**The Hong Kong Polytechnic University**

**Department of Applied Biology and Chemical Technology**

# Production of L-lactic acid from starch by recombinant *Bacillus subtilis* 1A304 (Φ105MU331)

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**A thesis submitted in partial fulfillment of the requirements for the degree of Master of Philosophy**

**October 2009**

# CERTIFICATE OF ORIGINALITY

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WONG YUK KI

**Abstract of thesis entitled**

**Production of L-lactic acid from starch by recombinant**

***Bacillus subtilis* 1A304 (Φ105MU331)**

Submitted by

**Wong Yuk Ki**

For the degree of Master of Philosophy in the Hong Kong Polytechnic University

## Abstract

The interest in the production of lactic acid has significantly increased recently. Besides the increasing demand, it is also due to the prospects of using environmental friendly, renewable resources instead of petrochemicals as feedstocks. Lactic acid is widely used in the food, cosmetic, pharmaceutical and chemical industries. One of the most promising applications of lactic acid is its use for biodegradable and biocompatible lactate polymers, such as polylactic acid. The worldwide demand of lactic acid is about 130,000 – 150,000 metric tones per year and it may increase to 500,000 metric tones per year by 2010.

An UV-induced mutant strain of recombinant *Bacillus subtilis* 1A304 (Φ105MU331) was used for the production of lactic acid. Shake flask experiments were performed to characterize the growth patterns and lactic acid production by recombinant *Bacillus subtilis* using glucose as the carbon source. Optimization of culture medium was conducted. The effect of complex nitrogen sources on the growth and lactic acid production of recombinant *Bacillus subtilis* was studied. 2% yeast extract and 3% martone was chosen for further studies. The effect of cell density and the oxygen transfer rate on the growth and lactic acid production of recombinant *Bacillus subtilis* was studied. It was found that the lactic acid concentration increased with increasing cell density and culture volumes in shake flasks (lower oxygen transfer). The optimal pH for the production of lactic acid was at pH 7.5.

Fed-Batch fermentation experiments were conducted to establish optimum operating conditions for the production of lactic acid. Two kinds of fermentation strategies were compared. One was two stage fermentation and the other one was one stage fermentation. It was shown that 40 g/L lactic acid was produced in 53.5 h in one stage fermentation. The effect of stir rate and air flow was studied. The optimum condition was achieved in 50 rpm and 2 vvm. Inorganic nitrogen was found to have some inhibitory effect on the production of lactic acid. The lactic acid production was

further improved by eliminating the inorganic nitrogen content in the medium. It was found that 79 g/L and 93 g/L lactic acid was produced in 25 h and 51 h respectively. Cultivation temperature was another important factor for lactic acid production. Higher lactic acid production was obtained at 40°C. The effect of different yeast extract concentrations on lactic acid production has been investigated. When 8% yeast extract was used in the medium, the lactic acid concentration was 125.6 g/L, 158.6 g/L and 170 g/L in 24 h, 51 h and 77 h respectively. The maximal lactic acid productivity was 16 g/L/h in 6 h. The lactic acid yield was about 89%.

Starch was also used as the substrate for lactic acid production. This recombinant *Bacillus subtilis* was able to produce alpha amylase under heat induction. There was nearly no lactic acid produced when liquefied starch was used as the carbon source for lactic acid production by recombinant *Bacillus subtilis*. Thus, saccharification of liquefied starch to glucose was needed. Saccharification process was optimized by using different temperatures and forms of *Aspergillus niger* (mycelium, pellet and supernatant of the culture). The effect of starch concentration on saccharification was also studied. Simultaneous saccharification and fermentation (SSF) of starch to lactic acid was conducted using the recombinant *Bacillus subtilis* and *Aspergillus niger*. The effect of temperature and starch concentration on SSF was studied. The result shown

that 84.8 g/L and 136.9 g/L lactic acid was produced in 24 h and 65 h respectively. Lactic acid productivity was 4.38 g/L in 13 h. The optical purity of L-lactic acid was greater than 96%. The results suggested that starch can be used as the carbon source to produce lactic acid by a mix culture of the recombinant *Bacillus subtilis* and *Aspergillus niger*.

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### Chapter 1



### Chapter 3

## Abbreviation

| | |
|---|---|
| BHY | Brain heart infusion with yeast extract |
| *E. coli* | *Escherichia coli* |
| *B. licheniformis* | *Bacillus licheniformis* |
| *B. subtilis* | *Bacillus subtilis* |
| $ddH_2O$ | Distilled deionized water |
| EDTA | Ethylenediamine tetra acetic acid |
| g | Gram (s) |
| h | Hour(s) |
| L | Liter (s) |
| kDa | Kilo-daltons |
| LB | Luria-Bertani medium |
| M | Molarity |
| min | Minute(s) |
| nm | Nanometer |
| OD | Optical density |
| PAGE | Polyacrylamide gel electrophoresis |
| RCF | Relative centrifugal force |
| rpm | Revolutions per minute |

| | |
|---|---|
| s | Second(s) |
| SDS | Sodium dodecyl sulphate |
| Tris | Tris(hydroxymethyl) aminomethane |
| UV | Ultra violet light |
| Vol | Volume |
| vvm | Volume per volume per minute |

# Chapter One

# Introduction

## 1.1 Lactic acid

Lactic acid (2-hydroxypropionic acid), $CH_3$-CHOHCOOH, is the most widely occurring hydroxycarboxylic acid. It has a prime position due to its versatile applications in the food, pharmaceutical, textile, leather, and chemical industries (Vickroy 1985). Lactic acid was first isolated from sour milk by CW Scheele in 1780 and was first commercially produced in 1881 by CE Avery in Littleton, MA, USA (Vickroy 1985). Lactic acid was identified as a microbial metabolite by Pasteur, Lister and Delbrueck (Gregoe 1999).

Lactic acid is present in almost every form of organized life. Its most important function in animals and humans is related to the supply of energy to muscle tissues. It is a water soluble and highly hygroscopic aliphatic acid and an enigmatic chemical. Lactic acid is a product of natural fermentation processes occurring in buttermilk, cheese, beer, sourdough and many other fermented foods. It is non-volatile and an odorless organic acid. There are two optical isomers of lactic acid: L(+) lactic acid and D(–) lactic acid (Figure 1.1).

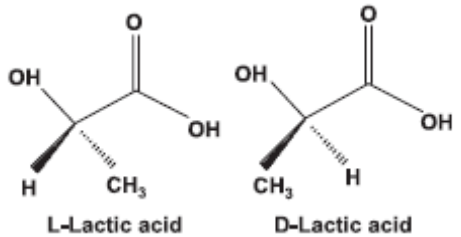



Figure 1.1 Optical isomers of lactic acid (Gupta et al., 2007)

Lactic acid is classified as GRAS (generally recognized as safe) for use as a food additive by the US FDA (Food and Drug Administration). However, D(–) lactic acid is found to be harmful to human metabolism and can result in acidosis and decalcification. L(+) lactic acid is therefore the preferred isomer in food and pharmaceutical industries as humans have only L-lactate dehydrogenase that can metabolize L(+) lactic acid (Akerberg et al., 1998 ; Hofvendahl and Hahn-Hägerdal, 2000).

Lactic acid is a naturally occurring organic acid that can be produced by either chemical synthesis or microbial fermentation. Chemical synthesis of lactic acid is mainly based on the hydrolysis of lactonitrile by strong acids. However, the racemic mixture of D(–) and L(+) lactic acid are produced. Other possible chemical synthesis methods for lactic acid include base-catalyzed degradation of sugars, oxidation of propylene glycol, reaction of acetaldehyde, carbon monoxide, and water at elevated temperatures and pressures, hydrolysis of chloropropionic acid, and nitric acid oxidation of propylene (Datta et al., 1995).

In order to produce lactic acid more effectively and economically, biological production of lactic acid is preferred. Biological production has several advantages

compared to chemical synthesis. This process can use cheap raw materials such as whey, molasses, starch waste, beet, cane sugar and other carbohydrate rich materials (Anuradha et al., 1999; Richter and Berthold, 1998; Tsao et al., 1999; Vishnu et al., 1998, 2000). The biological process can lower the cost of substrates, the temperature of production and energy consumption. High product specificity is another advantage of lactic acid fermentation as it produces a desired stereoisomer, optically pure L(+) or D(−) lactic acid (Pandey et al., 2001). The optical purity of lactic acid is crucial to the physical properties of poly(lactic acid) (PLA). An optically pure L(+)- or D(–)-lactic acid, rather than racemic DL-lactic acid, can be polymerized to a high crystalline PLA that is suitable for commercial uses (J. Lunt 1998, A. Sodergard, M. Stolt 2002). Therefore, the biological production of lactic acid has received a significant amount of interest recently as it offers an alternative to environmental pollution caused by the petrochemical industry and the limited supply of petrochemical resources.

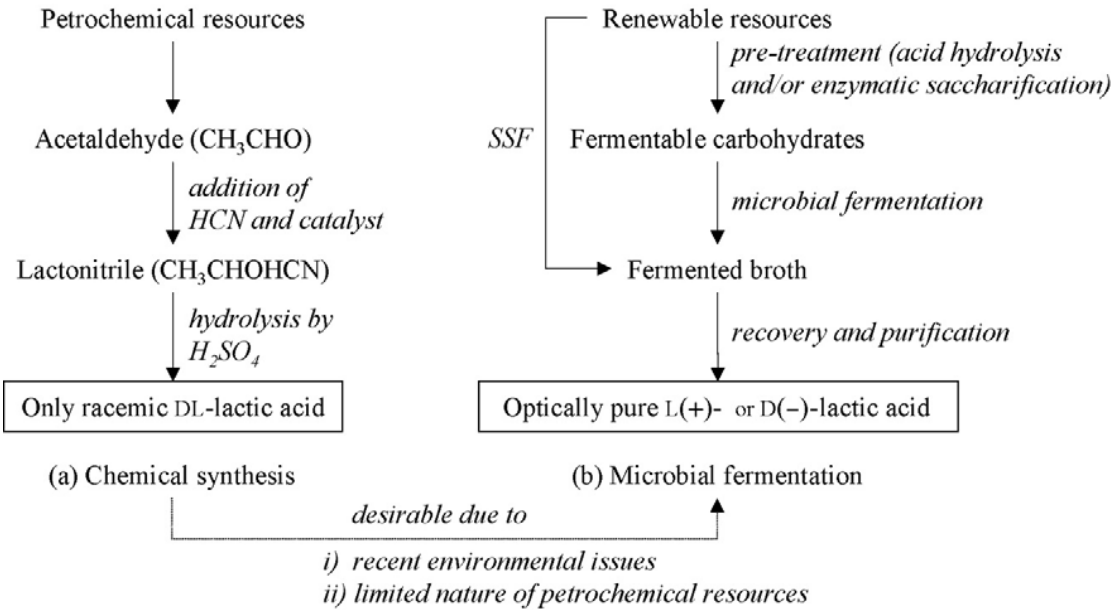



Figure 1.2 Overview of two kinds of lactic acid production (a) chemical synthesis (b) microbial fermentation. SSF represents simultaneous saccharification and fermentation (Wee et al., 2006)

## 1.2 Microbial sources for lactic acid

Lactic acid bacteria and some filamentous fungi are the major microbial sources of lactic acid (Litchfield 1996). The microorganisms selected for recent investigations of lactic acid production are listed in Table 1.1. On the basis of the nature of fermentation, lactic acid bacteria are classified into homofermentative and heterofermentative. Homofermentative lactic acid bacteria produce a single product such as lactic acid only. Heterofermentative lactic acid bacteria produce other products such as ethanol, diacetyl, formate, acetoin or acetic acid, and carbon dioxide along with lactic acid.

The major homofermentative lactic acid bacteria used in lactic acid production are *Lactococcus lactis* (Nolasco-Hipolito et al., 2002), *Lactobacillus delbrueckii* (John et al., 2006a; Kadam et al., 2006), *L. helveticus* (Tango and Ghaly 2002) and *L. casei* (Hujanen et al., 2001; Rojan et al., 2005). Some of the homofermentative bacteria like *L. amylophilus, L. manihotivorans* can directly consume complex carbohydrates like starch (Naveena et al., 2005; Ohkouchi and Inoue 2006). The amylolytic bacteria *Lactobacillus amylovorus* ATCC 33622 had the efficiency of full conversion of liquefied cornstarch to lactic acid (Zhang and Cheryan 1991).

Table 1.1 Microorganisms used for recent investigations of lactic acid production (Wee et al., 2006 ; Osawa et al., 2009)

| Organism | Lactic acid concentration (g/L) | Yield (g/g) | Productivity (g/L/h) | Reference |
|---|---|---|---|---|
| *Rhizopus oryzae* ATCC 52311 | 83.0 | 0.88 | 2.6 | Zhou et al., 1999 |
| *Rhizopus oryzae* NRRL 395 | 104.6 | 0.87 | 1.8 | Park et al., 1998 |
| *Enterococcus faecalis* RKY1 | 144.0 | 0.96 | 5.1 | Yun et al., 2003 |
| *Lactobacillus rhamnosus* ATCC 10863 | 67.0 | 0.84 | 2.5 | Berry et al., 1999 |
| *Lactobacillus helveticus* ATCC 15009 | 65.5 | 0.66 | 2.7 | Schepers et al., 2002 |
| *Lactobacillus bulgaricus* NRRL B-548 | 38.7 | 0.90 | 3.5 | Burgos-Rubio et al., 2000 |
| *Lactobacillus casei* NRRL B-441 | 82.0 | 0.91 | 5.6 | Hujanen and Linko et al., 1996 |
| *Lactobacillus plantarum* ATCC 21028 | 41.0 | 0.97 | 1.0 | Fu and Mathews, 1999 |

| Organism | Lactic acid concentration (g/L) | Yield (g/g) | Productivity (g/L/h) | Reference |
|---|---|---|---|---|
| *Lactobacillus pentosus* ATCC 8041 | 21.8 | 0.77 | 0.8 | Bustos et al., 2004 |
| *Lactobacillus amylophilus* GV6 | 76.2 | 0.70 | 0.8 | Vishnu et al., 2000 |
| *Lactobacillus delbrueckii* NCIMB 8130 | 90.0 | 0.97 | 3.8 | Kotzanmanidis et al., 2002 |
| *Lactococcus lactis ssp. lactis* IFO 12007 | 90.0 | 0.76 | 1.6 | Roble et al., 2003 |
| *Lactobacillus rhamnosus* | 61 | 0.97 | 1.56 | Sakai and Ezaki, 2006 |
| *Lactobacillus delbrueckii ssp.* DSM 20073 | 52 | 0.72 | 2.2 | Michelson et a., 2006 |
| Recombinant *Saccharomyces cerevisiae* | 82.3 | 0.82 | 0.38 | Ishida et al., 2006a |
| Recombinant *Kluyveromyces lactis* | 109 | 1.19 | 0.80 | Porro et al., 1999 |
| Recombinant *Pichia stipitis* | 41.0 | 0.44 | 0.91 | Ilmen et al., 2007 |
| Recombinant *Candida boidinii* | 85.9 | 1.01 | 1.79 | Osawa et al., 2009 |
| Recombinant *Candida utilis* | 103.3 | 0.95 | 3.13 | Ikushima et al., 2009 |
| *Bacillus coagulans* SIM-7 DSM 14043 | 101.7 | 0.71 | 8.5 | Michelson et a., 2006 |
| *Bacillus coagulans* CCM 4318 | 77.5 | 0.97 | 3.2 | Rosenberg et al., 2005 |
| *Bacillus coagulans* | 86 | 0.98 | 1.36 | Sakai and Ezaki, 2006 |
| *Bacillus subtilis* JCM 1465 | 3.7 | 0.49 | 0.19 | Ohara and Yahata, 1996 |
| *Bacillus subtilis* CHI alsS- | 105.6 | 1.09 | 0.54 | Romero-Garcia et al., 2009 |
| *Bacillus sp 2-6* | 182 | - | 3.03 | Qin et al., 2009 |

Most of the lactic acid-producing organisms are anaerobic, utilizing pyruvic acid which is the end product of Embden–Meyerhof pathway. The conversion of pyruvic acid to lactate can be effected by either of the two enzymes: L-lactate dehydrogenase or D-lactate dehydrogenase. The stereospecificity of the lactic acid depends on the

type of organism, whose enzyme is involved in the process of lactic acid production. About 90% of the literature on lactic acid production is focused on bacterial fermentation. *Lactobacillus, Streptococcus, Leuconostoc, and Enterococcus* (Naveena 2004) are the most common bacterial producers. Fungal strains such as *Mucor, Monilia, and Rhizopus* (Prescott and Dunn 1959) also produce lactic acid. The best-known fungal source of lactic acid is *Rhizopus oryzae* (Yu and Hang 1989).

Efforts have been made to improve the production of lactic acid through metabolic engineering approaches. Kyla-Nikkila et al. (2000) attempted to express L-lactate dehydrogenase and D-lactate dehydrogenase genes in *L. helveticus* for the production of pure D(–)- and L(+)-lactic acids. Dien et al. (2001, 2002) constructed recombinant *Escherichia coli* for the conversion of hexose sugar and pentose sugar into L(+)-lactic acid. They metabolically engineered the *E. coli* for the construction of carbon catabolite repression mutants. Chang et al. (1999) constructed recombinant *E. coli* for the production of optically pure D(–)- or L(+)-lactic acid. Their results suggested that the central fermentation metabolism of *E. coli* can be reoriented to the production of D(–)- or L(+)-lactic acid.

Lactic acid bacteria are fastidious bacteria typically having complex nutritional requirements. It is due to their limited ability to synthesize their own growth factors such as B vitamins and amino acids. They require some elements for growth, such as carbon and nitrogen sources, in the form of carbohydrates, amino acids, vitamins, and minerals (Axelsson 1993; Niel and Hahn-Hägerdal, 1999 ; Amrane 2000). These will increase the cost of the production process.

Fungal fermentation has some advantages in that R. oryzae requires only a simple medium and produces L(+)-lactic acid. However, it requires vigorous aeration because R. oryzae is an obligate aerobe (Tay and Yang, 2002). In fungal fermentation, the production rate was low (below 3 g/L/h) which is probably due to the low reaction rate caused by mass transfer limitation (Park et al., 1998). The lower product yield from fungal fermentation is attributed partially to the formation of by-products, such as fumaric acid and ethanol (Tay and Yang, 2002).

Yeast fermentation has the advantages that yeasts are more resistant to low pH environment than lactic acid bacteria and they are genetically engineered with ease. Dequin and Barre (1994) and Porro et al. (1995) genetically modified *S. cerevisiae* by the introduction of exogenous L-LDH genes for the conversion of pyruvic acid to

L-lactic acid. However, the production of L-lactic acid was relatively low (20 g/L) for industrial use and a considerable amount of ethanol was produced in the medium as one of the main by-products. In order to reduce alcohol production, genes encoding pyruvate decarboxylase (PDC), which converts pyruvic acid to acetaldehyde and is a key enzyme of the alcohol fermentation pathway, have been disrupted (Adachi et al., 1998; Ishida et al., 2005, 2006a, 2006b; Saitoh et al., 2005). The production of L-lactic acid using the Crabtree-negative yeasts *Kluyveromyces lactis* (Bianchi et al., 2001; Porro et al., 1999), *Pichia stipitis* (Ilmen et al., 2007), *Candida boidinii* (Osawa et al., 2009) and *Candida utilis* (Ikushima et al., 2009) has been reported. The results found that the yield and productivity of these yeasts were relatively low for industrial production of L-lactic acid.

Lactic acid production by *Bacillus* species has been reported recently. More information will be discussed at the later section.

### 1.3 Raw materials for lactic acid production

There have been various attempts to produce lactic acid efficiently from inexpensive raw materials. The carbon source for microbial production of lactic acid can be either sugar in pure form such as glucose, sucrose, lactose etc. or sugar-containing raw

materials such as molasses, whey, sugarcane bagasse, cassava bagasse, and starchy materials from potato, tapioca, wheat, barley, and carrot (Pandey et al., 2001; Anuradha et al., 1999). The economics of production of lactic acid is dependent on many factors of which the cost of raw material is very significant (Nolasco-Hipolito et al. 2002).

Cheap raw materials are another alternative for the biotechnological production of lactic acid. It is because polymer producers and other industrial users usually require large quantities of lactic acid at a relatively low cost. Raw materials for lactic acid production should have the following characteristics: cheap, low levels of contaminants, rapid production rate, high yield, little or no by-product formation, ability to be fermented with little or no pre-treatment, and year-round availability (Vickroy 1985). Using refined materials for lactic acid production can significantly reduce the costs for product purification. However, expensive refined carbohydrates eventually result in higher production costs (Hofvendahl 2000). There have been various attempts to screen for cheap raw materials for the economical production of lactic acid. Literatures of recent investigations using cheap raw materials are listed in Table 1.2.

Table 1.2 Literatures of recent investigations on lactic acid production from cheap raw materials (Wee et al., 2006)

| Raw material | Microorganism | Lactic acid concentration (g/L) | Productivity (g/L/h) | Reference |
|---|---|---|---|---|
| Molasses | *Lactobacillus delbrueckii* NCIMB 8130 | 90 | 3.8 | Kotzanmanidis et al., 2002 |
| | *Enterococcus faecalis* RKY1 | 95.7 | 4 | Wee et al., 2004 |
| Rye | *Lactobacillus paracasei* No.8 | 84.5 | 2.4 | Richter and Berthold 1998 |
| Sweet sorghum | *Lactobacillus paracasei* No.8 | 81.5 | 2.7 | Richter and Berthold 1998 |
| | *Lactobacillus paracasei* No.8 | 106 | 3.5 | Richter and Träger 1994 |
| Wheat | *Lactococcuslactis* ssp. *Lactis* ATCC 19435 | 106 | 1 | Hofvendahl and Hahn-Hägerdal 1997 |
| | *Enterococcus faecalis* RKY1 | 102 | 4.8 | Oh et al., 2005 |
| Corn | *Enterococcus faecalis* RKY1 | 63.5 | 0.5 | Oh et al., 2005 |
| | *Lactobacillus amylovorus* ATCC 33620 | 10.1 | 0.8 | Xiaodong et al., 1997 |
| Cassava | *Lactobacillus amylovorus* ATCC 33620 | 4.8 | 0.2 | Xiaodong et al., 1997 |
| Potato | *Lactobacillus amylovorus* ATCC 33620 | 4.2 | 0.1 | Yun et al, 2004 |
| Rice | *Lactobacillus* sp. RKY2 | 129 | 2.9 | Yun et al, 2004 |
| Barley | *Lactobacillus casei* NRRL B-441 | 162 | 3.4 | Linko and Javanainen 1996 |
| | *Lactobacillus amylophilus* GV6 | 27.3 | 0.3 | Vishnu et al., 2002 |
| Cellulose | *Lactobacillus coryniformis* ssp. *torquens* ATCC 25600 | 24 | 0.5 | Yáñez et al., 2003 |
| Corncob | *Rhizopus* sp. MK-96-1196 | 24 | 0.3 | Miura et al., 2004 |
| Waste paper | *Lactobacillus coryniformis* ssp. *Torquens* ATCC 25600 | 23.1 | 0.5 | Yáñez et al., 2005 |
| | *Rhizopus oryzae* NRRL 395 | 49.1 | 0.7 | Park et al., 2004 |
| Wood | *Lactobacillus delbrueckii* NRRL | 108 | 0.9 | Moldes et al., 2001 |

| | | | | |
|---|---|---|---|---|
| | B-445 | | | |
| | *Enterococcus faecalis* RKY1 | 93 | 1.7 | Wee et al., 2004 |
| Whey | *Lactobacillus helveticus* R211 | 66 | 1.4 | Schepers et al., 2002 |
| | *Lactobacilluscasei* NRRL B-441 | 46 | 4 | Büyükkilci and Harsa 2004 |

Different food/agro-industrial products become the cheaper alternatives to refined sugars for lactic acid production. Sucrose-containing materials such as molasses are commonly used raw materials for lactic acid production. The utilization of starchy materials in the place of expensive refined sugars is most economical (Yumoto and Ikeda 1995). Sugarcane bagasse is reported to be used as support for lactic acid production by *R. oryzae* and *Lactobacillus* in solid-state fermentation (SSF) by supplementing sugars or starch hydrolyzate as carbon source (Soccol et al. 1994; Rojan et al. 2005). Some agricultural by-products that are potential substrates for lactic acid production are cornstarch (Cheng et al. 1991; Hang 1990), cassava starch (Yumoto and Ikeda 1995), lignocellulose/hemicellulose hydrolyzates (Karel et al. 1997), cottonseed hulls, Jerusalem artichokes, corn cob, corn stalks (Vickroy 1985), beet molasses (Goksungur and Guvenc 1999; Kotzamanidis et al. 2002), wheat bran (Naveena et al. 2005), rye flour (Raccach and Mamiro 1997), sweet sorghum (Richter and Trager 1994), sugarcane press mud (Xavier and Lonsane 1994), cassava (Xiaodong et al. 1997; Rojan et al. 2005; John et al. 2006a,b), barley starch (Linko and Javaneinen 1996), cellulose (Venkatesh 1997), carrot processing waste (Pandey et

al. 2001), molasses spent wash (Sharma et al. 2003), corn fiber hydrolyzates (Saha and Nakamura 2003), and potato starch (Yumoto and Ikeda 1995; Anuradha et al. 1999).

Cheap raw materials such as starchy and cellulosic materials, whey, and molasses, have been used for lactic acid production (Hofvendahl 2000). Starchy and cellulosic materials are currently receiving a great deal of attention since they are cheap, abundant, and renewable (Akerberg et al. 2000; Richer et al. 1998; Venkatesh 1997). The starchy materials have to be hydrolyzed into fermentable sugars before fermentation as they consist mainly of α(1,4)- and α(1,6)-linked glucose. (Richter and Trager 1994 ; Hofvendahl and Hahn-Hagerdal 1997 ; Oh et al.,2005) This hydrolysis can be carried out simultaneously with fermentation (Linko and Javanainen 1996). Amylase-producing *L. amylophilus* and *L. amylovorus* are often used for the direct fermentation of starch into lactic acid (Xiaodong et al., 1997; Vishnu et al., 2002; Chatterjee et al., 1997).

Cellulosic materials have been used for lactic acid production in similar ways as starchy materials (Hofvendahl 2000). These materials consist mainly of β(1,4)-glucan, and often contain xylan, arabinan, galactan, and lignin (Hofvendahl 2000; Litchfield

et al. 1996). Venkatesh (1997) and Yánez et al. (2003) have previously attempted to produce lactic acid from pure cellulose through simultaneous saccharification and fermentation (SSF).

Some industrial waste materials of animal origin are also used as the carbon sources for the lactic acid fermentation. Whey is a by-product from cheese industry that contains lactose as carbon source, and it contains proteins, vitamins, and minerals. For complete utilization of whey lactose, it is necessary to supplement whey with an additional nitrogen source (Hofvendahl 2000). Amrane and Prigent (1998), Kulozik and Wilde (1999), and Schepers et al. (2002) supplemented whey with yeast extract for rapid production of lactic acid with *L. helveticus.* Yeast extract is the most common nutrient for lactic acid production. However, this may contribute significantly to an increase in production costs (Hofvendahl 2000). As an alternative to yeast extract, corn steep liquor, a by-product from the corn steeping process, has been used successfully for lactic acid production (Oh et al. 2005).

### 1.4 Processes in lactic acid fermentation

Lactic acid can be produced from various renewable resources such as sugars or sugar-containing hydrolyzates. Single-step conversion of starchy or cellulosic wastes

to lactic acid by amylolytic lactic acid-producing microorganisms was used. Simultaneous hydrolysis and fermentation adding enzymes and inoculum together was another commonly used method for lactic acid production. Different lactic acid fermentation processes adopted by the researchers are shown in Figure 1.3.

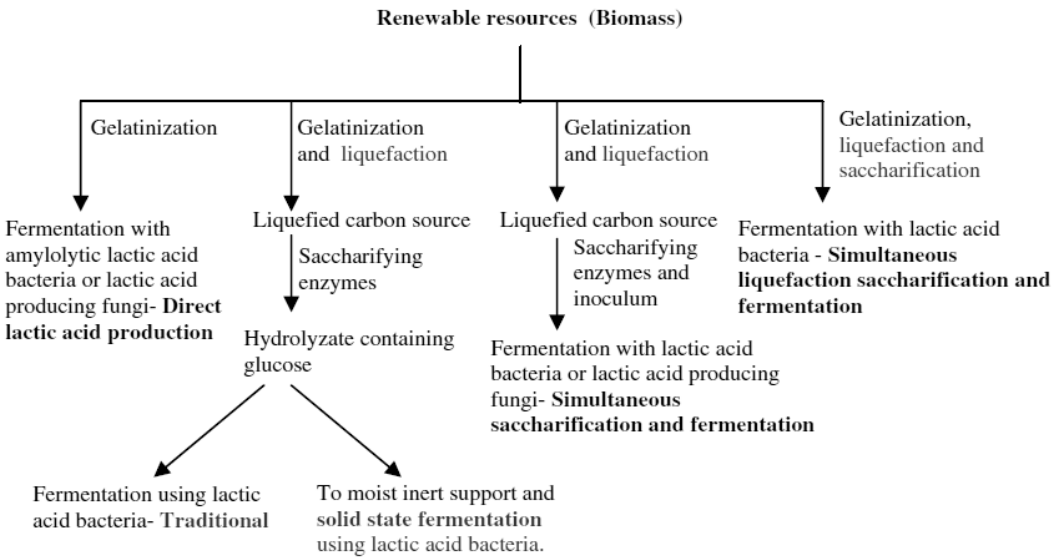



Figure 1.3 Different processes for lactic acid fermentation using renewable resources. (John et al., 2007)

The selection of mode of fermentation may vary with respect to different processes. Batch, fed-batch, repeated batch, and continuous fermentations are the most frequently used methods for lactic acid production. Higher lactic acid concentrations may be obtained in batch and fed-batch cultures than in continuous cultures but higher productivity may be achieved by the use of continuous cultures (Hofvendahl and Hahn-Hagerdal 2000). Another advantage of the continuous culture compared to the

batch culture is the possibility to continue the process for a longer period of time. A successful approach to continuous production of lactic acid with cell retention has been reported by Kwon et al. (2001) who attempted to produce lactic acid by a two-stage cell-recycle culture of *L. rhamnosus*. Immobilization of cells has been one of the methods for high cell retention in the bioreactor (Senthuran et al., 1999). The production of lactic acid by mixed cultures of free and coimmobilized *L. casei* and *L. lactis* cells in batch and fed-batch culture was investigated by Roukas and Kotzekidou (1998).

Lactic acid production processes traditionally suffer from end-product inhibition. An undissociated lactic acid passes through the bacterial membrane and dissociates inside the cell. The inhibition mechanism of lactic acid is probably related to the solubility of the undissociated lactic acid within the cytoplasmic membrane and the insolubility of dissociated lactate, which causes acidification of cytoplasm and failure of proton motive forces. It eventually influences the transmembrane pH gradient and decreases the amount of energy available for cell growth (Axelsson 1993 ; Goncalves et al., 1997). Therefore, lactic acid should be removed selectively *in situ* from the fermentation broth in order to alleviate the inhibitory effect of lactic acid during the fermentation.

Various attempts have been carried out to remove the lactic acid simultaneously. Hano et al. (1993) studied the reactive extraction of lactic acid from the fermented broth. They indicated that *in situ* extraction was feasible with the use of di-n-octylamine and with the pH adjustment of the fermentation broth. Iyer and Lee (1999) attempted to extract lactic acid simultaneously with the use of a two-zone fermentor-extractor system. The system was operated under a fed-batch mode with *in situ* removal of lactic acid by solvent extraction. Electrodialysis fermentation with ion exchange membranes was often used for removal of lactic acid (Nomura et al., 1998 ; Kim and Moon 2001). Min-Tian et al. (2005) had previously developed a continuous electrodialysis fermentation system for the production of lactic acid. Continuous removal of lactic acid with extraction or electrodialysis results in higher lactic acid concentrations and yields. However, the extracting material must be biocompatible in order not to harm the microorganism.

## 1.5 Recovery and purification of lactic acid

### 1.5.1 Traditional technologies

Excess calcium hydroxide/carbonate is added to the medium to neutralize the acid, maintaining a constant pH, and produce a calcium salt of lactic acid in the broth. It is usually desired to keep the calcium lactate in solution so that it can be easily handled

with the cell biomass and other insolubles and the final concentration achieved is around 10 wt%. The calcium lactate-containing broth is firstly filtered to remove cells, carbon treated, evaporated and acidified with sulfuric acid to convert the salt into lactic acid and insoluble calcium sulfate, which is removed by filtration. The filtrate can be further purified using carbon columns and ion exchange and evaporated to produce technical-grade lactic acid, but not a high-purity, heat-stable product, which is required for the stearoyl lactylates, polymers, solvents and other value-added applications. For the high-purity product, the technical-grade lactic acid is esterified with methanol or ethanol and the ester is recovered by distillation, evaporated and the alcohol recycled. This separation process produces a highly pure product which is water-white and heat stable and. The process is summarized in Figure 1.4.

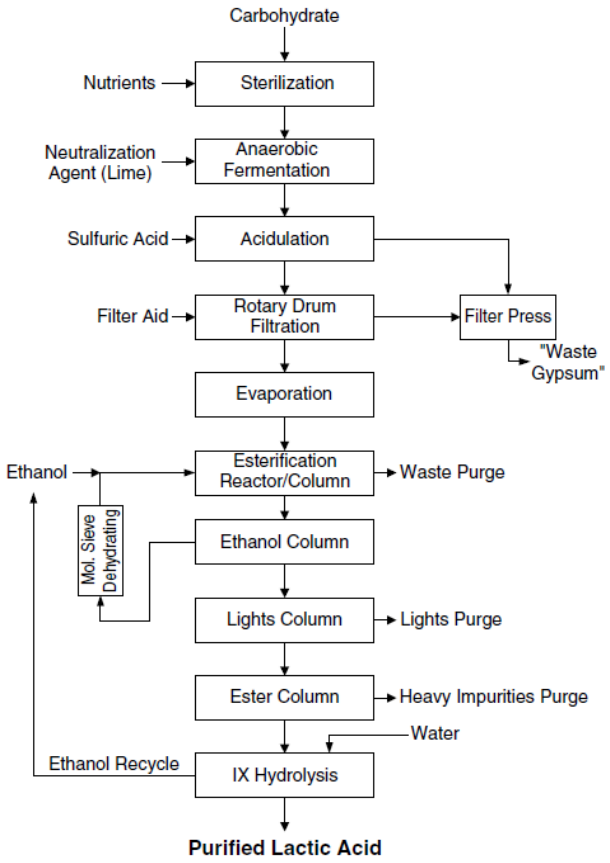



Figure 1.4 Conventional process for lactic acid manufacture from carbohydrate (Datta and Henry 2006)

Approximately 1 t of crude gypsum, CaSO4, is produced and as a waste byproduct needs to be disposed of for every ton of lactic acid produced by the conventional fermentation and recovery process (Prescott and Dunn 1959 ; Lee 1982). These factors have made large-scale production by this conventional route economically and ecologically unattractive.

### 1.5.2 Advanced technologies

Advances in membrane-based separation and purification technologies, particularly in microfiltration, ultrafiltration and electrodialysis (ED), have led to the inception of new processes for lactic acid production that do not produce a salt waste. Successful commercial development of bipolar ED membranes has recently occurred (Mani 1991 ; Hanada et al., 1993 ; Gillery et al., 2002). These membranes can split and separate water to protons ($H^+$) and hydroxyl ($OH^-$) ions. This technology advance now enables the $H^+$ to transport to the acid anion to form the free acid and the $OH^-$ ion to transport to the cation compartment to form the free base. Despite the recent advances in bipolar membranes, they have the fundamental problem of intolerance to multivalent cations such as calcium and magnesium that form insoluble hydroxides at the critical interface of the bipolar membrane where the ions separate. The tolerance limit for these divalent cations is about 1 ppm. Fermentation broths usually contain much higher concentrations, often in the region of 1000 ppm. Hence a successful process with bipolar membranes requires critical integration of key process steps that would work efficiently and economically.

One particular process configuration, termed the ‘double ED’ process has given promising results in the recovery and purification of lactic acid and is shown in

Figure 1.5. It has been developed and piloted at Michigan Biotechnology Institute (MBI) and at Argonne National Laboratory (ANL) (Datta 1989 ; Glassner and Datta 1990). This process uses a desalting ED unit to remove the multivalent cations and concentrate the lactate salt, followed by a 'watersplitting' ED unit with bipolar membranes to produce concentrated lactic acid and alkali for recycle. The desalting ED is a critical step that enables the process to operate efficiently and economically.

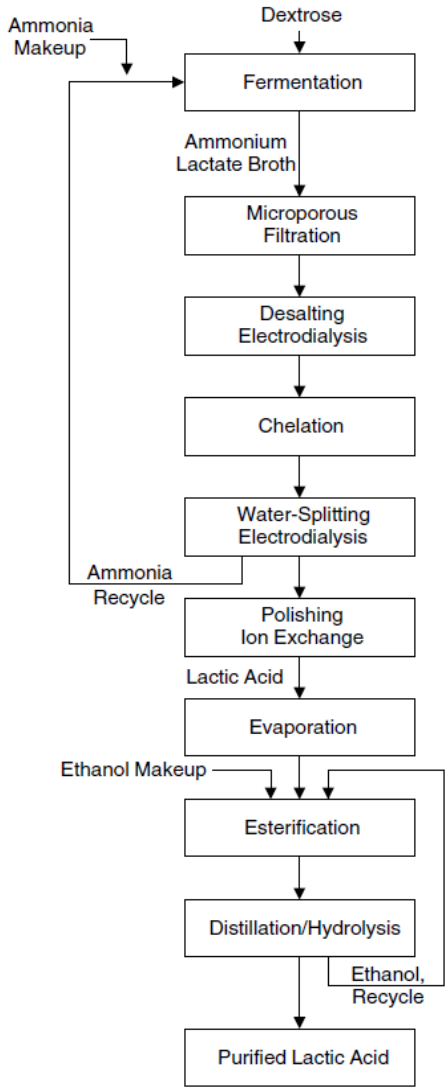



Figure 1.5 Double-ED process (Datta and Henry 2006)

## 1.6 Applications of lactic acid

Lactic acid has a wide range of applications in many different industries. The current uses and applications of lactic acid can be generally classified into four categories: food, cosmetic, pharmaceutical and chemical applications. The applications of lactic acid are illustrated in Figure 1.6.

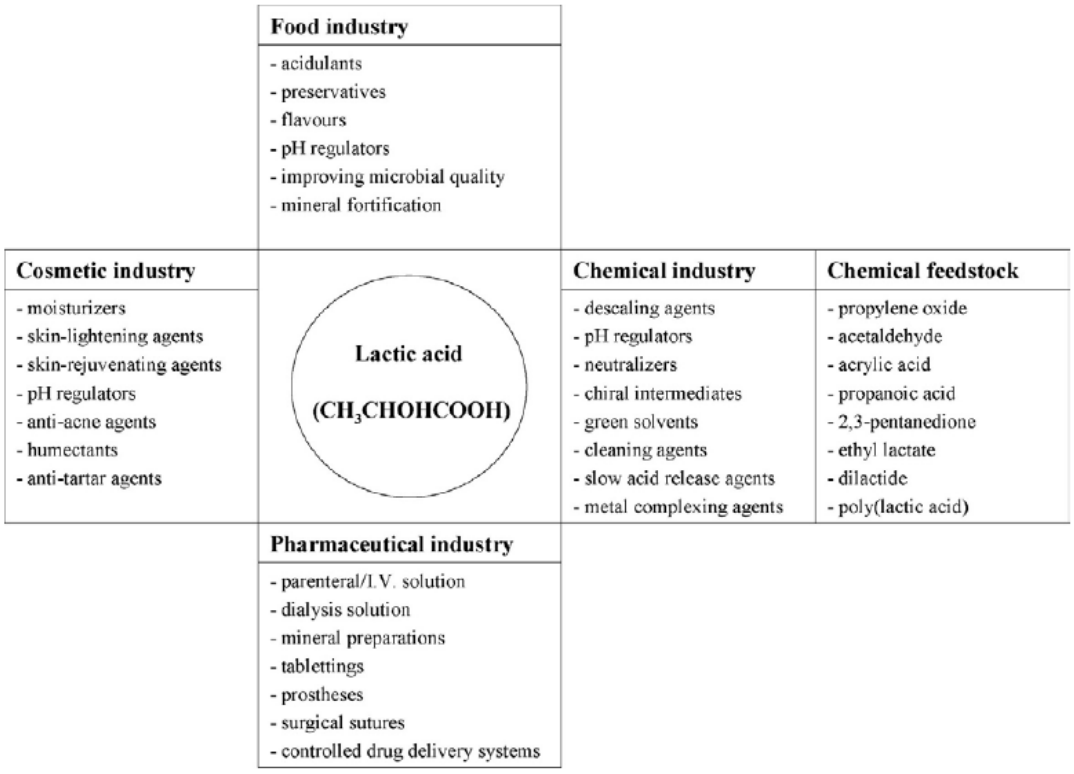



Figure 1.6 Commercial uses and applications of lactic acid and its salt (Wee et al., 2006)

### 1.6.1 Food industry

The lactic acid consumption market is dominated by the food and beverage sector since 1982. It account for approximately 85% of the demand for lactic acid. Lactic

acid has been used as a preservative and acidulant in food and beverage sector for several decades. Calcium lactate is a good dough conditioner, whereas sodium lactate acts both as conditioner and as emulsifier. Lactic acid is used as acidulant/flavoring/pH-buffering agent or inhibitor of bacterial spoilage in a wide variety of processed foods, such as candy, breads and bakery products, soft drinks, soups, sherbets, dairy products, beer, jams and jellies, mayonnaise, and processed eggs, often in conjunction with other acidulants (Datta et al. 1995). Lactic acid or its salts are now used in the disinfection and packaging of carcasses, particularly those of poultry and fish (Datta et al. 1995). The esters of calcium and sodium salts of lactate with longer chain fatty acids have been used as very good dough conditioners and emulsifiers in bakery products.

### 1.6.2 Cosmetic industry

Lactic acid can be used as moisturizer in cosmetic formulations due to its water-retaining capacity. The ability of lactic acid to suppress the formation of tyrosinase is responsible for the effect of skin lightening and rejuvenation. As humectants, the lactates are often superior to natural products and more effective then polyols (Datta et al. 1995). Ethyl lactate is the active ingredient in many anti-acne

preparations. The natural occurrence of lactic acid in human body makes it very useful as an active ingredient in cosmetics (Wee et al. 2006).

### 1.6.3 Pharmaceutical industry

Lactic acid has long been used in pharmaceutical formulations, mainly in topical ointments, lotions, and parenteral solutions. It is used in a wide variety of mineral preparations, which include tablets, prostheses, surgical sutures, and controlled drug delivery systems. It also finds applications in the preparation of biodegradable polymers for medical uses such as surgical sutures, prostheses, and controlled drug delivery systems (Wee et al. 2006).

### 1.6.4 Chemical industry

Lactic acid and its salt are used in various types of chemical products and processes. Natural lactic acid has an emerging use as an excellent and safe solvent, which is an alternative in many fine mechanical cleaning applications. Due to the high solvency power and solubility of lactic acid, it is an excellent remover of polymer and resins. Lactic acid can be used as a starting material in the production of herbicides. Since lactic acid offers better descaling properties than conventional organic descalers do, it is often used in many decalcification products such as bathroom cleaners, coffee machines, and toilets. Ethyl lactate is used in many anti-acne preparations because it

combines excellent solvency power against oils and polymeric stains, with no environmental impact and toxicological effects (Datta et al., 1995 ; Bulletin of the Purac ; Bulletin of the Galactic). The presence of two reactive functional groups, a carboxylic group and a hydroxyl group, makes lactic acid the feedstock monomer with the most potential for chemical conversions to useful chemicals such as propionic acid, acetic acid, acrylic acid, acetaldehyde, dilactide etc. (Dimerci et al. 1993 ; Varadarajan and Miller 1999).

### 1.6.5 Polymer industry

Lactic acid has recently received a great deal of attention for the production of its polymer, the polylactic acid (PLA) which can be use as an environmental-friendly alternative to plastics derived from petrochemicals. As the physical properties of PLA depend on the isomeric composition of lactic acid, the production of optically pure lactic acid is essential for polymerization. The optically pure lactic acid can be polymerized into a high molecular mass PLA through the serial reactions of polycondensation, depolymerization, and ring-opening polymerization (Sodergard et al. 2002). The lactic acid polymers have extensive applications and tremendous advantages like biodegradability, thermo plasticity and high strength etc. They have potentially large markets in the packaging of goods, protective clothing, fabrication of

prosthetic devices, cardiovascular applications, dental applications and controlled delivery of drugs in humans. The recent huge growth of the PLA market will further enhance the demands on lactic acid considerably.

### 1.6.6 Other applications

Lactic acid can be used for products that potentially have extensive uses in industrial applications and consumer products. The primary classes of such products are polymers for plastics and fibers, solvents for formulations and cleaning and oxygenated industrial chemicals. Environmentally friendly, 'green' solvents are another potential area for lactic acid derivatives, particularly lactate esters of low-molecular-weight alcohols such as ethyl, propyl, and butyl lactate. Purac Inc. has developed and commercialized several specialized applications of lactate esters in electronics and precision cleaning. Vertec Biosolvents Inc. has been developing and commercializing blends of these esters with other biologically derived solvents such as fatty acid methyl esters or D-limonene that can have a wide range of solvating and cleaning properties (Opre et al., 2001 ; Henneberry et al., 2004)

The US Environmental Protection Agency (EPA) recently classified the lactate esters ethyl and butyl lactate as Class 4A inert ingredient for use in the formulation of

pesticides and other bioactive compounds. The Class 4A designation is given to compounds that have demonstrated negligible toxicity and an excellent environmental profile. Hence a good range of specialty applications and commercial uses could be developed with these non-toxic, environmentally friendly lactate ester solvents with other biologically derived solvents (Chang 2002).

Lactic acid could potentially be used for the manufacturing of large-volume oxygenated chemicals, such as propylene glycol, propylene oxide, acrylic acid, and acrylate esters, and other chemical intermediates such as lactate ester plasticizers. The advances made in hydrogenolysis technology can be further developed and integrated to make propylene glycol from lactic acid in the future (Datta and Henry 2006).

### 1.7 Current industrial production of lactic acid

World consumption of lactic acid stands enthused by its use in key industries such as cosmetics, biodegradable plastics and food additives. The recently potential of lactic acid as a pH balancer in shampoos and soaps, and other alpha hydroxyl acid applications, is expected to further enhance the consumption in this market. Use of lactic acid bacteria in anti microbial compounds, environmentally friendly packaging, food additives, flavoring agents, and as a substitute for hazardous solvents in

industrial applications, will all help ensure steady consumption into the future. As stated by the recent report published by Global Industry Analysts, Inc., the United States, Asia-Pacific and Europe dominate the world lactic acid market, with a combined share of nearly 80% estimated in 2008. In Europe, Germany ranks as the largest individual market for lactic acid with a 29.3% share estimated in 2008, followed by France and Italy.

Consumption in the biodegradable plastics end-use industry in Europe is projected to reach 6.7 thousand metric tons by the year 2015. Given the low toxicity and biodegradability advantages of lactic acid as a raw material, the demand for lactic acid in biodegradable polymers is forecast to post healthy gains. The lactic acid industry is additionally expected to benefit significantly from the issues arising out of growing environmental pollution caused by plastic disposal, rising oil prices and greater consumer interest towards the use of greener products.

Consumption of lactic acid in the world cosmetic products end-use market is expected to rise by 24.67 thousand metric tons between the periods 2008 to 2012. For the same period, consumption in the food additives end-use market is expected to rise by 22.96

thousand metric tons. In Japan, cosmetic products, and food additives, together, account for over 86% of the total consumption of lactic acid.

Leading global and regional players operating in the industry include CSM N.V., Purac, Galactic S.A., Henan Jindan Lactic Acid Co. Ltd., Musashino Chemical Laboratory Ltd., and Jiangxi Musashino Bio-Chem. Co. Ltd. They are all the worldwide producers of natural lactic acid and its derivatives. The major manufacturers of fermentative lactic acid include NatureWorks LLC, Purac (The Netherlands), Galactic (Belgium), Cargill (USA), and several Chinese companies (Wee et al. 2006). Currently, NatureWorks LLC is the leader in lactic polymer technology and markets and the development and implementation of their dilactide technology has contributed to their success. More than 100 US patents in this area were assigned to this company. Over the past 10 years, this company has done extensive work on the development of lactic acid-based products, which are of two types—the polydilactide-based resins (NatureWorks PLA®), used for plastics or packaging applications, and the Ingeo™ polydilactide-based fibers that are used in specialty textiles and fiber applications. The US Food and Drug Administration (FDA) and European regulatory authorities have approved the PLA resins for all food-type applications. NatureWorks LLC has constructed a major lactic acid plant in Blair, NE, USA, with a nameplate capacity of 300 million pounds per year for the production of

lactic acid and PLA, and it began operating in late 2002 (Wee et al. 2006; Datta and Henry 2006).

Lactic acid consumption has increased considerably because of its role as a monomer in the production of biodegradable PLA, which is well-known as a sustainable bioplastic material (Datta et al., 1995 ; Litchfield, 1996). The worldwide demand for lactic acid is estimated roughly to be 130 000 to 150 000 metric tones per year (Mirasol 1999). However, the global consumption of lactic acid is expected to increase rapidly in the near future. NatureWorks LLC, a major PLA manufacturer established in the US, expects that the global PLA market may increase to 500 thousand metric tones per year by 2010 (Wee et al., 2006).

## 1.8 *Bacillus subtilis*

### 1.8.1 General characteristics of *Bacillus subtilis*

*Bacillus subtilis* and its close relatives are among the most important industrial microorganisms. They are used for the production of antibiotics, enzymes such as proteases, amylases, lipases, or cellulases, vitamins such as riboflavin, and even insecticidal proteins. Due to this practical relevance, much research has been devoted to many aspects of these bacteria and make *Bacillus subtilis* one of the best-studied and best characterized living organisms.

The Gram-positive, spore-forming *Bacillus subtilis* has been considered as a strict aerobe for many years. However, studies have shown that it can also grow anaerobically either by using nitrate or nitrite as a terminal electron acceptor or by fermentation. (Nakano et al, 1997) Recent studies have also shown that the metabolism of *Bacillus subtilis* is affected by the presence of organic acids. (Schilling et al. 2006)

*Bacillus subtilis* have several advantages over the other lactic acid bacterial strains. It is a Gram-positive bacterium, which is a GRAS (generally regarded as safe) microorganism by the FDA (Food and Drug Administration). *B. subtilis* has simple nutrition requirements as well as spore-formation capacity that simplify maintenance of stock cultures and shorter generation time.

*Bacillus subtilis* grows faster and is much less fastidious than *Lactobacillus*. These bacteria prefer sugars and ammonia as carbon and nitrogen sources, respectively. The preferred source of carbon and energy is glucose. This sugar is taken up and concomitantly phosphorylated by the bacterial phosphoenolpyruvate: sugar phosphotransferase system. The further metabolism involves glycolysis, the pentose

phosphate pathway, and the Krebs cycle, resulting in the oxidation of glucose to carbon dioxide and the generation of ATP. (Schilling et al. 2006)

Some studies showed that *Bacillus subtilis* performed mixed acid-butanediol fermentation. (Nakano et al, 1997) *B. subtilis* produces L-lactate and 2,3-butanediol from glucose (Romero et al., 2007). Lactate, acetate and 2,3-butanediol were identified as the major anaerobic fermentation products of *Bacillus subtilis*. (Ramos et al. 2000)

Two modes of anaerobic growth have been demonstrated in *Bacillus subtilis*. One is anaerobic respiration using nitrate or nitrite as an election acceptor. The other mode is fermentation. The anaerobic growth of *B. subtilis* on nitrate was found to be truly respiratory since *B. subtilis* was able to grow anaerobically in the presence of nitrate with glycerol as a sole carbon source. (Nakano et al, 1997) In the process of anaerobic nitrate ammonification, nitrate is reduced by a respiratory nitrate reductase to nitrite, and further reduced to ammonia by cellular nitrite reductase (Ramos et al, 2000).

*Bacillus subtilis* lacks or has a very inefficient glucose fermentation pathway. However, it grows anaerobically by fermentation either when both glucose and pyruvate are provided or when glucose and mixtures of amino acids are present. (Nakano and Zuber, 1998) Under anaerobic fermentative conditions, lactic acid is usually produced by reduction of pyruvate in a single step (as shown in Figure 1.7). This reaction is catalyzed by lactate dehydrogenase, with the simultaneous oxidation of one molecule of NADH per molecule of pyruvate reduced.

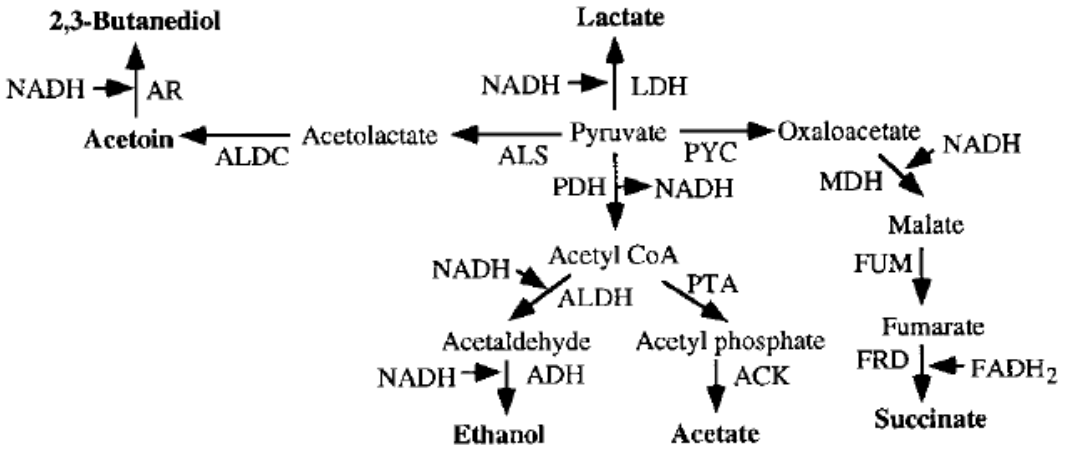



Figure 1.7 Fermentation pathways of *Bacillus subtilis* (Nakano et al, 1997)

Enzyme abbreviations: ACK, acetate kinase; ADH, alcohol dehydrogenase; ALDC, acetolacetate decarboxylase; ALDH, aldehyde dehydrogenase; ALS, acetolactate synthase; AR, acetoin reductase; FRD, fumarate reductase; FUM, fumarase; LDH, lactate dehydrogenase; MDH, malate dehydrogenase; PDH, pyruvate dehydrogenase; PTA, phosphotransacetylase; PYC, pyruvate carboxylase.

### 1.8.2 Production of lactic acid by *Bacillus* species

Some thermophilic *Bacillus* species were suggested to be new lactic acid producers. Michelson et al. (2006) reported lactic acid production using *B. coagulans* SIM-7 DSM 14043 and 101.7 g/L of lactic acid was obtained in high initial glucose fermenter culture. Rosenberg et al. (2005) reported lactic acid production using immobilized cells of *B. coagulans* CCM 4318 and 77.5 g/L of lactic acid could be produced. Sakai and Ezaki (2006) reported lactic acid production using *B. coagulans* NBRC12583 and 86 g/L of lactic acid was obtained after 5 days incubation.

Although there are numerous reports on lactic acid production by *B. coagulans*, there is limited information on lactic acid production by *Bacillus subtilis*. Ohara and Yahata (1996) reported that aerobic cultures of *B. subtilis* produced 3.7g/L lactic acid. Qin et al. (2009) reported lactic acid production using *Bacillus* sp. 2-6 and maximum L-lactic acid concentration of 182 g/L was obtained from 30-liter fed-batch fermentation with an average productivity of 3.03 g/liter/h. Romero-Garcia et al. (2009) also reported lactic acid production using *B. subtilis* that had the 2,3-butanediol biosynthetic pathways removed and 105.6 g/L of lactic acid was obtained. It eliminated the pyruvate competition between the L-lactate and the 2,3-butanediol pathway and caused more carbon to be directed to L-lactate production.

We reported a fermentative production of L-lactic acid by a UV-induced mutagenesis *Bacillus subtilis* with the higher yield and productivity of L-lactic acid when compared to the past studies in *B. subtilis*.

**1.9 Strain improvement by UV mutagenesis**

Random mutagenesis and fermentation screening have been reported as an effective way to improve the productivity of industrial microbial cultures (Parekh et al., 2000). 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. (Ng, 2006)

One of the more effective mutagenic agents is short wavelength ultraviolet (UV) radiation. The different ultraviolet (UV) wavelength components, UVA (320–400 nm), UVB (280–320 nm), and UVC (200–280 nm), have distinct mutagenic properties. A hallmark of UVC and UVB mutagenesis is the high frequency of transition mutations

at dipyrimidine sequences containing cytosine (Pfeifer et al., 2005). UV light induces a broad spectrum of point mutations in bacterial cells such as Escherichia coli (Miller, 1983).

Effective range of UV light for mutagenesis is between 200 - 300 nm with an optimum at 254nm, which is the absorption maximum of DNA. The major effect of UV light on DNA is the formation of covalent bonds between adjacent pyrimidines or between pyrimidines of complementary strands, which results in crosslinks. Dimerization between thymine-thymine, thymine-cytosine and cytosine-cytosine causes distortion of DNA double strands, resulting in an inhibitory effect of transcription and the cell dies eventually. 60-99% of the bacterial cells should be killed by the radiation indicating the chance of producing more desirable mutants.

UV radiation mainly induces transitions of GC to AT, transversions, frameshift mutations and deletions. It has been applied in the field of microbiology for a long time and there is an abundance of information about the effects and mechanisms of UV on microorganisms (Howard-Flanders et. al. 1964, Greenberg 1965, Hill 1972). Sedmera et al., 1986 reported the studies of boost up metabolites after UV mutagenesis. Improvement of lactic acid production in fungi by UV mutation (Bai et

al., 2004) and by chemical mutagen (Miura et al., 2004) had been studied. Ultraviolet light was employed for mutagenizing the parent strain *Bacillus subtilus* 1A304 (Φ105MU331). The purpose of UV mutagenesis was to mutate and screen for lactic acid overproduction strains.

**1.10 Starch hydrolysis**

Starch, a polyglycan (polymer of D-glucose units) and the major storage polysaccharide in plants, is composed of amylose and amylopectin. Amylose is mainly a linear polymer of D-glucosyl units (up to 6000 units) joined together through α-1,4 glycosidic linkages, and amylopectin is a highly branched polymer of D-glucosyl units (up to 2 million) joined together through α,1-4 and α,1-6 glycosidic linkages. For commercially interesting starch sources, the granule sizes range from 2–30 (maize starch) to 5–100 μm (potato starch) (Robyt, 1998). The molecules in starch granules are in a densely packed polycrystalline state with inter- and intra-molecular bonds. Hence, starch is insoluble in cold water and often resistant to chemical and enzyme treatments. Starches can be gelatinized by heating in water, which improve their chemical reactivity to amylolytic enzymes. An enzyme capable of digesting raw starch granules would be of value to reduce the cost of gelatinization and simplify the process of starch conversion.

Traditionally production of lactic acid from starch materials, for instance, requires pretreatment process 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. The aim of saccharification is to hydrolyze the oligosaccharides (mainly 8−12 glucose units) to form glucose syrup catalyzed by glucoamylase (Lynd, 1991).

Liquefaction and saccharification are carried out by biological catalysts. These biological catalysts are industrial enzymes which can reduce the time and cost to produce useful products. For industrial purposes, thermostable bacterial enzymes are commonly used. It is because the process may need to be maintained at a high temperature and the enzyme 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 α-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.

### 1.10.1 α- amylase

Starch can be treated into slurry with starch hydrolases such as α-amylase (EC 3.2.1.1), isoamylase (EC 3.2.1.68), pullulanase (EC 3.2.1.41), and amylopullulanase. α-amylase (EC 3.2.1.1) is a well-known endoamylase α-amylases. These are used in starch hydrolysis for sugar syrups and brewing, and are obtained from *Aspergillus oryzae, Bacillus amyloliquefaciens,* and *Bacillus licheniformis.* Calcium ions are added to improve the stability of the α-amylase. The major end products from the action of α-amylase on starch are glucose, maltose, maltotriose. maltotetraose. maltopentaose, and maltohexaose (Nigam and Singh, 1995).

α-amylase randomly cleaves the 1,4-α-D-glucosidic linkages between the adjacent glucose units of linear amylase chains (Ellaiah et al., 2002) and are able to operate at

temperatures as high as 105℃ and the process cannot be performed much lower than pH 5.9. α-amylase hydrolyzes the internal bond in starch granules to form oligosaccharides as maltodextrins. This process is also described as starch liquefaction for converting concentrated starch suspension into soluble dextrin solution (Crabb and Mitchinson, 1997).

Exoamylases act on the external glucose residues of amylose or amylopectin and thus produce only glucose (glucoamylase and α-glucosidase), or maltose and β-limit dextrin (β-amylase). Debranching enzymes such as isoamylase will hydrolyze only α,1-6 bond in amylopectin and pullulanase hydrolyzes α,1-6 glycosidic bond in pullulan and amylopectin. Amylopullulanase will hydrolyze α,1-4 and α,1-6 glycosidic bonds to yield mainly maltose and maltotriose (van der Maarel et al., 2002).

The source of α-amylase used in this project was recombinant *Bacillus subtilis* MU331 with α-amylase gene originally from *Bacillus licheniformis*, and it is a thermostable enzyme that is intense in starch liquefaction (Joel and Ana, 2003).

### 1.10.2 Glucoamylase

Glucoamylase (GA), also known as amyloglucosidase (EC 3.2.1.3), is a biocatalyst capable of hydrolyzing α-1,4 glycosidic linkages in raw or soluble starches and related oligosaccharides with the inversion of the anomeric configuration to produce β-glucose (Norouzian et al., 2006). 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).

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 inexpensive separation of filamentous or pellet biomass from the fermentation broth (Soccol, 1994; Rosenberg, 1995).

The process of removing single glucose residues from a soluble oligosaccharide is termed saccharification. This process is catalysed by glucoamylase which sequentially removes a glucose unit from the non-reducing end until all of the oligosaccharide is

degraded to glucose. The enzyme of choice is isolated from *Aspergillus niger* or a closely related species, for example *A. awamori* (Crabb and Mitchinson, 1997). *Aspergillus niger* has been used for a long time in industries and glucoamylase production was studied in carbon limited chemostat (Metwally et al., 1991). *A. niger* glucoamylase has a pH optimum near 4.2 and is extremely stable at 60°C, it is produced at high levels by industrial fermentation processes (Crabb and Mitchinson, 1997).

### 1.11 Simultaneous saccharification and fermentation (SSF) of lactic acid

Conventional fermentative production of lactic acid from starchy materials such as barley, corn, potato, or rice requires pretreatment by gelatinization and liquefaction, followed by enzymatic saccharification of starch to glucose and subsequently the conversion of glucose to lactic acid by fermentation. Microbial conversion of starch to lactic acid can be made much more economical by coupling the enzymatic hydrolysis of starchy substrates and microbial fermentation of the derived glucose into a single step, which is known as simultaneous saccharification and fermentation (SSF).

SSF is a cost-effective process for lactic acid production. In the SSF process, starch is simultaneously degraded to oligosaccharide and hydrolyzed to glucose by the

α-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 and 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. A 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 the total process time, reactor volume and capital costs (Takagi, 1977).

The first study on lactic acid production by SSF of cellulosic raw materials was reported by Abe and Takagi (1991), and since then some articles have been reported on this subject (Schmidt and Padukone, 1997 ; Parajo et al.,1997 ; Moldes et al., 2000). 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). During SSF, hydrolyzing enzymes are added to the substrates along with the inoculum. With simultaneous enzymatic liquefaction, saccharification and fermentation, amylolytic and

non-amylolytic bacteria and fungi can perform faster than the direct conversion by their own enzyme utilization. Co-immobilization of starch-degrading organisms like *Aspergillus awamori* and lactic acid-producing bacteria *Streptococcus lactis* was also studied for the simultaneous saccharification and lactic acid production (Kurusava et al., 1988). Simultaneous saccharification and lactic acid production was done with the addition of amylolytic enzymes and inoculated with *L. delbrueckii* (Anuradha et al., 1999).

Bacterial growth and lactic acid formation may become low in high concentrations of glucose due to the imbalance in osmotic pressure. In the early stages of fermentation, up to about 30 h, a high initial glucose concentration in a glucose-based medium markedly decreased lactic acid formation using *L. casei* inoculum (Linko and Javanainen, 1996). With the simultaneous liquefied, saccharified, and fermented barley starch, the lactic acid yield obtained in 30 h was 74% (130 g/L initial starch) to 86% (170 g/L starch), considerably higher than the 31–67% obtained in the fermentation of glucose with initial sugar concentrations of 143 g/L and 187 g/L (Linko and Javanainen, 1996).

SSF can also solve inhibition of high sugar concentration in the medium. Glucose are derived from saccharification of starch by amylases but the product, glucose inhibits the enzyme. In the SSF process, the hydrolyzing enzymes are added along with the inoculum. Enzymes release sugars from the substrate and the organism simultaneously uses it. Thus, SSF can prevent amylase inhibition caused by glucose accumulation and lead to an enhancement in the lactic acid productivity.

Many researchers have carried out SSF of starchy wastes using amylase and cellulosic wastes using cellulase. This approach can reduce the cost on energy consumption and it will reduce the negative influence of high glucose concentration in the media. In SSF, it has been shown experimentally that the inhibition can be overcome and starch can be used as a raw carbon source for lactic acid production.

The effect of lactic acid on the enzymatic hydrolysis of cellulose in SSF was studied by Iyer and Lee (1999). The extent of the inhibition is such that the enzymatic digestibility at 72 h decreases from 79% to 56% as the lactic acid concentration is increased from 0 to 90 g/l. The concentration of lactic acid for 50% inhibition is higher than 90 g/l. This inhibition by lactic acid on enzymatic hydrolysis is much lower than that caused by glucose build-up (Takagi, 1984).

Linko and Javanainen (1996) have reported the production of lactic acid from barley starch by simultaneous liquefaction and saccharification using α-amylase and glucoamylase and fermentation employing *L. casei*. A lactic acid concentration as high as 162 g/L was obtained and the yields were 90–98%. Amylolytic lactic acid-producing *Luctobacillus amylophilus* and *Luctobacillus amylovorus* have been reported to produce lactic acid from liquefied starch. Lactic acid production on barley flour without any additional nitrogen source, vitamins, or minerals employing *L. amylovorus* in mixed cultures with *L. casei* has been reported by Javanainen and Linko (1995). Simultaneous saccharification with glucoamylase effectively improved lactic acid production with *L. casei* alone, with the highest lactic acid concentration of 120 g/L (yield 667 g/kg barley flour fermented) on barley flour treated with barley malt without any additional nutrients.

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 inhibit 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.

### 1.12 Objectives

1. To develop a fermentation process for the production of L-lactic acid by recombinant *Bacillus subtilis* which use glucose as the carbon source

2. To study the feasibility of producing L-lactic acid by recombinant *Bacillus subtilis* which can directly use starch as the carbon source

### 1.13 Fermentation kinetics

Amount of glucose utilized (g/L) = (Initial – final) glucose concentration

$$\text{Rate of glucose utilization (g/L/h)} = \frac{\text{Amount of glucose utilized (g)}}{\text{Fermentation time (h)}}$$

$$Y_{LA/GLU}\ \text{(g/g)} = \frac{\text{Final lactic acid concentration (g/L)}}{\text{Amount of glucose utilized (g/L)}}$$

$$\text{Lactic acid productivity (g/L/h)} = \frac{\text{Final lactic acid concentration (g/L)}}{\text{Fermentation time (h)}}$$

# Chapter Two

# Materials and Methods

## 2.1 Microorganisms

### 2.1.1 *Bacillus subtilis*

The recombinant *Bacillus subtilus* 1A304 (Φ105MU331) was firstly provided by Dr. Leung Yun-chung, Thomas and further modified by Mr. Ng Chung-kei using UV mutagenesis. The strain was used for overexpression of α-amylase and was stored in brain heart infusion and yeast extract medium at -80℃. Recombinant *Bacillus subtilis* 1A304 (Φ105MU331) mutant strain R was chosen for the production of lactic acid.

### 2.1.2 *Aspergillus niger*

*Aspergillus niger* (ATCC 13496) was obtained from the Department of Applied Biology and Chemical Technology. The strain was maintained on potato dextrose agar slant at 4℃.

## 2.2 Lactic acid Production

### 2.2.1 Culture medium

BHY medium

37 g/L Brain Heart infusion and 5 g/L yeast extract

YE 20 medium

20 g/L glucose, 20 g/L industrial yeast extract Hy-Yest™ 444 (Kerry, Ireland), 1.5 g/L $KH_2PO_4$, 1.5 g/L $K_2HPO_4$, 6 g/L $(NH_4)_2SO_4$, 30 g/L Martone B-1 (Marcor, USA), 0.03 g/L $MgCl_2$ and 0.08 g/L $MgSO_4 \cdot 7H_2O$ (adjust pH to 7.5 by NaOH) Glucose was autoclaved separately. 2.3 ml/L microelement solution was added after medium sterilization.

YE 80 medium

20 g/L glucose, 80 g/L industrial yeast extract Hy-Yest™ 444 (Kerry, Ireland), 1.5 g/L $KH_2PO_4$, 1.5 g/L $K_2HPO_4$, 0.03 g/L $MgCl_2$ and 0.08 g/L $MgSO_4 \cdot 7H_2O$ (adjust pH to 7.5 by NaOH) Glucose was autoclaved separately. 2.3 ml/L microelement solution was added after medium sterilization.

The above media were sterilized at 121℃ for 20 min.

Microelement solution

10.75 g/L MgO, 2 g/L $CaCO_3$, 4.5 g/L $FeSO_4 \cdot 7H_2O$, 1.44 g/L $ZnSO_4 \cdot 7H_2O$, 1.12 g/L $MnSO_4 \cdot 7H_2O$, 0.25 g/L $CuSO_4 \cdot 5H_2O$, 0.28 g/L $CoSO_4 \cdot H_2O$, 0.06 g/L $H_3BO_3$, 51.3 ml/L concentrated HCl (The solution was filter sterilized by 0.22 μm filter.)

### 2.2.2 Inoculum

30 µl of recombinant *Bacillus subtilis* from 1.5 ml frozen vial was inoculated into a 1 L flask containing 100 ml selected medium (BHY medium or YE 20 medium) with 5 µg/ml chloramphenicol and was shaken overnight at 37℃ with 270 rpm. 20 g/L glucose was added into the medium before shaking. The inoculum medium was the same as the fermentation medium. The frozen seed culture was preserved in 1.5 ml vials containing 1 ml cell broth with 15% glycerol and was stored at – 80℃.

### 2.2.3 Culture conditions

#### 2.2.3.1 Shake flask fermentation

**A. One stage Fermentation (cell growth and lactic acid production in the same stage)**

3.5 ml culture (5% inoculum) was inoculated in 70 ml selected medium in a 100 ml flask and was shaken at 37℃ with 180 rpm for 12 hours. The pH of culture medium was maintained at 7.5 by 28% $NH_4OH$. 1 ml of sample was taken from each flask at two hour intervals for pH adjustment. Samples were collected to measure absorbance at 600 nm, cell dry weight, pH, glucose and lactic acid concentration.

**B. Two stage Fermentation (cell growth in the first stage followed by lactic acid production in the second stage)**

The first stage was started by inoculating 0.5 ml culture (0.5% inoculum) in 100 ml selected medium in a 1 L flask and was shaken at 37℃ with 270 rpm for 12 hours. After 12 hours of cell growth, the second stage was initiated. 40ml culture was transferred into 100 ml flask and shaken at 37℃ with 120 rpm. 15 g/L glucose and 5 g/L calcium carbonate were added at the beginning of the second stage. Samples were collected to measure absorbance at 600 nm, cell dry weight, pH, glucose and lactic acid concentration.

**2.2.3.2 Fed-batch fermentation in fermentor**

**A. One stage Fermentation**

5% inoculum was inoculated in 600 ml selected medium in a 1 L fermentor or 1 L selected medium in a 2 L fermentor (BIOSTAT® B plus, Sartorius BBI Systems, Germany). The medium was kept at selected temperature (37℃ or 40℃) and pH 7.5. The agitation speed was set at 50 rpm. Air flow was 2 vvm. The pH was controlled by 2M HCL and selected base solution (28% $NH_4OH$ or 8 M NaOH). 60% glucose solution was fed into the fermentor in order to maintain 20 g/L glucose level. Sigma antifoam 289 was added to minimize foaming. The dissolved oxygen tension was

monitored by a METTLER TOLEDO INGOLD dissolved oxygen sensor. The pH was measured by a METTLER TOLEDO INGOLD pH / redox electrode with gel-type electrolyte. The fermenter was interfaced by a graphical user interface with touch screen and controlled by MFCS/win 2.1 software. Exhaust gas from the tank was also analyzed by the exhaust gas analyzer for oxygen and carbon dioxide levels. Samples were taken out through a sampling tube. Samples were collected to measure absorbance at 600 nm, cell dry weight, glucose and lactic acid concentration.

**B. Two stage Fermentation**

The first stage was started by inoculating 0.5% inoculum in 1L selected medium in a 2L fermentor. (BIOSTAT® B plus, Sartorius BBI Systems, Germany) The medium was kept at 37°C and pH 7. DO was controlled at 30% by changing agitation speed from 400 rpm to 1300 rpm. Air flow was 4 vvm. The pH was controlled by 2M HCL and 5M NaOH. About 12 hours later, the second stage was initiated. The agitation speed was set at 400 rpm and the air flow was changed to 2 vvm. 60% glucose solution was fed into the fermentor in order to maintain 20 g/L glucose level. Sigma antifoam 289 was added to minimize foaming. The dissolved oxygen tension was monitored by a METTLER TOLEDO INGOLD dissolved oxygen sensor. The pH was measured by a METTLER TOLEDO INGOLD pH / redox electrode with gel-type electrolyte. The fermenter was interfaced by a graphical user interface with touch

screen and controlled by MFCS/win 2.1 software. Exhaust gas from the tank was also analyzed by the exhaust gas analyzer for oxygen and carbon dioxide levels. Samples were taken out through a sampling tube. Samples were collected to measure absorbance at 600 nm, cell dry weight, glucose and lactic acid concentration.

### 2.3 Starch liquefaction

#### 2.3.1 Overexpression of α-amylase from recombinant *Bacillus subtilis* Φ105 MU331

A loop of recombinant *Bacillus subtilis* from frozen stock was inoculated into a 100 ml flask containing 10 ml BHY medium with 5 μg/ml chloramphenicol and was shaken overnight at 37℃ with 280 rpm. 1 ml of overnight culture was transferred into another 100 ml flask containing 15 ml BHY medium and was shaken at 37℃ with 280 rpm. When absorbance at 600 nm reached 5, the culture was incubated at 50℃ for 5 minutes with vigorous shaking to induce α-amylase production. The heat shocked culture was re-incubated at 37℃ with 280 rpm. Samples were taken at regular intervals for the measurement of absorbance at 600 nm, α-amylase activity assay and running SDS-PAGE.

### 2.3.2 α-amylase activity assay

The enzyme activity of α-amylase was determined by iodine method (Park et al, 1995). 1 ml of sample taken after heat induction was centrifuged at 14000 x g for 3 minutes to obtain the culture supernatant. 50 µl of culture supernatant, 50 µl of 0.5 M glycine-NaOH buffer at pH 9.0 and 100 µl of 0.2% starch solution were mixed and incubated at 50℃ water bath for 10 minutes. The enzyme reaction was quenched by adding 200 µl of 1.5 M acetic acid. 200 µl of iodine reagent (freshly prepared by mixing 0.2% iodine solid and 2% KI) was added and the reaction mixture was diluted to a final volume of 5 ml by $ddH_2O$. The reaction blank was prepared by replacing 100 µl 0.2% starch solution with $ddH_2O$. For the negative control, 50 µl $ddH_2O$ was added instead of culture supernatant. Absorbance at 690 nm was measured for the calculation of enzyme activity. The amount of blue black complex produced was inversely proportional to the α-amylase activity. One unit of α-amylase activity is defined as the amount of enzyme reducing absorbance at 690 nm due to blue black starch-iodine complex by 2.5% in 10 min.

The α-amylase activity was calculated by the following equation (Park et al, 1995; Ng, 2006):

$$\text{α-amylase activity (Unit/ ml)} = \frac{\text{Abs}_{690}\text{ of -ve Control} - \text{Abs}_{690}\text{ of Sample}}{\text{Abs}_{690}\text{ of -ve Control}} \times \frac{1}{2.5\%} \times \frac{\text{Dilution Factor}}{\text{Sample Volume}}$$

### 2.3.3 SDS-PAGE

To detect the overexpression of α-amylase after heat induction, SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) was performed. A 7 cm x 8 cm x 0.75 mm (length x width x thickness) slab gel Mini-Protein II SDS-PAGE apparatus (Bio-Rad) was used. After polymerization, the gels were subjected to electrophoresis in 1 x running buffer (3 g/L Tris-base, 14.4 g/L glycine and 1 g/L SDS) at 200 volts for an hour. The gels were stained with Coomassie Brilliant Blue (250 ml $ddH_2O$, 250 ml methanol, 100 ml acetic acid and 0.6 g Coomassie Blue R-250) and destained with destaining solution (800 ml $ddH_2O$, 100 ml acetic acid and 100 ml methanol).

### 2.3.4 Liquefaction process

24 h after heat induction, the culture supernatant obtained from recombinant *Bacillus subtilis* Φ105 MU331 was used for starch liquefaction. The supernatant containing α-amylase enzyme was added to starch solution with 10 ppm calcium chloride to enhance enzyme stability. The liquefaction of starch was carried out at 105℃ for 30 minutes.

## 2.4 Starch saccharificatioin

### 2.4.1 Culture medium

FG medium

40 g/L sucrose, 10 g/L $NaNO_3$, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 0.5 g/L $KH_2PO_4$ and 0.01 g/L $FeSO_4 \cdot 7H_2O$, pH 6.0

Glucoamylase induction medium

40 g/L soluble starch, 10 g/L $NaNO_3$, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 0.5 g/L $KH_2PO_4$ and 0.01 g/L $FeSO_4 \cdot 7H_2O$, pH 6.0

Organic nitrogen growth medium

50 g/L glucose, 20 g/L corn steep liquor, 15 g/L peptone, 10 g/L yeast extract, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 0.5 g/L $KH_2PO_4$ , pH 6.0

Organic nitrogen glucoamylase induction medium

40 g/L soluble starch, 20 g/L corn steep liquor, 15 g/L peptone, 10 g/L yeast extract, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 0.5 g/L $KH_2PO_4$ , pH 6.0

The above media were sterilized at 121℃ for 20 min.

### 2.4.2 Inoculum

*Aspergillus niger* was grown on potato dextrose agar plate at 25℃ for 5 days to obtain cultures with dense sporulation.

### 2.4.3 Culture conditions

#### 2.4.3.1 Immobilization of *Aspergillus niger* in calcium alginate gel beads

*Aspergillus niger* spores was collected by using 5 ml 0.9% saline (NaCl) solution to form a spore suspension. 5 ml of spore suspension was then mixed with 20 ml of 3% sodium alginate solution and extruded through a 10 ml syringe into 0.5 M $CaCl_2$ solution with stirring of 100 rpm to form spore-entrapped gel beads. The gel beads were hardened for 20 minutes and washed with 0.5% $CaCl_2$ solution until no spores were present in the solution. For further hardening, the gel beads in sterile $ddH_2O$ were stored at 4℃ for an hour.

#### 2.4.3.2 Spores germination and glucoamylase induction of immobilized *Aspergillus niger*

3g (approximately 40 beads) of immobilized *Aspergillus niger* gel beads were grown in 250 ml flask containing 50 ml FG medium and shaken at 30℃ with 250 rpm for 48 hours. The immobilized *Aspergillus niger* gel beads were then washed with sterile

dd$H_2O$ and transferred to 50 ml enzyme production medium in 250 ml flask and shaken at 30℃ with 250 rpm for 48 hours.

#### 2.4.3.3 Spores germination and glucoamylase induction of *Aspergillus niger* culture

A loop of *Aspergillus niger* spores was inoculated in 50 ml FG medium in a 250 ml flask and was shaken at 30℃ with 250 rpm for 48 hours. The culture was centrifuged at 14000 x g for 20 minutes at 4˚C and resuspended in 50 ml enzyme production medium in 250 ml flask and shaken at 30℃ with 250 rpm for 48 hours.

#### 2.4.3.4 Saccharificatioin by immobilized *Aspergillus niger*

3g (approximately 40 capsules) of immobilized *Aspergillus niger* gel beads after enzyme induction were washed with sterile dd$H_2O$ and inoculated into 50 ml sterilized liquefied starch solution in 250 ml flask. The cultures were shaken at 43℃ with 250 rpm. Samples were taken at regular intervals for the measurement of glucose concentration.

#### 2.4.3.5 Saccharificatioin by *Aspergillus niger* culture

10 ml *Aspergillus niger* culture after enzyme induction was added into 40 ml sterilized liquefied starch solution in a 250 ml flask and was shaken at selected temperatures with 250 rpm. For the saccharification using *Aspergillus niger* cell pellets and culture supernatant, 10 ml *Aspergillus niger* culture after enzyme induction was centrifuged at 14000 x g at 4˚C. The cell pellets were washed by sterilized dd$H_2O$ thoroughly and the culture supernatant was filter-sterilized by 0.22 μm filter before adding into the sterilized liquefied starch solution for saccharification. Samples were taken at regular intervals for the measurement of glucose concentration.

### 2.5 Simultaneous saccharification and fermentation (SSF) of lactic acid

#### 2.5.1 Culture medium and conditions

<u>YE 20 medium</u>

Various concentrations (20 g/L or 50g/L or 120 g/L or 300 g/L) of potato starch, 20 g/L industrial yeast extract Hy-Yest™ 444 (Kerry, Ireland), 1.5 g/L $KH_2PO_4$, 1.5 g/L $K_2HPO_4$, 30 g/L Martone B-1 (Marcor, USA), 0.03 g/L $MgCl_2$ and 0.08 g/L $MgSO_4 \cdot 7H_2O$ (adjust pH to 7.5 by NaOH). 2.3 ml/L microelement solution was added after medium sterilization. Potato starch containing 20% moisture was used in the study.

The above media were sterilized at 121℃ for 20 min.

Microelement solution

10.75 g/L MgO, 2 g/L $CaCO_3$, 4.5 g/L $FeSO_4 \cdot 7H_2O$, 1.44 g/L $ZnSO_4 \cdot 7H_2O$, 1.12 g/L $MnSO_4 \cdot 7H_2O$, 0.25 g/L $CuSO_4 \cdot 5H_2O$, 0.28 g/L $CoSO_4 \cdot H_2O$, 0.06 g/L $H_3BO_3$, 51.3 ml/L concentrated HCl (The solution was filter sterilized by 0.22 μm filter.)

**2.5.2 SSF in shake flask**

3.5 ml recombinant *Bacillus subtilis* culture (5 % inoculum) and 14 ml *Aspergillus niger* culture after enzyme induction were mixed together and inoculated in 70 ml medium in a 100 ml flask. The flask was shaken at 37°C or 40°C with 180 rpm. The pH value was maintained at 7.5 by 5 M NaOH. Starch in medium was liquefied by α-amylase from recombinant *Bacillus subtilis* Φ105 MU331. The process was carried out at 105℃ for 30 minutes with the addition of 10 ppm calcium chloride.

**2.5.3 SSF in fermentor**

25 ml recombinant *Bacillus subtilis* culture (5 % inoculum) and 120 ml *Aspergillus niger* culture after enzyme induction were mixed together and inoculated in 600 ml

YE 20 medium in a 1L fermentor. (BIOSTAT® B plus, Sartorius BBI Systems, Germany) Starch in medium was liquefied by α-amylase from recombinant *Bacillus subtilis* Φ105 MU331. The process was carried out at 105℃ for 30 minutes with the addition of 10 ppm calcium chloride. The fermentation medium was kept at 37℃ or 40℃ and pH 7.5. The agitation speed was set at 50 rpm. Air flow was 2 vvm. The pH was controlled by 2M HCL and 8 M NaOH. Liquefied starch was fed into the fermentor in order to facilitate the SSF process. Sigma antifoam 289 was added to minimize foaming. The dissolved oxygen tension was monitored by a METTLER TOLEDO INGOLD dissolved oxygen sensor. The pH was measured by a METTLER TOLEDO INGOLD pH / redox electrode with gel-type electrolyte. The fermenter was interfaced by a graphical user interface with touch screen and controlled by MFCS/win 2.1 software. Exhaust gas from the tank was also analyzed by the exhaust gas analyzer for oxygen and carbon dioxide levels. Samples were taken out through a sampling tube. Samples were collected to measure absorbance at 600 nm, cell dry weight, glucose and lactic acid concentration.

## 2.6 Analytical methods

### 2.6.1 Measurement of absorbance at 600 nm

The cell growth was monitored by measuring the absorbance at 600 nm using spectrophotometer Biochrom Libra S11. Serial dilution of samples were needed when the absorbance was larger than 0.5. The average data of three measurements was shown.

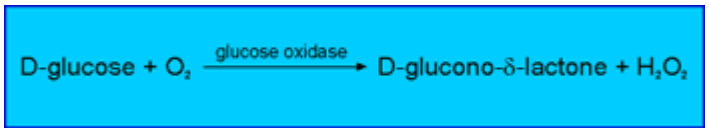
### 2.6.2 Measurement of cell dry weight

The eppendorf was pre-weighted. 1.5 ml of the cell broth was added into the weighted eppendorf. It was then centrifuged at 14000 x g for 3 minutes. The supernatant was discarded and the pellet was dried by a freeze-dryer until constant weight. The eppendorf with cell pellet was measured again and the dry weight was determined.

### 2.6.3 Measurement of glucose concentration

YSI 2700 SELECT Biochemistry Analyzer was used. 1.5ml sample was centrifuged at 14000 x g for 3 minutes and the amount of glucose in the supernatant was measured by the glucose membrane of the analyzer. Glucose oxidase was immobilized on the glucose membrane, which oxidized glucose to release hydrogen peroxide and the amount of hydrogen peroxide was detected by a probe. The resulting current was

proportional to the concentration of glucose which was compared with the standard solution. For glucose measurements, samples were diluted within 0 to 10 g/L. The average data of three measurements was shown.



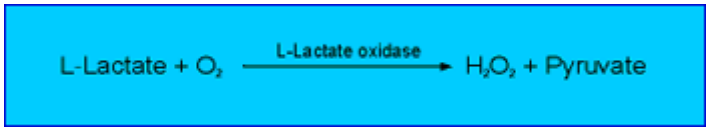
(YSI, 2009)

### 2.6.4 Measurement of lactate concentration

Lactate concentration was measured by YSI 2700 SELECT Biochemistry Analyzer and high performance liquid chromatography. 1.5ml sample was centrifuged at 14000 x g for 3 minutes and the amount of lactate in the supernatant was measured. The average data of three measurements was shown.

#### 2.6.4.1 YSI 2700 SELECT Biochemistry Analyzer

Lactate oxidase was immobilized on the lactate membrane, which oxidized lactate to release hydrogen peroxide and the amount of hydrogen peroxide was detected by a probe. The resulting current was proportional to the concentration of lactate which was compared with the standard solution. For lactate measurements, samples were diluted within 0 to 0.5 g/L.



(YSI, 2009)

### 2.6.4.2 High Performance Liquid Chromatography (HPLC)

Both the stereoselective and non-stereoselective assay utilized the same HPLC system with a UV detector.

For the non-stereoselective assay, the racemic lactic acids were separated by an Alltech IOA-1000 (7.8 mm x 300 mm) organic acid column. 0.005N $H_2SO_4$ was used as the mobile phase. The wavelength was monitored at 210 nm. 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. For the standard, 90% pure sodium lactate in liquid form was used and ran for 60 minutes. For the sample, the supernatant was collected and pass through a 0.2 µm filter. Each sample ran for 180 minutes until all the compounds elute out.

For the stereoselective assay, D-(-)- and L-(+)-lactic acids were separated by an Astec CLC-L (15 cm x 4.6 mm) analytical column (Supelco, USA). 5 mM $CuSO_4$ was used as the mobile phase. The wavelength was monitored at 254 nm. Flow rate of the pump was set at 1 ml/min. The whole system was allowed to equilibrate until a stable baseline was attained in the detector. 20µl of sample was injected. For the standard, D-(-)- lactic acid and L-(+)-lactic acid (Sigma, USA) were used. For the sample, the supernatant was collected and pass through a 0.2 µm filter.

### 2.6.5 Measurement of pH

The pH of medium and solution was determined by an Orion digital pH meter which was calibrated each time before use by standard pH buffer solutions of pH 4.0 and pH 7.01.

## 2.7 Determination of lactic acid tolerance by agar plate method

Nutrient agar plates containing lactic acid were prepared by mixing different proportion of lactic acid solution and nutrient agar solution with the addition of chloramphenicol as antibiotics. The nutrient agar solution was sterilized by autoclave at 121℃ for 20 minutes. Concentration of lactic acid was ranging from 10 to 120 g/L. 50% sodium lactate solution was used.

Inoculum prepared from seed culture was incubated at 37℃ with 270 rpm shaker speed for 6 hours. 20 μl of seed culture was spread on the agar plates. To have a better comparison on the colony forming units, serial dilution of the culture was done. The nutrient agar plates were incubated at 37℃ for 24 hours.

# Chapter Three

## Shake flask studies to optimize fermentation parameters for the production of lactic acid from glucose by recombinant *Bacillus subtilis* 1A304 (Φ105MU331)

## 3.1 Introduction

The performance of a fermentation process is affected by various factors including temperature, pH, dissolved oxygen, inoculum level, and the concentrations of medium components. Since the effects of these factors are very complex with possible interactions among them, they are often characterized by performing experiments. Optimization of a fermentation process needs to consider the productivity and the purity of the product. It is essential to balance the product yield and the duration of the fermentation process, and to consider the compatibility with the industrial scale operations such as low cost substrates and the ease of downstream process.

Fermentation studies on the production of lactic acid by recombinant *Bacillus subtilis* were carried out in shake flasks before using fermentor. The reason was that shake flask experiments were easier to handle and they could be used to characterize the growth patterns of the bacteria. Lactic acid production by recombinant *Bacillus subtilis* 1A304 (Φ105MU331) using glucose as the carbon source was studied.

## 3.2 Selection of UV-induced mutant

The parent strain of recombinant *Bacillus subtilis* 1A304 (Φ105MU331) was provided by Dr. Leung Yun-chung, Thomas. This strain was further modified by Mr.

Ng Chung-kei using classical mutagenesis by UV light (Ng Chung-Kei, 2006). Several mutants (K, L, P, Q, R and T) were selected after mutagenesis and their performance of lactic acid production was studied and compared to the lactic acid production by the parent strain. The mutant strain of recombinant *Bacillus subtilis* that produced the highest concentration of lactic acid was chosen for further investigation.

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 culture was grown in 100 ml BHY medium in 1L flask during 8 hours of growth phase and 40 ml culture was then transferred to 100 ml flask for the production phase. 15 g/L glucose and 5 g/L calcium carbonate were added at the beginning of production phase. The detailed protocol was listed in Chapter 2.

The cell density ($A_{600}$) of six mutants during shake flask fermentation was shown in Figure 3.1. The lactic acid production of six mutants was plotted in Figure 3.2

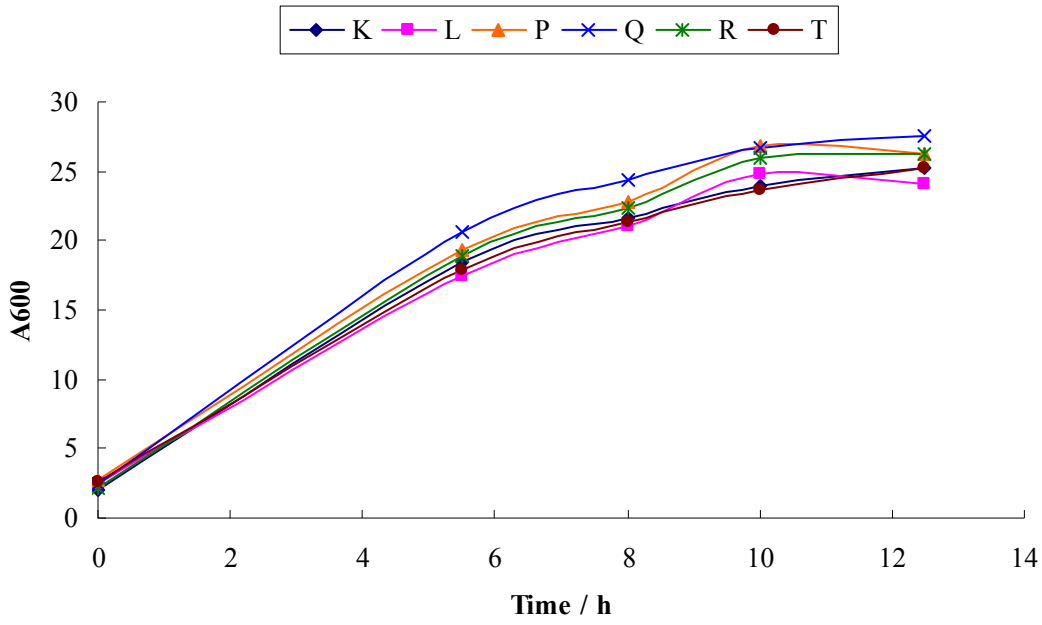



Figure 3.1 Cell density ($A_{600}$) of the six mutant strains of recombinant *Bacillus subtilis* during growth phase and production phase

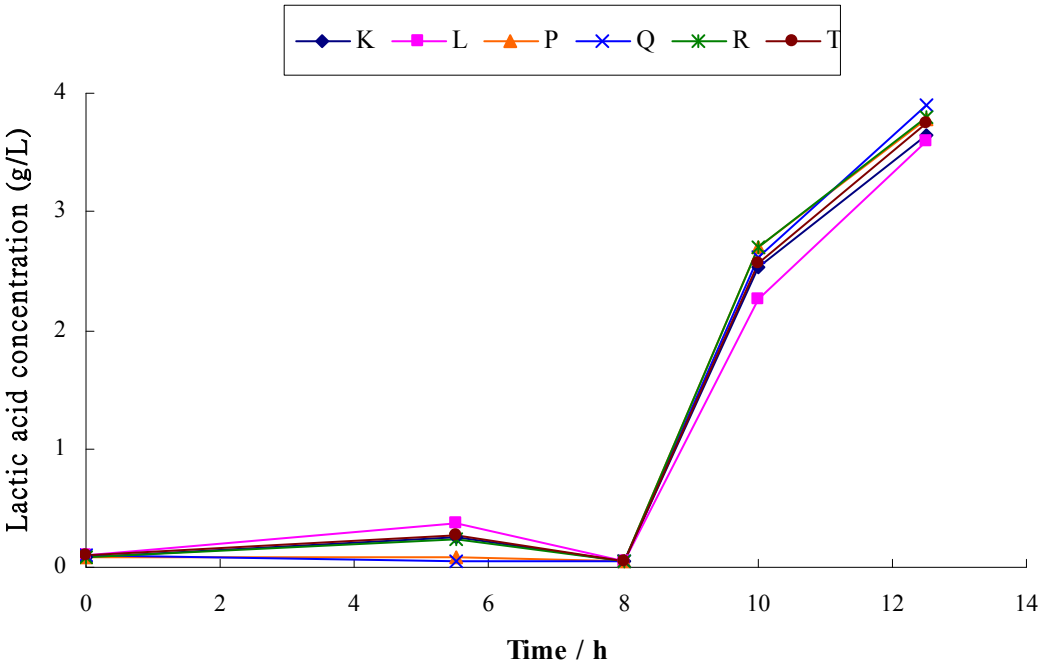



Figure 3.2 Lactic acid concentrations in the culture supernatant of six mutant strains of recombinant *Bacillus subtilis* during growth phase and production phase

The objective of this section was to compare the growth and lactic acid production among the six mutant strains (K, L, P, Q, R and T) of recombinant *Bacillus subtilis* using two stage fermentation. All strains were studied by using glucose as the carbon source. For the first stage fermentation, the cells were grown in aerobic conditions so that the cell growth was enhanced. For the second stage fermentation, the cells were grown under anaerobic conditions which lead to the production of lactic acid.

As shown in Figure 3.1, the cell density of the six mutant strains of recombinant *Bacillus subtilis* increased during 8 hours of the growth phase and remained almost the same during the production phase. The $A_{600}$ value of the mutant strains reached as high as 27.6. During the production phase, the shaking speed and the flask volume was reduced which created the anaerobic environment. The LctE gene of *Bacillus subtilis* was activated under anaerobic conditions and the enzyme lactate dehydrogenase was produced. This enzyme then catalyzed the production of lactic acid from pyruvate.

From Figure 3.2, it showed that the amount of lactic acid produced by the six mutant strains was similar during the production phase. The lactic acid concentration reached as high as 3.9 g/L. Since the mutant strain Q and R produced a higher concentration of

lactic acid, they were chosen to compare with the growth and lactic acid production of the parent strain of recombinant *Bacillus subtilis* 1A304 (Φ105MU331).

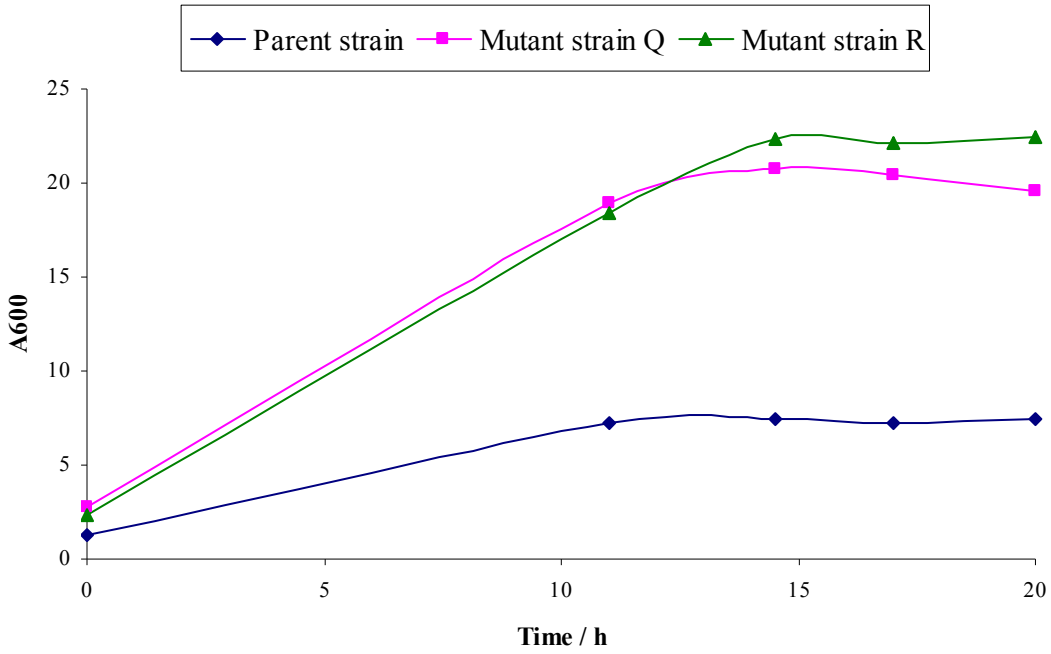



Figure 3.3 Cell density ($A_{600}$) of the parent strain and mutant strains of recombinant *Bacillus subtilis* during growth phase and production phase

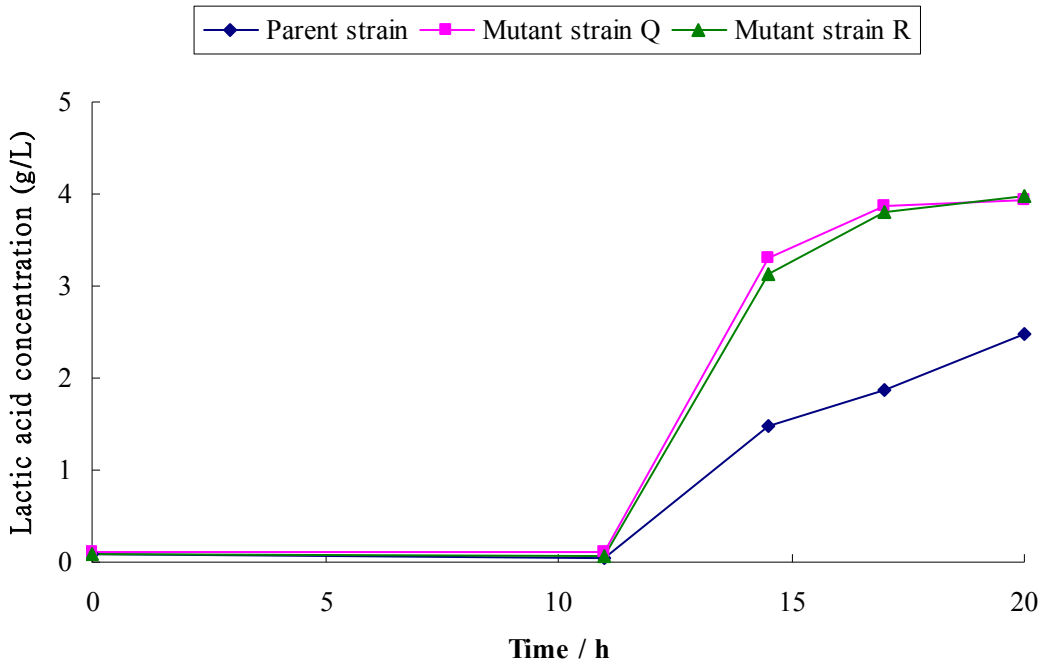



Figure 3.4 Lactic acid concentrations in the culture supernatant of parent strain and mutant strains of recombinant *Bacillus subtilis* during growth phase and production phase

Table 3.1 Glucose utilization rate of the parent strain and mutant strains of recombinant *Bacillus subtilis* during production phase

| Strain | Initial glucose concentration (g/L) | Final glucose concentration (g/L) | Amount of glucose utilized (g/L) | Rate of glucose utilization (g/L/h) (process hours=9 h) |
|---|---|---|---|---|
| Parent | 13.96 | 10.96 | 3.00 | 0.333 |
| Mutant Q | 15.58 | 7.06 | 8.52 | 0.946 |
| Mutant R | 17.24 | 8.58 | 8.66 | 0.962 |

Table 3.2 The lactic acid yield and productivity of the parent strain and mutant strains of recombinant *Bacillus subtilis* during production phase

| Strain | Amount of glucose utilized (g/L) | Highest lactic acid concentration (g/L) | $Y_{LA/GLU}$ (g/g) | Lactic acid productivity (g/L/h) (process hours=9 h) |
|---|---|---|---|---|
| Parent | 3.00 | 2.48 | 0.83 | 0.276 |
| Mutant Q | 8.52 | 3.94 | 0.46 | 0.438 |
| Mutant R | 8.66 | 3.98 | 0.46 | 0.442 |

Figure 3.3 showed that during the 11 hours of growth phase, the cell density of the mutant strain Q and R of recombinant *Bacillus subtilis* were higher than that of the parent stain by more than two times. The highest cell density was obtained in mutant strain R. The graph showed that the initial cell density of three strains were similar. During the production phase, the cell density of mutant strains was higher than the parent strain.

As shown in Figure 3.3, the cell growth of the parent strain and mutant strains were maintained constantly during the production phase. This indicated that changing the environment from aerobic to anaerobic may inhibit the cell growth of *Bacillus subtilis*. Glucose was used as the carbon source to support cell growth during fermentation and it was converted to lactic acid under anaerobic conditions. From Figure 3.4, the lactic acid concentration of the mutant strains was higher than that of the parent strain by

nearly 60 %. One possible reason could be due to the relatively higher cell density of the mutant strains than the parent strain. Mutant strain R was chosen for further studies.

### 3.3 Effect of complex nitrogen sources on lactic acid production by recombinant *Bacillus subtilis*

Nitrogen sources are one of the important factors for the cell growth and the lactic acid production. The effect of different nitrogen sources in the culture medium on lactic acid production was investigated. However, some of them were not completely soluble in the culture medium YE 20 including soy peptone, soy peptone D, corn steep powder and Edamin S. The cell density and dry weight were inaccurate and over-estimated and thus the lactic acid amount was also not satisfactory.

Other soluble nitrogen sources were used for comparison. Brain heart infusion (Oxoid) with 5 g/L yeast extract (BHY) was used as positive control. BHY medium is commonly used for the cultivation of *Bacillus subtilis*. But the cost of this medium is high and it seems not economical for lactic acid production. YE 20 medium is consisted of industrial grade nutrients and the cost of it is much lower than that of BHY medium. 0.2 ml of seed culture of mutant strain R of recombinant *B. subtilis* was inoculated in 100 ml BHY medium (0.2%) in a 1 L flask and was incubated at

37°C with 270 rpm for 12 hours. Then 40 ml culture was transferred into 100 ml flask and incubated at 37°C with 120 rpm. 15g/ L glucose and 5g/L calcium carbonate were added at the beginning of this stage. The cell densities of different flasks were recorded and listed in the Table 3.3.

Table 3.3: Cell density ($A_{600}$) of the mutant strain R of recombinant *B. subtilis* with different nitrogen sources in the medium during production phase

| | $A_{600}$ | | | | |
|---|---|---|---|---|---|
| Time | BHY | YE20 | YE20 + proteose peptone | YE20 + martone | YE20 + casamino acid |
| 0 | 8.76 | 6.64 | 11.16 | 12.68 | 5.97 |
| 3 | 8.64 | 5.83 | 10.84 | 12.76 | 5.79 |
| 6 | 8.64 | 5.84 | 10.28 | 11.92 | 5.68 |
| 9 | 9.04 | 6.07 | 10.2 | 11.36 | 5.72 |

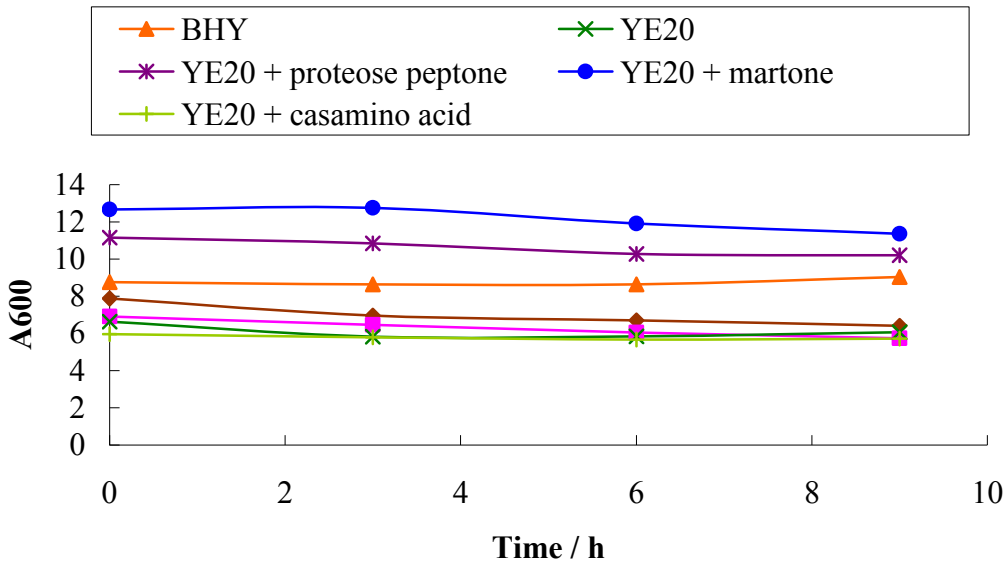



Figure 3.5 Cell density ($A_{600}$) of the mutant strain R of recombinant *B. subtilis* with different nitrogen sources in the medium during production phase

Figure 3.5 showed that the cell density in different combinations of media was quite similar to each other. YE 20 medium with proteose peptone or martone were relatively higher in cell density than the others. The lactic acid concentrations during the production phase were measured and listed in Table 3.4.

Table 3.4: The lactic acid concentration and productivity of the mutant strain R of recombinant *B. subtilis* with different nitrogen sources during production phase

| Lactic acid concentration (g/L) | | | | | |
|---|---|---|---|---|---|
| Time | BHY | YE20 | YE20 + proteose peptone | YE20 + martone | YE20 + casamino acid |
| 0 | 0.07 | 0.406 | 0.21 | 0.4 | 0.98 |
| 3 | 0.86 | 1.714 | 0.72 | 1.05 | 1.432 |
| 6 | 1.82 | 1.242 | 1.3 | 1.51 | 1.568 |
| 9 | 3.38 | 1.266 | 1.68 | 2.05 | 1.386 |
| Lactic acid productivity (g/L/h) | | | | | |
| | 0.376 | 0.141 | 0.187 | 0.228 | 0.154 |

Table 3.4 showed that higher lactic acid concentration was obtained in BHY medium. However, the high cost of the medium prohibits its industrial usage for lactic acid production. YE 20 medium with martone has the highest lactic acid amount as well as production rate among various combinations of YE 20 medium but lower than that of BHY medium. Martone is a kind of complex nitrogen source which is plant-derived protein hydrolysate. Due to its lower cost, YE 20 medium with martone was chosen for further studies.

### 3.4 Effect of cell density and oxygen transfer rate on lactic acid production by recombinant *Bacillus subtilis*

The amount of the cell biomass produced in the growth phase may affect the amount of lactic acid produced in the production phase. The cell biomass is generally directly proportional to the lactic acid produced by the recombinant *Bacillus subtilis.* However, this will also be affected by the environment conditions such as the limiting nutrients in medium and the availability of oxygen. The relationship between the oxygen transfer rate and lactic acid production by recombinant *Bacillus subtilis* was investigated. The oxygen transfer rate was varied by using different culture volumes in flasks. The mutant strain R of recombinant *Bacillus subtilis* was used in the study.

The procedure was done as previously described in Chapter 2 using YE 20 medium with martone. After the growth phase, the cell density was adjusted into the ratio of 0.5: 1: 2 by centrifuging down the cells for 15 min at ~3,000 x g at 4°C before resuspending in 40 ml of the supernatant. The oxygen transfer rate was varied by using three different culture volumes: 40 ml, 60 ml and 80 ml culture medium.

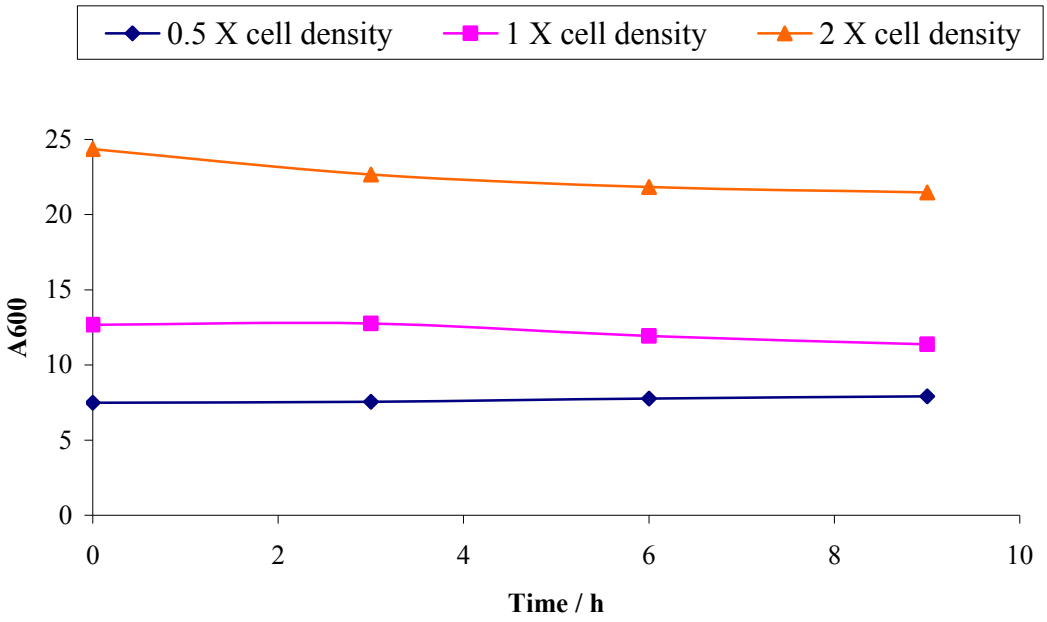



Figure 3.6 Cell density ($A_{600}$) of the mutant strain R using 0.5 X, 1 X and 2 X cell density during production phase

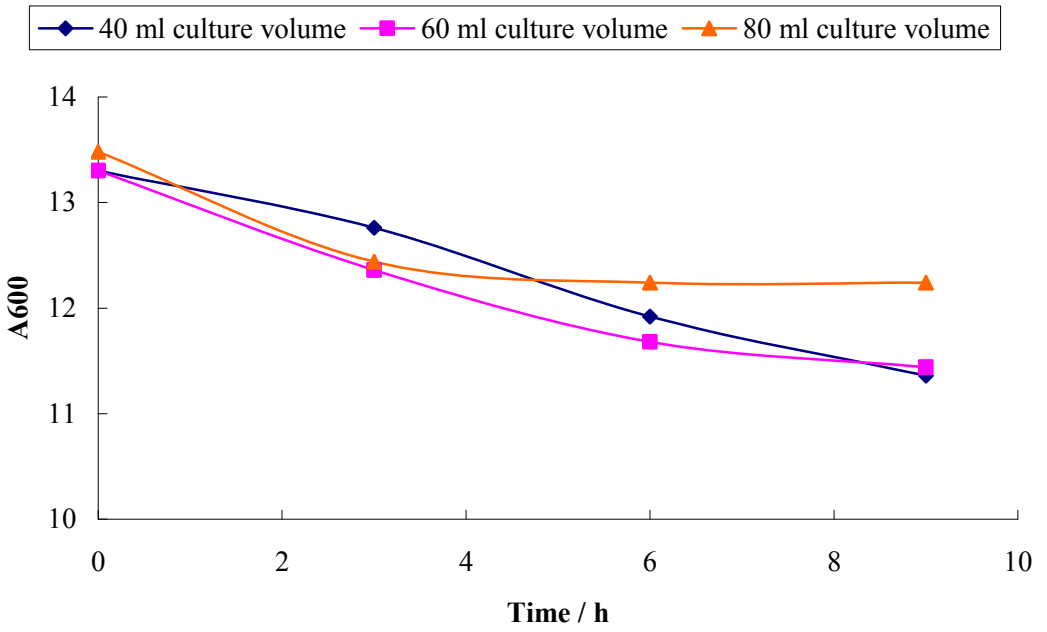



Figure 3.7 Cell density ($A_{600}$) of the mutant strain R using 40 ml, 60 ml and 80 ml culture volumes during production phase

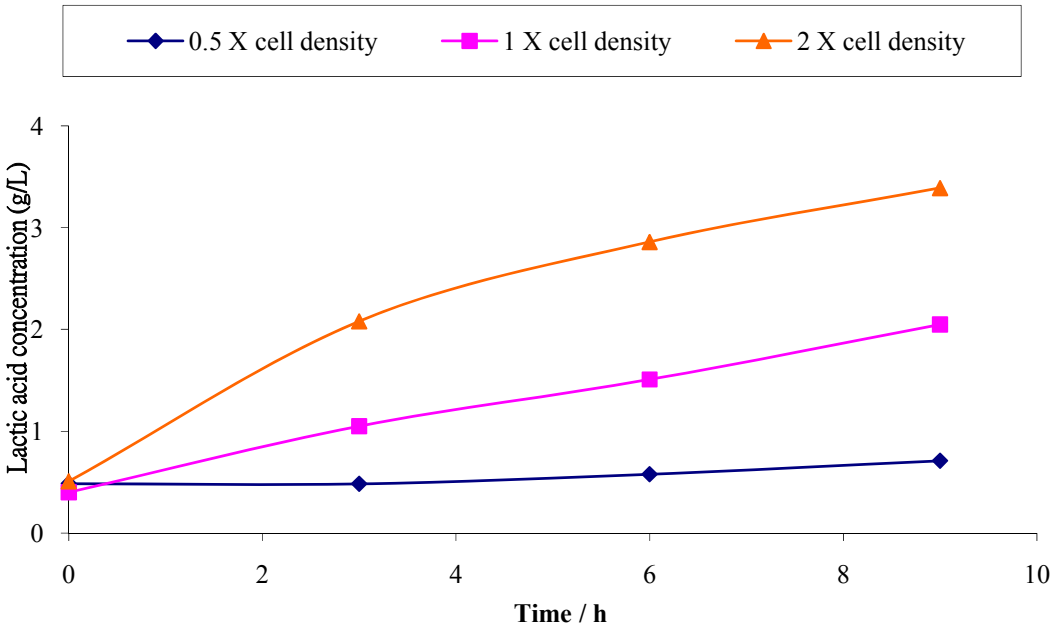



Figure 3.8 Lactic acid concentrations in the culture supernatant of mutant strain R using 0.5 X, 1 X and 2 X cell density during production phase

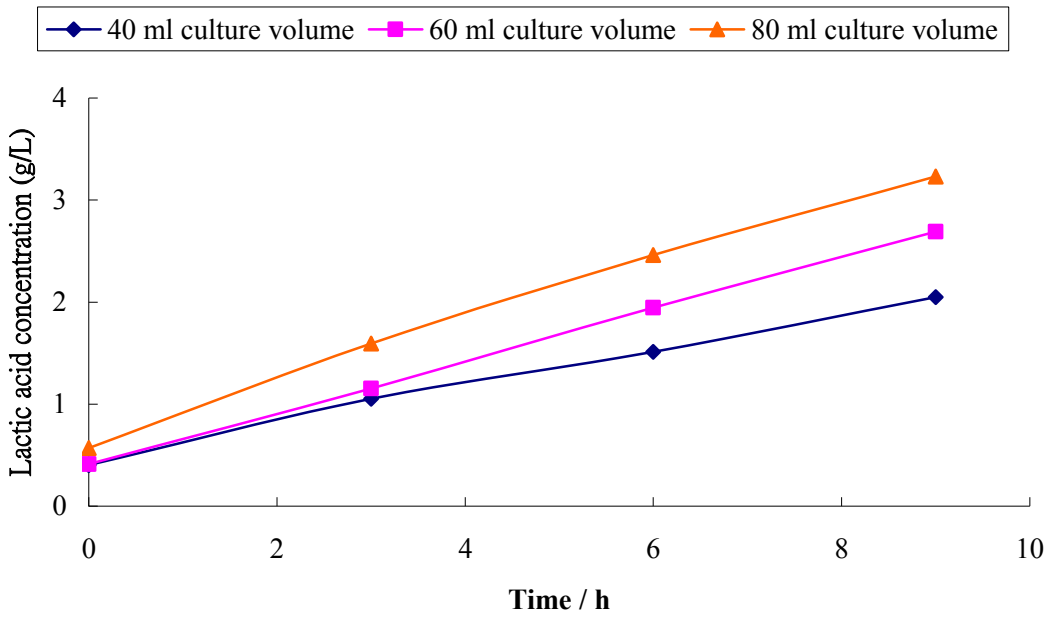



Figure 3.9 Lactic acid concentrations in the culture supernatant of mutant strain R using 40 ml, 60 ml and 80 ml culture volumes during production phase

Table 3.5 Glucose utilization rate of the mutant strain R using different cell densities and culture volumes during production phase

| Flask | Initial glucose concentration (g/L) | Final glucose concentration (g/L) | Amount of glucose utilized (g/L) | Rate of glucose utilization (g/L/h) (process hours=9 h) |
|---|---|---|---|---|
| 0.5 X cell density | 14.76 | 11.30 | 3.46 | 0.38 |
| 1 X cell density | 14.36 | 7.90 | 6.46 | 0.72 |
| 2 X cell density | 15.30 | 7.59 | 7.71 | 0.86 |
| 40 ml culture volume | 14.36 | 7.90 | 6.46 | 0.72 |
| 60 ml culture volume | 14.24 | 8.91 | 5.33 | 0.59 |
| 80 ml culture volume | 14.66 | 10.50 | 4.16 | 0.46 |

Table 3.6 The lactic acid yield and productivity of the mutant strain R using different cell densities and culture volumes during production phase

| Flask | Amount of glucose utilized (g/L) | Highest lactic acid concentration (g/L) | $Y_{LA/GLU}$ (g/g) | Lactic acid productivity (g/L/h) (process hours=9 h) |
|---|---|---|---|---|
| 0.5 X cell density | 3.46 | 0.71 | 0.21 | 0.08 |
| 1 X cell density | 6.46 | 2.05 | 0.32 | 0.23 |
| 2 X cell density | 7.71 | 3.39 | 0.44 | 0.38 |
| 40 ml culture volume | 6.46 | 2.05 | 0.32 | 0.23 |
| 60 ml culture volume | 5.33 | 2.69 | 0.50 | 0.30 |
| 80 ml culture volume | 4.16 | 3.23 | 0.78 | 0.36 |

The objective of this section was to study the relationship between cell density and oxygen transfer rate to the production of lactic acid. The oxygen transfer rate was varied by using three different culture volumes. This is based on the theory that the smaller the interfacial area between air and liquid for gas exchange, the lower would be the oxygen transfer rate. By increasing the culture volume in the flask, the

interfacial area for gas exchange would decrease and thus lowering oxygen transfer rate.

Figure 3.6 showed that the cell density ($A_{600}$) of the mutant strain in 0.5 X, 1 X and 2 X amount of cells remained almost the same during the production phase. Figure 3.8 showed that the lactic acid concentration increased with the increasing cell density from 0.5X (0.71 g/L) to 1X (2.05 g/L) and then to 2X (3.39 g/L). Doubling the cell density (2X) can produce 65% more lactic acid than 1X cell density. The result indicated that lactic acid production by recombinant *Bacillus subtilis* was directly affected by the cell density in the shake flask culture.

Figure 3.7 showed that the cell density ($A_{600}$) of the mutant strain R in 40 ml, 60 ml and 80 ml culture volumes decreased slightly during the production phase. Figure 3.9 showed that increasing the culture volume can enhance the lactic acid concentration. 80ml culture volume (3.23 g/L) also can produce nearly 60% more lactic acid than 40ml culture volume (2.05 g/L). Increasing the culture volume lowered the interface for gaseous exchange and thus lowered the oxygen transfer rate. The result indicated that oxygen limitation or depletion may stimulate more lactic acid production. It is probably due to the shift of respiration mode from aerobic to anaerobic. In aerobic

respiration, glucose is metabolized through glycolysis, Kreb's cycle and finally the electron transport chain. However, in anaerobic respiration, glucose is metabolized to pyruvate during glycolysis and is converted to lactic acid. These two metabolic pathways are mostly determined by the availability of oxygen.

Table 3.5 showed that the highest rate of glucose utilization was 0.86 g/L/h obtained in doubling the cell density in shake flask culture. More cells in the medium can utilize more glucose at the same time for cell growth and lactic acid production. Table 3.6 showed that the highest lactic acid yield was 0.78 obtained in 80 ml culture volume. The result indicated that increasing the culture volume can further enhance the conversion of glucose to lactic acid under anaerobic conditions.

The results indicated that lactic acid production increased with increasing cell density and culture volumes (less oxygen) in shake flask fermentation.

### 3.5 Comparison between one stage and two stage fermentation in cell growth and lactic acid production by recombinant *Bacillus subtilis*

In previous studies, two stage fermentation was used. Cells grow aerobically in the first stage and lactic acid was produced anaerobically in the second stage. This method was used because a higher cell density can be reached before the cells start to produce lactic acid. Cell growth was maintained at 37°C with 270 rpm for 12 h. After 12 h of growth, 40ml culture was transferred into a 100ml flask and incubated at 37°C with 120 rpm. 15 g/L glucose was added at the beginning of production stage. To investigate cell growth and lactic acid production in a single stage, the culture was grown in 70 ml medium in 100 ml flask and agitated at 180 rpm. The two different fermentation strategies were studied in order to choose a suitable strategy for the lactic acid production by recombinant *B. subtilis* mutant strain R. The cell density ($A_{600}$) and lactic acid concentration are shown in Figure 3.10. The zero hour of the two stage fermentation represents the beginning of the production phase after 12 h cultivation in the first stage.

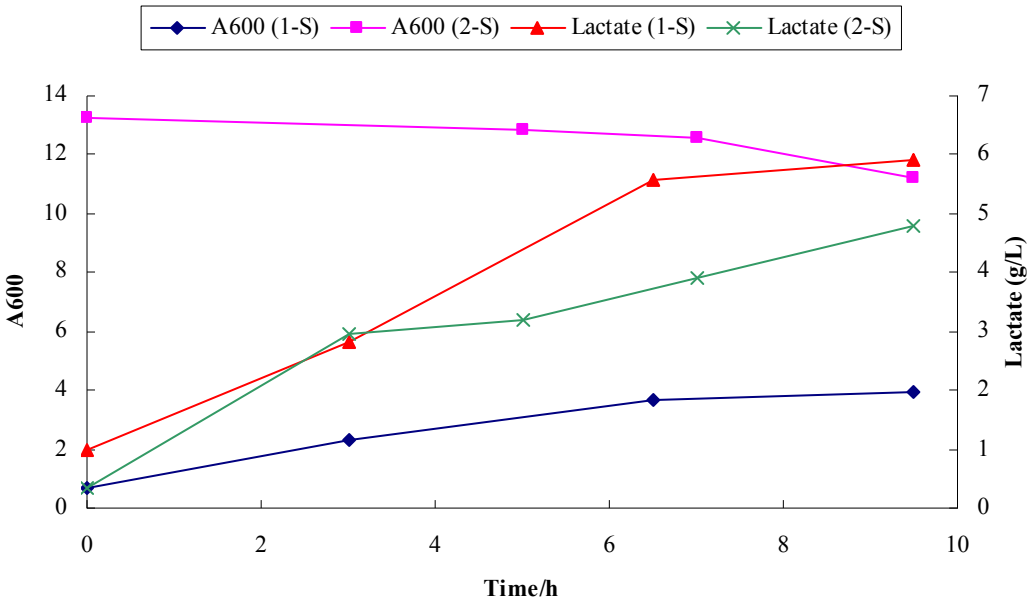



Figure 3.10 Cell density ($A_{600}$) and lactic acid production of recombinant *B. subtilis* mutant strain R in YE 20 medium in one stage (1-S) and two stage (2-S) fermentation

In Figure 3.10, the cell density of the mutant strain R in the two stage fermentation strategy after 9.5 h cultivation was much higher than that of the flask using the one stage fermentation. However, the recombinant *B. subtilis* mutant strain R in the one stage fermentation produced more lactic acid than the two stage fermentation and reached about 6 g/L. The recombinant *B. subtilis* mutant strain R in the two stage fermentation produced less than 5 g/L lactic acid and an extra 12 h was used for growth when compared to the one stage fermentation. The productivity of lactic acid in the two stage fermentation was about 0.50 g/L/h during the production phase whereas the productivity of lactic acid in the one stage fermentation was 0.62 g/L/h.

Higher lactic acid concentration and productivity was produced by using one stage fermentation and it shortened the process time of lactic acid production. Therefore, one stage fermentation was used for further studies in shake flask experiments.

### 3.6 Effect of pH on the cell growth and lactic acid production by recombinant *Bacillus subtilis*

To study the influence of pH on lactic acid production, one stage fermentation was performed with different pH values in the range of pH 5.5 to 8.5, by adjusting the culture medium pH to the required value with addition of 28% $NH_4OH$ or sterilized 2M HCl into the flasks at the start as well as during the fermentation. This is to investigate the effect of pH on cell growth and lactic acid production by *Bacillus subtilis*. The mutant strain R of recombinant *Bacillus subtilis* was used in the study.

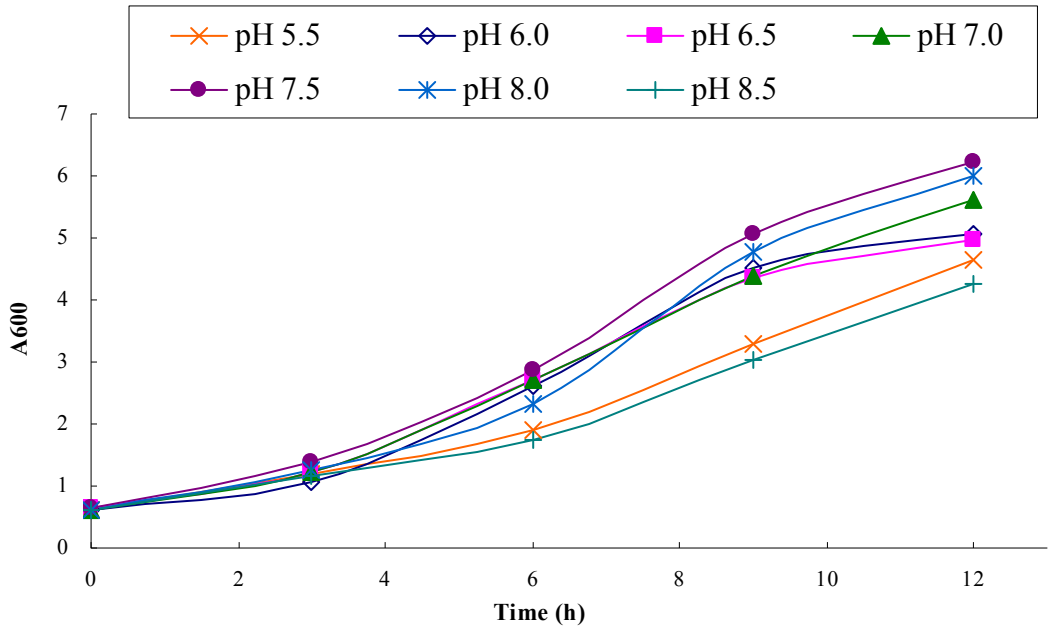



Figure 3.11 Cell density ($A_{600}$) of the mutant strain R grown at different pH in shake-flasks

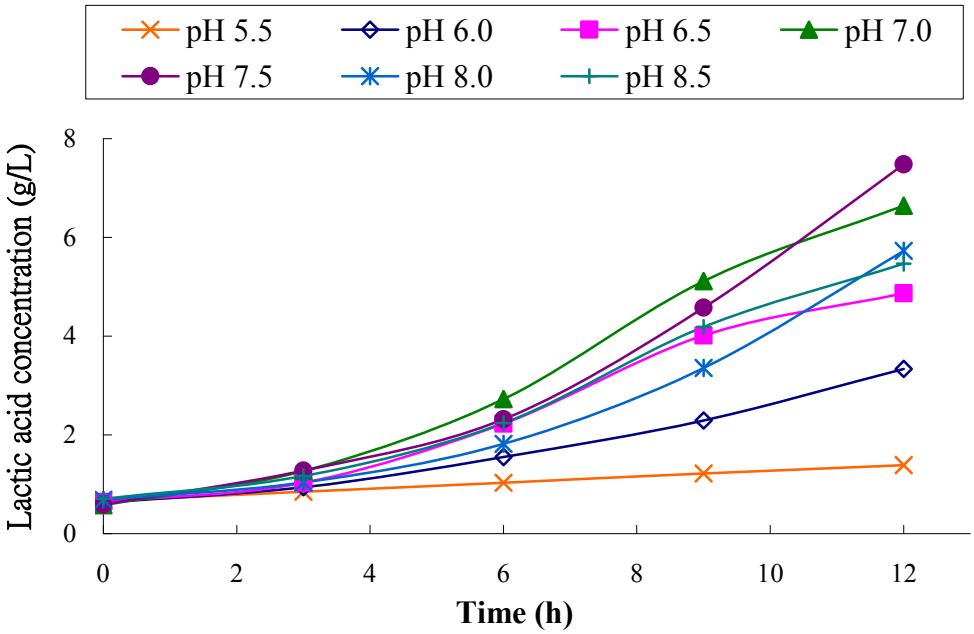



Figure 3.12 Lactic acid concentrations in the culture supernatant of mutant strain R grown at different pH in shake-flasks

Table 3.7 Glucose utilization rate of the mutant strain R grown at different pH in shake-flasks

| Flask | Initial glucose concentration (g/L) | Final glucose concentration (g/L) | Amount of glucose utilized (g/L) | Rate of glucose utilized (g/L/h) (process hours =12 h) |
|---|---|---|---|---|
| pH 5.5 | 19.20 | 6.30 | 12.90 | 1.075 |
| pH 6.0 | 19.20 | 5.43 | 13.77 | 1.148 |
| pH 6.5 | 20.40 | 3.64 | 16.76 | 1.397 |
| pH 7.0 | 20.30 | 2.05 | 18.25 | 1.521 |
| pH 7.5 | 18.20 | 1.94 | 16.26 | 1.355 |
| pH 8.0 | 17.70 | 2.76 | 14.94 | 1.245 |
| pH 8.5 | 19.40 | 4.81 | 14.59 | 1.216 |

Table 3.8 The lactic acid yield and productivity of the mutant strain R grown at different pH in shake-flasks

| Flask | Amount of glucose utilized (g/L) | Highest lactic acid concentration (g/L) | $Y_{LA/GLU}$ (g/g) | Lactic acid productivity (g/L/h) (process hours = 12 h) |
|---|---|---|---|---|
| pH 5.5 | 12.90 | 1.39 | 0.108 | 0.116 |
| pH 6.0 | 13.77 | 3.34 | 0.243 | 0.278 |
| pH 6.5 | 16.76 | 4.87 | 0.291 | 0.406 |
| pH 7.0 | 18.25 | 6.64 | 0.364 | 0.553 |
| pH 7.5 | 16.26 | 7.48 | 0.460 | 0.623 |
| pH 8.0 | 14.94 | 5.73 | 0.384 | 0.478 |
| pH 8.5 | 14.59 | 5.47 | 0.375 | 0.456 |

The objective of this section was to investigate the optimal pH for the growth and lactic acid production of mutant R. Figure 3.11 showed that the highest cell density was obtained at pH 7.5 in the culture medium. The lowest cell density was obtained at pH 5.5 and pH 8.5 in the culture medium. The result indicated that extreme acidic and alkaline pH was not favorable for the cell growth of *B. subtilis* and thus had an inhibitory effect to the growth of mutant R.

Figure 3.12 showed that the highest lactic acid concentration was obtained at pH 7.5 in the culture medium and then followed by pH 7. The amount of lactic acid was 7.48 g/L. When bacteria were growing in an acidic environment, either due to intrinsic pH of culture medium or accumulation of lactic acid, due to acid resistance of the bacteria, lactic acid production would be less efficient as fermentation proceeds (Hyronimus et al, 2000; Akerberg et al, 1998). Therefore at pH 5.5, the lactic acid concentration was 1.39 g/L which was the lowest amount of lactic acid produced.

Table 3.7 showed that the rate of glucose utilization was higher in the pH range of 6.5 to 7.5. Table 3.8 showed that the highest lactic acid yield was 0.46 and the highest productivity was 0.623 g/L/h which were obtained at pH 7.5.

The result showed that the optimal pH for the cell growth and lactic acid production by mutant R in shake flask fermentation was at 7.5.

### 3.7 Optimization of industrial yeast extract and martone concentrations as the nitrogen supply for cell growth and lactic acid production by recombinant *Bacillus subtilis*

Nitrogen is an important source for protein synthesis as well as cell growth and lactic acid production in *Bacillus subtilis.* Two nitrogen sources, industrial yeast extract (YE) and martone with different concentrations were added into the culture medium to study the effect of nitrogen supply for cell growth and lactic acid production in *Bacillus subtilis*. The optimal combination of industrial yeast extract and martone concentration for better cell growth and lactic acid production was investigated.

One stage fermentation was done as previously described in Chapter 2 using YE 20 medium. A series of combinations of 0 to 30 g/L industrial yeast extract Hy-Yest™ 444 (Kerry, Ireland) and martone B-1 (Marcor, USA) was added into YE 20 medium with 20 g/L glucose, 1.5 g/L $KH_2PO_4$, 1.5 g/L $K_2HPO_4$, 6 g/L $(NH_4)_2SO_4$, , 0.03 g/L $MgCl_2$ and 0.08 g/L $MgSO_4 \cdot 7H_2O$ and 2.3 ml/L microelement solution. The mutant strain R of recombinant *Bacillus subtilis* was used in the study.

Table 3.9 Combinations of industrial yeast extract and martone with different concentrations added to the culture medium in shake-flasks

| Flask | Industrial Yeast Extract (g/L) | Martone (g/L) |
|---|---|---|
| 1 | 20 | 30 |
| 2 | 0 | 0 |
| 3 | 20 | 0 |
| 4 | 20 | 5 |
| 5 | 20 | 10 |
| 6 | 20 | 20 |
| 7 | 0 | 30 |
| 8 | 5 | 30 |
| 9 | 10 | 30 |
| 10 | 30 | 30 |

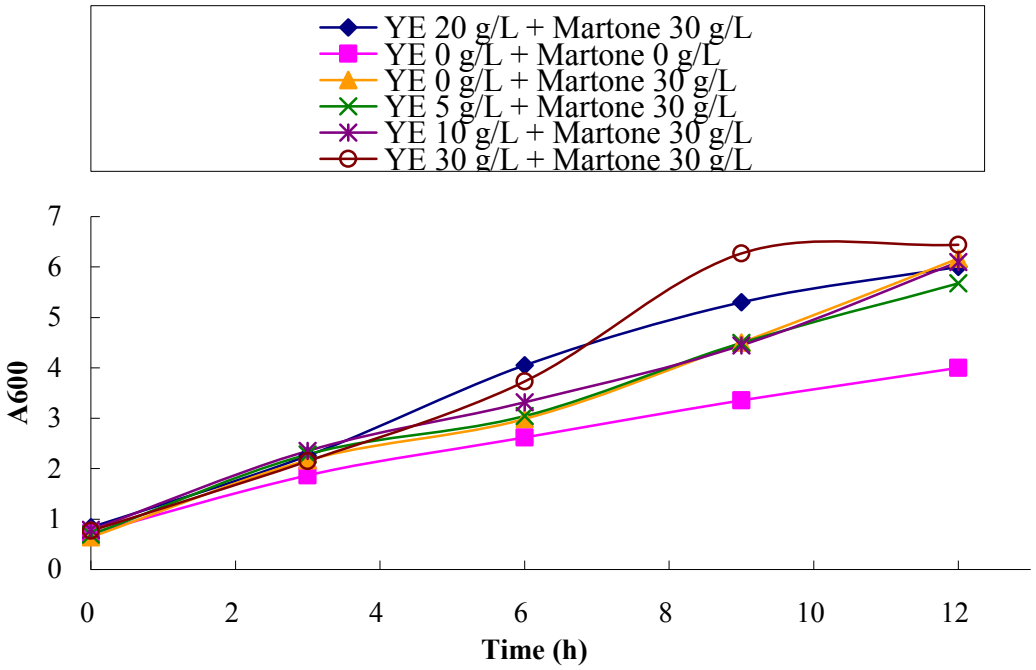



Figure 3.13 Cell density ($A_{600}$) of the mutant strain R grown with different industrial yeast extract concentrations in shake-flasks

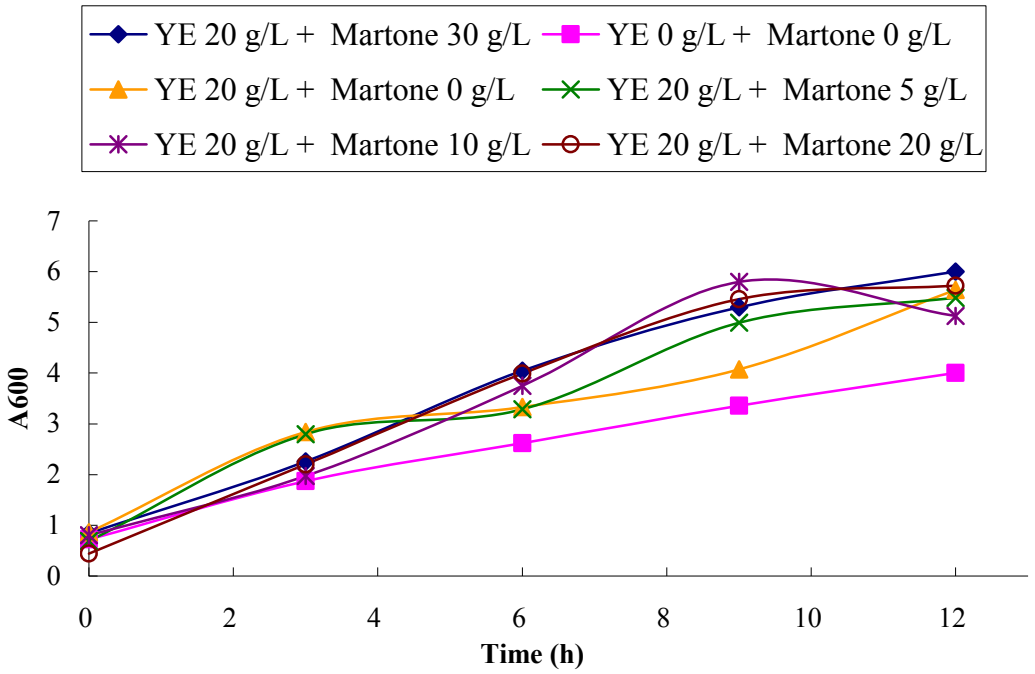



Figure 3.14 Cell density ($A_{600}$) of the mutant strain R grown with different martone concentrations in shake-flasks

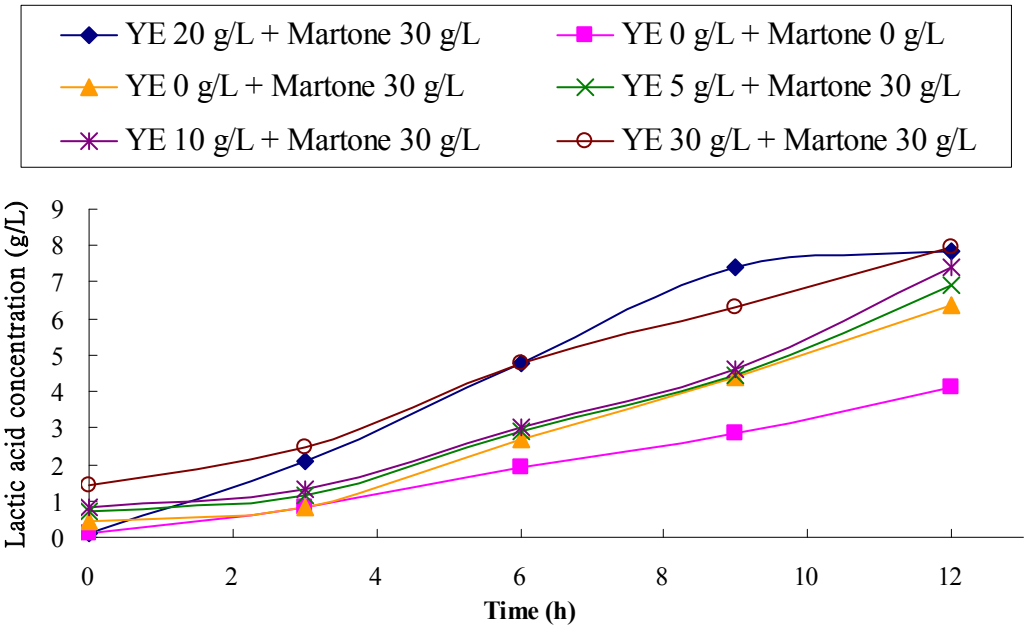



Figure 3.15 Lactic acid concentrations in the culture supernatant of mutant strain R grown with different industrial yeast extract concentrations in shake-flasks

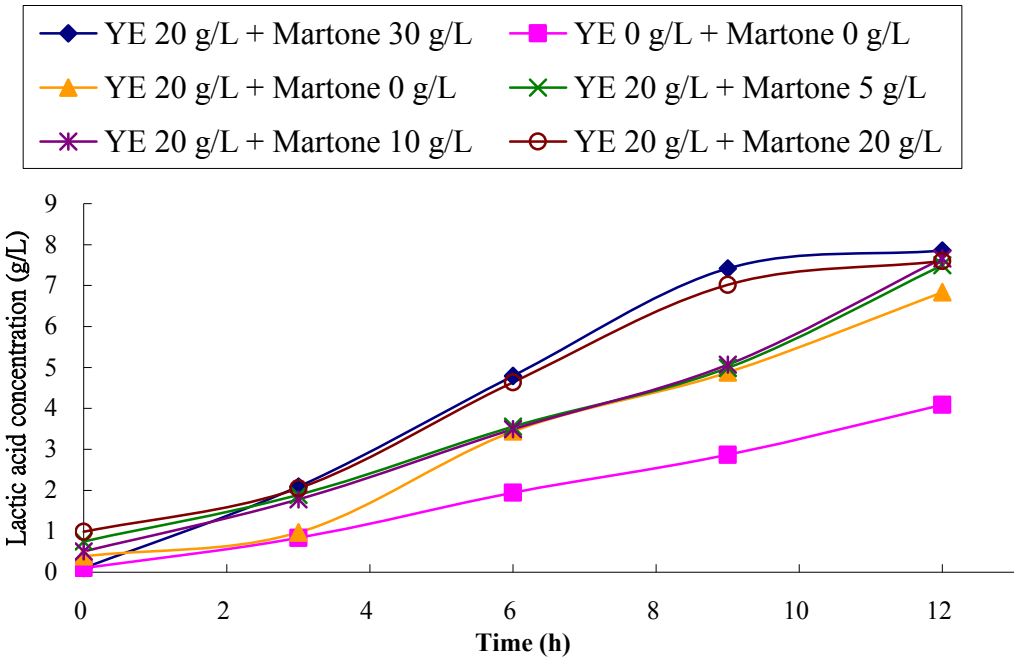



Figure 3.16 Lactic acid concentrations in the culture supernatant of mutant strain R grown with different martone concentrations in shake-flasks

Table 3.10 Glucose utilization rate of the mutant strain R grown in different industrial yeast extract and martone concentrations

| Flask | Initial glucose concentration (g/L) | Final glucose concentration (g/L) | Amount of glucose utilized (g/L) | Rate of Glucose utilized (g/L/h) (process hours = 12 h) |
|---|---|---|---|---|
| 20 g/L YE + 30 g/L Martone | 19.14 | 1.49 | 17.65 | 1.471 |
| 0 g/L YE + 0 g/L Martone | 19.4 | 3.17 | 16.23 | 1.353 |
| 20 g/L YE + 0 g/L Martone | 17.9 | 7.34 | 10.56 | 0.880 |
| 20 g/L YE + 5 g/L Martone | 17.7 | 7.08 | 10.62 | 0.885 |
| 20 g/L YE + 10 g/L Martone | 16.98 | 1.29 | 15.69 | 1.308 |
| 20 g/L YE + 20 g/L Martone | 18.7 | 2.23 | 16.47 | 1.373 |
| 0 g/L YE + 30 g/L Martone | 18.1 | 9.18 | 8.92 | 0.743 |
| 5 g/L YE + 30 g/L Martone | 18.1 | 9.16 | 8.94 | 0.745 |
| 10 g/L YE + 30 g/L Martone | 17.14 | 8.18 | 8.96 | 0.747 |
| 30 g/L YE + 30 g/L Martone | 19.0 | 1.6 | 17.4 | 1.450 |

Table 3.11 The lactic acid yield and productivity of the mutant strain R grown in different industrial yeast extract and martone concentrations

| Flask | Amount of glucose utilized (g/L) | Highest lactic acid concentration (g/L) | $Y_{LA/GLU}$ (g/g) | Lactic acid productivity (g/L/h) (process hours = 12 h) |
|---|---|---|---|---|
| 20 g/L YE + 30 g/L Martone | 17.65 | 7.86 | 0.445 | 0.655 |
| 0 g/L YE + 0 g/L Martone | 16.23 | 4.09 | 0.252 | 0.341 |
| 20 g/L YE + 0 g/L Martone | 10.56 | 6.84 | 0.648 | 0.570 |
| 20 g/L YE + 5 g/L Martone | 10.62 | 7.50 | 0.706 | 0.625 |
| 20 g/L YE + 10 g/L Martone | 15.69 | 7.66 | 0.488 | 0.638 |
| 20 g/L YE + 20 g/L Martone | 16.47 | 7.60 | 0.461 | 0.633 |
| 0 g/L YE + 30 g/L Martone | 8.92 | 6.34 | 0.711 | 0.528 |
| 5 g/L YE + 30 g/L Martone | 8.94 | 6.92 | 0.774 | 0.577 |
| 10 g/L YE + 30 g/L Martone | 8.96 | 7.40 | 0.826 | 0.617 |
| 30 g/L YE + 30 g/L Martone | 17.4 | 7.93 | 0.456 | 0.661 |

The objective of this section was to optimize the concentrations of industrial yeast extract and martone in the culture medium. Figure 3.13 and Figure 3.14 shows that the growth of recombinant *B. subtilis* mutant R increased with increasing concentrations of industrial yeast extract and martone. The cell density of mutant R was the lowest in the culture medium without the addition of industrial yeast extract and martone. The results indicated that organic nitrogen source was important for the cell growth of mutant R.

Figure 3.15 and figure 3.16 showed that the lactic acid concentration increased with increasing concentration of industrial yeast extract and martone. The highest lactic acid concentration was produced at 30 g/L industrial yeast and 30 g/L martone. Industrial yeast extract and martone are complex nitrogen sources of proteins and amino acids supplemented with vitamin B-complex and carbohydrates. High supplementation of growth factors such as vitamin B complex has positive effect on lactic acid production (Altaf et al, 2005).

Table 3.10 showed that the highest rate of glucose utilization was 1.471 g/L/h obtained at 20 g/L industrial yeast and 30 g/L martone. Table 3.11 showed that the highest lactic acid productivity was 0.661 g/L/h at 30 g/L industrial yeast and 30 g/L

martone. In order to lower the cost of lactic acid production, it is important to minimize the amount of expensive nutrients used for optimal performance. Among all the combinations of industrial yeast extract and martone concentration tested, the combinations of 20 g/L industrial yeast extract and 30 g/L martone concentration can produce a comparable high lactic acid concentration and productivity. Therefore, 20 g/L industrial yeast extract and 30 g/L martone in YE 20 medium was chosen for further studies.

### 3.8 Effect of inoculum size on the cell growth and lactic acid production by recombinant *Bacillus subtilis*

The inoculum size of a culture may affect the physiological state of cells. The cells will be affected by the growth environment and lag phase may occur when the cells are not physiologically active. The effect of inoculum size on lag phase may be due to an inhibitory effect of high cell concentrations which greatly reduce the oxygen tension or the effects at low cell concentrations. These will also affect the performance of lactic acid production. Therefore, the effect of initial inoculum size on the cell growth and lactic acid production by *Bacillus subtilis* was investigated. The optimal inoculum size which can enhance the lactic acid production was determined. The mutant strain R of recombinant *Bacillus subtilis* was used in the study.

Different size of inoculum (0.5%, 1%, 2% and 5%) was inoculated in 70 ml YE20 medium in a 100 ml flask and was agitated at 37°C with 180 rpm for 12 hours. The pH value was maintained at 7.5 by 28% $NH_4OH$

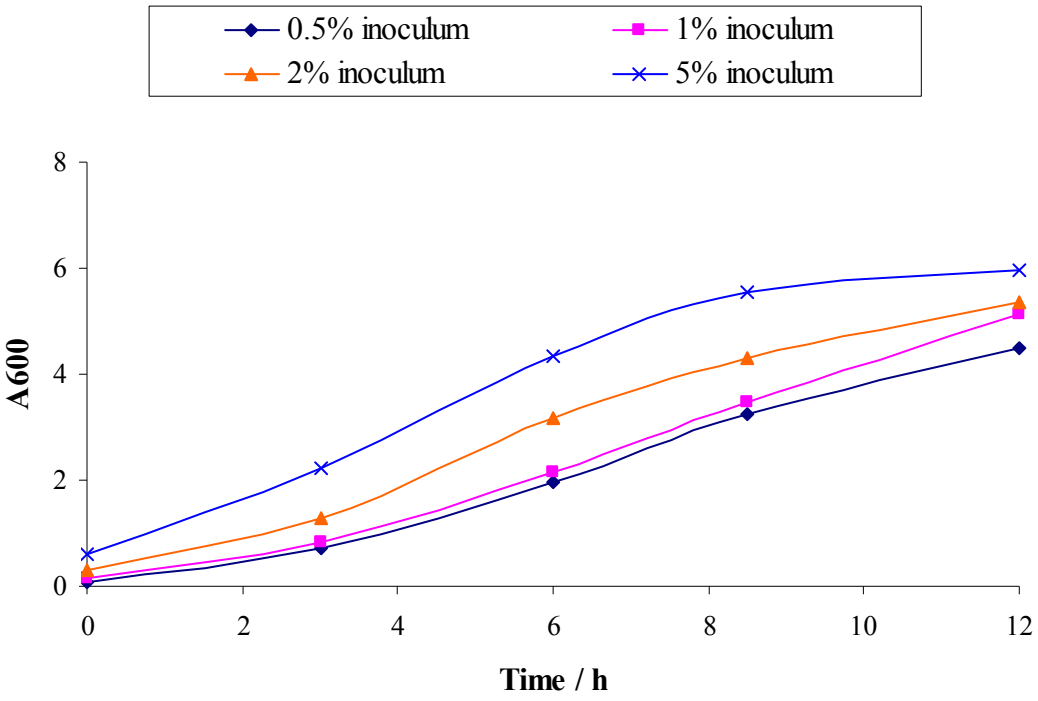



Figure 3.17 Cell density ($A_{600}$) of mutant strain R using different size of inoculum in shake-flasks

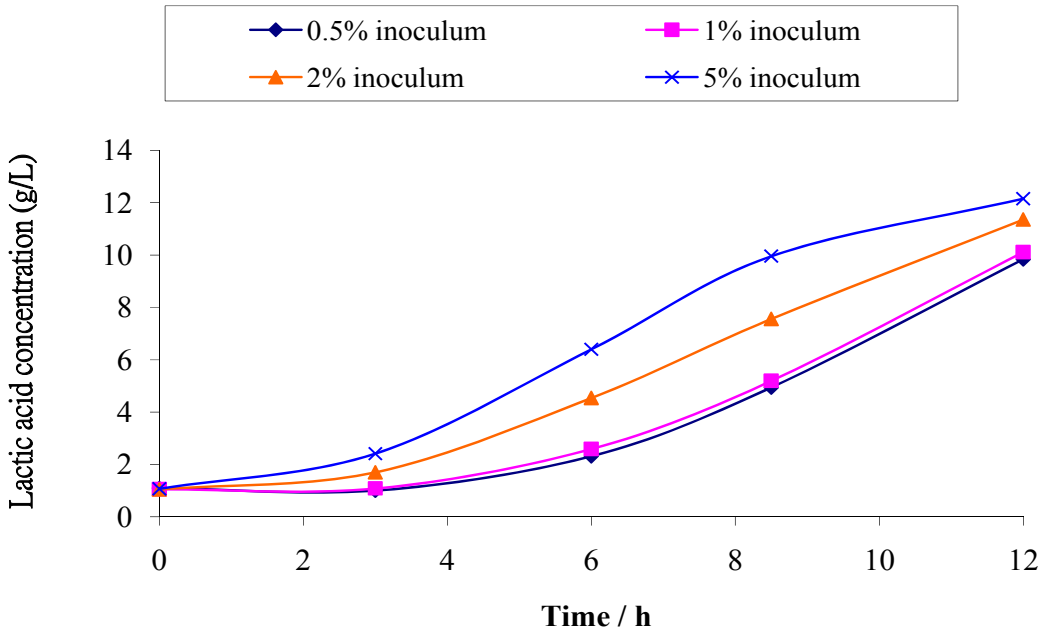



Figure 3.18 Lactic acid concentrations in the culture supernatant of the mutant strain R using different size of inoculum in shake-flasks

Table 3.12 Glucose utilization rate of the mutant strain R using different size of inoculum

| Flask | Initial glucose concentration (g/L) | Final glucose concentration (g/L) | Amount of glucose utilized (g/L) | Rate of Glucose utilized (g/L/h) (process hours = 12 h) |
|---|---|---|---|---|
| 0.5% inoculum | 11.78 | 2.52 | 9.26 | 0.772 |
| 1% inoculum | 11.38 | 2.56 | 8.82 | 0.735 |
| 2% inoculum | 11.26 | 0.40 | 10.86 | 0.905 |
| 5% inoculum | 11.52 | 0.48 | 11.04 | 0.920 |

Table 3.13 The lactic acid yield and productivity of the mutant strain R using different industrial yeast extract and martone concentrations

| Flask | Amount of glucose utilized (g/L) | Highest lactic acid concentration (g/L) | $Y_{LA/GLU}$ (g/g) | Lactic acid productivity (g/L/h) (process hours = 12 h) |
|---|---|---|---|---|
| 0.5% inoculum | 9.26 | 9.84 | 1.063 | 0.82 |
| 1% inoculum | 8.82 | 10.12 | 1.147 | 0.84 |
| 2% inoculum | 10.86 | 11.36 | 1.046 | 0.95 |
| 5% inoculum | 11.04 | 12.16 | 1.101 | 1.01 |

The objective of this section was to study the effect of inoculum size on cell growth and lactic acid production. Figure 3.17 showed that increasing the inoculum size can increase the cell growth of mutant R. The highest cell density was obtained at 5% inoculum. Figure 3.18 showed that increasing the inoculum size can enhance the lactic acid production by mutant R. As higher inoculum size would give a higher cell population in the culture at the initial stage of fermentation, the increase in cell growth would be higher and more cells were available for lactic acid production. The results indicated that there was a direct relationship between the lactic acid production and the initial inoculum size.

Table 3.12 showed that the rate of glucose utilization was higher in 2% and 5% inoculum. Table 3.13 showed that the highest lactic acid yield and the highest productivity were obtained with 5% inoculum.

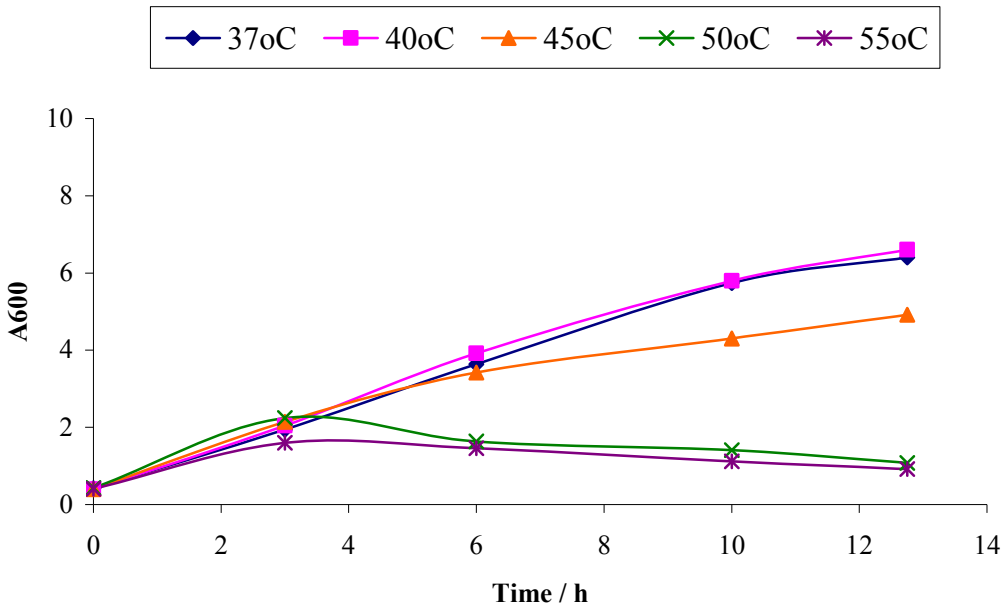
The result showed that higher lactic acid concentration and productivity was obtained with 5% inoculum. Therefore, the optimal inoculum size was 5% in one stage shake-flask fermentation.

### 3.9 Effect of cultivation temperature on lactic acid production by recombinant *Bacillus subtilis*

Temperature is a fundamental parameter affecting microbial activity and it is important to distinguish between the temperature requirements and the temperature tolerance of an organism. The temperature at which its enzymes can function properly is usually related to the growth temperature of an organism. *B. subtilis* is able to sustain growth in the temperature range from approximately 11°C (Nichols *et al.*, 1995) to 52°C (Holtmann & Bremer, 2004). In this study, the effect of temperature on the cell growth and production of lactic acid by mutant strain R of recombinant *Bacillus subtilis* was investigated.

Firstly, some preliminary studies were done by comparison of the lactic acid production by *B. subtilis* mutant R at 25°C, 30°C and 37°C and found that the amount of lactic acid produced at 37°C is better than that at the other two temperatures. Therefore further increase of the temperature was used to analyze the effect on the lactic acid production by *B. subtilis*. The mutant strain R of recombinant *Bacillus subtilis* was inoculated in flask to the final concentration of 5% and agitated at 37°C, 40°C, 45°C, 50°C, 55°C in YE 20 medium with 20 g/L glucose and with 180 rpm for 12 hours. The pH value was maintained at 7.5 by 28% $NH_4OH$. The cell density and corresponding cell dry weight of each flask were measured and shown in the Figure 3.19 (a) and Figure 3.19 (b).

(a)



(b)

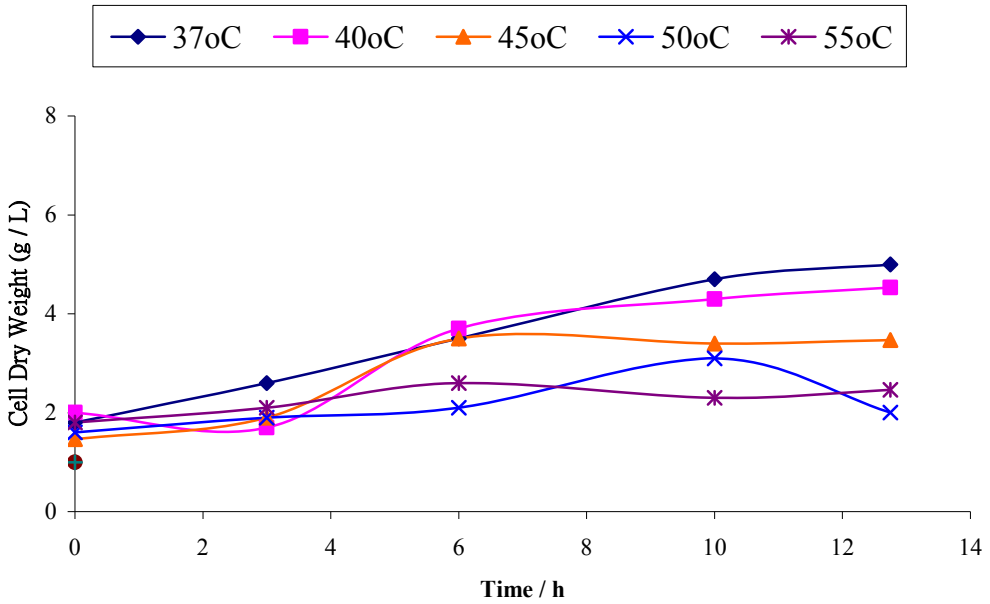



Figure 3.19 Recombinant *B. subtilis* mutant R was cultivated at different temperatures, 180 rpm and produced lactic acid in YE 20 medium containing 2% glucose. The cell growth (a) and cell dry weight (b) were monitored during the fermentation.

Figure 3.19 (a) and (b) showed that the temperature for better cell growth was 37℃ and 40℃. However, the cell growth was poor when the temperature exceeded 50℃. It may be due to the physiological enzymes that do not work well at high temperatures. High cell density may not result in higher lactic acid production. Further measurement of the lactic acid content in each sampling point was done and shown in Figure 3.20.

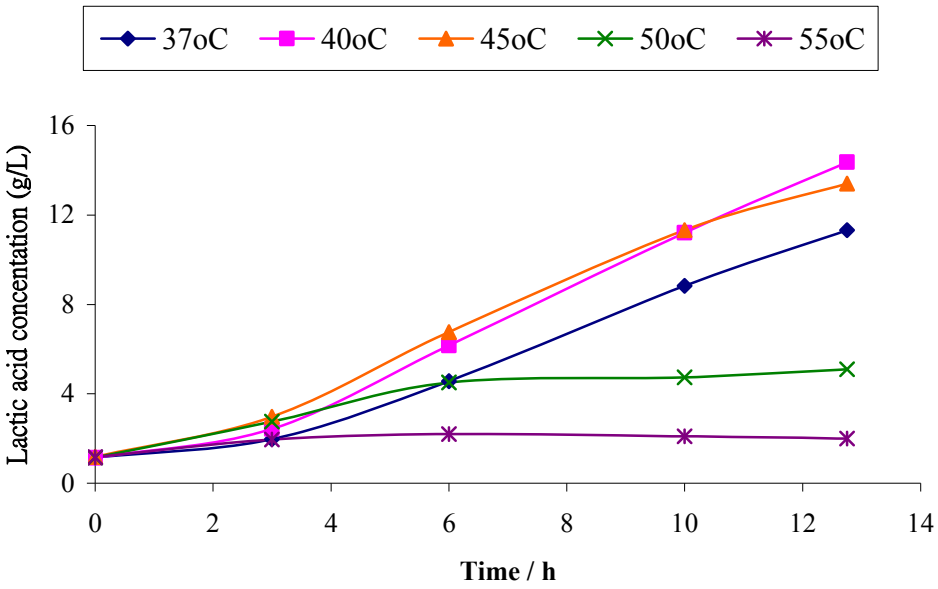



Figure 3.20 Lactic acid concentrations in the culture supernatant of mutant strain R grown at different temperatures in shake-flasks

Table 3.14 Glucose utilization rate of the mutant strain R grown at different temperatures

| Flask | Initial glucose concentration (g/L) | Final glucose concentration (g/L) | Amount of glucose utilized (g/L) | Rate of glucose utilization (g/L/h) (process hours=12.75 h) |
|---|---|---|---|---|
| 37°C | 20.5 | 6.44 | 14.06 | 1.103 |
| 40°C | 20.5 | 4.20 | 16.30 | 1.278 |
| 45°C | 21.0 | 3.80 | 17.20 | 1.349 |
| 50°C | 20.4 | 13.00 | 7.40 | 0.580 |
| 55°C | 21.0 | 16.50 | 4.50 | 0.353 |

Table 3.15 The lactic acid yield and productivity of the mutant strain R grown at different temperatures

| Flask | Amount of glucose utilized (g/L) | Highest lactic acid concentration (g/L) | $Y_{LA/GLU}$ (g/g) | Lactic acid productivity (g/L/h) (process hours=12.75 h) |
|---|---|---|---|---|
| 37°C | 14.06 | 11.32 | 0.81 | 0.888 |
| 40°C | 16.30 | 14.36 | 0.88 | 1.126 |
| 45°C | 17.20 | 13.40 | 0.78 | 1.051 |
| 50°C | 7.40 | 5.09 | 0.69 | 0.399 |
| 55°C | 4.50 | 2.00 | 0.44 | 0.157 |

The primary habitat of the Gram-positive bacterium *Bacillus subtilis* is the upper layer of the soil. Within this situation, *B. subtilis* experiences a wide variety of environmental temperature changes. *B. subtilis* produced lactic acid under anaerobic conditions and it was found that lactic acid can be produced within a wide range of temperatures. Figure 3.20 showed that the best temperature for the lactic acid production by recombinant *B. subtilis* mutant R was 40℃. Table 3.14 showed that the rate of glucose utilization was higher at 40℃ and 45℃. The lowest glucose utilization rate was obtained at 55℃. Table 3.15 showed that the highest lactic acid yield was 0.88 and the highest productivity was 1.126 g/L/h at 40℃.

The result showed that higher lactic acid concentration and productivity was obtained at the cultivation temperature of 40℃. Therefore, 40℃ was used in further studies of lactic acid production by *B. subtilis* in the fermenter.

# Chapter Four

# Fermentor studies to optimize the production of lactic acid from glucose by recombinant *Bacillus subtilis* 1A304 (Φ105MU331)

## 4.1 Introduction

Lactic acid production in shake flask fermentation was carried out and several parameters were optimized. However, the amount of lactic acid produced was still inadequate due to the limitation of the control and the volume in shake flask fermentation. Scale up of lactic acid production in fermentor was performed in order to find out the optimal operating conditions in lactic acid production which will be required for large-scale production.

The purpose of using a fermentor is to provide a stable and optimal environment for microbes in which they can reproduce and yield a desirable substance. When a suitable microorganism has been chosen, the pH, temperature, dissolved oxygen and nutrient concentrations that would give that microorganism optimal growth and product production rates are selected.

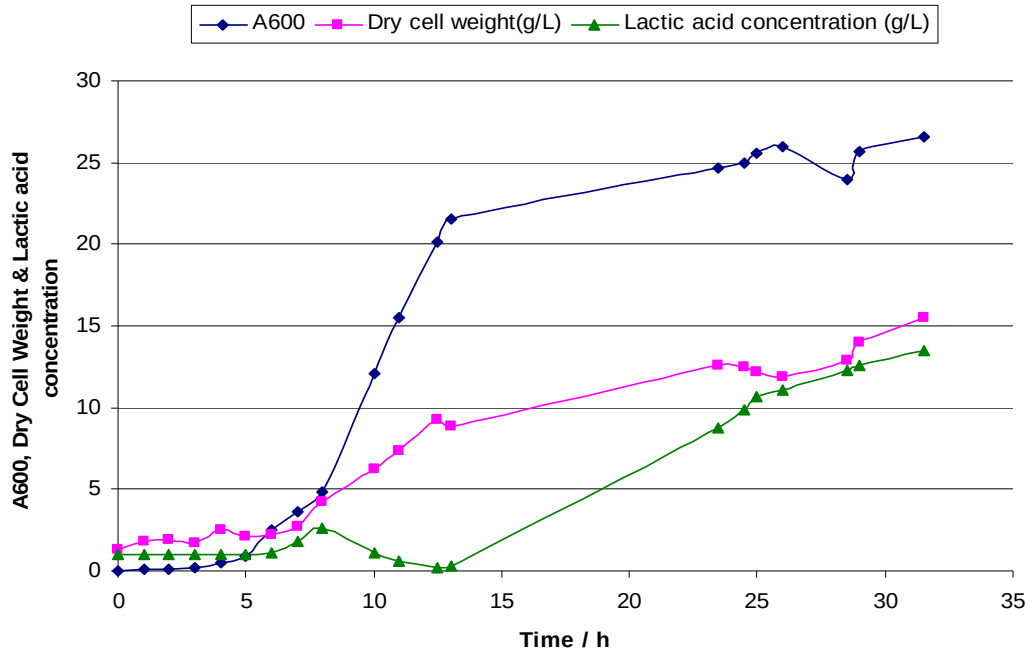
In order to maximize lactic acid production, the selection of fermentation strategy and optimization of controlled fermentation conditions are necessary. In this chapter, the study of fed-batch lactic acid production in the fermentor was described. Lactic acid production by recombinant *Bacillus subtilis* 1A304 (Φ105MU331) using glucose as the carbon source was studied.

## 4.2 Comparison between one stage and two stage fermentation in cell growth and lactic acid production by recombinant *Bacillus subtilis*

In some fermentation process, product yield is directly proportional to the cell density. The fermentation process may also divide into growth phase and production phase. The high cell density is achieved in the growth phase and the product is induced and produced in the production phase. It can prevent the product inhibition effect on growth if the product is toxic to the cells. Two different fermentation strategies were used in lactic acid production by *B. subtilis* mutant strain R, one is a two stage fermentation and the other is a one stage fermentation.

0.5 % inoculum was inoculated in 1L YE20 medium in a 2 L fermentor. The medium was kept at 37°C. DO was controlled at 30% by changing agitation speed from 400 rpm to 1300 rpm. Air flow was 4 L/min. The pH was controlled by 2 M HCL and 5 M NaOH. After about 12 h of growth, glucose was fed each hour into the fermentor. The agitation speed was set at 400 rpm and the air flow was reduced to 2 L/min in order to induce the lactic acid production by partial anaerobic conditions. Two trials were used to determine the lactic acid yield of the *B. subtilis* mutant strain R. Samples were collected to measure the cell density ($A_{600}$), cell dry weight and lactic acid concentration and were shown in Figure 4. 1.

A)




B)

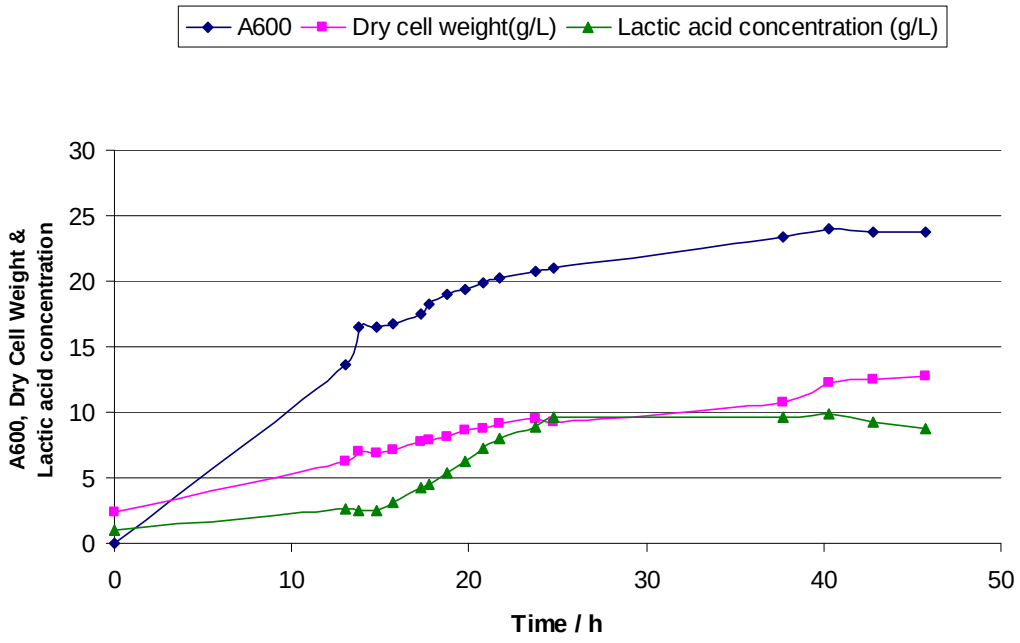
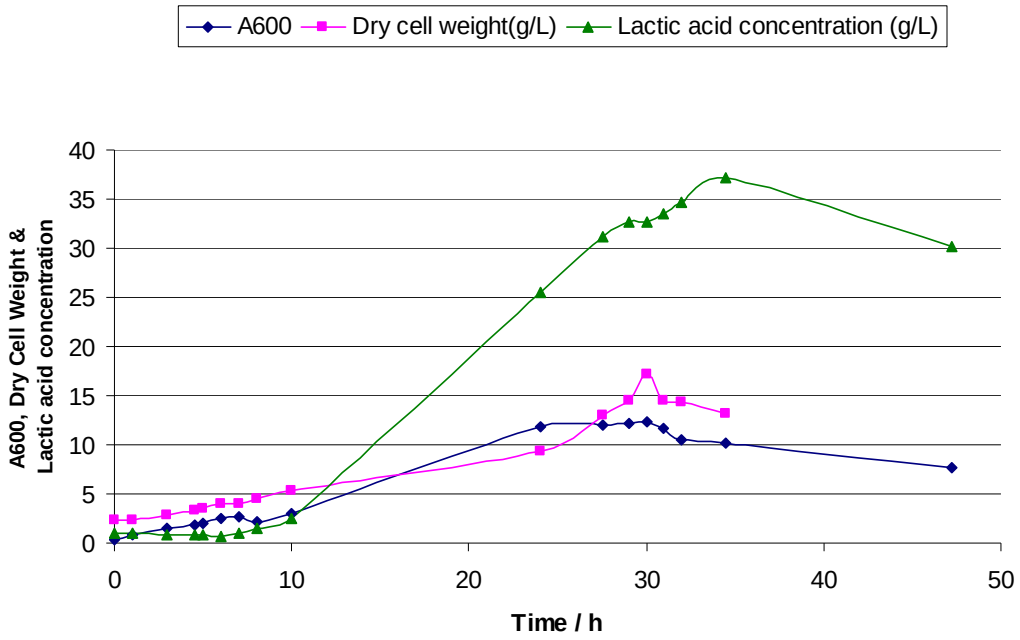



Figure 4.1 Cell density ($A_{600}$), cell dry weight and lactic acid concentration of *B. subtilis* mutant strain R during the two stage fed-batch fermentation. The cells were cultivated at 37°C. Similar conditions were used in the two trials A and B.

The lactic acid content in both fermentation batches shown in Figure 4.1 was about 10-13.5 g/L after 40 h of fermentation. The lactic acid yield was similar to that of shake flask fermentation, although the cell density was higher. The higher cell density did not enhance the lactic acid production in the two stage fermentation.

A one stage fermentation in lactic acid production was studied. The growth and lactic acid production were carried on simultaneously. Inoculum was inoculated in 1 L YE20 medium in a 2 L fermentor. The medium was kept at 37°C and pH 7.5. DO was not controlled and the agitation speed was set at 400 rpm. Air flow was 2 L/min. The pH was controlled by 2M HCL and 28% $NH_4OH$. Glucose was fed each hour into the fermentor in order to maintain 20g/L glucose level. Again, two trials were used to determine the lactic acid yield of the *B. subtilis* mutant strain R. Samples were collected to measure cell density ($A_{600}$), cell dry weight and lactic acid concentration and were shown in Figure 4.2.

A)




B)

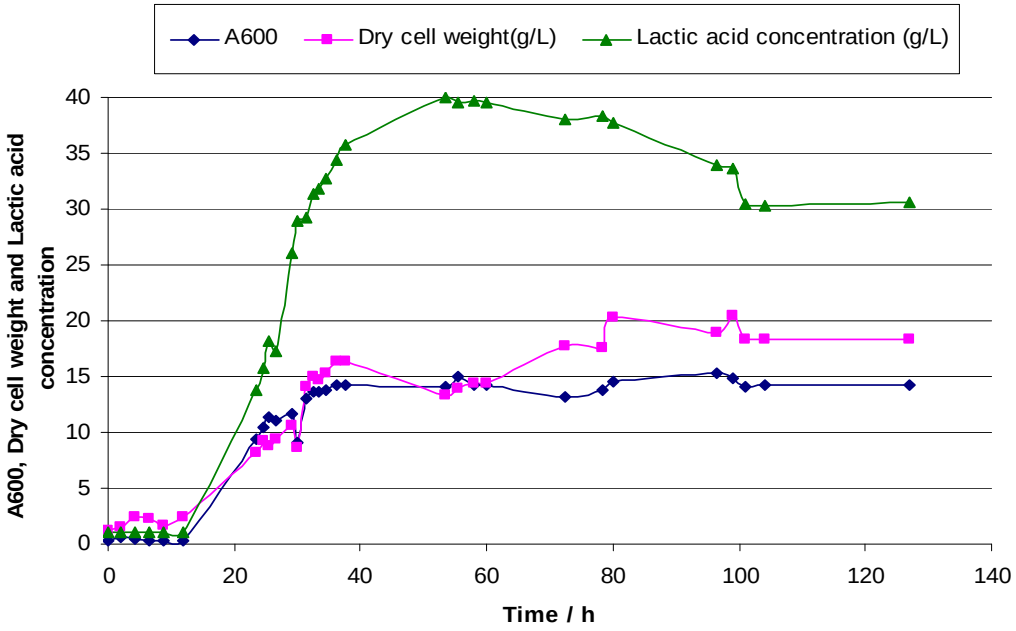



Figure 4.2 Cell density ($A_{600}$), cell dry weight and lactic acid concentration of *B. subtilis* mutant strain R during the one stage fed-batch fermentation. The cells were cultivated at 37°C. Similar conditions were used in the two trials A and B.

The cell density in the one stage fermentation was not high. The $A_{600}$ of both batches shown in Figure 4.2 were lower than 15 which were much less than the $A_{600}$ in the two stage fermentation shown in Figure 4.1. However, the lactic acid content in both fermentation batches shown in Figure 4.2 was up to 40 g/L after 40 h of fermentation and it was much higher than the lactic acid produced in the two stage fermentation batches which was about 13 g/L. The lactic acid produced seems not to be directly proportional to cell density in the fermentations. The overall process time in the one stage fermentation was less than that in the two stage fermentation due to the combination of the growth and production phases and hence lower process cost can be achieved due to shorter fermentation time. One stage fermentation was chosen for further studies due to the higher lactic acid yield and the shorter process time.

### 4.3 Effect of dissolved oxygen and agitation speed on the cell growth and lactic acid production by recombinant *Bacillus subtilis*

In fermentation technology, the improvement in productivity of the microbial metabolite is achieved by manipulating the nutritional and physical parameters and improving the strain by mutation selection. These can significantly alter the product yield. Since lactic acid production is an anaerobic process, it is very important to control the partial pressure of the oxygen in the medium. Dissolved oxygen was maintained at 5% by computer control of the agitation speed and its effect on lactic acid production was studied.

High concentration of oxygen may cause the toxicity for cells. It is important to control the oxygen level during fermentation. 50 ml culture (5 % inoculum) was inoculated in 1 L YE 20 medium in a 2 L fermentor. The medium was kept at 37°C and pH 7.5. DO was controlled at 5% in the fermentor by changing agitation speed from 400 rpm to 1300 rpm. Air flow was 2 L/min for the fermentor. The pH was controlled by 2 M HCL and 28% $NH_4OH$. 3 g/L glucose was continuously fed each hour into the fermentor. Figure 4.3 showed the cell density ($A_{600}$), cell dry weight, the lactic acid concentration and the glucose concentration throughout the fermentation.

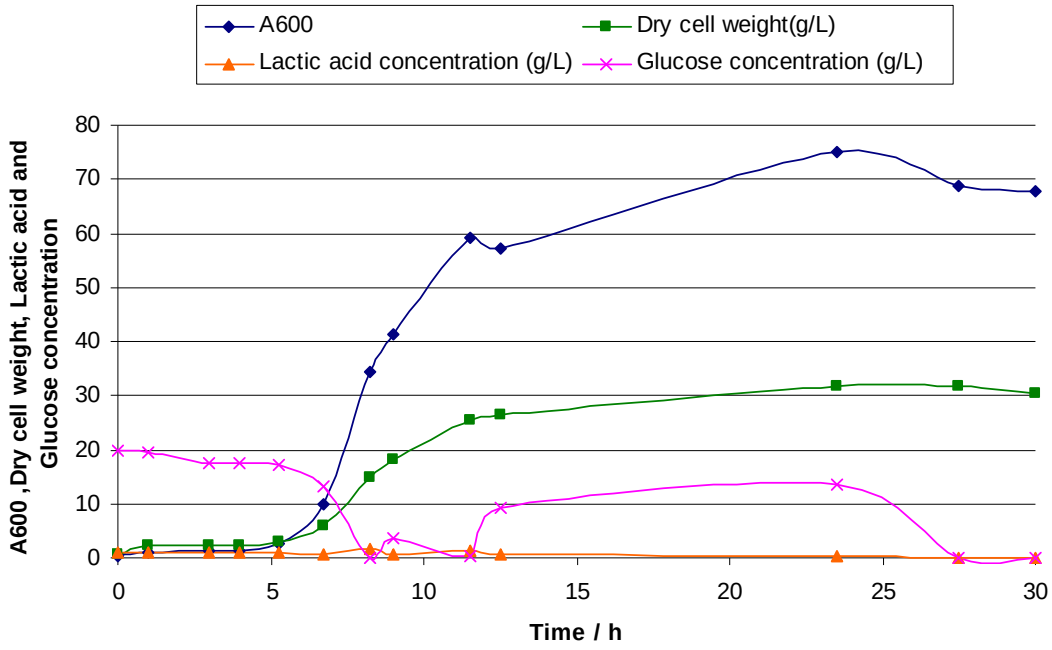
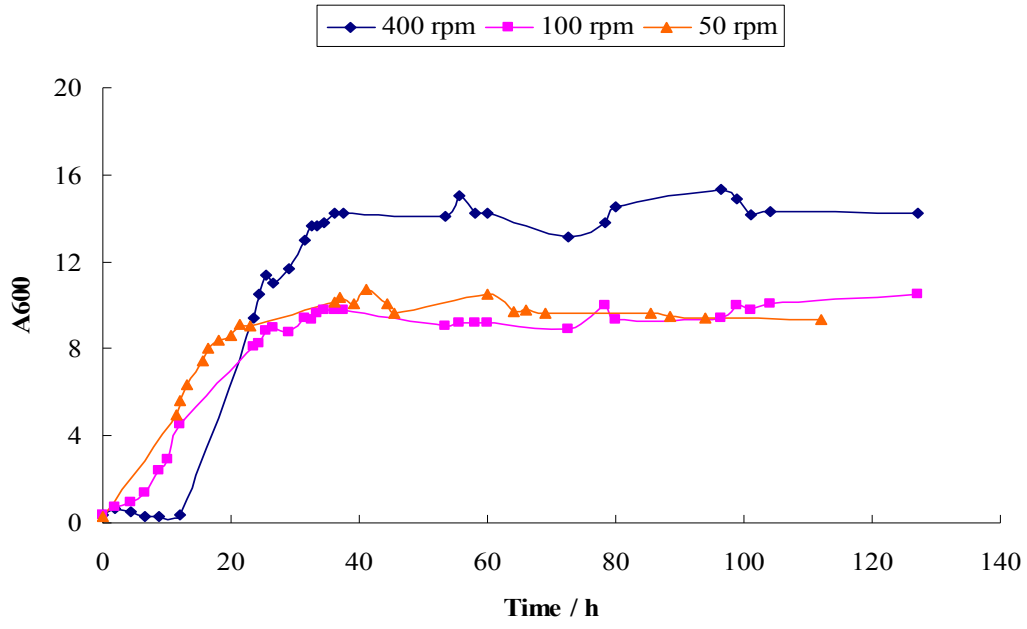



Figure 4.3 Cell density ($A_{600}$), cell dry weight, lactic acid and glucose concentration of *B. subtilis* mutant strain R during fed-batch fermentation. The cells were cultivated at 37°C and the DO was controlled at 5%.

There was absence of lactic acid production by *B. subtilis* mutant strain R throughout the fermentation. 5% DO level may be high for lactic acid production. The cell density ($A_{600}$) increased sharply throughout the fermentation process due to sufficient oxygen content being present in the culture medium. The cell density ($A_{600}$) reached the highest value 68.8 at 27.5 h. The nutrients were used for cell growth instead of being used for lactic acid production. Biomass increased under aerobic conditions, but lactic acid concentration did not increase. This distinctive feature was described for *B*.sp.SHO-1 by Ohara and Yahata in 1996 and was also confirmed by this experiment

Apart from this, there are many fermentation parameters that may significantly affect the growth and metabolic production of lactic acid such as agitation speed. The DO was not controlled and different agitation speeds were used for the lactic acid production by *B. subtilis* mutant strain R in fed-batch fermentation. The pH was controlled at 7.5 by 2 M HCL and 28% $NH_4OH$. Glucose was fed intermittently each hour into the fermentor in order to maintain the glucose level in culture medium. 50 rpm, 100 rpm and 400 rpm were used in three separate fermentations and the cell densities ($A_{600}$) and cell dry weight of the three fermentations with different agitations were shown in Figure 4.4.

A)



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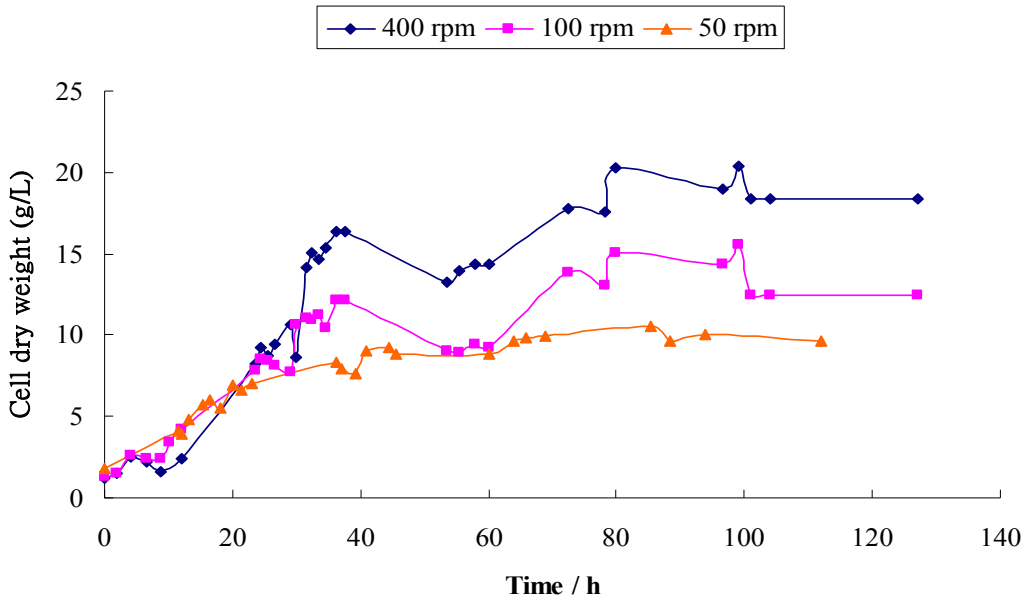



Figure 4.4 (A) Cell density ($A_{600}$) and (B) cell dry weight of *B. subtilis* mutant strain R during three fed-batch fermentations with different agitation speeds. The cells were cultivated at 37°C and 50 rpm, 100 rpm and 400 rpm agitations were used in the fermentation.

Figure 4.4 showed that the cell density ($A_{600}$) was proportional to the agitation speed. Higher agitation speed provided higher dissolved oxygen levels, which provided sufficient oxygen content for the cell growth during the fermentation. However, lactic acid production is an anaerobic process. The higher the oxygen content, the lower the amount of lactic acid was obtained (Figure 4.5).

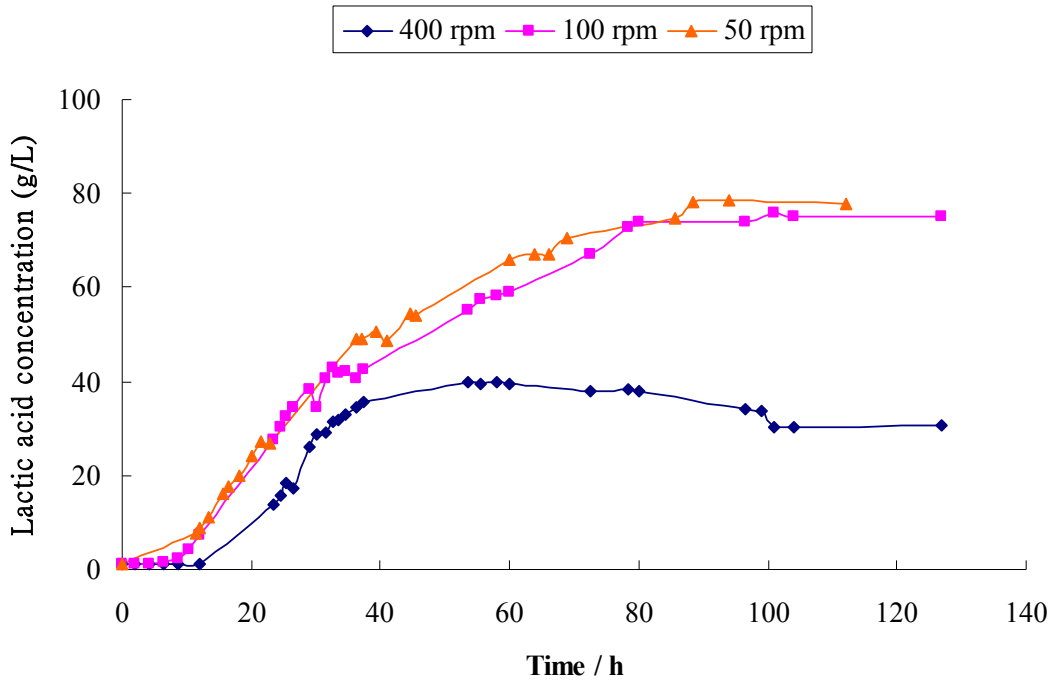



Figure 4.5 Lactic acid concentrations in the culture supernatant of mutant strain R during the fermentation with different agitation speeds

Figure 4.5 showed that the highest lactic acid concentration was 40 g/L obtained at 53.5 h of fermentation when the agitation speed was fixed at 400 rpm. The relatively lower lactic acid production may be due to the higher agitation speed providing higher oxygen content for the cell growth instead of lactic acid production. The lactic acid concentration was increasing throughout the fermentation when the agitation speed was fixed at 100 rpm and started to level off at around 75 h of fermentation. The lactic acid concentration was 72.8 g/L at 78.25 h and the highest concentration was 75.5 g/L obtained at 101 h of fermentation. Finally, the amount of lactic acid produced when

the agitation speed was fixed at 50 rpm was similar to the result obtained when the agitation speed was fixed at 100 rpm. The production of lactic acid was increasing throughout the process and the highest lactic acid concentration was 78.7 g/L obtained at 94 h of fermentation. Lower oxygen amount during the fermentation was found to be favorable to the lactic acid production.

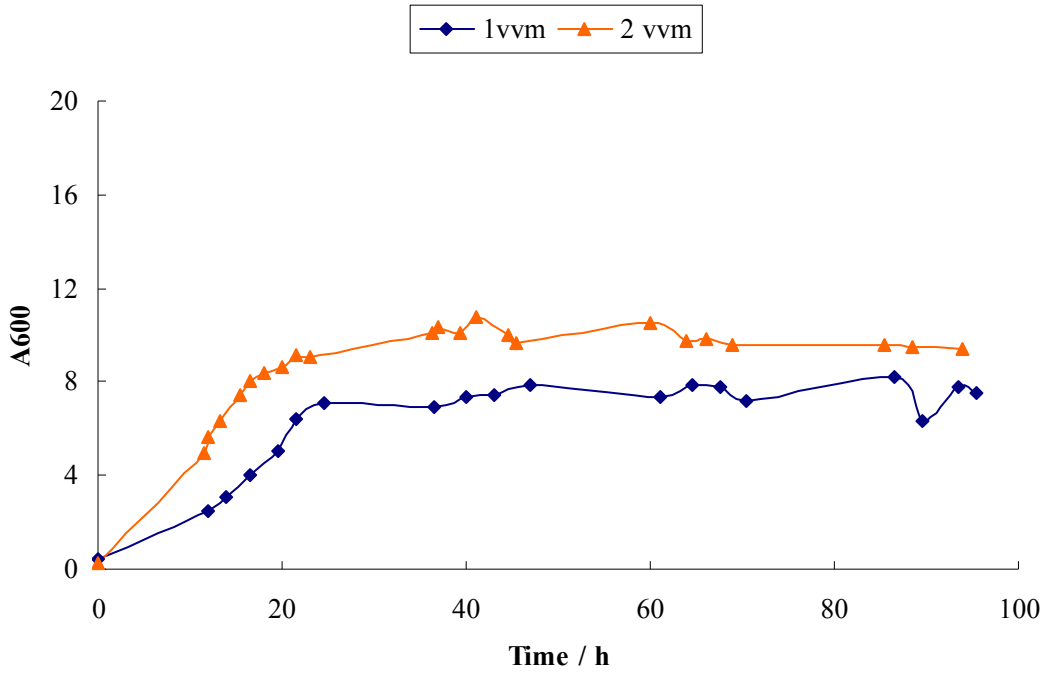
These results showed that more lactic acid can be produced when a lower agitation speed was used in the fermentation. The highest productivity of lactic acid (0.837 g/L/h) was obtained in fed-batch fermentation using 50 rpm agitation, which was higher than that of the fermentation using 100 rpm (0.75 g/L/h) and 400 rpm (0.748 g/L/h). Therefore, agitation speed 50 rpm was chosen for further studies.

## 4.4 Effect of aeration rate on the cell growth and lactic acid production by recombinant *Bacillus subtilis*

*Bacillus subtilis* has been considered as a strict aerobe for many years. However, studies have shown that it can also grow anaerobically by fermentation. (Nakano et al, 1997) Aeration rate is one of the fermentation parameters that can affect the cell growth and production of lactic acid. The effect of two aeration rates (1 vvm and 2 vvm) on lactic acid production by the mutant strain R of recombinant *Bacillus subtilis* was investigated.

5% inoculum was inoculated in 1 L YE 20 medium in a 2 L fermentor (BIOSTAT® B plus, Sartorius BBI Systems, Germany). The medium was kept at 37℃ and pH 7.5. The agitation speed was set at 50 rpm. The pH was controlled by 2M HCL and 28% $NH_4OH$. 60% glucose solution was fed into the fermentor in order to maintain a glucose level of 20 g/L.

A)

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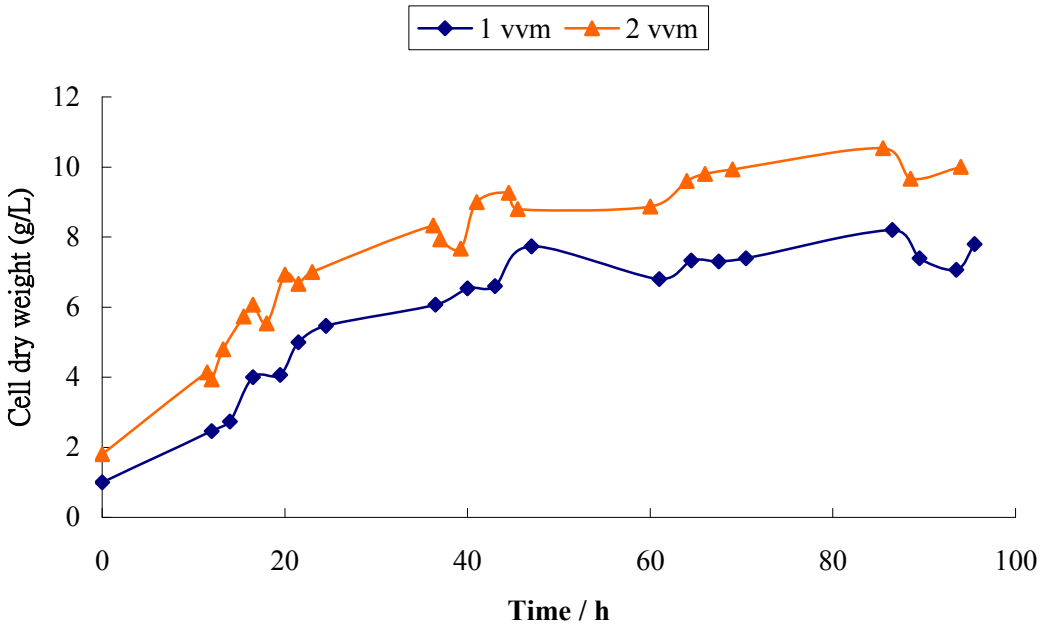



Figure 4.6 (A) Cell density ($A_{600}$) and (B) cell dry weight of *B. subtilis* mutant strain R during fed-batch fermentation. The cells were cultivated at 37°C and 1 vvm or 2 vvm aeration were used in the fermentation.

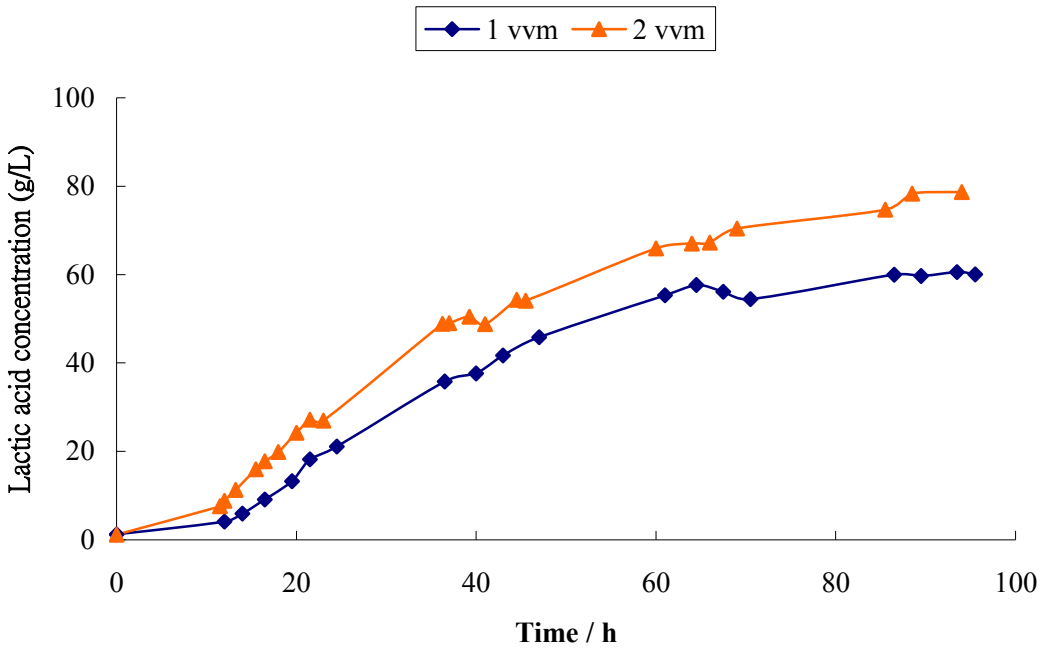



Figure 4.7 Lactic acid concentrations in the culture supernatant of mutant strain R during the fermentation at different aeration rates

Table 4.1 The lactic acid yield and productivity of the mutant strain R grown at different aeration rates

| Aeration rates | Amount of glucose utilized (g/L) | Highest lactic acid concentration (g/L) | $Y_{LA/GLU}$ (g/g) | Lactic acid productivity (g/L/h) | Duration of fermentation (h) |
|---|---|---|---|---|---|
| 1 vvm | 99.3 | 60.53 | 0.610 | 0.647 | 93.5 |
| 2 vvm | 111.9 | 78.7 | 0.703 | 0.837 | 94 |

As shown in Figure 4.6, fermentation at aeration rate of 2 vvm had higher cell density than that of 1 vvm. Growth rate during the exponential phase was faster at aeration rate of 2 vvm than that of 1 vvm. The highest cell density obtained at aeration rate of 2 vvm and 1 vvm was 10.53 g/L and 8.2 g/L, respectively. This indicated that mutant

R of recombinant *Bacillus subtilis* grow faster in the environment with aeration rate of 2 vvm than that of 1 vvm.

Figure 4.7 showed that the lactic acid concentration was 70.4 g/L after 69 h of fermentation using aeration rate of 2 vvm and 54.48 g/L after 70.5 h of fermentation using aeration rate of 1 vvm. The highest lactic acid concentration at aeration rate of 2 vvm and 1 vvm was 78.7 g/L and 60.53 g/L, respectively. This may be related to the cell density obtained at different aeration rates. As the cell density at aeration rate of 2 vvm was higher than that of 1 vvm, higher concentration of lactic acid was produced by mutant R in aeration rate of 2 vvm. This may also be attributed to the enhancement of cell viability. Andersen and Stier (1954) showed that oxygen appears to be involved in the synthesis of oelic acid and ergosterol which are essential membrane components and stimulate growth under anaerobic conditions.

Table 4.1 showed that higher lactic acid yield and lactic acid productivity was obtained at the aeration rate of 2 vvm. The yield was 0.703 g/g and productivity was 0.837 g/L/h.

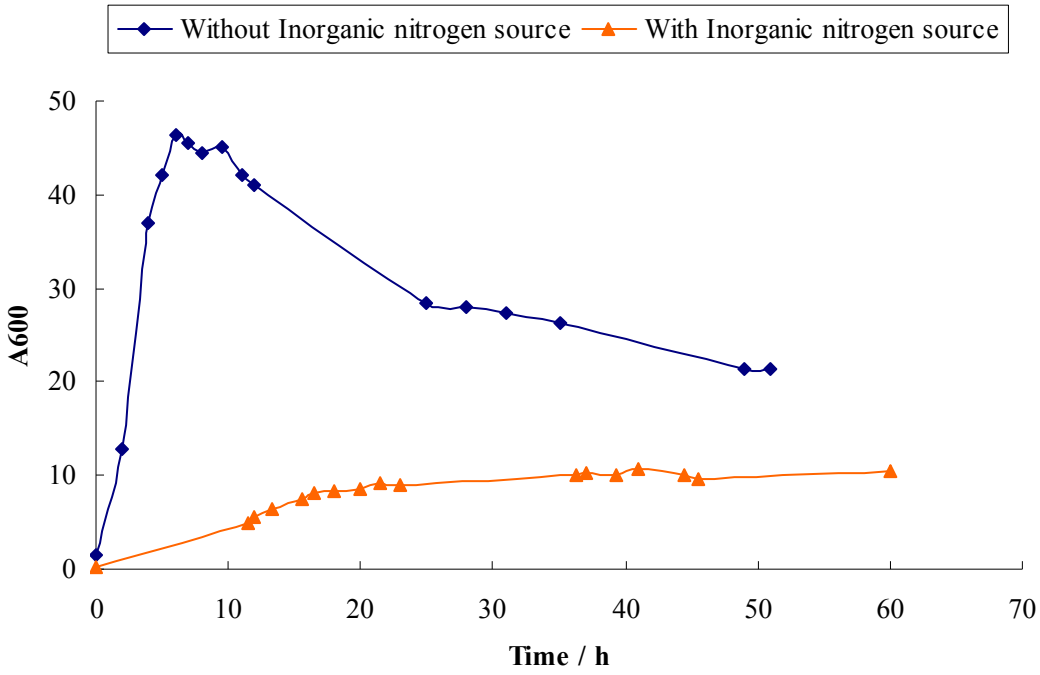
It was found that higher lactic acid concentration and productivity was obtained at the aeration rate of 2 vvm. Therefore, 2 vvm was used in further studies of lactic acid production by *B. subtilis* in fermenter fermentation.

## 4.5 Effect of inorganic nitrogen source on the cell growth and lactic acid production by recombinant *Bacillus subtilis*

Ammonium salt addition into culture medium was recommended by many authors (Zayed and Winter 1995 ; Heriban et al., 1993).The inorganic nitrogen content in YE 20 medium included ammonium sulphate and ammonium hydroxide solution used to adjust the pH of the medium during fermentation. The effect of inorganic source on lactic acid production was studied by removing the ammonium sulphate content in YE 20 medium and using sodium hydroxide solution for pH control during fermentation.

5% inoculum was inoculated in 600 ml YE 20 medium (with or without the inorganic nitrogen source) in a 1 L fermentor (BIOSTAT® B plus, Sartorius BBI Systems, Germany). The medium was kept at 37℃ and pH 7.5. The agitation speed was set at 50 rpm. Air flow was 2 vvm. The pH was controlled by 2M HCL and 28% $NH_4OH$ or 8 M NaOH. 60% glucose solution was fed into the fermentor in order to maintain a glucose level of 20 g/L.

A)

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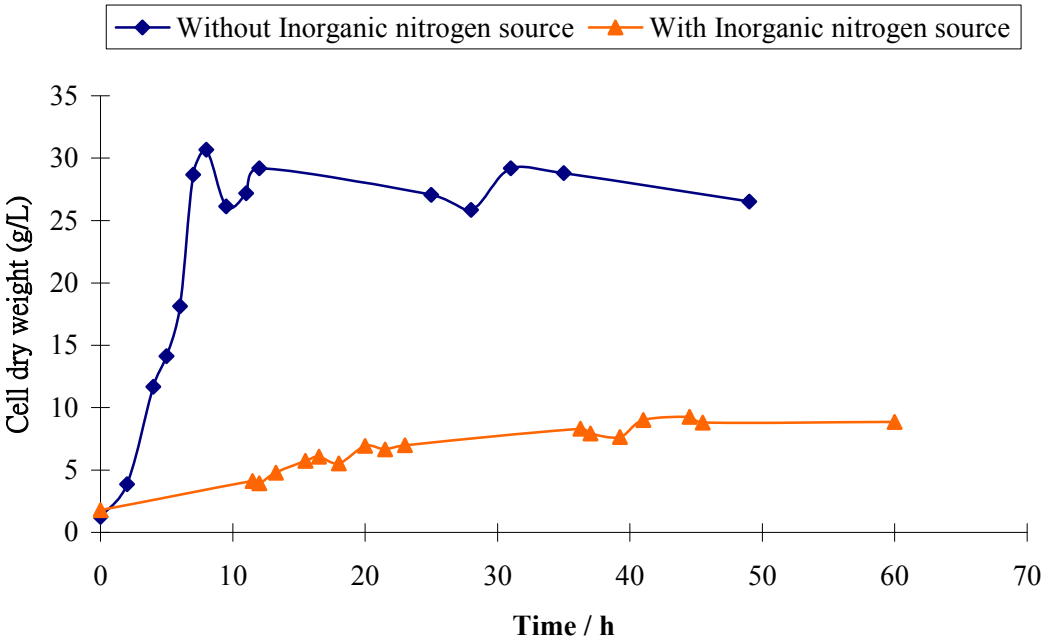



Figure 4.8 (A) Cell density ($A_{600}$) and (B) cell dry weight of *B. subtilis* mutant strain R during fed-batch fermentation. The cells were cultivated at 37°C and the medium with or without inorganic nitrogen source were used in the fermentation.

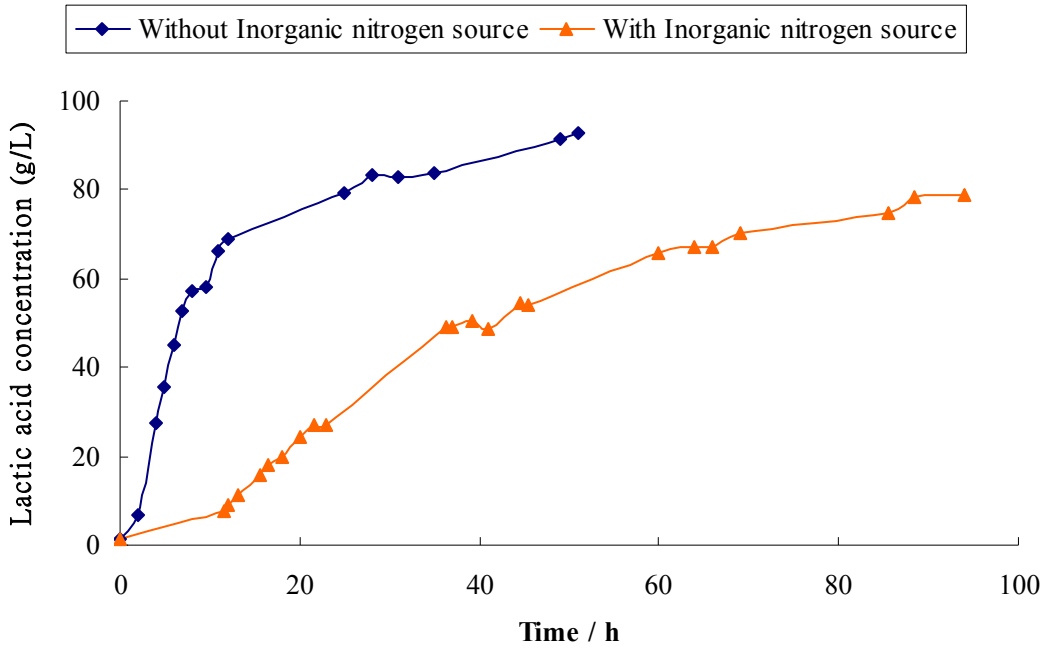



Figure 4.9 Lactic acid concentrations in the culture supernatant of mutant strain R during the fermentation using medium with or without inorganic nitrogen source

Table 4.2 The lactic acid yield and productivity of the mutant strain R grown in medium with or without inorganic nitrogen source

| Fermentor | Amount of glucose utilized (g/L) | Highest lactic acid concentration (g/L) | $Y_{LA/GLU}$ (g/g) | Lactic acid productivity (g/L/h) | Duration of fermentation (h) | Maximal lactic acid productivity (g/L/h) |
|---|---|---|---|---|---|---|
| With Inorganic nitrogen source | 97.07 | 78.7 | 0.811 | 0.837 | 94 | 2.18 ($20^{th}$ h)* |
| Without Inorganic nitrogen source | 112.06 | 93 | 0.830 | 1.824 | 51 | 10.42 ($4^{th}$ h)* |

* ( ) The fermentation time at which maximal lactic acid productivity was obtained.

As shown in Figure 4.8, YE 20 medium without inorganic nitrogen source had higher cell density than that of medium with inorganic nitrogen source. Growth rate during the exponential phase was much faster in the medium without inorganic nitrogen source than with inorganic nitrogen source. The highest cell density obtained in the medium without inorganic nitrogen source and with inorganic nitrogen source were 30.67 g/L and 10.53 g/L, respectively. This indicated that mutant strain R of recombinant *Bacillus subtilis* grow faster in the YE 20 medium without inorganic nitrogen source.

Figure 4.9 showed that higher lactic acid was produced in the medium without inorganic nitrogen source within a shorter period of fermentation time than medium with inorganic nitrogen source. After 12 h of fermentation, the lactic acid concentration in the medium without inorganic nitrogen source and with inorganic nitrogen source were 69 g/L and 8.86 g/L, respectively. This was likely due to the higher cell density obtained in the medium without inorganic nitrogen source. The highest lactic acid concentrations in the medium without inorganic nitrogen source and with inorganic nitrogen source were 93 g/L and 78.7 g/L, respectively. By removing the inorganic nitrogen content in the medium, the fermentation time was shortened from 94 h to 51 h.

Inorganic nitrogen content in YE 20 medium included ammonium sulphate and ammonium hydroxide solution used to adjust the pH of medium during fermentation. The effect of ammonium sulphate on lactic acid production was also studied by Kadam et al., 2006 and Payot el al., 1998. The results showed that supplementation of medium with ammonium sulphate did not support lactic acid production and was not suitable for lactic acid production.

Table 4.2 showed that higher lactic acid productivity was obtained in the medium without inorganic nitrogen content. The productivity was 1.834 g/L/h, which was higher than that of the medium with inorganic nitrogen content by more than two times. The maximum productivity in the medium without inorganic nitrogen source was 10.42 g/L/h. The lactic acid yield was similar in these two medium.

The results indicated that higher lactic acid concentration and productivity was obtained in the medium without inorganic nitrogen source. Therefore, YE 20 medium without inorganic nitrogen source was used in further studies of lactic acid production by *B. subtilis* in fermenter.

### 4.6 Effect of fermentation temperature on the cell growth and lactic acid production by recombinant *Bacillus subtilis*

Shake flask fermentation has been carried out to study the effect of cultivation temperature on lactic acid production in Chapter 3. The result showed that higher lactic acid concentration and productivity was obtained at the cultivation temperature of 40℃. Therefore, the effect of fermentation temperatures (37℃ and 40℃) on lactic acid production by mutant strain R of recombinant *Bacillus subtilis* in the fermentor was investigated.

5% inoculum was inoculated in 600 ml YE 20 medium (without inorganic nitrogen source) in a 2 L fermentor (BIOSTAT® B plus, Sartorius BBI Systems, Germany). The medium was kept at selected temperature (37℃ or 40℃) and pH 7.5. The agitation speed was set at 50 rpm. Air flow was 2 vvm. The pH was controlled by 2 M HCL and 8 M NaOH. 60% glucose solution was fed into the fermentor in order to maintain 20 g/L glucose level.

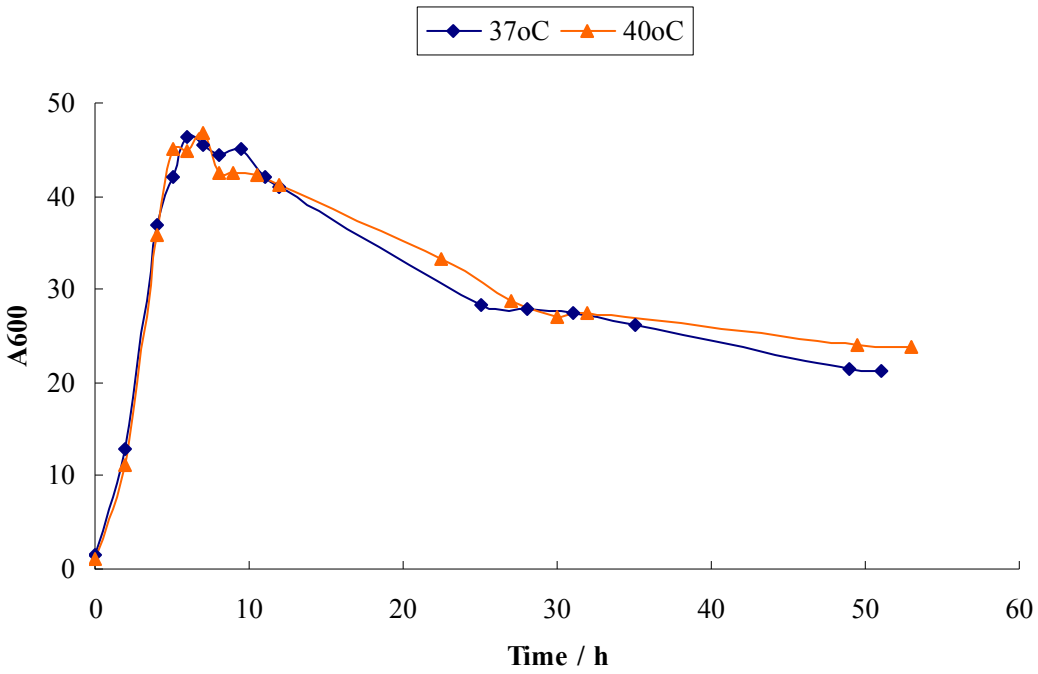



Figure 4.10 Cell density ($A_{600}$) of *B. subtilis* mutant strain R during fed-batch fermentation. The cells were cultivated at pH 7.5 and 37°C or 40°C were used in the fermentation.

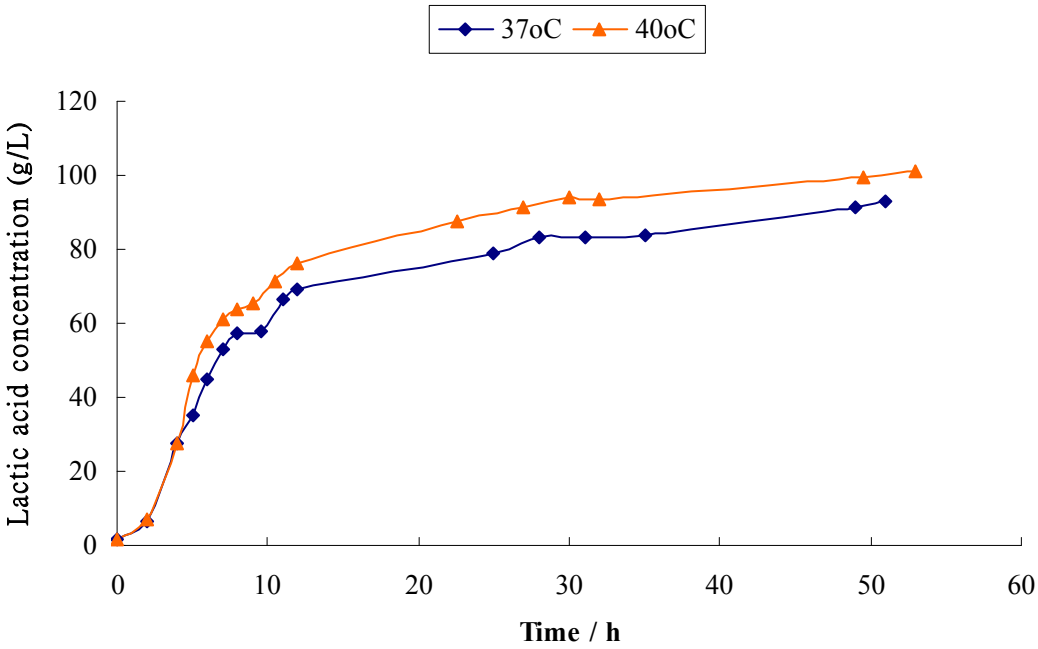



Figure 4.11 Lactic acid concentrations in the culture supernatant of mutant strain R grown at different temperatures in the fermentor

As shown in Figure 4.10, the cell density obtained at fermentation temperature of 37°C and 40°C was similar. The growth rate during the exponential phase at 37°C and 40°C were seen to be similar where the cell density was rapidly increased from 0 h to 5 h. The highest cell density obtained at 37°C and 40°C was around 26.4 g/L. This indicated that mutant R of recombinant *Bacillus subtilis* had similar growth pattern at 37°C and 40°C.

Figure 4.11 showed that the curve of lactic acid production at 37°C and 40°C can be divided into two parts. Before 12h, the lactic acid concentrations at 37°C and 40°C were 69 g/L and 76.4 g/L, respectively and the productivity at 37°C and 40°C reached 5.75 g/L/h and 6.37 g/L/h, respectively. The maximal lactic acid productivity at 40°C was 18.85 g/L/h obtained after 5 h of fermentation, which was higher than that of 37°C with productivity of 10.42 g/L/h. From 12 h to the end of fed-batch fermentation, the lactic acid concentration increased slowly. The final lactic acid concentration at 40°C was 101.2 g/L which was higher than that of at 37°C. The average productivity at 37°C and 40°C were 1.824 g/L/h and 1.909 g/L/h, respectively. The result indicated that higher lactic acid concentration and productivity was obtained at 40°C than at 37°C. It may be due to the physiological enzymes that work better at 40°C than at 37°C. Similar conclusions were obtained in shake flask

fermentations. This confirmed that fermentation temperature of 40°C was better for lactic acid production by recombinant *Bacillus subtilis* mutant strain R.

Further increase in fermentation temperature to 45°C was investigated (data not shown). The result showed that cell growth of mutant R increased within short period of time. However, the growth of mutant R at 45°C was not stable and inhibited for relatively longer period of the fermentation. The amount of lactic acid produced was not satisfactory. Ying et al (2009) showed that the growth of *B. subtilis* was completely inhibited when incubated at 45°C.

The results indicated that higher lactic acid concentration and productivity was obtained at the fermentation temperature of 40°C. Therefore, 40°C was used in further studies of lactic acid production by *B. subtilis*.

## 4.7 Effect of YE 50 and YE 80 medium on the cell growth and lactic acid production by recombinant *Bacillus subtilis*

Nitrogen source is essential for the lactic acid fermentation process. Yeast extract is one of the commonly used nitrogen source for the production of lactic acid. In this study, different quantities of industrial yeast extract were added into YE 20 medium. 30g/L martone in YE 20 medium was substituted by 30 g/L industrial yeast extract to form the YE 50 medium. Further addition of 30 g/L industrial yeast extract in YE 50 medium form the YE 80 medium.

5% inoculum was inoculated in 600 ml YE 20, YE 50 and YE 80 medium (without inorganic nitrogen source) in a 2 L fermentor (BIOSTAT® B plus, Sartorius BBI Systems, Germany). The medium was kept at 40℃ and pH 7.5. The agitation speed was set at 50 rpm. Air flow was 2 vvm. The pH was controlled by 2 M HCL and 8 M NaOH. 60% glucose solution was fed into the fermentor in order to maintain 20 g/L glucose level.

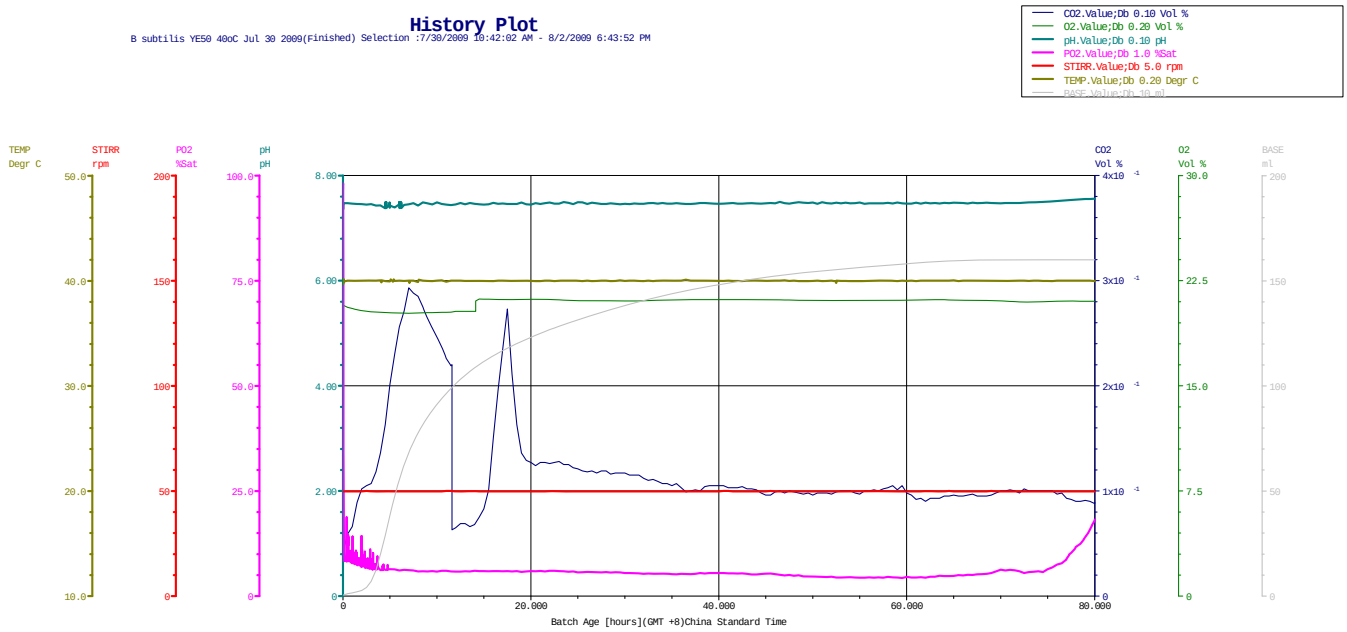



Figure 4.12 On-line monitoring of different parameters during fermentation

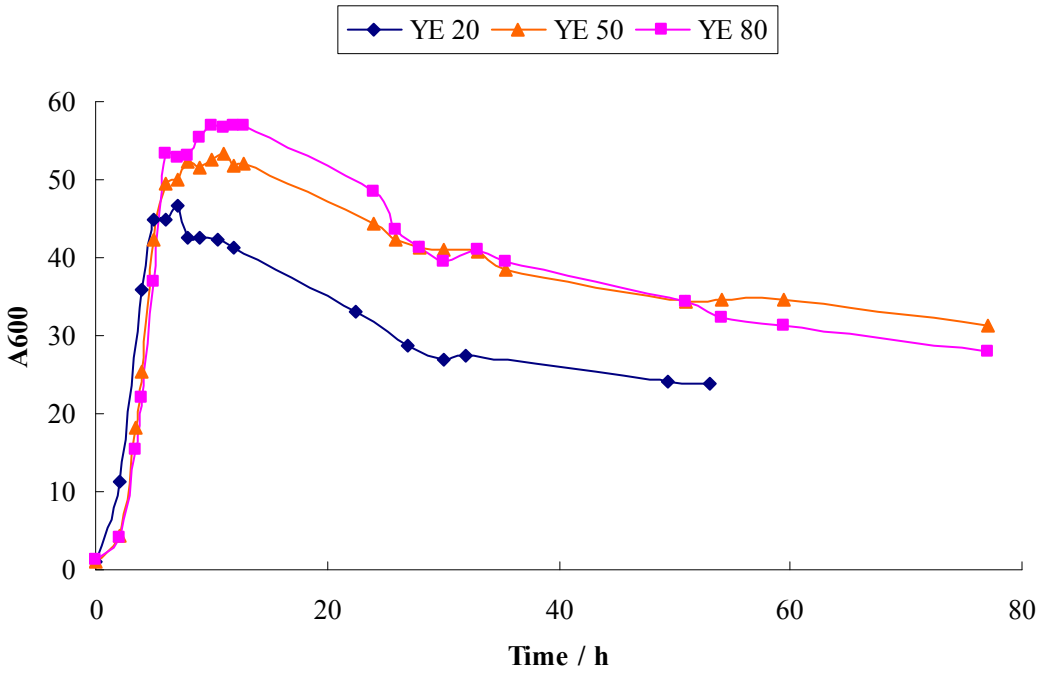



Figure 4.13 Cell density ($A_{600}$) of *B. subtilis* mutant strain R during fed-batch fermentations with different amounts of yeast extract. The cells were cultivated at 40°C and YE 20, YE 50 and YE 80 medium were used in the fermentation.

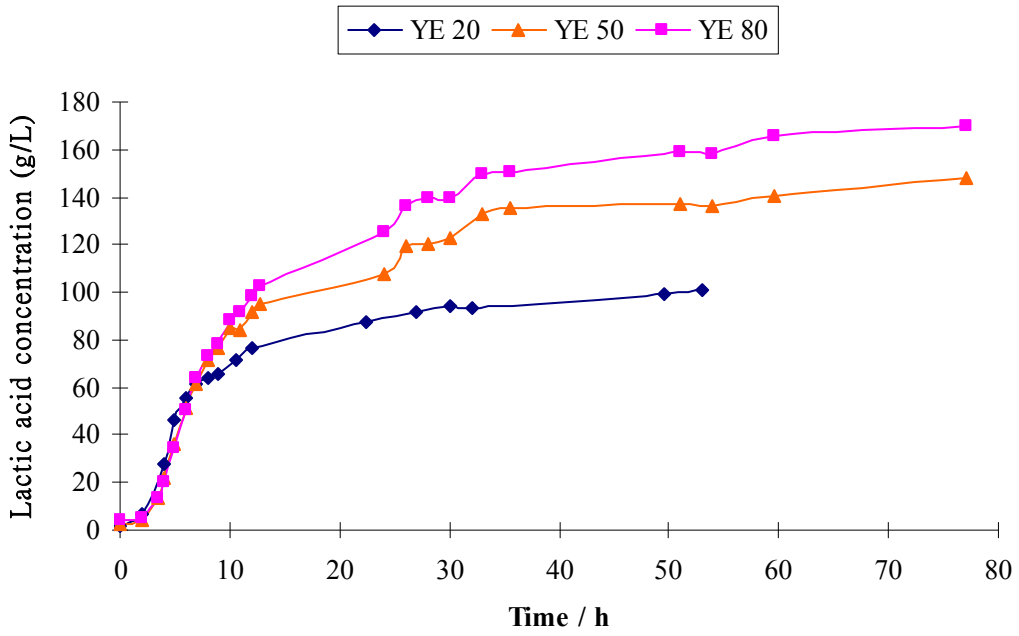



Figure 4.14 Lactic acid concentrations in the culture supernatant of mutant strain R grown in YE 20, YE 50 and YE 80 medium

Table 4.3 The lactic acid yield and productivity of the mutant strain R grown in YE 20, YE 50 and YE 80 medium

| Fermentor medium | Amount of glucose utilized (g/L) | Highest lactic acid concentration (g/L) | $Y_{LA/GLU}$ (g/g) | Lactic acid productivity (g/L/h) | Duration of fermentation (h) | Maximal lactic acid productivity (g/L/h) | Dilution factor |
|---|---|---|---|---|---|---|---|
| YE 20 | 129.07 | 76.4 | 0.784 | 6.367 | 12 | 18.85 ($5^{th}$ h)* | 1.472 |
| | | 87.5 | | 3.889 | 22.5 | | |
| | | 101.2 | | **1.909** | 53 | | |
| YE 50 | 185.12 | 91.65 | 0.802 | 7.638 | 12 | 16.4 ($4^{th}$ h)* | 1.683 |
| | | 107.4 | | 4.475 | 24 | | |
| | | 137.2 | | 2.690 | 51 | | |
| | | 148.4 | | **1.927** | 77 | | |
| YE 80 | 206.76 | 98.7 | 0.822 | 8.225 | 12 | 15.95 ($6^{th}$ h)* | 1.802 |
| | | 125.6 | | 5.233 | 24 | | |
| | | 158.6 | | 3.110 | 51 | | |
| | | 170 | | **2.208** | 77 | | |

* ( ) The fermentation time at which maximal lactic acid productivity was obtained.

Figure 4.12 showed the change (on-line) of several fermentation parameters during lactic acid production. The fermentation temperature was controlled at 40°C and the pH of medium was controlled at 7.5 by 8M NaOH throughout the whole fermentation process. The agitation speed was set at 50 rpm and air flow was 2 vvm in order to provide optimal conditions for lactic acid production. The dissolved oxygen level dropped to nearly 0% very quickly after the start of fermentation. It is because mutant R grew rapidly from 0 h to 6 h and consumed large amount of oxygen for growth and lactic acid production. The volume of base added increased with the amount of lactic acid produced in order to maintain at constant pH 7.5.

As shown in Figure 4.13, the cell density obtained in YE 80 medium was the highest, followed by YE 50 medium and then YE 20 medium. The growth rate during the exponential phase with the three medium were seen to be similar where the cell density was rapidly increasing from 0 h to 6 h. Higher cell density was obtained in YE 50 and YE 80 medium than that of YE 20 medium. This indicated that the higher the amount of yeast extract in the fermentation medium, the higher will be the cell density.

Figure 4.14 showed that lactic acid concentration increased with enhanced yeast extract concentration in the medium. The highest lactic acid concentration was obtained in YE 80 medium. At 12h of fermentation, the lactic acid concentration in YE 20, YE 50 and YE 80 medium were 76.4 g/L, 91.65 g/L and 98.7 g/L, respectively and the productivity reached 6.367 g/L/h, 7.638 g/L/h and 8.225 g/L/h, respectively. The maximal lactic acid productivity in YE 80 medium was 15.95 g/L/h obtained after 6 h of fermentation. From 12 h to the end of fed-batch fermentation, the lactic acid concentration increased slowly and the productivity decreased with the increased in lactic acid production . The final lactic acid concentration in YE 80 medium was 170 g/L which was higher than that of the YE 20 and YE 50 medium. The average productivity in YE 20, YE 50 and YE 80 medium were 1.909 g/L/h, 1.927 g/L/h and 2.208 g/L/h, respectively. Higher productivity of lactic acid in fed-batch fermentation was suppressed. It may due to the end-product inhibition by lactic acid. It was seen that over 100 g/L lactic acid was produced in 24 h of fermentation in YE 50 and YE 80 medium. This high concentration of lactic acid would inhibit the growth of recombinant *B. subtilis* mutant strain R leading to a decrease in productivity as fermentation continued.

Table 4.3 showed that the lactic acid yield was similar in the three medium, reaching 0.784 g/g, 0.802 g/g and 0.822 g/g for YE 20, YE 50 and YE 80 medium, respectively.

Among the medium used, YE 80 medium was more efficient for cell growth and lactic acid production than YE 20 and YE 50 medium. One of the most important parameters for lactic acid production by *B. subtilis* mutant strain R is the nitrogen source. Yeast extract is essential for a good fermentation performance. It is assimilated as the nitrogen source and contains Vitamin B complex content in addition to organic nitrogen, and cofactors for growth. (Aeschlimann and Stockar 1990 ; Kadam et al., 2006 ) The maximum biomass, final lactic acid concentration and lactic acid productivity increased with enhanced yeast extract supplementation in the fermentation medium.

In this study, the average productivity of lactic acid production in YE 80 medium was 2.208 g/L/h. It compares favorably with two representative lactic acid producers, *L.casei* LA-04-1 and *L.lactis* BME5-18M, which had the average productivity of 2.14g/L/h and 2.2 g/L/h, respectively (Ding and Tan, 2006 ; Bai et al., 2003 ; Li et al., 2006). The final lactic acid concentration of 170 g/L for recombinant *Bacillus subtilis*

mutant strain R exceeds the performance of previously reported microorganisms such as *L. lactis* (Bai et al., 2004), *E. coli* SZ194 (Zhou et al., 2006), *B. subtilis* (Garcia et al.,2009) and engineered biocatalysts (Zhou et al.,2005,Saitoh et al., 2005, Skory 2004).

*Bacillus subtilis* mutant strain R could be a potential candidate for lactic acid production from glucose because of the faster growth rates and higher lactic acid production.

# Chapter Five

# Shake flask and fermentor studies to optimize fermentation parameters for the production of lactic acid from starch by recombinant *Bacillus subtilis* 1A304 (Φ105MU331)

## 5.1 Introduction

In order to have an economically competitive fermentation process for lactic acid production, an inexpensive and abundant carbon source should be used. There have been various attempts to produce lactic acid efficiently from inexpensive raw materials. Production of lactic acid using glucose has been demonstrated in the previous chapters in shake flask and fermentor studies. In this chapter, lactic acid production by recombinant *Bacillus subtilis* 1A304 (Φ105MU331) using starch as the carbon source was studied.

Simultaneous saccharification and fermentation (SSF) is a combined process of saccharification of a renewable bioresource and fermentation process to produce products such as lactic acid. SSF has been used for the conversion of starch to various fermentative products. In this study, SSF of starch to lactic acid was conducted using the recombinant *Bacillus subtilis* and *Aspergillus niger*.

## 5.2 The effect of different carbon sources on the cell growth and lactic acid production by recombinant *Bacillus subtilis*

Xylose is one of the major fermentable sugars present in cellulosic biomass, second to glucose only. Cellulosic biomass, which includes agriculture residues, paper wastes, wood chips, etc., is an ideal inexpensive, renewable, abundantly available source of sugars for fermentation to lactic acid. Starch is obtained predominantly from starch-rich plants such as wheat, potatoes, maize, rice, peas and tapioca. Starch is the material in which plants store reserves of energy, which is a polysaccharide consisting of glucose units. Starch is a renewable and low cost carbon source.

The *B. subtilis* mutant strain R was cultivated with various economical carbon sources such as starch and xylose to compare the lactic acid production yields to the medium with glucose. Liquefied starch was also used to determine the ability of *B. subtilis* mutant strain R on liquefied starch utilization and the YE 20 medium without carbon source was used as a control. 5% inoculum was inoculated in 70 ml YE20 medium with 20 g/L carbon sources in a 100 ml flask and was agitated at 37°C with 180 rpm for 12 hours. The pH value was maintained at 7.5 by 28% $NH_4OH$.

The cell density ($A_{600}$) was shown in Figure 5.1. It was found that the cell densities from growth on various carbon sources throughout the fermentation did not increase much and were similar except for the YE 20 medium with glucose. The absorbance of the flask culture using glucose ($A_{600}$ = 6.74) was much higher than that in the other flasks ($A_{600}$ < 4) with the other carbon sources and in the control flask using YE 20 medium without carbon sources. The cell dry weight of the different samples has similar pattern as the absorbance. The results showed that *B. subtilis* mutant strain R cannot use liquefied starch and thus saccharification of starch to glucose is needed.

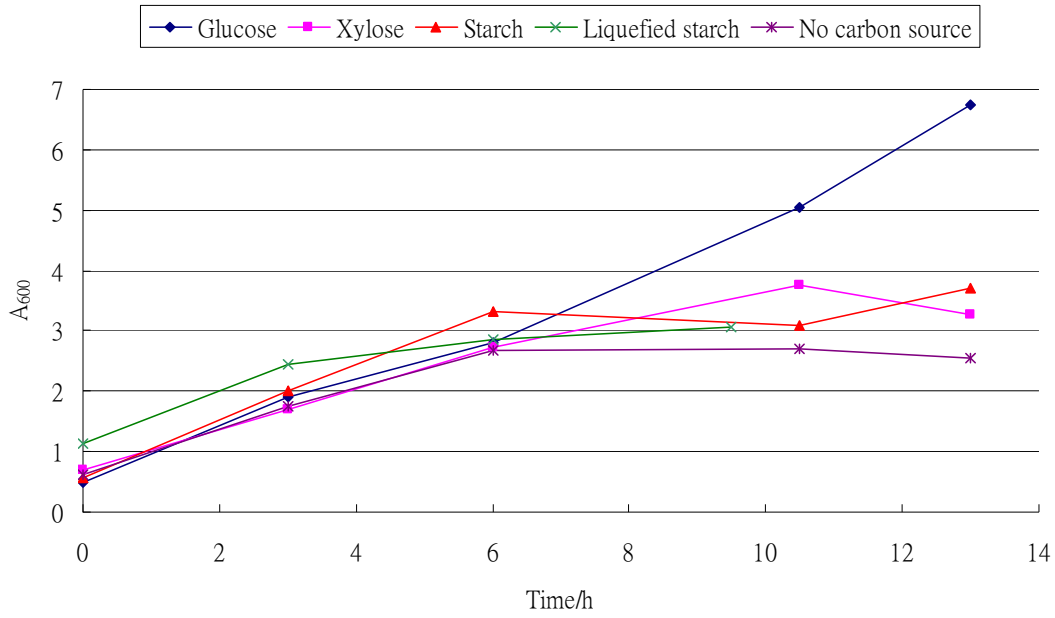



Figure 5.1 Cell density ($A_{600}$) of *B. subtilis* mutant in different carbon sources, the cells were cultivated at 37°C with agitation speed of 180 rpm

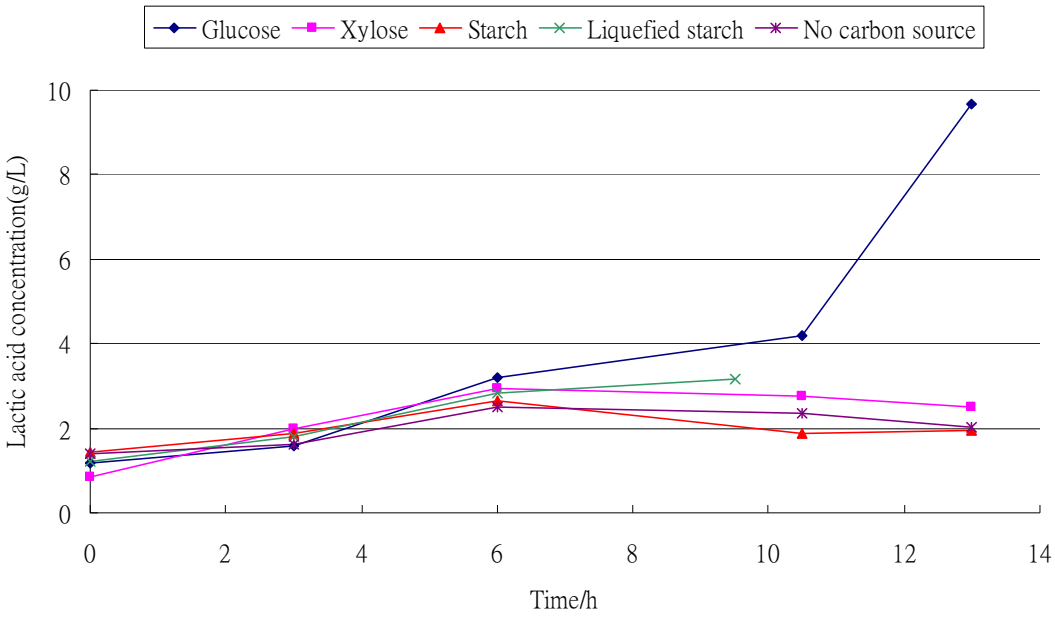



Figure 5.2 The lactic acid produced by *B. subtilis* mutant strain R in different carbon sources and the cells were cultivated at 37°C with agitation speed of 180 rpm

Figure 5.2 showed that the highest lactic acid concentration was obtained from the flask culture using glucose as the carbon source. The flasks using other carbon sources had much lower lactic acid concentration when compared to the medium using glucose as the carbon source. This indicated that the starch, liquefied starch and xylose cannot be metabolized by *B. subtilis* mutant strain R to produce lactic acid. It also showed that saccharification is essential when starch is to be used as the carbon source for lactic acid production.

## 5.3 Effects of the heat shock process on *B. subtilis* mutant strain R in lactic acid production

The bacteriophage ϕ105 was incorporated into the *B. subtilis* chromosomal DNA of which the bacteriophage is only in the mode of lysogenic state. The ϕ105 MU331 prophage is an efficient expression vector based on *B. subtilis* phage ϕ105 (Birdsell et al., 1969) and has been proven for high-level protein overproduction in *B. subtilis*. The promoter is controlled by a temperature sensitive phage repressor and offers temperature inducibility (Errington J., 1986). 5% *B. subtilis* mutant strain R inoculum was inoculated in 70 ml YE 20 medium with 20 g/L glucose in three 100 ml flask and was agitated at 37°C with 180 rpm for 12 hours. Two flasks were heat shocked at 50 ℃ for 5 min at 7.5 hours after inoculation, the third flask was not heat shocked and used as a control. Additional *B. subtilis* cells were added to one of the flask containing heat shocked cells and glucose. Two other flasks with 70 ml YE 20 medium and 10 g/L glucose were inoculated. After heat shock, the cells were centrifuged and resuspended in YE 20 medium with 2.5 g/L starch. Additional *B. subtilis* cells were added to one of these flasks containing starch. The intention of this experiment is to observe the ability of lactic acid production by *B. subtilis* after the heat shock process. The pH values of flasks were maintained at 7.5. The cell density ($A_{600}$) of the cells was shown in Figure 5.3.

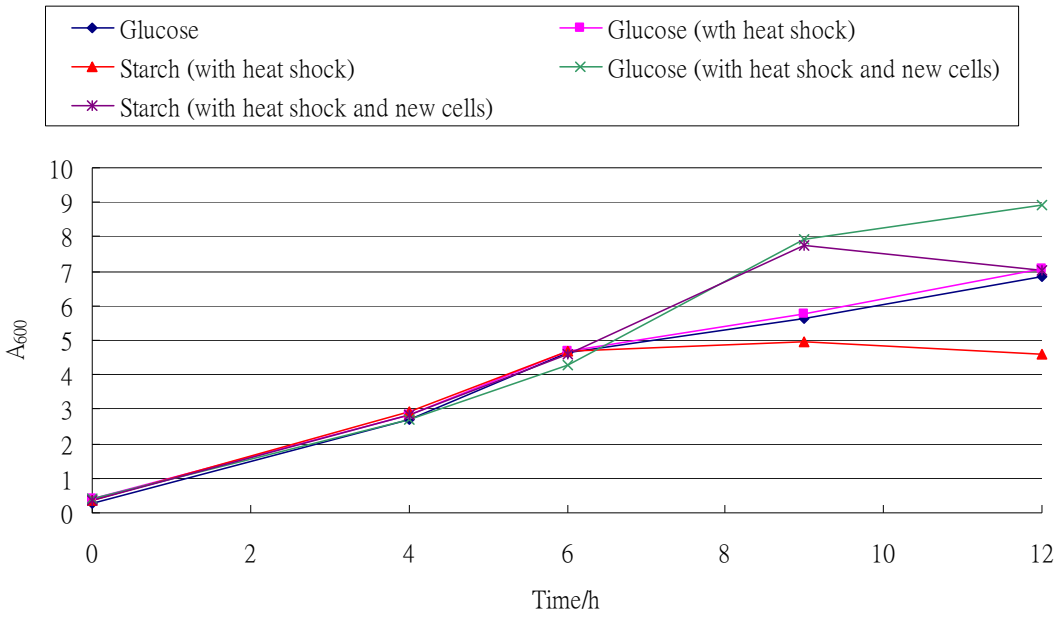



Figure 5.3 The effect of the heat shock process on growth of *B. subtilis* mutant strain R in glucose and starch. *B. subtilis* cells were heat shocked at 7.5 hours after inoculation. The cells were cultivated at 37°C with agitation speed of 180 rpm

The cell density ($A_{600}$) obtained in flasks without additional cells after heat shock were about the same and shown in Figure 5.3. The growth of the *B. subtilis* mutant strain R slowed down after the heat shock process. This was because the phage promoter of the *B. subtilis* mutant strain R was triggered and the alpha-amylase gene behind the promoter was expressed. Some of the energy was shift to the protein expression and the growth slowed down or stopped. The lowest absorbance was obtained in flask using starch as carbon source after the heat shock process. *B. subtilis* cannot utilize starch as the carbon source directly and the absorbance leveled off or

slowly decreased after the heat shock process of the culture. For the two flasks with new *B. subtilis* cells added after the heat shock, the newly added cells were replicating and the cell density was continuously increasing. The highest cell density ($A_{600}$) was obtained in the flask containing YE20 medium with glucose and additional cells added. The production of lactic acid by the cultures was showed in Figure 5.4.

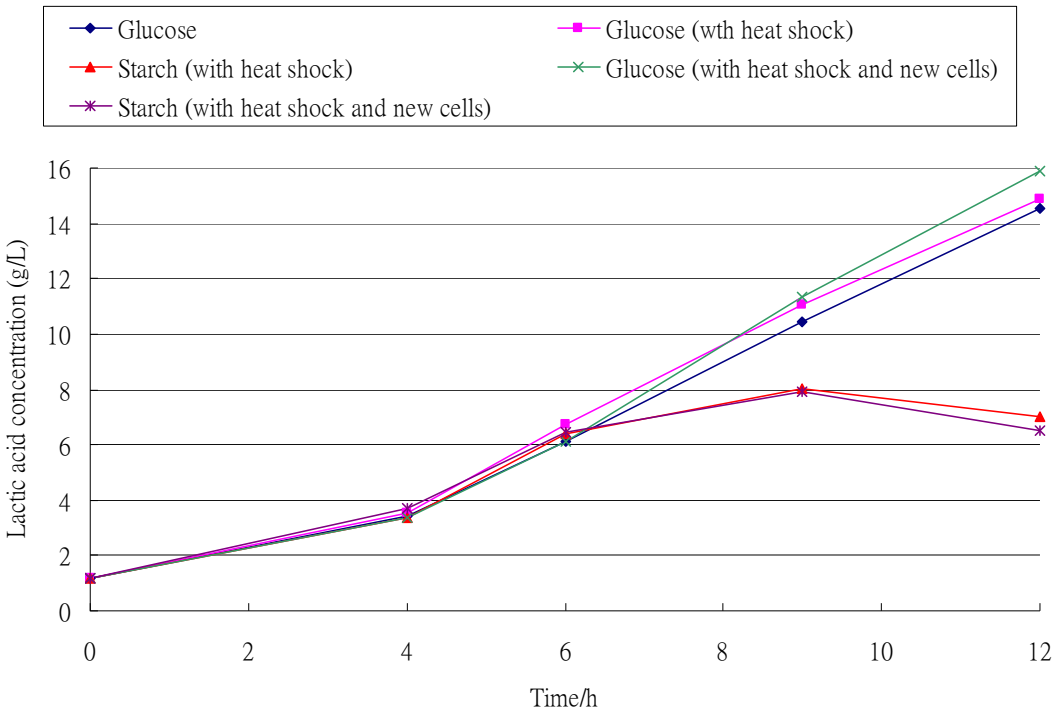



Figure 5.4 Production of lactic acid by *B. subtilis* mutant strain R using glucose and starch, with and without heat shock at 7.5 h and the addition of fresh *B. subtilis* cells. The cells were cultivated at 37°C with agitation speed of 180 rpm

Starch cannot be utilized by the *B. subtilis* mutant strain R and hence the lactic acid concentration was low (< 6 g/L) in the two flasks with starch as carbon source. The lactic acid concentrations of the other three flasks using glucose as carbon source are quite similar. It indicates that the heat shock did not have any adverse effect on lactic acid production. Although the cells after heat shock slow down in growth, it did not affect the ability of lactic acid production by mutant R. Moreover, the higher cell densities in flasks with the additional cells did not have higher lactic acid production. This may be due to the various environmental and/or nutritional factors which can affect the lactic acid production as well.

## 5.4 Lactic acid production by *B. subtilis* mutant strain R using saccharified glucose from various sources of starch

Starch is a major source of carbohydrates in human diets and can be obtained from seeds, fruits, nuts, corn, potatoes, and so on. The difference between a starch from corn or one from potato depend on the structure of the starch, controlled by the kinds of polymers found in the grain or tuber and the way the polymers are packed into the starch granule. Therefore, different sources of starch have been used for the lactic acid production and the differences in the ability of the lactic acid production from the *B. subtilis* mutant strain R was studied

Starch was liquefied by using α-amylase from *B. subtilis.* 1 ml of α-amylase from *B. subtilis* mutant after heat shock was added to 10 g of starch. The process was carried out during autoclaving with 10 ppm calcium chloride. Spores of *Aspergillus niger* were immobilized in calcium alginate gel beads using 3 % sodium alginate and 0.5 M calcium chloride. Ten grams of gel beads were incubated in 50 ml FG medium in a 250 ml flask and was agitated at 30°C with 250 rpm for 24 hours. Glucoamylase was then induced by replacing the sucrose content in FG medium with 20 g/L starch for the next 24 hours. Three types of starch have been used for saccharification. One was potato starch, and the other two were soluble starch (ADH) and corn starch. Glucose

production after liquefaction and saccharification of 6 % soluble starch, potato starch and corn starch were shown in figure 5.5. 5% inoculum was inoculated in 70 ml Y E20 medium in a 100 ml flask and was agitated at 37°C with 180 rpm for 12 hours. The pH value was maintained at 7.5. The cell density ($A_{600}$) of the cultures and the lactic acid production in flasks containing glucose from different sources of starch are shown in Figure 5.6 and Figure 5.7.

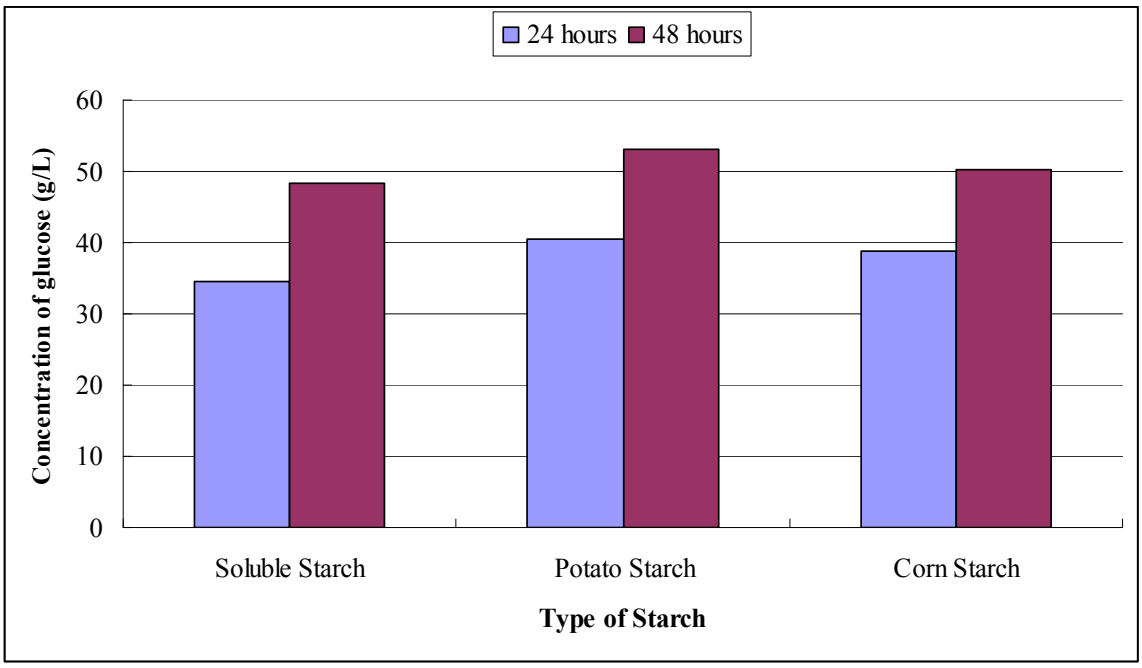



Figure 5.5 The amount of glucose from saccharification of different sources of starch

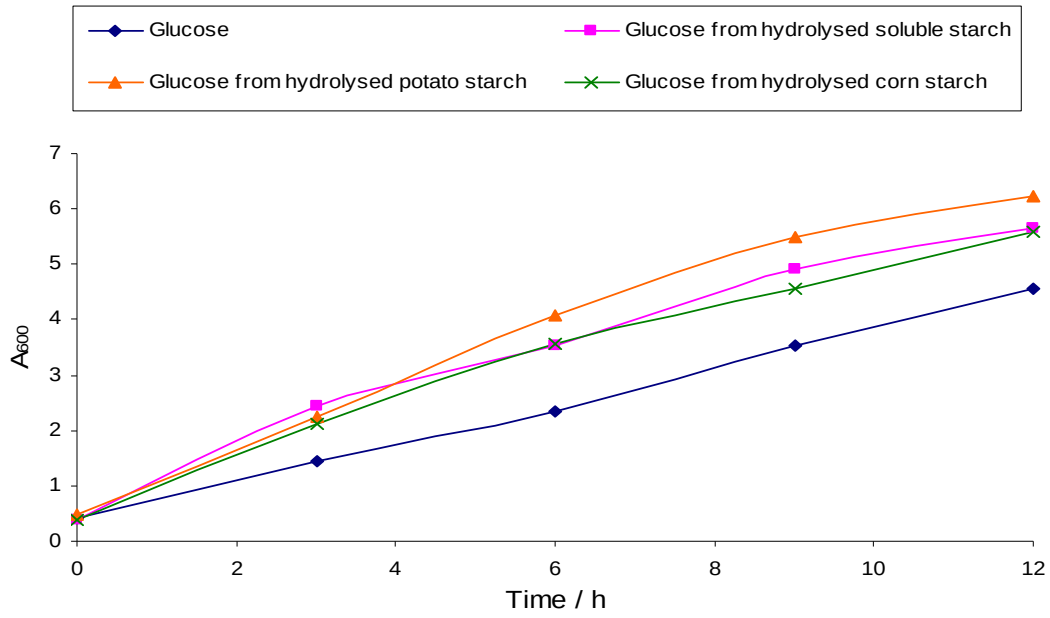



Figure 5.6 Cell density ($A_{600}$) of *B. subtilis* mutant strain R in flasks with glucose from different sources of starch

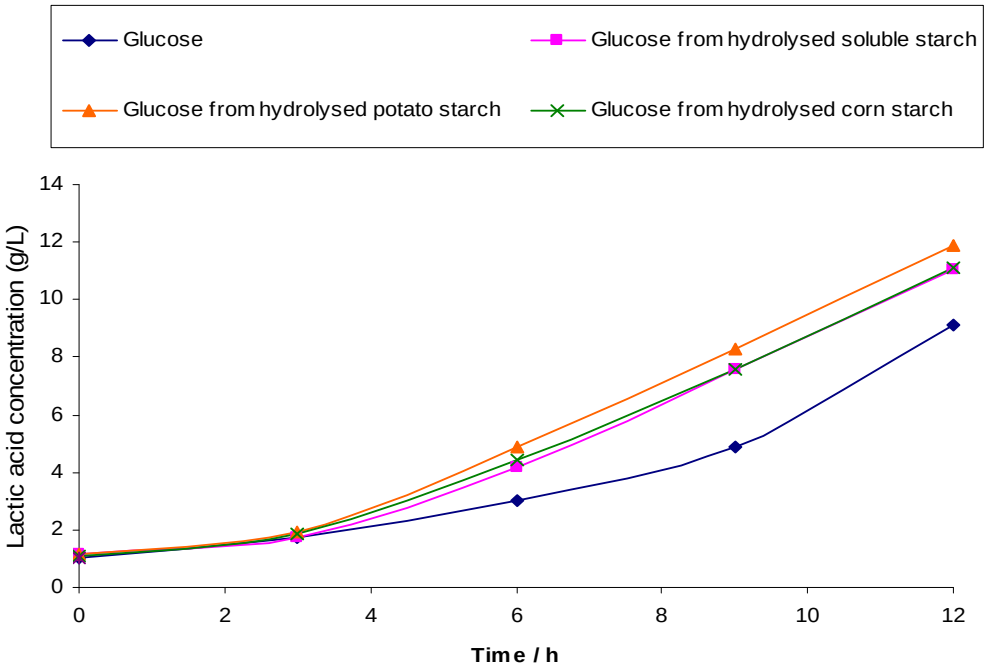



Figure 5.7 Lactic acid production by *B. subtilis* mutant strain R in flasks with glucose from different sources of starch

Figure 5.5 showed that the saccharification of liquefied starch was similar in different types of starch. The efficiency of the glucose conversion from starch was similar. The cell density and lactic acid production by *B. subtilis* mutant strain R in Figure 5.6 and Figure 5.7 showed that the conversion efficiency of lactic acid using glucose from different sources of starch was similar.

### 5.5 Effect of forms of *Aspergillus niger* culture on starch saccharification

*Aspergillus niger* is the source of glucoamylase in this project. *Aspergillus niger* has been used for decades in the production of glucoamylase in fed-batch cultivations. Glucoamylase production in *A. niger* has been studied in carbon-limited chemostats and in shake flasks (Pedersen et al., 2000). The spores of *A. niger* can be immobilized in sodium alginate to form beads. Immobilization has the advantage that high cell density of *A. niger* can be obtained as the spores are concentrated and trapped in sodium alginate. In order to investigate the performance of starch saccharification by immobilized and free culture *Aspergillus niger*, two forms of inoculum are used to saccharify 20 g/L liquefied soluble starch. The effect of immobilization of *A. niger* culture on starch saccharification was studied.

3g (approximately 40 capsules) of immobilized *A. niger* gel beads or *A. niger* culture after enzyme induction were washed with sterile dd$H_2O$ and inoculated into 50 ml sterilized liquefied starch solution in 250 ml flask. The cultures were agitated at 43℃ with 250 rpm.

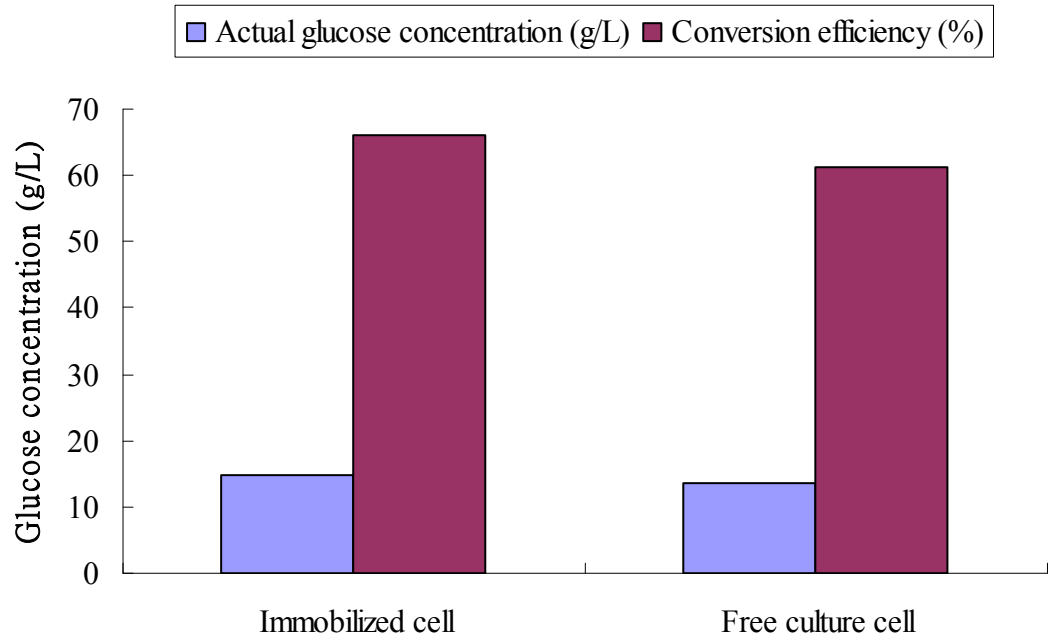



Figure 5.8 Comparison between immobilized and free culture *A. niger* on starch saccharification

Table 5.1 Conversion efficiency of starch to glucose in saccharification by different forms of *A. niger* culture

| Forms of *A. niger* culture | Starch concentration (g/L) | Theoretical glucose concentration (g/L) | Actual glucose concentration (g/L) | Conversion efficiency (%) |
|---|---|---|---|---|
| immobilized cell | 20 | 22.2 | 14.65 | 65.99 |
| free culture cell | 20 | 22.2 | 13.60 | 61.26 |

For the theoretical glucose concentration, as 1 g of starch can give 1.11 g of glucose on complete hydrolysis (Roy et al , 2001), it is used as the standard to evaluate the efficiency of conversion from starch to glucose in hydrolysis.

Figure 5.8 showed that the amount of glucose saccharified from starch in immobilized cell and free culture cell of *A. niger* was similar. Immobilized cell produced a slightly higher concentration of glucose than free culture cells. It may due to the higher cell density of *A. niger* trapped in sodium alginate gel beads. Table 5.1 showed that the conversion efficiency of starch to glucose was similar in both immobilized cell and free culture cell of *A. niger*, reaching 65.99% and 61.26% respectively.

The result showed that similar concentration of glucose was saccharified from starch in immobilized *A. niger* and *A. niger* culture. In order to shorten the process time and simplify the preparation steps, *A. niger* culture was chosen as the inoculum form for starch saccharification.

Different forms of *A. niger* culture may also affect the performance of starch saccharification. The morphology of *A. niger* can be affected by the composition in fermentation medium. Many studies have suggested various minerals and other

nutrients as predominant factors in the induction of particular morphological forms of *A. niger* in submerged fermentations (Clark et al., 1966 ; Barnett and Lilly, 1966 ; Kisser et al., 1980 ; Steel et al., 1952). For example, Kisser et al (1980) reported that manganese deficiency in the medium influenced the development of pellet morphologies of *A. niger* in submerged fermentations. In spore-inoculated fermentations a high initial spore concentration usually tends to produce dispersed growth while a low one favours pellet formation. However, evidence has been presented that pellets can arise even at high spore concentrations due to spore agglomeration during the early stage of germination (Galbraith and Smith, 1969).

Nitrogen may be supplied as ammonia, as nitrate or in organic compounds, such as amino acids or proteins. Nitrogen such as yeast extract plays an important role in metabolite overproduction and affects fungal morphology. In this study, the effect of organic nitrogen on starch saccharification was studied.

A loop of *Aspergillus niger* spores was inoculated in 50 ml FG medium or organic nitrogen growth medium in a 250 ml flask and was agitated at 30˚C with 250 rpm for 48 hours. The culture was centrifuged at 12,000 rpm at 4˚C and resuspended in 50 ml glucoamylase induction medium for 48 hours. The induction medium was made by replacing the sucrose content in medium with 30 g/L starch. 4 ml *A. niger* culture was

added into 16 ml liquefied starch solution in a 100 ml flask and was agitated at 40˚C with 250 rpm for saccharification. 20 g/L potato starch (contained 20% moisture) were used in the study.

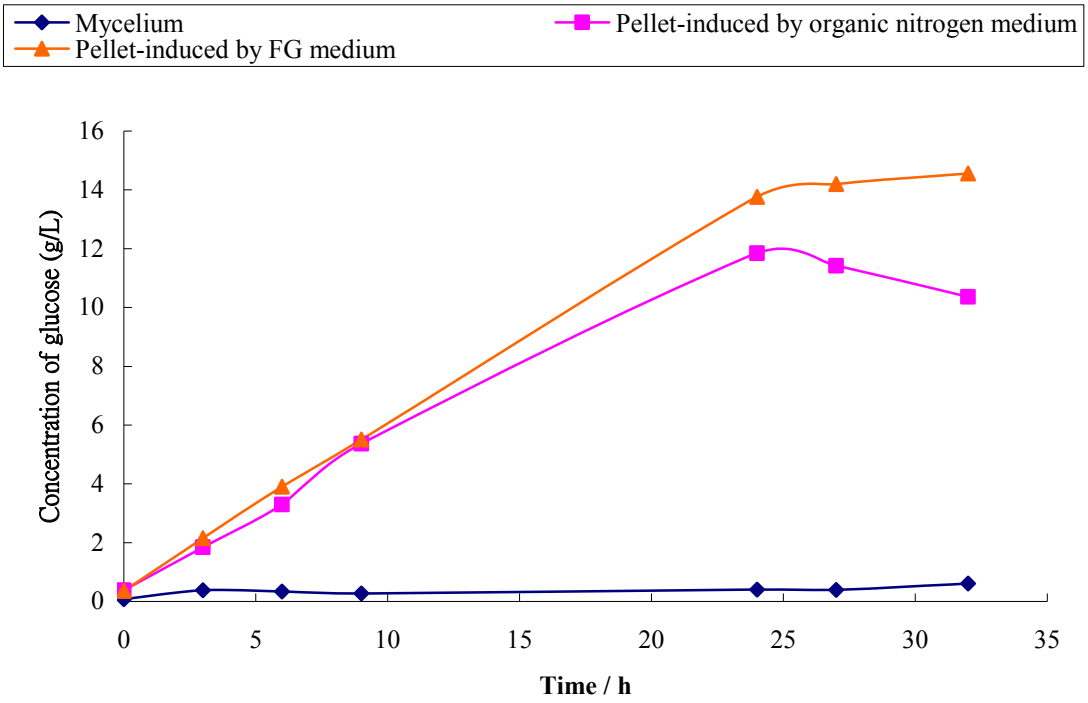



Figure 5.9 Glucose concentrations in the culture supernatant during starch saccharification using different forms of *A. niger* culture

Table 5.2 Conversion efficiency of starch to glucose in saccharification by different forms of *A. niger* culture

| Flask | Starch concentration (g/L) | Theoretical glucose concentration (g/L) | Actual glucose concentration (g/L) | Conversion efficiency (%) |
|---|---|---|---|---|
| Mycelium | 20 | 17.76 | 1.46 | 8.22 |
| Pellet-induced by organic nitrogen medium | 20 | 17.76 | 11.84 | 66.67 |
| Pellet-induced by FG medium | 20 | 17.76 | 14.56 | 81.98 |

Figure 5.9 showed that the amount of glucose saccharified from starch in pellet form of *A. niger* was much higher than that of mycelium form of *A. niger*. Mycelium form of *A. niger* was observed in the medium containing organic nitrogen and pellet form of *A. niger* was observed in FG medium. Among the factors considered to induce pellet formation in filamentous fungi is the limitation of particular nutrients, including nitrogen (Braun and Vecht-Lifshitz, 1991)

The difference in glucose concentration after starch saccharification in the mycelium form and pellet form of *A. niger* was probably due to the production of glucoamylase by *A. niger*. Differentiation of mycelia during pellet formation results in striking effects on enzyme production. For example, polygalacturonidase synthesis is well associated with the fungal morphology of *A. niger*. The more compact the pellet, the greater the polygalactorunidase synthesis. An increase of two orders of magnitude in enzyme concentration and rate of production between the free filamentous mycelium and the pelleted type was observed (Hemmersdorfer et al., 1987). Similar increases were observed in glucoamylase production rates by pellets of *A. niger* (Papagianni and Moo-Young, 2002). Such phenomena may be related to diffusional limitations in pellets, which either reduce the extent of catabolic repression in pellets or limit the oxygen supply, preventing an oxidative inactivation of a specific set of enzymes.

In the process of glucoamylase production by *A. niger*, fungal morphology was manipulated by means of inoculum level and quality. Different levels of spore inocula and vegetative inocula (Papagianni 2004) were used for the development of distinctive morphological forms in the main culture, and it was found that large pellets were associated with increased specific glucoamylase activities and lower specific protease activities compared with filamentous morphologies.

Table 5.2 showed that the highest conversion efficiency of starch to glucose was obtained in pellet form of *A. niger* in FG medium, followed by pellet form of *A. niger* in organic nitrogen medium. The lowest conversion efficiency was found in the mycelium form of *A. niger*. The disadvantages of dispersed mycelial growth included a reduction in efficiency of mixing and oxygen supply as well as increased wall growth which may lead to a low level of glucoamylase production by *A. niger*.

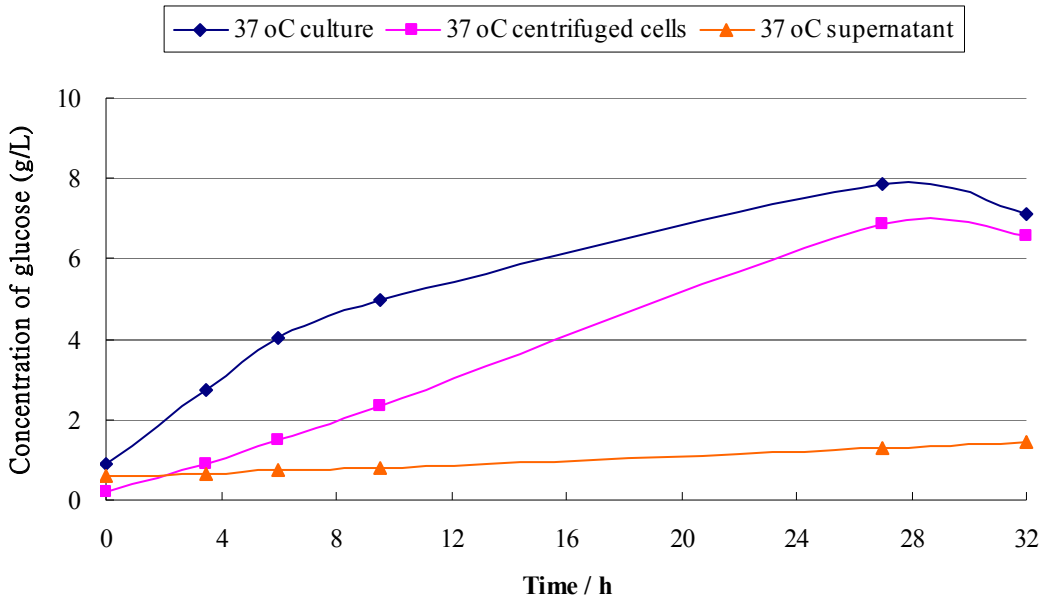
It was found that the pellet form of *A. niger* culture in FG medium had the highest conversion efficiency of starch to glucose. Therefore, pellet form of *A. niger* culture was chosen for further investigations.

### 5.6 Effect of temperature on starch saccharification

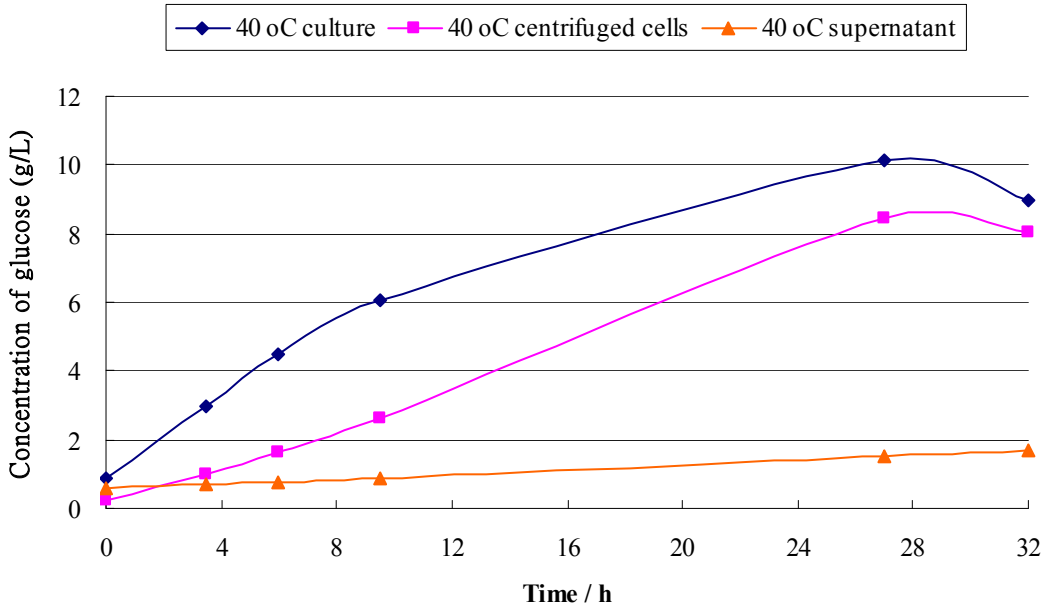
The efficiency of this fermentation process depends on the glucose concentration produced by saccharification process. Optimization of the saccharification and the fermentation processes is needed to achieve high lactic acid yield and low production cost. In spite of the economical advantage of simultaneous saccharification and fermentation (SSF) over separate hydrolysis and fermentation, the critical problem of SSF is the difference in temperature optimum of the enzyme and the fermenting microorganisms (Kadar et al., 2004).

A loop of *Aspergillus niger* spores was inoculated in 50 ml FG medium in a 250 ml flask and was agitated at 30˚C with 250 rpm for 24 hours. The culture was centrifuged at 4˚C and resuspended in 50 ml glucoamylase induction medium for 24 hours. The induction medium was made by replacing the sucrose content in FG medium with 20 g/L starch. The culture or centrifuged cells or supernatant of *A. niger* was added into 40 ml liquefied starch solution in a 250 ml flask and was incubated at 37˚C, 40˚C and 43˚C with 250 rpm for saccharification. Conversion efficiency is directly proportional to the glucose concentration in the medium. The glucose concentrations of the flasks incubated at different temperature were shown in Figure 5.10.

A)


37 oC culture
37 oC centrifuged cells
37 oC supernatant
Concentration of glucose (g/L)
0
2
4
6
8
10
0
4
8
12
16
20
24
28
32
Time / h

B)


40 oC culture
40 oC centrifuged cells
40 oC supernatant
Concentration of glucose (g/L)
0
2
4
6
8
10
12
0
4
8
12
16
20
24
28
32
Time / h

C)

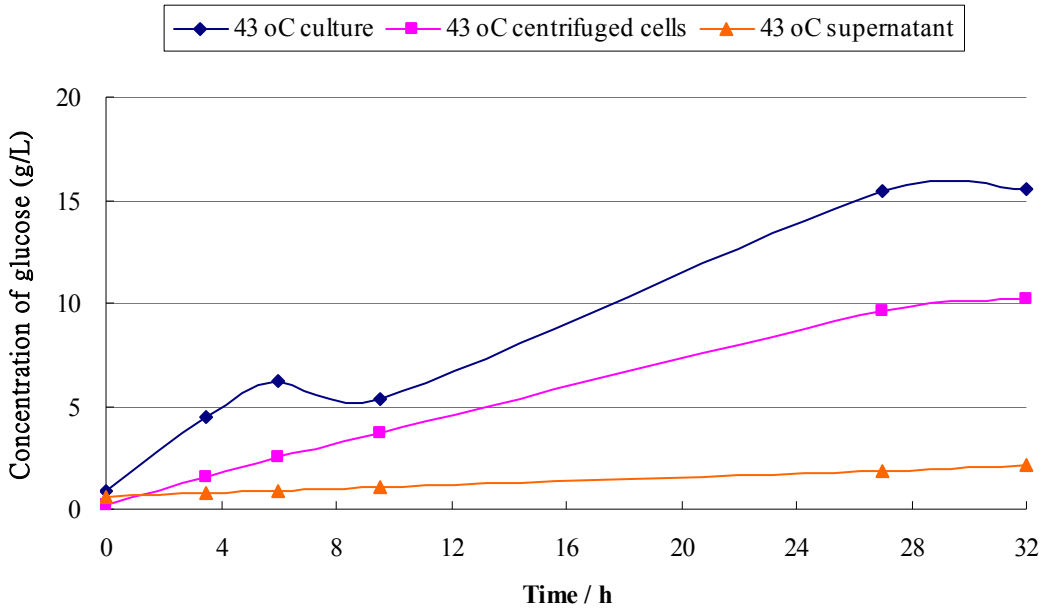



Figure 5.10 The glucose produced by saccharification of liquefied starch by *Aspergillus niger* spores at A) 37˚C, B) 40˚C and C) 43˚C

The conversion efficiency of starch to glucose was increased with increasing temperature as shown in Figure 5.10. Nearly 16 g/L glucose was produced from 20 g/L liquefied starch at 43˚C. However, only around 10 g/L glucose was saccharified in the flask incubated at 40˚C.

Although 43˚C was better for *Aspergillus niger* in the saccharification process, the survival of *B. subtilis* mutant strain R at high temperature (43˚C) had to be considered. In the previous section on "temperature effect on *B. subtilis* mutant strain R", 37°C,

40°C, 45°C, 50°C, 55°C in YE 20 medium were tested and it was found that 40°C was the best temperature for growth and lactic acid production. Poor growth of *B. subtilis* mutant strain R was observed at 45°C which might be due to cell death at the higher temperature.

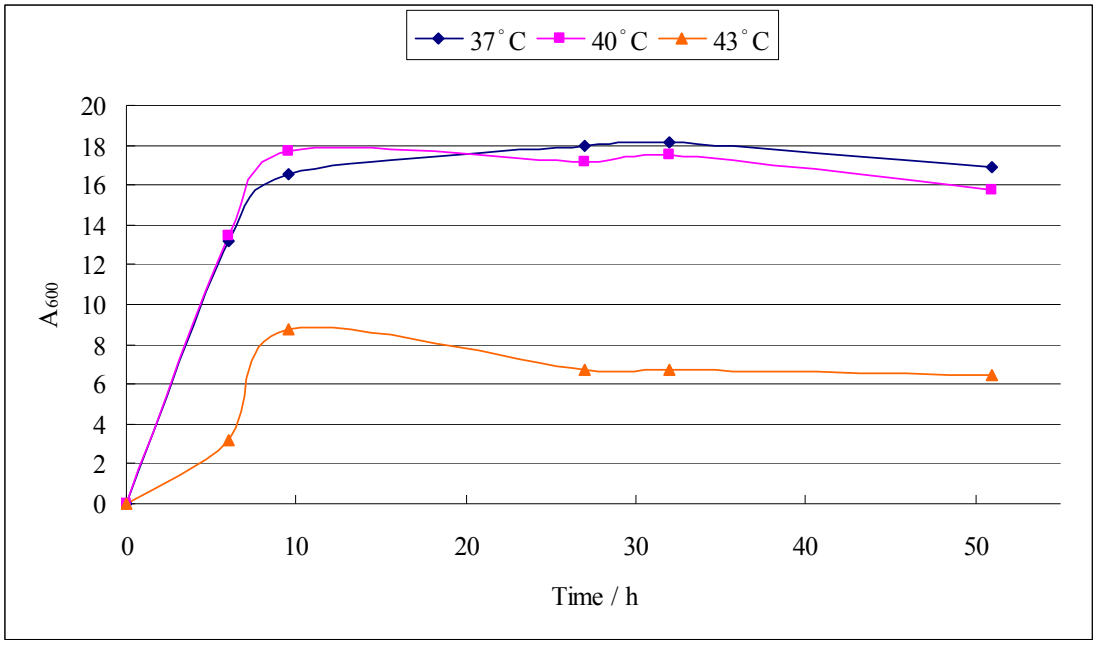



Figure 5.11 Cell density ($A_{600}$) of *B. subtilis* mutant strain R grown at different temperatures with agitation speed of 250 rpm in flasks

*B. subtilis* mutant strain R in YE 20 medium was incubated at 37°C, 40°C and 43°C to identify a suitable temperature for the SSF process. The growth of the *B. subtilis* mutant strain R was shown in Figure 5.11. The cell growth of *B. subtilis* mutant strain R at 43°C was poor and nearly two times lower than *B. subtilis* mutant strain R

cultivated at 37°C and 40°C. For co-culturing *Aspergillus niger* and *B. subtilis* mutant strain R in SSF, a compromised temperature of 40°C was chosen for further studies.

**5.7 Effect of starch concentration on starch saccharification**

Starch was liquefied by using α-amylase from *Bacillus subtilis.* The supernatant containing α-amylase (64,000 unit/ml) was added to starch solution. The process was carried out during autoclaving with 10 ppm calcium chloride.

A loop of *Aspergillus niger* spores were inoculated in 50 ml FG medium in a 250 ml flask and was agitated at 30˚C with 250 rpm for 48 hours. The culture was centrifuged at 12,000 rpm in 4˚C and resuspended in 50 ml glucoamylase induction medium for 48 hours. The induction medium was made by replacing the sucrose content in FG medium with 40 g/L starch.

6 ml *Aspergillus niger* culture was added into 24 ml liquefied starch solution in a 50 ml tube and was agitated at 40˚C with 250 rpm for saccharification. Potato starch (contained 20% moisture) was used in the study. The effect of starch concentration (50 g/L, 100 g/L, 150 g/L and 200 g/L) on starch saccharification was studied.

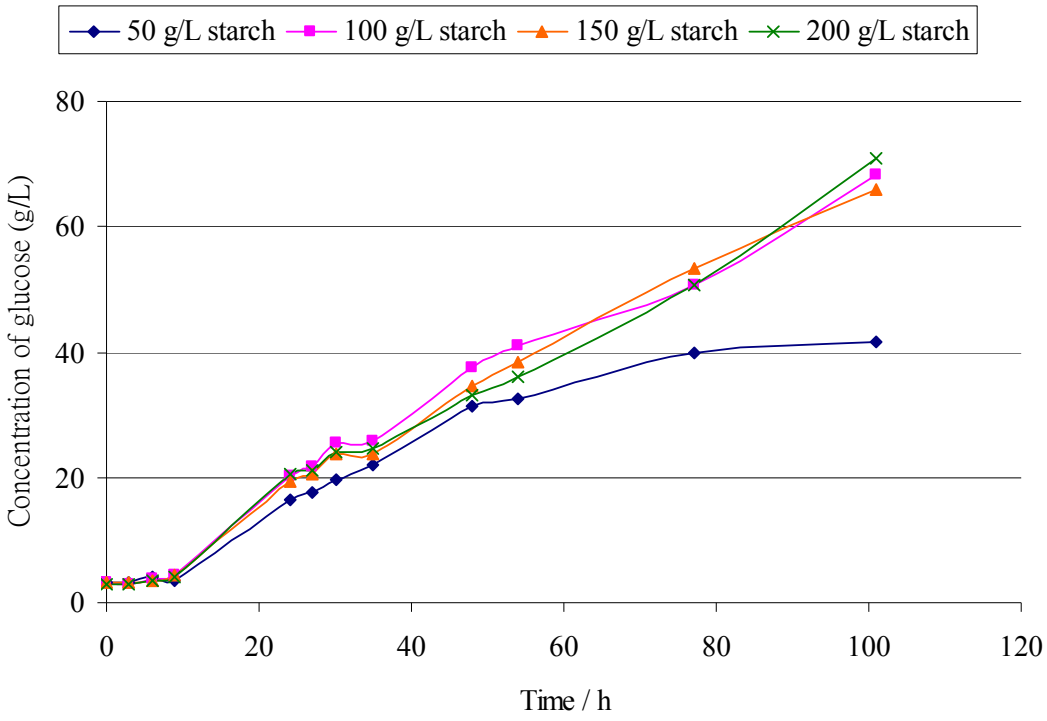



Figure 5.12 Glucose concentrations in the culture supernatant during starch saccharification using different concentrations of starch

Table 5.3 Conversion efficiency of starch to glucose in saccharification with different concentrations of starch

| Starch concentration (g/L) | Theoretical glucose concentration (g/L) | Actual glucose concentration (g/L) | Conversion efficiency (%) |
|---|---|---|---|
| 50 | 44.4 | 41.60 | 93.69 |
| 100 | 88.8 | 68.20 | 76.80 |
| 150 | 133.2 | 66.00 | 49.55 |
| 200 | 177.6 | 70.80 | 39.86 |

Figure 5.12 showed that the glucose concentration increased with time in all concentrations of starch. It was found that the amount of glucose saccharified from 100 g/L, 150 g/L and 200 g/L starch was 68.2 g/L, 66 g/L and 70.8 g/L respectively, which was higher than that of 50 g/L starch. Around 70 g/L of glucose was saccharified from 100 g/L, 150 g/L and 200 g/L starch and no further increase in glucose concentration was observed. The glucose concentration did not increase with the increase in starch concentrations. The reason was because high glucose concentration may inhibit the activity of glucoamylase which would affect the starch saccharification process.

Table 5.3 showed that the conversion efficiency of starch to glucose decreased with the increase in starch concentrations. This may also be due to the accumulation of glucose which would inhibit the activity of glucoamylase.

## 5.8 Effect of temperature on simultaneous saccharification and fermentation (SSF) to produce lactic acid by *Aspergillus niger* and recombinant *Bacillus subtilis* in shake flasks

Conventional biotechnological production of lactic acid from starch materials requires pretreatment by gelatinisation and liquefaction, which is carried out at high temperatures followed by enzymatic saccharification to glucose and subsequent conversion of glucose to lactic acid by fermentation (Anuradha et al., 1999). 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 (Cheng et al., 1991 ; Zhang and Chenyan 1994 ; Vishnu et al., 2002). In this study, mixed culture of *Aspergillus niger* and recombinant *Bacillus subtilis* were used in SSF to produce lactic acid from starch and the effect of temperature on SSF was studied.

3.5 ml recombinant *Bacillus subtilis* culture (5 % inoculum) and 14 ml *Aspergillus niger* culture after enzyme induction were mixed together and inoculated in 70 ml medium in a 100 ml flask. The flask was agitated at 37°C or 40°C with 180 rpm. The pH value was maintained at 7.5 by 5 M NaOH. Starch in the medium was liquefied by α-amylase from recombinant *Bacillus subtilis* Φ105 MU331. The process was carried out at 105℃ for 30 minutes with 10 ppm calcium chloride.

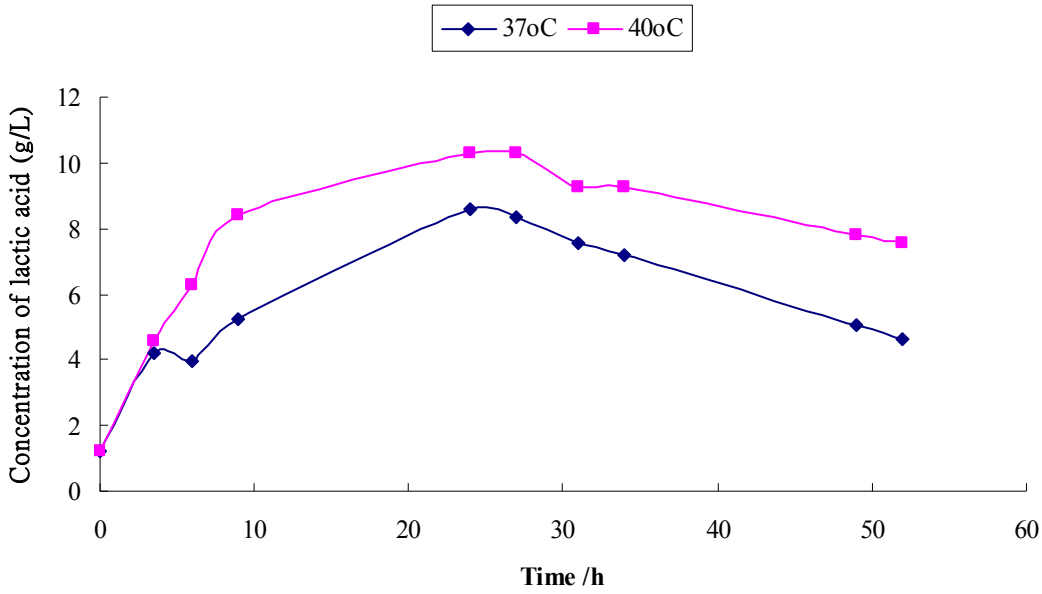



Figure 5.13 SSF to produce lactic acid from 20 g/L potato starch at 37°C and 40°C

Table 5.4 The lactic acid yield and productivity of the mutant strain R in SSF of lactic acid from starch at 37°C and 40°C

| Flask | Time (h) | Starch concentration (g/L) | Theoretical glucose concentration (g/L) | Lactic acid concentration (g/L) | Yield $_{LA/GLU}$ (g/g) | Lactic acid productivity (g/L/h) |
|---|---|---|---|---|---|---|
| 37°C | 24 | 20 | 17.76 | 8.58 | 0.48 | 0.358 |
| 40°C | 24 | 20 | 17.76 | 10.32 | 0.58 | 0.430 |

During the SSF, potato starch was saccharified to glucose by glucoamylase from *A. niger*, and glucose was metabolized by recombinant *B. subtilis* mutant strain R and converted to lactic acid. Figure 5.13 showed that higher lactic acid was obtained in SSF at 40°C. The fermentation rate was affected by the saccharification rate. When the saccharification rate was greater than the fermentation rate, glucose accumulation was observed. The results showed that only very small amounts of glucose accumulated during the SSF process. This indicated that the whole process was mainly affected by the saccharification rate.

In the initial 24h of SSF, the lactic acid concentration at 37°C and 40°C was 8.58 g/L and 10.32 g/L, respectively. From 24 h to the end of SSF, the lactic acid concentration decreased slowly. It was because the glucose concentration was close to zero and the cells started to metabolize lactic acid for maintenance. Therefore, a decrease in lactic acid concentration was observed.

Table 5.4 showed that the average lactic acid productivity at 37°C and 40°C was 0.358 g/L/h and 0.43 g/L/h with the lactic acid yield of 0.48 g/g and 0.58 g/g, respectively.

The results indicated that higher lactic acid concentration was obtained at 40°C in SSF to produce lactic acid from potato starch in shake flask.

## 5.9 Effect of temperature and initial starch concentration on simultaneous saccharification and fermentation (SSF) to produce lactic acid by *Aspergillus niger* and recombinant *Bacillus subtilis* in fermentor

In this study, mixed culture of *Aspergillus niger* and recombinant *Bacillus subtilis* were used in SSF of lactic acid from potato starch and the effect of temperature and initial starch concentration on SSF was studied in a fermentor. The initial starch concentration of 50 g/L and 300 g/L potato starch were used. The starch contained 20% moisture.

25 ml recombinant *Bacillus subtilis* culture (5 % inoculum) and 120 ml *Aspergillus niger* culture after enzyme induction were mixed together and inoculated in 600 ml YE 20 medium in a 1L fermentor. (BIOSTAT® B plus, Sartorius BBI Systems, Germany) Starch in the medium was liquefied by α-amylase from recombinant *Bacillus subtilis* Φ105 MU331. The process was carried out at 105℃ for 30 minutes with 10 ppm calcium chloride. The fermentation medium was kept at 37℃ or 40℃ and pH 7.5. The agitation speed was set at 50 rpm. Air flow was 2 vvm. The pH was controlled by 2M HCL and 8 M NaOH. Liquefied starch was fed intermittently into the fermentor in order to facilitate the SSF process.

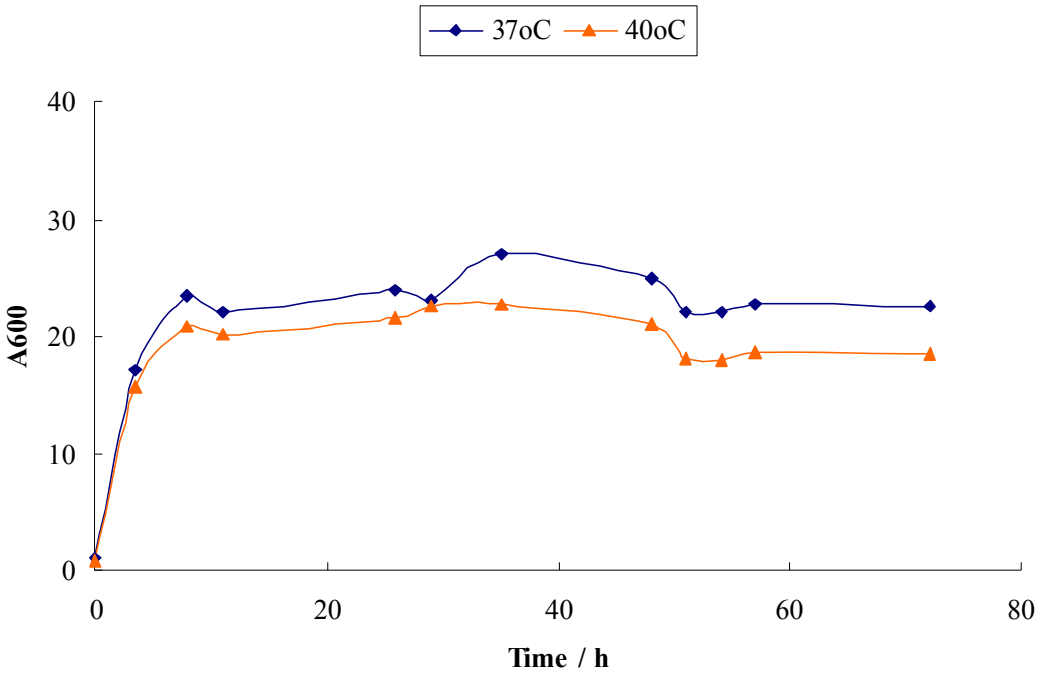



Figure 5.14 Cell density ($A_{600}$) of *B. subtilis* mutant strain R during the SSF. The cells were cultivated at pH 7.5 and 37°C or 40°C were used in the fermentation, respectively. Initial potato starch concentration was 50 g/L.

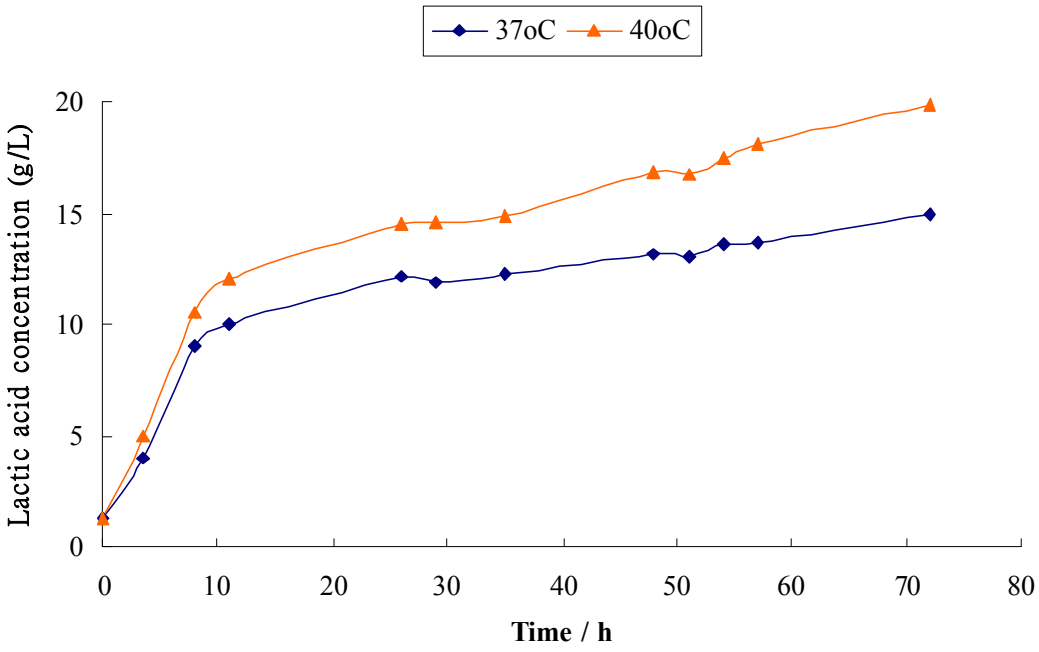



Figure 5.15 Lactic acid concentrations in the culture supernatant of mutant strain R during the SSF at different temperatures. Initial potato starch concentration was 50 g/L.

Table 5.5 The lactic acid yield and productivity of the mutant strain R in SSF of lactic acid from starch at 37°C and 40°C using initial starch concentration of 50 g/L

| Fermentor | Time (h) | Starch concentration (g/L) | Theoretical glucose concentration (g/L) | Lactic acid concentration (g/L) | Yield $_{LA/GLU}$ (g/g) | Lactic acid productivity (g/L/h) |
|---|---|---|---|---|---|---|
| 37°C | 11 | 40 | 44.4 | 10.04 | 0.226 | 0.91 |
| 37°C | 57 | 64 | 71.04 | 13.72 | 0.193 | 0.24 |
| 40°C | 11 | 40 | 44.4 | 12.04 | 0.271 | 1.09 |
| 40°C | 57 | 64 | 71.04 | 18.12 | 0.255 | 0.32 |

During the SSF, potato starch was saccharified to glucose by glucoamylase from *A. niger*, and glucose was metabolized by recombinant *B. subtilis* mutant strain R and converted to lactic acid. The effect of cultivation temperature on the SSF was investigated by controlling the growth temperature at 37°C or 40°C using initial potato starch concentration of 50 g/L.

As shown in Figure 5.14, the cell density obtained at the fermentation temperature of 37°C and 40°C was similar. The growth rate during the exponential phase at 37°C and 40°C were seen to be similar where the cell density rapidly increased from 0 h to 8 h. The highest cell density obtained at 37°C and 40°C was around 12 g/L. This indicated that the mutant strain R of recombinant *Bacillus subtilis* had similar growth pattern at 37°C and 40°C.

Figure 5.15 showed that the curve of lactic acid production at 37°C and 40°C during SSF can be divided into two parts. In the initial 12h, the lactic acid concentration at 37°C and 40°C were 10.04 g/L and 12.04 g/L, respectively and the productivity at 37°C and 40°C reached 0.91 g/L/h and 1.09 g/L/h, respectively. From 12 h to the end of SSF process, the lactic acid concentration increased slowly. The final lactic acid concentration at 40°C was 18.12 g/L which was higher than that of at 37°C. Table 5.5 showed that the average productivity at 37°C and 40°C were 0.24 g/L/h and 0.32 g/L/h, respectively. The results indicated that higher lactic acid concentration and productivity was obtained at 40°C than at 37°C. It may be due to the activity of glucoamylase that work better at 40°C than at 37°C. The same results were obtained in shake flask studies.

The productivity of lactic acid using initial starch concentration of 50 g/L was low. It may be due to the limitation of substrate concentration for SSF to produce lactic acid. In order to investigate the effect of starch concentration on SSF, a higher initial starch concentration of 300 g/L was used and the result was shown in Figure 5.17.

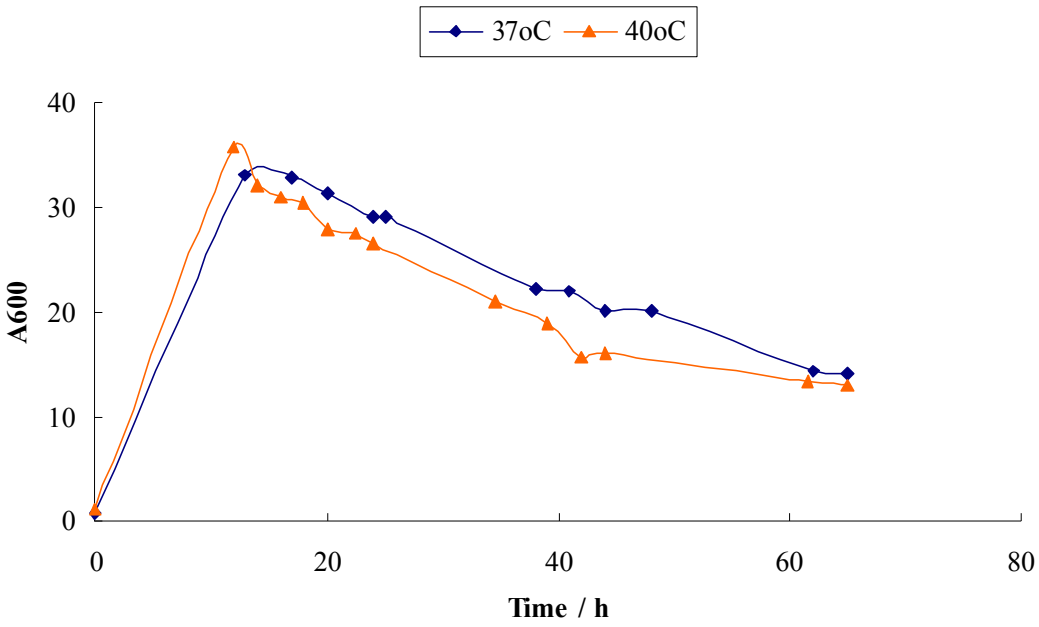



Figure 5.16 Cell density ($A_{600}$) of *B. subtilis* mutant strain R during the SSF. The cells were cultivated at pH 7.5 and 37°C or 40°C were used in the fermentation, respectively. Initial potato starch concentration was 300 g/L.

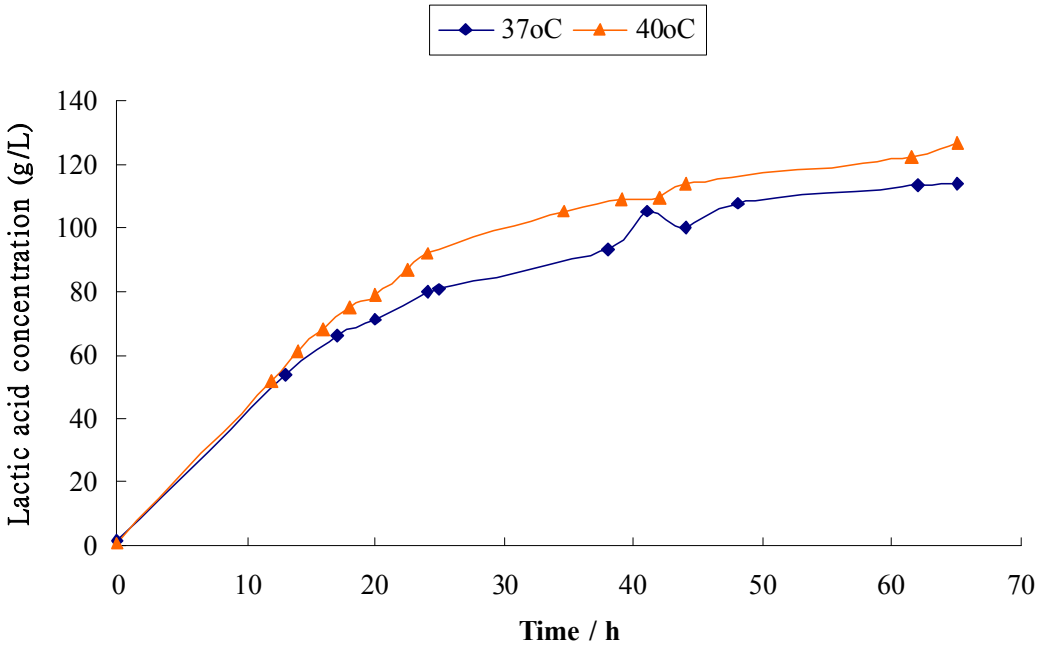



Figure 5.17 Lactic acid concentrations in the culture supernatant of mutant strain R during the SSF at 37°C or 40°C. Initial potato starch concentration was 300 g/L.

Table 5.6 The lactic acid yield and productivity of the mutant strain R in SSF to produce lactic acid from starch at 37°C and 40°C using initial starch concentration of 300 g/L

| Fermentor | Starch concentration (g/L) | Theoretical glucose concentration (g/L) | Lactic acid concentration (g/L) | $Y_{LA/GLU}$ (g/g) | Lactic acid productivity (g/L/h) | Duration of fermentation (h) | Maximal lactic acid productivity (g/L/h) |
|---|---|---|---|---|---|---|---|
| 37°C | 330 | 366.3 | 80.25 | 0.311 | 3.34 | 24 | 4.01 (13h) |
| | | | 114 | | 1.75 | 65 | |
| 40°C | 310.64 | 344.81 | 92.2 | 0.368 | 3.84 | 24 | 4.85(14h) |
| | | | 126.75 | | 1.95 | 65 | |

By comparing Figure 5.14 and Figure 5.16, it was found that the maximal cell density was higher with an initial starch concentration of 300 g/L than an initial starch concentration of 50 g/L. This indicated that the starch saccharification process was enhanced in higher starch concentration and more glucose was saccharified from starch to support the growth of mutant strain R of recombinant *B. subtilis*.

Figure 5.16 showed that the cell density obtained at fermentation temperature of 37°C and 40°C in initial starch concentration of 300 g/L was similar. The growth rate during the exponential phase at 37°C and 40°C were seen to be similar where the cell density rapidly increased from 0 h to 12 h. The highest cell density obtained at 37°C

and 40°C was around 35.6 g/L. This indicated that mutant R of recombinant *Bacillus subtilis* had similar growth pattern at 37°C and 40°C.

By comparing Figure 5.15 and Figure 5.17, it was found that higher lactic acid was produced in initial starch concentration of 300 g/L than that of in initial starch concentration of 50 g/L. This was probably due to the higher cell density obtained in increased starch concentration. The concentration of lactic acid at 40°C increased from around 12 g/L to 92.2 g/L after 24 h of SSF, as a result of increasing initial starch concentration from 50 g/L to 300 g/L.

Figure 5.17 showed that the curve of lactic acid production at 37°C and 40°C can also be divided into two parts. In the initial 24h, the lactic acid concentration at 37°C and 40°C were 80.25 g/L and 92.2 g/L, respectively and the productivity at 37°C and 40°C reached 3.34 g/L/h and 3.84 g/L/h, respectively. The maximal lactic acid productivity at 40°C was 4.85 g/L/h obtained after 14 h of fermentation, which was higher than that of 37°C with productivity of 4.01 g/L/h. From 24 h to the end of SSF process, the lactic acid concentration increased slowly. The final lactic acid concentration at 40°C was 126.75 g/L which was higher than at 37°C, at 114 g/L.

Table 5.6 showed that the average productivity at 37°C and 40°C were 1.75 g/L/h and 1.95 g/L/h, respectively. The result indicated that higher lactic acid concentration and productivity was obtained at 40°C than at 37°C. It may be due to the physiological enzymes that work better at 40°C than at 37°C. Similar conclusions were obtained from the SSF to produce lactic acid with initial starch concentration of 50 g/L. This confirmed that fermentation temperature of 40°C was an optimum cultivation temperature for both saccharification and fermentation for the SSF of lactic acid production by recombinant *Bacillus subtilis* mutant strain R.

Result indicated that higher lactic acid concentration and productivity was obtained at the fermentation temperature of 40°C. Therefore, 40°C was used in further studies of SSF of lactic acid by *B. subtilis*.

### 5.10 Effect of immobilization of *A. niger* culture on simultaneous saccharification and fermentation (SSF) to produce lactic acid by *Aspergillus niger* and recombinant *Bacillus subtilis* in fermentor

*Aspergillus niger* is the source of glucoamylase in this project. The spores of *A. niger* can be immobilized in sodium alginate to form beads. Immobilization has the advantage that high cell density of *A. niger* can be obtained as the spores are concentrated and trapped in sodium alginate. In order to investigate the performance of SSF by immobilized and free culture *Aspergillus niger*, two forms of inoculum are used. The effect of immobilization of *A. niger* culture on SSF was studied.

25 ml recombinant *Bacillus subtilis* culture (5 % inoculum) and 120 ml *Aspergillus niger* culture or ~20 g of immobilized *Aspergillus niger* gel beads after enzyme induction were mixed together and inoculated in 600 ml YE 50 medium in a 1L fermentor. (BIOSTAT® B plus, Sartorius BBI Systems, Germany) Initial starch concentration of 300 g/L in the medium was liquefied by α-amylase from recombinant *Bacillus subtilis* Φ105 MU331. The process was carried out at 105℃ for 30 minutes with 10 ppm calcium chloride. The fermentation medium was kept at 40℃ and pH 7.5. The agitation speed was set at 50 rpm. Air flow was 2 vvm. The pH was controlled by 2M HCL and 8 M NaOH. Liquefied starch was fed intermittently into the fermentor in order to facilitate the SSF process.

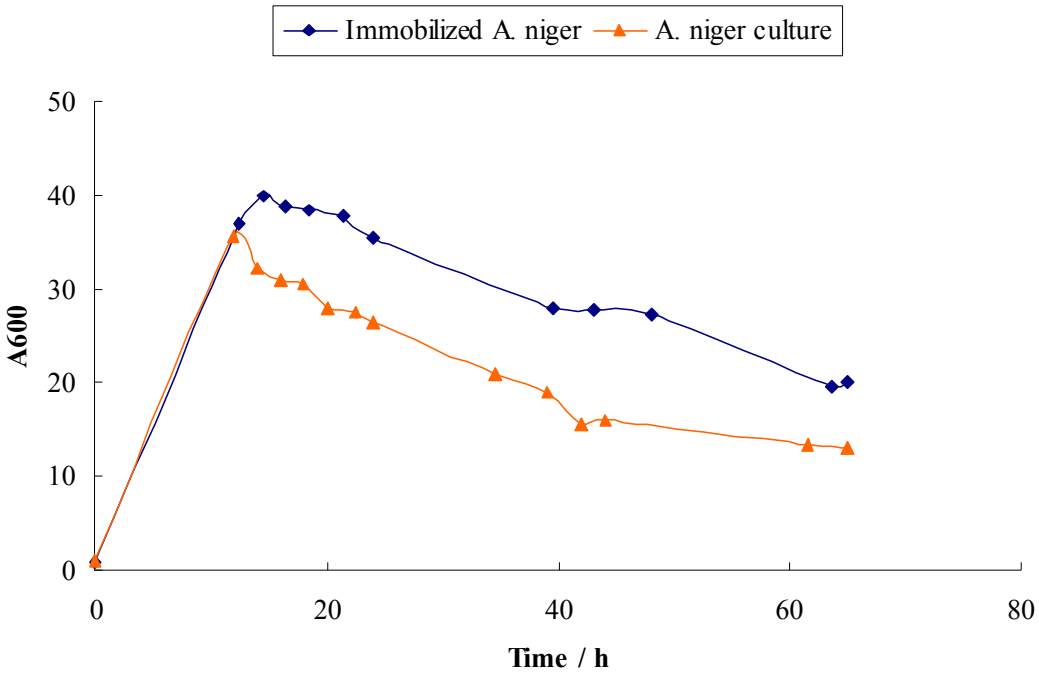



Figure 5.18 Cell density ($A_{600}$) of *B. subtilis* mutant strain R during the SSF. The cells were cultivated at pH 7.5 and 40°C. Immobilized *A. niger* and *A. niger* culture were used in the fermentation, respectively. Initial potato starch concentration was 300 g/L.

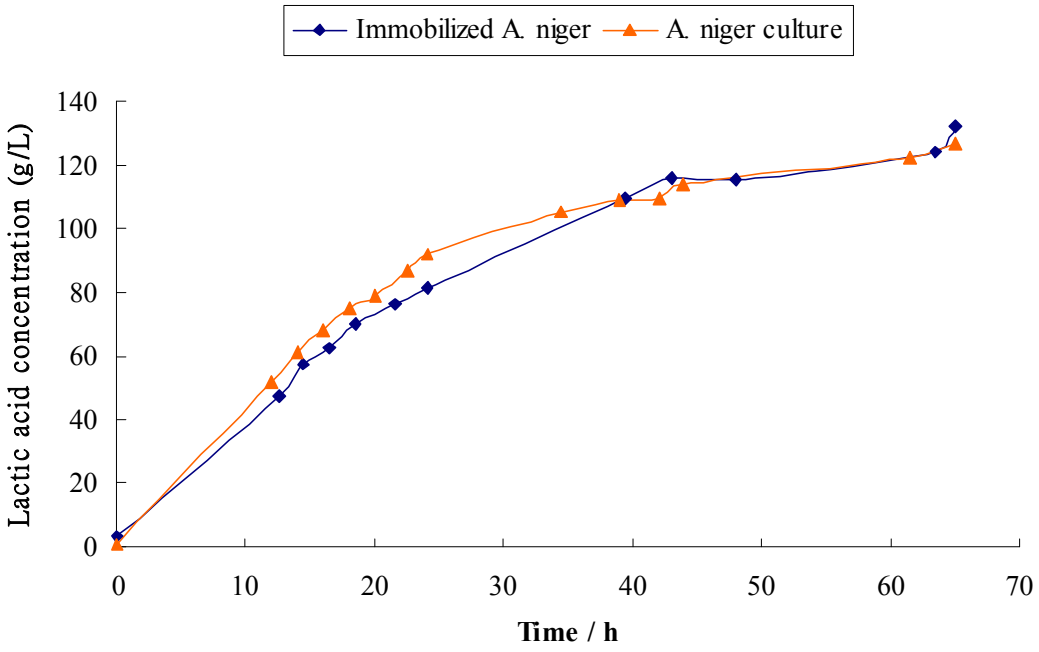



Figure 5.19 Lactic acid concentrations in the culture supernatant of mutant strain R during the SSF at 40°C. Immobilized *A. niger* and *A. niger* culture were used in the fermentation, respectively. Initial potato starch concentration was 300 g/L.

Table 5.7 The lactic acid yield and productivity of the mutant strain R in SSF of lactic acid from starch at 40°C using immobilized *A. niger* and *A. niger* culture with initial starch concentration of 300 g/L

| Fermentor | Starch concentration (g/L) | Theoretical glucose concentration (g/L) | Lactic acid concentration (g/L) | $Y_{LA/GLU}$ (g/g) | Lactic acid productivity (g/L/h) | Duration of fermentation (h) | Maximal lactic acid productivity (g/L/h) |
|---|---|---|---|---|---|---|---|
| Immobilized *A. niger* | 350.4 | 388.94 | 81.5 | 0.340 | 3.40 | 24 | 4.9 (14.5h) |
| | | | 132.4 | | 2.04 | 65 | |
| *A. niger* culture | 310.64 | 344.81 | 92.2 | 0.368 | 3.84 | 24 | 4.85 (14h) |
| | | | 126.75 | | 1.95 | 65 | |

As shown in Figure 5.18, the cell density obtained in *B. subtilis* with immobilized *A. niger* or *A. niger* culture was similar. The growth rate during the exponential phase in the two types of inoculum was seen to be similar where the cell density rapidly increased from 0 h to 12 h. This indicated that mutant strain R of recombinant *Bacillus subtilis* had similar growth pattern when it was mixed with immobilized *A. niger* or *A. niger* culture.

Figure 5.19 showed that the lactic acid production increased with time during SSF in both types of *A. niger* inoculum. In the initial 24h, the lactic acid concentration using immobilized *A. niger* and *A. niger* culture were 81.5 g/L and 92.2 g/L, respectively and the productivity reached 3.4 g/L/h and 3.84 g/L/h, respectively. From 24 h to the

end of SSF process, the lactic acid concentration increased slowly. The final lactic acid concentration using immobilized *A. niger* was 132.4 g/L which was similar to that of *A. niger* culture, reaching 126.75 g/L. Table 5.7 showed that the average productivity using immobilized *A. niger* and *A. niger* culture were 2.04 g/L/h and 1.95 g/L/h, respectively. The results indicated that similar lactic acid concentration and productivity was obtained when immobilized *A. niger* or *A. niger* culture was used with recombinant *B. subtilis* mutant strain R.

## 5.11 Effect of pH on simultaneous saccharification and fermentation (SSF) to produce lactic acid by *Aspergillus niger* and recombinant *Bacillus subtilis* in fermentor

During the SSF of lactic acid from starch, glucoamylase from *A. niger* was required for the saccharification of starch to glucose. The optimum pH and temperature of *A. niger* glucoamylase was 4.2 and 60$^{o}$C. However, it was found that the growth of recombinant *B. subtilis* mutant strain R was not stable at temperature over 43$^{o}$C in the previous section. It was believed that there could be an increase in saccharification rate when the cultivation pH was closer to the optimum pH of glucoamylase. Therefore, the effect of pH 6.5 and pH 7.5 on SSF to produce lactic acid was studied.

25 ml recombinant *Bacillus subtilis* culture (5 % inoculum) and 120 ml *Aspergillus niger* culture after enzyme induction were mixed together and inoculated in 600 ml YE 50 medium in a 1L fermentor. (BIOSTAT® B plus, Sartorius BBI Systems, Germany) Initial starch concentration of 300 g/L in medium was liquefied by α-amylase from recombinant *Bacillus subtilis* Φ105 MU331. The process was carried out at 105℃ for 30 minutes with 10 ppm calcium chloride. The fermentation medium was kept at 40℃ and pH 6.5 or pH 7.5. The agitation speed was set at 50 rpm. Air

flow was 2 vvm. The pH was controlled by 2M HCL and 8 M NaOH. Liquefied starch was fed intermittently into the fermentor in order to facilitate the SSF process.

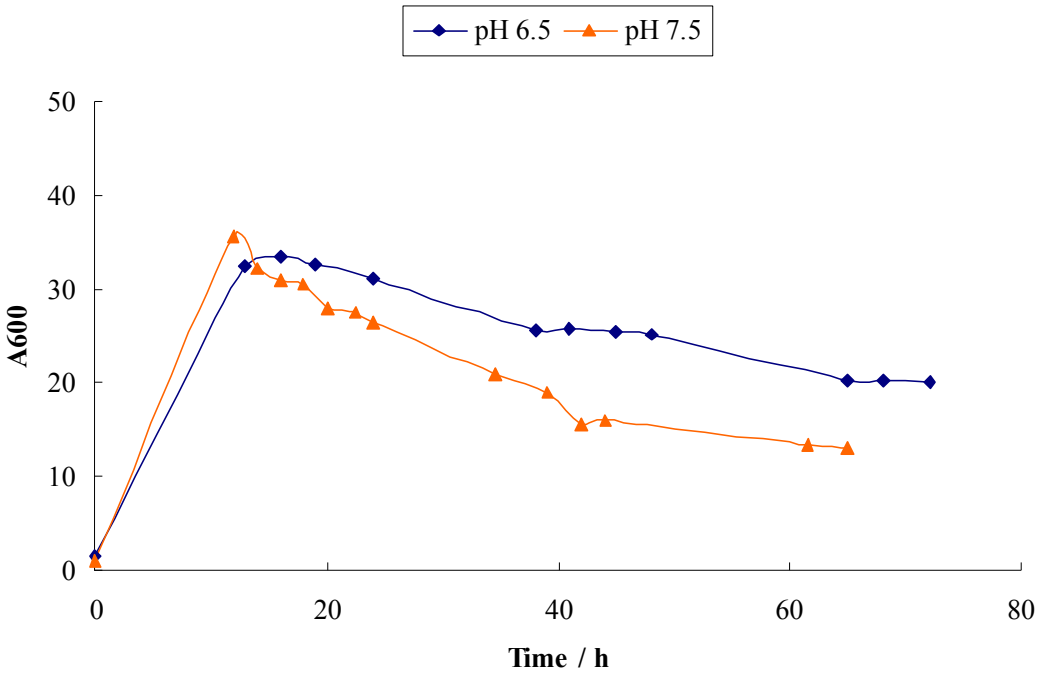



Figure 5.20 Cell density ($A_{600}$) of *B. subtilis* mutant strain R during the SSF. The cells were cultivated at 40°C and pH 6.5 or pH 7.5 was used in the fermentation. Initial potato starch concentration was 300 g/L.

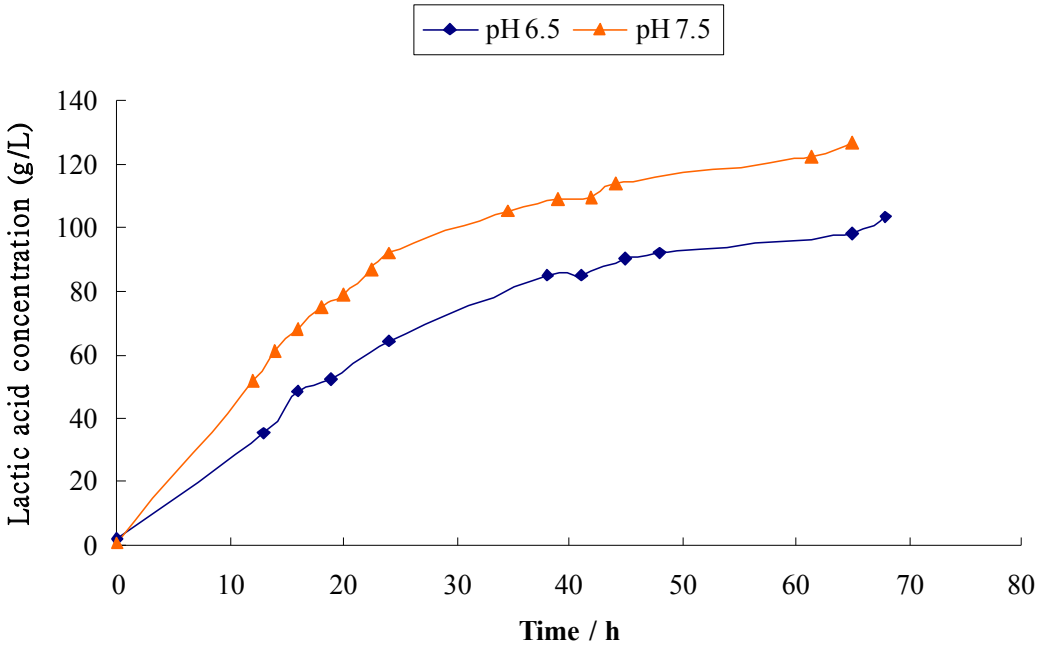



Figure 5.21 Lactic acid concentrations in the culture supernatant of mutant strain R during the SSF at 40°C. pH 6.5 or pH 7.5 were used in the fermentation, respectively. Initial potato starch concentration was 300 g/L.

Table 5.8 The lactic acid yield and productivity of the mutant strain R in SSF of lactic acid from starch at pH 6.5 and pH 7.5 with initial starch concentration of 300 g/L

| Fermentor | Starch concentration (g/L) | Theoretical glucose concentration (g/L) | Lactic acid concentration (g/L) | $Y_{LA/GLU}$ (g/g) | Lactic acid productivity (g/L/h) | Duration of fermentation (h) | Maximal lactic acid productivity (g/L/h) |
|---|---|---|---|---|---|---|---|
| pH 6.5 | 320.08 | 355.29 | 64.33 | 0.277 | 2.68 | 24 | 4.3 (16h) |
| | | | 98.5 | | 1.52 | 65 | |
| pH 7.5 | 310.64 | 344.81 | 92.2 | 0.368 | 3.84 | 24 | 4.85 (14h) |
| | | | 126.75 | | 1.95 | 65 | |

As shown in Figure 5.20, the cell density obtained at pH 6.5 and pH 7.5 was similar. The growth rate during the exponential phase in the two types of inoculum was similar where the cell density rapidly increased from 0 h to 12 h. This indicated that the mutant strain R of recombinant *Bacillus subtilis* had similar growth pattern at pH 6.5 and pH 7.5 during the SSF process.

Figure 5.21 showed that higher lactic acid concentration was produced at pH 7.5 than that of pH 6.5 during SSF. In the initial 24h, the lactic acid concentration at pH 6.5 and pH 7.5 were 64.33 g/L and 92.2 g/L, respectively and the productivity reached 2.68 g/L/h and 3.84 g/L/h, respectively. From 24 h to the end of SSF process, the lactic acid concentration increased slowly. The final lactic acid concentration at pH 7.5 was 126.75 g/L which was higher than that of pH 6.5, at 98.5 g/L. Table 5.8 showed that the average productivity at pH 6.5 and pH 7.5 were 1.52 g/L/h and 1.95 g/L/h, respectively. It was found that lower amounts of lactic acid was produced at pH 6.5 than at pH 7.5 even though pH 6.5 was closer to the optimal pH of glucoamylase. The results indicated that fermentation process of lactic acid was affected when the pH of medium was changed. In the SSF process, the temperature and pH for enzymatic saccharification and the fermentation process should be optimal for both in order to produce the fermentation products effectively.

It was found that higher lactic acid concentration and productivity was obtained at pH 7.5 in SSF of lactic acid from starch by *B. subtilis*.

### 5.12 Effect of induction time of glucoamylase on simultaneous saccharification and fermentation (SSF) to produce lactic acid by *Aspergillus niger* and recombinant *Bacillus subtilis* in fermentor

*A. niger* glucoamylase was induced in the medium containing starch. The induction period of glucoamylase may affect the SSF of lactic acid. The effect of shortening the induction period of glucoamylase from 48 h to 24 h on SSF was studied.

25 ml recombinant *Bacillus subtilis* culture (5 % inoculum) and 120 ml *Aspergillus niger* culture after 24 h or 48 h enzyme induction were mixed together and inoculated in 600 ml YE 50 medium in a 1L fermentor. (BIOSTAT® B plus, Sartorius BBI Systems, Germany) Initial starch concentration of 300 g/L in medium was liquefied by α-amylase from recombinant *Bacillus subtilis* Φ105 MU331. The process was carried out at 105℃ for 30 minutes with 10 ppm calcium chloride. The fermentation medium was kept at 40℃ and pH 7.5. The agitation speed was set at 50 rpm. Air flow was 2 vvm. The pH was controlled by 2M HCL and 8 M NaOH. Liquefied starch was fed intermittently into the fermentor in order to facilitate the SSF process.

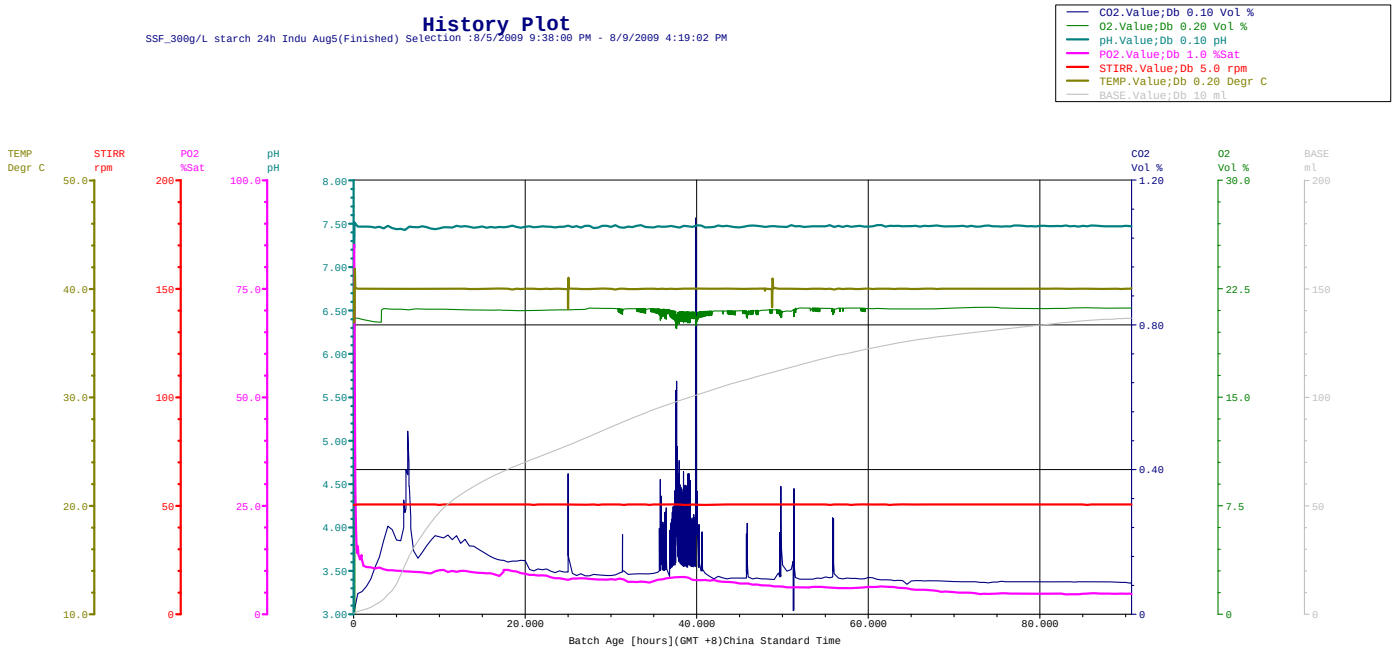



Figure 5.22 On-line monitoring of different parameters during SSF

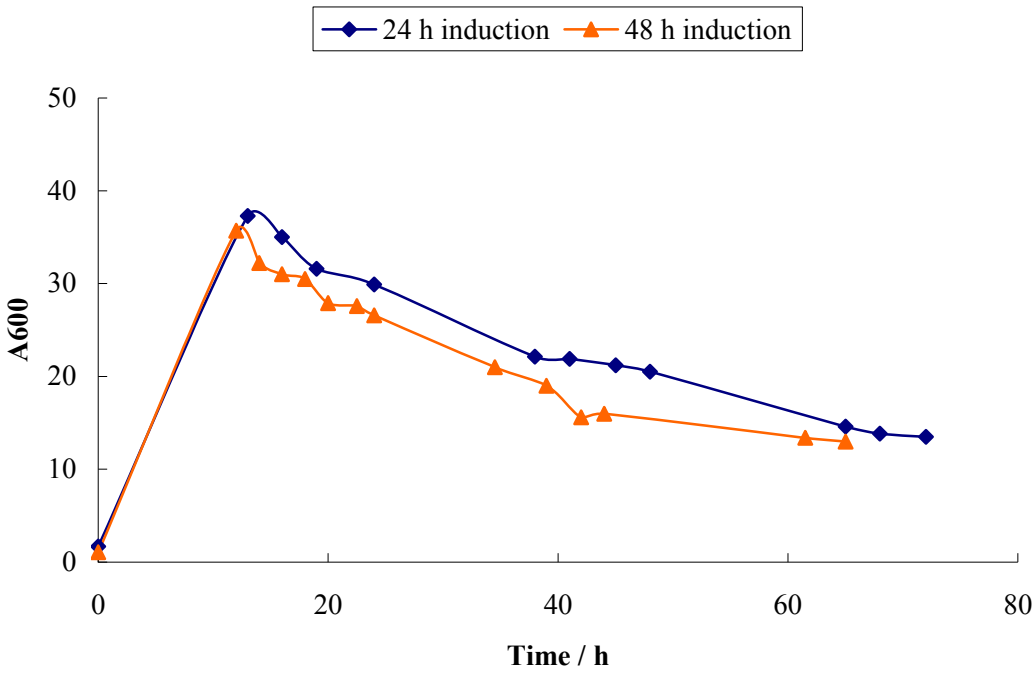



Figure 5.23 Cell density ($A_{600}$) of *B. subtilis* mutant strain R during the SSF. The cells were cultivated at 40°C and *A. niger* culture after 24 h or 48 h glucoamylase induction was used in the fermentation, respectively. Initial potato starch concentration was 300 g/L.

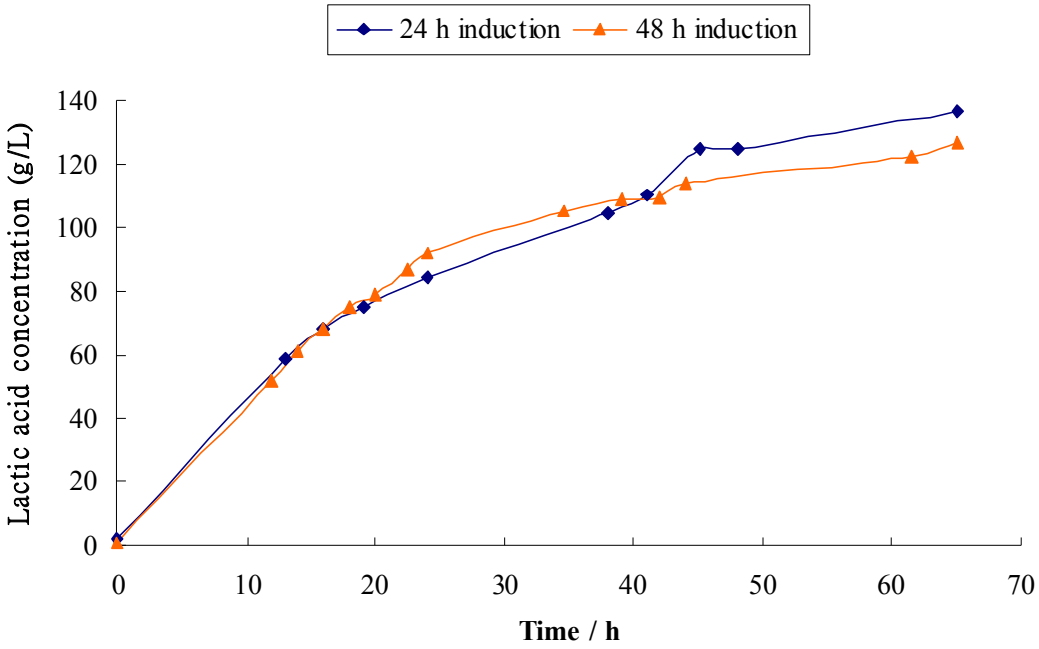



Figure 5.24 Lactic acid concentrations in the culture supernatant of mutant strain R during the SSF at 40°C. *A. niger* culture after 24 h or 48 h glucoamylase induction were used in the fermentation, respectively. Initial potato starch concentration was 300 g/L.

Table 5.9 The lactic acid yield and productivity of the mutant strain R in SSF of lactic acid from starch using *A. niger* culture after 24 h or 48 h induction

| Fermentor | Starch concentration (g/L) | Theoretical glucose concentration (g/L) | Lactic acid concentration (g/L) | $Y_{LA/GLU}$ (g/g) | Lactic acid productivity (g/L/h) | Duration of fermentation (h) | Maximal lactic acid productivity (g/L/h) |
|---|---|---|---|---|---|---|---|
| 24 h induction | 320.08 | 355.29 | 84.8<br>136.9 | 0.385 | 3.53<br>2.11 | 24<br>65 | 4.38 (13h) |
| 48 h induction | 310.64 | 344.81 | 92.2<br>126.75 | 0.368 | 3.84<br>1.95 | 24<br>65 | 4.85 (14h) |

Figure 5.22 showed the monitoring of several fermentation parameters on-line during SSF of lactic acid. The fermentation temperature was controlled at 40°C and the pH of medium was controlled at 7.5 by 8M NaOH throughout the whole fermentation process. The agitation speed was set at 50 rpm and air flow was 2 vvm in order to provide optimal conditions for lactic acid production. The dissolved oxygen level dropped to nearly 0% very quickly after the start of fermentation. It is because mutant R grew rapidly from 0 h to 6 h and they consumed large amount of oxygen for growth and lactic acid production. The volume of base added was increasing with the amount of lactic acid produced in order to maintain at constant pH 7.5.

As shown in Figure 5.23, the cell density of *B. subtilis* mixed with *A. niger* culture after 24 h or 48 h glucoamylase induction was similar. The growth rate during the exponential phase in the two types of inoculum was similar where the cell density rapidly increased from 0 h to 12 h. This indicated that mutant strain R of recombinant *Bacillus subtilis* had similar growth pattern when it was mixed with *A. niger* culture after 24 h or 48 h glucoamylase induction.

Figure 5.24 showed that similar lactic acid concentration was obtained in *A. niger* culture after 24 h or 48 h glucoamylase induction during SSF. In the initial 24h, the

lactic acid concentration in *A. niger* culture with 24 h or 48 h glucoamylase induction were 84.8 g/L and 92.2 g/L, respectively and the productivity reached 3.53 g/L/h and 3.84 g/L/h, respectively. From 24 h to the end of SSF process, the lactic acid concentration increased slowly. The final lactic acid concentration in *A. niger* culture with 24 h glucoamylase induction was 136.9 g/L which was slightly higher than that of in *A. niger* culture with 48 h glucoamylase induction, at 126.75 g/L.

Table 5.9 showed that the average productivity in *A. niger* culture with 24 h or 48 h glucoamylase induction were 2.11 g/L/h and 1.95 g/L/h, respectively. The maximum lactic acid productivity in *A. niger* culture with 24 h or 48 h glucoamylase induction were 4.38 g/L/h and 4.85 g/L/h, respectively. The lactic acid yield was similar in the *A. niger* culture with 24 h or 48 h glucoamylase induction, reaching 0.385 g/g and 0.368 g/g respectively. A higher yield can be obtained by increasing the SSF time but it would lead to a lower productivity. It was found that the induction time of glucoamylase can be shortened from 48 h to 24 h without affecting the SSF performance in the fermentor.

In this study, the average productivity of lactic acid production in SSF from potato starch was 2.11 g/L/h. It compares favorably with *Lactobacillus* sp. RKY2 and

*Lactobacillus paracasei* No.8, which had the average productivity of 2.9 g/L/h and 2.4 g/L/h, respectively (Yun et al, 2004 ; Richter and Berthold 1998). The final lactic acid concentration of 136.9 g/L for recombinant *Bacillus subtilis* mutant strain R in SSF from starch exceeds the performance of previously reported microorganisms such as *Lactobacillus* sp. RKY2 (Yun et al, 2004), *Enterococcus faecalis* RKY1 (Wee et al., 2004 ; Oh et al., 2005), *Lactobacillus delbrueckii* NRRL (Moldes et al., 2001) and *Rhizopus oryzae* NRRL 395 (Park et al., 2004).

*Bacillus subtilis* mutant strain R could be a potential candidate for lactic acid production from starch because of the faster growth rates and higher lactic acid produced.

# Chapter Six

# High-Performance Liquid Chromatography (HPLC) analysis of lactic acid

## 6.1 Introduction

Lactic acid is the simplest hydroxycarboxylic acid and exists as two enantiomers due to its asymmetric C2 atom. These two forms are optical isomers, and rotate light in different directions. Typically, an enantiomer that rotates light in the clockwise direction is called d, for dextrorotary or (+), and the enantiomer that rotates light in the counter-clockwise direction is called l, for levorotary, or (-). In addition, compounds are also classified as D- or L- based on the absolute configuration of D- and L-glyceraldehyde. However, lactic acid is an exception to these rules, existing as a levorotary D-isomer and a dextrorotary L-isomer. Both enantiomers have similar physical and chemical properties. Each isomer can contribute to metabolic acidosis, but since the origins of L-lactic acid and D-lactic acid are different, distinguishing between the two isomers is important in understanding their relative contribution.

D-lactic acid is found to be harmful to human metabolism and can result in acidosis and decalcification. L-lactic acid is therefore the preferred isomer in food and pharmaceutical industries as humans have only L-lactate dehydrogenase that can metabolize L(+) lactic acid (Akerberg et al., 1998 ; Hofvendahl and Hahn-Hägerdal, 2000).

The concentration of lactic acid was measured by YSI 2700 SELECT Biochemistry Analyzer and HPLC. For lactate measurements by YSI 2700 SELECT Biochemistry Analyzer, samples were diluted within 0 to 0.5 g/L.

For the HPLC analysis, D- and L-lactic acids were separated by an Astec CLC-L (15 cm x 4.6 mm) analytical column (Supelco, USA). 5 mM $CuSO_4$ was used as the mobile phase. The wavelength was monitored at 254 nm. Flow rate of the pump was set at 1 ml/min. 20 μl of sample was injected. For the standard, D-(-)- lactic acid and L-(+)-lactic acid (Sigma, USA) were used. For the sample, the supernatant was collected and pass through a 0.2 μm filter.

## 6.2 Measurement of L-lactic acid by YSI 2700 SELECT Biochemistry Analyzer and HPLC using Astec CLC-L analytical column

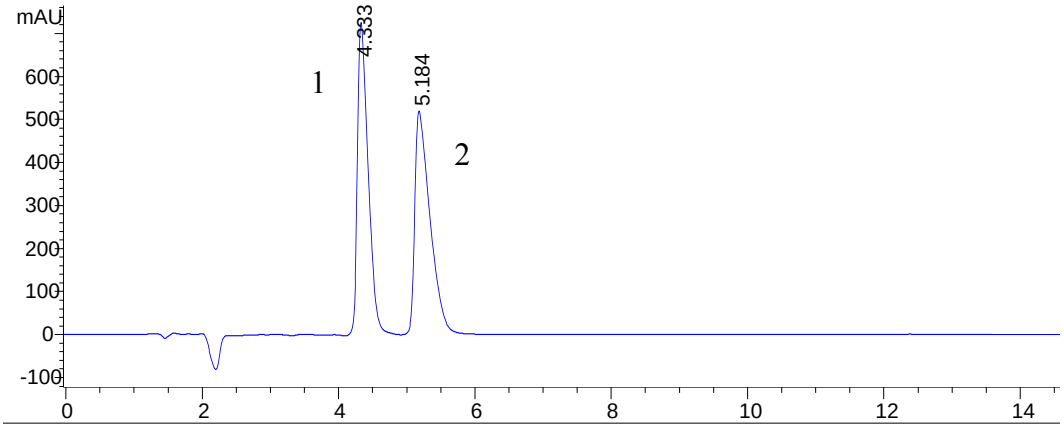



Figure 6.1 Separation of 1 g/L lactic acid enantiomers. Conditions: Column, Astec CLC-L (15 cm x 4.6 mm) analytical column, ambient temperature; mobile phase, 5 mM $CuSO_4$, 1 ml/min. UV detection at 254 nm. Peak identification: (1) D-lactic acid; (2) L-lactic acid.

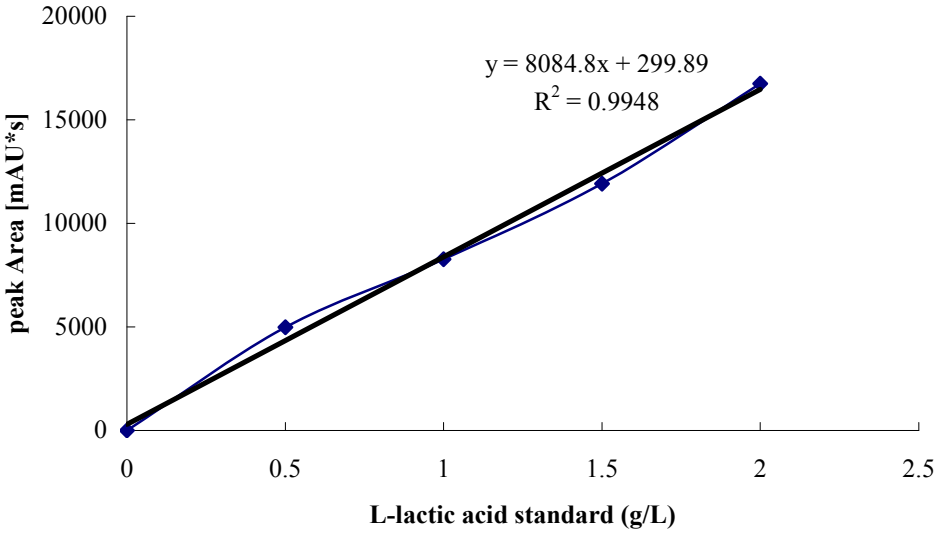



Figure 6.2 Calibration curve of L-lactic acid

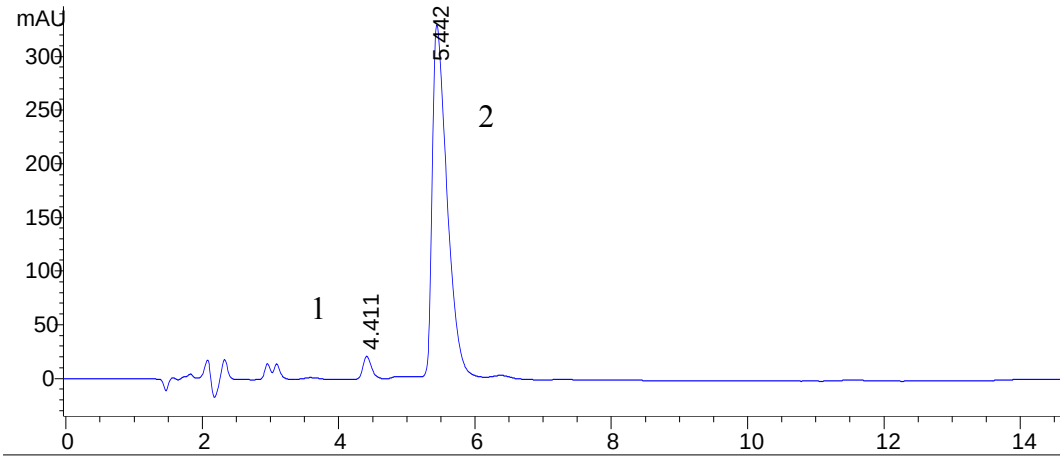
Table 6.1 The percentage difference of L-lactic acid measured by HPLC and YSI 2700 SELECT Biochemistry Analyzer

| Sample | L-lactic acid concentration measured by HPLC (g/L) | L-lactic acid concentration measured by YSI Analyzer (g/L) | The percentage difference of L-lactic acid measured by HPLC and YSI Analyzer (%) |
|---|---|---|---|
| SSF 24 h induction | 112.7 | 107.4 | 4.7 |
| SSF 300 g/L starch | 91.8 | 95.9 | 4.5 |

Figure 6.1 showed the separation of D- and L-lactic acid standards by Astec CLC-L analytical column. The D-lactic acid eluted before L-lactic acid. D-lactic acid and L-lactic acid were eluted at 4.3 and 5.2 min, respectively. In Figure 6.2, calibration curve of L-lactic acid was plotted and used to calculate the concentration of L-lactic acid in samples. Table 6.1 showed that the L-lactic acid concentration measured by HPLC and YSI 2700 SELECT Biochemistry Analyzer was similar. It was found that the percentage difference of L-lactic acid measured by HPLC and YSI Analyzer was less than 5%. Therefore, the L-lactic acid concentration in samples can be measured by YSI 2700 SELECT Biochemistry Analyzer in order to shorten the process time.

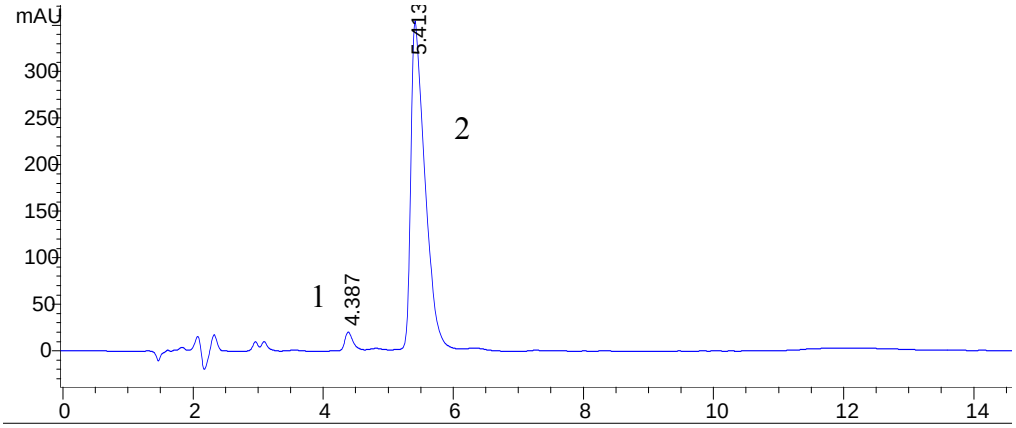
## 6.3 Measurement of L-lactic acid produced by recombinant *B. subtilis* using glucose and starch by HPLC

**(A)**




| Peak # | RetTime [min] | Type | Width [min] | Area [mAU*s] | Height [mAU] | Area % |
|---|---|---|---|---|---|---|
| 1 | 4.411 | MM | 0.1403 | 176.54021 | 20.97084 | 3.4997 |
| 2 | 5.442 | MM | 0.2472 | 4867.91748 | 328.15268 | 96.5003 |

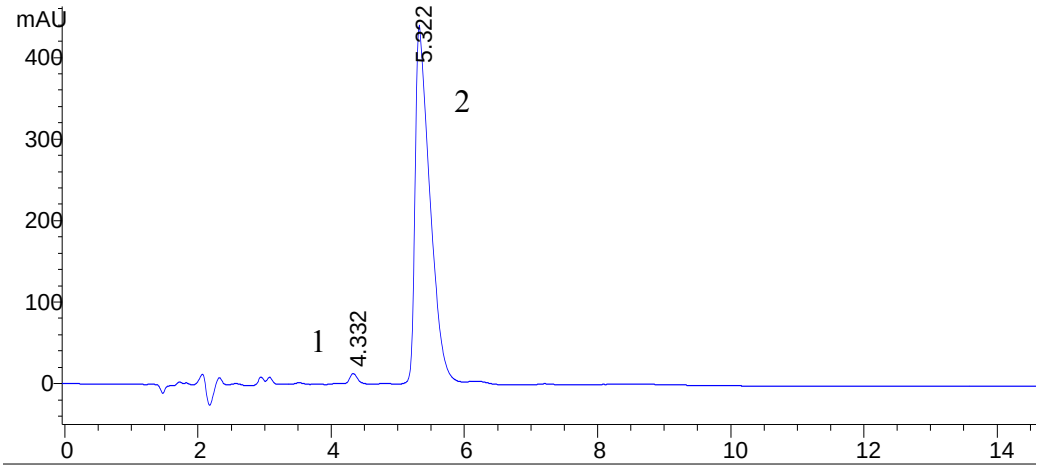
**(B)**




| Peak # | RetTime [min] | Type | Width [min] | Area [mAU*s] | Height [mAU] | Area % |
|---|---|---|---|---|---|---|
| 1 | 4.387 | BV | 0.1413 | 195.70895 | 20.51232 | 3.4687 |
| 2 | 5.413 | VB | 0.2268 | 5446.42725 | 355.31152 | 96.5313 |

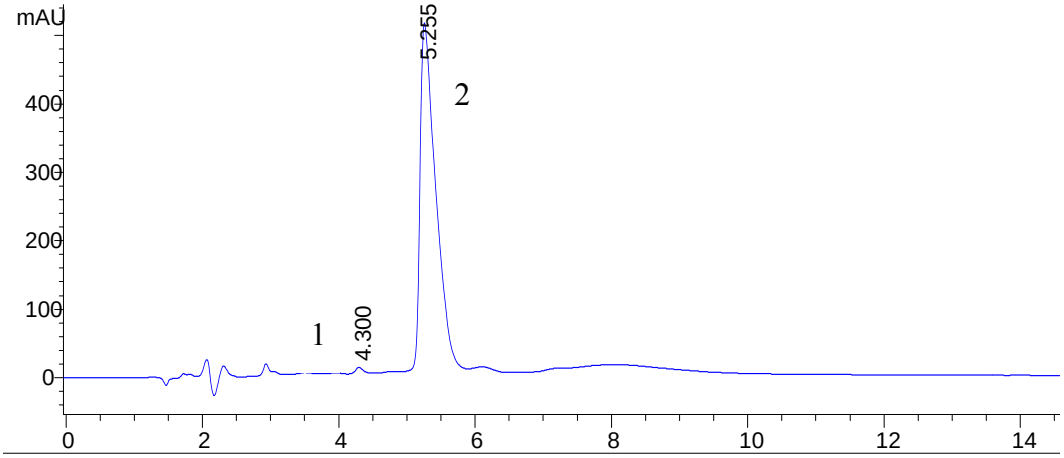
Figure 6.3 Separation of lactic acid enantiomers produced by recombinant *B. subtilis* mutant strain R using glucose as the carbon source in YE 20 medium under 37 $^{o}$C (A) and 40$^{o}$C (B). Peak identification: (1) D-lactic acid; (2) L-lactic acid.

**(A)**




| Peak # | RetTime [min] | Type | Width [min] | Area [mAU*s] | Height [mAU] | Area % |
|---|---|---|---|---|---|---|
| 1 | 4.332 | MM | 0.1311 | 103.14346 | 13.11701 | 1.5115 |
| 2 | 5.322 | MM | 0.2554 | 6720.65771 | 438.52347 | 98.4885 |

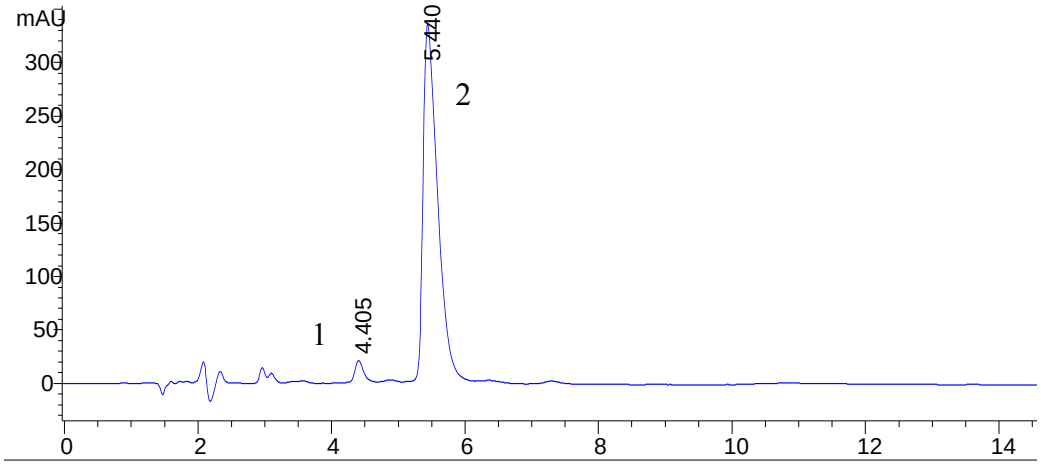
**(B)**




| Peak # | RetTime [min] | Type | Width [min] | Area [mAU*s] | Height [mAU] | Area % |
|---|---|---|---|---|---|---|
| 1 | 4.300 | MM | 0.1422 | 79.67116 | 9.33953 | 0.9750 |
| 2 | 5.255 | MM | 0.2651 | 8091.54102 | 508.62225 | 99.0250 |

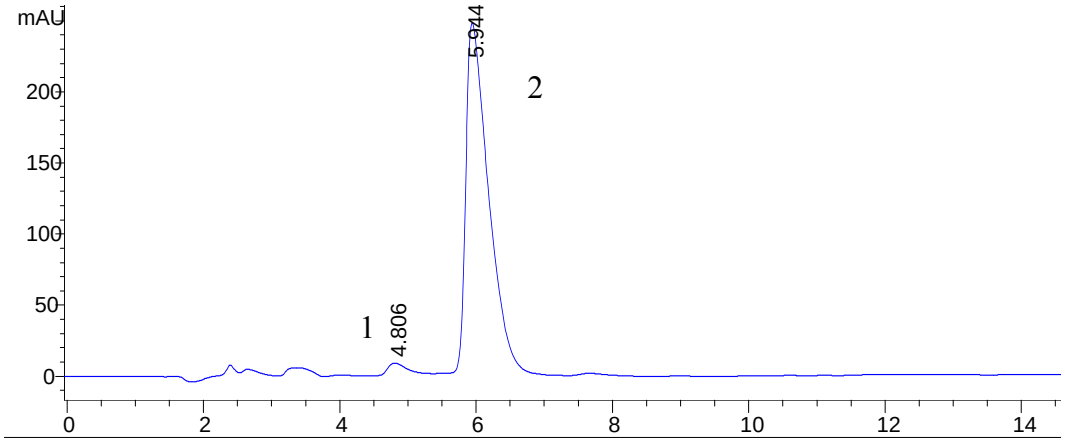
Figure 6.4 Separation of lactic acid enantiomers produced by recombinant *B. subtilis* mutant strain R using glucose as the carbon source in YE 50 (A) and YE 80 (B) medium under 40$^{o}$C. Peak identification: (1) D-lactic acid; (2) L-lactic acid.

**(A)**




| Peak # | RetTime [min] | Type | Width [min] | Area [mAU*s] | Heiqht [mAU] | Area % |
|---|---|---|---|---|---|---|
| 1 | 4.405 | MM | 0.1481 | 183.10052 | 20.60947 | 3.5617 |
| 2 | 5.440 | MM | 0.2480 | 4957.66553 | 333.21106 | 96.4383 |

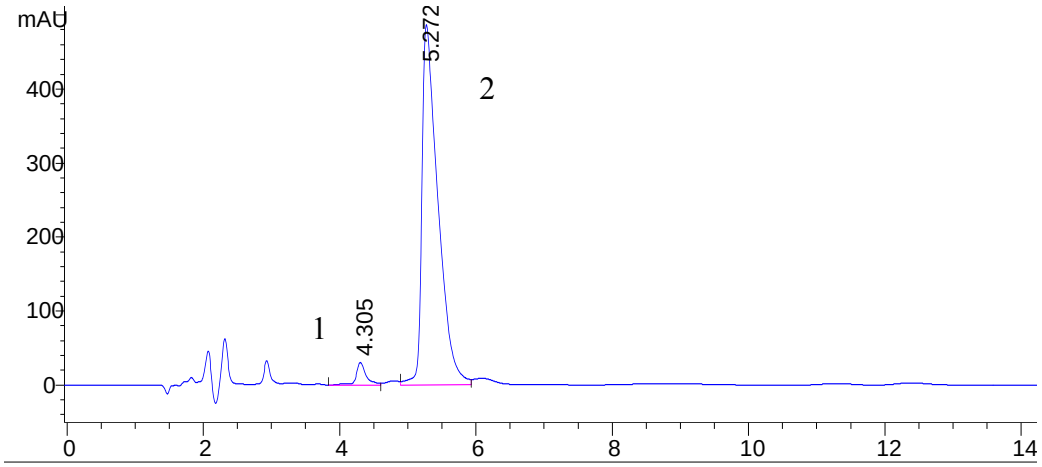
**(B)**




| Peak # | RetTime [min] | Type | Width [min] | Area [mAU*s] | Heiqht [mAU] | Area % |
|---|---|---|---|---|---|---|
| 1 | 4.806 | MM | 0.2909 | 141.00761 | 8.08001 | 2.4377 |
| 2 | 5.944 | MM | 0.3819 | 5643.48145 | 246.26746 | 97.5623 |

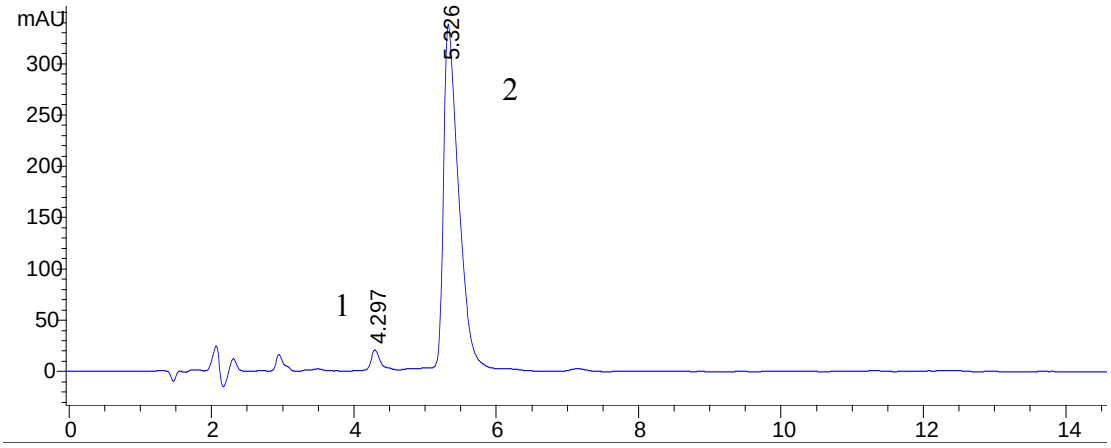
Figure 6.5 Separation of lactic acid enantiomers produced by recombinant *B. subtilis* mutant strain R using starch as the carbon source in SSF under 37 $^{o}$C (A) and 40$^{o}$C (B). Peak identification: (1) D-lactic acid; (2) L-lactic acid.

**(A)**




| Peak # | RetTime [min] | Type | Width [min] | Area [mAU*s] | Height [mAU] | Area % |
|---|---|---|---|---|---|---|
| 1 | 4.305 | VV | 0.1498 | 311.76431 | 30.36119 | 3.7543 |
| 2 | 5.272 | VV | 0.2375 | 7992.53027 | 487.02664 | 96.2457 |

**(B)**




| Peak # | RetTime [min] | Type | Width [min] | Area [mAU*s] | Height [mAU] | Area % |
|---|---|---|---|---|---|---|
| 1 | 4.297 | MM | 0.1396 | 165.28885 | 19.73140 | 3.2909 |
| 2 | 5.326 | MM | 0.2416 | 4857.35498 | 335.07159 | 96.7091 |

Figure 6.6 Separation of lactic acid enantiomers produced by recombinant *B. subtilis* mutant strain R using starch as the carbon source in SSF under pH 6.5 (A) or 24 h glucoamylase induction (B). Peak identification: (1) D-lactic acid; (2) L-lactic acid.

Figure 6.3 showed that the optical purity of L-lactic acid produced by recombinant *B. subtilis* mutant strain R using glucose as the carbon source in YE 20 medium under 37$^{o}$C and 40$^{o}$C was 96.5%, measured by HPLC using Astec CLC-L analytical column. Only 3.5% D-lactic acid was measured in the samples.

Figure 6.4 showed that the optical purity of L-lactic acid produced by recombinant *B. subtilis* mutant strain R using glucose as the carbon source in YE 50 and YE 80 medium under 40$^{o}$C was around 99%, measured by HPLC using Astec CLC-L analytical column. Only less than 2% D-lactic acid was measured in the samples.

Figure 6.5 showed that the optical purity of L-lactic acid produced by recombinant *B. subtilis* mutant strain R using starch as the carbon source in SSF under 37$^{o}$C and 40$^{o}$C was around 97%, measured by HPLC using Astec CLC-L analytical column. Only less than 4% D-lactic acid was measured in the samples.

Figure 6.6 showed that the optical purity of L-lactic acid produced by recombinant *B. subtilis* mutant strain R using starch as the carbon source in SSF under pH 6.5 or 24 h glucoamylase induction was around 96.5%, measured by HPLC using Astec CLC-L analytical column. Only less than 4% D-lactic acid was measured in the samples.

It was found that the optical purity of L-lactic acid produced by recombinant *B. subtilis* mutant strain R using glucose and starch as the carbon source was more than 96%. Only little amount of D-lactic acid was measured which may be due to the medium components. The high optical purity of L-lactic acid may be due to the undetectable activity or lack of an NAD-dependent D-lactate dehydrogenase in *B. subtilis* (Qin et al., 2009) and *B. subtilis* has only one gene (IctE) that exclusively

encodes L-lactate dehydrogenase (Romero-Garcia et al.,2009). It was found that recombinant *B. subtilis* mutant strain R can produced L-lactic acid with high optical purity from glucose and starch.

# Conclusions

The objective of this project was to optimize conditions for the production of lactic acid by recombinant *Bacillus subtilis* in shake flasks and fermentor in order to maximize the lactic acid production.

Shake flask experiments were performed to characterize the growth patterns and lactic acid production by recombinant *Bacillus subtilis.* A strain of recombinant *Bacillus subtilis* that could produce a higher yield of lactic acid was selected for further investigation. The results showed that the UV-mutated strain R of recombinant *B. subtilis* had a faster growth rate and higher lactic acid productivity than the parent strain and thus was selected for further studies.

The effect of using YE 20 medium with additional nitrogen sources on the growth and lactic acid production of recombinant *B. subtilis* was studied. Among the nitrogen sources added, the lactic acid concentration was comparatively higher in YE20 medium added with casamino acid, proteose peptone and martone than that added with other nitrogen sources. YE 20 medium with martone had the highest lactic acid amount as well as production rate among various combinations of YE 20 medium

The effect of cell density and the oxygen transfer rate on the growth and lactic acid production of recombinant *B. subtilis* was studied. It was found that the lactic acid concentration increased with increasing cell density and culture volumes.

An one stage fermentation was performed. The result showed that the glucose utilization rate, lactic acid concentration and productivity was increased in this one stage fermentation at a lower cell density than two stage fermentation. The highest productivity of lactic acid was 6 g/L/h using 5% inoculum.

The effect of pH on the growth and lactic acid production of recombinant *B. subtilis* was studied. The result showed that a relatively higher pH value (pH 7.5) could increase the lactic acid production.

Optimization of concentration of industrial yeast extract and martone as nitrogen supply for cell growth and lactic acid production by recombinant *B. subtilis* in one stage fermentation was studied. The combination of 20 g/L industrial yeast extract and 30 g/L martone concentration can produce a comparable high lactic acid concentration and productivity in various combinations of industrial yeast extract and

martone. Therefore, 20 g/L industrial yeast extract and 30 g/L martone in YE 20 medium was chosen for the studies.

The effect of inoculum size on the cell growth and lactic acid production by recombinant *B. subtilis* in one stage fermentation was studied. It was found that higher lactic acid concentration and productivity was obtained in 5% inoculum. Therefore, the optimal inoculum size was 5% in one stage shake flask fermentation.

The effect of cultivation temperature on lactic acid production by recombinant *B. subtilis* in one stage fermentation was studied. It was found that higher lactic acid concentration and productivity was obtained at the cultivation temperature of 40℃. Therefore, 40℃ was used in further studies of lactic acid production by *B. subtilis* in fermentors.

After the characterization of lactic acid production in shake flasks, fermentation of lactic acid was carried out in fermentors to investigate the production of lactic acid under controlled conditions. In two stage fermentation, aerobic growth was enhanced in the first stage of growth phase and lactic acid was produced in the second stage of production phase. However, only around 13.5 g/L lactic acid was produced. In one

stage fermentation, aerobic growth and lactic acid production occurred at the same time. The result showed that more lactic acid can be produced in one stage fermentation than two stage fermentation. One stage fermentation was chosen for further studies due to the higher lactic acid yield and the shorter process time.

The effect of dissolved oxygen and agitation speed on the cell growth and lactic acid production by recombinant *B. subtilis* was studied in the fermentor. These results showed that more lactic acid can be produced when a lower agitation speed was used in fermentation. The highest productivity of lactic acid (0.837 g/L/h) was obtained in fed-batch fermentation using 50 rpm agitation, which was higher than that of fermentation using 100 rpm (0.75 g/L/h) and 400 rpm (0.748 g/L/h). Therefore, agitation speed 50 rpm was chosen for further studies.

The effect of aeration rate on the cell growth and lactic acid production by recombinant *B. subtilis* was studied in the fermentor. It was found that higher lactic acid concentration and productivity was obtained at the aeration rate of 2 vvm. Therefore, 2 vvm was chosen for lactic acid production by *B. subtilis* in fermentor.

The effect of inorganic nitrogen source on the cell growth and lactic acid production by recombinant *B. subtilis* was studied in the fermentor. It was found that higher lactic acid concentration and productivity was obtained in medium without inorganic nitrogen source. Therefore, YE 20 medium without inorganic nitrogen source was used.

The effect of temperature on the cell growth and lactic acid production by recombinant *B. subtilis* was studied. It was found that higher lactic acid concentration and productivity was obtained at temperature of 40°C.

The effects of YE 50 and YE 80 medium on the cell growth and lactic acid production by recombinant *B. subtilis* were studied. It was found that 170 g/L lactic acid was produced in YE 80 medium and the average productivity of lactic acid in YE 80 medium was 2.208 g/L/h. It compares favorably with two representative lactic acid producers, *L. casei* LA-04-1 and *L. lactis* BME5-18M

Shake flask and fermentor studies were carried out to optimize the fermentation parameters for the production of lactic acid from starch. The effect of heat shock process on *B. subtilis* mutant strain R in lactic acid production was studied. It was

found that the heat shock process did not impose any adverse effect on lactic acid production.

Saccharified glucose from various sources of starch was carried out. It was found that the saccharification of liquefied starch was similar in different types of starch. It also showed that the same amount of lactic acid was produced by immobilized *A. niger* and *A. niger* culture from the glucose saccharified from starch. In order to shorten the process time and simplify the preparation steps, *A. niger* culture was chosen as the inoculum form for starch saccharification.

The effect of temperature on starch saccharification was studied. The cell growth of *B. subtilis* mutant strain R at 43°C was poor and nearly two times lower than that at 37°C and 40°C. For co-culturing *Aspergillus niger* and *B. subtilis* mutant strain R in SSF, a compromised temperature of 40°C was chosen for further studies..

The effect of temperature on simultaneous saccharification and fermentation (SSF) to produce lactic acid by *Aspergillus niger* and recombinant *B. subtilis* in shake flask and fermentor were studied. It was found that higher lactic acid concentration was

obtained at 40°C in SSF to produce lactic acid from potato starch in shake flask and fermentor.

The effect of immobilization of *A. niger* culture on SSF to produce lactic acid by *A. niger* and recombinant *B. subtilis* in the fermentor was studied. The results indicated that similar lactic acid concentration and productivity was obtained with co-cultures of immobilized *A. niger* or *A. niger* with recombinant *B. subtilis* mutant strain R.

The results also showed that higher lactic acid concentration and productivity was obtained at pH 7.5 in SSF to produce lactic acid by *B. subtilis* from starch. The induction time to produce glucoamylase in the flask can be shortened from 48 h to 24 h without affecting the SSF performance in the fermentor.

The average productivity of lactic acid production in SSF from potato starch was 2.11 g/L/h. It compares favorably with *Lactobacillus* sp. RKY2 and *Lactobacillus paracasei* No.8. The final lactic acid concentration of 136.9 g/L for recombinant *B. subtilis* mutant strain R in SSF from starch exceeds the lactic acid produced from previously reported microorganisms such as *Lactobacillus* sp. RKY2, *Enterococcus*

*faecalis* RKY1, *Lactobacillus delbrueckii* NRRL and *Rhizopus oryzae* NRRL 395 (Park et al., 2004).

*B. subtilis* mutant strain R could be a potential candidate for lactic acid production from glucose and starch because of the faster growth rates and higher lactic acid produced. It was found that the optical purity of L- lactic acid was greater than 96% identified from HPLC. The lactic acid tolerance of recombinant *B. subtilis* was between 50 to 70 g/L.

# Appendix

**Determination of lactic acid tolerance by agar plate method**

Nutrient agar plates containing lactic acid were prepared by mixing different proportion of lactic acid solution and nutrient agar solution with the addition of chloramphenicol as antibiotics. The nutrient agar solution was sterilized by autoclave at 121℃ for 20 minutes. Concentration of lactic acid was ranging from 10 to 120 g/L. 50% sodium lactate solution was used.

Inoculum prepared from seed culture was incubated at 37℃ with 270 rpm shaker speed for 6 hours. 20 μl of seed culture was spread on the agar plates. To have a better comparison on the colony forming units, serial dilution of the culture was done. The nutrient agar plates were incubated at 37℃ for 24 hours.

Table Number of colony forming unit of *Bacillus subtilis* at different lactic acid concentrations

| Lactic Acid Concentration (g/L) | 24-hours Incubation | | 42-hours Incubation | |
|---|---|---|---|---|
| | Original Concentration | $1 \times 10^{-2}$ Dilution | Original Concentration | $1 \times 10^{-2}$ Dilution |
| 0 (control) | TNTC | TNTC | TNTC | TNTC |
| 10 | TNTC | TNTC | TNTC | TNTC |
| 20 | TNTC | TNTC | TNTC | TNTC |
| 40 | 2 | 33 | 5 | 110 |
| 50 | 1 | 0 | 1 | 20 |
| 70 | 0 | 0 | 0 | 0 |
| 80 | 0 | 0 | 0 | 0 |
| 90 | 0 | 0 | 0 | 0 |
| 100 | 0 | 0 | 0 | 0 |
| 110 | 0 | 0 | 0 | 0 |
| 120 | 0 | 0 | 0 | 0 |

TNTC: Too numerous to be counted (over 100 CFU in 1/4 of agar plate)

The inhibitory effect of lactic acid concentration on growth of *Bacillus subtilis* start beyond 20 g/L, with a decrease in colony forming units in 40 and 50 g/L. The presence of colony forming units in 40 and 50 g/L lactic acid indicated that growth and reproduction of *Bacillus subtilis* is not totally inhibited. However, as the bacterial cells only begin to grow in 40 and 50 g/L lactic acid after 42 hours of incubation, the growth rate was lower than that in 0 to 20 g/L. Therefore, a higher lactic acid concentration had increasing inhibitory effect on cell growth.

From 70 g/L onwards, no colony was formed, showing that *Bacillus subtilis* cannot survive in high lactic acid level and growth was inhibited. Therefore, the tolerance to lactic acid of recombinant *Bacillus subtilis* strain R was considered to be within 50 to 70 g/L.

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