木质纤维素和菊芋生物质生产乳酸的生物炼制技术研究

摘要

生物可降解聚乳酸材料需求量的显著增加极大地促进了乳酸生产行业的发展。利用价格低廉且资源丰富的农业废弃物作为原料进行生物发酵乳酸的生产技术更是受到了极大关注。这些生物质原料主要包括菊芋、玉米秸秆、小麦秸秆以及稻秆等。本论文主要对利用菊芋和纤维素生物质为原料生产乳酸的生物炼制工艺进行了深入系统的研究。

首先，从用于玉米秸秆原料进行乙醇发酵的体系中分离得到了一株非常特异的乳酸菌*Pediococcus acidilactici* DQ2，并利用16S DNA测序和微生物实验方法对其进行菌种鉴定。研究证明，其具有超级耐热和耐受木质纤维素来源抑制物的特性。

其次，将这株新分离得到的乳酸菌*P. acidilactici* DQ2用于以菊芋块茎为原料的乳酸发酵工艺中。同时，在该研究过程中发现三种商业糖化酶存在较高的菊粉酶活性。对其根本原因进行研究，进一步鉴定发现在工业糖化酶中存在两种具有菊粉酶活性的蛋白。利用Genencor公司生产的一种GA-L New工业糖化酶对菊芋块茎进行糖化能够获得较高的果糖糖化得率。然后，以*P. acidilactici* DQ2作为乳酸发酵菌，GA-L New作为菊粉酶，利用菊芋块茎进行同步糖化和乳酸发酵（SSF）72 h，能够得到111.5 g/L的乳酸，其得率为0.46 g/g DM，并且生产速率可达1.55 g/L/h。对常规菊粉酶和以商业糖化酶替代菊粉酶进行的发酵过程中添加酶所占的成本进行了比较，发现利用商业糖化酶替代菊粉酶能够有效降低该发酵工艺成本。上述结果提出了一条利用价格低廉的商业糖化酶水解菊芋块茎进行乳酸发酵的现实可行的生产工艺。

最后，通过最新研发的干法生物炼制工艺，以玉米秸秆为原料，进行*P. acidilactici* DQ2的乳酸发酵。主要考察了生物脱毒菌*Amorphotheca resinae* ZN1对预处理原料中抑制物的去除能力，以及生物脱毒作用对后续*P. acidilactici* DQ2发酵乳酸的影响。在预处理后的玉米秸秆中残留的不同浓度的抑制物对乳酸的发酵性能存在严重影响。通过7-10天的生物脱毒过程能够完全去除原料中的抑制物，从而使同步糖化和乳酸发酵过程的乳酸浓度和得率显著提高。同时，利用盘磨对预处理后的玉米秸秆进行磨浆处理，并考察了磨浆过程对乳酸发酵的影响。以经过上述磨浆和深度生物脱毒处理的玉米秸秆作为原料，在带有螺带式搅拌桨的5 L发酵罐中，固含量为25% DM、pH5.5、温度48 ^{o}C条件下进行SSF，最终乳酸浓度可达74.2 g/L，相应得率为59.76%，并且生产速率可达1.03 g/L/h。该结果表明以生物质原料资源链条中的农业木质纤维素废弃物作为原料进行纤维素乳酸的生产过程是一条实际可行的生物炼制工艺。

通过以上结果可以得出以下结论，本论文分离鉴定了一株耐热耐抑制物乳酸发酵菌*P. acidilactici* DQ2，并研究开发了一种利用该乳酸菌分别以菊芋和纤维素生物质为原料进行高浓度乳酸生产的生物炼制工艺。这些研究将为以后通过价格低廉资源丰富的农业生物质生产高附加值的化工产品的产业化发展铺平道路。

关键词：乳酸、*Pediococcus acidilactici* DQ2、同步糖化发酵、菊芋块茎、玉米秸秆磨浆、高固含量。

Study on biorefinery processing technology on lactic acid production from lignocellulose and inulin biomass

Abstract

The sharp increase in manufacturing biodegradable polylactic acid (PLA) has significantly stimulated the lactic acid production from low value and abundant agricultural residues materials such as inulin, corn stover, wheat straw, and rice straw. In this thesis, the biorefinery processing technology for fermentative production of lactic acid using inulin and lignocellulose biomass as the raw materials were intensively studied.

First, an interesting lactic acid bacteria strain, *Pediococcus acidilactici* DQ2, was isolated from the corn stover slurry of ethanol fermentation. *P. acidilactici* DQ2 was identified using 16S DNA sequencing and microbial experimentation methods. The unique properties of *P. acidilactici* DQ2, the excellent thermo- and inhibitor- tolerance, were demonstrated and carefully investigated.

Then, the newly isolated *P. acidilactici* DQ2 strain was applied to the lactic acid fermentation using Jerusalem artichoke tubers (Jat) as the raw materials. A high inulinase activity was found in three commercially available glucoamylase enzymes. Its origin was investigated and two proteins in the commercial glucoanylases were identified as the potential enzymes showing inulinase activity. One of the commercial glucoamylases, GA-L New from Genencor, was used for Jerusalem artichoke tubers (Jat) hydrolysis and a high hydrolysis yield of fructose was obtained. The simultaneous saccharification and lactic acid fermentation (SSF) of Jat was carried out using GA-L New as the inulinase and *P. acidilactici* DQ2 as the fermenting strain. A high lactic acid titer, yield, and productivity of 111.5 g/L, 0.46 g/g DM, and 1.55 g/L/h, respectively, were obtained within 72 hours. The enzyme cost using the commercial glucoamylase as inulinase was compared to that using the typical inulinase and a large profit margin was identified. The results provided a practical way of Jat application for lactic acid production using cheap commercial glucoamylase enzyme.

Finally, corn stover was used as the raw materials for lactic acid production using the same strain *P. acidilactici* DQ2 through the newly developed dry biorefinery processing technology. The effect of the inhibitor removal intensity by the biodetoxification strain *Amorphotheca resinae* ZN1 on the lactic acid fermentation of *P. acidilactici* DQ2 was carefully investigated. Different amount of inhibitor residues in the pretreated corn stover materials severely affected the performance of lactic acid fermentation. The complete removal of all the inhibitor substances after 7-10 days of bio-detoxification led to the higher lactic acid yield and titer in the simultaneous saccharification and lactic acid fermentation process. The effect of disk milling of the pretreated corn stover on the lactic acid fermentation was also investigated. After the deep biodetoxification and disk milling, the SSF at 48 ^{o}C, pH 5.5, and 25% (w/w) solids loading in 5L of helical agitated bioreactor resulted in the lactic acid titer, yield, and productivity of 74.2 g/L, 59.76%, and 1.03 g/L/h, respectively. The result provided a practical process option for cellulosic lactic acid production using agro-lignocellulose residual in the chain of biomaterial resources.

Conclusively, the biorefinery processing technology of inulin and lignocellulose biomass for production of lactic acid were established with high lactic acid titer and yield using new microorganisms and new processing technology in this thesis. These studies paved the way of industrial application of value added chemicals from cheap and abundant agricultural biomass.

Key words: Lactic acid, *Pediococcus acidilactici* DQ2, simultaneous saccharification and fermentation (SSF), Jerusalem artichoke tubers, corn stover milling, high solids loading.

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CHAPTER I

Review on lactic acid manufacture and applications

Lactic acid was widely used in food, leather, cosmetic, pharmaceutical, textile and chemical industries, and much work has been done to improve the production of lactic acid from inexpensive raw substrates in a cheap and energy-saving process using genetically modified organisms and multi-stage fermentation system (Maas et al., 2008b; Yun et al., 2004; Gao et al., 2008; Romani et al., 2008). Lactic acid fermentation is caused by some fungi and bacteria. The most important lactic acid producing bacteria is *Lactobacillus*. Lactic acid is also found in large quantitities in the micro-organism world. Several bacterial species produce large quantities of lactic acid, among which the best known are *Lactobacillus, Sporolactobacillus, Enterococcus, Lactococcus, Bacillus, Streptococcus, Pediococcus, Leuconostoc or bifidobacteria* which widely differ from the others by the metabolic path they go through.

Lactic acid is a bulk chemical demonstrating strong market growth. Global production capacity is over 500,000 tonnes with current production between 300,000 - 400,000 tonnes per year (Nattrass and Higson, 2011). The world market for lactic acid was forecasted to reach 367.3 thousand metric tons by the year 2017 according to Global Industry Analysts, Inc., (GIA). The production of lactic acid, as well as various its applications and future prospects of biologically derived lactic acid, is becoming a potential production due to their high demand. In biotechnological processes for the production of lactic acid, lactic acid fermentation by microorganisms that can produce lactic acid from sources of biomass. Sources as raw materials (Jerusalem artichoke, cassava, etc.), other agricultural residues (corn stover, wood, etc.), and waste have already been proposed as a possible substrate for production of lactic acid in a simultaneous saccharification and fermentation process (SSF).

Cheap raw materials are necessary for the feasible economic production of lactic acid 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 not only be of low cost, but with low levels of contaminants and low toxic materials capable of being fermented with little or no pre-treatment resulting in rapid production rate and high yield with little or no by-product formation and be available throughout the year.

The Jerusalem artichoke crop contains large amounts of carbohydrates, mainly inulin, which can be converted to sugars such as glucose, fructose. Fructose is used as a sweetener in the food industry and has several health benefits. The inulin, other extractable sugars, and cellulose can also be used to produce ethanol. Inulinases can be used in a wide range of industrial applications: for ultra-high fructose syrup obtaining from inulin, bioethanol production, inulo-oligosaccharide production, single-cell oil, and single-cell protein production, some chemicals production, like citric acid, butanediol, alcohols, and lactic acid (Chi et al., 2009; Chi et al., 2011; Pandey et al., 1999; Liu et al., 2010).

Lignocellulosic biomass represents the World's most abundant carbohydrate reserve, and it has the potential to become a major source of fermentable sugars for production of second generation fuel ethanol. The conversion of native lignocellulosic biomass, in which three polymeric components cellulose, hemicellulose, and lignin are interpenetrated, to fermentable sugars involves two steps feedstock pretreatment and enzymatic saccharification. A major drawback of dilute acid pretreatment is the generation of sugar degradation products such as furfural and hydroxymethyl furfural (HMF), which are toxic to the fermentative microorganisms. The generation of these fermentation inhibitors depends on a number of factors such as acid dose, temperature, and duration of pretreatment. After acid pretreatment, the pH is to be adjusted to within the optimal activity range of the hydrolytic enzymes.

To find and isolate a new lactic acid bacteria from corn stover using for the simultaneous saccharification and fermentation (SSF); research development of innovative anaerobic cultivation systems to convert agricultural residues to bioproducts; study the inhibitory effect of the investigated on, good antimicrobial metabolite producer strains against them; reveal the inhibitory mechanism of the new lactic acid bacteria, which have shown good bacterium activity; optimize fermentation parameters for the production of lactic acid from glucose, fructose and xylose by a new lactic bacteria; fermentor studies to optimize the production of lactic acid from inulin of Jerusalem artichoke tubers using a new lactic bacteria by simultaneous saccharification and fermentation (SSF); study tolerance of a new lactic bacteria to the inhibitor compounds derived from lignocellulose degradation in the pretreatment; study biological and chemical pretreatment of lignocellulosic from corn stover in agricultural residues for lactic acid; study pretreatment and biodetoxification of corn stover; survey different low-high solid loading of milled corn stover for lactic acid production using a new lactic bacteria by the SSF process.

Increasing lactic acid production demands in the industrial production of lactic acid and reduce the cost of lactic acid for numerous industries. The demand of organic acids is steadily increasing due to larger production volumes of organic acid containing foods. Lactic acid fermentation seems to be better choices for production from the industrial production of lactic acid because of their increasing demand in food, cosmetic and pharmaceutical industries.

1.1 Lactic acid

Lactic acid (2-hydroxypropionic acid), CH_3-CHOHCOOH, is the most widely occurring hydroxycarboxylic acid. It was first discovered in 1780 by the Swedish chemist Scheele. In 1857, however, Pasteur discovered that it was not a milk component, but a fermentation metabolite generated by certain microorganisms by Benninga (1990).

Lactic acid (LA) is widely used by the pharmaceutical, biomaterial, detergent, leather, food, chemical, textile industries, and over the past few years it has received increasing attention as a building block for biodegradable plastics such as polylactic acid and polylactic-co-glycolic acid (Secchi et al., 2012; Iqbal et al., 2012).

Lactic acid is present in almost every form of organism. Its most important function in animals and humans is related to the supply of energy to muscle tissues. It is a water solution, highly hygroscopic aliphatic acid and an enigmatic chemical. It is present in many foods, either naturally or as a product of in situ microbial fermentation (as in sauerkraut, yogurt, buttermilk, sourdough breads, and many other fermented foods). There are two optical isomers of lactic acid L (+) and D (-) lactic acid (Figure 1.1).

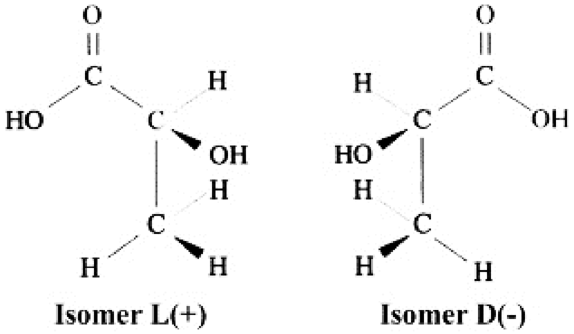

Figure 1.1 Optical isomers of lactic acid

Lactic acid exists as two enantiomeric forms: L-lactic acid and D-lactic acid, which can be produced either by chemical synthesis or by fermentation. Chemical synthesis is based on raw materials from the bulk chemical industry and results in mixtures of L- and D-lactic acid. In contrast, the biotechnological production of lactic acid may lead to the desired pure enantiomeric form and requires lower temperatures and energy consumption in comparison with chemical approaches.

It can be produced by either biotechnological fermentation or chemical synthesis, but the former route has received considerable interest recently, due to environmental concerns and the limited nature of petrochemical feedstocks. Lactic acid is 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 lacto-nitrile 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).

Lactic acid is produced by lactic acid bacteria (LAB). Biotechnological process can use cheap biomass materials such as corn stover, natural inulin and/or other carbonhydrate rich materials. Fermentation routes to lactic acid can produce optically pure L (+) or D (-) lactic acid, depending on the bacteria selection. Lactic acid producing microorganisms use pyruvic acid as the precursor for lactic acid production. The conversion of pyruvic acid to lactic acid can be catalyzed by 2 types of enzymes: NAD-dependent L-lactate dehydrogenase and NAD-dependent D-lactate dehydrogenase by Garvie (1980). The stereospecificity of lactic acid produced by microorganisms depends on the type of enzymes involved in the lactic acid production. Because the optical purity of lactic acid is a crucial factor in lactic acid based industries, numerous studies have investigated the biotechnological production of optically pure lactic acid (Gao et al., 2011).

Biological production has several advantages compared to chemical synthesis, the biotechnological process for lactic acid production offers several advantages: low substrate costs, production temperature and energy consumption (Datta and Henry, 2006). Lactic acid bacteria comprise a heterogeneous group of microorganisms. Isolates from food include representatives of the genera *Lactobacillus*, *Streptococcus*, *Pediococcus* and *Leuconostoc*. As no universal selective medium exists for the cultivation of all lactic acid bacteria the well-known media for *Lactobacilli* may be modified by changing the pH value, the concentrations of inhibitory agents and the incubation time and temperature. It may be necessary to select media for special fields of application, e.g. milk and milk products, meat and meat products. Surface plating under microaerophilic or anaerobic conditions is generally recommended. Generally suitable as selective media for lactic acid bacteria are MRS medium, adapted to a lower pH (e.g. 5.7) with the addition of an inhibitor (e.g. sorbic acid) or a modified Rogosa medium with increased pH (e.g. 6.2).

Table 1.1 Identification and physical chemical properties (Source: Ren J. 2010)

Identification		Physical & chemical properties	
CAS number	D/L: [50-21-5] L: [79-33-4] D: [lO326-4l-7]	Melting point	L: 53 ^{o}C D: 53 ^{o}C D/L: l6.8 ^{o}C
Einecs	No. 200-018-0	Boiling point	122 ^{o}C (12 mmHg)
H.S. Code	2918.11	Specific gravity	1.2 g/mL
Formula	$CH_3CH(OH)COOH$	Molar mass	90.08 g/mol

Table 1.2 Thermodynamic characteristics of lactic acid (Source: Ren J. 2010)

Items	Characteristics
Dissociation constant (Ka)	0.000 137 (at 25 ^{o}C)
Heat of dissociation (ΔH)	-63 caVmol (at 25 ^{o}C)
Free energy of dissociation (ΔF)	5000 cal/mol
Heat of solution (ΔH)	1868 cal/mol (for crystalline L(+) lactic acid at 25 ^{o}C)
Heat of dilution (ΔH)	-1000 cal/mol (for dilution with a large volume of water)
Heat of fusion (ΔH)	2710 cal/mol (for racemic lactic acid) 4030 caVmol (for L(+) lactic acid)
Entropy of solution (ΔS)	6.2 cal/mol/ ^{o}C
Entropy of dilution (ΔS)	-3.6 cal/mol/ ^{o}C
Entropy of fusion (ΔS)	9.4 cal/mol/ ^{o}C (for racemic lactic acid) 12.2 cal/mol/ ^{o}C (for L(+) lactic acid)
Heat of combustion (ΔHc0)	-321220 cal/mol (for crystallineL(+) lactic acid at 25 ^{o}C) -325600 cal/mol (for liquid racemic lactic acid at 25 ^{o}C)
Heat of formation (ΔHf0)	-165 890 cal/mol (for crystalline L(+) lactic acid at 25 ^{o}C) -163000 cal/mol (for liquid lactic acid) -164020 cal/mol (for lactic acid in dilute solution) -164 080 cal/mol (for dissociated and diluted lactic acid)
Heat capacity (Cp)	0.338 cal/g/ ^{o}C (for crystalline lactic acid at 25 ^{o}C) 0.559 al/g/ ^{o}C (for liquid lactic acid at 25 ^{o}C)
Absolute entropy (S0)	34.0 cal/mol/ ^{o}C (for crystalline L(+) lactic acid at 25 ^{o}C) 45.9 cal/mol/ ^{o}C (for liquid racemic lactic acid at 25 ^{o}C)
Entropy off ormation (ΔSf0)	-137.2 cal/mol/ ^{o}C (for crystalline lactic acid at 25 ^{o}C) -125.3 cal/mol/ ^{o}C (for liquid lactic acid at 25 ^{o}C)
Free energy off ormation (ΔFf0)	-124980 cal/mol (for crystalline L(+) lactic acid at 25 ^{o}C) -126500 cal/mol (for liquid racemic lactic acid at 25 ^{o}C)

Properties: Lactic acid (LA) has a hydroxyl group adjacent to the carboxyl group, making it an alpha hydroxy acid (AHA). In solution, it can lose a proton from the acidic group, producing the lactate ion $CH_3CH(OH)COO$-. It is miscible with water or ethanol, and is hygroscopic. It exhibits low volatility. Other identification and physicalchemical properties of lactic acid are summarized in Table 1, and the thermodynamic characteristics of lactic acid are listed in Table 2.

1.2 Microorganisms for lactic acid production

1.2.1 Background of lactic acid producing bacteria

Most investigations of lactic acid production were carried out with lactic acid bacteria (LAB). Lactic acid producing fermentation is caused by some fungi and bacteria. LAB are renowned for the potential of producing antimicrobial compound and other value added products. The concept of the group name 'lactic acid bacteria' was created for bacteria causing fermentation and coagulation of milk, and defined as those which produce lactic acid from lactose.

Lactic acid bacteria are a group of Gram-positive bacteria united by a constellation of morphological, metabolic, and physiological characteristics. They are non-sporing, carbohydrate fermenting lactic acid producers, acid tolerant of non-aerobic habitat and catalase negative. Typically they are non-motile and do not reduce nitrite. They are subdivided into four genera *Streptococcus, Leuconstoc, Pediococcus*, and *Lactobacillus*. LAB consists of bacterial genera within the phylum Firmicutes comprised of about 20 genera. The genera *Lactococcus, Lactobacillus, Streptococcus, Leuconostoc, Pediococcus, Aerococcus, Carnobacterium, Enterococcus, Oenococcus, Tetragenococcus, Vagococcus* and *Weisella* are the main members of the LAB (Reddy et al., 2008). Lactobacillus is largest of these genera, comprising around 80 recognized species (Axelsson L., 2004). The taxonomy of lactic acid bacteria has been based on the Gram reaction and the production of lactic acid from various fermentable carbohydrates. Lactobacilli vary in morphology from long, slender rods to short coccobacilli, which frequently form chains. Typical LAB are Gram-positive, nonsporing, catalase-negative, devoid of cytochromes, anaerobic but aerotolerant cocci or rods that are acid-tolerant and produce lactic acid as the major end product during sugar fermentation (Axelsson L., 2004).

1.2.2 Classification at genus level

According to Bergey's Manual of Determinative Bacteriology (Ninth edn.) the lactic acid bacteria are classified as follows:

1. Gram-positive cocci: *Enterococcus* spp., *Lactococcus* spp., *Pediococcus* spp., *Saccharococcus* sp., and *Streptococcus* spp.
2. Endospore-forming Gram-positive rods and cocci: *Bacillus* spp. and *Sporolactobacillus* sp.
3. Regular, nonsporing Gram-positive rods: *Lactobacillus* spp.

Lactic acid can be produced by several microorganisms classified into bacteria, fungi, yeast, cyanobacteria, and algae. Each biocatalyst has achieved one or more improvements over the others, such as a broader substrate range, improved yield, and productivity, reduction of nutritional requirements, or improved optical purity of lactic acid. Lactic acid-producing bacteria can be divided into 4 main producers, namely, lactic acid bacteria (LAB), *Bacillus* strains, *Escherichia* coli, and *Corynebacterium glutamicum*. In general, bacterial lactic acid fermentation suffers from several limitations, including (i) production of both L- and D-lactic acid via L-lactate dehydrogenase (L-LDH) and D-lactate dehydrogenase (D-LDH), respectively; (ii) low yield due to byproduct formation; (iii) use of nutritionally rich medium; and (iv) high risk of bacteriophage infection that results in cell lysis and subsequent cessation of lactic acid production (Budhavaram and Fan, 2009, Litchfield J.H., 2009).

LAB are classified mainly into two groups: homofermentative and heterofermentative. While homofermentative LAB produce only lactic acid through the Embden-Meyerhof pathway, heterofermentative LAB produce additional by-products, including ethanol, acetic acid, and carbon dioxide. Thus, the identification of a homofermentative LAB is an important factor in developing an economical bioprocess for the production of lactic acid. The major homofermentative LAB that can be used in the production of lactic acid include the genera *Lactobacillus, Lactococcus*, and *Enterococcus* (Moon et al., 2012). About 90% of the literature on lactic acid production is focused on bacterial fermentation.

Table 1.3 Homoferementative and heterofermentative lactic acid bacteria

(Source: Mohamed et al., 2013)

Characterization	Homofermentative LAB	Heterofermentative LAB
Products	Lactic acid	Lactic acid, ethanol, diacetyl, formate, acetoin or acetic acid, and carbon dioxide
Metabolic pathways	Hexose: Embden–Meyerhof pathway Pentose: Pentose phosphate pathway	Hexose: phosphogluconate and phosphoketolase pathway Pentose:phosphoketolase pathway
Theoretical yield of lactic acid to sugars	Hexose: 1.0 g/g (2.0 mol/mol) Pentose: 1.0 g/g (1.67 mol/mol)	Hexose: 0.5 g/g (1.0 mol/mol) Pentose: 0.6 g/g (1.0 mol/mol)
Genera	*Lactococcus, Streptococcus, Pediococcus, Enterococcus,* some *Lactobacillus*	*Leuconostoc, Oenococcus,* some *Lactobacillus species*
Availability for commercial lactic acid production	Available due to high selectivity	Not available due to high by-product formation

Lactic acid producing organisms, most of which are anaerobic, utilize pyruvic acid, which is the endproduct of Embden-Meyerhof pathway. The conversion of pyruvic acid to lactate can be effected by either of the two enzymes, 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 in Table 1.3.

The genus is divided into three groups based on fermentation patterns:

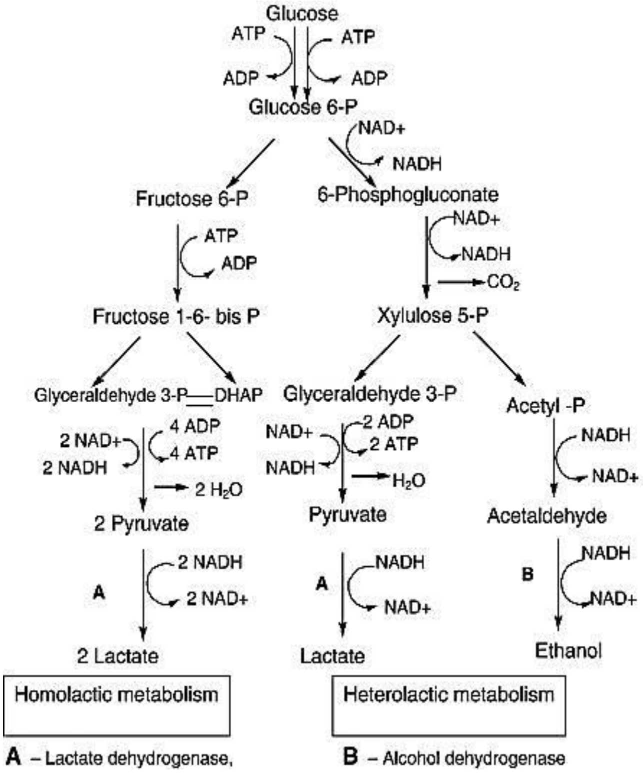

Figure 1.2 Metabolism of lactic acid bacteria

Homofermentative: produce more than 85% lactic acid from glucose. They ferment 1 mol of glucose to 2 mol of lactic acid, generating a net yield of 2 mol of ATP per molecule of glucose metabolized. Lactic acid is the major product of this fermentation (Fig. 1.2).

Heterofermentative: produce only 50% lactic acid. These ferment 1 mol of glucose to 1 mol of lactic acid, 1 mol of ethanol, and 1 mol of CO_2. One mole of ATP is generated per mole of glucose, resulting in less growth per mole of glucose metabolized (Fig. 1.2).

LAB comprise a group of Gram-positive, low-GC content, acid-tolerant, generally non-sporulating, non-respiring rods or cocci, which usually produce lactic acid from hexose as the major metabolic end-product of carbohydrate fermentation (Makarova & Koonin, 2007, O’Sullivan., 2011). The main groups include *Lactobacillus, Leuconostoc, Pediococcus, and Streptococcus*. Some of the LAB are homofermentative, and produce lactic acid as the main product of glucose fermentation, while others are heterofermentative and produce carbon dioxide and ethanol in addition to lactic acid (Blandino et al., 2003). LAB produce lactic acid and other antimicrobial substances that will inhibit the growth of harmful bacteria along with reducing the sugar content, thereby prolonging the shelf life of the product. An advantage of well-controlled biotechnical processes of this kind is not only the yield of a valuable end-product (in this case the “physiological” isomer of lactic acid), but also to prevent any significant CO_2 production by choosing a homofermentative LAB species.

A newly isolated *Enterococcus mundtii* was reported to produce 94.5 g/L of lactic acid from 104 g/L of xylose with a yield of 85% and a productivity of 1.3 g/L h (Abdel-Rahman et al., 2011). In contrast, *Lactobacillus* species converted glucose to as high as 198 g/L of lactic acid with a maximum productivity of 5.6 g/L h (Ge et al., 2011).

1.2.3 *Pediococcus* spp.

The lactic bacterium *Pediococcus* have diverse and unique morphological, physiological, nutritional and genetic characteristics. A variety of methods were developed to detect the organism and its production of meat and vegetables and has potential as a bio-preservation. The genus *Pediococcus*, a member of *Streptococcaceae*, is a Gram-positive lactic acid bacterium, which uniquely divides in to form tetrads. Cultures usually show cocci (0.4-1.4 μm in diameter) in pairs. (Moshe Raccach, 1999).

Following, Holland et al. (2011). Currently, 12 species of *Pediococci* (*Pc*) have been described: *Pc. acidilactici, Pc. argentinicus, Pc. cellicola, Pc. claussenii, Pc. damnosus, Pc. ethanolidurans, Pc. inopinatus, Pc. lolii, Pc. parvulus, Pc. pentosaceus, Pc. siamensis,* and *Pc. stilesii*. Strains from only two species, *Pc. acidilactici* and *Pc. pentosaceus,* are associated with dairy products. They are found in some cheeses as adventitious cultures, and are occasionally used in the fermentation of milk and during cheese manufacture. *Pediococci* are lactic acid bacteria commonly found in fermented vegetables, in dairy products, and in meat by Pederson (1949), and by Raccach (1987). *Pediococci* are homofermentative and produce DL-lactate from glucose (Haakensen et al., 2009a).

1.2.3.1 Characterizations of *Pediococcus acidilactici*

Pediococcus acidilactici is labeled as a Generally Regarded As Safe (GRAS) bacteria by the Food and Drug Administration (FDA). It is a species of gram positive coccus that is often found in pairs or tetrads. *Pediococcus acidilactici* is a homo-fermentative bacterium that can grow in a wide range of pH, temperature, and osmotic pressure, therefore being able to colonize the digestive tract. *Pediococcus acidilactici* was homo fermentative vegetable associated lactic acid bacteria (LAB) commonly used in several fermented products (McKay and Baldwin, 1990). These species are involved in the preparation of starter cultures in meat and in vegetable fermented products, and are present as secondary flora in different types of cheese (Bhowmik and Marth, 1989; Bhowmik et al., 1990).

Lactic acid bacteria are among the best studied microorganisms. Important new developments have been made in the research of lactic acid bacteria in the areas of multidrug resistance, bacteriocins, quorum sensing, osmoregulation, autolysins and bacteriophages. Progress has also been made in the construction of food grade genetically modified Lactic acid bacteria. These have opened new potential applications for these microorganisms in various industries.

1.2.3.2 Production, applications of *Pediococcus acidilactici* and Potential benefits

In 2003, *Pediococcus acidilactici* was 11% in total prevalent LAB, predominantly homofermentative (66%), consisted of *Lactobacillus plantarum* (24%), *Lactococcus lactis* (22%), *Leuconostoc pseudomesenteroides* (20%), *Lactobacillus brevis* (11%), *Enterococcus faecalis* (7%), *Weissella kimchii* (3%), and *Pediococcus pentosaceus* (2%) as following Ennahar et al. (2003).

The most identification of Pediococcus bacteria were applied to produce various bacteriocins such as: *Pediococcus acidilactici, Pediococcus pentosaceus,* and *Pediococcus parvulus* isolated from meat (Helena et al., 2007; Simha et al., 2012), from vegetables (Bennik et al., 1997), L-serine (Irmler et al., 2013).

Pediococcus acidilactici has a wide range of potential benefits of which and its production are still being studied such as: wine fermentation starters (Lv et al., 2012; Nguyen et al., 2013a,b), Silage Preparation (Cai et al., 1999), milk fermentation (Caldwell et al., 1996). Because of them accelerated the rates of lactic acid production and pH decrease (Fitzsimons et al., 1992).

Some genetic modifications have been successfully implemented to tailor made industrial strains. Recombinant plasmids containing the *Pediococcus acidilactici*, L (+)-lactate dehydrogenase gene (*ldh* L) were isolated by complementing for growth under anaerobiosis of an *Escherichia coli* lactate dehydrogenase-pyruvate formate lyase double mutant (Marugg et al., 1992). Fermentations of glucose and xylose to lactic acid were compared in M9 medium containing 5% sugar, using *E. coli* SZ85 expressing *P. acidilactici* ldhL, Lactic acid yields based on metabolized xylose were 0.93 g/g of xylose. Glucose was exhausted after 120 h with yields averaging 0.95 g/g of glucose metabolized (Zhou et al., 2003). Metabolism of selected cucumber secondary fermentation isolates incubated in cucumber juice medium, *Pediococcus thanolidurans* produced 92.6 mM of LA after 20 days under aerobic conditions (30 ± 2 ℃) and 12 months under anaerobiosis (25 ± 2 ℃) was 77.2 (Wendy and Ilenys, 2012).

In addition, identification and finding of *Pediococcus acidilactici*, for example: from Beer (Haakensen et al., 2009b), Chili Bo in a Malaysian Food Ingredient (Leisner et al., 1999), Hong Qu glutinous rice wine (Lv et al., 2012), fermented vegetables and uncooked fermented meat (Nguyen et al., 2013a,b), Tarhana is a mixture of spontaneously fermented yogurt and wheat flour, a traditional fermented product with *Pediococcus acidilactici* were found to constitute 27 % of the isolates (Sengun et al., 2009),

On other hand, to date, a few studies on high productivity of lactic acid use this strain from cheap materials. Zhao et al., 2013 studied production of this species.

There are a number of potential benefits that might be derived from *Pediococcus acidilactici*. However, in the lactic acid industry, we have known for many years that there was variation among strains and species of LAB with regard to their ability to produce the desired changes yield and productivity in the lactic acid fermented. Thus, it should not be surprising that there would be variation among strains and species of microorganisms with regard to their ability to produce lactic acid. Furthermore, we should not expect one strain or species of microorganisms to provide all of the potential benefits that might be possible from consumption of these organisms. More information will be discussed at later charpter.

1.3 Lactic acid fermentation by the fungus, yeast

Fungi and bacteria are the most widely employed microorganisms for lactic acid production. The main advantages of the use of fungi as fermenting agents are their ability to release extracellular amylases able to hydrolyze starchy materials, thus not requiring any prior stage of hydrolysis (Li et al., 2012; Jin et al., 2005), and the easy separation of biomass because of mycelium formation. These fungi, which usually belong to the genus *Rhizopus* and produce especially the L (+) isomer have been employed in flour fermentation with starches from rice (Fukushima et al., 2004), potato, wheat and pineapple (Jin et al., 2003; Jin et al., 2005), pine wood (Woiciechowski et al., 1999) and waste paper (Marques et a., 2008; Park et al., 2004). The genus *Aspergillus* in particular has been used with success as a host for the production of recombinant proteins of both fungal and non-fungal origins, e.g., glucoamylase, bovine chymosin, human lactoferrin, hen egg-white lysozyme, human interleukin-6, and thaumatin. Other host strains include *Trichoderma reesei, Chrysosporium lucknowense, Mortierella alpinis,* etc. (Wang et al. 2005).

Some fungi such as *Rhizopus oryzae* have been known to produce L-lactic acid homofermentatively from both glucose and xylose during aerobic growth with lactic acid yields of 71-79% on glucose and 54-65% on xylose due to the partial carbon oxidation into

CO_2 (Maas et al., 2008a). *Rhizopus oryzae* NBRC 5378 produced lactic acid from wheat straw powder by a simultaneous saccharification and fermentation process, with a yield of 0.23 g/g from cellulose and hemicellulose in wheat straw (Saito et al., 2012).

Fungal *Rhizopus* species have attracted a great interest, and have been recognized as suitable candidates for lactic acid production. Unlike the LAB, lactic acid producing *Rhizopus* strains generate L-lactic acid as a sole isomer of lactic acid. However, an increased research interest has been given to lactic acid fermentation by fungal species in recent decades. *Rhizopus* strains grow better under nitrogen-limited environments than the lactic acid producing bacteria.

Recently, yeasts such as *Saccharomyces cerevisiae* have been recognized as suitable hosts for lactate production because yeasts exhibit acid tolerance and can grow on inexpensive synthetic media. While wild *S. cerevisiae* does not produce lactate because of the absence of lactate dehydrogenase genes (*ldh*), the optical purity of L-lactate or D-lactate that is produced by a recombinant *S. cerevisiae* strain expressing a heterologous L-ldh or D-ldh gene is extremely high.

Rhizopus mutant using ammonia water as neutralizing agent, 93 g/l of lactic acid with a productivity of 2.6 g/l h and a yield of 87% was obtained (Miura et al., 2003). This result is similar to that of *R. oryzae* ATCC 9363 produced maximum lactic acid at 92.2 ± 2.7 g/l merely and the optimal pH was shifted to 5.25. But in the same pH, *R. oryzae* BCRC 33071 produced up to 102.4 ± 1.6 g/l of lactic acid with a productivity of 3.01 ± 0.05 g/l h and a yield of 85.3 ± 1.3%, which were much higher than that of *R. oryzae* ATCC 9363 (Wang et al., 2013).

Yeast fermentation has the advantages that yeasts are more resistant to low pH environment than LAB and they are genetically engineered with ease. Interest in yeast species as alternative microorganisms for lactic acid production has recently increased because yeasts are more resistant to low pH than lactic acid bacteria (Tamakawa et al., 2012). However, because yeasts exhibit a low pH tolerance, lactic acid production can be expected under the condition of low pH. *C. utilis* produced L-lactic acid with a yield of 70.8%.

The results found that the yield and productivity of these yeasts view relatively low for industial production of L-Lactic acid. The choice of the yeast host is of paramount importance for the success of a whole industrial process. *Saccharomyces cerevisiae*, the most important yeast because of its industrial significance, and has been used safely as an organism generally regarded as safe (GRAS) for centuries in the brewing and baking industries. Moreover, yeasts are free of toxins and their applications in food industry from times immemorial accredited them. *S. cerevisiae* was the first eukaryote to have its genome completely sequenced (Goffeau et al., 1996). However, it has to be said that sometimes this yeast is not an optimal host for large-scale production. Its technical fermentation needs require highly sophisticated equipment, the proteins produced by S. cerevisiae are often hyper-glycosylated and retention of the products within the periplasmic space is frequently observed. Additionally it is not highly resistant to severe stresses (pH, temperature, etc.) often imposed by the production process itself (Paola et al., 2008).

S. cerevisiae produces very little lactic acid because S. cerevisiae mainly converts pyruvate to ethanol; *S. cerevisiae* produces ethanol under anaerobic fermentation. However (Suzuki et al., 2013) examined the productivity of lactic acid in the disruptants harboring LDH without neutralization after 72 hours cultivation, at which time all glucose in medium was consumed in almost all disruptants. In particular, *S. cerevisiae* is useful as a host for genetic engineering, since it allows the folding and glycosylation of expressed heterologous eukaryotic proteins and can be subjected to many genetic manipulations

Applied research using the so-called ‘non-conventional’ yeasts has been developed. The most established or prominent examples of non-conventional yeasts are L-lactate respiration, are difficult to detect in living yeast cells due to the small activity of L-lactate oxidizing enzymes but some yeasts could produce lactic acid using the crabtree yeasts *Saccharomyces cerevisiae* (Tokuhiro et al., 2008; Garjonyte et al.; 2008, Gao et al., 2009b), *Hansenula polymorpha* (Shkil et al., 2009), *Kluyveromyces lactis* (Bianchi et al., 2001), *Kluyveromyces marxianus* (Plessas et al., 2008), *Candida utilis* (Tamakawa et al., 2012), *Pichia stipites* (Ilmen et al., 2007).

1.4 Biomass material for lactic acid production

1.4.1 Carbon sources for lactic fermentation

A number of different substrates have been used for the fermentative production of LA by LAB. The purest product is obtained when a pure sugar is fermented, resulting in lower purification costs. However, this is economically unfavorable, because pure sugars are expensive and lactic acid is a cheap product. Instead waste products from agriculture and forestry are utilized. Molasses, a by-product of the sugar manufacturing process, is used as an animal feed. The starchy materials not only contribute to a significant proportion of lactic acid production cost but also compete with food supply. Another common substrate for lactic acid production is starch from crops or wastes. It has to be hydrolyzed to glucose and maltose to be fermentable by LAB. Starch from various origins has been used for lactic acid production, including such as wheat, corn, and cassava (Oh et al., 2005; Lu et al., 2010), peanut meal (Meng et al., 2012).

Lignocellulosic materials have also been used for the production of LA in similar ways as starch. It consists mainly of the hexoses glucose, galactose, and mannose and the pentoses xylose and arabinose, and has to hydrolyze to monomers to be fermentable. In addition, inulin source from The Jerusalem artichoke (*Helianthus tuberosus* L.) is a tuberous plant belonging to the sunflower family. It accumulates inulin in its roots, tubers, and stalks. Thus, there are other hexoses such as fructose, glucose, which can be consumed by lactic acid bacteria.

1.4.2 Composition of lignocellulosic biomass

Recently, lactic acid has been produced from a variety of carbohydrates, including starchy materials, lignocellulosic biomass, agro-industrial and food wastes

There have been various attempts to produce lactic acid from cheap raw materials. The carbon source for microbial production of lactic acid could be either sugar in pure form such as glucose, fructose, sucrose, lactose etc., or sugar containing raw material. Pure sugars or edible crops have been a traditional substrate for lactic acid production that is advantageous in obtaining a pure lactic acid product and lowering costs of pretreatment and recovery. Since substrate cost cannot be reduced by process scale-up, extensive studies are currently underway to search for novel substrates for lactic acid production (Mohamed Ali et al., 2013). The demand for lactic acid has been estimated to grow yearly at 5-8% (Yadav et al., 2011). The search for low-cost raw materials to be used in the production of lactic acid by fermentation has been promoting the development of competitive processes. The materials most frequently used to this purpose can be classified into two groups, namely the monosaccharides and disaccharides and the polymeric substrates (Fabio et al., 2013). Some agricultural by products that are potential substrates for lactic acid production to reduce the nutrient cost of L-lactic acid production, pretreated wheat bran showed a better performance than that without treatment, especially for L-lactic acid yield (0.99 g/g) (Li et al., 2010), a rice washing drainage storage tank under the simultaneous saccharification and fermentation (SSF) process (Watanabe et al., 2013), corncob molasses (Wang et al., 2010b), recycled paper sludge by SSF (Marques et al., 2008), cassava powder (Wang et al., 2010b). The raw material cost for the fermentative production of lactic acid usually accounts for 68% of total manufacturing cost (Tejayadi and Cheryan, 1995). Åkerberg and Zacchi (2000) previously reported that the cost of raw materials for lactic acid fermentation account for more than 34% of total production cost. Cheap raw materials are necessary for the feasible economic production of lactic acid because polymer producers and other industrial users usually require large quantities of lactic acid at a relatively low cost.

However, high cost of raw materials, e.g. starch and refined sugars, which accounts for the highest portion of the production cost, represents one of the most serious obstacles for cost effective fermentative production of lactic acid (Talukder et al., 2012). Fortunately, lignocellulosic biomass represents, biomass is the third largest primary energy in the world, after coal and oil (Chen et al., 2009), the most abundant global source of biomass, and for this reason it has been largely utilized in many applications. These materials are not only an important cost factor for the economy; their utilization is also connected to environmental pressures. The economics of lactic acid production by fermentation is dependent on many factors, of which the cost of the raw materials is very significant. It is very expensive when sugars, e.g., glucose, sucrose, starch, etc., are used as the feedstock for lactic acid production.

Biomass consists of three types of polymers: cellulose, hemicellulose, and lignin. Cellulose has strong physico-chemical interaction with hemicelluloses and lignin. Cellulose, a linear glucose polymer, is highly ordered polymer of cellobiose (D-glucopyranosyl-β-1,4-D-glucopyranose) representing about 50% of the wood mass. Cellulose is degraded by acid or enzymes known as cellulases to its monomer, glucose that is fermented further to commodity chemicals such as lactic acid (Adsul et al., 2011). Zhang et al. (2011a) Cellulose, which is the main component of biomass, has become an attractive substrate because of its conversion useful chemical products, as well as for its potential as a clean fuel. Currently, the main methods to convert cellulose to useful chemicals are acid or enzymatic hydrolysis processes. However, there are many disadvantages of these two processes: (1) Acid hydrolysis of biomass needs to consume a large amount of acid and also results in serious secondary pollutions, and (2) the reaction rate of enzymatic hydrolysis is slow, and the efficiency is low because microorganisms cannot easily directly degrade cellulose. Thus, the development of an environmentally friendly and highly effective method for converting cellulose to useful chemicals is strongly desired. However, Venkatesh K.V. (1997), Hofvendahl and Bärbel (2000), Yánez et al. (2003), Marques et al. (2008) have previously attempted to produce lactic acid from pure cellulose though SSF process.

Hemicelluloses, are types of plant cell wall polysaccharides, are short, highly branched heteropolymer of mainly xylose, plus glucose, mannose, galactose, arabinose, and sometimes uronic acids (Fig. 1.3). Hemicelluloses are referred to as mannans, xylans or galactans depending upon the predominance of type of sugars present in the polymer. The C5 and C6 sugars that are linked through 1,3; 1,6, and 1,4 glycosidic bonds and often acetylated, form a loose, very hydrophilic structure acting as a glue between cellulose and lignin (Zhang et al., 2011b, Peng et al., 2012). Hemicellulose, in contrast to starch and cellulose, contains pentoses, which give rise to by-products such as acetate and ethanol, decreasing the LA yield (Hofvendahl and Bärbel, 2000). In 2001, Sreenath et al. was used liquid hot water (LHW)-pretreated alfalfa fibers, the “extract” soluble hemicellulosic fraction of alfalfa produced 0.38 to 0.62 g of lactic acid per g of dry matter extract during SSF and did not require nutrient supplementation. Enzymatic depolymerization of hemicellulose to monomer sugars needs the synergistic action of multiple enzymes, among them endo-xylanases (EC 3.2.1.8) and β-xylosidases (EC 3.2.1.37) (collectively xylanases) play a vital role in depolymerizing xylan, the major component of hemicellulose (Juturu et al., 2012). A hemicellulose hydrolysate: wheat straw (Garde et al., 2002), corn cobs (Cristina et al., 2012).

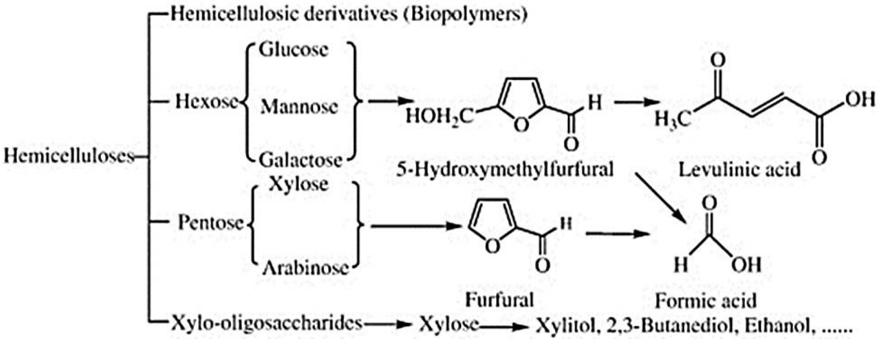

Figure 1.3 The potential products from hemicelluloses (Source: Peng et al., 2012)

Lignin is made up of phenylpropanoid units derived from the corresponding p-hydroxycinnapyl alcohols. These phenylpropanoid units are made up of dimethoxylated, monomethoxylated, and nonmethoxylated alcohols. Lignin is hydrophobic and highly resistant to chemical and biological degradation. It is present in the middle lamella and acts as cement between the plant cells. It is also located in the layers of the cell walls, forming, together with hemicelluloses, an amorphous matrix in which cellulose fibrils are embedded and protected against biodegradation. This association between cellulose and hemicelluloses and lignin makes the plant cell wall resistant to mechanical and biological degradations. The processing of lignocellulosic biomass will make the lignin enormously available for conversion into value added products, rather than its fuel value. The partially hydrolyzed lignin has excellent properties for use as substitutes for phenol-formaldehyde resins, polyurethane foams, adhesives, insulation materials, rubber processing, antioxidants, etc. It also provides a cheap source for vanillin and syringol for the flavor and fragrance industry (Zhang et al., 2011b).

Corn stover is a low cost, renewable, and easily available feedstock in China, but large quantities of them are unutilized and burnt out every year (Chen et al., 2009). Corn stover is rich in cellulose, hemicellulose, and lignin; the derived sugars are economically attractive carbohydrate feedstocks for fermentation of bulk chemicals. Therefore, the effective utilization of corn stover not only reduces environmental pollution, but also provides abundant cheap bioenergy.

1.4.3 Inulin source for lactic acid production

Raw materials such as lignocellulosics are cheap renewable substrate for lactic acid fermentation, but use of lignocellulosic biomass requires extensive pretreatment and saccharification (Abdel-Rahman et al., 2011). Therefore, an additional carbon resource like fructans that can be produced from non-grain crop plants such as Jerusalem artichoke should be considered for cost-effective production of commodity chemicals such as lactic acid. In addition to sucrose and starch, fructans contribute to the pool of storage carbohydrates in plants (Choi et al., 2012).

Inulin is a widespread naturally occurring polyfructan in various plants. In fact, it is produced naturally in over 36,000 plants worldwide, including 1200 grasses belonging to 10 families (Marchetti G., 1993). Inulin is a polyfructan, consisting of linear β-2,1-linked polyfructose chains terminated at the reducing end by a glucose residue (Vandamme and Derycke, 1983). This polymer is a reserve carbohydrate in the roots and tubers of plants such as Jerusalem artichoke, chicory, and dahlia. The inulinases are classified among the hydrolases and target on the β-2,1 linkage of inulin and hydrolyze it into fructose and glucose (Chi et al., 2011). In nature, inulin is a mixture of polysaccharides composed of 20-30 fructose unit chains (linked by β-(2,1)-D-fructosyl-fructose bonds) of various lengths with a glucose molecule at the end of each fructose chain and produced by some plants. However, the garlic fructan has a (2,1)-linked β-D-fructosyl backbone with (2,6)-linked β-D-fructosyl side chains (Baumgartner et al., 2000).

Mantzouridou et al. (2012), the degree of polymerization in the natural inulin mixture may range from 2 to 60 (average degree of polymerization around 12). The natural inulin mixture may be separated into an oligomeric fraction (degree of polymerization < 10) and a long-chain inulin fraction (degree of polymerization between 22 and 25). Inulin is used by some plants as a means of storing energy, degree of polymerization (DP) of inulin depends upon many factors, such as plant source, climate and growing conditions, harvesting maturity and storage time after harvest (Chi et al., 2011).

In recent years, Jerusalem artichoke has been found to be one of the most important candidates for use as a raw material for the industrial production of fructose and inulin (Li and Chan-Halbrendt, 2009). Chi et al. (2011), Yacon is a perennial herb, which belongs to Asteraceae, and stores inulin in its tubercles (3-10%). 75% of the dry matter of Garlic (*Alliumsativum* L.), (Baumgartner et al., 2000). Asparagus contain up to 15% inulin (Singh and Bhermi, 2008). Large amount of inulin (12-15%) of Dandelion (*Taraxacumofficinale syn*) in its tap roots (Kango, 2008).

Recently, it has been found that lactic acid can be used as the substrate for manufacture of the bio-degradable polylactide polymer, causing more demand for lactic acid using fermentative route. It was found that the synthesis of inulinase and invertase from *A. niger* SL-09 was enhanced significantly by the inoculation of *Lactobacillus* sp. G-02 at 12 h of culture, which reached 275.6 and 571.8 U/ml in 60 hours. In the following SSF procedure, the highest lactic acid concentration of 120.5 g/l was obtained in 36 hours of the fed-batch fermentation with high conversion efficiency of 94.5% (Ge et al., 2009). Choi et al. (2012), lactic acid fermentation of Jerusalem artichoke tuber and direct lactic fermentation of Jerusalem artichoke tuber extract at 111.6 g/L of sugar content with a supplement of 5 g/L of yeast extract by *L. paracasei* KCTC13169 in a 5 L jar fermentor produced 92.5 g/L of lactic acid with 16.8 g/L fructose equivalent remained unutilized in 72 h. The conversion efficiency of inulin-type sugars to lactic acid was 98% of the theoretical yield.

In recent years, the production of oligosaccharides has concentrated on “high-content” commercial products. In this regard, inulin is a promising source for oligosaccharide production, provided that strong endo-inulinase acts on inulin in the absence of exoinulinase or invertase activities. IOSs from inulin have similar chemical structures to fructo-oligosaccharides from sucrose (Chi et al 2011).

1.5 Processes for lactic acid fermentation

1.5.1 Lactic acid production by lactic acid bacteria from lignocellulosic biomass

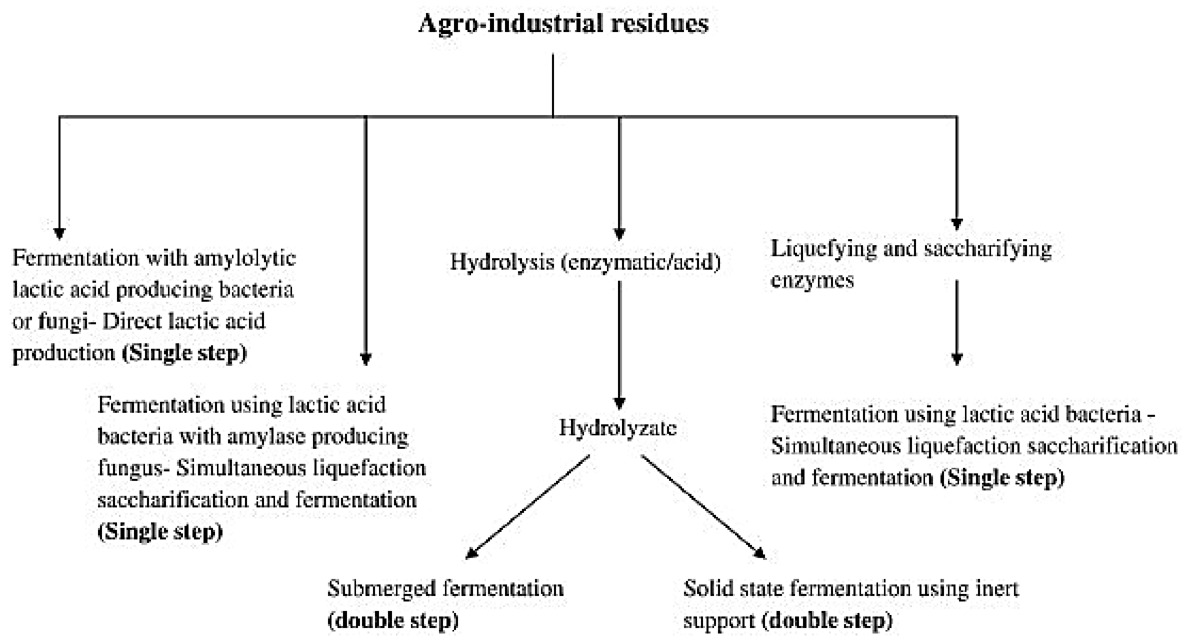

Figure 1.4 Different modes of fermentative production of lactic acid showing single and double step conversion (source: John et al., 2009)

Biomass conversion process involves five major steps, choice of suitable biomass, effective pretreatment, production of saccharolytic enzymes-cellulases and hemicellulases, fermentation of hexoses and pentoses and downstream processing.

Once the technologies are established and commercialized, a wide range of valuable products could be produced from lignocellulosic biomass. The conventional processes for producing lactic acid from lignocellulosic biomass include the following 4 main steps (John et al., 2009) as following:

- Pretreatment-breaking down the structure of the lignocellulosic matrix.
- Enzymatic hydrolysis-depolymerizing lignocellulose to fermentative sugars, such as glucose and xylose, by means of hydrolytic enzymes.
- Fermentation-metabolizing the sugars to lactic acid, generally by LAB.
- Separation and purification of lactic acid-purification of lactic acid to meet the standards of commercial applications.

Lactic acid can be produced by the fermentation of sugars or sugar containing hydrolyzates or the single step conversion of starchy or cellulosic wastes by direct conversion using amylolytic lactic acid producing microorganisms or by the simultaneous hydrolysis and fermentation with concomitant addition of saccharifying enzymes and inoculum together.

There are different processes for biotechnological production of lactic acid. Different lactic acid fermentation processes adopted by the researchers are shown in (Fig. 1.4).

The selection of mode of fermentation may be unlike with respect to different processes. Batch (Hofvendahl and Bärbel, 2000), fed-batch (Shi et al., 2012; Gao et al., 2012), repeated batch (Wee et al., 2009; Lu et al., 2012), continuous fermentation, separate hydrolysis and fermentation, or Simultaneous Saccharification and Fermentation (Zhao et al., 2013) are the most frequently used methods for lactic acid production.

1.5.2 Pretreatment methods and steam explosion pretreatment

The main goals of pretreatment are to remove lignin, separate cellulose and hemicellulose, increase the accessible surface area, partially depolymerize cellulose, and increase the porosity of the materials to aid in the subsequent access of the hydrolytic enzymes. Within the context of production of fuels from biomass, pretreatment has come to denote processes by which cellulosic biomass is made amenable to the action of hydrolytic enzymes. Some methods disrupt the lignin-carbohydrate complex, and other disrupts the highly ordered crystalline cellulose structure (Sun et al., 2012). The limited effectiveness of current enzymatic process on lignocellulose is thought to be due to the relative difficulties in pretreating the feedstocks (Menon and Rao, 2012).

The hemicellulose should be removed or altered without degradation for a high ultimate yield of sugars (Mosier et al., 2005). Pretreatment includes physical (milling and grinding), chemical (alkali, dilute acid, oxidizing agents, and organic solvents), physicochemical (steam explosion/autohydrolysis, hydrothermolysis, and wet oxidation), and biological methods (Mohamed Ali et al., 2011).

Different pretreatment methods have been extensively developed, including acid (hydrochloric or sulfuric) hydrolysis (Laopaiboon et al., 2010), diluted acid (Shi et al., 2012, Zhao et al., 2013), alkaline wet oxidation (Helene et al., 2002), alkaline neutralizing reagent (Cristina et al., 2012, Su et al., 2012), steam explosion (Su et al., 2012), diluted acidic steam (Zhang et al., 2013b), lime pretreatment (Kim and Mark, 2005, Xu and Jay, 2011), ammonia fiber explosion (Kim and Mark, 2005, Lee et al., 2010), microbial, 2-step: 2-step pretreatment first with acid then alkaline catalyst (MIC) (Lim et al., 2011), integrated utilization (Li et al., 2010), etc. In addition to choice of pretreatment strategy is a compromise between positive and negative effects because of the complex and sometimes opposing effects of various pretreatment parameters and considerations.

The steam explosion pretreatment is physicochemical; combining the destructing physical effect with chemical hydrolysis. The raw material is exposed to saturated steam at elevated temperature and pressure followed by the rapid reduction to atmospheric pressure, which causes lignocellulosic material to be broken down. Therefore, the accessibility of enzyme to cellulose component was enhanced and the digestibility was increased (Cara et al., 2008; Zimbardi et al., 2007).

In Fenske et al. (1998) research, total phenols conversion of corn stalk was 0.11 by acid steaming pretreatment (180 ℃, 1% (w/w) H_2SO_4, 1 min). Du et al. (2010) reported that total inhibitor conversion of corn stover were 0.067 and 0.0473 by 0.7 % H_2SO_4 and wet oxidation at 180 ℃ for 8 min, respectively. Zhang et al. (2012) reported that under 1.1 MPa/4 min-ISFC-1.2 MPa/4 min conditions, where the separation degree of the corn stalks is 1.6, the inhibitor conversion is the lowest as 0.0397.

1.6 Role of enzyme in lactic acid fermentation

1.6.1 Enzymatic hydrolysis

Enzymatic hydrolysis is the most promising means to yield fermentable sugars from pretreated lignocellulosic biomass, Jerusalem artichoke, other sources as a low cost feedstock and is necessary to allow LAB to utilize poly-(mono-) saccharides as a carbon source for SSF. The enzyme hydrolysis step remains a major technico-economical challenge in the way of biomass conversion (Menon and Rao, 2012).

1.6.2 Hydrolysis of cellulosic materials by cellulases

The hydrolysis of insoluble cellulose by microorganisms requires the production of either free or cell-associated extracellular cellulases. The biochemical analyses of cellulase systems from aerobic and anaerobic bacteria and fungi performed during the past two decades have revealed that multiple enzymatic activities are needed to hydrolyze cellulose into soluble sugar monomers that can be metabolized by microorganisms. In SSF, cellulose has to be hydrolysed to produce glucose and the glucose formed is immediately fermented to various chemicals. Previous studies by various workers have reported the conversion of both cellulose- and hemicellulose-derived sugars from corn stover for lactic acid production by SSF (Cui et al., 2011). Jacquet et al. (2012) the hydrolysis of lignocellulosic materials and to obtain high concentrated hydrolysate to improve the hydrolysis of cellulose fiber and to obtain highly concentrated hydrolysate, two methods based on successive addition of enzyme and substrate were assessed. The first method, which required only substrate addition, allowed to increase by 50% the hydrolysate concentration and to decrease by 30% enzyme units needed. The second method highlighted the ability to reach very high concentrated hydrolysate (up to 170 g/L) by simultaneous addition of enzyme and substrate.

At least three major types of enzymes are required for hydrolyzing cellulose:

- Endoglucanase (EG) or 1,4-*β*-D-glucan-4-glucanohydrolase;
- Exoglucanase, including 1,4-*β*-D-glucan glucanohydrolase (also known as cellodextrinase) and 1,4-*β*-D-glucan cellobiohydrolase (cellobiohydrolase; CBH); and
- β-glucosidase (BGL) or *β*-glucoside glucohydrolase.

EG randomly hydrolyzes the β-glycoside linkages of internal amorphous regions in cellulose to produce oligosaccharides of various degrees of polymerization and generate new chain ends. Exoglucanases hydrolyze cellulose in a processive manner from the reducing or non-reducing ends of cellulose chains to generate either glucose or cellobiose as major products. Exoglucanases can also hydrolyze microcrystalline cellulose, conceivably by peeling cellulose chains from the microcrystalline structure. BGL cleaves soluble cellodextrins and cellobiose into glucose. The correct combination of the activities and production level of each cellulase enzyme is critical for efficient lignocellulosic biomass utilization (Chandel et al., 2012).

1.6.3 Non-complexed and Complexed cellulase systems

Acremonium cellulolyticus, which efficiently produces both cellulase and β-glucosidase in addition to carboxymethyl cellulose-hydrolyzing enzyme and small amounts of xylanase, β-1,3-glucanase and amylase because of its high accessibility to cellulose, is an alternative cellulase producer, (Park et al., 2011), and noted that the level of cellulase produced from pretreated waste milk pack in cultures of A. cellulolyticus is similar to those obtained with pure cellulose.

In the complexed cellulase (cellulosome) systems are multi-enzymatic complexes produced by anaerobic bacteria that efficiently degrade plant biomass (Fontes and Gilbert, 2010). In these systems, different types of cellulose-degrading enzymes are assembled on the structural scaffoldin subunits through strong non-covalent protein-protein interactions between the docking modules and complementary modules. In addition, scaffoldin contains a carbohydrate-binding module, which binds the entire enzymatic complex to the cellulose surface. As the scaffoldin subunits are covalently bound to the cell walls of microbes by their anchoring proteins, microbes expressing cellulosomes can utilize cellulose as a source of carbon and energy. The efficient synergistic degradation of plant biomass results from the combination of targeting the enzymatic complex to the substrate and the spatial proximity of the different types of cellulases to each other.

1.6.4 Enzymatic hydrolysis of inulin

Inulin is a polysaccharide consisting of linear *β*-2,1 linked polyfructose units, terminated by a glucose residue through a sucrose-type linkage at the reducing end, that can be found in Jerusalem artichoke, dahlia tubers, chicory roots garlic, asparagus root and salsify. The use of this polyfructan as raw material for the production of high fructose syrups or inulooligosaccharides. The total or partial hydrolysis of inulin, leading to syrups with high fructose content, usually designated ultra-high fructose syrups (UHFS) or to functional sweeteners, respectively, is achieved by the action of exoinulinase (EC 3.2.1.80) or the synergistic action of exoinulinase and endoinulinase (EC 3.2.1.7), if the goal is UHFS; or by the action of endoinulinase, if short chain fructans, namely oligofructose, are aimed at, as a result of partial enzymatic inulin hydrolysis. Inulin containing plant species are found in a number of mono and dicotyledonous families such as Liliaceae, Amaryllidaceae, Gramineae, and Compositae. In *Liliaceae*, *Amaryllidaceae* and Compositae, inulins are usually stored in organs such as bulbs, tubers and tuberous roots. The two species currently used by the industry to produce inulin belong to Compositae: Jerusalem artichoke (*Helianthustuberosus*) and chicory (*Cichoriumintybus*) (Leroy et al., 2010).

Inulinases are fructofuranosyl hydrolases produced by a wide array of microorganisms, comprehending bacteria, fungi and yeast. Among these, the most common sources for inulinases are *Aspergillus* spp. and *Kluyveromyces* spp. (Pendey et al., 1999), a dual endoinulinase system originated from *Xanthomonas* sp. and *Pseudomonas* sp. (Cho et al., 2001), It has been known that *Aspergillus niger* SL-09 can produce a considerable amount of inulinase and invertase while *Lactobacillus* sp. G-02 can produce a large amount of lactic acid (Ge et al., 2009), *Klebsiella pneumoniae* (Gao et al., 2010), *Arthrobacter aurescens* (Zhao et al., 2011), *Saccharomyces cerevisiae* could utilize almost completely the fructo-oligosaccharides present in Jerusalem artichoke (Lim et al., 2011) or Lactobacillus paracasei and *Saccharomyces cerevisiae* was found to efficiently ferment the inulin-containing carbohydrates in Jerusalem artichoke without acidic or enzymatic inulin hydrolysis prior to fermentation (Lim et al., 2011; Choi et al., 2012).

Specially, glucoamylase (GA), also known as amyloglucosidase (EC 3.2.1.3; 1, 4-*α*, D-glucan glucohydrolase), is an exoglucosidase, is a biocatalyst capable of *α*-1,4 glucosidic linkages in raw or soluble starches. However, glucoamylase catalyse the hydrolysis of inulin into sugar (glucose and fructose). More information will be discussed at the later section in SSF.

1.6.5 Biomass comminution and enzymatic hydrolysis of the cellulose component

The physical pretreatment of the biomass, involves size reduction by chipping, grinding or milling of the biomass materials for some pretreatment methods. The high costs on milling pretreatment have promoted a research line, usually used hammer mill, knife mill and disc mill were the most appropriate equipment for biomass processing. In this area, larger biomass particle size achieved the better enzymatic hydrolysis performance to convert them to producing high sugar, which reflected the higher pretreatment efficiency. During biomass size reduction process can be considered as the best ones for biomass conversion process. These methods make the sugars more accessible to cellulase enzymes for saccharification. Such as rice straw by planetary and attrition milling (Kim et al., 2013), corn stover (Liu et al., 2013), (Cynara) size of pellets ranges from (2-5) cm in length by a hammer mill (Gil et al., 2012), pine, beanstalk, rice straw and reed by knife mill and different particle size ranges: 83-106 μm, 106-150 μm, 150-180 μm, 180-300 μm and 300-425 μm (Guo et al., 2012), Jerusalem artichoke tubers were milled, and passed through a 60 mesh sieve (Wang et al., 2013), Miscanthus (commonly known as Elephant Grass) by a hammer mill with 0.08, 2.0 or 6.0 mm screens (Khullar et al., 2013) , etc.,…For example, Bitra et al. (2010) measured size distribution of particles milled using four different sieve sizes with three types of biomass (corn stover, switchgrass, and wheat straw) for knife mill screen openings from 12.7 to 50.8 mm. Ballesteros et al. (2000) reported that, after 8 min steam-explosion pretreatment at 210 ℃, the relationship was negative, because the sugar yield of smaller particles (2-5 mm) was about 10% higher than that of larger particles (5-8 mm) if sugar yield was defined as glucose/pre-hydrolysis cellulose ratio.

Particle shape and particle size distribution are both important factors which could influence the physical properties of granular materials. Due to the high content of cellulose, hemicellulose, and lignin, biomass material is anisotropic in spatial structure, which induces the evident difference in mechanical property in different directions (Guo et al., 2012). Size reduction of biomass before pretreatment is an energy-intensive and expensive process, is a crucial factor in transportation logistics (Khullar et al., 2013), but it is necessary for cellulose bioconversion (Zhu et al., 2009). In addition, particle size reduction processes have a significant positive effect on handling parameters such as angle of repose, internal friction coefficient, wall friction coefficient, or bulk density due to size decrease and shape and size homogeneisation (Gil et al., 2012). In this context, it is possible that different relationships between particle size and sugar yield could be obtained from the same test by using pre-hydrolysis cellulose based or pre-hydrolysis biomass based sugar yield definitions. Liu et al.

(2013) has reported size reduction is an essential process for corn stover biomass utilization. Sugar recoveries and conversions of the biomass with particle sizes at 2.5, 2.0, 1.5, 1.0 and 0.5 cm were compared. The highest sugar recovery reached 99.6% for glucan and 67.0% for xylan at the particle size of 1.0 and 0.5 cm, respectively, but the highest sugar conversion (100% for glucan and 8% for xylan) was observed at the particle size of 2.5 cm. Or (Wang et al., 2013) the conditions for inulinase hydrolysis are: the enzyme dosage of 30 U/g Jerusalem artichoke powder, the response surfaces and corresponding contour plots described by the multiple regression model showed that the maximum value of reducing sugars (16.9 g/L) was predicted using an optimized condition at pH value and temperature of 5.2 and 35 ℃.

The optimizing biomass particle size is crucial in terms of achieving high sugar conversion and low production cost (Zhu et al., 2009; Ballesteros et al., 2000). The concentration of soluble glucose (monomer plus oligomers) was found increasing with reduced biomass particle size except for the particle size of 2.5 cm which gave the highest value; however, pretreating particle size in biomass particle size (0.5-2.5 cm) on steam explosion pretreatment performance for improving the enzyme digestibility of corn stover. It was interesting to note that the conditions generating high amount of glucose and xylose in the liquid fraction typically also generated high amount of HMF (0.46-0.78 g/100 g feedstock) and furfural (0.82-1.12 g/100 g feedstock) , respectively (Liu et al., 2013).

1.7 Milling energy and cost evaluation

Today, the cheap materials in lactic acid fermentation were used, beside optimal side fraction of materials such as etc., corn stover, starch, non-grain to interact with enzymatic hydrolysate in SSF process is necessary. Decreasing side fractions usually include milling, sieving, grinding. Choice of an appropriate material for a particular product is one of the critical tasks for the designers. Designers need to identify materials with specific functionalities in order to find feasible design concepts and fulfill the product’s end requirements (Chatterjee and Chakraborty, 2012). In that milling offers other obvious advantages with regards to cost and energy consumption, including the need for no additional chemicals or water to rinse the biomass. For chemical pretreatments, such as an acid treatment, the costs will be incurred not only by the catalysts being used, but also by the equipment requirements and the higher formation of degradation. For each combination of mill type and biomass type, six sieves (with sieve size of 0.25, 1, 2, 4, 6, and 8 mm respectively) will be used to produce particles. The particles milled using the 2-mm sieve will be separated into five size ranges (0.2-0.4, 0.4-0.6, 0.6-0.8, 0.8-1, and 1-1.2 mm), and the particles milled using the 8-mm sieve will be separated into another five size ranges (1.4-1.7, 1.7-2, 2-2.4, 2.4-2.8, and 2.8-3.3 mm) using screens (Zhang et al., 2013a). Energy requirements, on dry ton basis, for (1 and 2) mm target sizes are about 20 kWh/ ton for pellets and (50-60) kWh/ ton for stems or whole Cynara. For target size 5 mm, the specific energy to grind pellets is as low as 6.12 kWh/ ton, and for stems or whole Cynara is below 15 kWh/ ton (Gil et al., 2012).

Material selection has great importance in design and development of the products. The success and competitiveness of the producers also depends on the selected material (Chatterjee and Chakraborty, 2012). The objectives of performance, cost and environmental sensitivity drive engineering design, and are generally limited by materials. Selection of the materials that best meet the requirements of the design, give maximum performance and minimum cost is the goal of optimum product design (Thakker et al., 2008). However, some conflicting situations are generally observed between these objectives and criteria and there is a necessity to decide which property is more important than others (Çalışkan et al., 2013).

Finally, saccharification could simultaneously be performed with the milling process, which would reduce both the processing time and labor.

1.8 Simultaneous saccharification and fermentation for lactic acid

Various carbohydrate raw materials are used for lactic acid production by bacteria and molds such as refined carbohydrate raw materials; glucose, sucrose, and starch; complex carbohydrates-cellulose, cereal grains, corn, Jerusalem artichokes, and potatoes; and waste materials-cheese whey and permeate, municipal solid waste, and wood molasses. However, Simultaneous saccharification and fermentation can replace the classical double step fermentation by the saccharification of agricultural biomass and conversion to lactic acid concurrently by adding inoculum along with the substrate degrading enzymes. It not only reduces the cost of production by avoiding high energy consuming biomass saccharification, but also provides the higher productivity than the single step conversion by the providing adequate sugar release.

Simultaneous saccharification and fermentation (SSF) is a combined process of saccharification of a renewable bioresource and fermentation process to produce lactic acid products in Fig. 1.4. Recently, SSF has been extensively used to convert various sources of Agro-industrial residues and agricultural production into fermentative products. There were many reports that the SSF was superior to the traditional saccharification and subsequent fermentation in the production of lactic acid because the SSF process increased the yields of lactic acid by minimizing product inhibition as well as eliminated the need for separate reactors for saccharification and fermentation and the sugars could be assimilated rapidly by bacteria during SSF as Wang et al. (2010a), Watanabe et al. (2013), Zhao et al (2013). When the carbohydrates exist as polysaccharides, they must be hydrolyzed by inorganic acids or enzymatic catalysis to release fermentative sugar (Nguyen et al., 2013a).

Table 1.4 Lactic acid concentration, yield, and productivity using different materials

Raw material	Microorganism	Type	Lactic acid conc. (g/L)	Yield (g/g)	Productivity (g/L/h)	Reference
Corn stover	*Pediococcus acidilactici* DQ2	SSF	101.9	0.77	1.06	Zhao et al. (2013)
Hydrolysate of Jerusalem artichoke	*Lactococcus lactis* ATCC19435	fed-batch	140.5a	0.84 to 1.01	0.71 to 2.85	Shi et al. (2012)
Corn stover	*L. rhamnosus* + *L. brevis*	SSF	14.80	0.73	0.40	Cui et al.(2011)
Cassava powder	*Lactobacillus rhamnosus strain* CASL	SSF	175.4	0.71 based on initial starch	1.8	Wang et al. (2010a)
Paper sludge	*Lactobacillus rhamnosus* ATCC 7469	SSF	73.0	2.9	0.97	Marques et al. (2008)
Paper sludge	*Bacillus coagulan strains* 36D1	SSCF	92.0c	0.96	0.77	Budhavaram and Fan (2009)
Rice bran	*Lactobacillus rhamnosus* M-23	SSF	59a	0.85	1.23	Watanabe et al. (2013)
Corncob molasses	*Bacillus* sp. strain	Fed batch (SSF)	74.7a	0.38	0.50	Wang et al. (2010b)
Broken rice	*Lactobacillus delbrueckii*	SSF	79.0b	0.81	3.59	Nakano et al. (2012)

SSF: Simultaneous saccharification and fermentation. SSCF: Semi-continuous simultaneous saccharification and co-fermentation. a: L-lactic acid; b: D-lactic acid; c: DL-lactic acid.

The first study by SSF on lactic acid production of cellulosic raw materials was reported by Abe and Takagi (1991), and since then some articles have been reported on SSF using different sources for lactic acid. Agricultural biomass is another carbohydrate source that has recently drawn a lot of interest for lactic acid production because of (Mohamed Ali et al., 2013) higher production costs have hindered the large-scale application of PLA because of the high price of lactic acid. For example, Jerusalem artichoke, cassava, paper sludge, rice bran, corncob molasses, corn stover (Shi et al., 2012; Wang et al., 2010a; Marques et al., 2008; Wang et al. 2010b; Watanabe et al., 2013; Zhao et al., 2013). Besides, simultaneous saccharification and fermentation (SSF) process with hydrolytic enzymes has been studied to reduce the time and steps of bioprocess for the production of lactic acid (Watanabe et al., 2013).

The utilization of cellulosic resources such as cellulose, corncob, and waste paper for lactic acid production is considered to be a promising approach. However, to date, many technical problems, such as inhibition of the enzymes involved in cellulose hydrolysis by intermediate products, have hindered the commercial application of these processes. Using simultaneous saccharification and fermentation (SSF) operation, the enzyme inhibition could be removed (Wang et al., 2010a, Zhao et al., 2013).

1.9 Environmental factors affecting lactic acid fermentation

1.9.1 Fermentation conditions

Several factors were reported to improve lactic acid production efficacy including nitrogen sources, pH, neutralizing agents, and aeration. There are lots of factors that affect fermentative lactic acid production such as carbon source, substrate concentration, pH, temperature, etc.

Lactic fermentation is relatively fast, has high yields, and can lead, selectively, to one of the two stereoisomers of lactic acid or to their racemic mixture. After supplementation of nutrients, sugar solutions are inoculated with the selected microorganism, and the fermentation takes place. It is necessary to select the most favorable fermentation conditions, in terms of temperature, pH, aeration, agitation, and so on, which vary depending on the microorganism (Fabio et al., 2013). Chemical and physical properties of the acid followed by its commercial uses (John H.L., 1999)

1.9.2 Effect of inorganic cations on fermnentation

On other hand, the lactic acid yield from cellulose can be improved greatly by adding Zn, Ni and activated carbon under alkaline hydrothermal conditions. The highest lactic acid yield of 42% was obtained at 300 ℃, 5 min with 0.02 g Zn, 0.03 g Ni, 0.07 g activated carbon and 2.5 mol/L NaOH, which was much higher than that with only NaOH (15%), (Zhang et al., 2011b).

In this regard, improvements in process performance may be achieved by supplementation with organic or inorganic nutrients, quantitate inorganic cations in biological culture media are often helpful for medium optimization and comprehensive process monitoring and control. Analysis of inorganic cations found in culture media was accomplished by ion chromatography for several types of microbial fermentations as well as cell culture samples.

This analysis accurately monitored eight inorganic cations, several of which are commonly present in fermentation media (Na^+, NH_4^+, K^+, Mg^{2+}, and Ca^{2+}). The relationship of H^+, Na^+, and K^+ is especially critical in the maintenance of membrane physiology and in the role of various cytoplasmic membrane ATPases in the uptake of nutrients mediated by proton antiport or symport.

1.9.3 Effect of neutralizing agents on fermentation

Trapping lactic acid as calcium lactate during the fermentation of *Lactobacillus delbrueckii* has been shown to be an effective means to improve lactic acid production (Nakano et al., 2012). Therefore, $Ca(OH)_2$ (35%, w/v) was tested as the neutralizing agent to control the pH during the fermentations of *B. coagulans* C106 and the results were compared with those obtained using NaOH as the neutralizing agent. $Ca(OH)_2$ was found to be a better neutralizing agent than NaOH in terms of its giving higher lactic acid titer (1.2-fold) and productivity (1.8-fold) under the same conditions (Ye et al., 2013). Calcium carbonate is usually used, but it would bring out calcium lactate which required further processing to be converted to lactic acid by H_2SO_4. This complicates down-stream processing and generates by-product $CaSO_4$. Instead, NaOH is a simpler and cleaner alternative to $CaCO_3$ (Jia et al., 2013).

Removal of acetic acid and sulfuric acid from biomass hydrolyzate using a lime addition. $Ca(OH)_2$ is an effective neutralizing agent in the SSF process of broken rice using *L. delbrueckii*. $Ca(OH)_2$ and it seems that low molarity of D-(-)-calcium lactate would enhance the growth of lactic acid bacteria and lactic acid productivity (Nakano et al., 2012). This hybrid process was able to remove 98.08% of sulfuric acid and 76.97% of acetic acid from the mixture of glucose, xylose, acetic acid, and sulfuric acid. The process was able to recover almost all sugar (>99%) at high purity (97.53%), (Kim et al., 2012).

1.9.4 Physical factors

Several studies concerned the effect of temperature and pH on different lactic acid bacteria, examined the optimal pH and temperature range for the specific strains and integrated the effect of these parameters to the kinetic description of growth and lactic acid production.

Temperature: Temperature is one of the fundamental thermodynamic state variables and surely one of the most important properties of thermal systems such as in power and process engineering.

Lactic acid bacteria can grow at temperatures from 5 to 45 ℃ (Reddy et al., 2008). For example, The temperature of 41 ℃ selected here for the fermentation by *L. rhamnosus* ATCC 7469 is within an optimal range of 35-47 ℃ (Aleksandra et al., 2012). Extremes of fermentation temperature have previously been demonstrated to induce fermentation arrest under growth conditions that would otherwise result in complete sugar utilization at normal temperatures and nutrient levels. Among these factors, temperature affects microbial activity, conversion rate of substrate, and economic analysis (Jung et al., 2011). The optimal temperature for growth varies between the genera from 20 to 45 ℃. Thus, *B. coagulans* is moderately thermophilic and capable of fermenting at 50-55 ℃, which reduces the risk of microbial contamination as the conventional mesophilic contaminants can hardly grow at this temperature range. But Ye et al. (2013) shown that *Bacillus coagulans* C106 was isolated from environment and used to produce L-lactic acid from xylose at 50 ℃ in batch fermentation with 85 g/L of xylose, lactic acid titer and productivity reached 83.6 g/L and 7.5 g/L/ h, respectively.

An experimental study was carried out for the corn cobs thermal conversion to obtain the maximum content in lactic acid. For this purpose, under the same conditions (275 ℃ and 30 min) different concentrations of $Ca(OH)_2$ as alkaline catalyst were used (from 0.32 M to 1 M). The maximum content of lactic acid (6.72 ± 0.31 g/L) was obtained with 0.7 M of $Ca(OH)_2$. With this catalyst concentration, different reaction conditions were used (250, 275 and 300 ℃ and 15, 30 and 45 min). The optimal conditions to produce the highest yield of lactic acid from corn cobs in alkaline conditions were determined at 300 ℃ and 30 min, achieving 44.76 ± 2.59% respect to the total cellulose and hemicellulose contained in the initial corn cobs (7.38 ± 0.43 g/L of lactic acid) (Cristina et al., 2012).

pH control: Kata (2012) and Kaur (2013), pH is one of the most important chemical environmental measurements used to indicate the course of the fermentation process. It detects the presence of specific chemical factors that influence growth, metabolism, and final product. To maintain the constant pH for optimal growth and for optimal lactic acid production several agents can theoretically be applied. Current industrial lactic acid fermentations are performed with lactic acid bacteria. Although several strategies have been applied in order to improve the productivity of these bacteria, the problem of their pH sensitivity has not been solved. During the fermentation process, the pH declines, affecting the growth of the bacteria and their productivity (Valli et al., 2006). To neutralize the produced lactic acid, alkali addition or pH buffering was applied to avoid the inhibitory effect of the undissociated lactic acid (Hetényi et al., 2011).

Besides the appropriate medium components the success of a lactic acid fermentation technology depends on other technological parameters such as temperature or pH. The pH is either set at the beginning and left to decrease by lactic acid formation, or it can be controlled via addition of neutralizing agent, or via lactic acid removal. The optimal pH range for lactic acid bacteria varies between 5.0 and 7.0 (Reddy et al., 2008; Hetényi et al., 2011).

The most commonly used alkaline neutralizing reagent is $CaCO_3$. These results support opinion of Gao et al. (2009a), who observed that in low lactate concentration calcium lactate had higher inhibitory effect than ammonium lactate but over a certain concentration its inhibitory effect was less. However, in case of $CaCO_3$ as pH buffering agent an additional problem arises namely the already mentioned flocculation of the fermentation broth at high calcium lactate concentration which caused a consolidated mass. Because of this phenomenon, fermentation became incomplete, although nutrient components were sufficient and they remained in the broth, and for sampling or for lactic acid recovery from this flocculated broth heating or acidifying should be applied (Hetényi et al., 2011).

Alkaline neutralizing reagent must be added to maintain a stable pH in lactic acid fermentations using either, Sodium hydroxide (NaOH) and lime $Ca(OH)_2$, (Kim and Mark, 2005; Xu and Jay, 2011) or NH_4OH have been reported. However, side by side comparative evaluation of biochemical effects of $Ca(OH)_2$ with other soluble neutralization agents (e.g., NH_4OH or NaOH) in D-lactic acid fermentation by *Lactobacillus delbrueckii* using any of the three alkaline suspension or solution (25%, w/v). Calcium carbonate is usually used, but it would bring out calcium lactate which required further processing to be converted to lactic acid by H_2SO_4. This complicates downstream processing and generates by product $CaSO_4$. Instead, NaOH is a simpler and cleaner alternative to $CaCO_3$ (Jia et al., 2013).

1.9.5 Nutritional requirements of lactic acid bacteria

However, it is known that lactic acid producing bacteria (LAB) such as Lactobacilli need a large number of nutrients like amino acids, vitamins, etc., for their growth and lactic acid production. The organisms have complex nutritional requirements for amino acids, peptides, nucleotide bases, vitamins, minerals, fatty acids and carbohydrates (Reddy et al., 2008; Cui et al., 2011).

Yeast extract (YE) as the organic carbon source, is the most commonly used nutrient source in fermentations, the largest contributor for lactic acid production was found to be YE, which accounted for about 38% of total production medium cost (Altaf et al., 2007). YE is advantageous over the conventional lactic acid bacteria for cost-effective production of lactic acid. The conventional lactic acid bacteria such as *Lactobacillus* and *Enterococcus* require a medium rich in organic nitrogen sources for their growth and fermentation (Ye et al., 2013).

Lactic acid bacteria are traditionally fastidious microorganisms and have complex nutrient requirements due to their limited ability to biosynthesize B-vitamins and amino acids (Fitzpatrick and O’Keeffe, 2001). Furthermore, a considerable amount of expensive complex nitrogen source, such as yeast extract, must be added to the medium to produce lactic acid in a reasonable time. However, these are economically unfavorable because pure sugars and pure complex nitrogen sources are expensive but lactic acid is a relatively cheap product (Oh et al., 2005).

Nitrogen, which is implied either in anabolic or catabolic processes, is available in the form of amino acids, peptides and inorganic compounds that can be added to the culture media as peptone, yeast extract, urea or ammonium sulfate (Nancib et al., 2001; Zhang et al., 2010a; Zhao et al., 2013). Mineral elements (Mg, Mn, and Fe) are provided in the medium in the form of salts as $MgSO_4$, $MnSO_4$ and $FeSO_4$ (Büyükkileci & Harsa, 2004).

Thus, economic analysis for lactic acid production showed that the cost of YE contributed over 30-38% to the total production cost independent of lactic acid fermentation types, which implied an urgent need for a cheaper alternative.

1.9.6 Inhibition on lactic acid fermentation

The effects of inhibitors on subsequent fermenting microorganisms have become one of the main for lignocellulose bioconversion processes. Although the composition of the released compounds depends are not only on the type of lignocellulosic material and the chemistry but also on the characteristics of the pretreatment process. These thermochemical pretreatment processes often result in the generation of by products such as acetic, levulinic acids, 5-hydroxymethyl furfural (HMF), furfural and phenolic compounds, which significantly inhibit enzymatic hydrolysis and fermentation (Klinke et al., 2004; Kim et al., 2011; Zhao et al., 2013).

The effects on both cellulose conversion rate and lactic acid yield were studied by adding inhibitors, including formic acid, acetic acid, furfural, and vanillin into SSF (Zhao et al., 2013). Recirculation of process streams is necessary to minimize the amount of fresh water required and also decreases the energy demands for the distillation and evaporation steps. However, recirculation will give rise to an accumulation of compounds which are potential inhibitors (Larsson et al., 1997).

Recently, wild type *Lactobacillus brevis* S_3F_4 was shown to have strong resistance to fermentation inhibitors such as ferulic acid and furfural (Guo et al., 2010). Biological delignification with white rot fungi, which selectively degrade lignin and leave cellulosic materials, also has potential advantages such as low capital cost, low-energy input, no chemical requirements, mild environmental conditions, and high yields without generating polluting by products. However, at high substrate loading, also SSF processes may suffer from inhibition due to inhibitors from the pretreatment step. Effective measures must be taken to reduce or to eliminate the inhibitors. A number of detoxification methods, have been proposed to transform inhibitors into inactive compounds or to reduce their concentration.

1.10 Applications of lactic acid

1.10.1 Food industry

Fermented foods have been prepared for thousands of years and adopted in various ways depending on the properties of available raw materials and desired features of final products. Although the microbial and enzymatic processes responsible for the food manufacture have not been fully elucidated until now, it is generally accepted that LAB contributes significantly to the properties of many fermented foods. Lactic acid is used as acidulant, flavoring or pH-buffering agent or inhibitor of bacterial spoilage in a wide variety of processed foods, such as candy, breads, 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). LA is also present in wheat beers, especially Lambic, due to the activity of *Pediococcus damnosus* (Ren J., 2010).

Lactic acid has long been widely, is naturally available in various food products, has long been widely used in food applications, including bakery products, beverages, meat products, confectionery, dairy products, salad dressings, ready meals. Generally, lactic acid severs as a pH regulator, preservative, or flavoring agent in food products. In meat, poultry, and fish products, lactic acid is used in the form of sodium lactate or potassium lactate. It helps extend the product shelf life, improve food safety by inhibiting pathogenic bacteria, protect and enhance meat flavor, and improve water binding capacity. In pickled vegetables, lactic acid is effectively used in preventing the spoilage of olives, gherkins, pearl onions, and other vegetables preserved in brine. Since lactic acid provides the milder taste compared with other organic acids used in food application, it is used as an acidulant and an antimicrobial agent in beverages such as soft drinks and fruit juices and many dairy products. In addition, lactic acid is used as a preservative in salad dressings, giving the milder flavor of products while maintaining microbial stability and safety. Lactic acid not only provides a mild acid taste in formulation hard boil candy, fruit gums, and other confectionery products, it also helps improve quality reduce stickiness, and prolong shelf life. Besides the application in food, lactic acid can be used as an additive in animal nutrition because of health promoting properties.

1.10.2 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).

Ren J. (2010), both racemic and D-Iactic acid are less suitable for cosmetic applications because they are not as mild as the L form. The L form is the form which is present in the human body (e.g., skin, hair, and muscles). Every day the human body produces about 120 g of lactic acid (LA). LA is also part of the natural moisturizing factor that retains moisture in the skin. Being a natural ingredient and a natural constituent of the human body, LA and lactates fit perfectly in today's trend towards natural and safer formulations. With their multi-functionality (moisturizing, pH-regulation, humectancy, antimicrobial), LA and lactates are ideal standard ingredients in the formulation of a good base for cosmetic products. In addition to their multi-functionality, L-Iactic acid and lactates also possess special features (e.g., skin lightening) that make them very useful as active ingredients.

1.10.3 Detergents, Chemical, pharmaceutical industry and medical field

Lactic acid (LA), an important carboxylic acid, can participate in a wide variety of chemical reactions leading to a host of products for use in the fields of textiles, pharmaceuticals, medical industy.

LA has gained importance in the detergents industry in the last couple of years. Being a good descaler, soap-scum remover and being a registered anti-bacterial agent, lactic acid becomes a safer and natural ingredientes (Ren J., 2010).

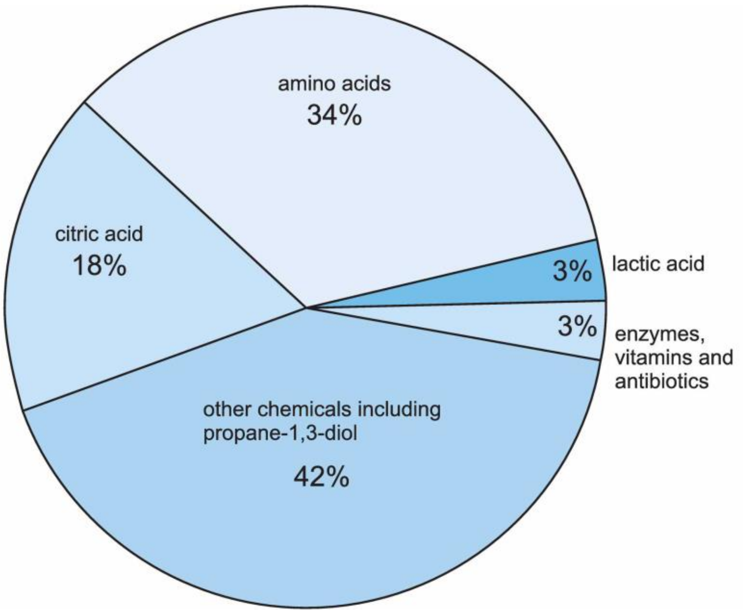

Figure 1.5 The compounds produced by fermentation reactions in the chemical industry

Source: http://www.essentialchemicalindustry.org

Producing more chemicals using biotechnology could reduce our dependence on natural gas and oil and reduce the environmental impact of the chemical industry. Such as lactic acid, have for many years been routinely produced on the million-tonne scale using biotechnology, as the chemical synthetic routes are complex and expensive. Other notable examples are described in this unit but there are many other processes still in the developmental stage, with chemicals being produced in small reactors on the scale of a few tonnes.

Lactic acid has long been used in pharmaceutical formulations, pharmaceutical grade D (L)-lactic acid, medical field , mainly in topical ointments, lotions, based vaccine adjuvants, and parenteral solutions. It is used in a wide variety of mineral preparations, which include tablets, prostheses, surgical sutures, and controlled drug delivery. It also finds application in the preparation of biodegradable polymers for medical (Wee et al., 2006; Modi et al., 2006, Hamdy et al., 2007; Anne and Samuli, 2010; Anne and Alan, 2011). Since lactic acid and its salts are readily accepted by the body, lactate is an ideal vehicle for introducing therapeutic minerals. In general these preparations have advantages of good tolerance and pleasant or neutral taste. Some of the more important mineral lactates are calcium lactate (for osteoporosis, hypertension, tooth decay), ferrous lactate (anemia), magnesium lactate (hypertension, muscle weakness), manganese lactate (glucose intolerance), and zinc lactate (skin disorders).

1.10.4 Poly-lactic acid industry and others application

Industrial the poly-lactic acid (PLA, lactic acid) is mostly composed of poly (L-lactic acid) (PLLA) and poly (L, D-lactic acid) (PLDLA), being the l-isomer the main fraction. PLA possesses interesting physical properties together with biocompatibility and biodegradability properties, which are all strongly influenced by its stereochemistry and molecular weight. In addition, it is easily processed into a desired configuration on standard plastics equipment to yield moulded parts, films, packaging or fibres and used in apparel, fiberfill (pillows, comforters), carpet, and nonwoven applications such as wipes (Auras et al., 2004, Lim et al., 2008, Miller & Soucek. 2012). One of the most promising biopolymers able to replace the petroleum-derived polymers for industrial applications is poly (lactic acid) (PLA). PLA is a linear aliphatic thermoplastic polyester derived from 100% renewable resources such as sugar, corn, potatoes, cane, beet, etc. The most common route for industrial production of high molecular weight PLA is the ring opening polymerisation of lactide monomer formed from lactic acid, which is produced by fermentation of renewable agricultural resources (Auras et al., 2004). Owing to these properties, PLA has a wide range of potential industrial applications. Due to its initial production costs, the starting applications of PLA have been focused on high value products, particularly medical devices (Lasprilla et al., 2012). More, among the biomaterials (biopolymers) used in the medical field; the poly (lactic acid) (PLA) has received significant attention. Poly-lactic acid (PLA) is at present one of the most promising biodegradable polymers for this purpose and has convincingly demonstrated the proof of concept for using in bioabsorbable polymer as bone fixation devices, owing to its mechanical property profile, thermoplastic possibility and biological properties, such as biocompatibility and biodegradability (Lopes et al., 2012). Lactic acid, a naturally occurring organic acid found in many different biological systems, is environmentally safe. Industrial fermentation of lactic acid produces a salt byproduct that is landfilled or incorporated into building materials or used in agriculture. It also produces an aqueous stream that can be treated in a typical wastewater treatment plant. As more lactic acid goes into making PLA, there are considerable environmental benefits of using PLA over conventional oil-based polymers, including lower CO_2 emissions and wider choice of disposal options that includes composting. The lactic acid polymers have extensive applications and tremendous advantages like bio degradability, thermo plasticity, mechanical, thermal, thermo-mechanical, barrier, optical, morphological properties, and high strength etc. Application in the field of commodity and industry product: Packaging materials, fiber and nonwoven, engineering plastic, disposable ware, biodegradable HMAs are prepared mainly based on biodegradable polyester (e.g., PLA, PCL, PHBV and PEA) and natural polymers (e.g., starch etc.). The Field of biomedical materials: Tissue engineering scaffold, controllable drug delivery, PLA used for surgical sutures, ideal filler for soft tissue augmentation, mesh insertion for groin hernia repair, etc. (Ren J. 2010). They have potentially large and the recent huge growths of the PLA market further enhance the demands on lactic acid considerably.

1.11 The past, current and future industrial production of lactic acid and values

In the past, efficient and economical technologies for the recovery and purification of lactic acid from crude fermentation broths and the conversion of lactic acid to the chemical or polymer intermediates had been the key technology impediments and main process cost centers. The process economics of some of these processes and products can also be quite attractive. The recent technical advances in lactic and polyactic acid processes are discussed on the economic potential and manufacturing cost estimates of several products and process options are presented.

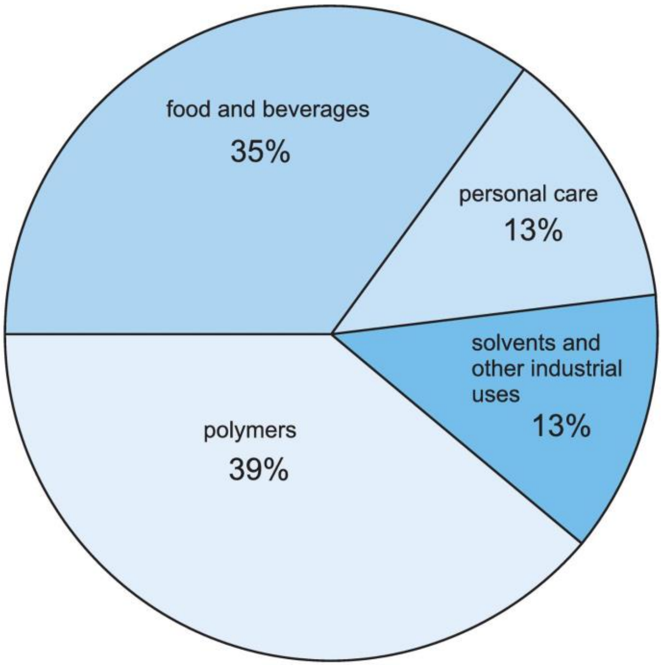

Figure 1.6 Valuable uses of lactic acid

Source: http://www.essentialchemicalindustry.org

The global market for lactic acid, largely immune to the recent turmoil in the global economy, is presently experiencing steady growth. Volume growth, achieved by rise in demand from existing end use markets and emergence of new product applications, is expected to continue driving growth for the next few years. Lactic acid, with a variety of purity levels, is used in food, beverages, cosmetics, pharmaceutical, biodegradable plastics and other chemical sectors. Over the last few years, demand for lactic acid in industrial applications has far surpassed the demand in food and beverages market. Industrial application market is set to become one of the largest consumers of lactic acid and is expected to consume over half of the world lactic acid production in the near future.

Annually, about 275,000 tonnes of lactic acid are produced globally. The worldwide demand of lactic acid, its production should increase significantly over the coming years mainly to provide the polylactic acid manufacturing sites, and is expected to reach 259,000 metric tons in 2012 (Iqbal et al., 2012). The Global Industry Analyst Inc. announced in January 2011 that the global market for lactic acid is forecast to reach approximately 329,000 metric tons by the year 2015 (Fabio et al., 2013).

Lactic acid ranks among the high-volume chemicals produced microbially, with an annual world production volume in the range of 370,000 MT. World lactic acid production has expanded 10-fold in the last decade due, in large part, to increased demand for green products derived from lactic acid, including ethyl lactate and polylactic acid (PLA). In order for these products to be competitive in the marketplace, their cost of production must be minimized. Research efforts to improve the economics of lactic acid production have most recently focused on genetic engineering of yeast to produce lactic acid at low pH while at the same time maintaining high rates and yields of production. Many yeasts offer advantages over the traditional bacterial strains, including the ability to grow rapidly on simple defined salt media and the lack of susceptibility to phage infections - features that contribute to a more robust and cost-competitive process. Yeasts also offer the advantage of tolerance to low-pH conditions, which allows the process to avoid costs associated with fermentation neutralization and downstream acidification. Metabolic engineering of yeast has been utilized successfully by several groups to block ethanol formation and replace the metabolic outlet with a lactic acid production pathway. Cargill has taken this approach farthest by engineering and improving lactic acid production in proprietary yeast all the way to commercially feasible rates, yields, and titers, then developed a process and implemented it at a commercial scale (Miller et al., 2011).

Lactic acid has the potential of becoming a very large volume, commodity chemical intermediate produced from renewable carbohydrates for use as feedstocks for biodegradable polymers, oxygenated chemicals, plant growth regulators, environmentally friendly ‘green’ solvents, and specialty chemical intermediates.

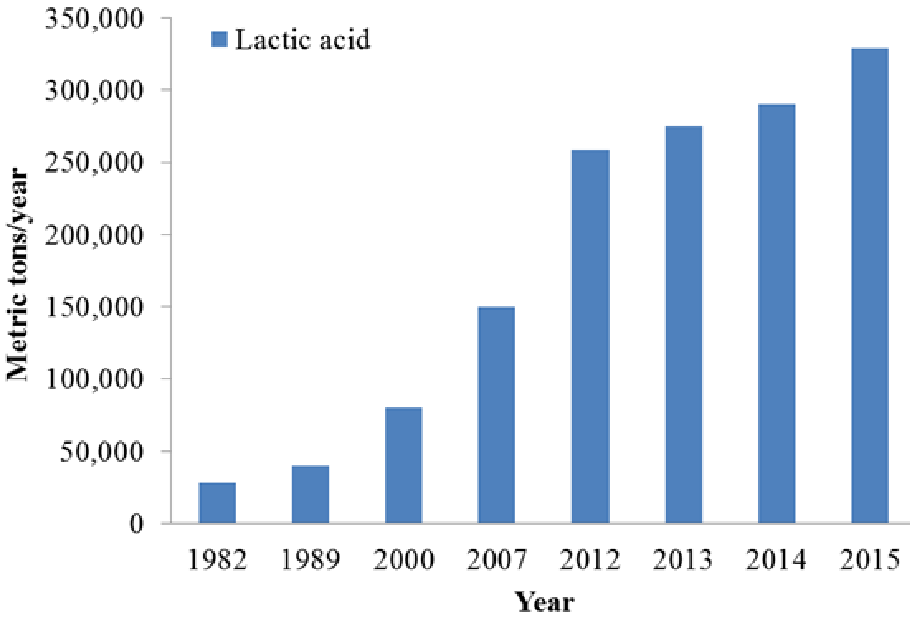

Figure 1.7 Potentially large and the global market for lactic acid.

Source: Chahal S.P. 1989; Iqbal et al., 2012; Fabio et al., 2013.

Lactic acid, the most widely occurring hydroxycarboxylic acid, is an enigmatic chemical. It was discovered a long time ago and its use in food preservation and processing and as a specialty chemical has grown over the years with current production of about 300,000 t/year. Its potential as a major chemical feedstock derived from renewable carbohydrates by sustainable technologies, to make plastics, fibers, solvents and oxygenated chemicals had also been recognized. Recently, new biotechnologies have emerged that can overcome major barriers in separations and purification and processing.

Food & Beverages Industry constitutes a major market, driving growth in the lactic acid market. China as well as other Asian countries is expected to display strong demand for lactic acid in food and beverages, as the acid would be largely used in various local-made food items, pH adjusters and food fortifiers. Use of lactic acid in various personal care and pharmaceutical products such as intravenous solutions, moisturizers, shampoos, anti-aging alpha-hydroxy skin creams and soaps would continue to be on the rise in the matured as well as emerging nations. The United States represents the largest region for lactic acid worldwide, as stated by the new market research report on lactic acid. Growth-wise, Europe is projected to be the fastest growing regional market for lactic acid, with a Compounded Annual Growth Rate (CAGR) of more than 8.5% over the analysis period. In terms of end-use, food additives constitutes the largest application area for lactic acid globally. On the other hand, lactic acid production represents the fastest growing end use application. With demand for lactic acid expanding at the cost of conventional polymers on counts of environmental friendliness, easy recyclability and cost effectiveness, emergence of new lucrative opportunities are portended for lactic acid consumption in the coming years.

Another major trend in the lactic acid market includes the emergence of polylactide (PLA) as a key biodegradable polymer. PLA, a chemical product manufactured using lactic acid as the primary feedstock, is an important raw material for the production of biodegradable plastics. Although presently still a niche market, biodegradable plastics is expected to merge into the mainstream market in the upcoming years, driven largely by rising oil prices and stringent government regulations and greater consumer interest towards the use of greener products. Lactic acid market is expected to benefit significantly from the backlash arising out of growing environmental pollution caused by plastic disposal, escalating costs of petroleum feedstock and wide scale availability of naturally occurring cheap feedstock. The raw material cost for the fermentative production of lactic acid usually accounts for 68% of total manufacturing cost. Thus, cost cheap renewable material is set to become one of the most promising use markets for lactic acid. As a result, leading lactic acid manufactures worldwide are engaged in developing various new and innovative PLA technologies with an intention to benefit from this growing market.

CHAPTER II

Isolation and characterization of lactic acid producing strain *P. acidilactici* DQ2

Abstract

P. acidilactici DQ2 isolated from the corn stover slurry used for ethanol fermentation was studied. This strain had 16S rDNA sequence of the isolated LAB strain *P. acidilactici* DQ2 containing 1640 nucleotides. The result shows that the 16S rDNA sequence of the isolated bacterium had a 99.9% similarity to that of *P. acidilactici*. *P. acidilactici* DQ2 is a gram-positive coccus that is facultatively anaerobic. Biside, effect of inhibitors on lactic acid productivity of *P. acidilactici* DQ2 were investigated in optimal fermentation condition at 42 ^{o}C, 150 rpm and the pH was controlled by addition 12 g/L of $CaCO_3$. A lactic acid bacterium with high tolerance of temperature and lignocellulose derived inhibitor was isolated and characterized as *Pediococcus acidilactici* DQ2.

2.1 Introduction

Lactic acid bacteria LAB consist of the Gram-positive genera: *Carnobacterium, Enterococcus (Ent), Lactobacillus (Lb), Lactococcus (Lc), Leuconostoc (Leu), Oenococcus, Pediococcus (Ped), Streptococcus (Str), Tetragenococcus, Vagococcus,* and *Weissella* (Stiles and Holzapfel, 1997). Most LAB are facultatively anaerobic, catalase negative, nonmotile and nonspore forming (Hofvendahl and Bärbel, 2000). *Pediococcus* spp. are Gram-positive cocci belonging to the group of lactic acid bacteria (Carr et al., 2002). In that, most commonly, strains of *Pediococcus acidilactici* and *P. pentosaceus* have been reported to produce bacteriocins (Anastasiadou et al., 2008) were used as a starter culture. Currently, 12 species of *Pediococci* are found in some cheeses as adventitious cultures, and are occasionally used in the fermentation of milk and during cheese manufacture (Holland et al., 2011). *Pediococci* are often found living in association with plant material, dairy products, and foods produced by LAB. Candidates for this genetic area in bacteria included the genes that code for the 5S, the 16S (also called the small subunit), and the 23S rRNA and the spaces between these genes.

The part of the DNA now most commonly used for taxonomic purposes for bacteria is the 16S rRNA gene. In addition, molecular techniques for identification of *Pediococcus* have been described, including DNA target probes, ribotyping, randomly amplified polymorphic DNA PCR (Simpson et al., 2002). 16S ribosomal RNA currently represents the most important target of study in bacterial ecology genomes to explore the variability of 16S rRNA sequences and copy numbers at various taxonomic levels and apply it to estimate bacterial genome and DNA abundances, (Větrovský and Baldrian, 2013). Whereas 16S rRNA gene sequence data can be used for a multiplicity of purposes (Janda and Sharon, 2007).

Lactic acid fermentation of other carbon sources to lactic acid is affected by the different substances present in the cultivation media. To achieve fully the utilization of biomass, a concentration of sugars (glucose, fructose, and xylose) is desirable in all lactic acid processes, to have a higher concentration of lactic acid. However, concentrations of toxins inhibit the lactic acid fermentation process. Furthermore, concentration of the various sugars available in most of the biomass such as corn stover, wheat straw, barley straw, and molasses can also produce high productivity of lactic acid by the fermenting microorganisms.

The choice of pretreatment and/or hydrolysis of lignocellulosic materials can lead to a number of severe inhibiting compounds, including furans, carboxylic acids, and phenolic compounds. The inhibition effects of all these components can be reduced or removed by choosing a suitable concentration of the substrates and choosing a proper method for the pretreatment and/or hydrolysis steps to reduce the inhibitors. If the toxic compounds follow the culture, detoxification could be an option, although it has some expenses and might lead to partial loss of the sugars. However, choosing the right LAB for the fermentation and a tolerant organism or an organism that can convert the inhibitors, might prevent the need for prior detoxification.

The aim of this study is determine the relative sensitivity of lactic acid producing bacteria from the Man, Rogosa and Sharpes (MRS) medium-derived fermentation inhibitors representing weak acids, furans and phenolics as valinin. A further aim was to measure the lactic acid production of select organisms on lignocellulosic-derived monosaccharides, xylose, glucose, and fructose.

2.2 Materials and Methods

2.2.1 Isolation of lactic acid bacteria

The original lactic acid bacteria (LAB) strain was isolated from the corn stover slurry used for ethanol fermentation. Ten grams of the biodetoxified corn stover samples were diluted with 90 ml of sterilized water and incubated for 2 h at 37 ^{o}C and 150 rpm to obtain the 1 x 10^{-1} suspension. The suspension was further diluted into 10^{-2}, 10^{-3}, and 10^{-4} suspensions. The 10^{-4} dilution was streaked onto the Lysogeny Broth (LB) agar and the simplified MRS gels for enrichment culture (Fig. 2.1). The culture was incubated for 3 days at 25, 37 and 42

^{o}C, respectively under anaerobic conditions, then the colonies were re-streaked onto the YPD and the simplified MRS gels based on the morphology of the colonies. The single colony was isolated, restreaked and transferred for five times to obtain the purified single colony. The single colony was inoculated onto the YPD medium and the simplified MRS medium for lactic acid fermentation, respectively.

The stock cultures were maintained at -80 ^{o}C freezer in the simplified MRS broth containing 30% (v/v) glycerol solution. One stock vial was inoculated into the simplified MRS broth and cultured at 42 ^{o}C, 150 rpm for 12 hours for activation and seeds culture. The culture was carried out in a 250 mL flask containing 50 mL simplified MRS medium with 10% (v/v) inoculation ratio.

The simple MRS (w/v): 2.0% glucose; 1.0% peptone; 0.4% yeast extract; 0.5% sodium acetate trihydrate; 0.2% dipotassium hydrogen phosphate; 0.2% triammonium citrate; 0.02% magnesium sulfate heptahydrate; 0.005% manganese sulfate tetrahydrate; 1.0% agar. YPD (Yeast Extract Peptone Dextrose) (g/L): 20 glucose; 10 yeast extract; 10 Peptone; 15 agar. LB (Lysogeny Broth) (g/L): 10 tryptone; 5 yeast extract; 10 NaCl; 15 agar.

2.2.2 DNA extraction

DNA was extracted using the E.Z.N.A Bacterial DNA Kit (Omega Biotek, Norcross, GA, USA). The DNA was stored at -20 ℃ for subsequent use in experiments. The DNA was analyzed by 1% (w/v) agarose gel electrophoresis at a constant voltage of 75 V for 60 min until the methylene blue dye reached approximately 10 mm from the base of the gel.

2.2.3 PCR amplification of 16S rDNA gene

PCR amplification was performed on an Eppendorf-Mastercycler (Eppendorf, Germany). Primers used to aplify the 16S rRNA gene sequences are the universal primers TH1f (5’-AGAGTTTGATCMTGGCTCAG-3’) and TH1r (5’-ACGGCTA CCTTGTTACGACTT-3’). The reaction mixture of 50 μL consisted of 1 μL of genomic DNA, 5 μL of 10X *Pfu* Buffer with $MgSO_4$, 5 μL of dNTP, 1 μL of *Pfu* DNA polymerase. PCR amplification was done by initial denaturation at 90 ℃ for 30 second, annealing at 49 ℃ for 30 second, and extension at 72 ℃ for 2 min for 25 cycles, followed by a final extension step at 72 ℃ for 10 min, and then held at 4 ℃.

2.2.4 Agarose gel electrophoresis and Purification of PCR product

10 μl of the reaction mixture was then analyzed by submarine gel electrophoresis using 1.0 % agarose with ethidium bromide at 85 V and the reaction product was visualized under Gel doc/UV trans-illuminator. After agarose gel electrophoresis, the PCR products were

purified using the DNeasy procedure to purify DNA from Gram-positive bacteria according to the manufacturer's instructions. The DNA fragment was shown in Fig. 2.2.

2.2.5 DNA sequencing of 16S rRNA fragment

The 16S rRNA amplified PCR product (100ng concentration) was used for the sequencing with the single 16S rDNA TH1f (5'-AGAGTTTGATCMTGGCTCAG-3') by Sangon biotech Co., Ltd. (Shanghai, China).

2.2.6 Determination of phylogenetic relationships

The 16S rRNA gene sequences were blasted in the NCBI GenBank and the phylogenetic tree was constructed based on the neighbor-joining method using Bioedit 7.0 (http://www.mbio.ncsu.edu/bioedit/bioedit.html) and Mega 4 (http://www.megasoftware.net/).

2.2.7 Cell morphology and Gram stain

Gram staining was carried out according to the routine procedure, and cell morphology was examined by light microscopy.

In the Gram stain, the cells are first heat fixed and then stained with a basic dye, crystal violet, which is taken up in similar amounts by all bacteria. The slides are then treated with an I_2-KI mixture (mordant) to fix the stain, washed briefly with 95% alcohol (destained), and finally counterstained with a paler dye of different color (safranin).

2.2.8 Optimal temperature for microbial growth development

The microbial growth was performed under anaerobic conditions for 32 h. Inoculate flasks with a loopful of the test organism into the MRS broth at a different temperature: 38 ^{o}C, 42 ^{o}C, 44 ^{o}C, 46 ^{o}C, 48 ^{o}C, 50 ^{o}C, 52 ^{o}C. The Beckman coulter spectrophotometer DU 800 was applied. Experiments were carried out in 250 ml flasks containing 50 ml of medium.

2.2.9 Effect of nutrient concentration on the growth and lactic acid concentration

Experimental design: The effects of sugar concentration 10 g/L of Glucose; 10 g/L of Fructose; 20-70 g/L of Xylose. The effect of sodium acetate of different volumes (g/L): 0; 2; 4; 6; 8; 10. The sugar utilization for lactic acid production was also carried out in the same way at 42 ^{o}C, 150 rpm for 48 hours in simplify MRS medium.

2.2.10 Effect of inhibitors on lactic acid production by *P. acidilactici* DQ2

5-Hydroxymethylfurfural (HMF) (g/L): 0-0.5-1.0-2.0-3.0; Furfural (g/L): 0-0.25-0.5-1.0-2.0; Acetic acid (g/L): 0-1-2-4-6-8; Formic acid (g/L): 0-0.25-0.5-0.75-1.0-1.5-2.0; Levulinic acid (g/L): 0-0.5-1-2.5: Vanillin (g/L): 0-0.25-0.5-1.0. Effect of inhibitors were carried out at 42 ^{o}C, 150 rpm for 24 h in simplify MRS medium.

Samples were autoclaved at 115 ^{o}C for 20 minutes. All culture was carried out in a 250 mL flask containing 50 ml of medium, with shaking (150 rpm, HZ-2111 K-B, Shaking incubator, Hualida Laboratory Equipment Co., Ltd., China) in anaerobic condition. The pH was controlled by addition 60 g of $CaCO_3$ per 100 g of glucose. All experiments were done in triplicate.

2.2.11 Analytical methods

Glucose, fructose, xylose, lactic acid and inhibitors were analyzed using HPLC (LC-20AD, refractive index detector RID-10A, Shimadzu, Japan) with a Bio-Rad Aminex HPX-87H column at 65 ^{o}C. The mobile phase setup 5 mM H_2SO_4 with the rate of 0.6 mL/min. All samples were diluted and filtered before analysis.

2.3 Results and discussion

2.3.1 Isolation and characterization of *Pediococcus acidilactici* DQ2

Differential characteristics of lactic acid bacteria based on morphology and physiology determined from various microbial and analytical confirmation testing is shown in rod shape, the best available microscopic results of the experiments are shown in Fig. 2.1. Illustrated in this image is the Gram stain of Bacteria, illustrating gram-positive cocci arranged in pairs and short chains. In Fig. 2.1 shown these strains have round colonies with cream coloring. Thus Gram-positive organisms retain the initial violet stain, while gram-negative organisms are decolorized by the organic solvent and hence show the pink counterstain. The difference between gram-positive and gram-negative bacteria lies in the ability of the cell wall of the organism to retain the crystal violet.

The sequence data for the 16S rRNA gene is highly conserved for different organisms and has also been shown to be very accurate for genus and species identification of eubacteria (Barney et al., 2001). Some isolates from forage crops and silage have been identified as *P. acidilactici* and *P. pentosaceus* (Cai et al., 1999). The corn stover slime producing used for ethanol fermentation isolate had 16S rDNA gene sequence homology to the *P. acidilactici* reference strain. Although 16S rDNA gene sequencing correctly identified the genus and species of the test *Pediococcus acidilactici* isolates.

The present study demonstrated that the universal primers are a very useful tool for identifying corn stover slurry *Pediococcus* isolates. 16S ribosomal DNA sequencing of a number of isolates showed that *P. acidilactici* isolates. The neighbor-joining method gave the correct genus and species and also allowed the subspeciation of many strains. The 16S rDNA sequence analysis identified it as *P. acidilactici*. The sequence analysis method was very good at identifying the organisms by genus and species. Fig. 2.2 shows the original gel from which bands were excised which sequencing was attempted.

However, the phenotypic procedures to assign isolates to known species are difficult, because it is hard to differentiate readily between species of *Pediococcus pentosaceus* and *Pediococcus acidilactici* (Simpson et al., 2002; Cai et al., 1999).

The 16S rDNA fragment of the isolated LAB bacterium was amplified using the universal primers TH1f and TH1r, where the sequence was shown (in Table 2.1) 16S rDNA sequence of the isolated LAB strain *P. acidilactici* DQ2 containing 1640 nucleotides. The sequence was blasted in the NCBI genebank and the phylogenetic tree was constructed as shown in Fig. 2.3 Phylogenetic tree of 16S rDNA sequence. Morphological, physiological and biochemical identification suggest that all of isolates belong to lactic acid bacteria. The result shows that the 16S rDNA sequence of the isolated bacterium had a 99.9% similarity to that of *P. acidilactici*, thus the isolated LAB strain was designated as *P. acidilactici* DQ2 based on the analysis. *P. acidilactici* DQ2 is a lactic acid bacterium (LAB) strain, but rarely used for production of lactic acid (Okano et al., 2010). Instead, the strain was usually used for production of bateriocin, an antibiotic for animal feed use. Currently, the *P. acidilactici* DQ2 strain is in the process of the registration into the China General Microorganism Collection Center (CGMCC, Beijing, China) and it will be open to the academic community after the registration.

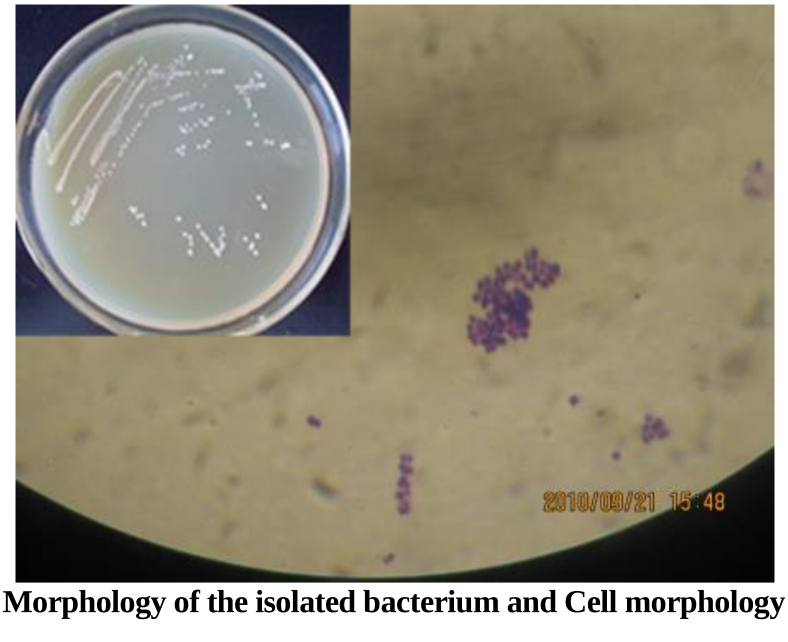

Figure 2.1 Morphology of the isolated bacterium and Cell morphology and Gram stain

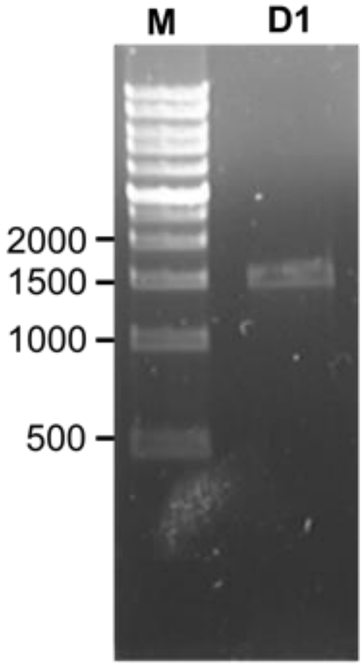

Figure 2.2 Agarose gel electrophoresis of DNA fragment.

M: Wide range DNA Marker 500-12.000 bp; D1: DNA fragment

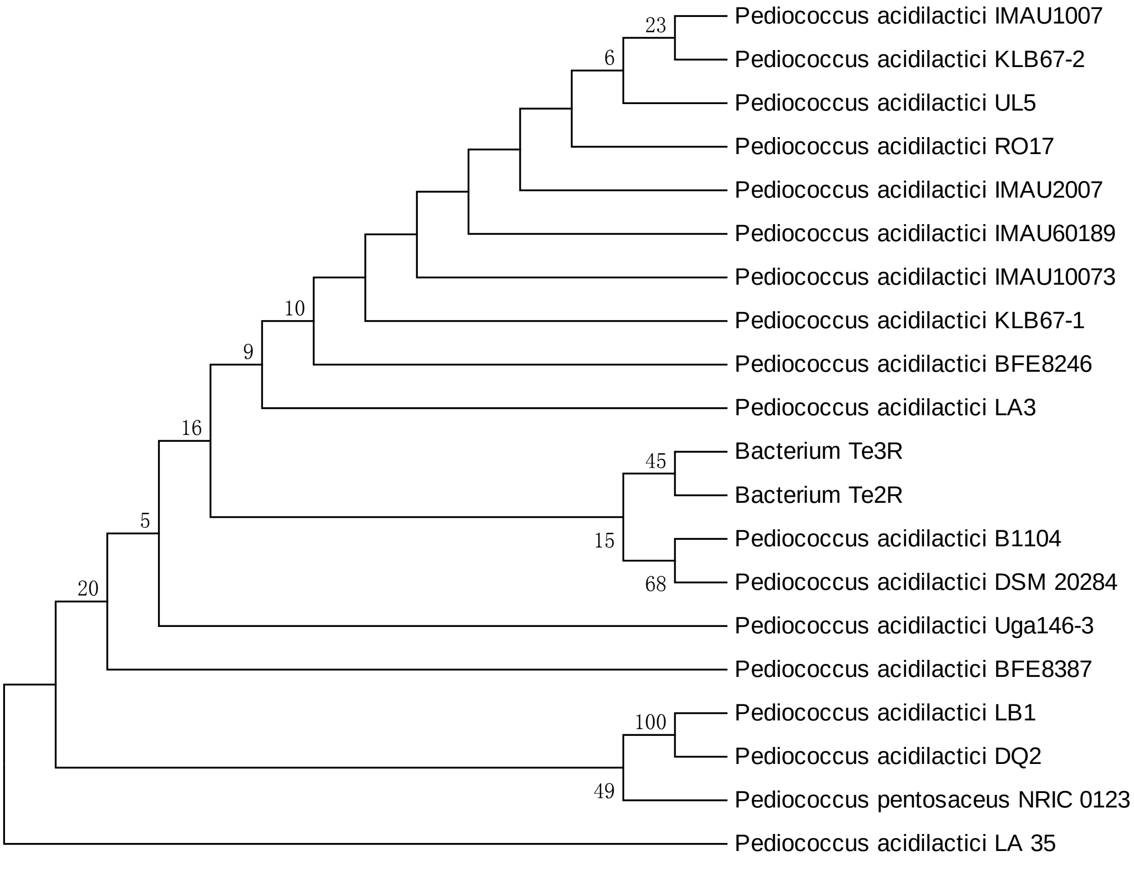

Figure 2.3 Phylogenetic tree of 16S rDNA sequence

Table 2.1 16S rDNA sequence of the isolated LAB strain *P. acidilactici* DQ2

```
GGGCGGCGGCAGTGATTCGAGCTCGGTACCCGGGGATCCTCTAGAGATTACGGCT
ACCTTGTTACGACTTCACCCTAATCATCTGTCCCACCTTAGACGGCTAGCTCCTAA
AAGGTTACCCCACCGGCTTTGGGTGTTACAAACTCTCATGGTGTGACGGGCGGTG
TGTACAAGGCCCGGGAACGTATTCACCGCGGCATGCTGATCTGCGATTACTAGCG
ATTCCGACTTCGTGTAGGCGAGTTGCAGCCTACAGTCCGAACTGAGAATGGTTTT
AAGAGATTAGCTAAACCTCGCGGTTTCGCAACTCGTTGTACCATCCATTGTAGCA
CGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTCGTCCCCACCTTCC
TCCGGTTTGTCACCGGCAGTCTCACTAGAGTGCCCAACTGAATGCTGGCAACTAG
TAATAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTG
ACGACAACCATGCACCACCTGTCATTCTGTCCCCGAAGGGAACGCCTAATCTCTT
AGGTTGGCAGAAGATGTCAAGACCTGGTAAGGTTCTTCGCGTAGCTTCGAATTAA
ACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCTTTTGAGTTTCAACCTT
GCGGTCGTACTCCCCAGGCGGATTACTTAATGCGTTAGCTGCAGCACTGAAGGGC
GGAAACCCTCCAACACTTAGTAATCATCGTTTACGGCATGGACTACCAGGGTATC
TAATCCTGTTCGCTACCCATGCTTTCGAGCCTCAGCGTCAGTTACAGACCAGACA
GCCGCCTTCGCCACTGGTGTTCTTCCATATATCTACGCATTTCACCGCTACACATG
GAGTTCCACTATCCTCTTCTGCACTCAAGTCTCCCAGTTTCCAATGCACTTCTTCG
GTTGAGCCGAAGGCTTTCACATTAGACTTAAAAGACCGCCTGCGCTCGCTTTACG
CCCAATAAATCCGGATAACGCTTGCCACCTACGTATTACCGCGGCTGCTGGCACG
TAGTTAGCCGTGGCTTTCTGGTTAAATACCGTCACTGGGTGAACAGTTACTCTCAC
CCACGTTCTTCTTTAACAACAGAGCTTTACGAGCCGAAACCCTTCTTCACTCACGC
GGCGTTGCTCCATCAGACTTGCGTCCATTGTGGAAGATTCCCTACTGCTGCCTCCC
GTAGGAGTCTGGGCCGTGTCTCAGTCCCAATGTGGCCGATTACCCTCTCAGGTCG
GCTACGCATCATCGCCTTGGTGAGCCGTTACCTCACCAACTAGCTAATGCGCCGC
GGGTCCATCCAGAAGTGATAGCAGAGCCATCTTTTAAAAGAAAACCAGGCGGTTT
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AGTGCAAGCACGTCATAATCAATTAACGGAAGTTCGTTCGACTTGCATGTATTAG
GCACGCCGCCAGCGTTCATCCTGAGCCATGATCAAACTCTAATCGTCGACCTGCA
GGCATGCAAGCTTGGCGTAATCATGTCATTGTCCCC
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2.3.2 Optimal *P. acidilactici* DQ2 growth temperatures

Although the technologically important parameters like optimal pH and temperature for industrially used strains are well known, the behavior of the quantitative growth characteristics like growth rate, the production of bacteria based on ATP production with changing pH, temperature and other environmental factors are relatively poorly studied.

In this study: Effect of various temperatures on *P. acidilactici* DQ2 growth was shown in Fig. 2.4, detected growth of this strain within 48 hours of bacterial growth when the pH was control by calcium carbonate. It was apparent to see that *P. acidilactici* DQ2 was well increased from 38 ^{o}C to 46 ^{o}C; cells are similar to that of growth. Specially, at 42 ^{o}C, the number of new cells and the rate of the organism by testing OD600 nm increased with consecutive in short time from 12 to 16 hours and a stationary growth for 24 hours is the highest bacterial growth, slight increase of cells was observed up to 42 ℃ at 44 hours followed by a decrease at 48 hours of bacterial growth. However, starting-growth of this strain at 46 ^{o}C was determined earlier on 8 hours in their log phase but stationary phase result finished from 28 to 44 hours contain of all experimental temperatures (38-52 ^{o}C) then a bacterial growth limiting decreased. This situation could be explained as the depletion of an essential nutrient. Following Adamberg (2003) increase of temperature suggested that ATP production capacity was an important factor in determining the maximum growth rate of lactic acid bacteria. LAB constitute a diverse group of Gram-positive microorganisms and the optimal growth conditions vary depending on the producers, since these bacteria can grow in the temperature of 5-45 ℃ (Mohamed Ali et al., 2013).

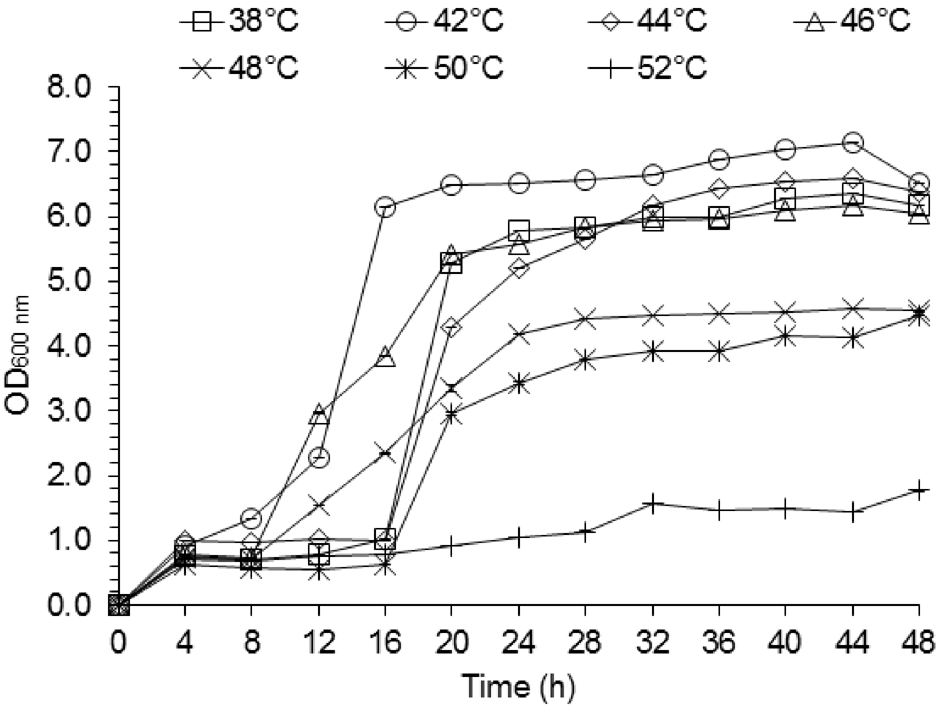


Figure 2.4 Effect of various temperatures on *P. acidilactici* DQ2 growth

2.3.3 Effect of sodium acetate concentrations on yield of lactic acid

In the lactic acid fermentation most sodium acetate substrate is added in the MRS medium. Figure 2.5 show effect of sodium acetate concentrations on yield of lactic acid by *P. acidilactici* DQ2, was observed different 0-10 g/L of sodium acetate concentrations to find out optimal the highest yield of lactic acid in the anaerobic fermentation.

The results of lactic fermentation indicated that effect of alternative sodium acetates contents is obvious in comparison of without added this substrate. Specially, it also increased content of cells when presented in MRS medium. It is the same synonym with increasing lactic acid yield. Takao (2003) indicated that the specific activity of LDHs (the total activity of L-LDH plus D-LDH) from the cells cultivated in the presence of sodium acetate, the amount of LDHs in the cells of *Lactobacillus sakei* NRIC 1071^T cultivated in the presence of 50 mM sodium acetate was much more than that in the cells cultivated in the absence of sodium acetate.

This testing leads to the conclusion that effect of yield of lactic acid on sodium acetate from 4-6 g/L and close to 1.0 of conversion of 1 Mold of glucose produce 2 Mold lactate by *P. acidilactici* DQ2, The stimulatory effect of sodium acetate on growth of lactic acid bacteria was also demonstrated and significantly. Beside, to improve cutting down cost of chemical substrates to decrease price on lay out of lactic acid production. Despite all sodium acetate concentrations test, the results in the lactic fermentation indicated that high both cells and yield of lactic acid oscillate from 4-6 g/L of sodium acetate.

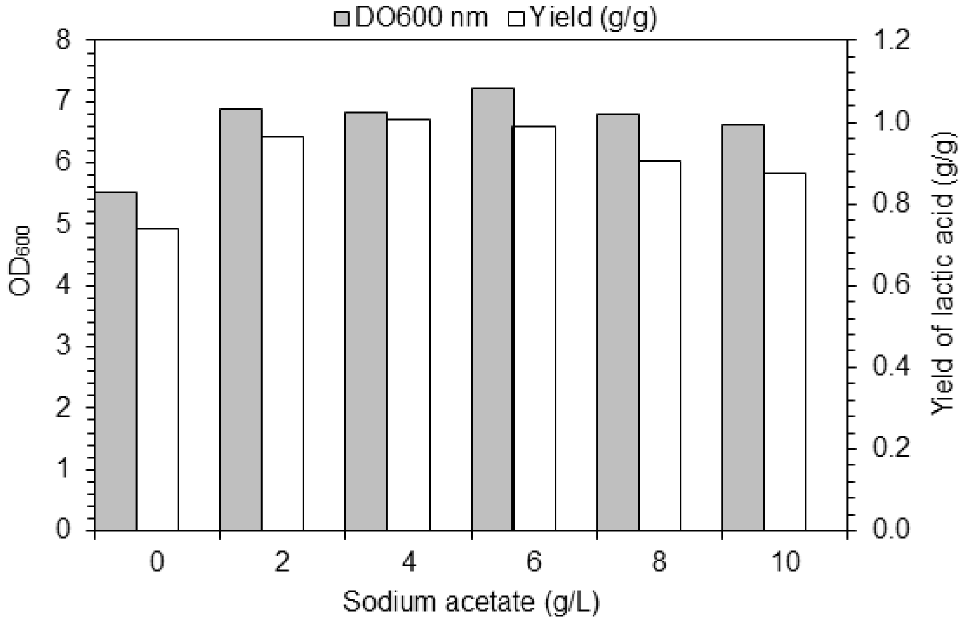


Figure 2.5 Effect of sodium acetate concentration on yield of lactic acid *by P. acidilactici* DQ2

2.3.4 Utilization of glucose, fructose, and xylose by the strain DQ2 and its lactic acid production

P. acidilactici DQ2 was observed with different initial concentrations of sugars, such as glucose, fructose, and xylose to serve as sole carbohydrates sources were used for lactic acid production. Fig. 2.6 shows that effect of utilization of glucose, fructose concentration by *P. acidilactici* DQ2, and its production of lactic acid. The results show that strain DQ2 utilized speacial sugars (glucose and fructose) to convert them into production of lactic acid with the highest lactic acid concentration was 9.73 g/L and 7.53 g/L and lactic acid yield was 0.98 g/g and 0.77 g/g, respectively within 48 hours at 42 ^oC, 150 rpm and pH was controlled by addition 12 g/L of $CaCO_3$. The result implies that *P. acidilactici* DQ2 could metabolize both of glucose and fructose into only lactic acid by the homo-fermentation pathway through the

Embden-Meyerhof-Parnas pathway (EMP, or glycolysis), which 1 mole of glucose should produce 2 moles of lactic acid (Gottshalk, 1986).

The utilization of xylose by *P. acidilactici* DQ2 is shown in Fig. 2.7. No limitation with xylose concentration was observed of 20 g/L, 30 g/L, 40 g/L, 50 g/L, 60 g/L, 70 g/L, and the highest lactic acid concentration of 0.19 g/L was obtained at the xylose concentration of 70 g/L. In Fig. 2.7, results indicated that carbon source as xylose could ferment to lactic acid by this strain DQ2 but its lactic acid production is very low. In xylose fermenting bacteria, xylose is firstly converted into xylulose-5-phosphate, metabolized through either the pentose phosphate pathway, or through the phosphoketolase pathway converted to fructose-6-phosphate and glycoeraldehyde-3-phosphate (Wang et al., 2010a). Overall, the profiled metabolites were similar in concentration at the same MRS medium and anaerobic fermentation conditions. Figure 2.8 showed production of lactic acid by *P. acidilactici* DQ2 using xylose in the time course of lactic fermentation. However, profiles of metabolites involved in the glycerol pathway did show significantly lower intracellular concentrations in Fig. 2.8.

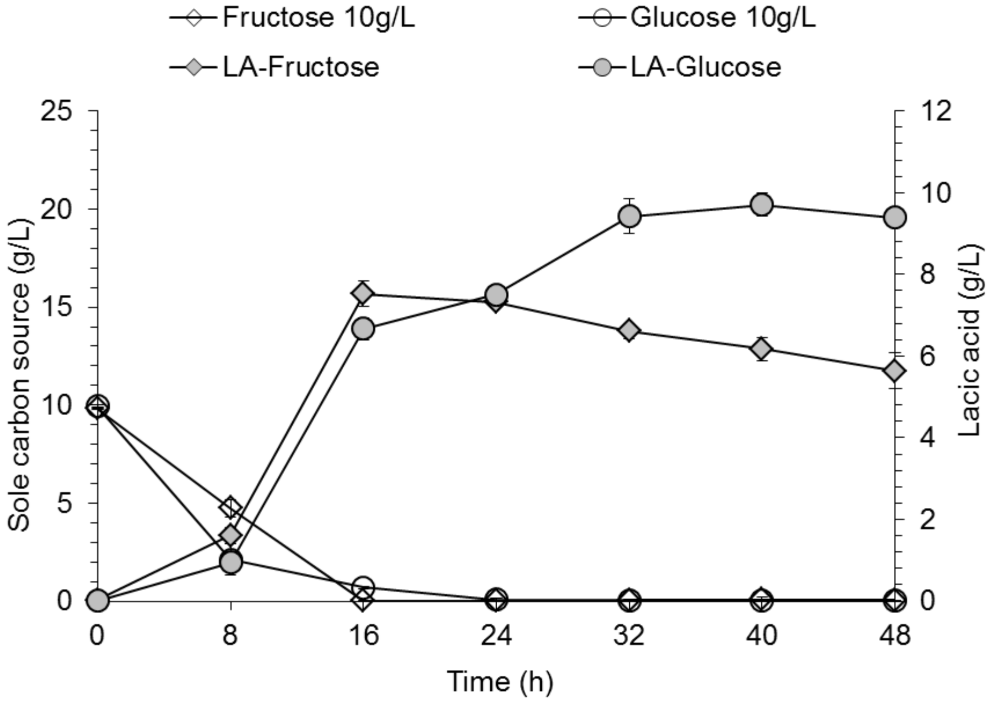


Figure 2.6 Effect of utilization of glucose, fructose concentration by *P. acidilactici* DQ2, and its production of lactic acid in homolactic fermentation condition

Some strains can utilize the pentoses present in xylose (a five carbon aldose) such as *Lactobacillus plantarum, Enterococcus mundtii,* some *Bacillus species*, including *Bacillus coagulans, Bacillusstearothermophilus, Bacillus licheniformis, Bacillus* sp. Strain XZL9, has also been reported (Okano et al., 2010; Abdel-Rahman et al., 2011; Wang et al., 2010b). From these results of xylose fermentation is not signification in the industrial production of lactic acid by *P. acidilactici* DQ2.

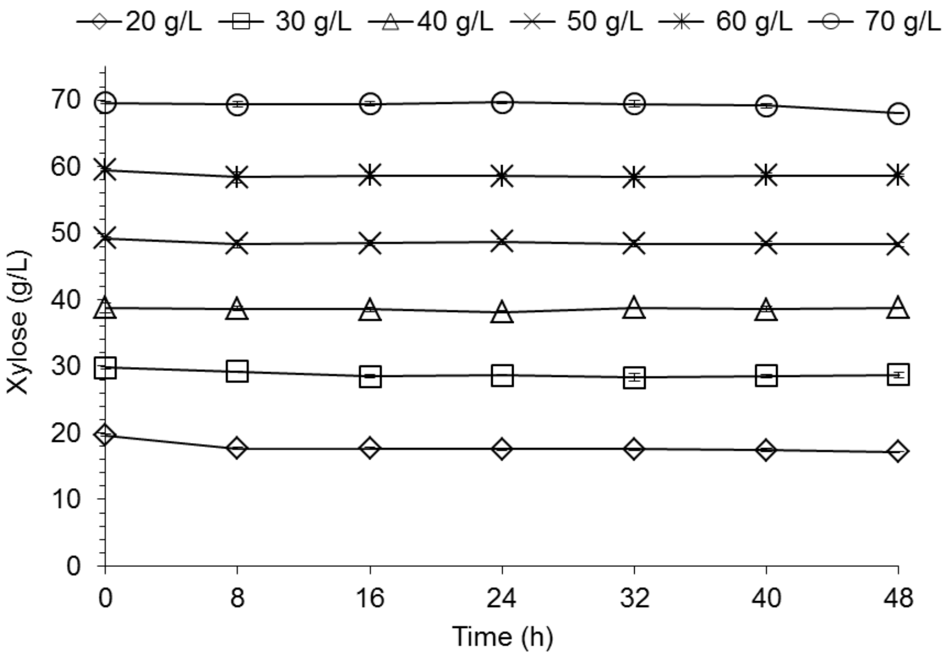


Figure 2.7 Time course of xylose consumption by *P. acidilactici* DQ2

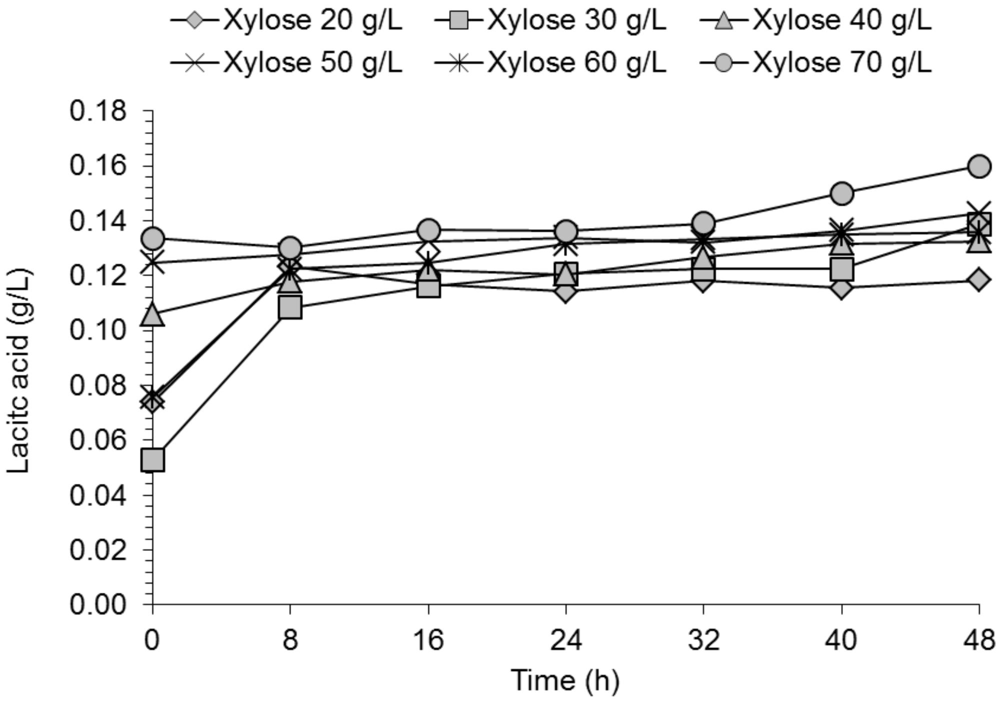


Figure 2.8 Production of lactic acid by *P. acidilactici* DQ2 using xylose

2.3.5 Effect of initial single inhibitors on lactic acid productivity

The generation of by products from the pretreatment is strongly dependent on the feedstock and the pretreatment method. Substances that may act as inhibitors of microorganisms include phenolic compounds and other aromatics, aliphatic acids, furan aldehydes, inorganic ions, and bioalcohols or other fermentation products.

Inhibitory compounds in lignocellulose hydrolysates comprise aliphatic acids, furaldehydes, aromatic compounds, and extractives. Aliphatic acids, such as acetic, formic and levulinic acid are formed as degradation products from carbohydrates. Furaldehydes, such as furfural and 5-hydroxymethylfurfural (HMF), are also derived from carbohydrates. A variety of different aromatic compounds, primarily phenolics, formed by the partial breakdown of lignin are also present (Alriksson et al., 2010).

Fermentation experiments were performed to evaluate the effectiveness of the substantial additions. For comparison, inhibitors were added, gave rise to pretreating lignocellulose hydrolysates, in the fermentation experiments as well as reference the growth of LAB with an amount of initial arrangement of toxins. The goals of these experiments find out effectiveness of toxins content on productivity of lactic acid. Fig. 2.9 Inhibitor tolerance of *P. acidilactici* DQ2 to the lignocellulose degradation compounds was investigated.

The results of addition of the furan aldehydes HMF (0-3 g/L) and furfural (0-6 g/L) in the fermenting culture shown that both of them present most effect on growth of cells and lactic acid productivity. Different volume of HMF and furfural decreased the lactic acid production of *P. acidilactici* DQ2 in 24 hours of lactic fermentation. Illustration by Fig. 2.9a shown at 1.5 and 3.0 g/L of HMF, which remarkable decreasing production of lactic acid and cell numbers were produced. Beside small 0.5 (g/L) of furfural, in Fig. 2.9b, also reduced productivity of lactic acid in SSF process.

The impact of HMF and furfural is the most important inhibitors in the lactic acid fermentation and on the anaerobicogy physiology of *P. acidilactici* DQ2 was studied by subjecting the cells to a continuous stress by including the inhibitors. In this experiment, the cells are expected to adapt to the toxins environment, which means that only strain DQ2 required for growth with its lactic acid production in the presence of HMF and furfural are likely to be differentially expressed, in contrast without pulsed cultivations where an instantaneous in comparison of low or high stress response is expected to tolerate HMF and furfural expression at different concentrations in the lactic fermentation.

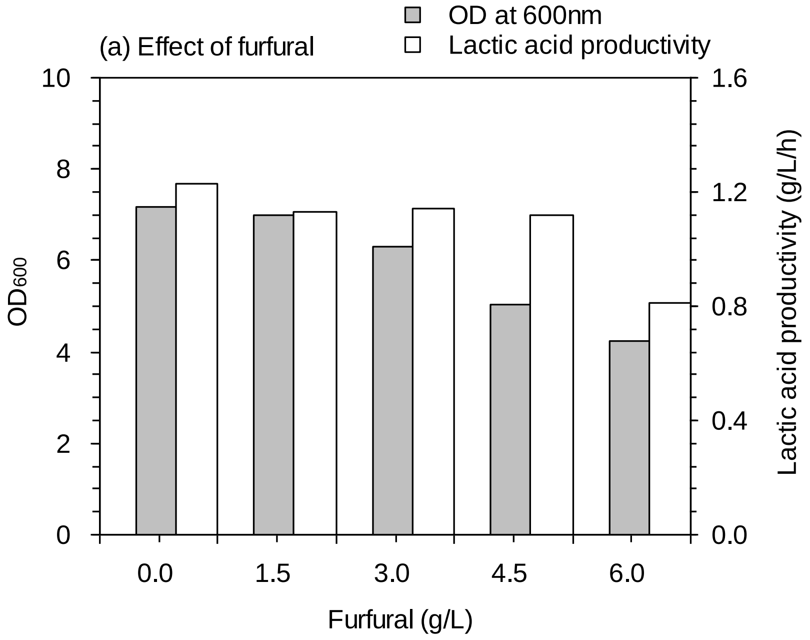
Hence, in the SSF by *P. acidilactici* DQ2 using glucose as a carbon source with the presentation of HMF and furfural compounds to grow and to product lactic acid at high yield, HMF and furfural have to remove or close to zero about concentration under anaerobic conditions. Cell growth, on the other hand, was quite limited. Both the lactic acid productivity and the OD600 nm rate were significantly lower than the values obtained for the cultivations in which only furfural from xylose degradation and HMF from glucose degradation were added (Zhang et al., 2010b).

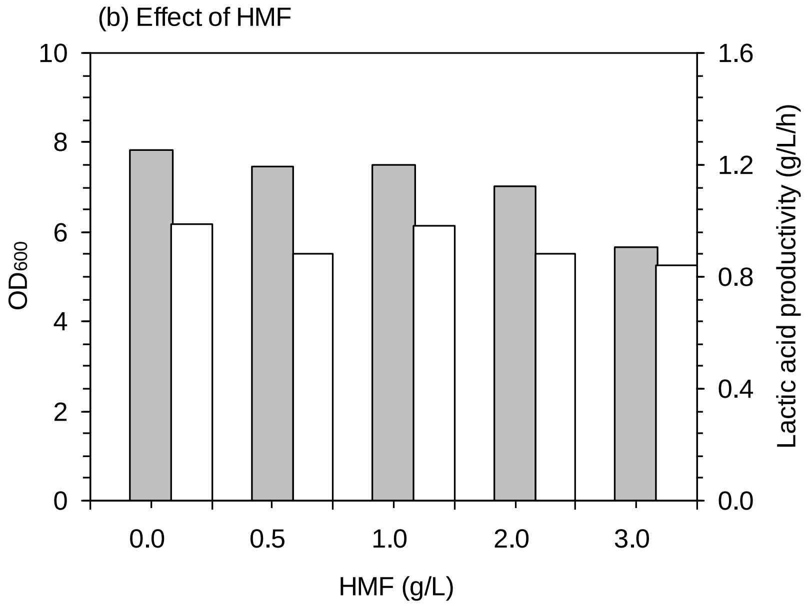
According to Quéméneur (2012), these inhibitors can affect microbial growth with three distinct modes of action: undissociated weak organic acids penetrate microbial cells and decrease the intracellular pH, furfural derivatives interfere with glycotic and/or fermentative enzymes, while macromolecules and phenolic compounds are damaging the microbial cellular membranes. However, weak acids inhibit cell proliferation by uncoupling and intracellular anion accumulation.

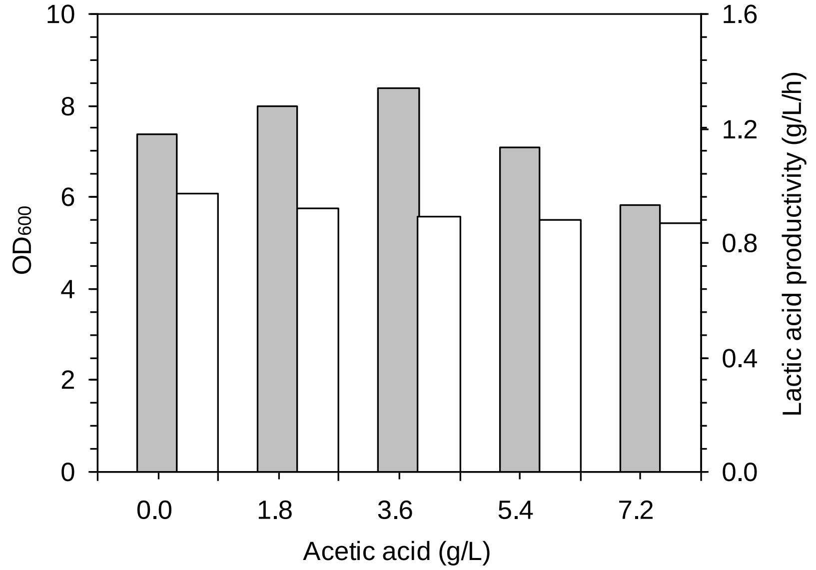
To study the level of inhibitors as weak acid where are produce in the lactic fermentation on of *P. acidilactici* DQ2. Weak acids such as acetic acid, formic acid, and levulinic acid were carried out experiment to determine effect of them on lactic acid productivity of *P. acidilactici* DQ2. Fig. 2.9c, 2.9d, and 2.9e show effect of different acetic acid and formic acid concentration on lactic acid productivity. Acetic acid (0-7.2 g/L) shows that growth of *P. acidilactici* DQ2 was not affected until the acetic acid reached 3.6 g/L, and the lactic acid productivity was affected up to a high level of 7.2 g/L. Acetic acid (3 g/L) were found to be stimulatory about growth of cells rather than other concentrations and formic acid (0-3 g/L) shows both the cell growth and the lactic acid productivity of *P. acidilactici* DQ2 was relatively sensitive to formic acid but still maintained a normal performance to 0.5 g/L of formic acid, which was already a high value in the pretreated lignocellulose, was checked evidence with impact of them to production of lactic acid. Fig. 2.9e indicated that shows that levulinic acid had almost no obvious effect on both the cell growth and the fermenting performance of *P. acidilactici* DQ2 up to 3.2 g/L and under 0.8 g/L of levulinic acid have the stimulus of increasing numerous cells but lactic acid was produced lower than control where without addition of weak acids.

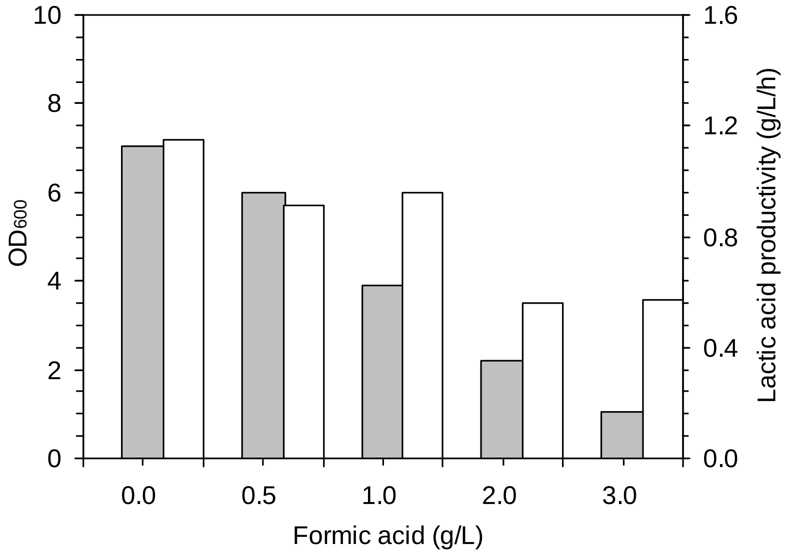
According to Dietrich et al. (2013), aliphatic acids such as levulinic and formic acid result from further degradation of these furan aldehydes, while acetic acid is released by hydrolysis during dilute acid pretreatment of lignocellulosic feedstock. Lactic acid yields, which are defined as the ratio between production of lactic acid and sugars consumption, non-inhibitors were higher than those obtained in the fermentation inhibitors.

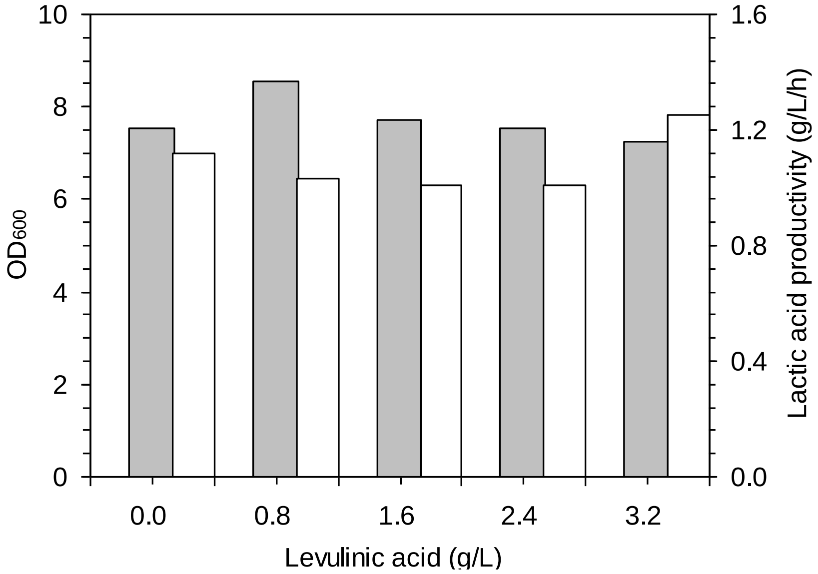
Vanillin is used as a flavouring agent and it is generally regarded as safe (GRAS). *P. acidilactici* DQ2 was relatively sensitive to vanillin in both the cell growth and lactic acid fermentation performance, but when vanillin was below 0.2 g/L, both the cell growth and the lactic acid yield maintained satisfactory. Vanillin or other lignin derivatives were minor in most of the pretreatment processing and the level of 0.2 g/L vanillin was hardly detected in Fig. 2.9f.






(c) Effect of acetic acid


(d) Effect of formic acid


(e) Effect of levulinic acid

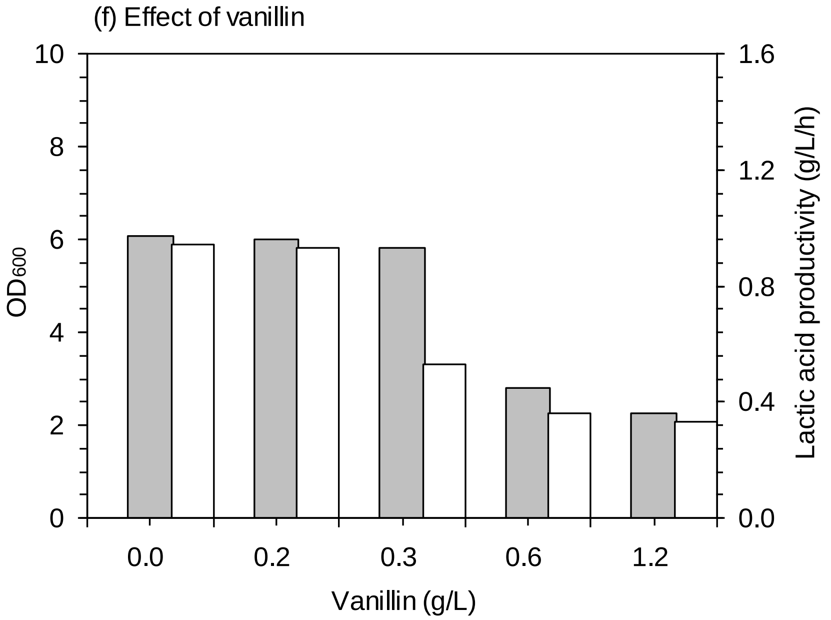


Figure 2.9 Inhibitor tolerance of *P. acidilactici* DQ2 to the lignocellulose degradation compounds

2.4 Conclusions

The DNA sequence based analysis is a central method to understand not only the microbial diversity within and across the group but also to identify new strains. Bacterial species have the 16S rRNA gene containing highly conserved regions together with hyper variable regions, which is used for identification of new strains. However a considerable variation can occur between species in both the length and the sequence of 16S rDNA therefore this region is useful in characterization of bacterial species. We used the 16S rDNA gene sequence to characterize the bacterial isolate *P. acidilactici* DQ2 from corn stover slurry of ethanol fermentation. Accurate and definitive microorganism identification is essential for a wide variety of application to produce lactic acid.

A decrease in the content of lactic acid productivity, accompanied by an increase in the content of toxins compounds, was observed during fermentation with the *P. acidilactici* DQ2 strain, illustrating that this strain was active even at the very low levels of inhibitors supplied. Our results demonstrate the importance of three classes that include furans, aliphatic acids, and phenolics as fermentation inhibitors and the advantage of using *P. acidilactici* DQ2 strains for producing lactic acid production from carbon source as glucose, fructose.

CHAPTER III

Simultaneous saccharification and lactic acid fermentation of Jerusalem artichoke tubers (Jat) using commercial glucoamylase as inulinase enzyme

Abstract

A high inulinase activity was found in three commercially available glucoamylase enzymes. Its origin was investigated and two proteins in the commercial glucoamylases were identified as the potential enzymes showing inulinase activity. The simultaneous saccharification and lactic acid fermentation of Jat was carried out using GA-L New as the inulinase and *Pediococcus acidilactici* DQ2 as the fermenting strain. A high lactic acid titer, yield, and productivity of 111.5 g/L, 0.46 g/g DM, and 1.55 g/L/h, respectively, were obtained within 72 hours. The results provided a practical way of Jat application for lactic acid production using cheap commercial glucoamylase enzyme.

3.1 Introduction

Although current industrial production of lactic acid overwhelmingly uses starch feedstock, future need of lactic acid for biodegradable polylactide acid (PLA) certainly requires various feedstock for replacing petroleum based polymer materials, other than food-based starch only (Nampoothiri et al., 2010; Gao et al., 2011). Among many feedstock options, Jerusalem artichoke tubers (Jat) is a promising one because of its desirable growing traits such as cold and drought tolerance, wind and sand resistance, saline tolerance, strong fecundity, and high pest and disease resistant (Li and Chan, 2009). Jat contains high carbohydrates (20%, w/w), in which 70-90% (w/w) are inulin, a polysaccharide composed of fructose unit chains and minor glucose unit (Szambelan et al., 2005). Jat had been widely planted in North America, Europe, and Asia countries besides its advantages (Chi et al., 2011; Li et al., 2013). In recent few years in China, Jerusalem artichoke started the large scale cultivation in the drought and coast regions as well as in the polluted regions by oil drilling or coal mining to replace the corn and wheat as feedstock of fermentation industry for production of ethanol, butanol, sugar alcohols and organic acids (Li et al., 2013). Although its total quantity is not comparable to lignocellulose biomass, Jat could be processed easily using the available technologies, or even easier than starch processing (Chi et al., 2011; Li et al., 2013), while lignocellulose processing is still a technology challenge and only used in the small scale demonstration stage (John et al., 2009; Zhao et al., 2013). Therefore, Jat has been considered as a readily available feedstock at present and a promising one in the future for industrial lactic acid production.

Jat can be hydrolyzed into fructose and glucose by inulinase enzyme or acid catalyst (Chi et al., 2011). Inulinase includes two enzymes, endo-inulinase (EC 3.2.1.7) cleaving middle sites of inulin oligos into even smaller oligos, and exo-inulinase (EC 3.2.1.80) cleaving fructose or glucose from the ends of inulin oligos. The recent studies showed that invertase (EC 3.2.1.26) from yeast and fungi also demonstrated strong inulin hydrolysis property (Wang and Li, 2013; Guo et al., 2013). However, these inulinase enzymes are expensive and not available as industrial enzyme for Jat hydrolysis at large scale (Sigma Product ID 16285, Novozym 960, $350 for 250mL). Therefore, the cost reduction of inulinase is a key step for industrial application of Jat for production of biofuels and biochemicals.

In this study, a high inulinase activity was found from a typical commercial glucoamylase GA-L New of Genencor produced by *Aspergillus niger* fermentation. The inulin hydrolyzing enzyme(s) in glucoamylase GA-L New were characterized and the results showed that the activity was from glucoamylase itself, other than other protein components in this mixed commercial enzyme, although the catalytic properties of the two enzymes were different (glucoamylase cleaves 1,4-α-D-glucosidic linkage, while inulinase cleaves 1,2-β-D-fructosidic linkage). Then the glucoamylase was used for Jat hydrolysis and a high yield of fructose from Jat was obtained. The simultaneous saccharification and lactic acid fermentation (SSF) of Jat was carried out using glucoamylase GA-L New as the inulinase and *Pediococcus acidilactici* DQ2 as the lactic acid fermenting strain. A high lactic acid titer, yield, and productivity of 111.5 g/L, 0.46 g/g DM, and 1.55 g/L/h, respectively, were obtained within 72 hours. The results provided a practical way of Jat application for lactic acid production using cheap commercial glucoamylase enzyme.

3.2 Materials and Methods

3.2.1 Enzyme and strain

The commercial glucoamylase enzymes include glucoamylase GA-L New from DuPont Genencor Science, Wuxi, China, (http://biosciences.dupont.com/duponttm-genencorr-science/), amyloglucosidase A107823 from Aladdin Industrial Co., Ltd., Shanghai, China (http://www.aladdin-e.com), and a local product, glucoamylase YY0515 from Shanghai Yuanye Biological Technology Co., Ltd., Shanghai, China (www.shyuanye.com). All these glucoamylases were produced using *Aspergillus niger* as the production strain.

The lactic acid fermentation strain, *Pediococcus acidilactici* DQ2 (CGMCC 7471), was isolated in our previous study (Zhao et al., 2013) and stored in China General Microbial

Collection Center (CGMCC), Beijing, China. The simplified MRS medium contained 10 g/L of peptone, 5 g/L of yeast extract, 1 g/L of $(NH_4)_2SO_4$, 2 g/L of KH_2PO_4, 0.58 g/L of $MgSO_4 \cdot 7H_2O$, 0.25 g/L of $MnSO_4 \cdot 4H_2O$, 5 g/L of sodium acetate, and 2 g/L of diammonium hydrogen citrate. The medium was autoclaved at 115 ^oC for 20 min. One vial of *P. acidilactici* DQ2 was inoculated into simplified MRS broth and cultured at 42 ^oC, 150 rpm for 12 hours as inoculum seeds with the inoculum was 10%. pH was maintained by adding 60 g of $CaCO_3$ per 100 g of glucose. All experiments were repeated twice.

3.2.2 Raw materials and chemicals

Jerusalem artichoke tubers (Jat) were purchased from Xinnong Technology Co., Ltd. (Qinghai, China). The Jat was washed with water, sliced into pieces of 0.3-0.5 grams each, and dried at 60 ℃ for 24 hours. The clean Jat was ground to make smaller than 60 mesh fine powder using a pulverizer (HR 2094, Philips, the Netherlands) for this experiment. The Jat was washed, sliced, and dried at 60 ℃ until constant weight, then ground to fine powder. The composition of the dried Jat was determined using the same method by Kaldy et al. (1980). One gram of the dried Jat composed of 0.75 g inulin (glucose and fructose), 0.03 g cellulose, 0.02 g hemicellulose, 0.02 g protein, 0.01 g fat, and 0.05 g water.

The inulin powder was purchased from Langrui Fine Chemical Co., Ltd. (Shanghai, China). One gram of inulin powder was composed of 0.78 g of fructose and 0.23 g of glucose obtained from 1 g of inulin (dry base) according to the method by Gao et al. (2010).

All chemicals including peptone, yeast extract, $(NH_4)_2SO_4$, KH_2PO_4, $MgSO_4 \cdot 7H_2O$, $MnSO_4 \cdot 4H_2O$, sodium acetate, diammonium hydrogen citrate, were purchased from Lingfeng Chemical Reagent Co., Ltd. (Shanghai, China). Albumin, Fraction V (CAS Number: PB10056, 69 kDa) was purchased from Beijing Probe bioscience Co., Ltd (Shanghai, China). Coomassie Brilliant Blue G-250 (CAS Number: 0006192525), Coomassie Brilliant Blue R-250 (CAS Number: 0006104592) was obtained from Sigma-Aldrich.

3.2.3 Inulinase assay

The inulinase activity in the glucoamylase enzymes was measured using the modified method of Jing et al. (2003). 100 μL of the crude enzyme solution was mixing with 900 μL of 2% inulin powder in a 0.1 M sodium acetate buffer (pH 4.5). The solution was incubated at 60 ^oC for 10 minutes, then mixed with 3 mL of 3.5 dinitrosalicylic acid (DNS) solution. The mixture was placed in an ice bath, heated for 5 minutes at 100 ^oC to deactivate the enzyme, and the reducing sugars were assayed by observing the change of absorbance at 540 nm. One unit (U) of inulinase activity was defined as the amount of enzymes that produced one micromole of reducing sugars per minute from inulin under the assay conditions.

The protein concentration of the three commercially available glucoamylases was measured using Bradford method.

3.2.4 Purification of glucoamylase proteins

The crude glucoamylase GA-L New solution was filtered through a 0.22 μm membrane and purified using a chromatography column packed with Sephadex G-100 (Life Sciences, Uppsala, Sweden). The column was pre-equilibrated and eluted with citrate buffer (0.1 M, pH 5.0) at a flow rate of 1.0 mL/min and the eluent protein monitored at 280 nm. The SDS-PAGE electrophoresis of the fractions were performed with 12% polyacrylamide denaturing SDS gel and stained with Coomassie Brilliant Blue R-250. The inulinase activity of the fractions was measured using the method above in section 2.5.

3.2.5 SSF Bioreactor and its operation

SSF operation was carried out in 5-L bioreactor with pH and temperature control. A specially designed helical ribbon impeller was installed for well mixing at high Jat solids loading as described by Zhang et al. (2010a) and Zhao et al. (2013). The SSF operation continued to 72 hours and samples were withdrawn at regular intervals.

3.2.6 Data calculations

$$\text{Amount of glucose utilized (g/L)} = \text{(Initial-Final) glucose concentration}$$

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

$$\text{Yield of lactic acid fermentation (g/g DM)} = \frac{\text{Final lactic acid concentration (g/L)}}{\Sigma\text{DM (g/L) x 0.749}}$$

Where: ΣDM is total dry material in 1 L total liquid (g/L), 0.749 is the conversion factor for glucoamylase GA-L New to equivalent sugar (fructose and glucose).

3.2.7 Analytical methods

Glucose, fructose and lactic acid was analyzed using HPLC (LC-20AD, refractive index detector RID-10A, Shimadzu, Japan) with a Bio-Rad Aminex HPX-87H column at 65 ^oC. The mobile phase setup 5 mM H_2SO_4 with the rate of 0.6 mL/min. All samples were diluted and filtered before analysis.

3.3 Results and Discussion

3.3.1 Characterization of inulin hydrolyzing enzyme(s) in commercially available glucoamylases

To find a cheap inulin hydrolyzing enzyme for replacement of expensive inulinase, various available industrial hydrolase enzymes were screened, and a high inulinase activity was found from a typical glucoamylase GA-L New, a product of Genencor widely used in starch processing industry (www.genenco.cn). The inulinase activity data of glucoamylase GA-L New were using inulin powder as substrate in Table 3.1, indicates that the inulinase activity of glucoamylase GA-L New maintained high at pH from 3.6 to 5.0, but decreased gradually with increasing pH; the stability was also relatively stable at pH from 3.6 to 5.0, then decreased when pH increased to 5.6. Inulinase activity of glucoamylase GA-L New increased with increasing temperature from 30-60 ^{o}C, then decreased at 70 ^{o}C and sharply decreased at 80 ^{o}C; the inulinase stability showed an optimal range from 30-60 ^{o}C, then decreased quickly above 60 ^{o}C was shown in Table 3.1.

The inulinase activity of three commercially available glucoamylase enzymes was tested to further confirm the inulinase activity in glucoamylase. The three commercially available glucoamylase enzymes include glucoamylase GA-L New from DuPont Genencor Science, Wuxi, China, (http://biosciences.dupont.com/duponttm-genencorr-science/); amyloglucosidase A107823 from Aladdin Industrial Co., Shanghai, China (http://www.aladdin-e.com); and a local product, glucoamylase YY0515 from Shanghai Yuanye Biological Technology Co., Shanghai, China (www.shyuanye.com). The inulinase activity per milliliter enzyme solution and the specific activity per milligram of total protein were measured and the results were shown in a newly added Table 3.2.

Table 3.2 indicates that all the three commercially available glucoamylase enzymes demonstrated high inulinase activity, with the close activity of the first two enzymes (GA-L from Genencor and A107823 from Aladdin, and the relatively low activity of YY10515 from the local Shanghai Yuanye. The finding suggests that this phenomenon of inulinase activity in commercial glucoamylase enzymes might be a universal property of general glucoamylase enzymes, instead of a unique property of a specific glucoamylase such as GA-L New of Genencor product.

The glucoamylase GA-L New is the crude enzyme product extracted from *Aspergillus niger* fermentation broth without further purification. Multiple enzyme components were secreted into the crude enzyme product, besides the major glucoamylase component. These enzyme components may include inulinase enzyme(s) such as endoinulinase (EC 3.2.1.7), exoinulinase (EC 3.2.1.80), and invertase (EC 3.2.1.26) based on the information in the Brenda Enzyme Database (http://www.brenda-enzymes.org/). One evidence is that the inulinase properties were similar to that of the typical inulinase from *Aspergillus niger*, with the pH and temperature optima at pH 4.0-7.0 and at 35-60 ℃, respectively (Kango, 2008;

Derycke and Vandamme, 1984; Gaye et al., 1994). Still, glucoamylase itself might behave a substrate promiscuity of inulin hydrolysis and then degrade the Jat into fructose and glucose.

Table 3.1 Effect of pH and temperature on inulinase activity and stability of glucoamylase GA-L New

pH	Relative activity (%)	Relative stability (%)
3.6	88.7±0.18	74.2±0.32
4.0	100.0±0.00	100.0±0.00
4.6	91.6±3.28	95.1±7.49
5.0	87.2±4.10	84.5±4.56
5.6	76.1±2.64	27.9±3.75
Temperature (°C)	**Relative activity (%)**	**Relative stability (%)**
30	38.13±2.22	95.48±4.74
40	49.34±0.72	98.20±6.9
45	62.69±2.49	100.91±5.87
50	84.85±3.67	94.79±2.11
55	89.07±4.00	91.83±1.59
60	100.00±0.00	96.39±1.86
65	95.59±3.07	52.76±5.44
70	91.47±1.62	17.18±2.66
80	40.90±3.01	5.36±0.00

pH stability was tested by pre-incubating 120 min at 4 °C and reaction mixture containing 0.9 mL of 1% inulin and 0.1 mL enzyme solution in sodium acetate buffer at 60 °C for 10 min. The thermo stability was tested by pre-incubating the enzyme at different temperatures (30-80 ℃) for 2 h. The inulinase activity of the final concentrated elute with setting time and pH or temperature was regarded as 100%.

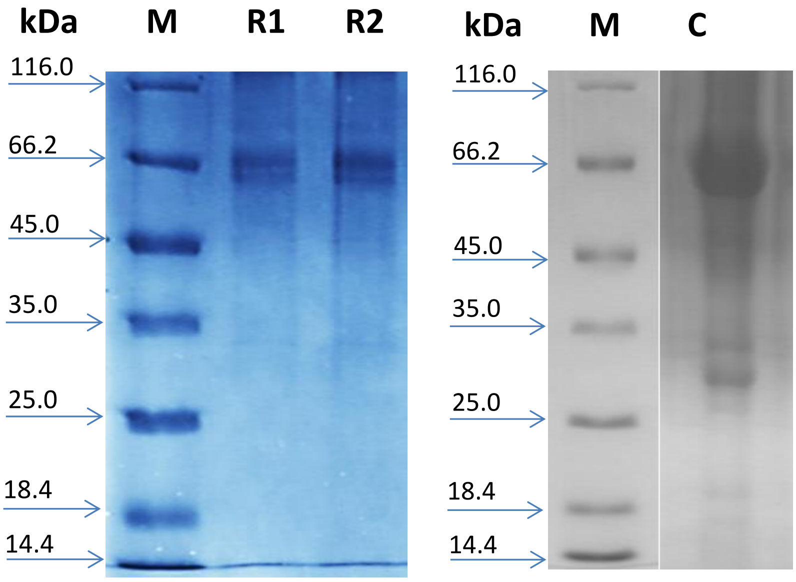
Table 3.2 Inulinase activity of three commercially available glucoamylase enzymes

Glucoamylase type	Inulinase activity per mL (U/mL)	Protein concentration (mg/ml)	Specific inulinase activity (U/mg protein)
GA-L New (Genencor)	18.50	40.75	0.45
A107823 (Aladdin)	19.58	46.12	0.42
YY10515 (Shanghai Yuanye)	5.86	40.03	0.15

100 μL of the crude enzyme solution was mixing with 900 μL of 2% inulin powder in a 0.1 M sodium acetate buffer (pH 4.5). The solution was incubated at 60 °C for 10 minutes.

Identifying source of inulinase activity in Fig. 3.2, glucoamylase GA-L New, its crude enzyme solution was purified chromatographically by Sephadex G-100 gel and fractions were sent to test its proteins and the inulinase activity, respectively. Fig. 3.2, there were two protein peaks at 50 min and 150 min of the chromatography purification, respectively; in addition to two inulinase activity peaks at 50 min and 80 min, both were included in the first protein peak, while almost no inulinase activity was found in the second protein peak. Fig. 3.1(a), the SDS-PAGE of the fraction at 50 min of the chromatography purification, and two protein bands appeared and both were close to the molecular weight of 66.2 kDa. Fig. 3.1(b) shows the SDS-PAGE of the crude glucoamylase GA-L New and the result indicates that the major protein component of the crude enzyme also had the molecular weight close to 66.2 kDa, which should be the major enzyme of glucoamylase (EC 3.2.1.7).

To identify the protein close to 66.2 kDa in Fig. 3.3, the molecular weight data of the related proteins from *Aspergillus niger*, including glucoamylase (EC 3.2.1.3), endoinulinase (EC 3.2.1.7), exoinulinase (EC 3.2.1.80), and invertase (EC 3.2.1.26), were mined from the Brenda Enzyme Database (http://www.brenda-enzymes.org/) and the data were placed in order in Fig. 3.3. Fig. 3.3 shows that among the three typical inulinase enzymes and glucoamylase from *Aspergillus niger*, the molecular weights of both exoinulinase (EC 3.2.1.80) and invertase (EC 3.2.1.26) were far from the sample value of 66.2 kDa, thus could be deleted from the possible inulin hydrolyzing enzymes. Only endoinulinase (EC 3.2.1.7) and glucoamylase (EC 3.2.1.3) had the molecular weight close to 66.2 kDa. Therefore, the high inulinase activity in GA-L New should come from either the major glucoamylase fraction, or the minor endoinulinase fraction, or the both. Glucoamylase generally cleaves 1,4-α-D-glucosidic linkage of starch, and the mechanism of cleaving 1,2-β-D-fructosidic linkage is under investigation.



(a) (b)

Figure 3.1 Purification of glucoamylase GA-L New.

(a) SDS-PAGE of the chromatograph Sephadex G-100 fractions at 50 min; Lane M: protein ladders; Lanes R1 and R2: samples; (b) SDS-PAGE of crude solution of glucoamylase GA-L New; Lane M: protein ladders; Lanes C: original crude enzyme samples.

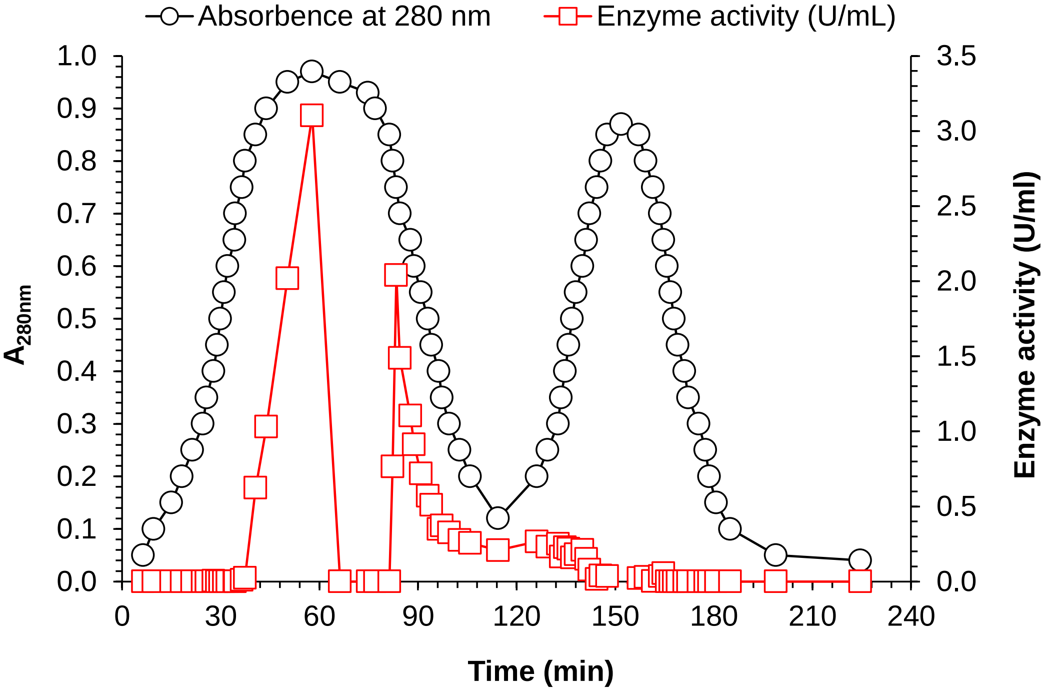


Figure 3.2 Purification of protein and inulinase activity assays of glucoamylase GA-L New using the chromatograph Sephadex G-100.

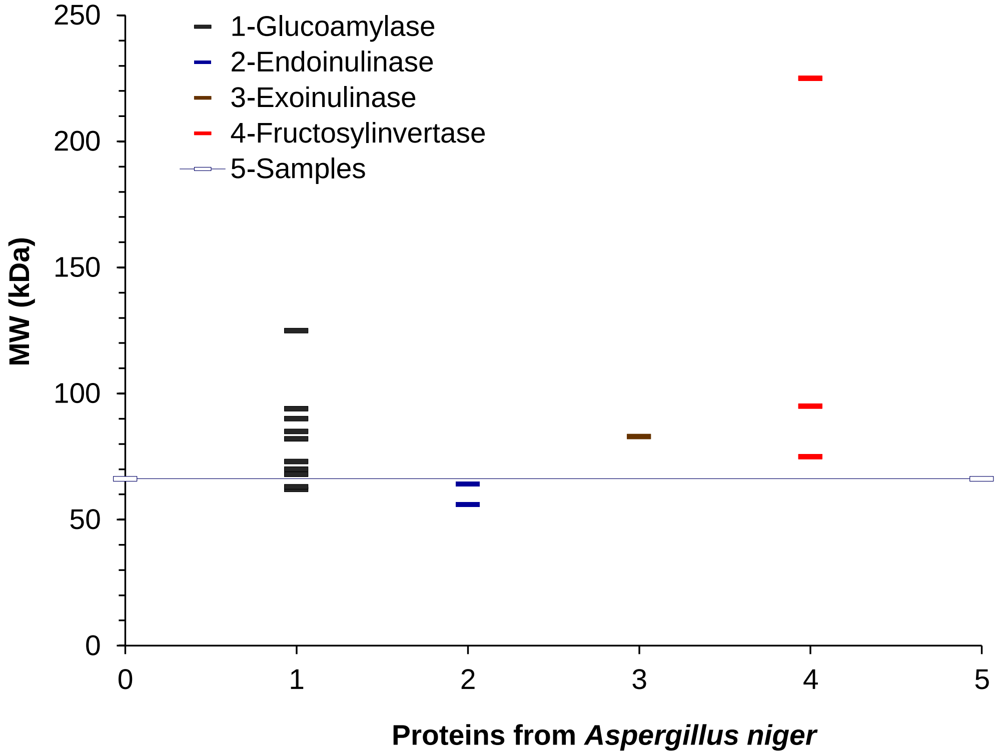


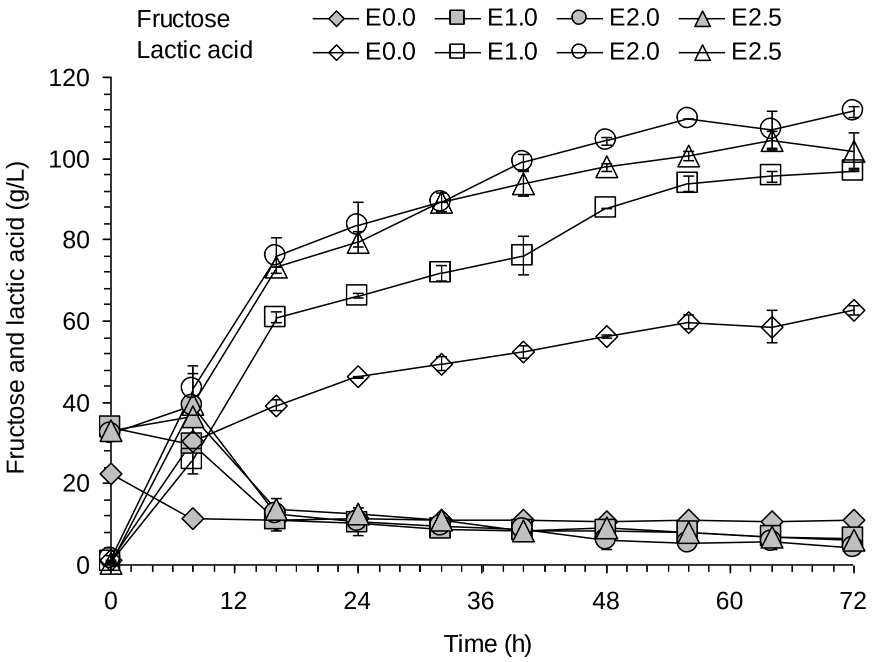
Figure 3.3 Molecular weight analysis of proteins from *Aspergillus niger*

3.3.2 Saccharification of Jat by glucoamylase GA-L New and subsequent lactic acid fermentation

The findings suggest that glucoamylase GA-L New, a cheap, commercially available, and widely used industrial enzyme, could be used for practical Jat hydrolysis. In Fig. 3.4 shows the simultaneous saccharification and lactic acid fermentation (SSF) of Jat using glucoamylase GA-L New at the dosage from 1.0-2.5 U/g dry Jat (DM). Fructose accumulated slightly in the first 8 hours of SSF, and then consumed completely in the next 16 hours at all the enzyme dosage, while lactic acid constantly and rapidly increased in the first 16 hours. In the next 56 hours, fructose maintained at a low level with slight decrease, while lactic acid steadily increased to its maximum. The results indicate that fructose and glucose were released from Jat continuously by glucoamylase GA-L New, then the released fructose and glucose were converted into lactic acid by *P. acidilactici* DQ2 simultaneously. The results suggest that the optimal glucoamylase dosage was 2.0 U/g DM, and the further increase of enzyme dosage led to the decrease of lactic acid fermentation performance. Interestingly, 62 g/L of lactic acid was produced from 30 g/L of fructose and glucose when no glucoamylase was added to the SSF system. The results may suggest that the lactic acid bacteria (LAB) used in this study, *P. acidilactici* DQ2, exhibited some natural inulinase activity.

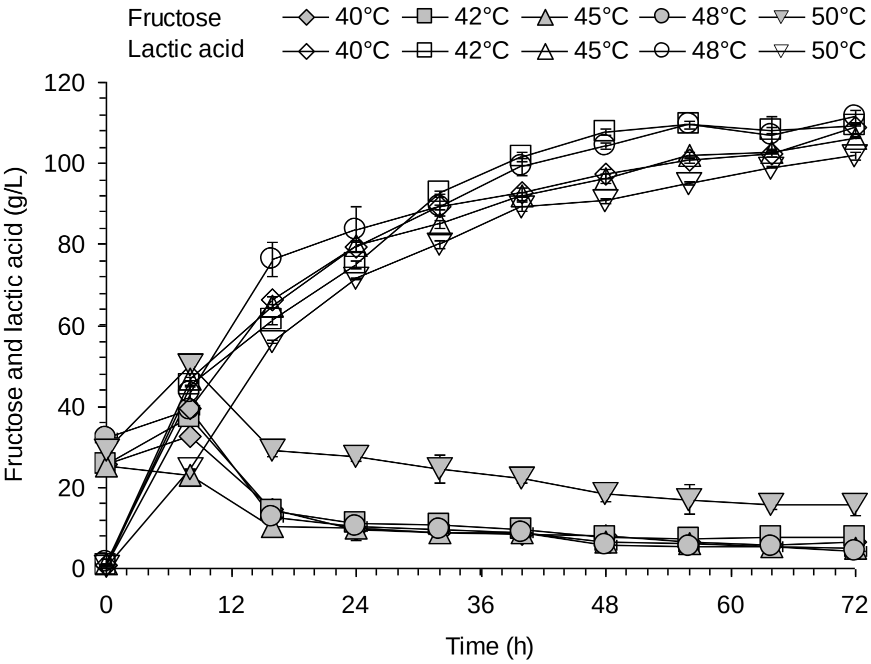
Generally, lactic acid bacteria (LAB) strains including are mesophiles at elevated temperature (Rojan et al., 2009; Zhao et al., 2013). Glucoamylase GA-L New is also thermo-tolerant enzyme with high activity and constant stability up to 60 ^{o}C as shown in Table 3.1. Fig. 3.5 shows the SSF of Jat at different temperature profiles from 40 to 50 ^{o}C. The results indicate that the lactic acid production performance maintained high in the range of 40 to 50 ^{o}C, suggesting that both *P. acidilactici* DQ2 and glucoamylase GA-L New were strong thermo-tolerant. Similar results were obtained at the temperature range of the experiments, suggesting that the effect of temperature on the lactic acid fermentation was not significant. When SSF temperature increased to 50 ^{o}C, fructose began to accumulate and 15.6 g/L of fructose left at the end of the 72 hours' SSF. Among the temperature ranges tested, the SSF performance at 48 ^{o}C and 42 ^{o}C was a slight advantage over others. The lower temperature of 42 ^{o}C preferred the cell growth and metabolism of *P. acidilactici* DQ2, while 48 ^{o}C preferred the Jat hydrolysis to give more fructose and glucose sugars. The higher temperature was selected because of the contamination control and energy saving.

Fig. 3.6 shows the SSF performance of Jat at different pH values and the results indicates that the effect of pH on the SSF performance of Jat was complicated. The fermenting strain *P. acidilactici* DQ2 showed poor performance at low pH (below 5.0) (Zhao et al., 2013), while the saccharification enzyme GA-L New showed a poor activity and stability at high pH (above 6.0) (Table 3.1). Clearly, there should be a trade-off between the efficiency of saccharification and lactic acid fermentation. In Fig. 3.5, a significant fructose accumulation occurred and led to a low lactic acid production when pH was less than or equal to 5.0, mainly due to the bad fermentability of *P. acidilactici* DQ2 at low pH levels. When pH increased to 6.0, no fructose accumulation was found but lactic acid formation was very limited, mainly due to the low activity and stability of glucoamylase GA-L New. Only when pH value maintained at 5.5, the satisfying performance of both Jat saccharification and lactic acid formation was obtained.



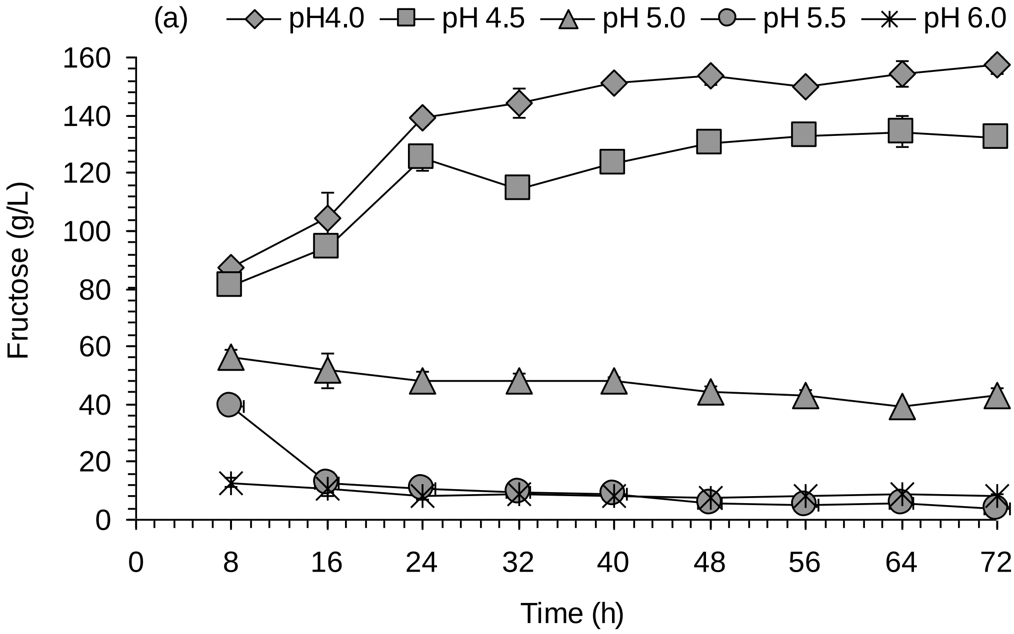
Glucoamylase GA-L New (U/g DM)	0.0	1.0	1.5	2.0	2.5
Lactic acid yield (g/g DM)	0.26	0.40	0.42	0.46	0.42
Lactic acid productivity (g/L/h)	0.87	1.34	1.41	1.55	1.40

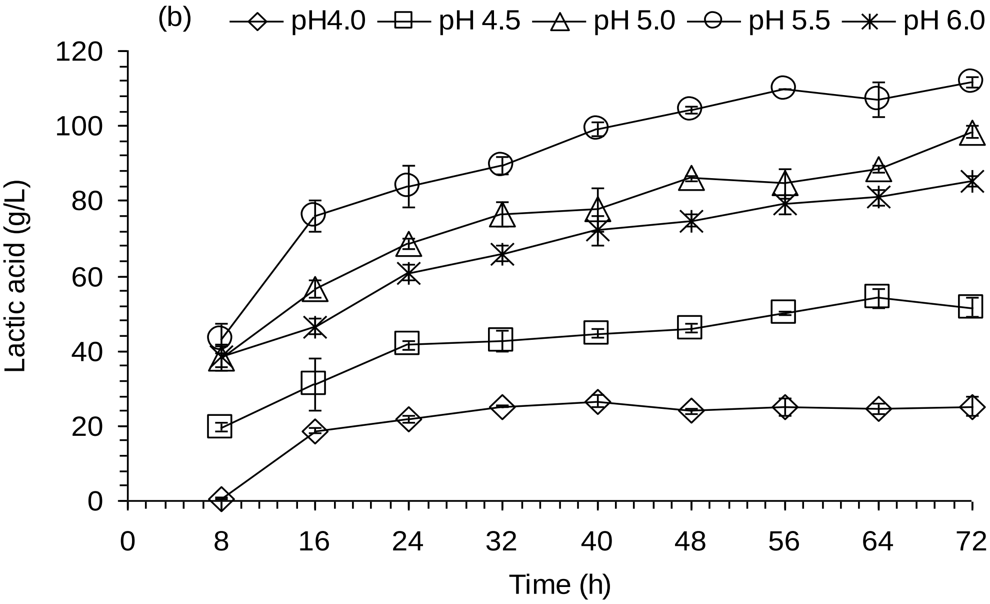
Figure 3.4 SSF of Jat at different glucoamylase GA-L New dosage



Temp (^oC)	40	42	45	48	50
Lactic acid yield (g/g DM)	0.45	0.46	0.44	0.46	0.44
Lactic acid productivity (g/L/h)	1.51	1.52	1.48	1.55	1.46

Figure 3.5 SSF of Jat at different temperature





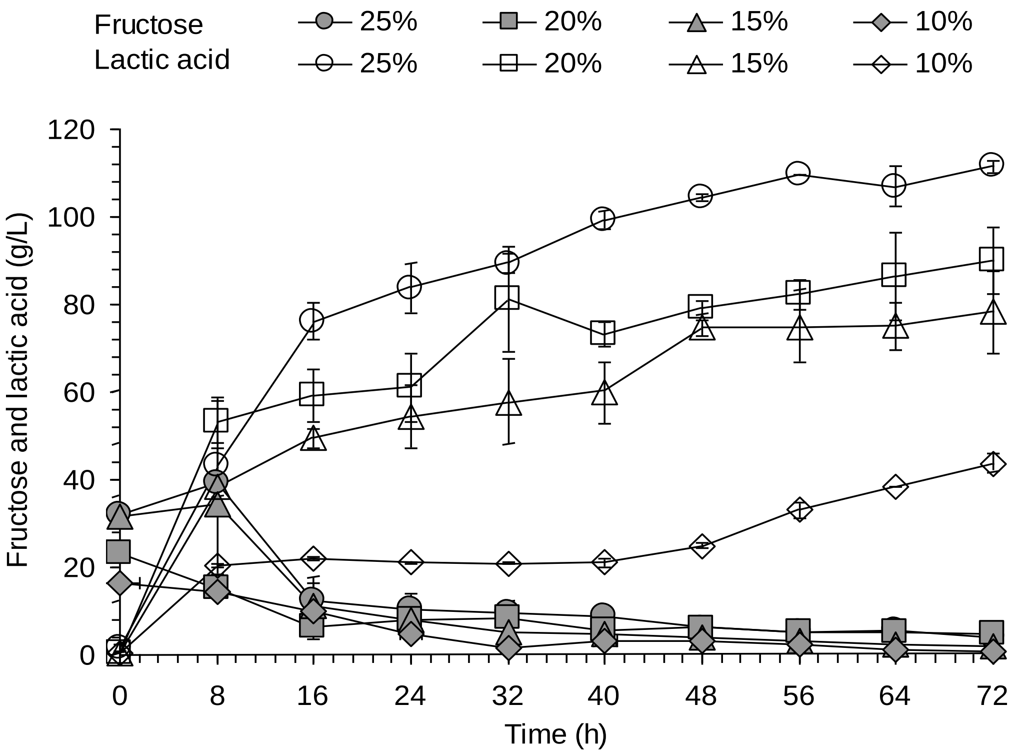
pH values	4.0	4.5	5.0	5.5	6.0
Lactic acid yield (g/g DM)	0.10	0.21	0.43	0.46	0.39
Lactic acid productivity (g/L/h)	0.33	0.72	1.37	1.55	1.18

Figure 3.6 SSF of Jat at different pH values

3.3.3 SSF at high solids loading of Jat for production of high titer lactic acid

A high lactic acid product concentration achievement, the solids loading of Jat feedstock should be increased to its maximum. In this study, a specially designed bioreactor with a helical ribbon stirrer for well mixing the high Jat solids loading was used and the possible high Jat content from 10% to 25% (w/v) in the bioreactor were investigated at the optimal enzyme dosage, pH, temperature conditions. In addition to reduce cost of lactic acid production only 5 g/L of yeast extract was used. Figure 3.7 shows that both lactic acid titer and productivity increased with increasing Jat solids content from 10% to 25%, and up to 111.5 g/L and 1.55 g/L/h after 72 hours' SSF operation at 25% Jat solids content. However, almost 30% of lactic acid yield was found when the lactic acid yield decreased with increasing Jat solids loading from 0.69 g/g DM at 10% solids (w/v) to 0.46 g/g DM at 25% solids (w/v). Balancing the yield loss and the increase of titer and productivity, 25% (w/v) might be a proper solids content in the practical SSF of Jat.

Glucoamylase is one of the most widely used and the cheapest industrial enzymes in starch processing industry (Nghiem et al., 2013). The price of glucoamylase GA-L New was approximately $3.0 per kg (Inquiry from Genencor China, Wuxi, China, www.genencor.cn). Based on the inulinase activity data of GA-L New, which was 20 U/mL, the cost for one unit (U) of inulinase was $3.0/1,000/20=$0.00015, when glucoamylase GA-L New was used as the inulinase enzyme. On the other hand, the only available commercial inulinase enzyme was on the Sigma catalog (Product ID 16285, equivalent name: Novozym 960) with the inulinase activity of 285 U/mL (www.sigma.com). Its cheapest price with the largest package (250 mL) was approximately $350 per 250 mL, thus the cost for on unit (U) of inulinase was calculated as $350/250/285=$0.005. The result indicates that the cost using glucoamylase GA-L New as inulinase enzyme was only 3% (=$0.00015/$0.005) than that using Novozyme 960, thus leaving a huge profit margin for glucoamylase use as inulinase enzyme in industrial application.



Solid conc. (%, w/v)	25	20	15	10
Lactic acid yield (g/g DM)	0.46	0.49	0.57	0.69
Lactic acid productivity (g/L/h)	1.55	1.25	1.09	0.78

Figure 3.7 SSF of Jat at different solids loading

3.4 Conclusions

High inulinase activity was observed and characterized in three commercial glucoamylases. One of the glucoamylase GA-L New from Genencor was applied to lactic acid production from Jat. A high lactic acid titer, yield, and productivity of 111.5 g/L, 0.46 g/g DM, and 1.55 g/L/h, respectively, were obtained within 72 hours. The cost using commercial glucoamylase was compared to that using inulinase and a large profit margin was identified.

CHAPTER IV

Disk milling of pretreated corn stover and consequent simultaneous saccharification and lactic acid fermentation

Abstract

Corn stover was pretreated using the dry sulphuric acid pretreatment, followed by a biological detoxification to remove the inhibitors produced in the pretreatment. The bioreactor with a novel helical impeller was used to the SSF operation of the pretreated and biodetoxified corn stover. The complete removal of all the inhibitor substances after 7-10 days of bio-detoxification led to the higher lactic acid yield and titer in the simultaneous saccharification and lactic acid fermentation process. After the deep biodetoxification and disk milling, the SSF at 48 ^{o}C, pH 5.5, and 25% (w/w) solids loading in the lactic acid titer, yield, and productivity of 74.2 g/L, 59.76%, and 1.03 g/L/h, respectively. The result provided a practical process option for cellulosic lactic acid production using agro-lignocellulose residual in the chain of biomaterial resources.

4.1 Introduction

Corn stover (CS) is a cheap raw material, the stalks, leaves, husks, tassels, and cobs of the corn plant, Approximately 200 million tons of corn stover is produced, in China per year, and approximated 56.6% of the total straw production (Sun et al., 2011; Chen et al., 2011) potential substrate for lactic fermentation. Lactic acid (LA) can be produced by chemical synthesis (mostly lactonitrile route) or by fermentation (from carbohydrates and agro industrial feedstock) and 90% of total LA is produced by the fermentation route, including the world largest producers (Aleksandra P. Djukić-Vuković et al., 2012), applied and asserted up to now in food, pharmaceutical, textile, and chemical industries following itself.

Many lactic acid bacteria (LAB), including *Lactobacillus* species, *Lactococcus* species, and *Sporolactobacillus* species, have been reported to be lactic acid producers and efficient utilization of both hexoses and pentoses in cellulose and hemicellulose is of significance for the economical conversion of lignocellulosic biomass to biochemical (Cui et al., 2011; Jia et al., 2013). CS mainly composed of lignin, cellulose, and hemicellulose but using of lignocellulosic feedstocks for bioproducts requires pretreatment (William et al., 2000, Zhang et al., 2010b). The goal of the pretreatment technologies including physical, chemical and thermochemical processes are necessary to break down the lignin and complex carbohydrates and consequently increase susceptibility of lignocellulosic biomass to hydrolytic enzymes

(Cui et al., 2011). As else reported by Ayse Avci (2013) exist on the pretreatment of corn stover using various methods such as dilute H_2SO_4, steam pre-treatment, and hot water pretreatment. Yields of the sugar released were dependent on the temperature and the time as well as the method applied. Yields obtained by other researchers varied from 73-91% for glucose and 70-82% for xylose for corn stover. To achieve the maximum lactic acid productivity and yield have been studied to improve fermentation efficiency such as optimum conditions (e.g. pH, temperature, new medium) but one hindrance fermentation of lactic acid is the existence of inhibitors.

Biological, physical, and chemical methods have been employed for detoxification of lignocellulosic hydrolysates. Different detoxification methods cannot be strictly compared when different lignocellulosic hydrolysates and different microorganisms have been used. (Review, Eva et al., 2000). The toxic effects of furfural, 5-hydromethylfurfural (5-HMF), and weak acids are generated as by-products during concentrated H_2SO_4 saccharification on the fermentation of glucose and xylose by *Pediococcus acidilactici* DQ2 was demonstrated in our previous study (Zhao et al., 2013). The toxic compounds are undesirable in simultaneous saccharification and lactic acid fermentation of CS. Fortunately, in 2010b, Zhang et al. founded, *Amorphotheca resinae* ZN1, was successfully isolated from the microbial community growing on the pretreated corn stover material. It was found that the solid-state culture of *A. resinae* ZN1 on various pretreated lignocellulose feedstock such as corn stover, wheat straw, rice straw, cotton stalk and rape straw degraded all kinds of toxins quickly and efficiently, was considered a promising option for the removal of toxins.

Besides the problems as above, in the fermentation process, the particle size reduction is a crucial factor in transportation logistics and conversion facilities. The composition of each anatomical fraction is slightly different, each fraction, and cell types is known to respond differently to enzymatic cellulose hydrolysis and fermentation performances (Noah et al., 2010; Chen et al., 2011). Biomass particle size impacts handling, storage, conversion, and dust control systems (Venkata et al., 2009). To solve this problem from inhomogeneity of CS, milled out with a fixed unit of uniform size reduction is an essential process for biomass utilization using enzymatic hydrolysis in lactic fermentation and also reduce transportation costs. Such reduced particle sizes can be achieved by fine grinders; however, these grinders were directly utilized to fibrous biomass for want of specialized size reduction machinery (Venkata et al., 2009; Igathinathanea et al., 2009).

In our previous study, *Pediococcus acidilactici* DQ2 was shown as a good lactic acid bacterium on corn stover for lactic acid production (Zhao et al., 2013). Little published data on lactic fermentation from corn stover milling. Hence, in this study, the corn stover milling (CSM) was bio-pretreated at different days, using as a renewable resource for lactic acid fermentation with a cheap cellulose enzyme. The simultaneous saccharification and lactic acid fermentation (SSF) operation type was carried out at 48 ℃, pH 5.5 with high solids loading.

4.2 Materials and methods

4.2.1 Biomass material, enzyme, and chemicals

Corn stover (CS) war purchased from agricultural market in Shandong province, China. Dry CS was packed and equipped with a screen stencil of 8-10 mm pore diameter. The materials were washed, dried following Zhao et al. (2013), and stored in air tight containers at room temperature prior to use with moisture content was less than or equal to 10%. The components of the corn stover (dry weight%) were as follows: The original CS contained 34.2% cellulose, 31.5% hemicellulose and 8.1% lignin (w/w, dry weight base).

The cellulase enzyme (Youtell #6) used was kindly provided by Hunan Youtell Biochemical Co. (Yueyang, China). The activity of Youtell #6 was 135.0 FPU/g in the filter paper unit (FPU). All other chemicals used in this study were analytical grade and purchased from Lingfeng Chemical Reagent Co., Shanghai, China. Inhibitors chemicals including Furfuryl alcohol, 5-hydroxymethylfurfural (5-HMF) purchased from J&K Scientific Co. (Beijing, China).

4.2.2 Medium and strains

In our previous study (Zhao et al., 2013). The lactic acid fermentation strain, *Pediococcus acidilactici* DQ2 (CGMCC 7471), was stored in China General Microbial Collection Center (CGMCC), Beijing, China. The stock culture was described in our previous study with the simplified MRS broth. The inoculum culture was carried out in a 250 mL flask containing 50 mL simplified MRS medium with 10% (v/v) inoculation ratio at 42 ℃, 150 rpm for 24 hours.

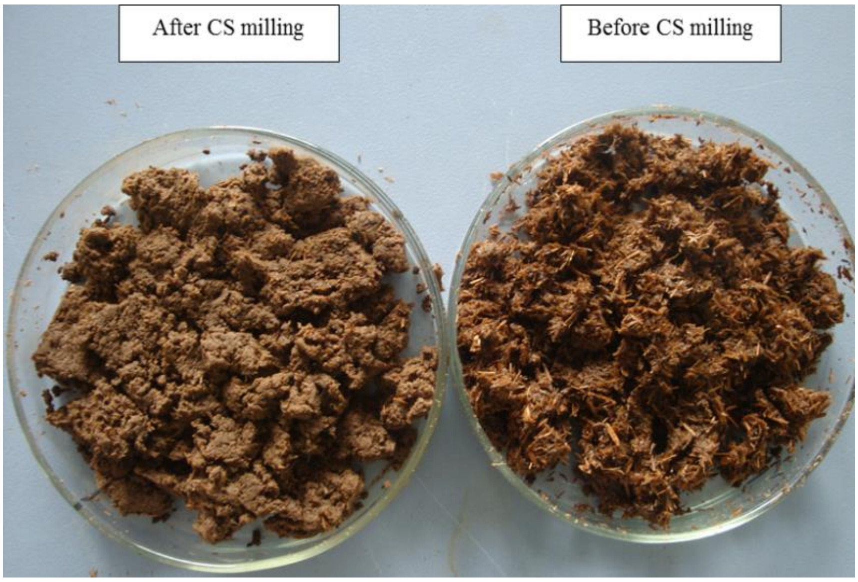
Amorphotheca resinae ZN1 was obtained in our laboratory and used to detoxify inhibitor compounds in the pretreated CS materials (Zhang et al., 2010b). Stock cultures and activation cultures were carried out by Zhao et al., 2013, respectively. The strain is under the processes of the registration in CGMCC and Chinese Patent application before open to the academic community.

4.2.3 Double disc grinder and operating variables

All CS were mill in the double disc grinder, which instrumentation set up content of a double disc mill and a double face cutting knife (Mode: DSX-32, handing capacity, China

Qidong Dongcheng Chemical machinery factory Jiangsu) with a three phase asynchronous motor, type: Y132S2-2, No. Y0203069 with an engine rated at 7.5 kW (Shanghai Huabin motor Co. Ltd), various engine throttle settings (on/off) operated the double disc grinder (DSG), was used for milling, at speeds ranging from 2900 rpm. The DSG included an input feeding funnel, the cutting and grinding synchronization (a knife and double disc), an output milling, a set of cooling by water, a motor, and a platform in Picture 4.1 Illustrate before and after milling of corn stover.

The performance of the cutting and grinding sync: Length and thickness of each knife shear (three knifes blade included 15^{o} leading edges and angular distribution is 120^{o}) was 2cm and 0.35 cm, respectively. It was turn on a pivot center of motor, rotate left with the second disc together, and is placed in the middle of the first disc. On the first disc held by a fast flange and fasten a point of fixed depression on the inner wall. On the first disc, upper face is flat surface and under face included teethes with three round teethes (inside, middle, outside), which the number of each round teethes were 20, 60 and 60, respectively. The second disc pinned on the shaft of motor content of two face and rotate with knife shear, upper side had lot of teethes fit on the first under face included three round teethes (inside, middle, outside), in which the number of it were 47, 90 and 120, respectively, and four curved bar pinned to under side. All of them are placed in the inox steel box with one outdoor.



Picture 4.1 Illustrate before and after milling of corn stover

4.2.4 Steam explosion pretreatment and bio-detoxification of corn stover procedure

Corn stover (CS) was pretreated with 2.5 g sulfuric acid per 100 g of the dried CS (Zhao et al., 2013) and the dry dilute acid pretreatment followed the procedure by Zhang (2011a). Steam explosion pretreatment of CS were carried out in a vessel rotating reactor system equipped with stainless steel reactors. This system is heated and conjunction with steam heating systems through an iron pipe. The reactors were heated to 185 ℃ (approximately 10 min) and incubated at 185 ℃, 1.2 MPa for 3 min in the reactor was rotated continuously at 50 rpm.

The inhibitor compounds were rejected in the pretreated CS followed the bio-detoxification procedure by *A. resinae* ZN1 (Zhang et al., 2010b), were inoculated onto the solids pretreated CS containing 50% solids (w/w) for the removal of the inhibitors generated in the pretreatment. The bio-detoxification of CS process was incubated from 1 to lastly 10 days at 25 ℃ and marked in label order in separate boxes following days, in which was neutralized sulfuric acid in the corn stover after steam explosion pretreatment by 20 g/L of calcium carbonate at pH 6.0, with 60% (w/w) moisture until 90% of furfural and HMF were removed. Then the bio-detoxified CS was stored at 4 ℃ for use to mill CS. The milling CS was autoclaved for 20 min at 115 ℃ before SSF operation.

The composition of CS was performed following NREL laboratory analytical procedure. The original CS contained 44.7% cellulose, 27.1% hemicellulose and 7.16% lignin (w/w, dry weight base). The moisture content of CS was measured at 105 ^{o}C until the weight was constant.

4.2.5 Bio-detoxification of corn stover by *Amorphotheca resinae* ZN1

As following Chai et al. (2013), the results showed that these furan compounds were reversible inhibitors of the enzyme, the order of inhibiting ability was furfural>furoic acid>furfuryl alcohol and the functional groups on the furan ring play a crucial role in the inhibition on the enzyme. The inhibitors, in Figure 4.1, were pretreated detoxified CS by *Amorphotheca resinae* ZN1 from 1 to 10 days. The samples were tested as follows: adding 50 ml deionized water to 5 g of corn stover (the moisture content measured precisely), then shaking at 150 rpm, 30 ^{o}C for about 2 hours. And then the mixture was centrifuged at 10,000 rpm for 5 min, and the supernatants were used for HPLC analysis.

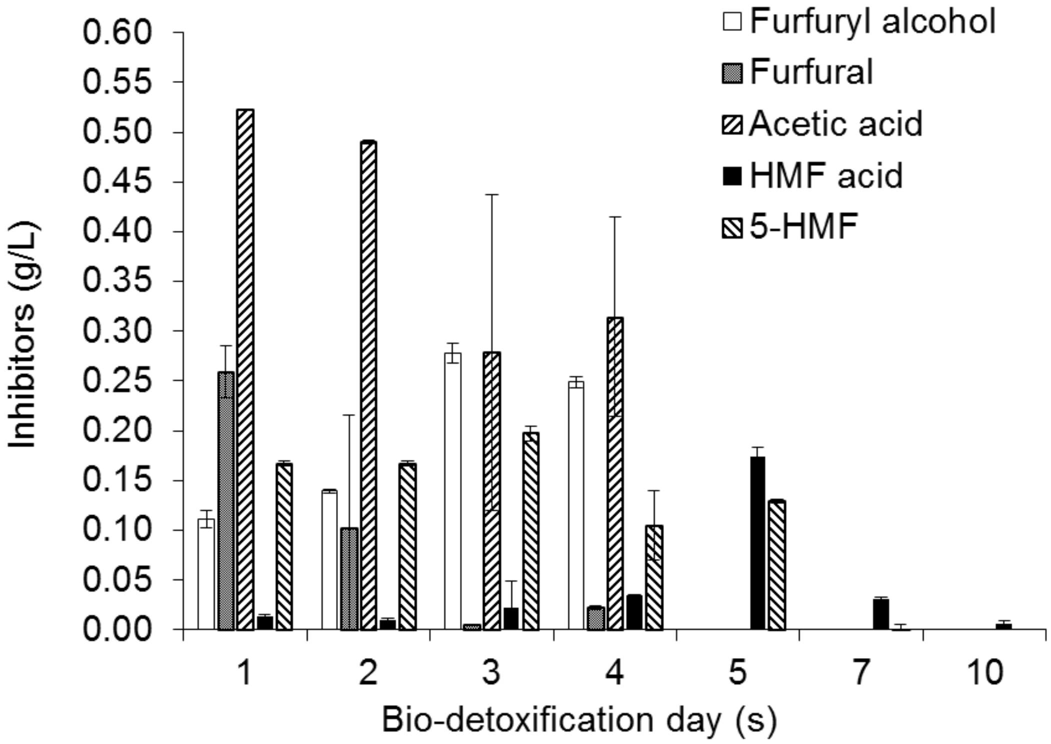


Figure 4.1 The time of bio-detoxified toxins in corn stover by *Amorphotheca resinae* ZN1

4.2.6 Simultaneous saccharification and lactic acid fermentation of corn stover milling

The SSF operations of milling corn stover were conducted in a helical stirring bioreactor (HSB) and details of the operation were in described in Zhang (2010a). The SSF was operated at two stages, the prehydrolysis stage started at the beginning and then the real SSF stage was operated according to methods previously our reported by Zhao (2013). The real SSF stage was carried out in 72 hours with the extra nutrients contained 1.0 g/L of ammonium sulfate; 2.0 g/L of dipotassium phosphate, 1.0 g/L of magnesium sulfate heptahydrate, and 5.0 g/L of yeast extract at pH 5.5. The pH was maintained during the hydrolysis and SSF stages by addition of 5 M NaOH. Samples were periodically taken and centrifuged at 13,000 rpm for 5 min. The samples were stored at -20 ℃ before analysis on HPLC. All experiments were repeated for two times and the error ranges were given in the tables and figures.

4.2.7 Caculation of lactic acid yield

The lactic acid yield of the SSF based on the pretreated CS was calculated according the following equation:

$$\text{Lactic acid yield (\%)} = \frac{\text{Lacf x Vf -Lac0 x V0}}{\text{f x WCS x 1:111 x 1:0}} \text{ x 100\%}$$

Where: Lacf and Lac0 are the lactic acid concentrations at the end and the beginning of the fermentation (g/L). Vf and V0 are the volume of liquid at the end and the beginning of the fermentation (L), WCS is the weight of the dry CS used in the SSF (g), f is the cellulose fraction of the dry CS (g/g), 1.111 is the conversion factor for cellulose to equivalent glucose, 1.0 is the conversion factor for glucose to lactic acid on the mass basis of stoichiometric biochemistry. The unit g/L refers to the volume of the liquid fraction calculated based on the water mass balance of the SSF operation.

4.2.8 Analysis of sugars, lactic acid, and inhibitors

Glucose, xylose, lactic acid, acetic acid were analyzed using high performance liquid chromatography (LC-20AD, refractive index detector RID-10A, Shimadzu, Japan) with a Bio-Rad Aminex HPX-87H column at the column temperature 65 ℃. The mobile phase was 5 mM H_2SO_4 at the rate of 0.6 mL/min. Furfural, Furfuryl alcohol, 5-HMF and vanillin were analyzed using the reversed-phase HPLC (LC-20AT, UV/VIS detector SPD-20A, Shimadzu, Japan) with a YMC-Pack ODS-A column at ambient temperature. The mobile phase was 50% methyl cyanide solution at the rate of 1.0 mL/min. All samples were centrifuged to remove the cell mass and other water insoluble substances, and then samples were filtered through a 0.22 μm filter before analysis.

4.3 Results and discussion

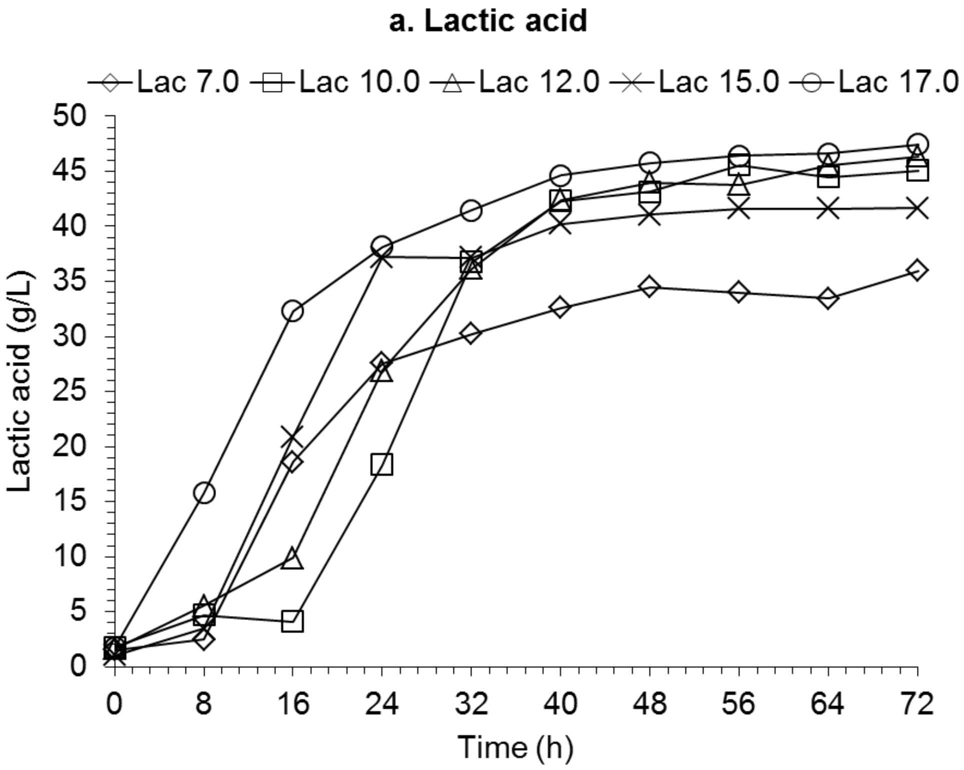
4.3.1 Effect of different cellulase concentrations on lactic acid production

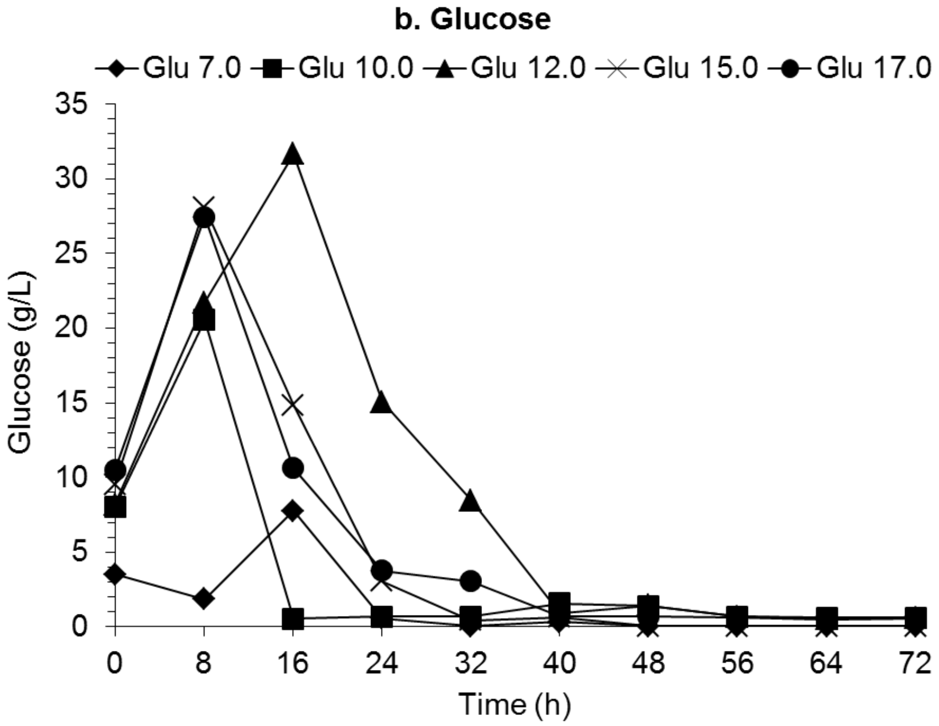
Corn stover is a residue of corn grain and involves basically three different fractions: stem, leaf, and cob. This material is heterogeneous because the composition of each anatomical fraction is slightly different, and each fraction is known to respond differently to enzymatic cellulose hydrolysis and fermentation performances (Noah et al., 2010; Chen et al., 2011).

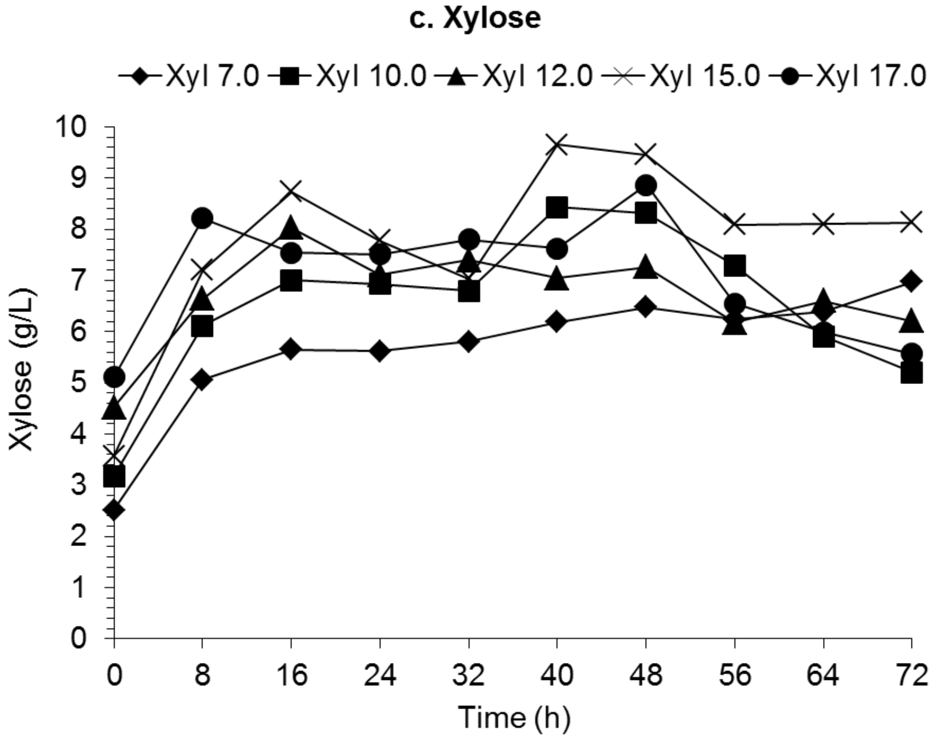
The usability of cheap CS and low price of Youtell#6 enzyme (approximately $1.60 per kg of cellulase, Zhang et al., 2013c) as inexpensive resource for lactic acid production using *P. acidilactici* DQ2 was investigated with diffirent cellulase doses (7 to 17 FPU/g DM). the goal of using corn stover milling (CSM) increases uniform size reduction of inhomogeneity of CS following enzymatic digestion while minimizing formation of furans (FA and 5-HMF), which are potent inhibitors of SSF to enhance lactic acid yield.

The inhomogeneity of CS chemical compositions and cell types affects its enzymatic hydrolysis and fermentation performances (Chen et al., 2011). It has been shown experimentally that if the amount of the enzyme is kept constant and the substrate concentration is then gradually increased, lactic acid yield and productivity will increase until it reaches a maximum.

To achieve the maximum lactic acid titer, the two step SSF operation (Zhao et al., 2013), in which content of the first step was in 8 hours of the pre-hydrolysis stage started at the beginning and then the real SSF stage was followed until the end of the operation, as along with reported, the optimum pH and temperature for SSF were 5.5 and 48 ℃, respectively. 15% (w/w) of solids loading was performed at different cellulase dosages using for CSM. Fig. 4.2a shows that the lactic acid concentration increased with the increasing cellulase dosages from 7 to 17 FPU/g DM. The result indicates that, at the present SSF conditions, the cellulase dosage up to 15 FPU/g DM might be a harmful dosage to cell viability. However, the highest yield of lactic acid only reached 60.77% at 17 FPU/g DM, and next 58.21% at 12 FPU/g DM. The results in Fig. 4.2 indicate that in the second step temperature swing operation improved the SSF process at high cellulase dosage and 10% solids loading. It would result in higher lactic acid yield if xylose in Fig. 4.2c was converted to lactic acid. In our previous study, Zhao et al., (2013) indicated that *P. acidilactici* DQ2 strain, which could not utilize xylose from hemicellulose in SSF. This utilization of xylose is the same in chapter 2 to test xylose consumption in MRS medium. At the same pretreatment severity, a 2-fold increase in monomeric xylose yield and a 1.5-fold increase in enzymatic cellulose digestibility from their lowest values were found (Noah et al., 2010) in the impact of corn stover compositional variability on xylose conversion yields during dilute acid pretreatment and on enzymatic cellulose digestibility of the resulting pretreated solids.







Cellulase Activity (FPU/g DM)	7.0	10.0	12.0	15.0	17.0
Final lactic acid concentration (g/L)	35.96	45.05	46.38	41.64	47.46
Lactic acid yield (%)	39.99	56.33	58.21	52.72	60.77
Lactic acid productivity(g/L/h)	0.50	0.63	0.64	0.58	0.66

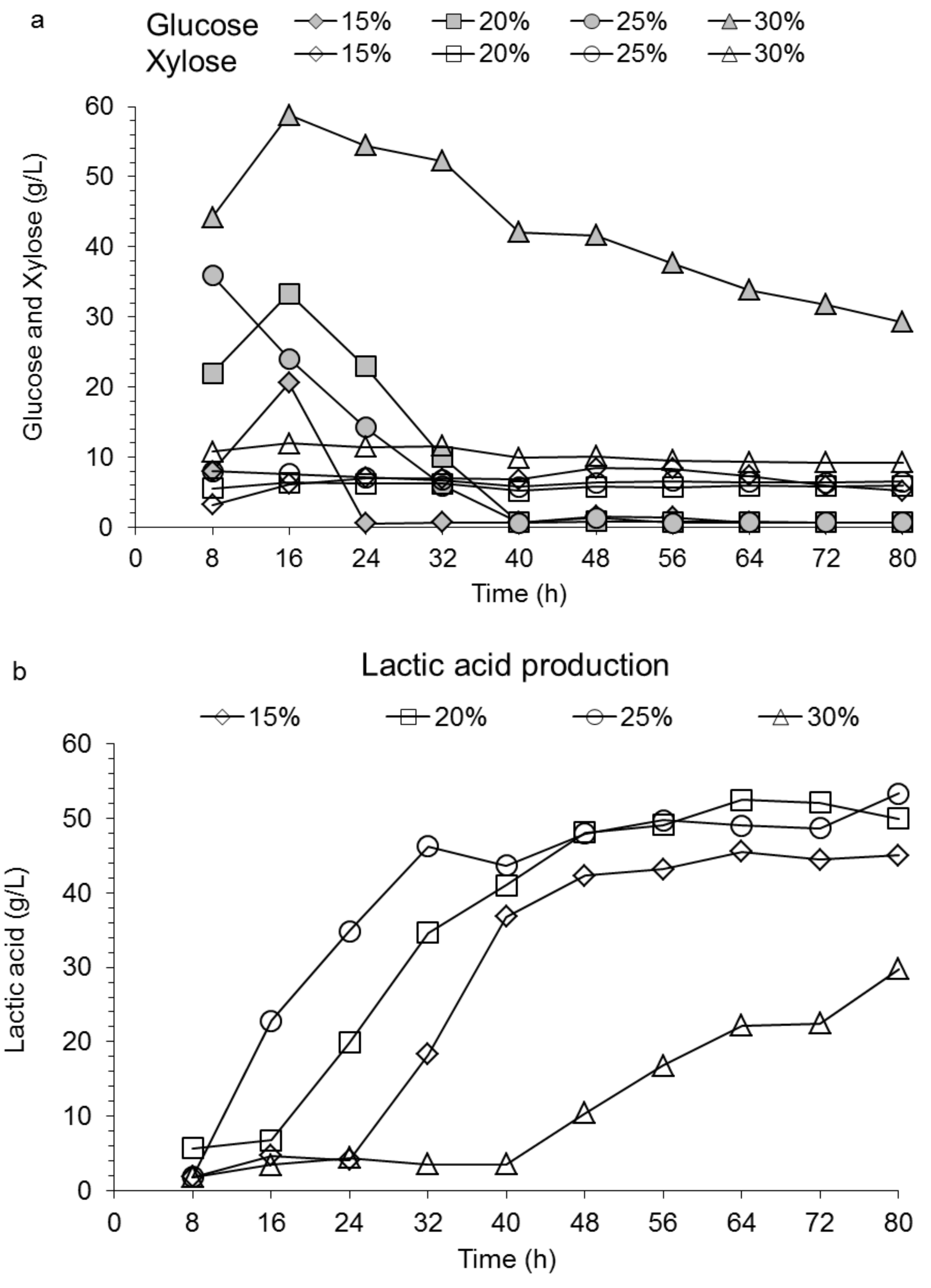
Figure 4.2 Effect of different Youtell #6 enzyme activity concentration on lactic acid production

Conditions: 50 ℃, pH 5.0 for 8 h pre-hydrolysis (before inoculation) and Youtell #6 enzyme activity concentrations were from 7 to 17 FPU/g DM. At Real SSF was carried out at 48 ℃, pH 5.5, solids loading 10.0% (w/w) and 10% of inoculation ratio with 150 rpm stirring rate.

4.3.2 Effect of different solids loading on the SSF of the Bio-Pretreated Corn Stover

Temperature at 48 ℃ and Enzyme activity was 10 FPU/g DM in a 5-L helical stirring reactor according to the two-step SSF procedure with 4 days bio-detoxification. Conditions: 50 ℃, pH 5.0 for 8 hours pre-hydrolysis (before inoculation). At Real SSF was carried out at 48 ℃, pH 5.5, and 10% of inoculation ratio with 150 rpm stirring rate.

The percent of solids loading was carried out and investigated from 15 to 30% (w/w) in the helical stirring reactor (HSB) in Figure 4.3, which shows the SSF performance at different solids loading with low dosage of enzyme activity. The glucose almost was agglomerated at 8 h, consumed out without 24 h after real SSF operation (Fig. 4.3a) and then remained at a low level in all solids loading case, individual glucose volume of solid loading of 25% (w/w) was used from start of real SSF and lactic acid production increasing (g/L) was 45.0, 50.0, and 53.3 at 15, 20, and 25% (w/w) solids loading, respectively on 72 hours later in Fig. 4.3b. Specially, at 30% (w/w) solid loading had high accumulated glucose (44.2 g/L) but this strain consumed them to be very slowly and kept glucose concentration at high level was 29.3 g/L after 72 h real SSF to lead lactic acid production was lower (29.7 g/L) than different solids loading (shown at Fig. 4.3b). The same SSF result (Zhao et al., 2013), the residual glucose of 30% solids loading appeared and the SSF was not complete. Besides, in 4 days for bio-detoxification CS with the inhibitors present were still in being at high level. These could explain that their present was effect on lactic acid production. Specially, when SSF was experiment at high solid concentration, inhibitory substrates such as FA and 5-HMF will high accumulate to be also caused effect of them on lactic acid yield and productivity. In our previous study, Zhao et al., 2013 indicated the lactic acid productivity was affected up to a high level of from 5.2 to 7.2 g/L of acid acetic and more than 0.5 g/L of 5-HMF, 0.5 g/l formic acid. In the experiment of Zhang et al., 2010b, for furfural, the concentration range was limited to less than 20 mg/g DM, indicating that furfural might be the most toxic toxin for *A. resinae* ZN1 growth.



Solid conc. (%, w/v)	15	20	25	30
Final lactic acid concentration (g/L)	45.05	50.02	43.81	29.75
Lactic acid yield (%)	62.67	51.51	43.81	17.28
Lactic acid productivity(g/L/h)	0.63	0.69	0.61	0.41

Figure 4.3 SSF of corn stover milling at different solids loading from 15 to 30%

In our previous study (Zhao et al., 2013). The different solids loading (15%, 20%, 25%, and 30% (w/w)) were designed for lactic acid fermentation. The result indicates that the SSF of *P. acidilactici* DQ2 could be operated at high solids loading of the pretreated and bio-detoxified CS and possessed the potential to achieve the high lactic acid titer.

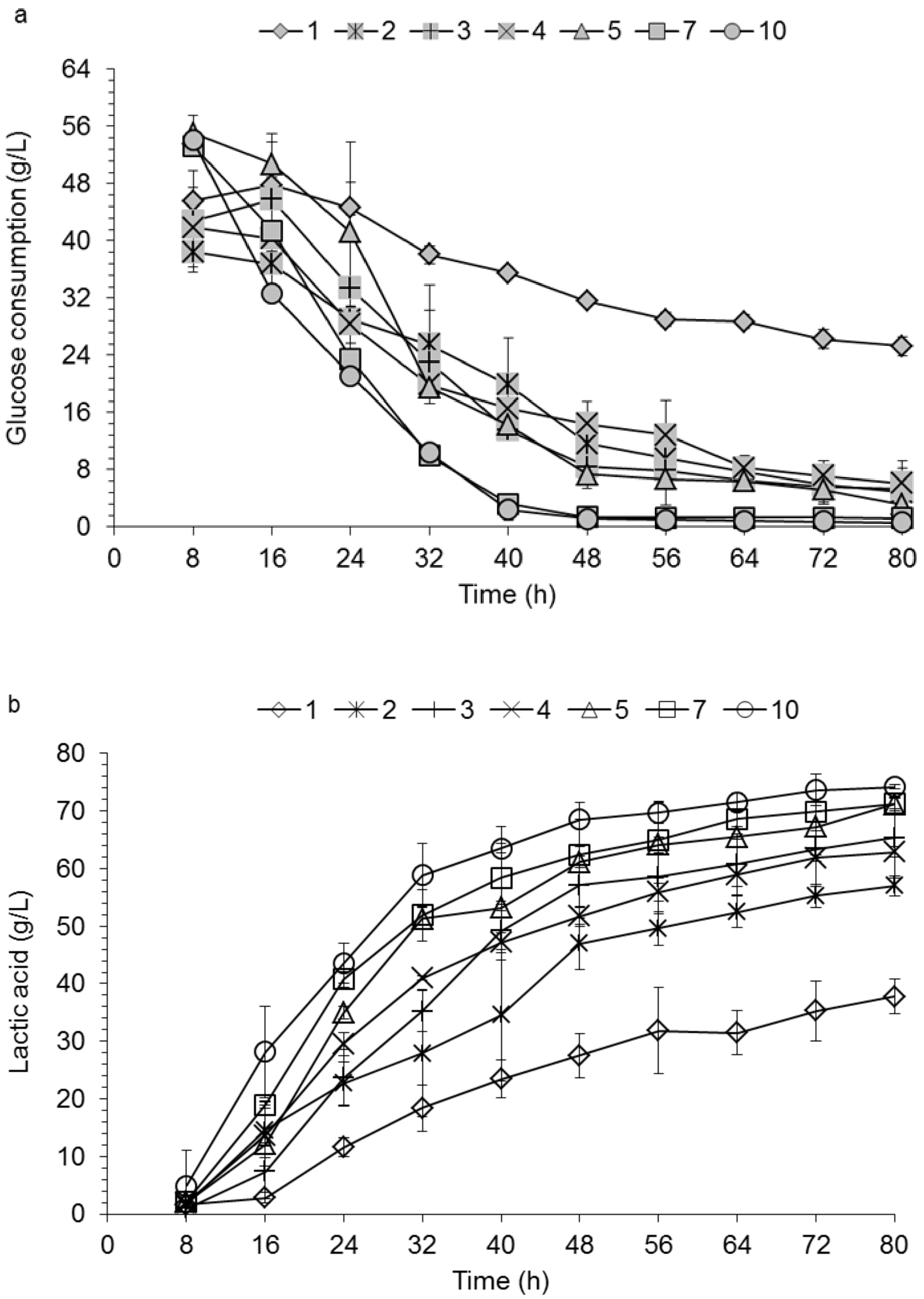
From our study could indicate that high solid loading of 25% (w/w) to be a rate of optimal lactic acid fermentation and it was also noticed that increasing the lactic acid titer did not worsen the other fermentation performances.

4.3.4 High solids loading for lactic acid production with various bio-detoxification days

Increasing lactic acid titer in the fermentation as a slurry type is crucially important for cost reduction of lactic acid production due to the great demand of lactic acid yield and productivity. The higher lactic acid titer inevitably requires higher lignocellulose solids loading in the simultaneous saccharification and fermentation. The pretreated CS was biologically detoxified to remove inhibitor substances using *Amorphotheca resinae* ZN1 following procedure in Zhang et al. (2010b) from 1 to 10 days. SSF at the solids loading of 25% (w/w) was carried out in a 5 L helical stirring bioreactor (Zhang et al., 2010a) for 72 hours. Inhibition of fermentation can be relieved upon treatment with the ligninolytic enzyme laccase, pre-fermentation by the filamentous fungus *Trichoderma reesei*, removal of non-volatile compounds, extraction with ether or ethyl acetate, and treatment with alkali or sulfite (Palmqvist, Bärbel Hahn-Hägerdal., 2000).

Jian Zhang et al. (2010b). Bio-detoxification used the solid-state culture of *A. resinae* ZN1 for 4 days. The results indicated that the toxin removal by bio-detoxification lessened the toxin inhibition to the fermenting yeast to a large extent and thus enhanced ethanol productivity. If the solids loss in the water washing is considered, the overall ethanol yield using the bio-detoxified CS was approximately 8% greater than the best result using the water washing CS.

Fig.4.4a shows that from bio-detoxification days, most of glucose yields were utilized by *P. acidilactici* DQ2. However, Glucose consumption of each day is different at 40 h after lactic fermentation by the strain DQ2 with different days of bio-detoxification. This illustrate indicate that glucose concentration was produced from 1-4 days of bio-detoxification using the cellulase enzyme (Youtell #6) lower than after 5-10 days of bio-detoxification in the same lactic fermentation condition. High selective conversion of glucose to lactic acid was obtained with *P. acidilactici* DQ2. The glucose consumption concentration at the end of 48 hours fermentation with this strain was almost using up at 7 and 10 days after bio-detoxification CS in Fig.4.4a. In the same time the glucose content could not exhausted after 48 and at 72 hours of the lactic acid fermentation using CS milling from 1 to 5 days of bio-detoxification CS by the SSF stage.



Bio-detoxification day(s)	1	2	3	4	5	7	10
Final lactic acid concentration (g/L)	21.60	57.02	65.28	62.89	71.20	71.28	74.21
Lactic acid yield (%)	21.59	48.65	52.97	50.76	57.43	57.29	59.76
Lactic acid productivity(g/L/h)	0.30	0.79	0.91	0.87	0.99	0.99	1.03

Figure 4.4 Comparison of lactic acid titers of *P. acidilactici* DQ2 strain at different bio-detoxified days (1-10 days) in the MRS simplify and SSF operation

a: Glucose consumption and b: lactic acid production. Conditions: The experiment was carried out in a 5-L helical stirring reactor at 25% (w/w) solids loading, 12 FPU/g DM according to the two-step SSF procedure, pH 5.5, and 150 rpm at 48 ^{o}C.

The profile of SSF could be divided into two distinct phases. During the first 6 hours, enzymatic saccharification was conducted sugar was released in the end. And then, the lactic acid concentration increased along with the fermentation time when *P. acidilactici* DQ2 was inoculated and added to SHB. After fermentation for 72 hours, the byproducts of the lactic acid fermentation yield by *P. acidilactici* DQ2 were calculated following Zhao et al. (2013) about lactic acid yield of the SSF and most of the glucose was converted into lactic acid.

The effect of bio-detoxified CS days from 1 to 10 days on the lactic acid titer of the two-step SSF and high solids loading was investigated, the results were shown in Fig. 4.4 at 48 ^{o}C and 25% (w/w) of solids loading. In our previous researched with the two-step SSF, as the first step SSF of the pretreated CSM, at first set-out, the pretreating temperature was elevated at 50 ^{o}C in 6 h. The glucose, released was accumulated at 6 h of the real SSF (step two), in which from cellulose hydrolysate stage and the lactic acid production

The effect of inhibition at different bio-detoxified CS days may be mainly reason for the decreasing lactic acid yield. In Figure 4.4 shows that the glucose consumption and the lactic acid production rates using CSM with 12 FPU/g DM of enzyme dose in the HSB after 10 days of bio-detoxification pretreatment CS were better than bio-detoxified days (from 1 to 7 days) with 25% solid loading thus leading to higher lactic acid yield and productivity was from 59.76% and 1.03 (g/L/h), respectively in comparison with all bio-detoxification CS, respectively. A lactic yield and productivity were obtained during 72 hours of fermentation (Fig. 4.4b). This result was comparable with previous reported by Zhao et al. (2013) in the SSF of CS was 60.6% lactic acid yield and 0.9 g/L/h lactic acid productivity.

The performance of *P. acidilactici* DQ2 strain for lactic acid from bio-pretreatment of CS for 1 to 3 days was unfeasible because of inhibitors present still keep high level from as Zhang et al. (2010a) have shown the inhibitor effect of for furfural and 5-HMF on *A. resinae* ZN1 growth, for furfural, the concentration range was limited to less than 20 mg/g DM, indicating that furfural might be the most toxic toxin for *A. resinae* ZN1 growth. But glucose was almost used after 24 hours in the same xylose was not used by this strain, compatibly as Zhao et al., (2013) reported.

However, to improve fermentative efficiency such optimum conditions have been studied for lactic acid fermentation from MRS simplify with 5 g YE as temperature and pH. The maximum productivity and specific growth rate of *P. acidilactici* DQ2 in these media were obtained at temperature of 48 ^{o}C and pH 5.5. One more time, this study indicated that the strain used in the simultaneous saccharification and fermentation (SSF) for high titer lactic acid production at 25% (w/w) high solids loading of corn stover milling.

4.4 Conclusions

Production of lactic acid from corn stover milling, a low-cost, a potentially renewable and feedstock, by *P. acidilactici* DQ2 was shown in this study. Economy and convenience for enzyme hydrolysis uniform size reduction of corn stover milling. After the optimal bio-detoxification CS time and fermentation conditions, SSF of corn stover milling was established to produce lactic acid with high concentration and high yield at very high solids loading after 5 to 10 days for detoxification corn stover using the fungal mycelium (*A. resinae* ZN1). SSF stage use corn stover milling on 5, 7, and 10 day of bio-detoxified corn stover at 25% (w/w) solids loading with lactic acid yield of 71.2, 71.3 and 74.2% and productivity of 0.99, 0.99 and 1.03 g/L/h, respectively. The lactic acid productivity can be further increased with an increase in substrate loading.

CHAPTER V

General conclusions and Prospects

Conclusions

1. A new LAB strain, *Pediococcus acidilactici* DQ2, was isolated and identifie using 16S rDNA sequencing. Lactic acid fermentation performance of *P. acidilactici* DQ2 from different carbon sources such as glucose, fructose, and xylose was studied. The inhibitor tolerance of *P. acidilactici* DQ2 were studied with different inhibitor concentrations of furfural, HMF, acetic acid, formic acid, levulinic acid, vanillin etc. *P. acidilactici* DQ2 demonstrated the unique properties of excellent thermo- and inhibitor-tolerance.
2. The newly isolated *P. acidilactici* DQ2 strain was applied to the lactic acid fermentation using Jerusalem artichoke tubers (Jat) as the raw materials. A high inulinase activity was found in three commercially available glucoamylase enzymes. Its origin was investigated and two proteins in the commercial glucoanylases were identified as the potential enzymes showing inulinase activity. One of the commercial glucoamylases, GA-L New from Genencor, was used for Jerusalem artichoke tubers (Jat) hydrolysis and a high hydrolysis yield of fructose was obtained. The simultaneous saccharification and lactic acid fermentation (SSF) of Jat was carried out using GA-L New as the inulinase and P. acidilactici DQ2 as the fermenting strain. A high lactic acid titer, yield, and productivity of 111.5 g/L, 0.46 g/g DM, and 1.55 g/L/h, respectively, were obtained within 72 hours. The enzyme cost using the commercial glucoamylase as inulinase was compared to that using the typical inulinase and a large profit margin was identified. The results provided a practical way of Jat application for lactic acid production using cheap commercial glucoamylase enzyme.
3. Corn stover was used as the raw materials for lactic acid production using the same strain *P. acidilactici* DQ2 through the newly developed dry biorefinery processing technology. The effect of the inhibitor removal intensity by the biodetoxification strain *Amorphotheca resinae* ZN1 on the lactic acid fermentation of *P. acidilactici* DQ2 was carefully investigated. Different amount of inhibitor residues in the pretreated corn stover materials severely affected the performance of lactic acid fermentation. The complete removal of all the inhibitor substances after 7-10 days of bio-detoxification led to the higher lactic acid yield and titer in the simultaneous saccharification and lactic acid fermentation process. The effect of disk milling of the pretreated corn stover

on the lactic acid fermentation was also investigated. After the deep biodetoxification and disk milling, the SSF at 48 ^{o}C, pH 5.5, and 25% (w/w) solids loading in 5L of helical agitated bioreactor resulted in the lactic acid titer, yield, and productivity of 74.2 g/L, 59.76 %, and 1.03 g/L/h, respectively. The result provided a practical process option for cellulosic lactic acid production using agro-lignocellulose residual in the chain of biomaterial resources.

4. The biorefinery processing technology of inulin and lignocellulose biomass for production of lactic acid were established with high lactic acid titer and yield using new microorganisms and new processing technology in this thesis. These studies paved the way of industrial application of value added chemicals from cheap and abundant agricultural biomass.

Prospects

1. The effect of nutrients such as yeast extract (YE) and other nitrogen sources on the lactic acid production should be considered for cost reduction because lactic acid fermentation is highly nutrients adding process.
2. Jerusalem artichoke tubers need to be pretreated by enzyme so the sugar concentration (glucose, fructose, and sucrose) can be increased.
3. Different cellulase enzymes with high enzymatic catalysis performance should be tested for improving the hydrolysis yield of corn stover.
4. The simultaneous saccharification and lactic acid fermentation by *P. acidilactici* DQ2 need to be studied on different agriculture biomass as feedstocks.

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1. **Thai Ha Dao,** Jian Zhang, Jie Bao. 2013. Characterization of inulin hydrolyzing enzyme(s) in commercial glucoamylases and its application in lactic acid production from Jerusalem artichoke tubers (Jat). **Bioresource Technology** 148:157-162.
2. Kai Zhao, Qingan Qiao, Deqiang Chu, Hanqi Gu, **Thai Ha Dao**, Jian Zhang, Jie Bao. 2013. Simultaneous saccharification and high titer lactic acid fermentation of corn stover using a newly isolated lactic acid bacterium *Pediococcus acidilactici* DQ2. **Bioresource Technology** 135:481-489.
3. Deqiang Chu, Kai Zhao, Qian Wu, **Thai Ha Dao**, Jie Bao. 2011. Isolation of a highly tolerant lactic acid bacterium and high titer L-Lactic acid fermentation using lignocellulosic Feedstock. **Journal of East China University of Science and Technology (Natural Science Edition)** 37:521-522.

In Preparation

4. **Thai Ha Dao**, Yanqing He, Jian Zhang, Jie Bao. 2013. Corn stover milling (CSM) for lactic acid production using the simultaneous saccharification and lactic acid fermentation (SSF). In preparation.

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