxygen transfer rate. Higher the volume is, smaller the interface. Probably, respiration mode shift from aerobic (Kreb’s cycle) to anaerobic. In aerobic respiration, glucose (carbon) goes through glycolysis, then Kreb's cycle and finally the electron transport chain. But in anaerobic respiration, pyruvate (after glycolysis) is converted to lactic acid. These two metabolic pathways are determined by the

availability of oxygen.

However, the lactic acid yield was only 0.144 and 0.129 from the 500ml and 700ml culture volumes respectively. The conversion efficiency was very low.

Effect of additional nutrient supplements and lower shaker speed (rpm)

In this study, shake flask experiments had been used to demonstrate the relationship between oxygen transfer and lactic acid production by *Bacillus subtilis* by reducing the shaking speed. The cultures were grown in 1L flasks, in an orbital shaker at 120 rpm, in 100ml BHY medium, with and without nutrient supplements. The cultures were inoculated with a loop of bacteria from a nutrient agar plate, incubated at 37 ºC with initial pH 7.4.

Fig 5.1.3 Glucose concentrations with different nutrient supplements at 120rpm

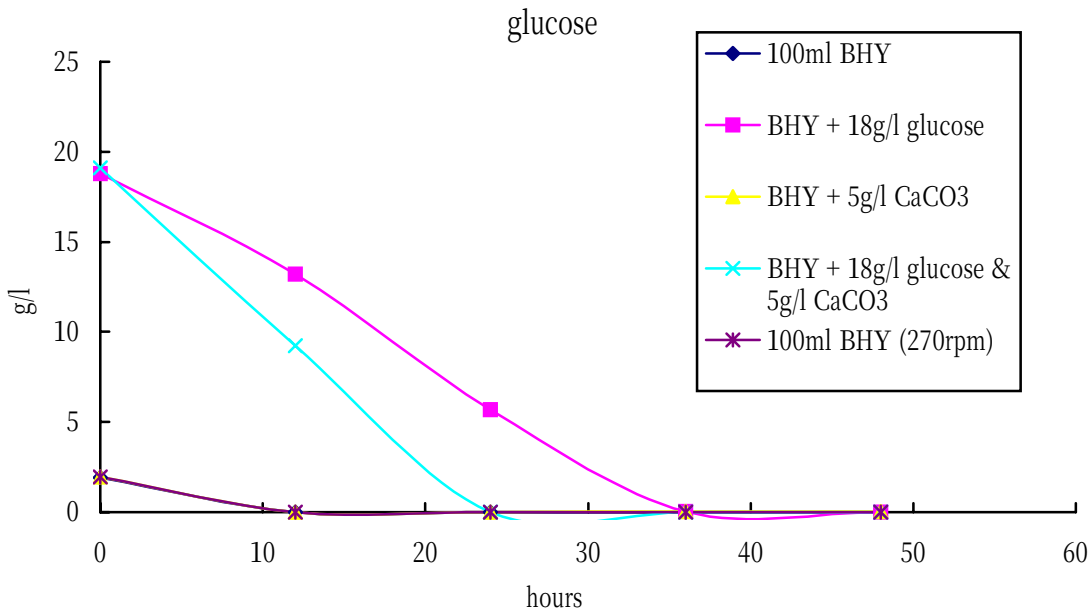


Fig 5.1.4 Lactate content with different nutrient supplements at 120rpm

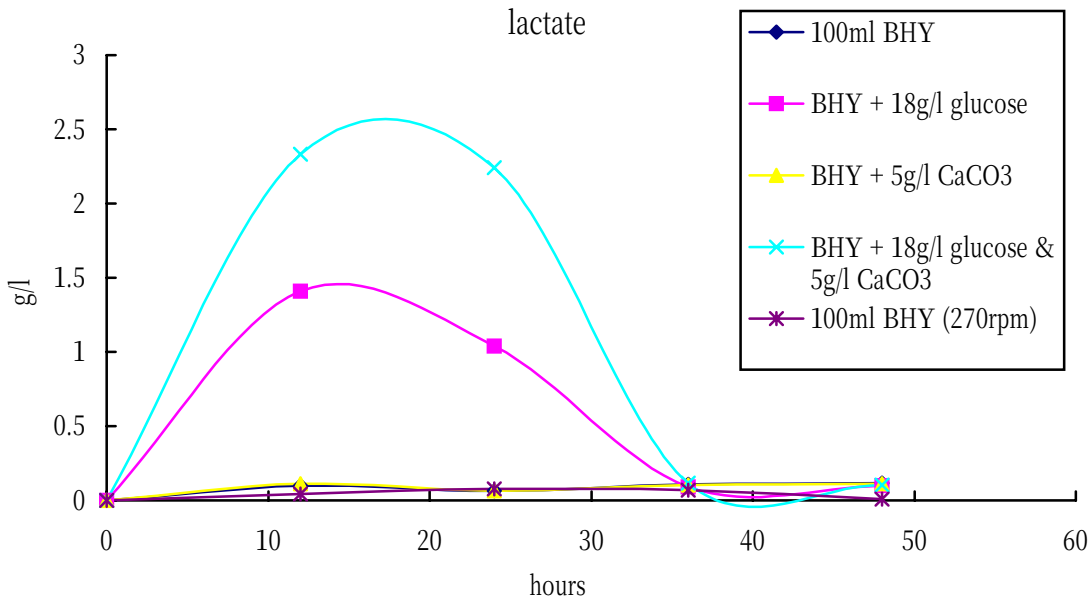


Table 5.1.3 Glucose utilization rate of Bacillus subtilis with different nutrient supplements at 120rpm

Culture	Initial glucose concentration (g/L)	Residual glucose concentration* (g/L)	Amount of glucose utilized (g/L)	Rate of glucose utilization (g/L/hr) [process hours]
100ml BHY	1.92	0.008	1.912	0.159 [12]
+ 18g/l glucose	18.8	13.2	5.6	0.467 [12]
+ 5g/l $CaCO_3$	1.95	0.007	1.943	0.162 [12]
+ 18g/l glucose & 5g/l $CaCO_3$	19.1	9.23	9.87	0.823 [12]
Control 270rpm	1.95	0.01	1.94	0.080 [12]

* Glucose concentration at the highest lactic acid concentration

Table 5.1.4 Conversion efficiency of glucose to lactic acid with different nutrient supplements at 120rpm

Culture	Amount of glucose utilized (g/L)	Highest lactic acid concentration (g/L)	$Y_{LA/GLU}$ (g/g)	Productivity (g/l/h) [process hours]
100ml BHY	1.912	0.1	0.052	0.008 [12]
+ 18g/l glucose	5.6	1.41	0.252	0.118 [12]
+ 5g/l $CaCO_3$	1.943	0.112	0.058	0.009 [12]
+ 18g/l glucose & 5g/l $CaCO_3$	9.87	2.33	0.236	0.194 [12]
Control 270rpm	1.94	0.079	0.041	0.003 [12]

Based on previous results, oxygen may affect the production of lactic acid. So, in this experiment, the shaker speed was reduced rather than using a large amount of culture volume.

Fig. 5.1.4 shows that the maximum lactate concentration was 2.33 g/L at the 12^{th} hour in the with additional glucose and calcium carbonate and 1.41 g/L in the medium with additional glucose only. Table 5.1.4 shows that glucose utilization was the highest in the medium containing addition glucose and calcium carbonate. Based on these results, glucose is limiting, additional carbon source in BHY media would increase lactic acid production by *Bacillus subtilis*.

Table 5.1.4 shows that the lactic acid yield 0.252 and productivity 0.194 obtained from the medium with excess glucose or glucose with calcium carbonate are better than the medium without these additions.

The highest lactic acid production was 2.33g/l and 1.41g/l for the medium containing excess glucose with and without calcium carbonate cultures respectively. Lactic acid is known to be a strong inhibitor for both cell growth and lactic acid production (Yin 1997, Moldes 2001 and Stenberg 2000). Calcium carbonate is a commonly used

reagent to neutralize lactic acid during fermentation. Its low solubility in water makes it possible to neutralize lactic acid as it is produced and maintain the pH at certain levels. In this study, $CaCO_3$ powder was added to the media at 5g/l instead of NaOH because calcium carbonate does not increase the pH but do react with acidic compounds. With calcium carbonate, the pH of the culture dropped to 5.823; without it, pH dropped to 5.01. The pH difference could be one of the reasons why lactic acid production increased to 2.33g/l.

Effect of two stage fermentation with additional nutrient supplements in the second stage on lactic acid production

Since *Bacillus subtilis* is an aerobic bacteria and the production of lactic acid is anaerobic, a two stage fermentation was studied to separate cell growth from product production. The cultures were grown in 1L flasks at 37°C and initial pH 7.4, in an orbital shaker at 270 rpm, with 100ml BHY inoculated with a loop of bacteria from nutrient agar plate. After 12 hours of growth (stage 1), the culture was transferred to four 100ml flasks with different volumes (i.e. 10ml, 20ml, 30ml and 40ml). 15g/l of glucose and/or 10g/l of yeast extract were added at the beginning of the second stage fermentation (120rpm at 37°C) with 5g/l calcium carbonate.

Table 5.1.5 Glucose and lactate content in the fermentation medium after 12 hours cultivation (end of first stage)

	Glucose	Lactate
After 12 hours	0	0.098

Second stage fermentation was designed as follows:

Set 1 15g/l glucose was added to each flask

Set 2 10g/l yeast extract was added to each flask

Set 3 15g/l glucose and 10g/l yeast extract were added to each flask

Set 1 15g/l of glucose was added

Fig. 5.1.5 Residue glucose concentration in the second stage fermentation (set 1)

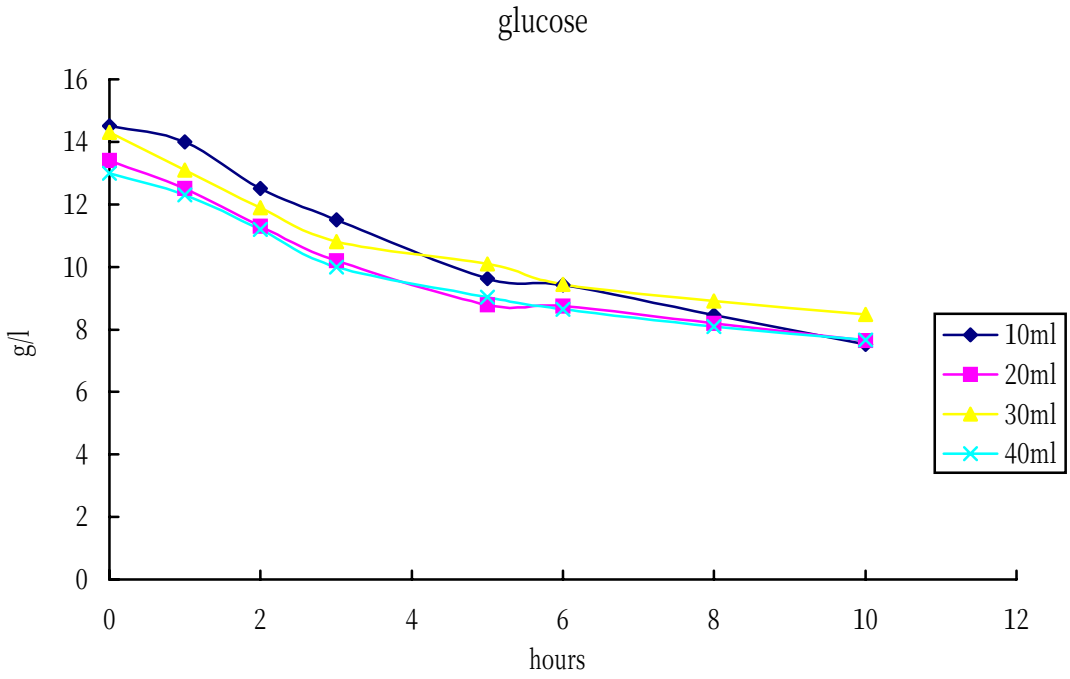


Fig. 5.1.6 Lactate content in the second stage fermentation (set 1)

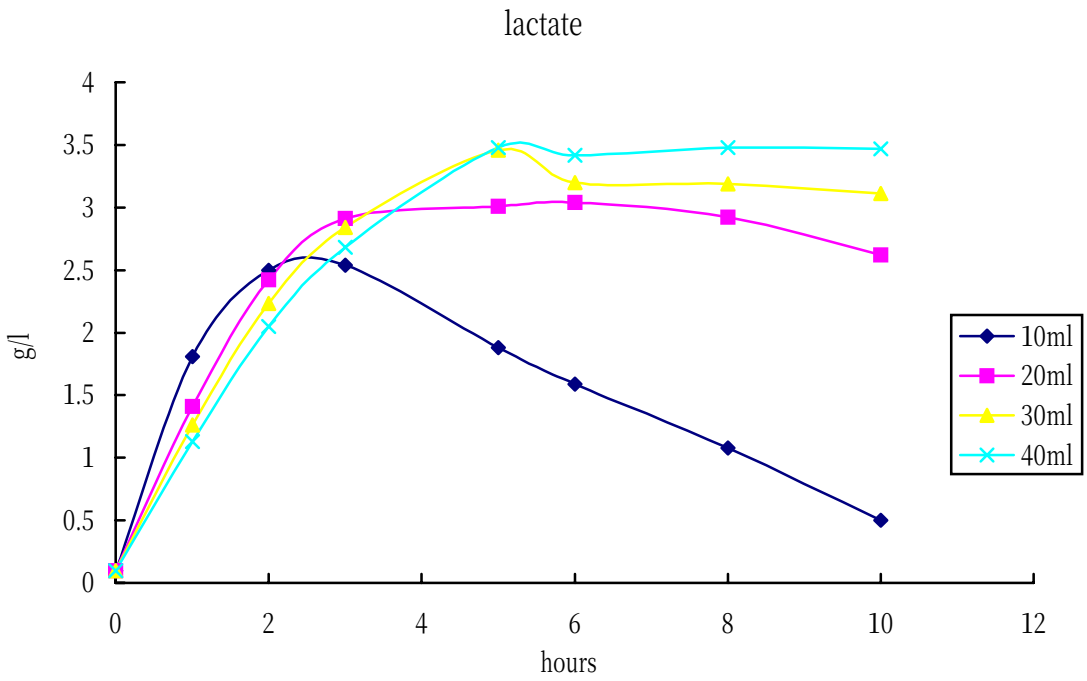


Table 5.1.6 Glucose utilization rate of Bacillus subtilis in the second stage with addition of 15g/l glucose (set 1)

Culture volume in 100ml flask	Initial glucose concentration (g/L)	Residual glucose concentration* (g/L)	Amount of glucose utilized (g/L)	Rate of glucose utilization (g/L/hr) [process hours]
10ml	14.5	11.5	3.0	1 [3]
20ml	13.4	8.74	4.66	0.777 [6]
30ml	14.3	10.1	4.2	0.84 [5]
40ml	13	7.66	5.34	0.534 [10]

*Glucose concentration at the highest lactic acid concentration

Table 5.1.7 Conversion efficiency of glucose to lactic acid in the second stage with addition of 15g/l glucose (set 1)

Culture	Amount of glucose utilized (g/L)	Highest lactic acid concentration (g/L)	$Y_{LA/GLU}$ (g/g)	Productivity (g/l/h) [process hours]
10ml	3.0	2.54	0.847	0.847 [3]
20ml	4.66	3.04	0.652	0.507 [6]
30ml	4.2	3.46	0.824	0.692 [5]
40ml	5.34	3.48	0.661	0.696 [10]

Set 2 only 10g/l of yeast extract was added

Fig. 5.1.7 Lactate content in the second stage fermentation (set 2)

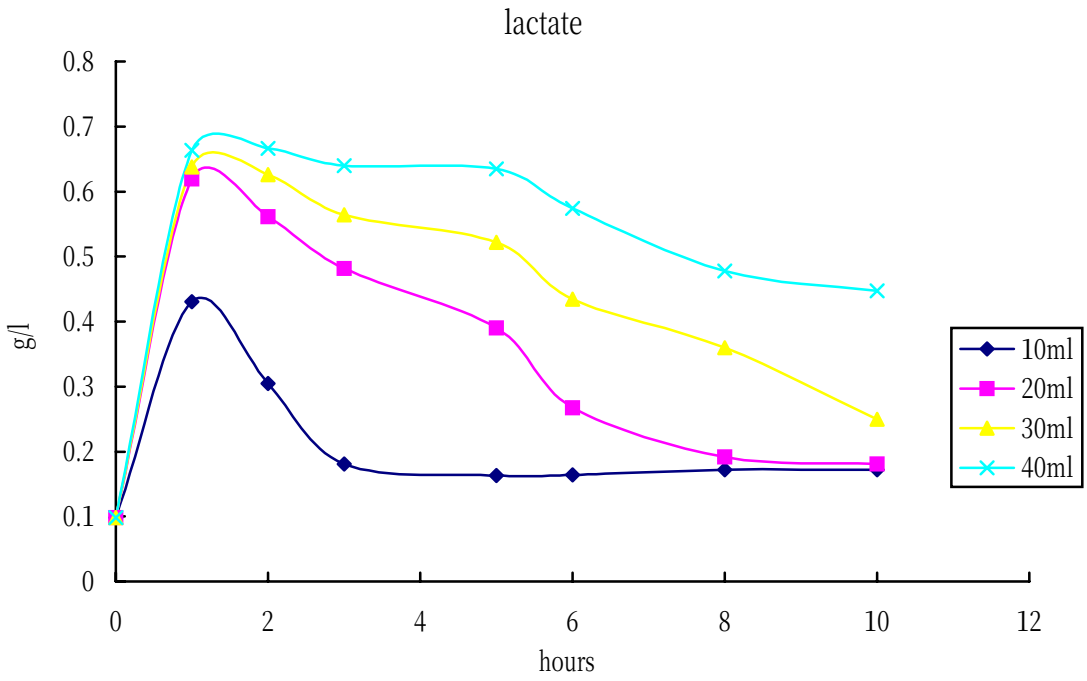


Table 5.1.8 Glucose utilization rate of Bacillus subtilis in the second stage with addition of 10g/l of yeast extract (set 2)

Culture volume in 100ml flask	Initial glucose concentration (g/L)	Residual glucose concentration * (g/L)	Amount of glucose utilized (g/L)	Rate of glucose utilization (g/L/hr)
10ml	As glucose was completely consumed in the first stage fermentation in all the cultures and was not added in the second stage to see whether nitrogen source can boost lactic acid production, rate of glucose utilization in the second stage was zero.			
20ml				
30ml				
40ml				

*Glucose concentration at the highest lactic acid concentration

Table 5.1.9 Conversion efficiency of glucose to lactic acid in second stage with addition of 10g/l yeast extract (set 2)

Culture	Highest lactic acid concentration (g/L)	Productivity (g/l/h) [process hours]
10ml	0.43	0.43 [1]
20ml	0.619	0.619 [1]
30ml	0.638	0.638 [1]
40ml	0.663	0.663 [1]

Set 3 ~15g/l of glucose and 10g/l of yeast extract were added

Fig. 5.1.8 Residue glucose concentration in the second stage fermentation (set 3)

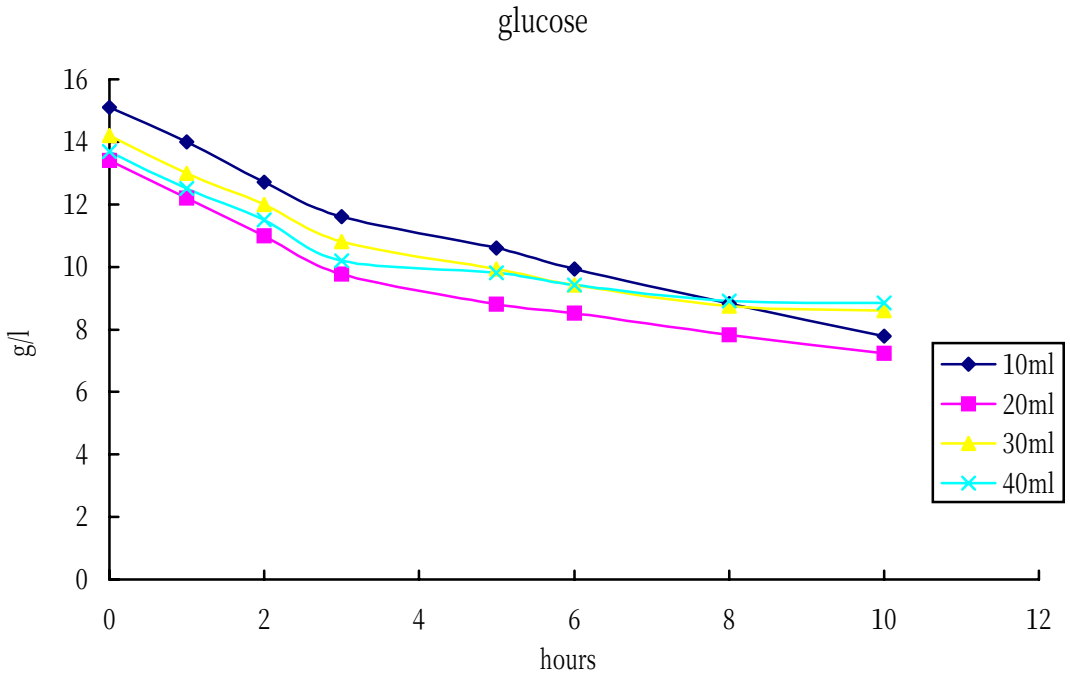


Fig. 5.1.9 lactate in the second stage fermentation (set 3)

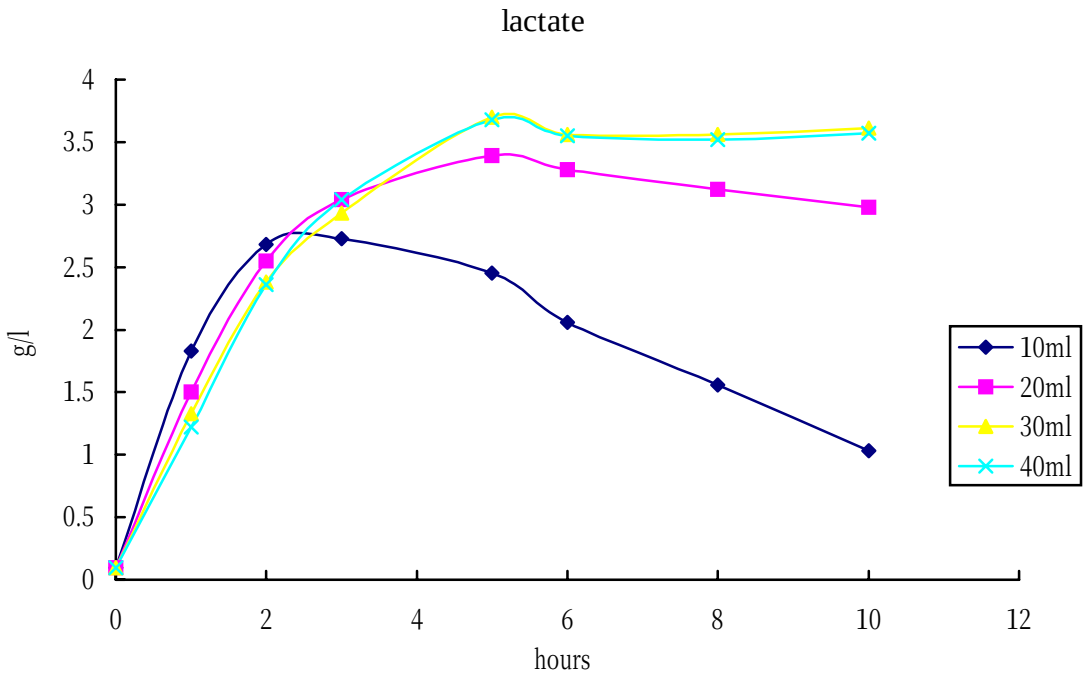


Table 5.1.10 Glucose utilization rate of Bacillus subtilis in the second stage with addition15g/l glucose and 10g/l yeast extract (set 3)

Culture	Initial glucose concentration (g/L)	Residual glucose concentration* (g/L)	Amount of glucose utilized (g/L)	Rate of glucose utilization (g/L/hr) [process hours]
10ml	15.1	11.6	3.5	1.16 [3]
20ml	13.4	8.81	4.59	0.918 [5]
30ml	14.2	9.93	4.27	0.854 [5]
40ml	13.7	9.81	3.89	0.778 [5]

*Glucose concentration at the highest lactic acid concentration

Table 5.1.11 Conversion efficiency of glucose to lactic acid in the second stage with addition of 15g/l glucose and 10g/l yeast extract nutrient supplements (set 3)

Culture	Amount of glucose utilized (g/L)	Highest lactic acid concentration (g/L)	$Y_{LA/GLU}$ (g/g)	Productivity (g/l/h) [process hours]
10ml	3.5	2.73	0.78	0.91 [3]
20ml	4.59	3.39	0.739	0.678 [5]
30ml	4.27	3.7	0.867	0.74 [5]
40ml	3.89	3.68	0.946	0.736 [5]

In the previous experiments, cell growth and lactic acid production were initiated at the same time. In this experiment, the maximum biomass dried weight 5.0g/l was achieved in the first stage fermentation (cell growth). Lactic acid concentration reached a high level after a few hours in the second stage.

Fig. 5.1.6 shows that for set 1 (only 15g/l glucose was added in the second stage) experiments, the maximum lactate concentration was 3.53 g/L in the 40ml culture volume flask and 3.46 g/L in the 30ml culture flask. Glucose utilization was the highest (5.34 g/L) in the 40ml culture volume flask (table 5.1.7). Based on these result, 2 stage fermentations with glucose addition and low oxygen transfer in the 2^{nd} stage were able to boost the lactic acid production.

The lactic acid yield in this experiment (table 5.1.7) had improved to 0.6 - 0.8. This indicates that carbon flux was directed more towards the anaerobic pathway. Compare to single stage fermentation, most carbon is used for cell replication. So, the yield was at a low level.

Fig 5.1.7 shows that set 2 (only 10g/l yeast extract was added in the second stage) experiments, the maximum lactate concentration was about 0.6 g/L among all the

culture flasks except 0.43 g/L in the 10ml culture volume flask. The low level of lactic acid was due to carbon source limitation in the media. Only nitrogen source was in excess in this case. The results showed that the culture should contain available carbon for lactic acid production.

Fig. 5.1.9 shows that for set 3 (15g/l glucose and 10g/l yeast extract were added in the second stage) experiments, the maximum lactate concentration was 3.7 g/L in the 30ml culture volume flask and 3.68 g/L in the 40ml culture volume flask. Glucose utilization was the highest (4.59 g/L) in 20ml culture volume flask (table 5.1.10). Based on these result, 2 stages fermentation were able to boost the lactic acid production with or without yeast extract supplements in the 2^{nd} stage.

Compare to set 1 experiments, the lactic acid yield value was slightly higher, about 0.7 to 0.9. For the 40ml culture volume flask, 0.946 is notably high (table 5.1.11).

Comparing results from set 1 and set 3, additional yeast extract seems to be not required for lactic acid production. Nitrogen source is not a limitation factor in the second stage fermentation.

The results also show that in some cultures after the lactate concentration had reached the maximum levels, it started to decrease. This phenomenon was only found in 100ml culture volume in the 1L flask (fig. 5.1.4) and 10ml culture volume in the 100ml flask (fig. 5.1.6). It may simply relate to the oxygen transfer rate in these flasks being higher than the others. In anaerobic respiration, lactic acid is temporary accumulated until oxygen is available again.

A similar experiment had been done (results not shown here), with shorter first stage: 3 hours (early log phase) and 7 hours (mid-log phase). However, lactic acid was not produced as high as shown here.

5.1.2 Lactic acid production by *Bacillus subtilis* in a fermentor

In this section, 2-stage fed-batch fermentation was carried out in a Bioengineering fermentor; a 3.7L jar fermentor with an initial culture volume of 2.0L was used. It was used to characterize biomass yield and lactic acid production.

Factors being determined in this section:

1) Medium development for scale up to fermentor.

2) Characterize the 2 stage fermentation.

Medium development for scale-up to fermentor

The purpose is to replace the traditional brain heart infusion broth (BHY) so that it can be easily scale-up into a larger fermentor and reduce the operational cost.

Generally, *Bacillus subtilis* can grow on a variety of carbon sources, 5 carbons or 6 carbons sugars. To develop a new medium (PNB), several nitrogen sources was screened including nutrient broth, yeast extract, casamino acid and Primatone RL. Casamino acid is the group of amino acids which results when casein, a protein found in milk, is broken down by enzymes. Primatone RL is a refined enzymatic digest of selected animal tissue. It is a high quality source of amino acids and peptides that is recommended for use as nutrient in laboratory media, fermentations and tissue culture. The use of Primatone as a substitute nitrogen source is also cited in literature (Yoo 1997).

The screening was performed in 1L flask with 100 medium at 37℃ and initial pH 7.4 in an orbital shaker at 270 rpm. The media was inoculated with a loop of bacteria from nutrient agar plate,. After 12 hours cultivation, samples were taken out and centrifuged. Supernatant was carefully removed and the cells placed in a 95℃ oven

until the weight remained constant. The performance of the media is analyzed through comparing the dried weight with the control (BHY). This parameter could reflect how well the bacteria grow. Based on the previous result, biomass may indirectly affect the lactic acid production. Table 5.1.12 shows that a medium containing Primatone, nutrient broth, yeast extract and 5g/l glucose supported a dried weight comparable to BHY.

Table 5.1.12 Dried weight of Bacillus subtilis with different nitrogen sources

Media (g/l)		Dry weight (g/l)
Control BHY		4.9
Glucose (2)	Nutrient broth (13) + yeast extract (3)	2.3
	Casamino acid (5)	2.5
	Primatone RL (5)	2.575
	Primatone RL (10)	3.45
Glucose (5)	Nutrient broth (13) + yeast extract (3)	2.725
	Casamino acid (5)	3.025
	Primatone RL (5)	3.05
	Primatone RL (10)	4.1
	Primatone RL (10) + Nutrient broth (13) + yeast extract (3)	4.8

Table 5.1.13 Comparison of BHY and PNB

Brain heart infusion broth + yeast extract (g/l)	**New medium (PNB) (g/l)**
Calf brain infusion solids (12.5)	
Beef heart infusion solids (5.0)	
Proteose peptone (10.0)	
Sodium chloride (5.0)	
Disodium phosphate (2.5)	
Glucose (2.0)	Glucose (5.0)
Yeast extract (5.0)	Yeast extract (3.0)
	Nutrient broth (13.0)
	Primatone RL (10.0)
pH 7.4 ± 0.2	pH 7.2 ± 0.2

2 stage fed batch fermentation in fermentor

In the 2 stage fed-batch fermentation of *Bacillus subtilis* with constant feeding, 2 liters

PNB media (table 5.1.13) was used. The medium was continuously stirred at a constant rate of 400rpm during all the runs. The temperature of the fermentor was automatically controlled at 37 ± 0.1°C. Air (4L /min) was supplied to the fermentor in order to attain aerobic conditions in the first stage, while 0.5.L/ min air was used during the second stage fermentation.

100 ml seed culture was used as inoculum. At the beginning of the second stage fermentation, glucose was added to a level of around 3g/l. Then, constant feeding of a 20% glucose solution (100g / 500ml) was started. Feeding continued until the end of the experiment. Glucose was fed at a rate of 50 ml/hour.

Fig. 5.1.10 Residue glucose in the second stage fed batch fermentation

Fig. 5.1.11 Lactate obtained in the second stage fed batch fermentation

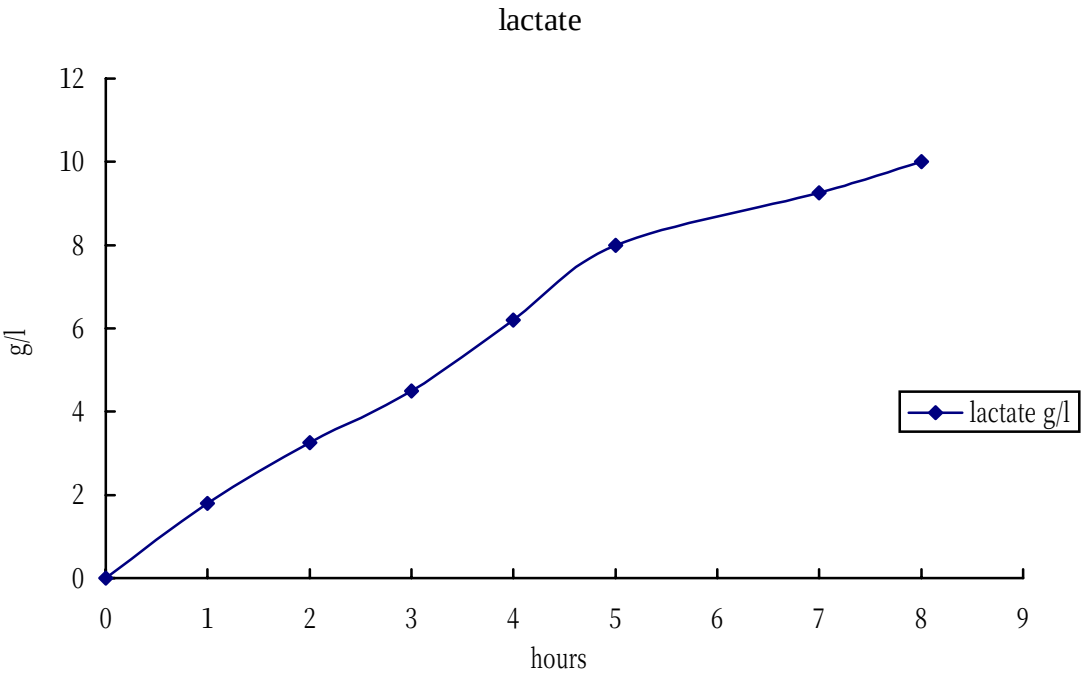


Table 5.1.14 Glucose utilization rate of Bacillus subtilis in the second stage fed batch fermentation

Culture	Glucose fed (g/L)	Final glucose concentration (g/L)	Amount of glucose utilized (g/L)	Rate of glucose utilization (g/L/hr) [process hours]
	33.3	4.4	28.933	3.617 [8]

Table 5.1.15 Conversion efficiency of glucose to lactic acid in the second stage fed batch fermentation

Culture	Amount of glucose utilized (g/L)	Highest lactic acid concentration (g/L)	$Y_{LA/GLU}$ (g/g)	Productivity (g/l/h) [process hours]
	28.933	10.0	0.346	1.25 [8]

In this 2 stage fed batch fermentation, the maximum biomass yield was 6.2 g/L obtained at 8 hours in the second stage fermentation. The maximum lactate concentration and yield were 10.0 g/l (fig. 5.1.11) and 0.346 g/g (table 5.1.15) at 8 hours. Glucose was monitored throughout the whole fermentation process. pH was controlled at 7.2 throughout the process.

From the residue glucose profile (fig. 5.1.10), glucose concentration was maintained at about 2g/l by constant feeding from 0 to 5 hours. The *Bacillus subtilis* adapted to the micro aerobic environment. The low oxygen content forced the cells to switch the metabolism from aerobic (pyruvate to acetyl CoA) to anaerobic (pyruvate to lactic acid). So, lactic acid (fig. 5.1.11) was produced, the product kept on increasing and accumulated. But, after the first 5 hours, glucose accumulated and lactate production level off at the end of the experiment.

There are several reasons behind this. The leveling off of production may be due to the ability of the cells themselves. They may not be able to produce more than 10g/l lactate. In other words, it can’t tolerate lactate at this concentration. Or, it might be due to overproduction other metabolites such as acetolactate, acetoin, ethanol, butanediol and acetate.

However, experiments had proved that this bacteria strain can grow on over 80g/l lactate. The bacteria were streaked on nutrient agar plate with various concentration of lactate (from 10 to 80g/l). All the plates showed positive result. So, the possibility of lactate intolerance could be excluded.

5.1.3 The effect of ultraviolet light (UV-C) on growth and lactic acid production by *Bacillus subtilis*

Mutations occur in vivo spontaneously or after induction with mutagenic agents. Mutations can also be induced in vitro by the use of genetic engineering techniques. All mutant types are found among spontaneous mutations, although deletions are relatively frequent. The causes of spontaneous mutations which are thus far understood include integration and excision of transposons, along with errors in the functioning of enzymes. The mutation frequency can be significantly increased by using mutagens.

One of the more effective mutagenic agents is short wavelength ultraviolet radiation. The wavelengths effective for mutagenesis are between 200 – 300 nm with an optimum at 254nm, which is the absorption maximum of DNA. The most important products of UV action are dimmers (thymine-thymine, thymine-cytosine and cytosine-cytosine) formed between adjacent pyrimidines or between pyrimidines of complementary strands, which results in crosslinks. Ultraviolet radiation mainly induces transitions of GC to AT, transversions, frameshift mutations and deletions are also found.

Early studies in the use ultraviolet radiation of microbiology field were mostly concerned with the effects and mechanisms on microorganisms (Howard-Flanders et. al. 1964, Greenberg 1965, Hill 1972). Then, the studies of boost up metabolites initiated (Sedmera et al., 1986). Improvement of lactic acid in fungi through UV mutation (Bai et al., 2004) and by chemical mutagen (Miura et al., 2004) had been studied recently.

Ultraviolet light was employed for mutagenizing the parent strain *Bacillus subtilis* MU 331 (pSG03+AMY), the purpose was to screen for lactic acid overproduction strains. UV irradiation was performed as follows: colonies of the parent strain grown on nutrient agar were transferred to BHY medium for 12 hours cultivation at 37°C with 270rpm. 10ml of the culture broth was transferred to a sterile Petri dish with a magnetic stirrer (for gently mixing). Then, it was exposed to UV irradiation at 254 nm for 0–45 min at a distance of 20 cm. The colonies forming units/ml (CFU/ml) on the culture broth were maintained at 47×10^6 cells/ml for UV-irradiation and further studies. Survival curve was prepared and time of exposure giving over 99% kill rate was selected for mutation of the organism. Samples were taken out at specific time point then spread over nutrient agar plate. The plates were placed in a 37°C incubator around 12 hours until colonies formed. The mutant derivatives were

selected (8-10 mutants, a total of 27 mutants for 3 set of experiments), characterized in liquid culture media as described in 2 stage fed batch shake flask fermentation previously. Glucose was added to 40ml culture volume in 100ml shake flask at 37℃ with 120rpm.

Results of UV mutation experiments

The selected 27 survivors after UV mutagenesis were subjected to 2 stage fed batch shake flask fermentation and compare with the parent strain. After the screening and characterization, 6 strains were selected and frozen stocks prepared with 20% glycerol in -80℃ for storage. They are named alphabetically ‘K’, ‘L’, ‘P’, ‘Q’, ‘R’ and ‘T’. Results that follows show the 6 selected strains only.

Fig. 5.1.12 Residue glucose in the second stage fed batch fermentation

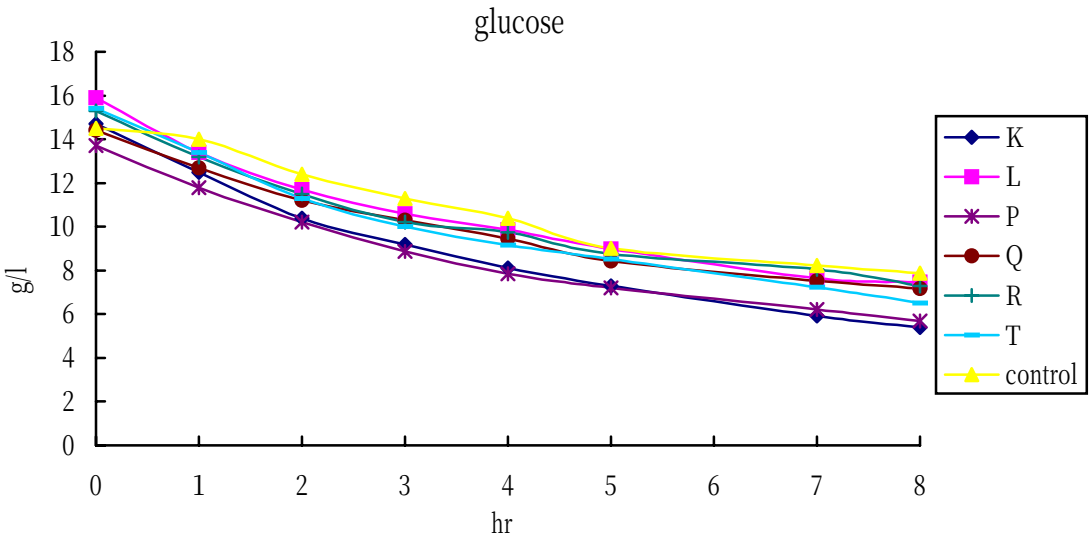


Fig. 5.1.13 Lactate obtained in the second stage fed batch fermentation

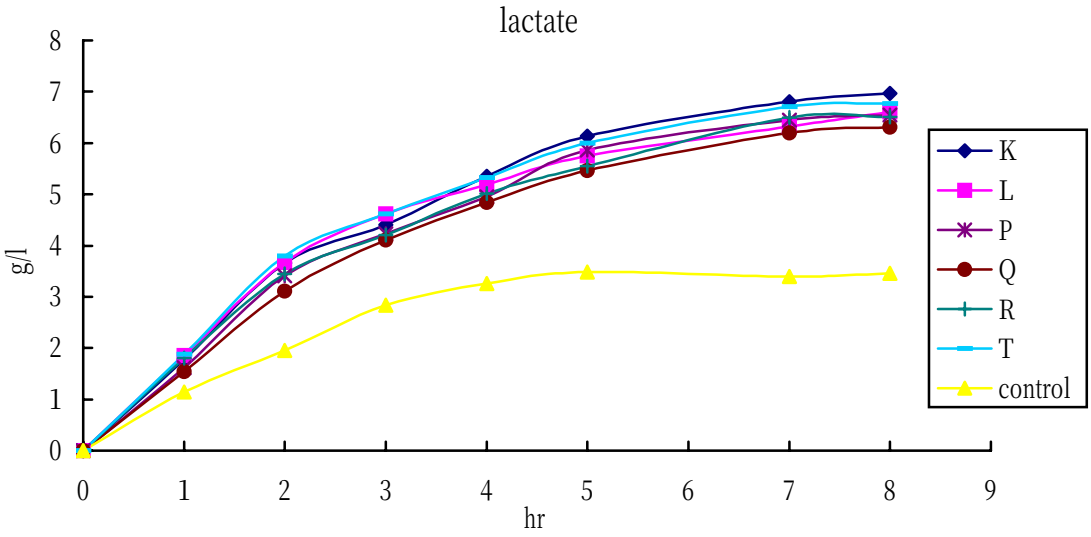


Table 5.1.16 Glucose utilization rate of the selected strains in the second stage fed batch fermentation

Culture	Initial glucose concentration (g/L)	Final glucose concentration (g/L)	Amount of glucose utilized (g/L)	Rate of glucose utilization (g/L/hr) [process hours = 8]
Control	14.5	9.02	5.48	1.096
K	14.7	5.41	9.29	1.161
L	15.9	7.47	8.43	1.054
P	13.7	5.69	8.01	1.001
Q	14.4	7.17	7.23	0.904
R	15.3	7.3	8	1
T	15.4	6.51	8.89	1.111

Table 5.1.17 Conversion efficiency of glucose to lactic acid in the second stage fed batch fermentation

Culture	Amount of glucose utilized (g/L)	Highest lactic acid concentration (g/L)	$Y_{LA/GLU}$ (g/g)	Productivity (g/l/h) [process hours = 8]
Control	5.48	3.48	0.635	0.696
K	9.29	6.97	0.750	0.871
L	8.43	6.61	0.784	0.826
P	8.01	6.54	0.816	0.818
Q	7.23	6.31	0.873	0.789
R	8	6.51	0.814	0.814
T	8.89	6.77	0.762	0.846

In this screening, the dry weight obtained was around 5.0 g/L in the first stage fermentation. The maximum lactate concentration and yield were 6.97 g/l and 0.873 g/g at 8 hours in the second stage fermentation (table 5.1.17). Glucose and lactate were monitored throughout the fermentation process. pH dropped to around 5.2 at the end of the experiment.

From the residue glucose profile (fig. 5.1.12), glucose was utilized by all the cultures. But the mutants showed a slightly higher utilization rate. As can be seen, the mutants use the glucose in more or less the same rate. In lactic acid production (fig. 5.1.13), all the strains showed a similar trend. Lactic acid was produced quickly in the first half of second stage fermentation, and leveled off afterwards. There was a big difference between the parent strain and the mutants. The parent strain stopped production of lactate at 4 to 5 hours in the 2^{nd} stage while the mutants extended the production to 8 hours. The most important thing is that the lactate concentration produced from the mutants nearly doubles that of the parent strain.

Apart from the improved volumetric concentration, yield and volumetric productivity also improved. The highest yield and volumetric productivity are 0.873 and 0.871 respectively (table 5.1.17). Compared to the parent strain 0.635 and 0.696, these

mutants showed a better production capability. The mutation might have modified the cell's metabolism but might not have change the cell growth, since there was no significant change in the cell dried weight of the mutants compare to the parent strain.

Nevertheless, the volumetric concentration is still not good enough when compared to other lactic acid bacteria. Next, these mutants will be scale up in the fermentor.

5.1.4 Lactic acid production by *Bacillus subtilis* (UV mutants) in the fermentor

These experiments are used to characterize the mutants' performance in a fermentor. The protocol is the same as described previously.

Fig. 5.1.14 Residue glucose in the second stage fed batch fermentation

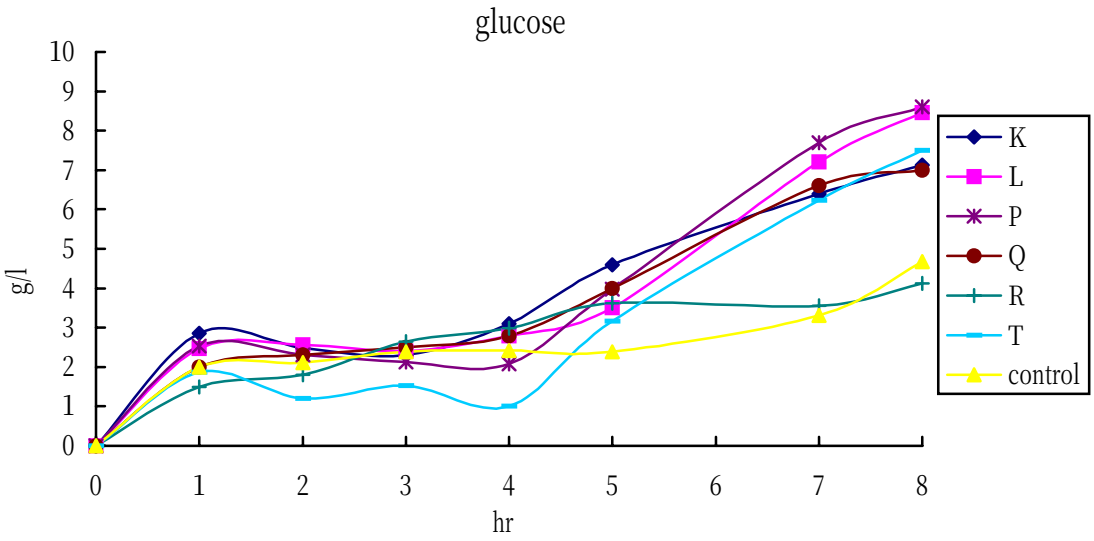


Fig. 5.1.15 Lactate obtained in the second stage fed batch fermentation

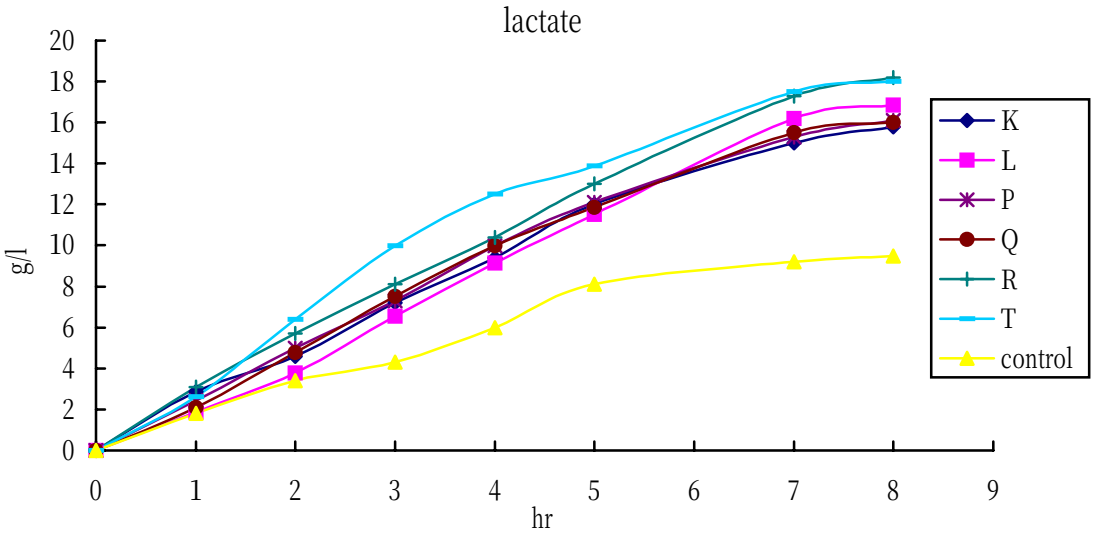


Table 5.1.18 Glucose utilization rate of mutants in the second stage fed batch fermentation

Culture	Glucose fed (g/L)	Final glucose concentration (g/L)	Amount of glucose utilized (g/L)	Rate of glucose utilization (g/L/hr) [process hours = 8]
Control	33.3	4.68	28.62	3.578
K		7.12	26.18	3.272
L		8.45	24.85	3.106
P		8.6	24.7	3.088
Q		7	26.3	3.288
R		4.12	29.18	3.648
T		7.5	25.8	3.225

Table 5.1.19 Conversion efficiency of glucose to lactic acid in the second stage fed batch fermentation

Culture	Amount of glucose utilized (g/L)	Highest lactic acid concentration (g/L)	$Y_{LA/GLU}$ (g/g)	Productivity (g/l/h) [process hours = 8]
Control	28.62	9.5	0.332	1.188
K	26.18	15.8	0.604	1.975
L	24.85	16.85	0.678	2.106
P	24.7	16.1	0.652	2.012
Q	26.3	16	0.608	2
R	29.18	18.2	0.624	2.275
T	25.8	18	0.698	2.25

In this 2 stage fed batch fermentation, the maximum dried weight obtained was around 6.2 g/L after 8 hours in the second stage fermentation. The maximum volumetric concentration and yield were 18.2 g/l and 0.698 g/g at 8 hours. The highest productivity was 2.275 g/g/l (table 5.1.19). Glucose and lactate were monitored throughout the fermentation process. pH was controlled at 7.2 during the process.

From the residue glucose profile (fig. 5.1.14), glucose was maintained at about 2g/l from 0 to 5 hours. But in the last few hours, glucose increased in the broth and accumulated. It showed that the utilization rate was decreasing. From the graph, it is obviously that the mutants except ‘R’ had a lower glucose utilization rate. Thus, the remaining glucose was higher. Mutant ‘R’ showed a similar utilization rate as the parent strain.

In fig. 5.1.15, lactate was produced but production flattened at hour 7. Among the mutants, there is no significant difference in volumetric concentration (around 16-18g/l). The concentrations are almost double when compared to parent strain. The improvement showed not only in the volumetric concentration, but also a doubling in yield and volumetric productivity.

The biomass yield obtained in this fermentation was higher when compared to the biomass obtained in the shake flask fermentation. At hour 8, the biomass yield was 6.2 g/L, which was 124% of the biomass obtained in shake flask experiments. It means that controlled growing conditions in the fermentor are better than in the shake flasks.

In figures 5.1.14 and 5.1.15, showing the glucose utilization and lactate production in fermentor, the pattern was similar to that obtained in shake flask. Although they had a similar pattern, they showed a higher value in glucose utilization rate, volumetric concentration and volumetric productivity. For glucose utilization rate and volumetric concentration, the values are nearly 3 fold that of the values obtained in shake flask; there is a doubling in the volumetric productivity.

Lactic acid production by *Bacillus subtilis* (strain 'R') in a fermentor by varying the mode of glucose feeding

In order to optimize the fermentation conditions, different modes of feeding was examined. 3 more fed batch fermentations were studied. The modes examined are as follows, (I) 33.3g/l glucose was quickly fed, (II) a controlled feed rate to maintain around 3 g/l glucose in the broth and (III) a controlled feed rate to maintain around 6 g/l glucose in the broth.

Table 5.1.20 Glucose utilization rate of strain 'R' in the second stage fed batch fermentation

Feeding mode	Glucose fed (g/L)	Final glucose concentration (g/L)	Amount of glucose utilized (g/L)	Rate of glucose utilization (g/L/hr) [process hours = 8]
(I)	33.3	8	25.3	3.163
(II)		3.2	30.1	3.763
(III)		6.2	27.1	3.388

Table 5.1.21 Conversion efficiency of glucose to lactic acid in the second stage fed batch fermentation

Feeding mode	Amount of glucose utilized (g/L)	Highest lactic acid concentration (g/L)	$Y_{LA/GLU}$ (g/g)	Productivity (g/l/h) [process hours = 8]
(I)	25.3	14.4	0.570	1.8
(II)	30.1	19.6	0.651	2.45
(III)	27.1	21	0.775	2.625

With these 3 modes of feeding, it was found that mode (III) was better that the others. In mode (I), the second stage fermentation was run at a very high residue glucose concentration. Tables 5.1.20 and 5.1.21 showed that glucose utilization, lactate concentration, yield and productivity were not as desirable as the others. Mode (II) showed a similar result as constant rate feeding. Only mode (III) showed an improvement of about 20% better than constant rate feeding. Nevertheless, the yield is only 77%. It might mean that carbon flux was directed to other pathways besides production of lactic acid. It may be used for cell maintenance or producing other metabolites.

Table 5.1.22 Comparison of kinetic parameter of Bacillus subtilis MU331 with other species of Bacillus in the literature

strain	Lactic acid (g/l)	Consumed glucose (g/l)	$Y_{LA/GLU}$ (g/g)
Bacillus sp. SHO-1	14.3	20.0	0.715
Bacillus cereus	11.9	18.2	0.654
Bacillus coagulans	0.7	2.2	0.318
Bacillus subtilis	3.7	7.5	0.493
Bacillus thuringiensis	11.9	19.1	0.623
Bacillus subtilis MU 331 (pSG03+AMY)	21	27.1	0.775

(Ohara 1996)

	Bacillus coagulans TB/04	*Bacillus subtilis* MU 331 (pSG03+AMY)
Growth temperature	52°C	37°C
Sugar	Molasses	Glucose
Biomass	3 g/l	6.2 g/l
Lactic acid	55 g/l	21 g/l
Fermentation time	48 hours	12 + 8 hours
Specific lactic acid production rate	0.382 g/lh / g/l cell	0.423 g/lh / g/l cell
Productivity	1.146 g/l/h	2.625 g/l/h

(Payot 1999)

Bacillus subtilis is an atypical strain for lactic acid production. The optimum temperature for growth and lactic acid production is 37℃. It's fully understood genome is useful and if it is used in the industrial production of lactic acid genetic engineering can be used to improve the strain and reduce the cost of the process.

The relationship between oxygen and lactic acid production was confirmed by this study and also stated in other literature (Ohara 1996). High dissolved oxygen levels showed a positive effect on biomass production, but lactic acid concentration dramatically decreased, and vice versa.

The study indicates that high concentrations of glucose led to diminished lactic acid production, indicating the inhibitory effect of high sugar concentrations. Fermentation under stressed conditions (low pH) will inhibit the bacteria growth as well as lactic acid production (data not shown).

One of the key factors for improving lactic acid production by *Bacillus subtilis* MU 331 (pSG03+AMY) in this study is the nitrogen source for cell growth. The biomass concentration affected the lactic acid production performance. So, a suitable nitrogen source is a crucial point for the whole process. Primatone RL and yeast extract

supported good cell growth. They are assimilated as the nitrogen source and contains also vitamins and cofactors for growth (Aeschlimann 1990). The biomass, specific growth rate, volumetric concentration of lactic acid, specific lactic acid production rate, and specific sugar consumption rate were increased with yeast extract supplementation (Aeschlimann 1990). Primatone RL is very promising as a partial substitute for yeast extract in order to reduce the production costs. During the screening of nitrogen source, casamino acid shows the potentiality to boost the biomass yield.

However, it still needs more evidence to prove that biomass is directly related to the lactic acid production. If the biomass is the key factor in this process, this limiting state, could be overcome by using a high cell density reactor coupling biomass recycling. Performance and productivity could be increased if the biomass of *Bacillus subtilis* is increased.

5.1.5 High Performance Liquid Chromatography (HPLC)

Nowadays, HPLC has become into a common analytical method as a result of crucial development of instrumentation and column packing. It is the preferred method for the separation and quantitative analysis of a wide range of sample.

Under HPLC, the mixture to be analyzed is dissolved in a suitable solvent, and then injected into the column through an injector. Samples are carried through the column by a continuous flow of a mobile phase. It is an advantage for the sample to be dissolved in a solvent that is the same as the mobile phase. The separation takes place with the stationary phase solid packing inside the column. Compounds in the sample that are injected reversibly interact with the stationary phase in a continuous manner. Thus, with the selection of a proper mobile phase and column packing material, different compounds of the mixture will move towards the column in different rates. When the compounds come out from the column, a suitable detector is used to monitor and transmit a signal to the recording device. The chromatogram is a record of the signal response as a function of time and presents the amount of the components as peaks.

HPLC provides the most sensitive, precise and reproducible method for quantitative analysis of metabolites, particularly when the instrumentation includes a computational integrator for measuring peak areas. The relative amounts of each compound in the chromatogram can be determined by the peak area. This can be calculated through result of injecting a known standard. An alternative strategy is to use as an internal standard,

the substance or one of the substances under investigation. The fermentation sample is first analyzed by HPLC and the peak areas are determined as normal. Then a known amount of a pure standard compound is added to the sample and a second trial of HPLC analysis performed. The increment in the peak area for the standard compound is then related to the amount that was added, to give a calibration that can be used to determine the amount of all substances that are present in the extract. This method can also be used to identify a specific component in the sample with the standard that is added.

In this chapter, HPLC was applied to identify some of the metabolites in the 2 stage fed batch fermentation of *Bacillus subtilis* UV mutant. As showed in the *Bacillus subtilis* chapter, the mutant under fed batch fermentation only have a glucose to lactic acid conversion yield of 77%, HPLC was used to identify some of the other overproduced metabolites in the carbon flux.

In this study, an alltech IOA-1000 organic acid column was used. 0.005N H_2SO_4 was used as the mobile phase. The wavelength was monitored at UV 210nm. Flow rate of the pump was set at 0.3 ml/min. The whole system was allowed to equilibrate until a stable baseline was attained in the detector. 20 µl of sample was injected. Each sample runs for 180 minutes until all the compounds come out. Standards run for 60 minutes.

For the standard, 90% pure sodium lactate in liquid form was purchased from Riedel-de Haen. Samples from fermentation broth were first centrifuge at 10000rpm for 3 minutes. The supernatant was saved and pass through a 0.45µ filter. The sample is ready for

chromatography.

Results of linearity of the standard by using different concentrations of the standard

Linear relationship: y =1611.5x – 457.63

R^2=0.9999

Table 5.1.23 Areas found at different concentrations of the lactic acid standard

Lactic acid Standard (g/l)	0.4	0.8	2	4
Area [mAU * s]	1237.604	2717.29077	7826.08008	15695.5

Fig 5.1.16 Calibration curve of lactate

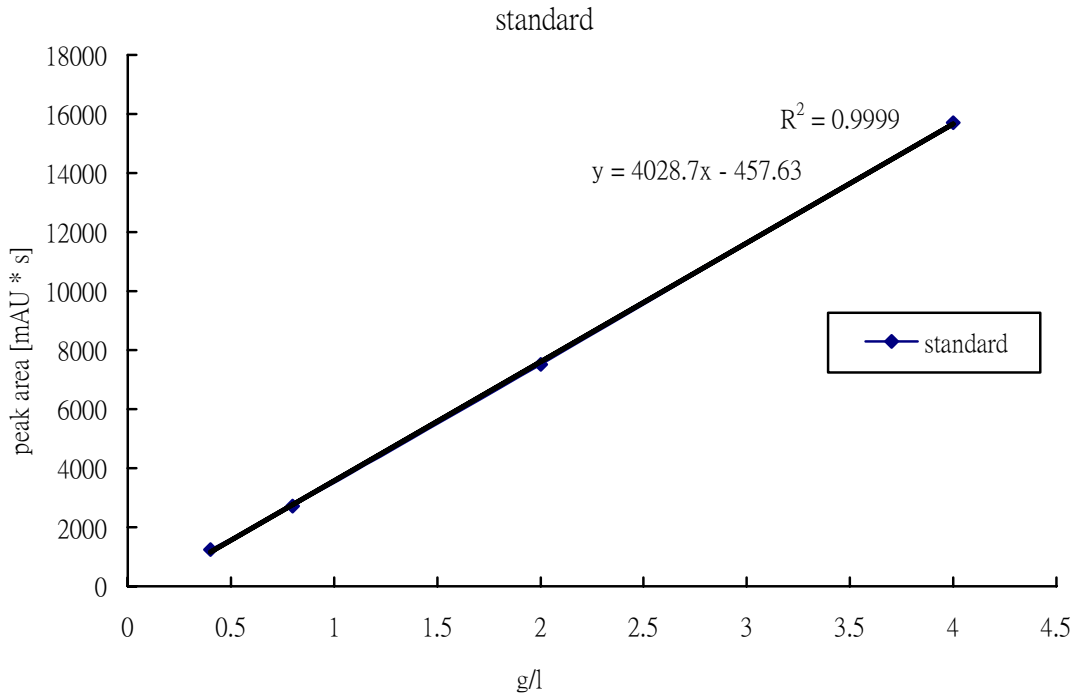
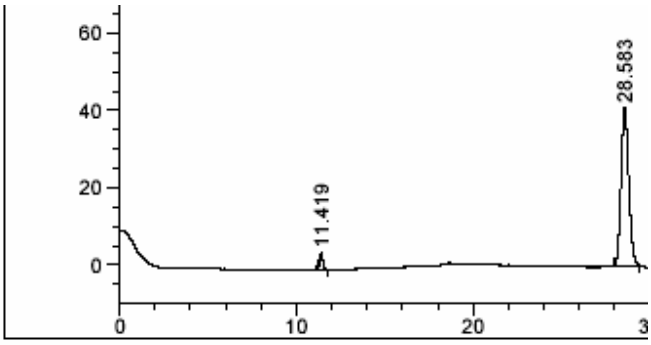
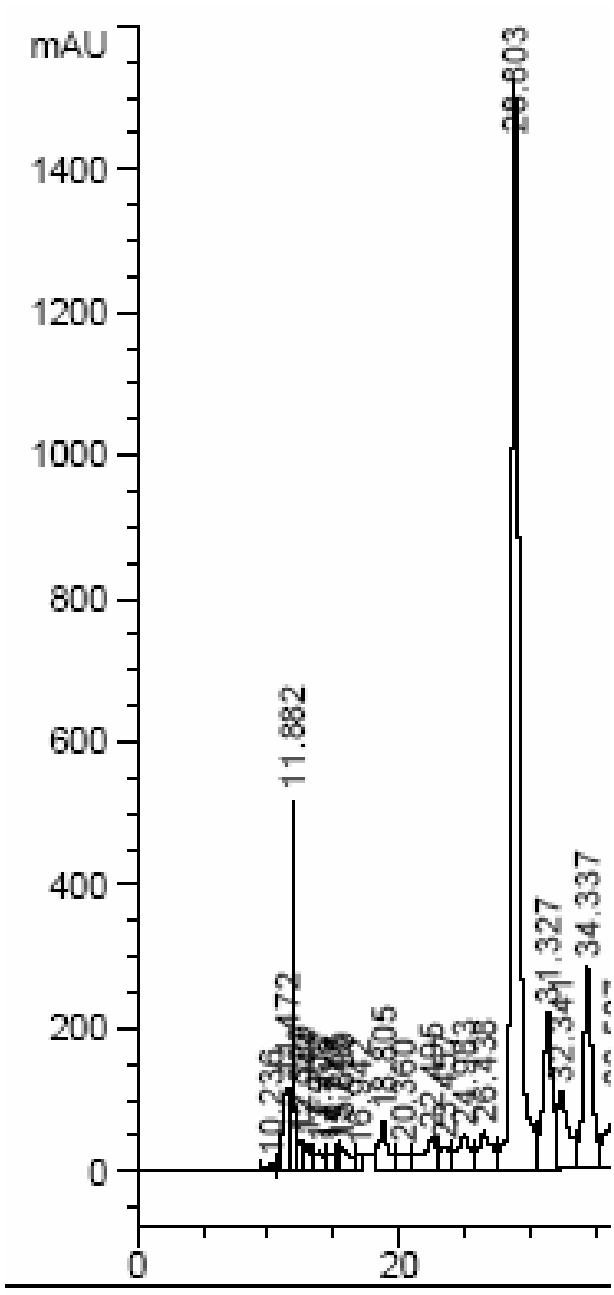


Fig 5.1.17 chromatogram of 0.4g/l lactate standard



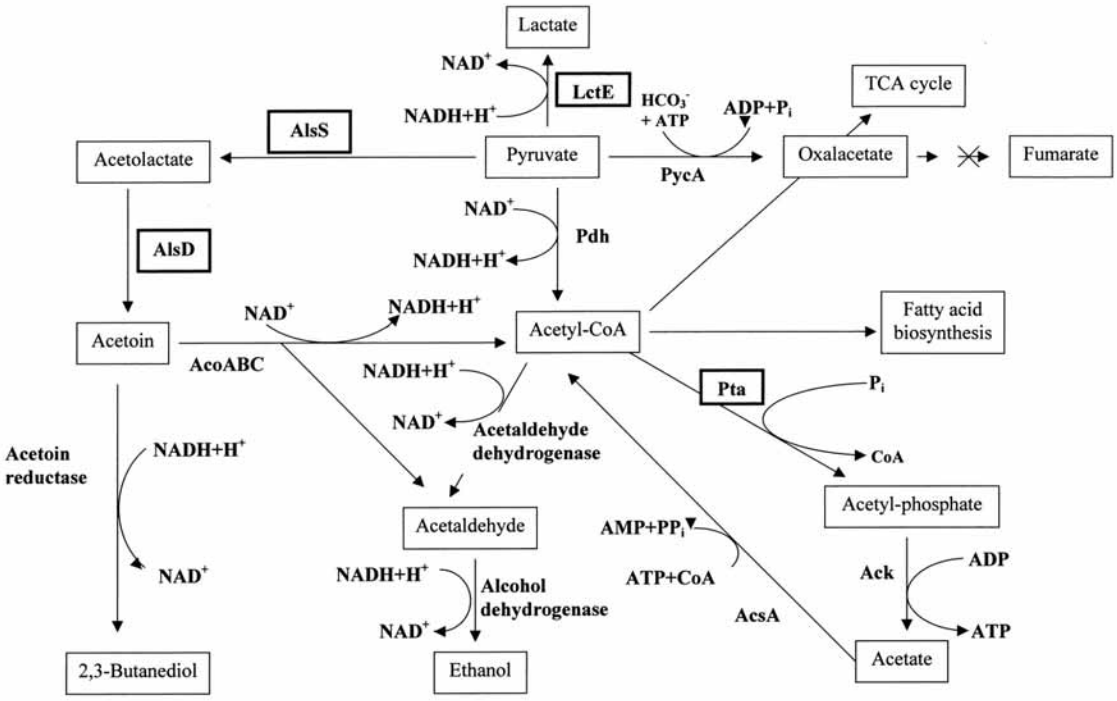
Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	11.419	VB	0.1765	52.90027	4.39418	0.4121
2	28.583	BB	0.4658	1237.60400	41.04528	9.6409

Fig 5.1.18 chromatogram of one of the sample



Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
17	28.803	VV	0.6247	6.31596e4	1520.27039	28.5888
20	34.337	VV	0.6863	1.28597e4	280.79053	5.8208

Fig 5.1.19 schematic diagram of metabolism of Bacillus subtilis



(Ramos et. al., 2000)

From the above diagram, lactate, acetolactate, acetoin, butanediol, ethanol and acetate are potential metabolites that can be found in the broth. From the HPLC results, several peaks are found in the broth obtained from the second stage fed batch fermentation. Among these peaks, two peaks stand out. One is lactate found at retention time 28 minutes, and the other is 34 minutes. It was identified by injecting standards such as acetoin, acetate etc. From the chromatograms, acetate comes out at a retention time of 34 minutes (fig. 5.1.18). Thus, the second overproduced metabolite is acetate.

Fig 5.1.20 chromatogram of acetate

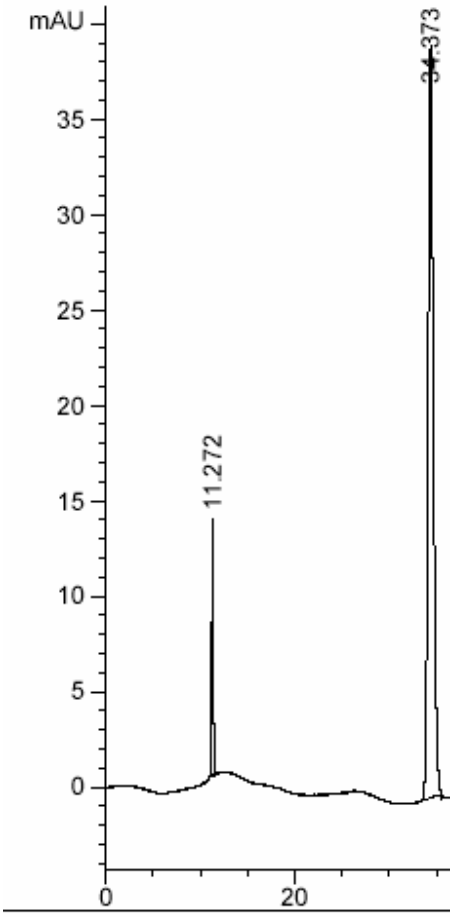


Fig 5.1.21 chromatogram of acetoin

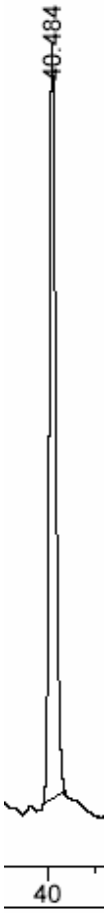


Fig 5.1.22 peak area of standard 1 g/l acetate

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	11.272	BB	0.1592	142.39458	13.48941	8.4614
2	34.373	BB	0.5959	1540.46997	39.41978	91.5386

Fig 5.1.23 peak area of standard 1 g/l acetoin

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	11.238	BP	0.1467	13.94614	1.46978	0.7891
2	34.391	BB	0.5497	288.96442	7.20832	16.3509
3	40.484	BB	0.7061	1464.35376	31.05433	82.8599

Table 5.1.20 and 5.1.21 has already shown that *Bacillus subtilis* strain 'R' with mode (III) glucose feeding in the second stage utilized 27.1g/l glucose and produced 21g/l lactate. HPLC results indicated that 5.4g/l acetate was produced. These results are combined and presented in table 5.1.24.

Table 5.1.24 summary of the carbon flux

Glucose utilized (g/l)	Products	Concentration (g/l)	$Y_{products/GLU}$ (g/g)
27.1	Lactate	21	0.775
	Acetate	5.4	0.199
	Lactate + acetate	Total 26.4	Total 0.974

In summary, the two major metabolites are identified which account for 97% glucose conversion. 77% is the target product, lactate while the other overproduces product acetate accounts for 20%. Based on this result, acetate may reach a certain level that inhibits the bacteria like acetate inhibition in *Escherichia coli.*

5.2 Lactic acid production by *Lactobacillus rhamnosus*

5.2.1 Lactic acid production by *Lactobacillus rhamnosus* in shake flasks

Since shake flask experiments are easier to handle than fermenter due to its smaller scale, they had been used to characterize the growth patterns and lactic acid production of *Lactobacillus rhamnosus.*

In this study, shake flask experiments and the fermentor have been used to demonstrate the growth of *Lactobacillus rhamnosus* using glucose as the carbon source. The cultures were grown in 250ml flasks, in an orbital shaker at 250 rpm, with 5% inoculum, at 37 °C and initial pH 6.0.

Factors being determined are:

1) Medium development to replace MRS.

2) Determine the ability of *Lactobacillus rhamnosus* to use various carbon sources.

3) Batch fermentation in the fermentor.

4) Strain improvement through continuous cultures.

Medium development

Lactic acid bacteria are generally recognized as nutritionally fastidious. MRS, an improved growth medium for the cultivation of *Lactobacilli* was developed by De Man J.C., Rogosa M. and Sharpe M.E. It supports good growth of *Lactobacilli* generally and also is particularly useful for a number of fastidious strains which grow only poorly in other general media. In this study, a new medium (YE10) was developed to replace MRS. 10g/l of yeast extract was used to replace all the nitrogen sources in MRS. This would be the simplest medium to support *Lactobacillus rhamnosus*, the remaining chemicals in MRS are essential for the growth of lactic acid bacteria.

Table 5.2.1 Comparison of MRS and YE10

MRS	**YE10**
Peptone	
`Lab-Lemco' powder	
Yeast extract	Yeast extract
Glucose	Glucose
Tween 80	Tween 80
K_2HPO_4	K_2HPO_4
Sodium acetate $3H_2O$	Sodium acetate $3H_2O$
Triammonium citrate	
$MgSO_4$	$MgSO_4$
$MnSO_4$	$MnSO_4$
pH 6.2 ± 0.2	pH 6.0 ± 0.2

This experiment is to determine whether YE10 is suitable for replacing MRS and the amount of nitrogen sources that should be used. Medium with various yeast extract content YE10 (10g/l), YE20 (20g/l) and YE30 (30g/l) were analyzed. Corn steep liquor (CSL) was examined also to see whether it will improve YE10 medium. CSL was used because it is a viscous concentrate of corn solubles, rich in vitamins, amino acids, minerals and other growth stimulants. It is commonly used in industrial fermentation medium, it is an easily available nutritional compound and it is inexpensive. It contains numerous vitamins and mineral in an appropriate concentration. The medium tested would be based on the YE10 formula with C10 (CSL 10ml/l), C20 (CSL 20ml/l) and C30 (CSL 30ml/l).

Fig 5.2.1 Residue glucose concentration in MRS and the other media

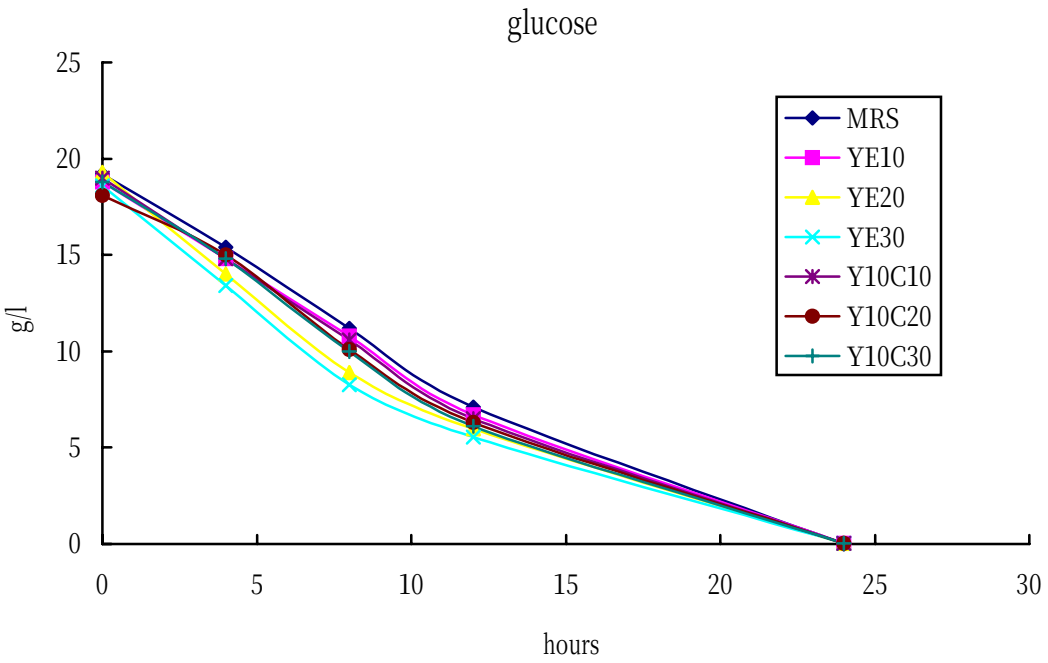


Fig 5.2.2 Lactate obtained from MRS and the other media

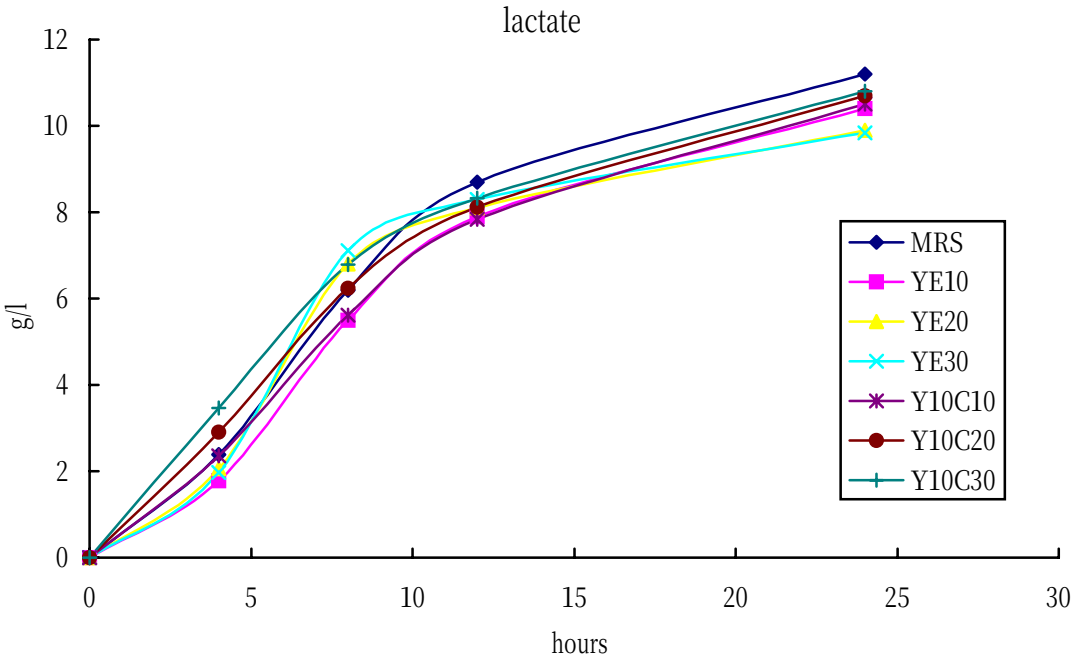


Table 5.2.2 Glucose utilization rate in MRS and the other media

Media	Initial glucose concentration (g/L)	Final glucose concentration (g/L)	Amount of glucose utilized (g/L)	Rate of glucose utilization (g/L/hr) [process hours = 24]
MRS	19.2	0.016	19.184	0.799
YE10	18.8	0.02	18.78	0.783
YE20	19.3	0.026	19.274	0.803
YE30	18.6	0.03	18.57	0.774
Y10C10	19	0.024	18.976	0.791
Y10C20	18.1	0.029	18.071	0.753
Y10C30	18.8	0.034	18.766	0.782

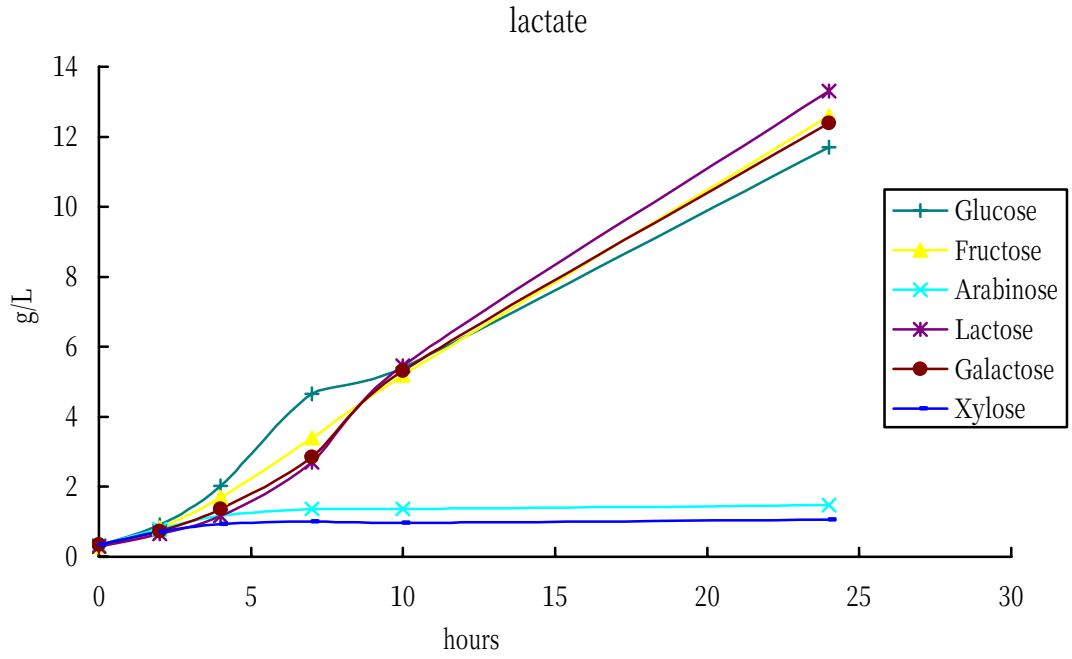
Table 5.2.3 Conversion efficiency of glucose to lactic acid in MRS and the other media

Media	Amount of glucose utilized (g/L)	Highest lactic acid concentration (g/L)	$Y_{LA/GLU}$ (g/g)	Productivity (g/l/hr) [process hours = 24]
MRS	19.184	11.2	0.584	0.467
YE10	18.78	10.4	0.554	0.433
YE20	19.274	9.89	0.513	0.412
YE30	18.57	9.83	0.529	0.410
Y10C10	18.976	10.5	0.553	0.438
Y10C20	18.071	10.7	0.592	0.446
Y10C30	18.766	10.8	0.576	0.45

Effect of various carbon sources on lactic acid production

In this study, shake flask experiments have been used to examine the growth of *Lactobacillus rhamnosus* in various carbon sources. The cultures were grown in 250ml flasks, on an orbital shaker at 250 rpm, with 5% inoculum, at 37 ºC and initial pH 6.0. YE10 medium was used with glucose or other carbon sources. The carbon sources can be divided into sugars with 6 carbons and 5 carbons. Glucose, fructose, galactose and lactose are 6 carbon monosaccharides (except lactose is disaccharide). Arabinose and xylose are 5 carbon monosaccharides.

Fig 5.2.3 Lactate obtained in YE 10 media with various carbon sources



5.2.2 Lactic acid production by *Lactobacillus rhamnosus* in a fermentor (batch fermentation)

In this section, batch fermentation was carried out in a Bioengineering (3.7L) fermentor to characterize biomass yield and lactic acid yield of *Lactobacillus rhamnosus* at 2 different temperatures. The initial conditions of the fermentations were pH 6, 37℃ or 43℃, dissolved oxygen (DO) content around 10% and an agitator rotation of 200 rpm without aeration.

In the batch fermentation of *Lactobacillus rhamnosus*, 2 liters of YE10 containing 20g/L glucose, 10 g/L yeast extract, 5 g/L sodium acetate, 2 g/L KH_2PO_4, 0.2 g/L $MgSO_4$, 0.05 g/L $MnSO_4$, Tween 80 1ml/L was used. 100ml seed culture was used as inoculum.

Fig 5.2.4 Residue glucose concentration in batch fermentations at 2 different temperatures

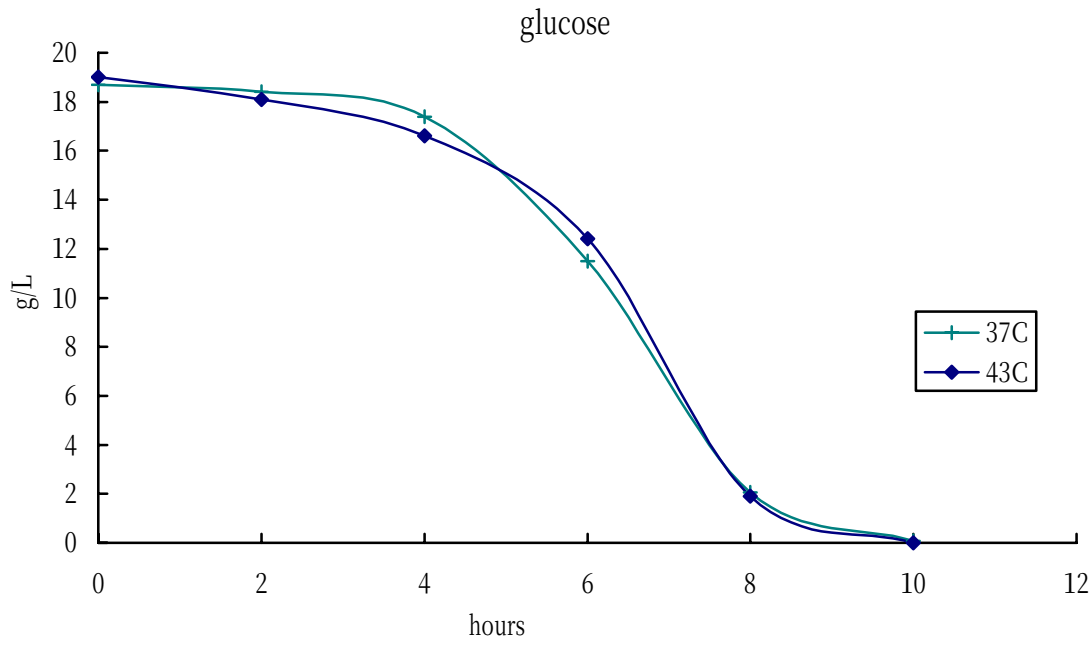


Fig 5.2.5 lactate content in batch fermentations at 2 different temperatures

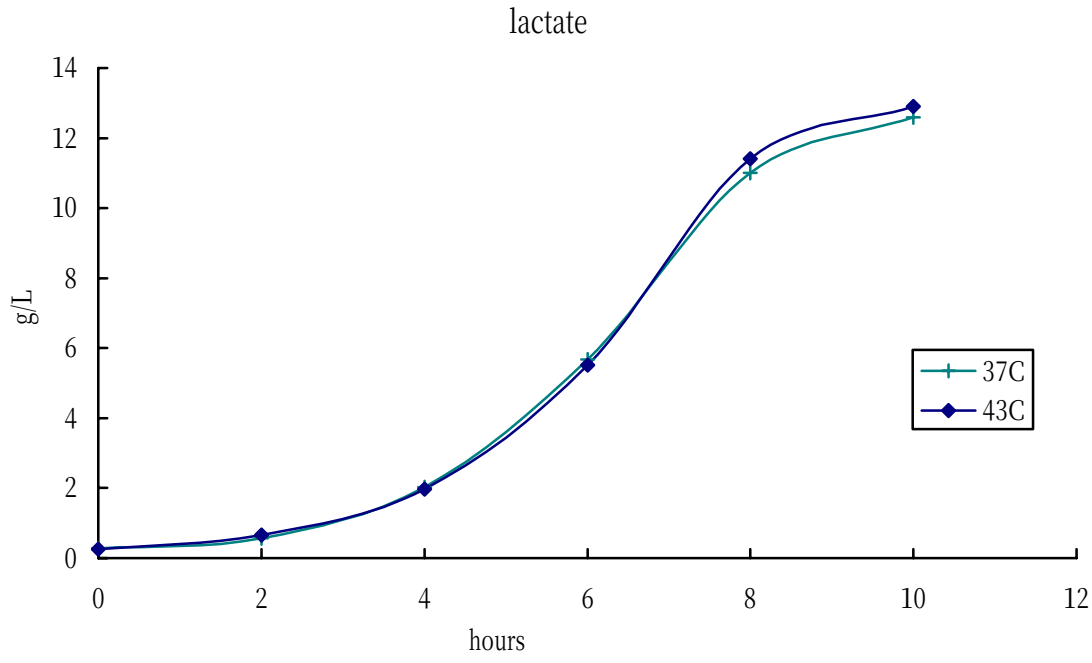


Table 5.2.4 dried weight produced in batch fermentations at 2 different temperatures

Hours	0	2	4	6	8	10
37°C	0.7	1.1	1.8	3.9	5.55	5.7
43°C	0.9	1	1.5	2.5	5.45	5.4

Fig. 5.2.6 dried weight produced in batch fermentations at 2 different temperatures

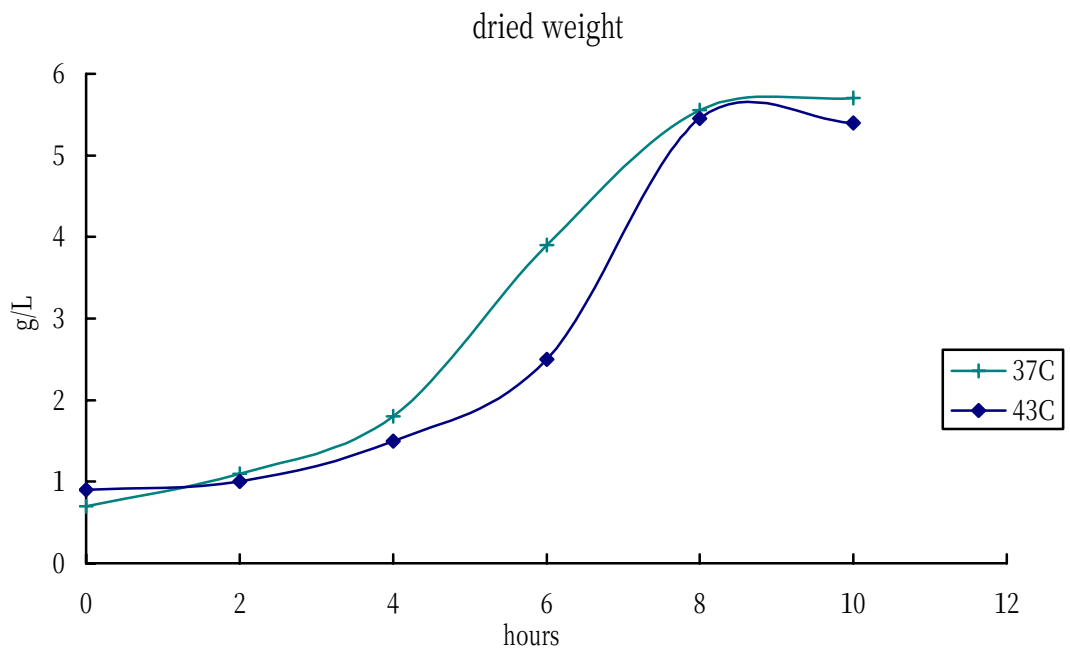


Table 5.2.5 Glucose utilization rate in batch fermentations at 2 different temperatures

Culture	Initial glucose concentration (g/L)	Final glucose concentration (g/L)	Amount of glucose utilized (g/L)	Rate of glucose utilization (g/L/hr) [process hours = 10]
37℃	18.7	0.08	18.62	1.862
43℃	19	0	19	1.9

Table 5.2.6 Conversion efficiency of glucose to lactic acid in batch fermentations at 2 different temperatures

Culture	Amount of glucose utilized (g/L)	Highest lactic acid concentration (g/L)	$Y_{LA/GLU}$ (g/g)	Productivity (g/l/hr) [process hours = 10]
37℃	18.62	12.6	0.677	1.26
43℃	19	12.9	0.679	1.29

In the first part of the medium development, results shows that YE10 can be used to replace MRS (table 5.2.3). 10.4g/l (93% of MRS) of lactic acid was produced in YE10 compared to 11.2g/l in MRS. The medium (YE10) contained: 20g/L glucose, 10 g/L yeast extract, 5 g/L sodium acetate, 2 g/L KH_2PO_4, 0.2 g/L $MgSO_4$, 0.05 g/L $MnSO_4$, Tween 80 1ml/L, pH 6.0. The addition of corn steep liquor did not improve the growth. It is an advantage that 10g/l of yeast extract can be used to replace the complex nitrogen sources in MRS which consists of 10 g/l of peptone, 8 g/l of 'Lab-Lemco' powder, and 4 g/l of yeast extract. It has been shown (results not shown here) that the addition of Tween 80 and acetate to media for *Lactobacilli* results in improved growth. Inorganic ions necessary for growth in MRS will be retained in the YE10 medium. Diminishing the amount of Tween 80 and manganese resulted in diminished growth and lactic acid production. In the experiment, lactic acid yield was around 0.55.

Results in fig. 5.2.3 shows that *Lactobacillus rhamnosus* was not able to use 5-carbon sugars. Arabinose and xylose are he two 5 carbon sugars examined. Moreover, these two 5-carbon sugars are the major carbohydrates found in some industrial carbon waste hydrolysates (e.g. malt waste).

In the fermentor experiment, 2 temperatures were examined, 37℃ and 43℃. Based on the results, there are no significant differences in glucose utilization, dried weight and lactic acid production. These results may be due to the fact that *Lactobacillus sp.* can grow from 30℃ to 45℃ when the fermentation conditions are optimum. That's why the production process finished at 10 hours. Compared to shake flask experiment, it takes a much shorter time. The reason might be due to pH being controlled at the optimum level and dissolved oxygen was kept at very low level which favors *Lactobacillus rhamnosus*. However, the lactic acid yield is only around 0.7. There are still room for improvement in this process.

5.2.3 Production of lactic acid by *Lactobacillus rhamnosus* in continuous cultures at D=0.03

In continuous fermentation, an open system is set up. Sterile nutrient solution is added to the fermentor continuously and an equivalent amount of converted nutrient solution with microorganisms is simultaneously taken out of the system. In a continuous culture under steady state conditions, cell loss as a result of outflow must be balanced by growth of the organism.

Continuous culture was performed under the same conditions as described in the batch fermentations, the working volume was 2.0L, pH was controlled at 6, 5, 4 and 3 at dilution rate (D) 0.03hr^{-1}. Medium YE10 was used for the continuous cultivation. Steady state was reached after 99 hours, 3 turnover of the culture. Samples were taken out and subjected to screening later.

Table 5.2.7 Continuous culture of Lactobacillus rhamnosus at different pH at D=0.03

pH	Dried weight (g/l)	Glucose (g/l)	Lactate (g/l)
6	5.3	0	10.7
5	4.65	0	13.4
4	2.5	7.76	6.62
3	1.3	14.5	1.89

This continuous fermentation was also used to isolate acid tolerant strains. Since lactic acid bacteria produces the primary metabolites lactic acid, this product would lower the pH until a level is reached that inhibits the bacteria. So, if a strain that can tolerate a lower pH can be isolated, it might be able to produce more lactic acid.

Several strains were isolated from the steady state cultures and screened. The screening was performed in YE10 medium with various glucose concentration 20g/l, 50g/l and 100g/l. Firstly, sample taken out at steady state at different pH were grown on agar plates. Single colonies were picked and screened. The parent strain was used as control. Unfortunately, none of the screened cultures showed an improved result whether in volumetric concentration, yield, volumetric productivity or specific productivity. Table 5.2.7 shows that in continuous cultures pH 5 is more favorable for lactic acid production, although the biomass was higher at pH 6.

5.2.4 Lactic acid production by *Lactobacillus rhamnosus* in a fermentor with 100 g/l and 150 g/l glucose (batch fermentation)

The experiment characterizes lactic acid production from high glucose concentration. The initial conditions of the fermentations were pH 5, 43℃, dissolved oxygen (DO) content around 10% and an agitator rotation of 200 rpm without aeration.

In the batch fermentation of *Lactobacillus rhamnosus*, 2 liters of YE10 containing 100 or 150 g/L glucose, 10 g/L yeast extract, 5 g/L sodium acetate, 2 g/L KH_2PO_4, 0.2 g/L $MgSO_4$, 0.05 g/L $MnSO_4$, Tween 80 1ml/L was used. 100ml seed culture was used as inoculum.

Fig. 5.2.7 Residue glucose concentration in YE10 media

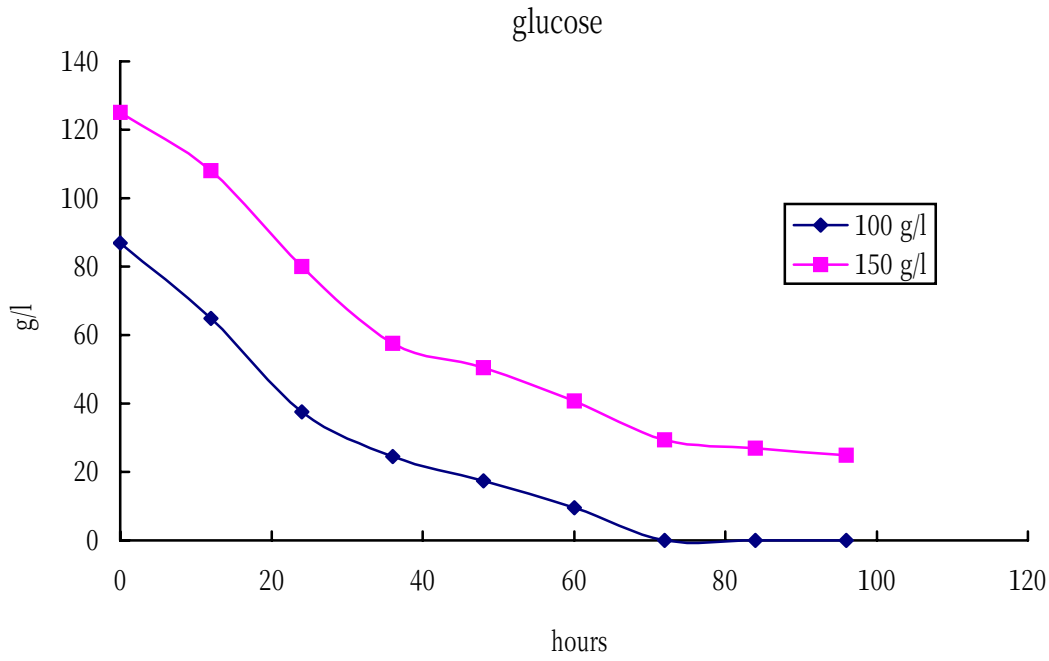


Fig. 5.2.8 Lactate obtained in YE10 media

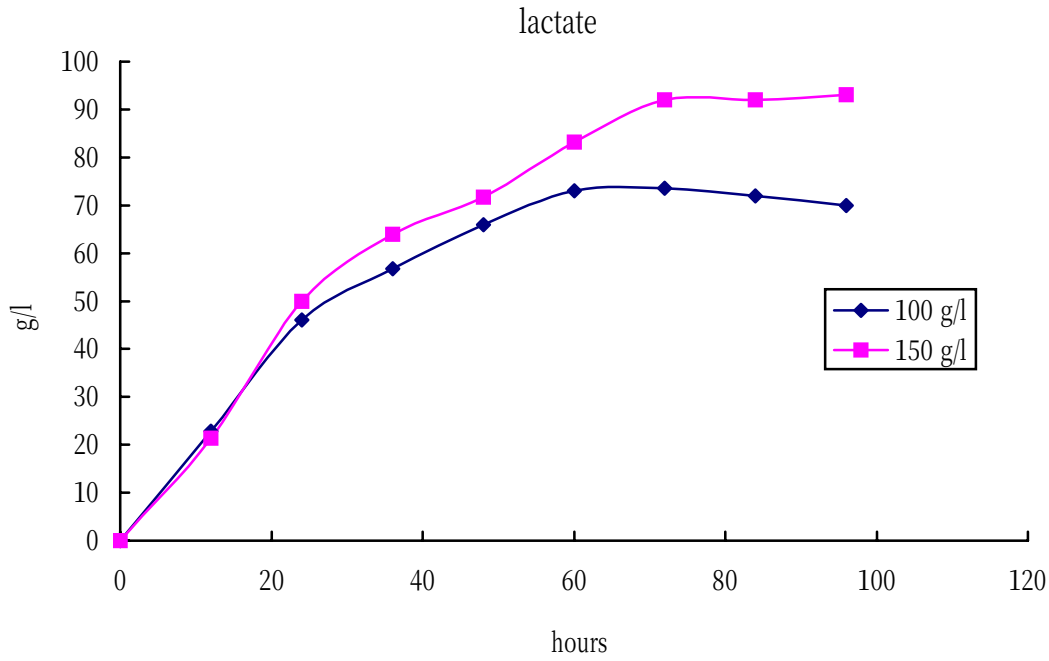


Table 5.2.8 Glucose utilization rate of in YE10 media

Culture	Initial glucose concentration (g/L)	Final glucose concentration* (g/L)	Amount of glucose utilized (g/L)	Rate of glucose utilization (g/L/hr) [process hours]
100 g/l	87	9.5	77.5	1.292 [60]
150 g/l	125	29.38	95.62	1.328 [72]

*Glucose concentration at the highest lactic acid concentration

Table 5.2.9 Conversion efficiency of glucose to lactic acid in YE10 media

Culture	Amount of glucose utilized (g/L)	Highest lactic acid concentration (g/L)	$Y_{LA/GLU}$ (g/g)	Productivity (g/l/hr) [process hour]
100 g/l	77.5	73	0.942	1.217 [60]
150 g/l	95.62	92.04	0.963	1.278 [72]

The maximum volumetric concentrations of lactic acid were 73 g/l and 92 g/l at 60 and 72 hours for 100 g/l and 150 g/l glucose respectively. The yield was over 94% for both cultures. pH was controlled at 5.0 throughout the process. Figures 5.2.7 and 5.2.8 show the glucose and lactate content throughout the fermentation process.

In the 100 g/l glucose culture, the glucose content dropped to zero, glucose consumption rate was relatively high, because the cells consume glucose throughout the lag and log phase. We can see that the rate is slightly higher in the first 24 hours than the remaining period, the cells were in an active state and were dividing exponentially, consuming glucose at a high rate. Afterwards, cells entered the stationary phase; cell growth nearly stopped but kept on producing the primary metabolite, lactic acid.

Figure 5.2.9 shows the lactic acid production throughout the fermentation process. The Lactic acid production curve showed a direct relationship to the glucose consumption throughout the process. This is because lactic acid production is growth-related. From 0 to 24 hours, lactic acid content increased rapidly from 0 g/L to 46 g/L, since cells were growing at exponential phase. Thus, lactic acid production rate is very high. After that, the cells entered the stationary phase, although cells

grow slowly, they are metabolically active and they kept on producing primary metabolites during this period.

In the 150 g/l glucose culture, similar profiles can be found except that there were excess glucose at the end of the fermentation and a higher amount of lactic acid was produced. Possible reason for the residue glucose may be it had reached its maximal production level and the metabolites produced were at inhibiting levels. Moreover, nutrients might have depleted, so the cells stopped production. However, these 2 cultures could be used as a reference for comparison in the mixed culture experiments presented in the later chapters.

5.3 Glucoamylase production and saccharification

Aspergillus niger is the glucoamylase source in this project. *Aspergillus sp.* is one of the most well known industrial microorganisms. It is able to produce numerous enzymes such as alpha amylase, beta amylase, glucoamylase, cellulase, cellobiase etc. Filamentous fungi have a number of properties which make them important both scientifically and industrially. Their applications range from production of organic acids and antibiotics to industrial enzymes.

5.3.1 Optimizing of the saccharification process

In this study, shake flask experiments have been used to study the growth and enzyme production of immobilized *Aspergillus niger* spores. 3g beads containing Aspergillus niger spores were grown in 50ml of MYG (sucrose medium) medium with initial pH 5.0, 6.0, 7.0 in 250ml flasks,, on an orbital shaker at 250 rpm, at 20 ºC for 24 hours. Then, the sucrose medium was replaced by 50ml of gelatinized 2% starch and continued to incubate at 20°C, 250rpm for 24 hours to induce the glucoamylase production. After that, another 50ml of 2% starch solution was added into the medium and shake at 43°C and 250rpm. Samples were taken out at intervals for analysis.

The above experiment was repeated at 25°C and 30°C.

Fig 5.3.1 Saccharification of 2% starch at 20°C, 250rpm at different pH

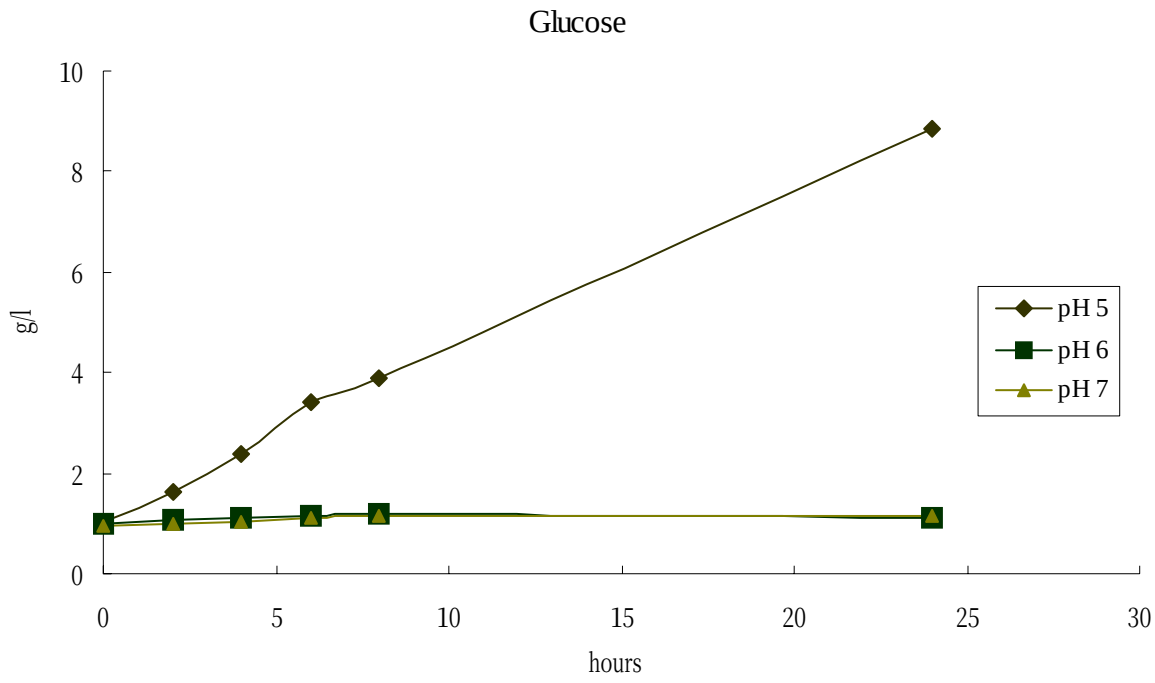


Fig 5.3.2 Saccharification of 2% starch at 25°C, 250rpm at different pH

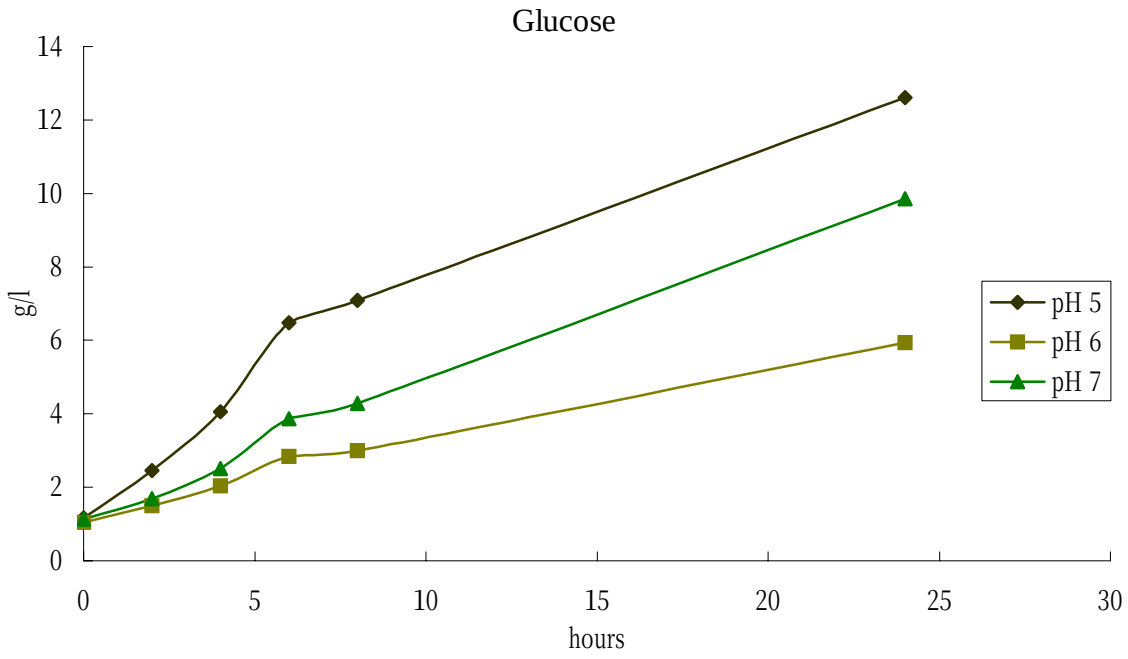
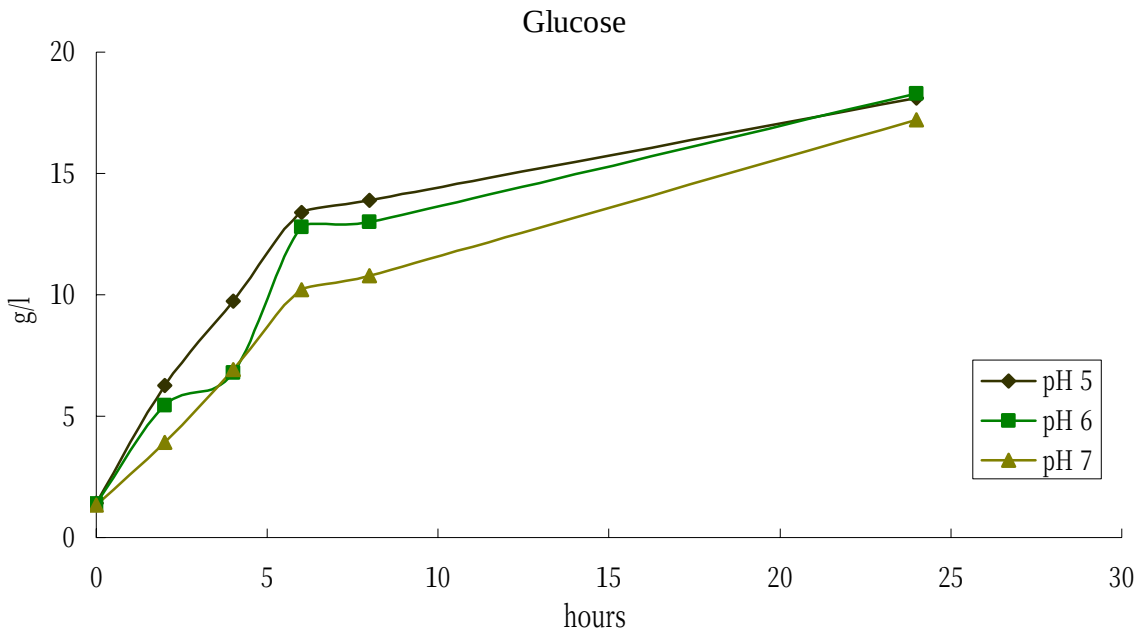


Fig 5.3.3 saccharification of 2% starch at 30^{o}C, 250rpm at different pH



From the previous figures, the conditions (both temperature and pH) that can saccharify the highest glucose concentration from 2% starch are grouped together in fig. 5.3.4.

Fig 5.3.4 Combined results of saccharification at different pH and temperature

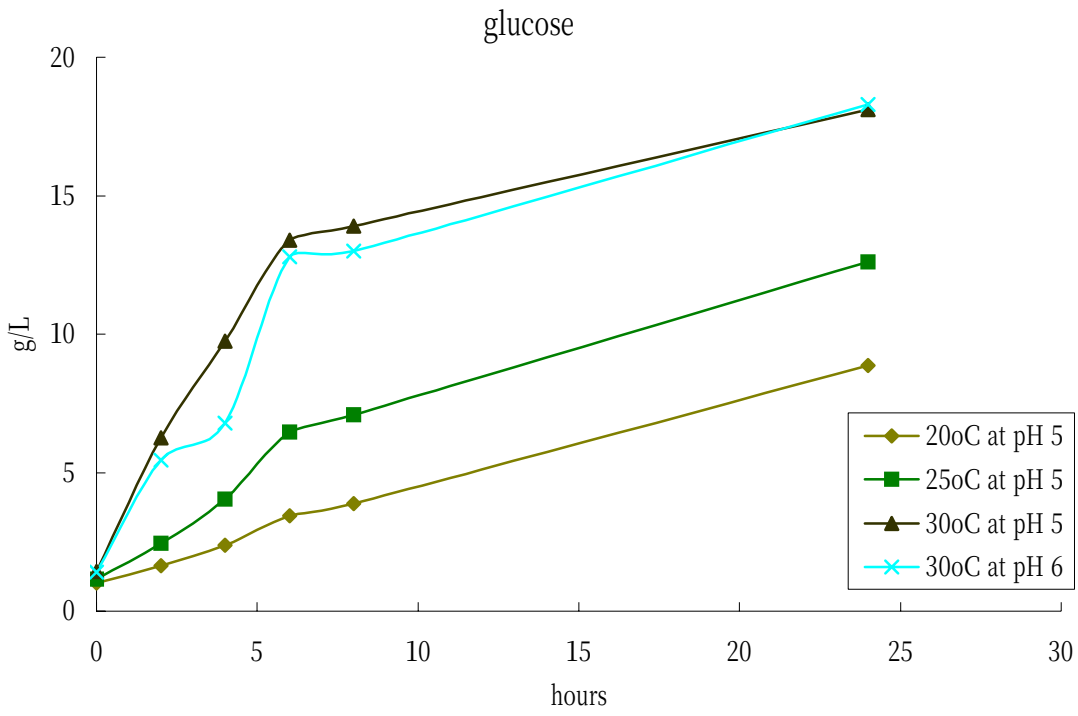


Table 5.3.1 the relative saccharifying activity of 2% starch under selected conditions

Temperature (℃)	pH	Theoretical glucose concentration (g/l)	Actual glucose concentration (g/l)	Relative saccharifying activity (%)
20	5	22.2	8.86	40%
	6		1.12	5%
	7		1.17	5.2%
25	5		12.6	57%
	6		5.93	27%
	7		9.85	44%
30	5		18.1	82%
	6		18.3	82%
	7		17.2	77%

The highest saccharifying activity can be achieved at 30℃, pH 5 or pH 6.

Optimizing the shaker speed for the saccharification process

This experiment determines the relationship between shaker speed and saccharification. The conditions used are the same as previous experiments except a shaker speed of 160 rpm was used.

Fig 5.3.5 Saccharification of 2% starch at 25^{o}C, 160rpm at different pH

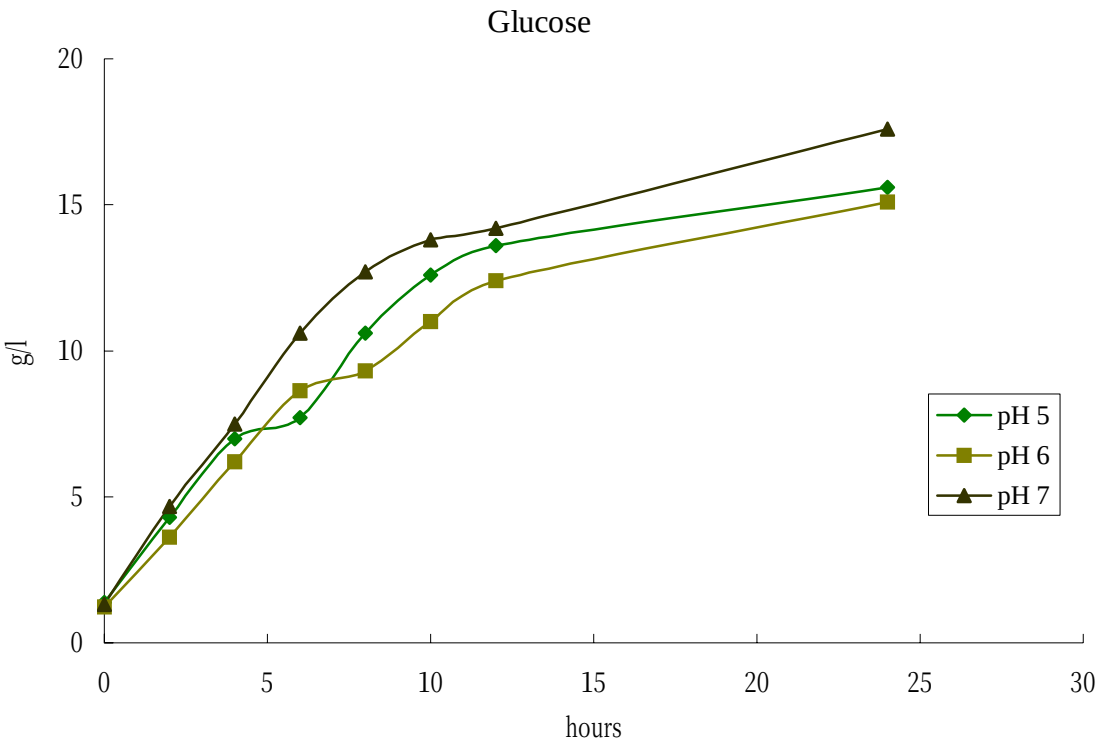


Fig 5.3.6 saccharification of 2% starch at 30°C, 160rpm at different pH

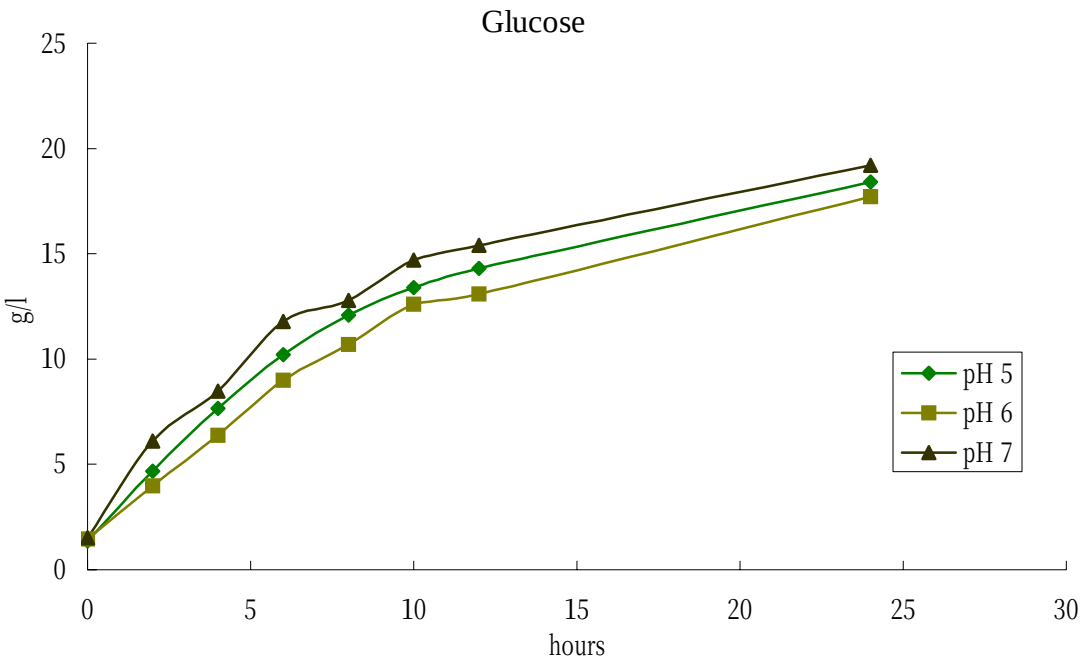


Fig 5.3.7 saccharification of 2% starch at 30°C, 250rpm at different pH

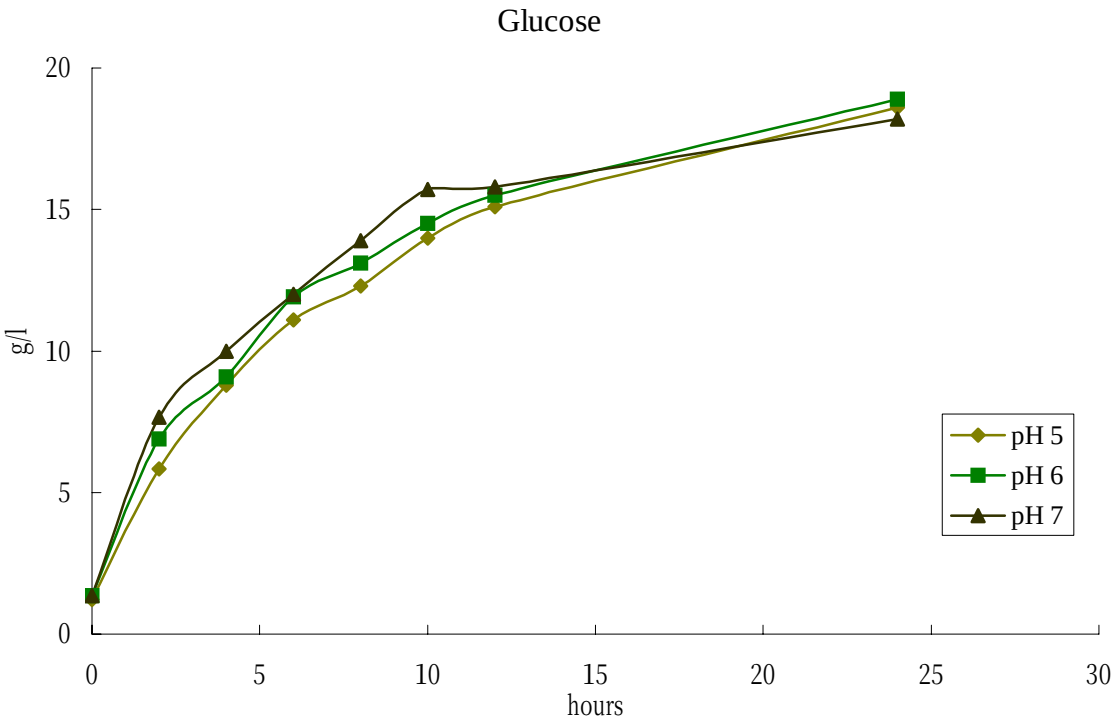


Fig 5.3.8 Grouped results of saccharification at different pH, temperature and rotational speed

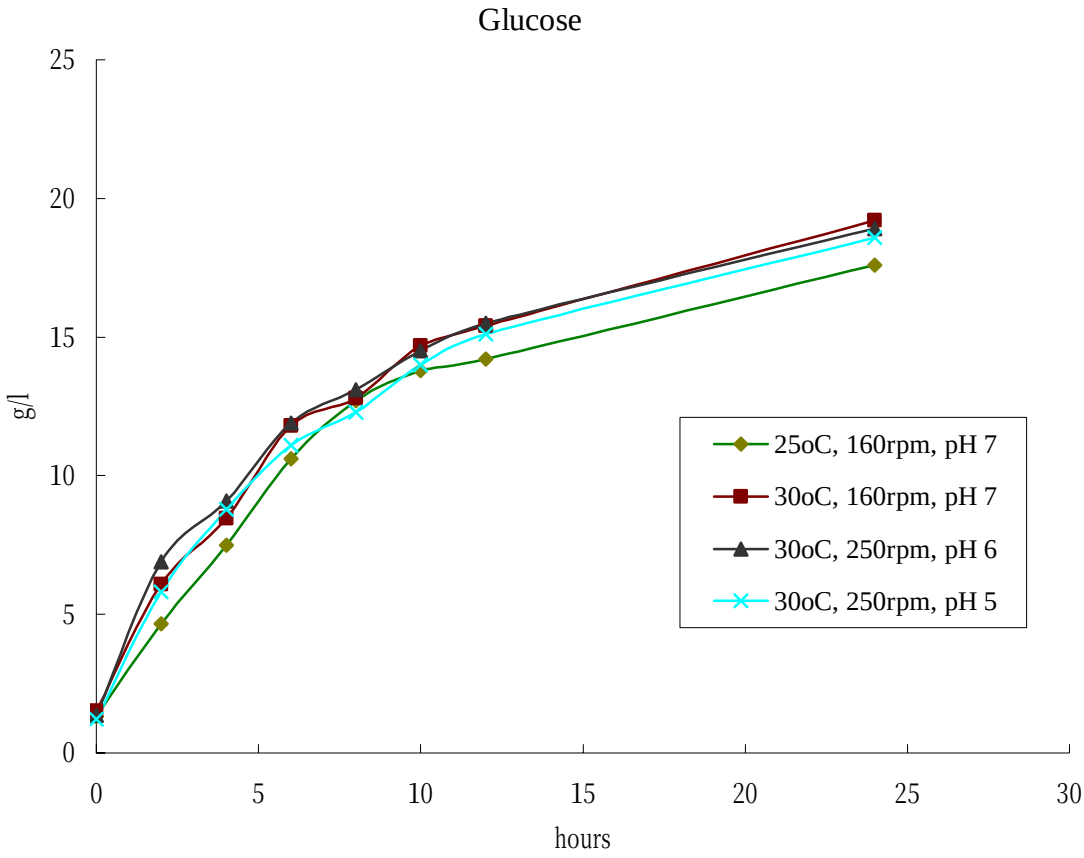


Table 5.3.2 The relative saccharifying activity with 2% starch under selected conditions

Temperature (°C)	Rotational speed (rpm)	pH	Theoretical glucose concentration (g/l)	Actual glucose concentration (g/l)	Relative saccharifying activity (%)
25	160	5	22.2	15.6	70%
		6		15.1	68%
		7		17.6	79%
30	160	5		18.4	83%
		6		17.7	80%
		7		19.2	86%
	250	5		18.6	84%
		6		18.9	85%
		7		18.2	82%

The highest saccharifying activity was achieved at 30°C, 160rpm, pH 7 as well as 30 °C, 250rpm and pH 5 or 6.

The spores of *Aspergillus niger* were first immobilized in sodium alginate and these immobilized beads were used for the whole project. Immobilized beads have several advantages. Firstly, high spore concentrations can be obtained as the spores were concentrated and trapped in sodium alginate. Secondly, it can provide favorable micro-environmental conditions for cells, subsequently a better performance of enzymes. Besides, cell immobilized can protect against shear damage.

To find the optimum saccharification conditions for *Aspergillus niger*, three temperatures (20°C, 25°C and 30°C) and pH (5, 6 and 7) with 250rpm were initially chosen for the investigation. The concentration of glucose saccharified from starch was measured as it is directly proportional to the glucoamylase activity. Different pH and temperatures provided different conditions for the spores to germinate and formation of mycelium. This mycelium would directly affect the second stage enzyme production. By combining the results which showed the higher glucose production in Figure 5.3.4, it can be seen that beads that were cultured at 30°C at pH 5 produced the more glucose than beads that were cultured at 20°C and 25°C. It can be concluded that saccharification is affected by temperature. The relative saccharifying activity was calculated and shown in Table 5.3.1. More than 80% saccharifying activity was obtained at 30°C at pH 5 or pH 6. As a result, pH 5 or pH

6 was considered suitable for cultivation of *Aspergillus niger* and later analysis was focused on this temperature. Figures 5.3.5 to 5.3.8 shows that two shaking speeds were studied and the results indicated that 160rpm and 250rpm at 30°C did not cause a significant difference in glucose production. The highest saccharifying activities are shown in table 5.3.2, 30°C, 250rpm at pH 6 was selected as the conditions to be used in the first stage for germination of immobilized *Aspergillus niger* spores. *Aspergillus niger* had a high enzymatic capability for saccharification of potato starch in the pH range from 6.0 to 7.0 at 30°C, and the starch hydrolysis and reducing sugar accumulation were influenced obviously by the pH and temperature used for cell growth. Cells grown at 20°Cwith pH higher than 5 was unfavorable for starch saccharification. However, pH higher than 5 can be used if the temperature is raised to 30°C.

Using different concentrations of starch in the saccharification process

Starch was liquefied by α-amylase in order to reduce its viscosity. 1ml of α-amylase (59kunit/ml) was added to gelatinized 1% w/v starch solutions. 10ppm of calcium was added in order to enhance the process. Liquefaction was carried out during autoclaving.

3g of beads containing *Aspergillus niger* spores were cultivated as described before at 30^{o}C, 250rpm for 24 hours at pH 6. Next, 50ml of 2% gelatinized starch solution or 2% liquefied starch solution were used to replace the sucrose medium and continued to incubate at 30^{o}C, 250rpm for 24 hours to induce the glucoamylase production. After 24 hours, another 50ml of 2% gelatinized starch solution or 2% liquefied starch solution were added to the medium at 43°C and 250rpm. Samples were taken out at intervals for analysis.

Solutions containing 3%, 4%, and 5% of starch were subjected to the same treatment and analysis.

Fig 5.3.9 Saccharification of 2% gelatinized or liquefied starch

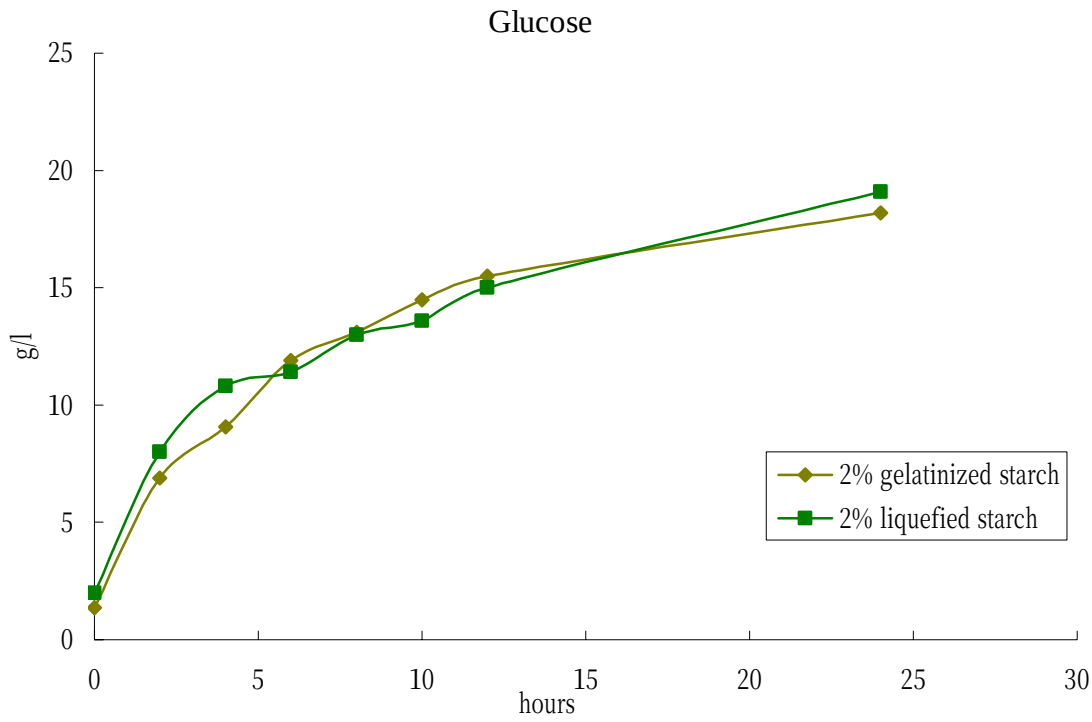


Fig 5.3.10 Saccharification of 3% gelatinized or liquefied starch

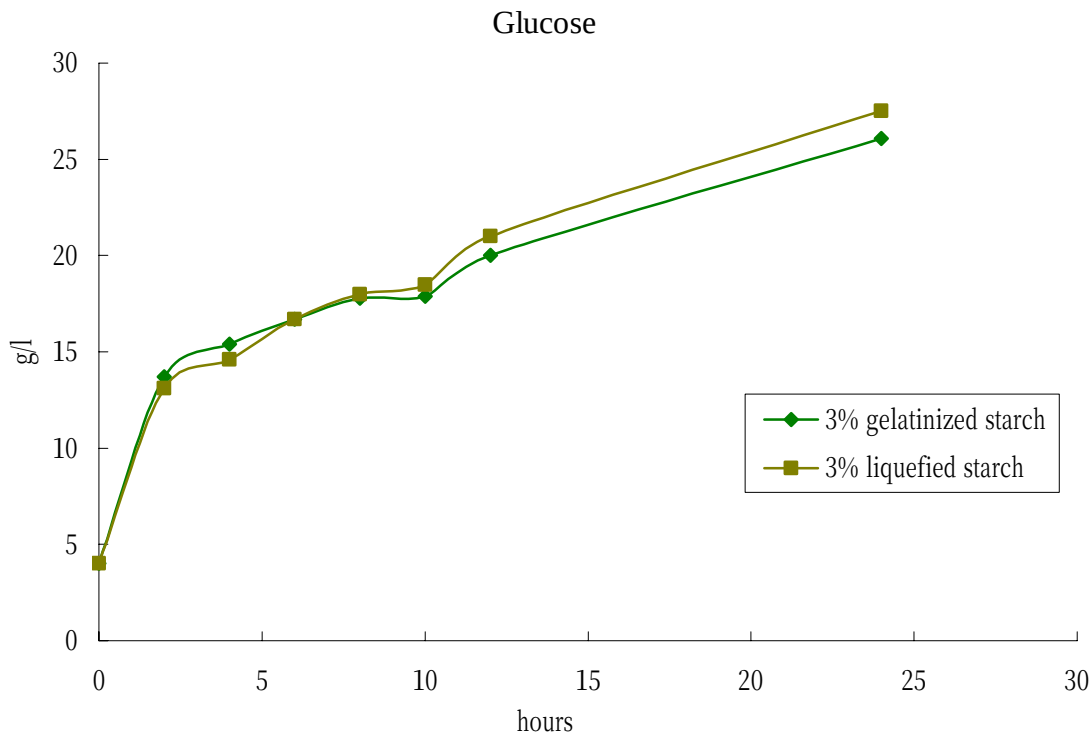


Fig 5.3.11 Saccharification of 4% gelatinized or liquefied starch

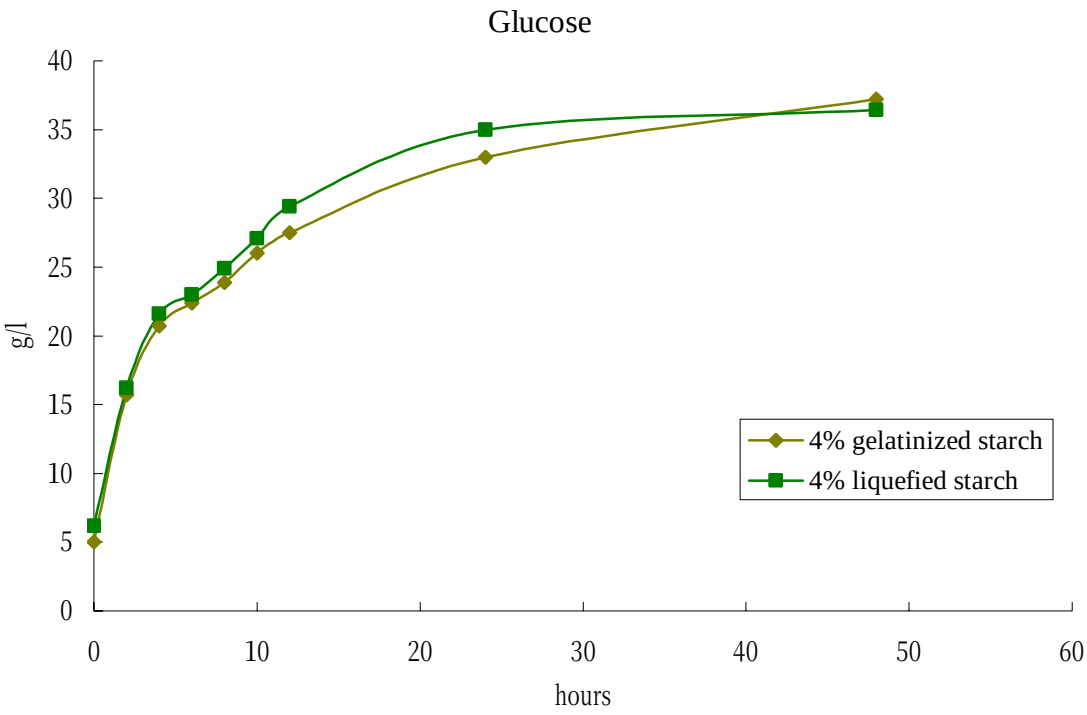


Fig 5.3.12 Saccharification of 5% gelatinized or liquefied starch

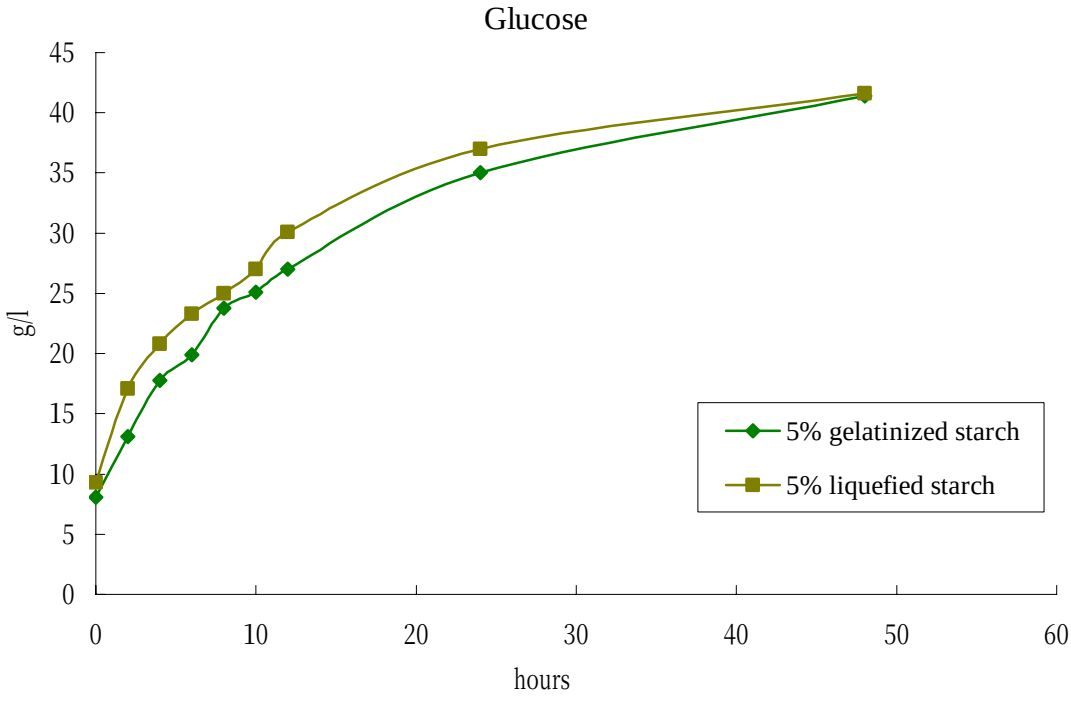


Fig 5.3.13 Saccharification of different concentrations of liquefied starch

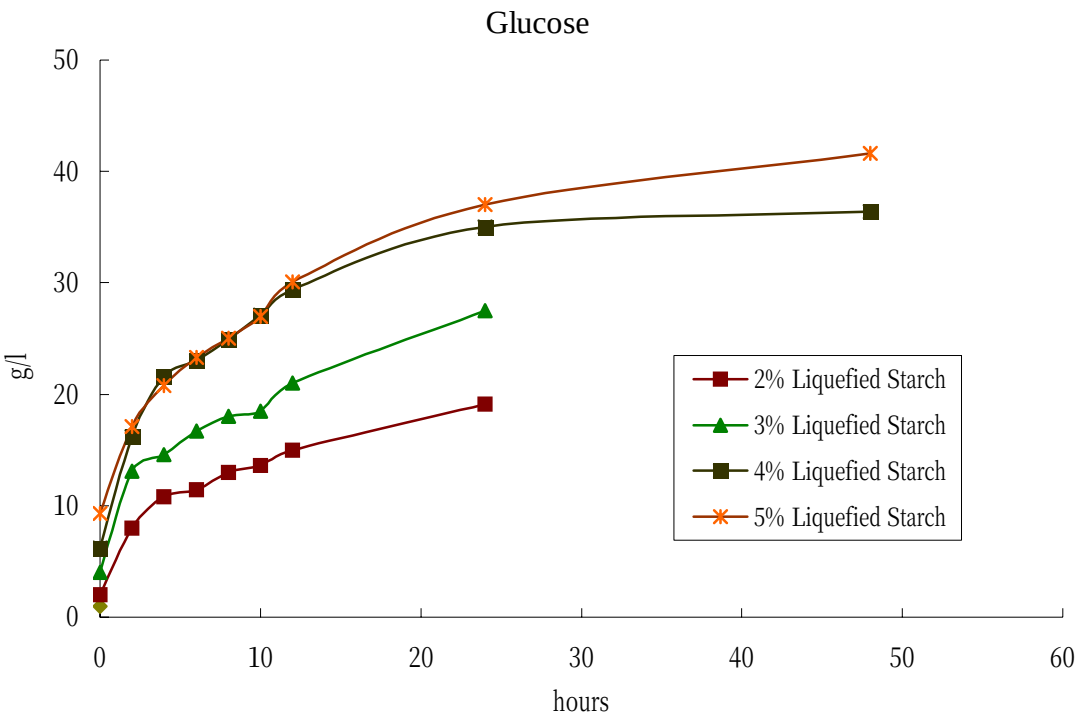


Table 5.3.3 The relative saccharifying activity with different concentrations of liquefied starch

Concentration of liquefied starch (%)	Theoretical glucose concentration (g/l)	Actual glucose concentration (g/l)	Relative saccharifying activity (%)
2%	22.2	19.1	86%
3%	33.3	27.5	83%
4%	44.4	36.4	73%
5%	55.5	41.6	75%

The effect of inoculation size on saccharification

Conditions used were the same as previous experiments: 30℃, 250rpm, pH6 for 2% and 5% starch. 1x (3g beads) and 2x (6g beads) inoculum size was compared.

Fig 5.3.14 Saccharification of 2% starch by different amounts of beads (immobilized cells)

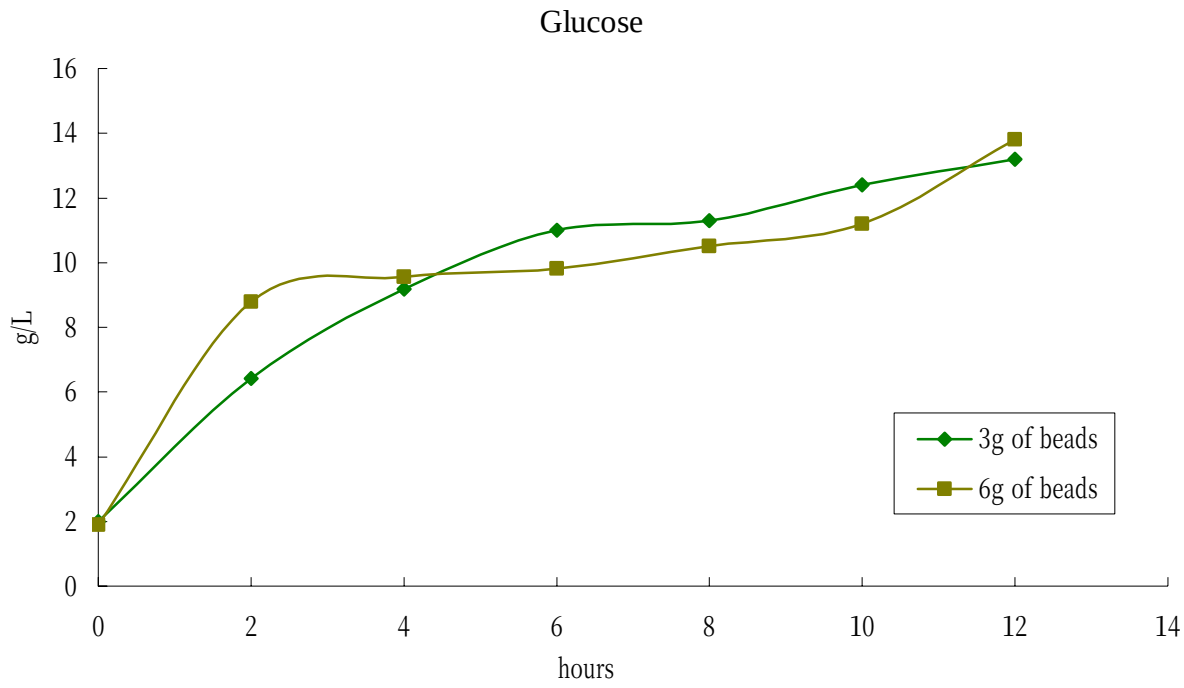
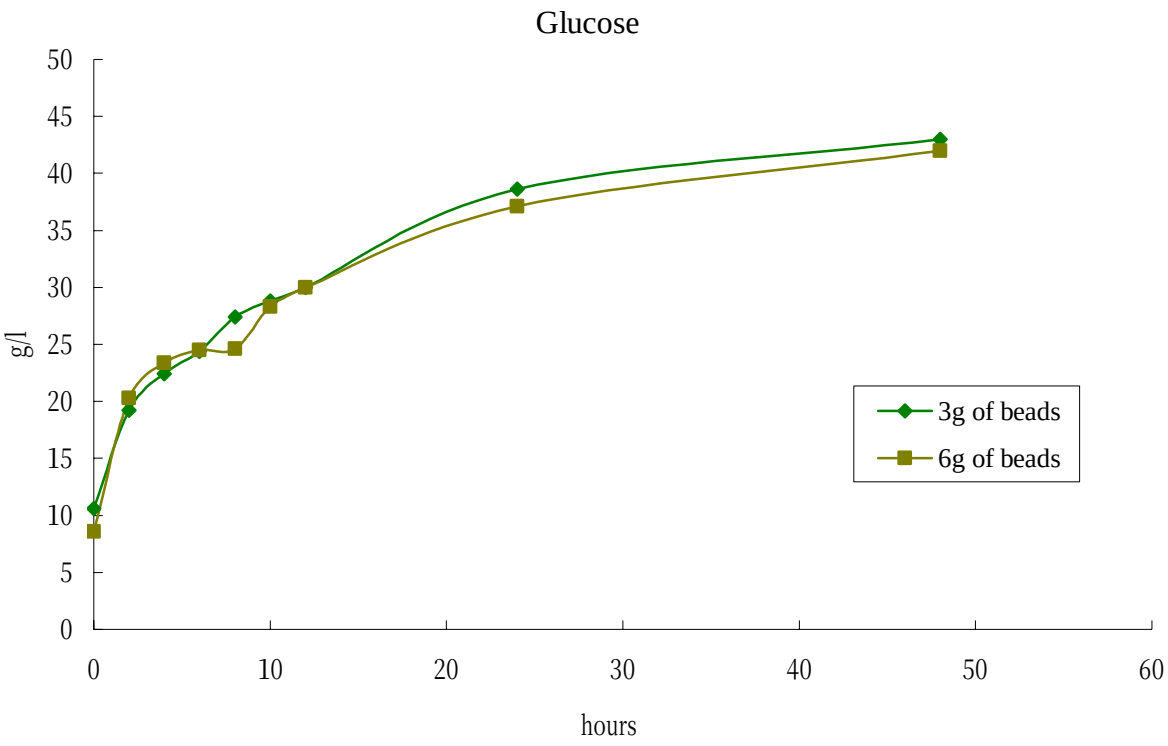


Fig 5.3.15 Saccharification of 5% starch by different amount of beads

(immobilized cells)



Aspergillus niger was investigated for its ability in starch saccharification, i.e. the production and activity of glucoamylase, with various concentration of starch.

The importance of α-amylase used to liquefy starch was investigated. Whether the saccharification process by immobilized *Aspergillus niger* can be enhanced if α-amylase is used in pretreatment of starch (liquefaction) was studied. Results in figure 5.3.9 to 5.3.13 shows that using α- amylase for starch liquefaction did not cause significant improvement in the production of glucose when compared to gelatinized starch. This is because *A. niger* can also produce α- amylase to hydrolyze the α 1→ 4 glycosidic bond from the non-reducing end of starch molecules. However, the main application of α-amylase is to reduce the viscosity of the starch solutions. Increasing the starch concentration up to 5% will make the solution very viscous, so α- amylase must be added prior to liquefying the starch substrate throughout the project in order to reduce its viscosity. Otherwise, it would form a gel when it cools down. 2% to 5% of liquefied starch was saccharified and the concentration of glucose analyzed. According to figure 5.3.13 and table 5.3.3, nearly 80% of liquefied starch can be saccharified into glucose. This represents a satisfactory level in this saccharification process.

The relative saccharifying activity is shown in table 5.3.3. Around 80% of glucoamylase saccharifying activity was obtained in 2% and 3% liquefied starch. This can contribute to a quick saccharification process. Also, about 70% glucoamylase saccharifying activity was obtained in the 4% and 5% liquefied starch. The reason that this saccharifying activity is lower than that of the 2% and 3% liquefied starch might be due to the product produced (i.e. glucose) that may cause some inhibition on glucoamylase.

The inoculum size of the immobilized cells was also studied to determine whether it can enhance the process of saccharification. However, results in figures 5.3.14 and 5.3.15 show that doubling the amount of beads did not improve the saccharification process. This phenomenon occurred in both starch concentrations (2% and 5%). This might be due to the accumulation of glucose which might inhibit the glucoamylase activity.

5.3.2 Simultaneous Saccharification and Fermentation (SSF) of lactic acid by *Aspergillus niger* and *Lactobacillus rhamnosus* in shake flasks

Starch was prepared and liquefied by α-amylase in order to reduce its viscosity. Calcium was added in order to enhance the process.

3g of beads containing *Aspergillus niger* spores were cultivated in 50ml sucrose medium at pH 6. The temperature and rotational speed were set at 30^{o}C and 250rpm respectively for 24 hours. 50ml of 2% liquefied starch solution was then used to replace the sucrose medium and incubation continued at 30^{o}C, 250rpm for 24 hours to induce the glucoamylase production. After 24 hours, another 50ml of 2% liquefied starch solution in YE10 medium (without glucose), 5% of *L. rhamnosus* inoculum and 20g/l $CaCO_3$ were added into the medium. The conditions for SSF were 43^{o}C at 250rpm. Samples were taken out at intervals for analysis. 5% and 10% liquefied starch containing 50g/l and 100g/l $CaCO_3$ respectively was also studied.

Fig 5.3.16 SSF of lactic acid from 2% liquefied starch

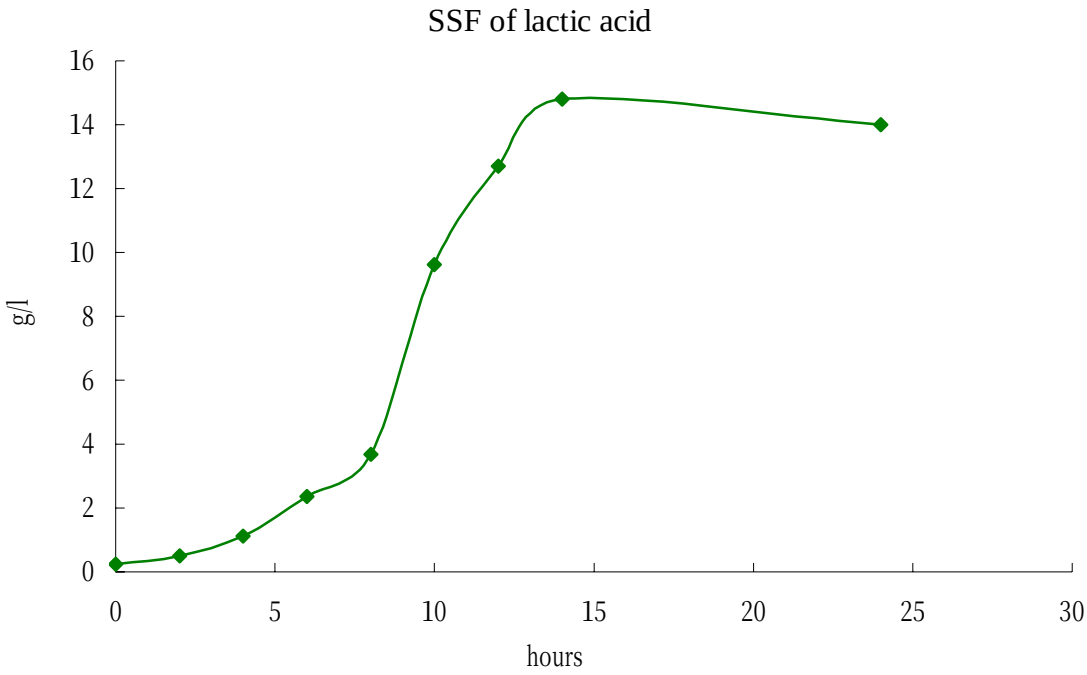


Fig 5.3.17 SSF of lactic acid from 5% liquefied starch

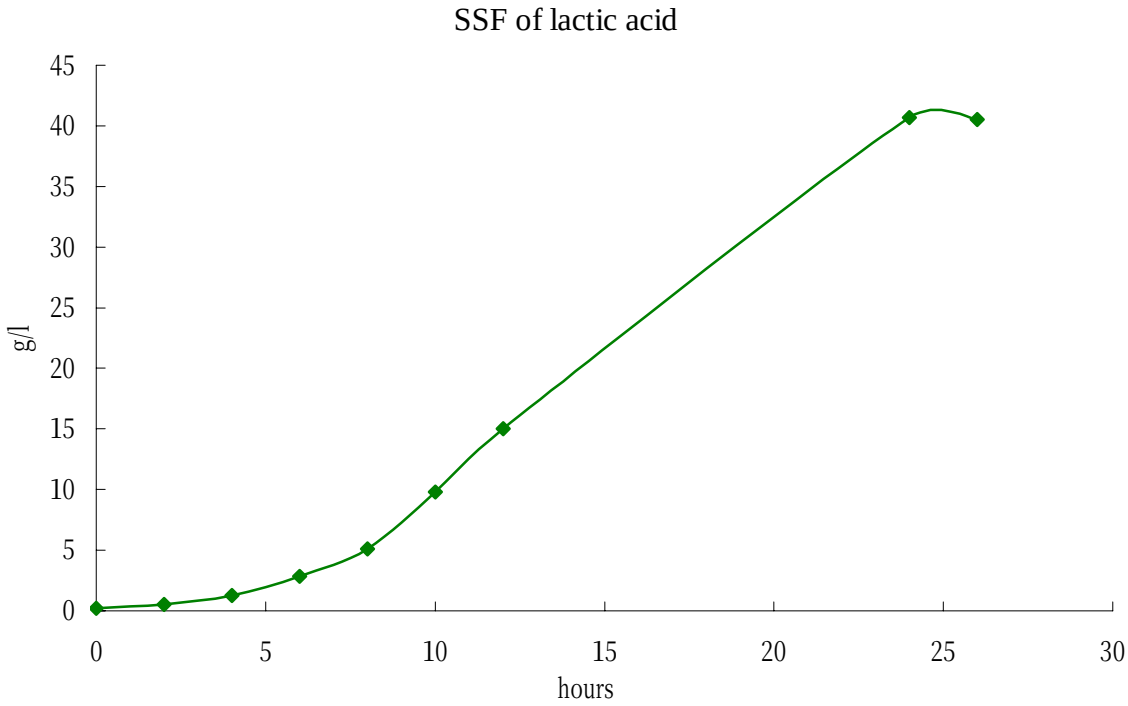


Fig 5.3.18 SSF of lactic acid from 10% liquefied starch

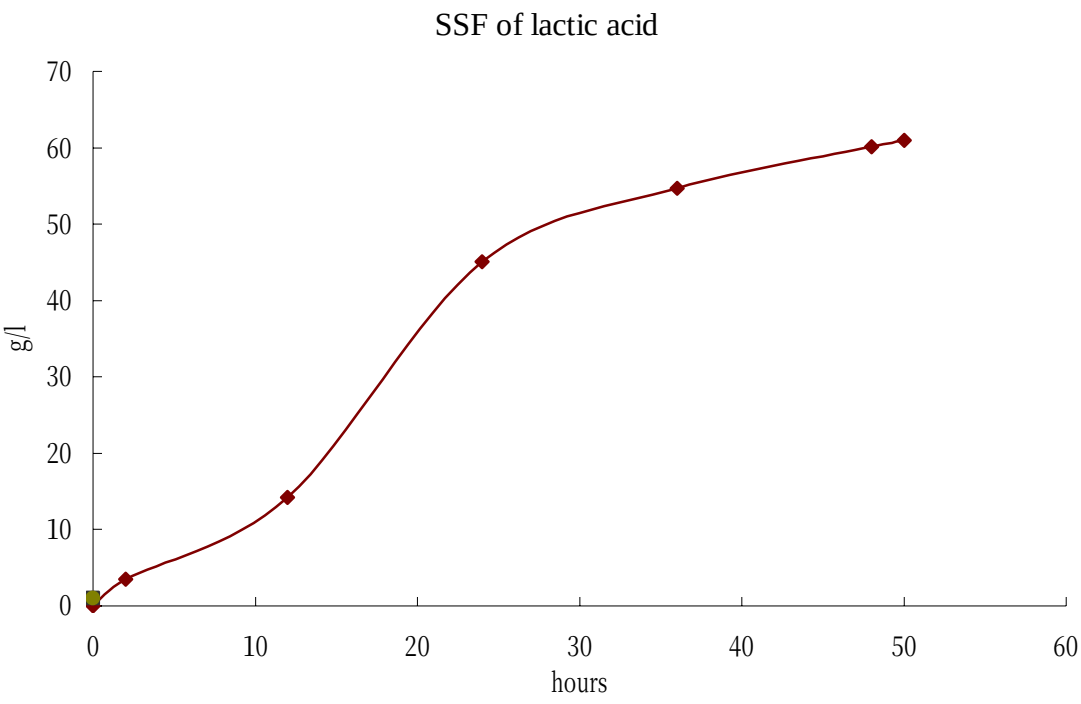


Table 5.3.4 Conversion efficiency of SSF of lactic acid

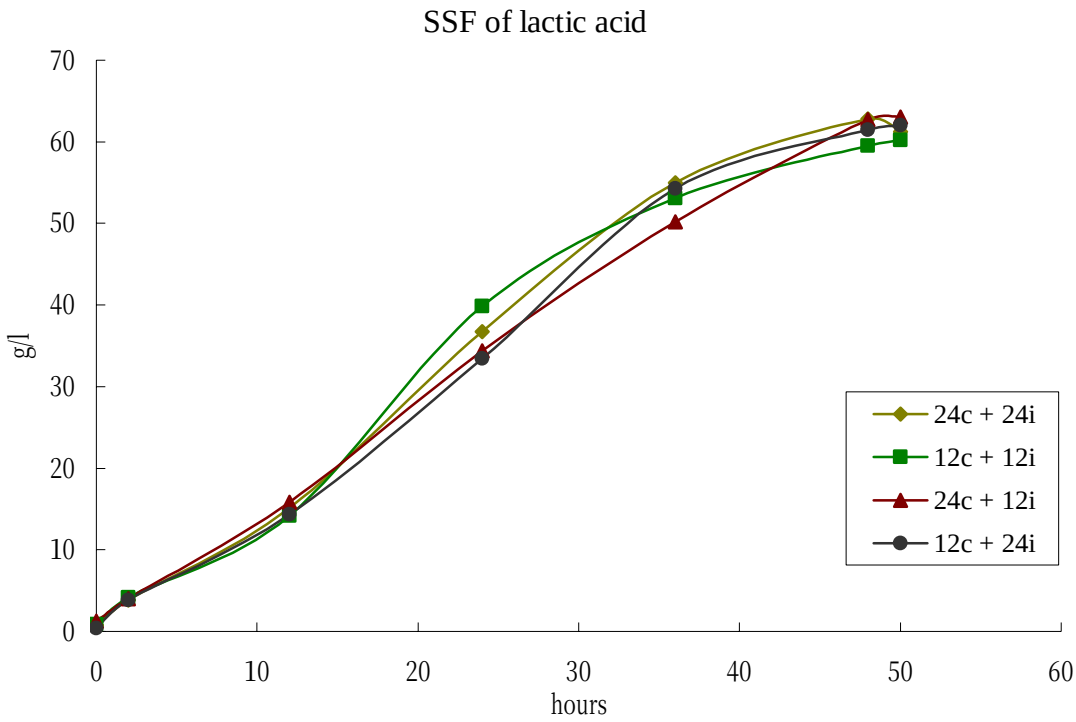
Concentration of liquefied starch (%)	Theoretical glucose concentration (g/l)	Lactic acid concentration (g/l)	Time of fermentation (h)	$Y_{LA/GLU}$ (g/g)	Productivity (g/l/h)
2%	22.2	14.8	14	0.67	1.057
5%	55.5	40.7	24	0.733	1.667
10%	111	60.1	48	0.541	1.252

Immobilized *Aspergillus niger* was used to saccharify starch which were then converted into lactic acid by *L. rhamnosus* at the same time. 2%, 5% and 10% of liquefied starch were used. Tale 5.3.4 shows that about 70% of 2% and 5% liquefied starch were simultaneously saccharified and fermented to lactic acid while 10% culture showed a 50% yield. The lactic acid productivity was quite high as 1.666 g/l/h of lactic acid was obtained from the 5% starch culture. However, due to the difficulty in controlling pH, the overall performance was not so good. One of the problems was having a large amount of calcium carbonate at the beginning of the fermentation. This increased the initial pH value which does not favor glucoamylase activity. It is also troublesome if the calcium carbonate have to be added manually.

Optimization of SSF of lactic acid by shortening the germination and induction stage

Since the saccharification process takes 24 hours, experiments were designed to determine whether the process time can be reduced. Various combinations of spore germination and enzyme induction time in the saccharification process was attempted (figure 5.3.19).

Figure 5.3.19 Optimization of SSF of lactic acid



Note:

c: spores germination time.

i: enzyme induction time.

The digits indicate the time used for that stage.

Table 5.3.5 conversion efficiency of SSF of lactic acid

Cultures	Concentration of liquefied starch (%)	Theoretical glucose concentration (g/l)	Lactic acid concentration (g/l)
24c + 24i	10%	111	62.8
12c +12i			59.5
24c + 12i			62.7
12c + 24i			61.5

Cultures	Time (h)	$Y_{LA/GLU}$ (g/g)	Lactic acid productivity (g/l/h)
24c + 24i	48	0.57	1.31
12c +12i		0.54	1.24
24c + 12i		0.57	1.31
12c + 24i		0.56	1.28

Results in table 5.3.5 shows that the time for cultivation of beads and enzyme induction in the saccharification process can be cut by half (from 48 to 24 hours) without affecting the SSF performance in the shake flasks. Lengthening the cultivation time in either of these 2 stages (spores germination and enzyme production), (results not shown here) would not improve the SSF but the beads burst

due to the pressure generated by the mycelium.

SSF and YE10 medium inhibition analysis

This experiment is to determine whether YE10 medium contains compounds that would inhibit the enzymes in the saccharification process. Conditions used were the same as described previously. A series of liquefied starch solution each containing one of the YE10 components: Tween 80 (1ml/l), sodium acetate (5g/l), KH_2PO_4 (2g/l) and $MgSO_4$ (0.05g/l) / $MnSO_4$ (0.2g/l) were examined.

Fig 5.3.20 SSF medium inhibition analysis

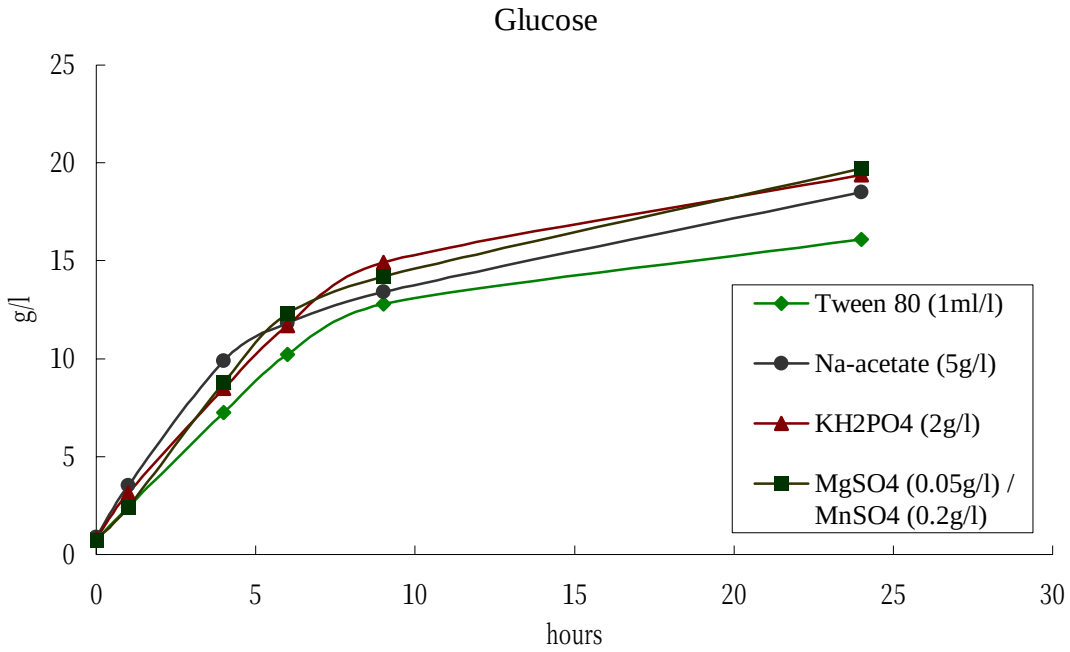


Table 5.3.6 the relative saccharifying activity

Chemical added in saccharification process	Theoretical glucose concentration (g/l)	Actual glucose concentration (g/l)	Relative saccharifying activity (%)
Tween 80 (1ml/l)	22.2	16.1	73%
sodium acetate (5g/l)		18.5	83%
KH_2PO_4 (2g/l)		19.4	87%
$MgSO_4$ (0.05g/l)/ $MnSO_4$ (0.2g/l)		19.7	89%

SSF medium was also investigated. Figure 5.3.20 shows that only Tween 80 shows a slightly lower value in saccharification. The relative saccharifying activity of glucoamylase in the medium which contained Tween 80 was just 73%. This may be due to the fact that Tween 80 is a detergent. Even though it has a slight adverse effect on saccharification; it has to remain in the medium because Tween 80 shows a positive effect on the growth of *Lactobacillus rhamnosus*. It helps to smoothen the transfer of nutrients and metabolites between cells and medium (De Man et. al. 1960).

5.3.3 SSF of lactic acid in a fermentor

Batch fermentation

In this section, batch fermentation was carried out in a Lh (1.0L) fermenter to characterize the SSF process. The initial conditions of all fermentations were pH 5.5, 43℃, dissolved oxygen (DO) content around 10% without aeration. The suspension medium was continuously stirred at a constant rate of 200rpm during all the runs.

3g of beads (a total of 12g of beads) were cultivated in 50ml sucrose medium in 250ml flask at 30^{o}C, 250rpm for 24 hours. To induce the glucoamylase production, the sucrose medium was replaced by 50ml of 2% of liquefied starch for 24 hours. After 24 hours, 12g of beads and 20ml of *Lactobacillus rhamnosus* inoculum were added to the fermentor. In the SSF, 400 ml of SSF medium that contained 2/10/15% of liquefied starch, 10 g/L yeast extract, 5 g/L sodium acetate, 2 g/L KH_2PO_4, 0.2 g/L $MgSO_4$, 0.05 g/L $MnSO_4$, Tween 80 1ml/L was used. $CaCO_3$ was used for neutralization of lactic acid. Samples were taken out at intervals for analysis.

In the fermentor runs, industrial grade starch was used. It contains 20% moisture.

Fig 5.3.21 SSF of lactic acid in the fermentor

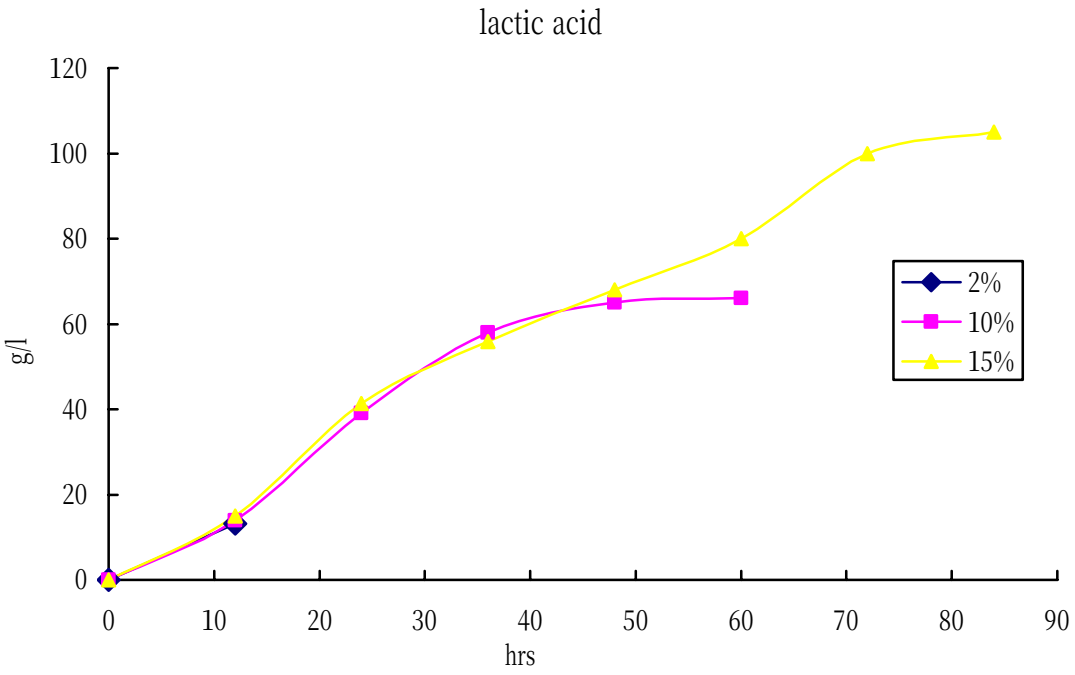


Table 5.3.7 conversion efficiency of SSF of lactic acid

Concentration of starch	Theoretical glucose concentration (g/l)	Lactic acid concentration (g/l)
2%	17.76	13.2
10%	88.8	65
15%	133.2	100

Concentration of starch	Time (h)	$Y_{LA/GLU}$ (g/g)	Lactic acid productivity (g/l/h)
2%	12	0.743	1.25
10%	48	0.732	1.354
15%	72	0.751	1.389

During the cultivation the potato starch was saccharified to glucose by enzymes, such as amylase and glucoamylase, generated by the *Aspergillus niger*, and the glucose was metabolized by the *Lactobacillus rhamnosus* and converted into the lactic acid. There was a lag phase for the fermentation within the first 12 hours. During this exponential saccharification phase, the reducing sugars were accumulated at a high rate. The reducing sugars remained at a high constant level at approximately 4 g/l between 0 to 4 hours. This may be due to the saccharification rate was greater than the fermentation rate, resulting in an accumulation of reducing sugars. Then, the bacteria enter the exponential phase and the lactic acid production was kept at a rate that is similar to the saccharification rate until the end of experiment. The reducing sugar content remains at below 1.0 g/l. There was no reducing sugar remaining in the culture at the end of the fermentation when 2%, 10% and 15% starch was used and starch was not detectable by the iodine colorimetric method except in the 15% starch culture.

In these fermentations, the volumetric concentrations are 13.2g/l, 65g/l and 100g/l for 2%, 10% and 15% starch respectively. They are all around 75% lactic acid yield and 1.3g/l/h volumetric productivity. A higher yield of lactic acid can be obtained by increasing the operational time but it showed a lower productivity. Compare with

using glucose as carbon source, there seems to be no difference in the volumetric concentration and productivity; but the yield is 20% lower than using glucose. This may be due to the difficulty in measuring how much reducing sugars are utilized accurately. So, when calculating the yield, it is assumed that all reducing sugars had been utilized.

During this study, my results showed that the pH of the culture media is one of the most essential factors that affected the lactic acid production. The experimental observations indicated that the pH of the culture decreased dramatically as lactic acid was produced, if no pH control was involved in the cultivation, the value would stay at a very low pH of around 4.3. A low pH not only affects the cell growth but also inhibits biochemical reactions for lactic acid production (Hofvendahl 2000). A pH control system was attempted in the present study by addition of NaOH solution. But it seems that sodium hydroxide affected the SSF system. Results from the experiment were not consistent and undesirable. A controlled growth pH of around 5 - 6 may be desirable to obtain a promising microbial performance for the starch saccharification and lactic acid production. A constant pH between 5 and 6 can be maintained by addition of certain amount of $CaCO_3$ during the lactic acid fermentation.

According to the literature, the optimum pH for starch saccharification by fungal amylase may somehow differ from the lactic acid fermentation by lactic acid bacteria. The growth pH also governed the metabolic reactions for lactic acid production and biomass formation using a limited carbon source (Huang 2005). However, more studies have to be done to study how pH affects saccharification by fungi and lactic acid production by lactic acid bacteria. Lactic acid is known to be a strong inhibitor for cell growth, enzymatic hydrolysis and microbial activity production in lactic acid fermentation (Hofvendahl 2000 and Iyer 1999). To prevent production inhibition and enzyme inhibition, thus, the addition of a neutralizing agent is a must. Sodium hydroxide has been often used to control the pH in fermentation. In this study, results showed that a neutralizing agent, $CaCO_3$ helps to maintain the pH of culture and achieve a maximum production of lactic acid. The addition of $CaCO_3$ neutralizes the cation H^+ released from the lactic acid which may reduce the synthesis reaction forward to lactic acid production (Rosenberg 1995 and Hang 1989). Apart from the production enhancement, the use of calcium carbonate brings into an extra cost for lactic acid purification and biomass recovery in the downstream process.

Table 5.3.8 Comparison of this process with others in the literature

	Rhizopus oryzae 2062 *	*Rhizopus arrhizus* 36017 *	*Lactobacillus rhamnosus*
Carbon source	Starch	Starch	Starch
Concentration	18.6 g/l	37.5 g/l	150 g/l
Lactic acid	13.62 g/l	21.25 g/l	100 g/l
$Y_{LA/GLU}$ (g/g)	0.45	0.57	0.751
Fermentation time	36 hours	36 hours	72 hours
Productivity	0.378 g/l/h	0.59 g/l/h	1.389 g/l/h

* (Huang, 2005)

Lactic acid-producing fungi, such as *Rhizopus oryzae and Rhizopus arrhizus*, have recently played a role in lactic acid production. *Rhizopus oryzae* is an important mold that produces L (+)-lactic acid. The major advantage of using the fungi over bacteria is the lower costs, they can use waste as substrates, have no specific nutrient requirements and have good tolerance to adverse conditions. Another important feature for using fungi is that some of them can supply all the enzymes for the SSF process. Table 5.3.8 compares the fermentation kinetics, yield and productivity of some published fungi data and the process developed in this project. It shows that

this process have 31% more in yield and 135% more in volumetric productivity. This may be due to the difference in carbon flux of fungal microorganism and lactic acid bacteria. A lot of carbon may be direct in fungi to biomass, enzyme production.

Table 5.3.9 Comparison of this process with others in the literature

	Lactobacillus delbrueckii NCIM 2365 **	*Lactobacillus bulgaricus* NRRL B-548 ***	*Lactobacillus rhamnosus*
Carbon source	Starch	Cellulose	Starch
Concentration	200 g/l	100 g/l	150 g/l
Lactic acid	139.47 g/l	62 g/l	100 g/l
$Y_{LA/GLU}$ (g/g)	0.691	0.62	0.751
Fermentation time	108 hours	144 hours	72 hours
Productivity	1.29 g/l/h	0.442 g/l/h	1.389 g/l/h

** (Roy, 2001), *** (Venkatesh, 1997)

In one study (Roy, 2001), a high concentration of starch was used; SSF was performed by lactic acid bacteria and commercial enzymes. A very high volumetric concentration of 139.47 g/l was obtained. A probable reason for this capability to withstand such high concentration of lactate, was that *Lactobacillus delbrueckii* can withstand lower intracellular pH without depending on the energy consuming proton pumps (Mcdonald 1990, Anuradha 1999). However, 100g/l of lactic acid produced by *Lactobacillus rhamnosus* seems to be the maximal capacity (Yoo 1997, Kwon

2000). In the *Lactobacillus rhamnosus* process, the yield and productivity are slightly higher (about 8%) than *Lactobacillus delbrueckii* NCIM process (table 5.3.9).

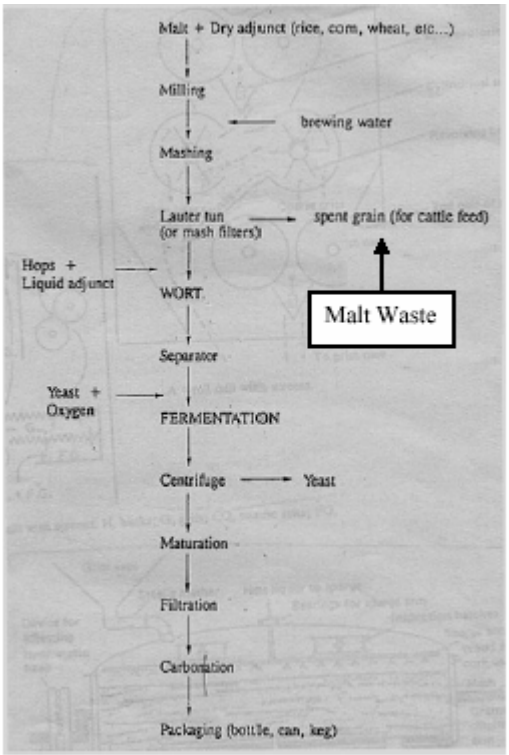
In the *Lactobacillus bulgaricus* process (Venkatesh, 1997), *Lactobacillus bulgaricus* and *Trichoderma reesei* are used together. It was a mixed culture process involving a lactic acid bacteria and fungi like this study. It used cellulose as the raw carbon source. However, the lengthy operational time of 6 days resulted in a very low productivity. Moreover, 100g/l cellulose needed 6 days to complete the process, making the process very time consuming.

5.4 Industrial waste

Malt waste

Malt waste is industrial waste taken from the brewery industry. Since the brewery industry is worldwide, so tons of malt waste are being produced every year all over the world. Since malt waste itself still contains a lot of nutrients, the usage of malt waste for fermentation process can be an attractive economic approach for the industry to use for different large-scale fermentations. The brewing process in which malt waste is produced is shown in figure 24.

Fig 5.4.1 Schematic outline of the brewing process



The material used in brewing is barley, which is used to produce Malt, by undergoing the Malting process that can be considered as the first step for making beer. Barley is used because of its high starch and protein content. In the barley corn, the bulk of the endosperm is made up of large non-living cells, packed with large and small starch grains. The thin cell walls comprise hemicellulose and glucan gums. On the periphery of the endosperm is a layer of small living cells that are rich in protein but free of starch grains that is the aleurone layer. The scutellum separating the starchy endosperm from the embryo has secretary function, permitting release of hydrolytic enzymes from the embryo into the starchy endosperm. Enzymatic degradation of protein, starch and cell wall provide soluble food for the embryo to grow. Malting is basically the initiation of germination, carrying it to a desired end point and then applying hot air to dry and stabilize the germinated grain so that the enzymatic activity that has developed will be preserved. So, it is a controlled germination process, in particular, for transforming and mobilizing food reserves in grain to substrates available for mashing in the brewery. The malting process consists of 3 steps: steeping, germination and kilning.

The spent grain (fig. 5.4.1) is used as animal feed supplements, and is the malt waste used in this study. The malt waste mainly contains the barley seed coat and other

compounds not soluble in the wort. Therefore, hemicellulose, cellulose, lignocelluloses, cellubiose, normally found in the barley seed, and other sugars such as glucose, fructose, maltose, sucrose and maltotriose that are remaining in the barley (not 100% efficiency in extracting all into the liquid wort) are always found in the malt waste. Among them, maltose has the highest proportion.

Malt waste commonly contains some nitrogenous constituents including ammonia, simple amines, amino acids, purines, and simple peptides to complex proteins. Other components include phosphates, chlorides, sulfates and the cations Na, K, Ca, Mg, Fe, Cu, and Zn.

However, to make use of the malt waste in a fermentation process, acid hydrolysis or solid state fermentation are needed to break down the complex compounds in the malt waste to release more readily fermentable carbon source for *Lactobacillus* to grow. Acid hydrolysis can be categorized under two general approaches, that of high acid concentration at a low temperature or that of low concentration at a high temperature.

Growth of *Lactobacillus rhamnosus* on hydrolysate from acid hydrolysis of malt waste in shake flasks

In this study, shake flask experiments have been used to study the growth of *Lactobacillus rhamnosus* on hydrolysate from acid hydrolysis of malt waste. The cultures were grown in 250ml flasks, on an orbital shaker at 250 rpm, with 100 ml culture medium containing 5% (5ml) inoculum, at 37º C and pH 6.0.

Factor being determined in these shake flask experiments:

1) Determine the concentration of acid hydrolysate of malt waste (between 20% - 100% with YE10 media) suitable for the growth and lactic acid production of *Lactobacillus rhamnosus.* Media YE10 without glucose was used as negative. Pure hydrolysate was used as 100% control.

Fig 5.4.2 Residue glucose concentration in acid hydrolysate cultures

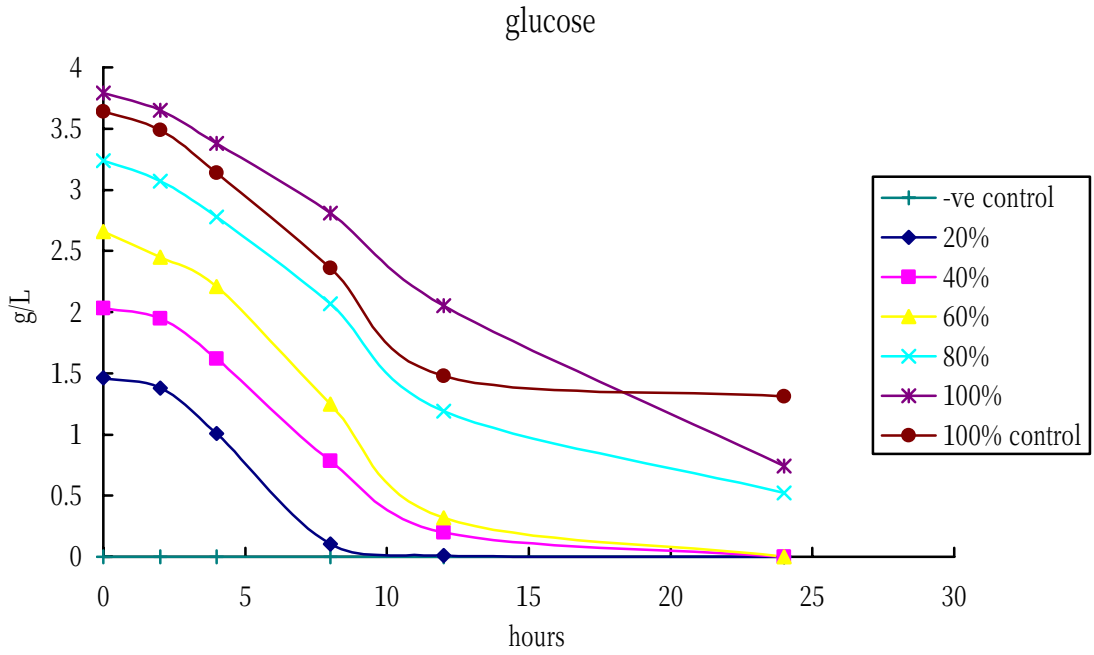


Fig 5.4.3 Lactate content from acid hydrolysate culture

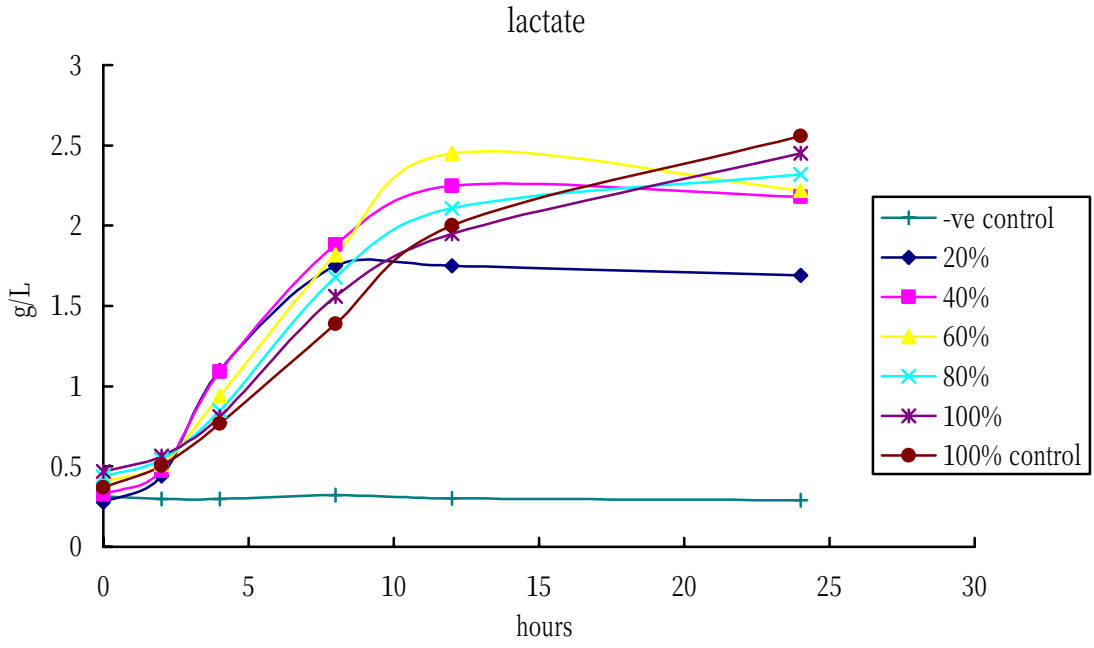


Table 5.4.1 Glucose utilization rate of acid hydrolysate cultures

Culture	Initial glucose concentration (g/L)	Final glucose concentration* (g/L)	Amount of glucose utilized (g/L)	Rate of glucose utilization (g/L/hr)
-ve control	0	0	0	0
20%	1.46	0.103	1.357	0.170
40%	2.03	0.196	1.834	0.153
60%	2.66	0.321	2.339	0.195
80%	3.24	0.523	2.717	0.113
100%	3.79	0.744	3.046	0.127
100% control	3.64	1.31	2.33	0.097

*Glucose concentration at the highest lactic acid concentration

Table 5.4.2 Conversion efficiency of glucose to lactic acid in acid hydrolysate cultures

Culture	Amount of glucose utilized (g/L)	Highest lactic acid concentration (g/L)	$Y_{LA/GLU}$ (g/g)
-ve control	0	0.321	0
20%	1.357	1.75	1.290
40%	1.834	2.25	1.227
60%	2.339	2.45	1.047
80%	2.717	2.32	0.854
100%	3.046	2.45	0.804
100% control	2.33	2.56	1.099

0.1 M sulphuric acid was used to hydrolyze the malt waste, the waste to acid ratio used was 1:5 (w/w), after autoclaving for 30 minutes at 121°C, the hydrolysate was brown in color. After neutralization of the hydrolysate to pH 6.0, glucose and lactate contents in the hydrolysate were measured. Then, *Lactobacillus rhamnosus* was used to inoculate the hydrolysate and cultured for 24 hours.

The hydrolysate medium was supplemented with components of the YE10 media except glucose. To determine a suitable concentration of the hydrolysate to be used in the medium, , 5 different concentrations (20%, 40%, 60%, 80%, 100%) were used. A negative (medium containing YE10 medium without glucose and hydrolysate) control and 100% pure hydrolysate (without any additional nutrients) were used as well. Table 5.4.2 shows that the yield and lactic acid content obtained by utilization of hydrolysate were higher than that of negative control; this indicates that the hydrolysate could support cell growth and lactic acid production. The 100% hydrolysate control (without YE10 media components) supported a similar lactic acid yield, as 100% hydrolysate + YE10 media, indicating that at this level of lactic acid production, components in YE10 medium is not required.

The maximum lactic acid produced was 2.56 g/L in the 100% control pure acid

hydrolysate flask at 24 hours and 2.45 g/L in 60% and 100% acid hydrolysate at 12 and 24 hours respectively. The maximum lactic acid yield was 1.290 g/g in the 20% acid hydrolysate flask and 1.227 g/g in the 40% acid hydrolysate flask. Based on these results, lactic acid concentration was found to be more or less the same from 40% to 100% control culture.

5.4.1 Optimization of process parameters for solid state fermentation by *Aspergillus niger*

Solid state fermentation is a process whereby an insoluble substrate is fermented with sufficient moisture but without free water (Hesseltine, C.W., 1976). In a solid state fermentation process the solid substrate not only supplies the nutrients to the culture but also serves as an anchorage for the microbial cells. The cost and availability are the important considerations. Thus, the selection of an appropriate solid substrate plays an important role in the development of efficient solid state fermentation processes (Selvakumar P. et al., 1998). Solid state fermentation has gained importance due to its several advantages over submerged fermentation (Aidoo K.E., 1982; Hesseltine, C.W., 1987; Pandey A., 1991; Lonsane B.K. et al., 1985).

Solid state fermentation process

For the solid state fermentation of malt waste, 25g of malt waste and a waste to distilled water ratio of 1:5 (w/w) was used. The soaked wastes (supplemented with minerals) were autoclaved at 121º C (15 p.s.i.) for 20 minutes in a 250ml shake flask. Minerals are used in following concentrations: 3.5g of KH_2PO_4, 0.5g of $MgSO_4$, 8.7mg of $FeSO_4$, 3.5mg of $CaCl_2$, 2.8mg of $MnSO_4$ and 2.5mg of $ZnSO_4$ per 100g of dried waste. Then, it was inoculated with 1ml spores suspension (~10^7 spores/ml). The inoculated culture was incubated in a 25℃ incubator for 24hours. Afterwards, 25ml of distilled water was added. The flask was shook on an orbital shaker at 250 rpm, at 60º C for 24 hours. After hydrolysis, the solid waste and the hydrolysate were separated by filtration to obtain a pale brown solution.

Then, diluted NaOH was used to neutralize the hydrolysate to pH 6.0. After neutralization, the hydrolysate contained a precipitate, which was removed by centrifuge at 4000rpm. A clear brown hydrolysate was obtained.

Factors that are being determined using these shake flask experiments:

1) Effect of incubation temperature in shaker

2) Effect of initial moisture content

3) Determine the concentration of hydrolysate of malt waste (between 20% - 100%) suitable for the growth and lactic acid production of *Lactobacillus rhamnosus.*

Effect of incubation temperature in shaker

This experiment determines the effect of incubation temperature in the shaker. 25℃, 37℃ and 60℃ were used. This step will affect the enzyme activity from the fungal mycelium.

Table 5.4.3 total organic carbon content in the hydrolysates from 3 different incubation temperatures

Temperature	25℃	37℃	60℃
TOC (g/l)	8.645	9.842	12.45

This indicates that there are more soluble carbon in the hydrolysate at higher incubation temperature. Then, 100% hydrolysate + YE10 components without glucose was prepared. The cultures were grown in 250ml flasks, on an orbital shaker at 250 rpm, with 100 ml culture medium containing 5% (5ml) *Lactobacillus rhamnosus* inoculum, at 37º C and pH 6.0.

Fig 5.4.4 Residue glucose concentration in Lactobacillus rhamnosus fermentation in fungal hydrolysate from 3 different incubation temperatures in SS

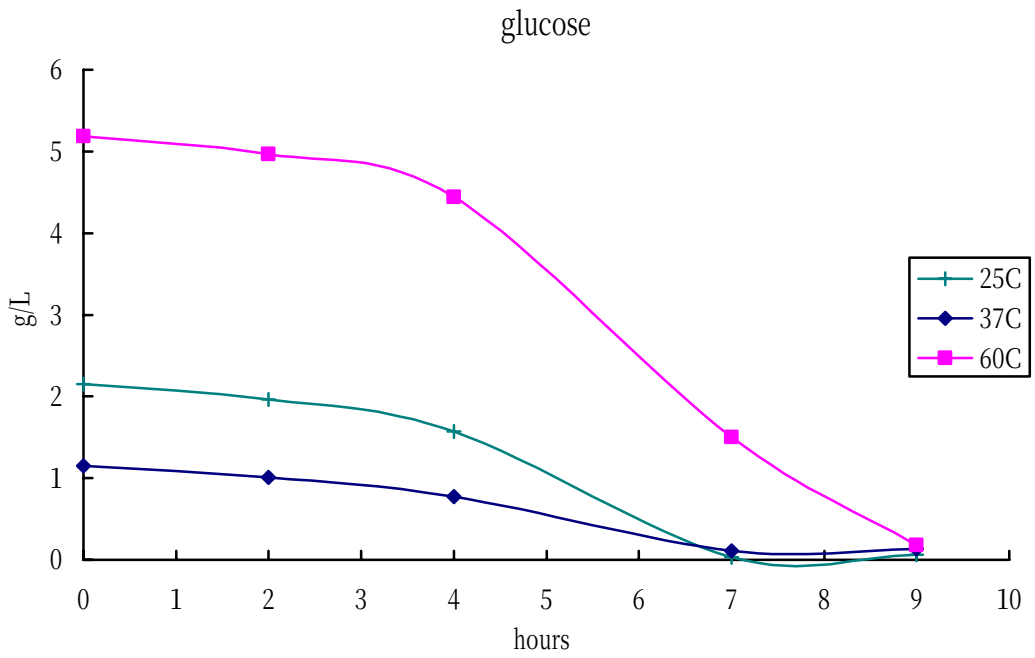


Fig 5.4.5 Lactate produced by Lactobacillus rhamnosus grown on fungal hydrolysate from 3 different incubation temperatures in SS

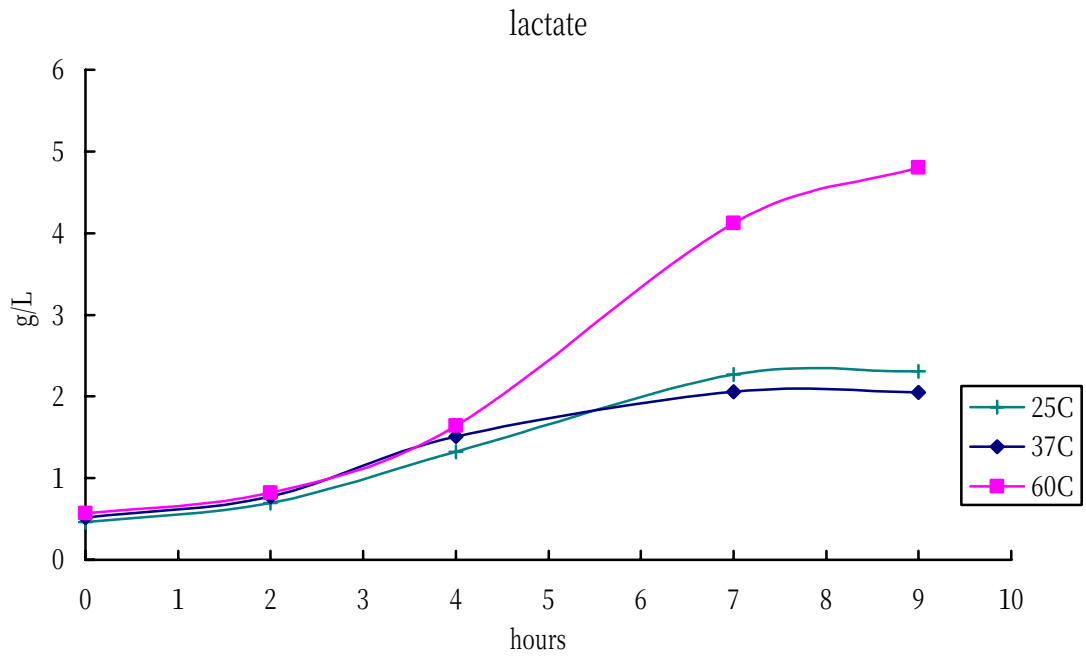


Table 5.4.4 Glucose utilization rate by Lactobacillus rhamnosus grown on hydrolysates from 3 different incubation temperatures

Process	Initial glucose concentration (g/L)	Final glucose concentration (g/L)	Amount of glucose utilized (g/L)	Rate of glucose utilization (g/L/hr)
25°C	2.15	0.03	2.12	0.303
37°C	1.15	0.11	1.04	0.149
60°C	5.19	0.18	5.01	0.557

Table 5.4.5 Conversion efficiency of hydrolysates from 3 different incubation temperatures to lactic acid

Process	Amount of glucose utilized (g/L)	Highest lactic acid concentration (g/L)	$Y_{LA/GLU}$ (g/g)
25°C	2.12	2.31	1.09
37°C	1.04	2.06	1.981
60°C	5.01	4.8	0.958

Effect of initial moisture content

To investigate the influence of the initial total moisture content of the substrate, the fermentation was carried out under various initial moisture content (dried, 1:3 and 1:5 waste to distilled water ratio) of malt waste. The other conditions were the same as described earlier.

Table 5.4.6 Total organic carbon content from 3 different initial moisture content

Moisture ratio	Dried	1:3	1:5
Toc (g/l)	3.91	11.58	10.04

From table 5.4.6, it is obvious that moisture would affect the solid state (SS) process. Soluble carbon from dried SS is around 4 folds less than the others. Water content is necessary for fungi to grow and produce enzyme to hydrolyze the malt waste. Then, the 100% of fungal hydrolysate + YE10 media without glucose was prepared. The cultures were grown in 250ml flasks, on an orbital shaker at 250 rpm, with 100 ml culture medium containing 5% (5ml) *Lactobacillus rhamnosus* inoculum, at 37º C and pH 6.0.

Fig 5.4.6 Residue glucose concentration in Lactobacillus rhamnosus fermentation on fungal hydrolysate with different moisture content in SS

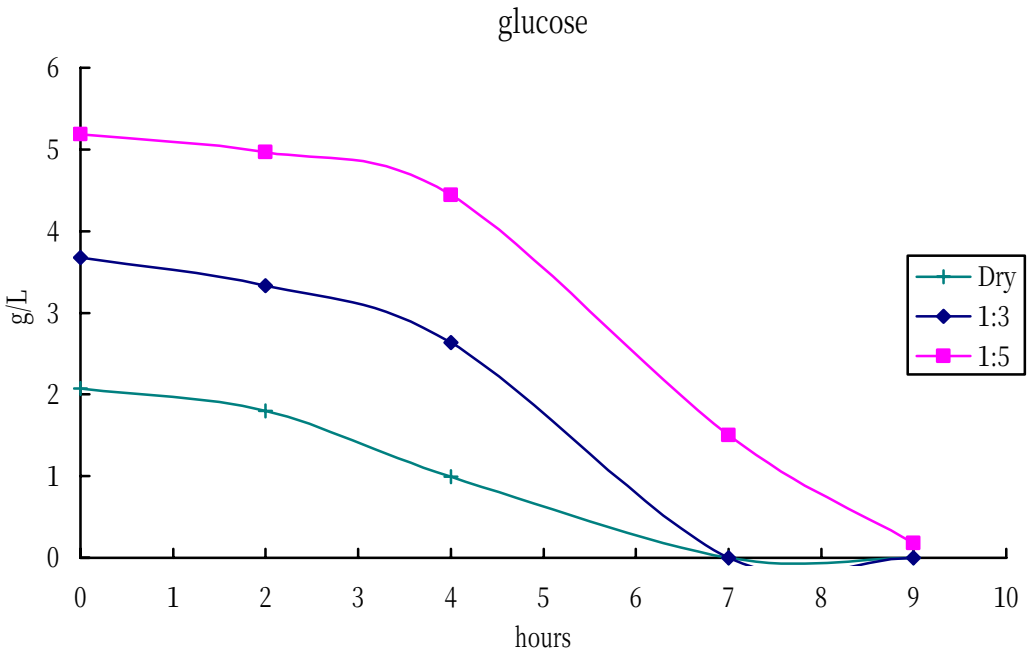


Fig 5.4.7 Lactate produced by Lactobacillus rhamnosus grown on fungal hydrolysate with different moisture content in SS

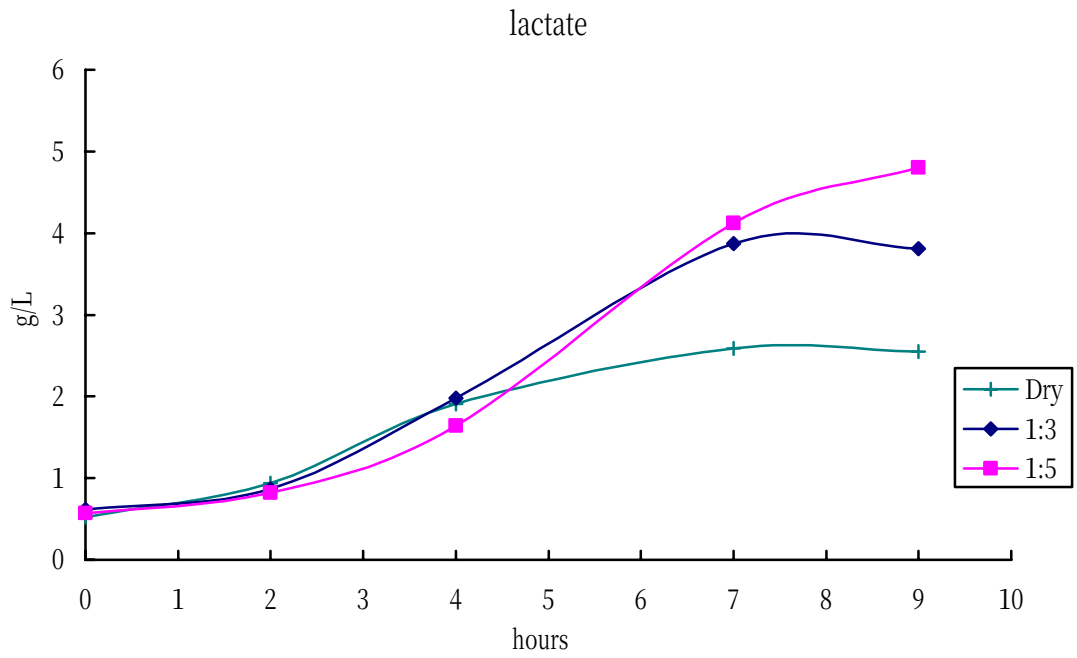


Table 5.4.7 Glucose utilization rate by Lactobacillus rhamnosus on fungal hydrolysate with different moisture content in SS

Moisture ratio	Initial glucose concentration (g/L)	Final glucose concentration* (g/L)	Amount of glucose utilized (g/L)	Rate of glucose utilization (g/L/hr)
Dry	2.07	0	2.07	0.296
1:3	3.68	0	3.68	0.526
1:5	5.19	0.18	5.01	0.557

*Glucose concentration at the highest lactic acid concentration

Table 5.4.8 Conversion efficiency of fungal hydrolysate with different moisture content in SS to lactic acid

Moisture ratio	Amount of glucose utilized (g/L)	Highest lactic acid concentration (g/L)	$Y_{LA/GLU}$ (g/g)
Dry	2.07	2.59	1.251
1:3	3.68	3.87	1.052
1:5	5.01	4.8	0.958

Growth of *Lactobacillus rhamnosus* on hydrolysate from fungal hydrolysis of malt waste in shake flasks

In this study, shake flask experiments have been used to demonstrate the growth of *Lactobacillus rhamnosus* on hydrolysate from fungal hydrolysis of malt waste as carbon sources. The cultures were grown in 250ml flasks, on an orbital shaker at 250 rpm, with 100 ml culture medium containing 5% (5ml) inoculum, at 37º C and initial pH 6.0.

Factor being determined using these shake flask experiments:

1) Determine the concentration of fungal hydrolysate of malt waste (between 20% - 100%) suitable for the growth and lactic acid production of *Lactobacillus rhamnosus.*

Fig 5.4.8 Residue glucose concentration from fungal hydrolysate as substrate

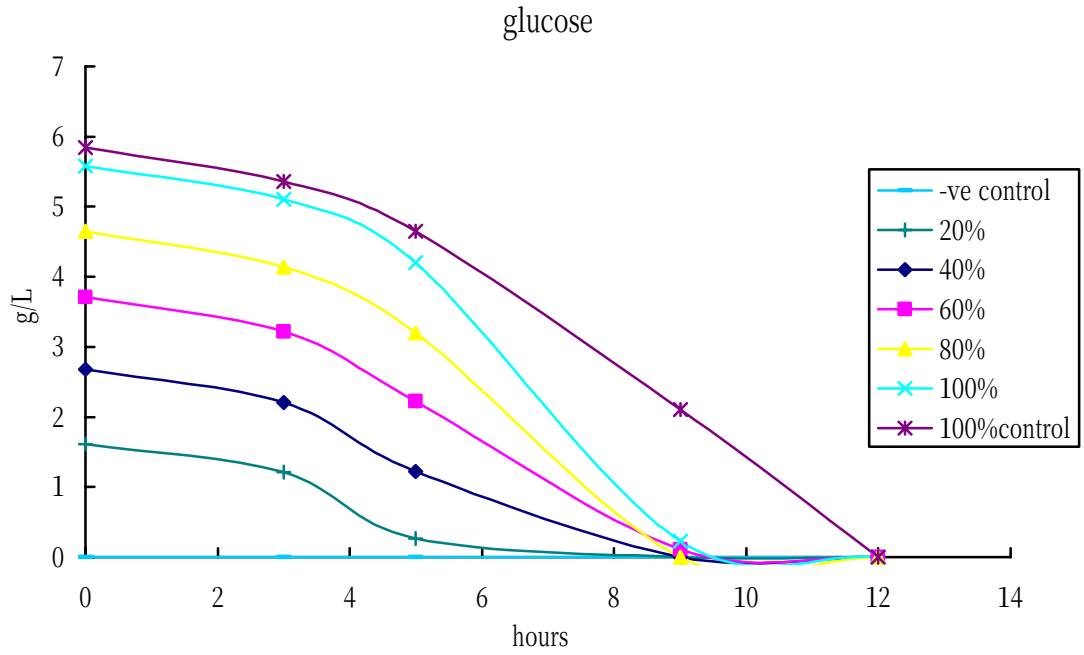


Fig 5.4.9 Lactate content from fungal hydrolysate as substrate

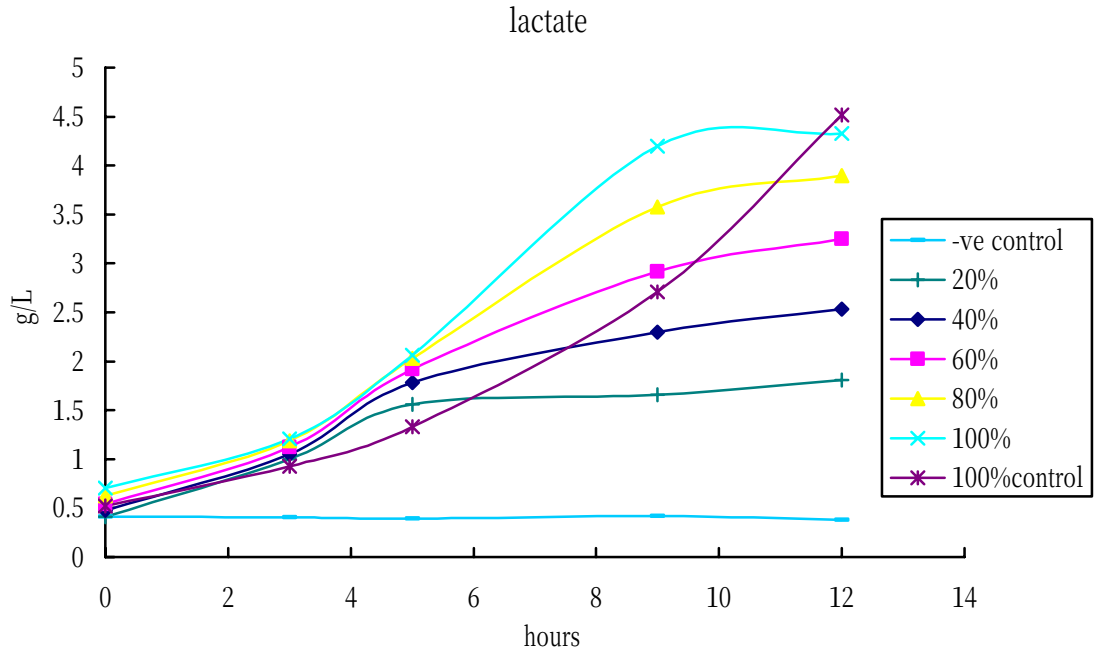


Table 5.4.9 Glucose utilization rate with fungal hydrolysate as substrate

Flasks	Initial glucose concentration (g/L)	Final glucose concentration* (g/L)	Amount of glucose utilized (g/L)	Rate of glucose utilization (g/L/hr)
-ve control	0	0	0	0
20%	1.61	0	1.61	0.134
40%	2.68	0	2.68	0.223
60%	3.71	0	3.71	0.309
80%	4.65	0	4.65	0.388
100%	5.58	0	5.58	0.465
100%control	5.84	0	5.84	0.487

*Glucose concentration at the highest lactic acid concentration

Table 5.4.10 Conversion efficiency of hydrolysate to lactic acid

Flasks	Amount of glucose utilized (g/L)	Highest lactic acid concentration (g/L)	$Y_{LA/GLU}$ (g/g)
-ve control	0	0.421	/
20%	1.61	1.81	1.124
40%	2.68	2.53	0.944
60%	3.71	3.25	0.876
80%	4.65	3.9	0.839
100%	5.58	4.33	0.776
100%control	5.84	4.52	0.774

Effect of additional carbon sources in malt waste

Malt waste was supplemented with different carbon sources during SS to study their effect on the final hydrolysate. The fermentation was carried out at 25℃ keeping all other conditions at their optimum levels.

Table 5.4.11 Total organic carbon from fungal hydrolysate with various supplements culture

	Control (malt waste only)	Starch 2% + malt waste	Xylose 2% + malt waste	Arabinose 2% + malt waste	Starch 4% + malt waste
TOC (g/l)	12.25	22.32	18.84	16.98	25.18

Fig 5.4.10 Residue glucose concentration of Lactobacillus rhamnosus fermentation with fungal hydrolysate and supplements as substrate

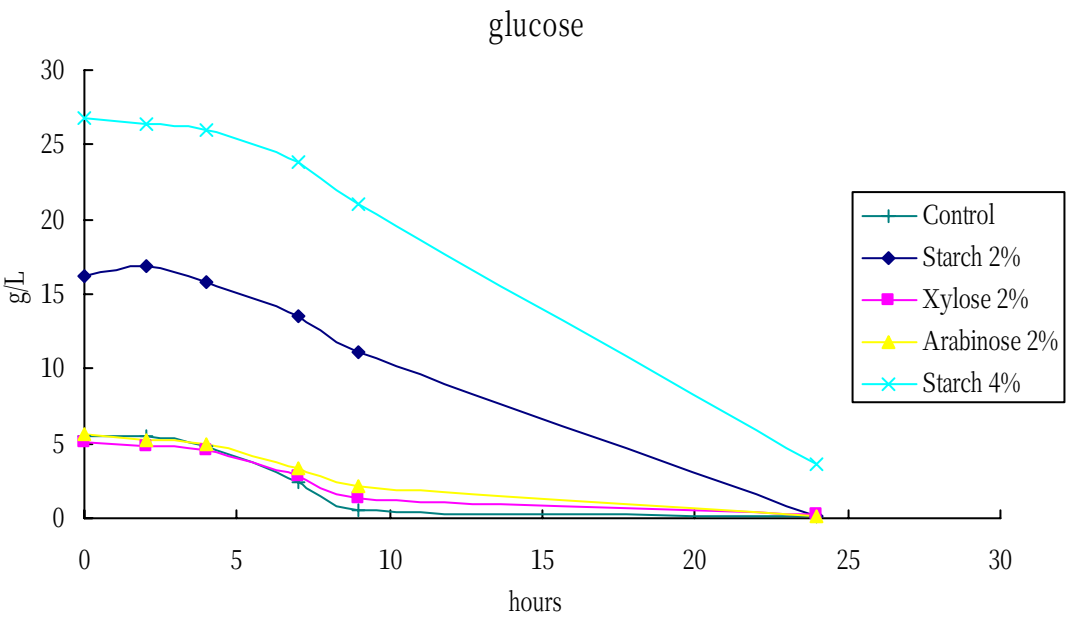


Fig 5.4.11 Lactate produced by Lactobacillus rhamnosus with fungal hydrolysate and supplements as substrate

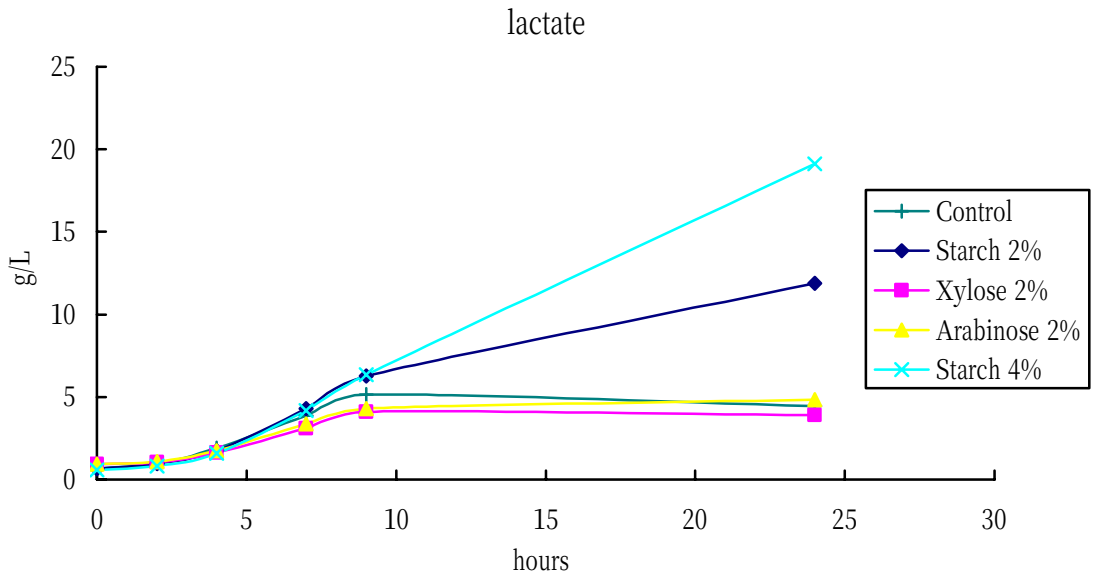


Table 5.4.12 Glucose utilization rate by Lactobacillus rhamnosus with fungal hydrolysate and supplements as substrate

Supplements	Initial glucose concentration (g/L)	Final glucose concentration* (g/L)	Amount of glucose utilized (g/L)	Rate of glucose utilization (g/L/hr)
Control	5.52	0.57	4.95	0.55
Starch 2%	16.2	0.13	16.07	0.670
Xylose 2%	5.14	1.4	3.74	0.416
Arabinose 2%	5.58	0.1	5.48	0.228
Starch 4%	26.8	3.66	23.14	0.964

*Glucose concentration at the highest lactic acid concentration

Table 5.4.13 Conversion efficiency to lactic acid with fungal hydrolysate and supplements as substrate

Supplements	Amount of glucose utilized (g/L)	Highest lactic acid concentration (g/L)	$Y_{LA/GLU}$ (g/g)
Control	4.95	5.15	1.04
Starch 2%	16.07	11.9	0.741
Xylose 2%	3.74	4.12	1.102
Arabinose 2%	5.48	4.85	0.885
Starch 4%	23.14	19.1	0.825

In general, to optimize the solid state fermentation of malt waste by Aspergillus niger; the process can be divided into two parts; first one is solid state fermentation responsible for spores' germination, growth of mycelium and enzyme secretion from mycelium. The second step is to let the enzymes react with the malt waste through shaking at a high temperature. The incubation temperature during shaking is important for the enzymes' reaction. Based on the results (table 5.4.4 and 5.4.5), 60℃ is better than 25℃ and 37℃. Volumetric glucose and lactic acid are the highest in 60℃ culture.

The highest glucose and lactic acid concentration were attained when the initial moisture level was 1:5 (waste to distilled water) ratio in comparison with that at dried and low moisture levels. The critical importance of moisture level in solid state fermentation and its influence on the biosynthesis and secretion of enzymes can be attributed to the interference of moisture in the physical properties of the solid particles. Increase in moisture level is believed to reduce the porosity of the malt waste, thus limiting oxygen transfer. The low moisture content causes reduction in the solubility of nutrients of the substrate and low degree of swelling.

Results (fig. 5.4.8 and 5.4.9) show that 100% fungal hydrolysate was the best

substrate for lactic acid production due to the highest glucose content among all the compositions tested. Notice that 100% control (fungal hydrolysate only without YE10 medium additive) also supported *Lactobacillus* growth. Malt waste maybe containing all the essential growth factors and nitrogen for the fastidious *Lactobacillus* growth.

Addition of different sugars as additives to malt waste resulted in better lactic acid production with starch. Amylases were produced during solid state fermentation and starch was hydrolyzed to dextrose. That's why the glucose level was higher with the additives. For xylose and arabinose, these 5 carbons are not suitable for *Lactobacillus*. So, they did not improve lactic acid production.

6. Conclusion

The main objectives of this study were to determine the possibility of using *Bacillus subtilis* as a lactic acid producer by fermentation; and using starch as substrate, to develop a process in which *Aspergillus niger* and *Lactobacillus rhamnosus* can be co-cultured to together to produce large amounts of lactic acid.

The optimum conditions for *Bacillus subtilis* to produce lactic acid were first determined. Shake flask experiments have been performed to determine the most suitable conditions for lactic acid production. The results showed that shake flasks using a lower shaking speed and lower oxygen transfer rates seemed to be more supportive for lactic acid production than the normal cultivation conditions of *Bacillus subtilis* (high shaking speed, a very high oxygen transfer rate). Then, addition of glucose and calcium carbonate showed a positive effect on production. Based on these findings, a 2 stage fed batch fermentation was developed, the process involved high oxygen transfer rate initially then switching to a lower rate. This process was scaled up in a fermentor in the later part of the study.

After the optimum conditions were determined in shake flasks, fermenter runs were made in order to investigate the production and yield under controlled conditions. The biomass produced was of 6.2 g/L and the highest lactic acid volumetric concentration was 10 g/L. the lactic acid yield was 34% and the volumetric productivity was 1.25 g/l/h. Comparing the shake flask and fermentor cultures studied, the volumetric concentration

and volumetric productivity in the fermentor were better than the shake flask cultures. But the lactate yield was relatively low compare to other studies

Next, UV irradiation was applied to improve the *Bacillus subtilis* strain. 6 mutants were isolated through screening. In the shake flask experiment, all parameters showed an improvement, there was an increase of nearly 200% of the volumetric lactic acid concentration, and around 130% of the product yield and the volumetric productivity comparing to the parent strain. When scaled up to the fermentor, the mutants showed a doubling of the fermentation yields of the parent strain. With the fed-batch fermentation method, a controlled feed rate to maintain around 6 g/l glucose in the broth was the best method for producing lactic acid. In one of the fed batch experiments, all the glucose was fed in at once. The glucose accumulated and showed a repression effect on *Bacillus subtilis*.

To trace the carbon flux, HPLC was used because lactate just accounted for about 77% of the carbon flow. The second major metabolite was identified as acetate which accounted for about 20% of the carbon flow. Acetate may cause inhibition of *Bacillus.*

In this study, a 2-stage fermentation process had been developed. This process involved two different cultivation conditions, one is aerobic and the other is micro aerobic. The reduction of oxygen in the second stage led to lactic acid production. With fed batch strategy, lactic acid production was improved. UV mutagenesis was applied to the parent strain *Bacillus subtilis* MU331, isolates were selected and screened. 6 mutants showed improved fermentation kinetics compared to the parent strain. Mutant “R” was the best

in term of volumetric concentration and volumetric productivity.

For the second part of my study, the growth of *Lactobacillus rhamnosus* was first characterized. YE10 medium was developed to replace MRS medium. YE10 mainly reduces the use of expensive complex nitrogen sources. In fermentor runs, incubation temperature of 37°C and 43°C were studied. Results showed that there was no difference between these two temperatures. 43°C was used for the latter part of the studies because the higher temperature was more suitable for the simultaneous saccharification and fermentation (SSF) process. In batch fermentations with 100 g/l and 150 g/l glucose, the concentration of lactic acid produced was 73 g/l and 92 g/l respectively, and the lactate yields were both over 90%.

In the cultivation of immobilized *Aspergillus niger* beads, 3 temperatures and pH were tested. Results showed that 30°C and pH 5 or 6 were the best conditions for the spores to germinate. Afterwards, 2 shaking speeds, 160 and 250 rpm were tested; results did not show any significant differences. The saccharifying activity was almost 80% with the optimized conditions. In experiments using very high starch concentrations (50g/l), it was found that alpha amylase had to be applied initially to reduce the viscosity.

To develop a process for the SSF of lactic acid, shake flask cultures were initially used with 2%, 5% and 10% of starch. However, the results except with the 5% starch were not impressive. The lactate yield is rather low, only around 50% for 10% starch. This might have due to the unfavorable environment in the flask such as nutrients, pH etc. In

scaling up to the fermentor, due to the high cost of analytical grade starch, a lower grade of starch was used. It contains 20% of moisture. In the batch fermentations, 2%, 10% and 15% starch were used. The results from the fermentor were better than those from flask cultures. 13.2 g/l, 65 g/l and 100 g/l of lactic acid were produced from 2%, 10% and 15% starch respectively. These 3 fermentations all had a lactate yield of over 70% and volumetric productivities of around 1.3 g/l/h.

A SSF process had been successfully developed. It involves the use of a fungus and a bacteria in the same culture. *Aspergillus niger* and *Lactobacillus rhamnosus* play important roles in the SSF. *Aspergillus* saccharifies the starch into glucose and *Lactobacillus* converts glucose into lactic acid. Compared with available literature on SSF, whether commercial enzymes or microbes were used to perform the SSF, the co-culture SSF method developed in this study showed a better performance.

To improve the lactic acid production process of *Bacillus subtilis* MU331 or *Lactobacillus rhamnosus*, genetic engineering technology could be applied. Any enzymes which the cell lacks, such as amylases, cellulase etc. can be cloned into the host, so that other polysaccharide based carbon sources like rice, bran, wheat etc. can be used by the cells. Apart from cloning the enzymes, metabolite engineering can be used to direct the carbon source to a desired metabolite pathway ad reduce undesired metabolite production. In term of fermentation technology, there is still room for process optimization. Different fed batch strategies, electrodialysis, and biomass recycling are examples that have been successfully developed in the literature. In term of economy, the SSF process developed in this study shows a better performance than *Bacillus*,

although if developed further, the *Bacillus* process should have a very high potential.

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8. Appendix

Oxygen transfer rate using sulfite oxidation

Reagents

0.1N I_2/KI

Dissolve 40g of potassium iodide (KI) completely in 250ml of deionized water. Add 12.7g of iodine (I_2) and mix to dissolve. Make up the final volume to 1L. keep in the dark.

0.1N sodium thiosulphate ($Na_2S_2O_3$)

Dissolve 24.82g of sodium thiosulphate.$5H_2O$ and 0.05f of sodium carbonate in 400ml of freshly boiled deionized water and make up volume to 1L. keep in the dark.

0.5N sodium sulfite (Na_2SO_3)

Add 31.5g of sodium sulfite to 1L of deionized water dissolve and add 0.28 of $CuSO_4.5H_2O$. Mix to dissolve completely.

Starch indicator

Make a paste by rubbing about 2g of soluble starch in about 30ml of water. Pour the

slurry into 1L of boiling water and heat until a clear solution results. Cool and store in a capped bottle.

Experiment 1 oxygen transfer rates of shake flasks at different rotational speeds

Add 25ml of sulphate solution to two 250ml conical flasks. Cover the flasks with cotton plugs. Place the flasks on a shaker rotating at 150rpm. After 10 minutes, remove one flask from the shaker, and immediately do a titration on duplicate samples. After 20 minutes, remove the second flask and do a titration on duplicate samples. Calculate the oxygen transfer rate and express it in mMO_2/L/hr. the 10 and 20 minute samples should give the same results. Repeat the above experiment with the shaker rotation speed at 300rpm, calculate the OTR.

Experiment 2 oxygen transfer rates of shake flasks at different liquid volumes

Add 80ml of sulphate solution to two 250ml conical flasks. Cover the flasks with cotton plugs. Place the flasks on a shaker rotating at 150rpm. After 10 minutes, remove one flask from the shaker, and immediately do a titration on duplicate samples. After 20 minutes, remove the second flask and do a titration on duplicate samples. Calculate the oxygen transfer rate and express it in mMO_2/L/hr. The 10 and 20 minute samples should give the same results. Repeat the above experiment with the shaker rotation speed at 300rpm, calculate the OTR.

Procedure

Add 25ml of iodine solution to a clean 250conical flask. Pipet 5ml of sulfite solution from the experiment into the iodine. Mix well by swirling. Titrate the excess iodine using the thiosulphate solution. Add 0.5ml of starch indicator near and end-point (light brown). A dark color should result. Titrate very carefully (drop by drop) until the solution clears. Make a note of the volume of thiosulphate used.

Table 1 result of the sulfite assay

Volume (ml)	Speed (rpm)	10 min (ml)			20 min (ml)			**OTR (mmol/L.hr)**	
		1	2	Aver.	1	2	Aver.	**0-10min**	**0-20min**
25	150	16.4	16.6	16.5	18.5	18.3	18.4	**52.5**	**40.5**
	300	17.5	17.7	17.6	20.5	20.7	20.6	**69**	**57**
80	150	13.9	13.7	13.8	14.2	14.4	14.3	**12**	**9.75**
	300	14.3	14.7	15	17	16.4	16.7	**30**	**27.75**