CHAPTER 4

Direct conversion of starch to lactic acid by *L. plantarum* S21

4.1 Introduction

Lactic acid (2-hydroxypropionic acid or 2-hydroxypropanoic acid, CH_3-CHOHCOOH) is ranked one of the most important organic acids firstly found in the early 1780 (Holten *et al*. 1971) according to world high production annually of approximately 370,000 MT (Miller *et al*. 2011). Approximately 85% of lactic acid is demanded for food and food related applications as food additives due to its wide range of functions such as flavoring enhancer in margarine and butter, gelling agent in jellies and jams, acidulant in dressing and beverages, pH regulator in candies and food preservatives for improvement of microbial quantity. The other 15% of lactic acid is demanded for non-food industries including cosmetic, pharmaceutical and chemical industry. Besides, lactic acid and its salts are served as chemical compositions in a wide variety of chemical products such as cleaning agents, neutralizer and descaling agents (John *et al*. 2009).

About 90% of global lactic acid comes from the biological production rather than producton by chemical synthesis (Vijayakumar *et al*. 2008) regarding to there are some limitations of chemical synthesis including the environmental issues and petrochemical resources, substrates for lactic acid synthesis and non-specified D- or L-form of lactic acid (Wee *et al*. 2006). The biological production is performed through fermentation of lactic acid producing microorganisms including bacteria, yeast, fungi, microalgae and cyanobacteria (Abdel-Rahman *et al*. 2013). Depending on the organisms, the biological fermentation provides many advantages in contrast to the chemical synthesis for example low production temperature, low energy consumption,

high optical purity of lactic acid and availability of various renewable substrate for the production (Abdel-Rahman et al., 2011). *Lactobacillus* sp. is known as the largest genera of lactic acid bacteria with 80 species (Wee *et al*. 2006; Abdel-Rahman *et al*. 2013) and it has long been utilized profitably for commercial lactic acid production (Reddy *et al*. 2008).

Cheap raw materials are alternative ways to use as substituted materials for lactic acid production and to reduce the cost effectiveness in the commercial application. Starch is an option to replace the use of refined sugars as substrate regarding the availability, renewable resource, plenty and cheapness (Wee *et al*. 2006; John *et al*. 2009) and it is therefore receiving attentions at this moment (John *et al*. 2009). Nevertheless, few lactic acid bacteria are able to produce lactic acid directly from starch. Although two steps of lactic acid fermentation (TSF), simultaneous saccharification and fermentation (SSF), and simultaneous liquefaction, saccharification and fermentation (SLSF) are recently trialed along with the use of starch degrading enzymes in which provided either single or two steps of fermentation (Wang *et al*. 2010), the enzyme cost still affected to the lactic acid production considerably and have investment of commercial lactic acid increased and is consequently inapplicable in commercial lactic acid production. To avoid using enzymatic methods in direct conversion of starch to lactic acid, amylolytic lactic acid bacteria (ALAB) are concerned to have the conversion become single step without addition of starch degrading enzymes. This strategy reduces both cost from the use of starch degrading enzyme and complicated process according direct lactic acid production. However, there are few high potential ALAB for direct lactic acid production in industrial scale using either cheap raw substrates or non-complex medium and no industrial production of lactic acid using this strategy is available commercially up to now (Miller *et al*. 2011). It is inferred that the difficulty of gelatilized starch when high concentration of starch is applied in order to produce high concentration of lactic acid. The reason probably causes from limitation of mass and energy transfer. In addition, attempts uses starchy wastes for direct lactic acid have less attention because low starch concentration is obtained from the wastes (Ohkouchi and Inoue 2006 and Pintado *et al.* 1999).

This chapter described feasibility in direct lactic acid production from starch by *L. plantarum* S21 was evaluated and applicability in production of lactic acid using high concentration of starch was also investigated. Moreover, feasibility determination of starchy wastewater utilization was investigated in order to produce high concentration of lactic acid from starchy wastewater.

4.2 Literature review

4.2.1 Lactic acid and its applications

Lactic acid, $CH_3CH(OH)COOH$, a water soluble, odorless, non-volatile organic acid (Reddy *et al*. 2008). It is classified as GRAS (Generally Recognized AS Safe) (Datta *et al*. 1995) and therefore utilized in many applications including food and non-food industry. In food industry, lactic acid is generally used to be food preservative agent because it gives low acid condition so food can be prevented from bacterial spoilage and food shelf life is increased. Favoring is also one of the applications in food. On the other hand, lactic acid is needed for non-food industry for instances, textile, medical, pharmaceutical and cosmetic industry. According to the acidulant property, lactic acid is used for deliming hides in leather industry (Reddy *et al.* 2008). In addition, it has water retaining capacity that is suitable for using as moisturizer in cosmetic formulation. As well as the application pharmaceutical, lactic acid is used as ingredient in ointments, lotion and parental solution. Interestingly, lactic acid is used as monomer to generate biopolymer called polylactic acid (PLA), a biodegradable polymer for many medical uses such as surgical sutures, prostheses and controlled drug delivery systems (Wee *et al.* 2006).

4.2.2 Synthesis of lactic acid

There are two forms of lactic acid occurring in nature, D and L-lactic acid which can be produced either by chemical and biological method. For chemical synthesis, it needs to hydrolyze lactonitrile, a derivative of petrochemicals, by strong acid which provides racemic mixture of D and L-lactic acid. There are other methods to produce lactic acid but it still needs high pressure and temperature that is non-economically

(Reddy *et al*. 2008). In contrast to the biological methods, the lactic acid is produced by lactic acid producing microorganisms through fermentation processes. Moreover, the fermentations provide a number of advantages rather than chemical synthesis for instances, mild condition for production, environmentally friendly, high optical purity of L- or D-lactic acid particularly various carbon sources for lactic acid fermentation. Alternative cheap raw materials are studied and developed for production of lactic acid by lactic acid producing microorganisms

4.2.3 Lactic acid bacteria (LAB)

Lactic acid bacteria is a group of lactic acid producing bacteria found to be Gram positive, rod and cocci shape and non-spore formation and catalase negative (Axelsson 2004). They produce lactic acid as a major product from refined sugars through fermentation processes. This group of bacteria has been divided into 3 main groups based on the fermentative pattern. However, the majority of lactic acid bacteria is generally known as *Lactobacillus* spp. (Reddy *et al*. 2008).

Homofermentative: this group of LAB produces almost 85 to 90% of lactic acid from carbohydrate metabolism such as *Lactobacillus plantarum, L. lactis*, *Enterococcus faecalis*, *Bacillus* sp. and *Kluyveromyces lactis*.

Heterofermentative: this group of LAB produces only 50% of lactic acid with other organic acids, alcohols, akdehydes, ketones and carbon dioxide as products of carbohydrate metabolism such as *Lactobacillus casei*.

Less well known heterofermentative species which produce DL-lactic acid, acetic acid and carbon dioxide Litchfield (2009) has also divided lactic acid bacteria into 3 groups according to Bergey's Manual of Determination Bacteriology (9th edition)

- Gram-positive cocci: *Enterococcus* spp., *Lactococcus* spp., *Pediococcus*spp., *Sacchrococcus* sp. and *Sterptococcus*spp.
- Endospore-forming Gram-positive rods and cocci: *Bacillus* spp. and *Sporolactobacillus* sp.

- Regular, nonsporing Gram-positive rods: *Lactobacillus* sp.

4.2.4 Substrates for lactic acid fermentation

A number of different substrates have been used as carbon sources for production of lactic acid through the fermentation. However, most publications and reports still attend in the use of refine sugars as substrate particularly glucose. Although agricultural wastes like molasses can be replaced those sugars, the demand of this carbon source remains to be increasing from time to time according to the requirement for ethanol production.

Agricultural wastes are become interesting because a large number are disposed. Sarkar *et al.* (2012) reviewed that there were approximately 14 billion tons of agricultural wastes worldwide including rice straw, wheat straw, corn straw and bagasse and some were served as substrate for ethanol production. However, these substrates needs pretreatment steps to produce refine sugar from biomass for example steam explosion, ammonium explosion, CO_2 explosion, microwave, acid or alkaline pretreatment or enzymatic saccharification (Sarkar *et al*. 2012). The additional steps consequently cause high investment of lactic acid production. Xue *et al*. (2012) pretreated corn stover by acid and saccharified with cellulose enzyme to obtained refined sugars for lactic acid production by *Bacillus* sp. XZL4. Newly interesting alternative substrates for lactic acid production have been studying and recently Nguyen *et al.* (2012a) used hydrolyzate from green microalga, *Hydrodictyon reticulum* as carbon source for lactic acid production.

4.2.5 Starch as substrate for lactic acid production

Regarding to the limitations of using agricultural wastes, starch biomass is suitable, abundant and available annually. Although starch saccharification and liquefaction by acid/alkaline hydrolysis or enzymatic methods are somehow necessary to hydrolyze starch like substrate hydrolysis in agricultural wastes, there is a great choice without any pretreatment steps for lactic acid production from starch. Several

past decades, the amylolytic lactic acid bacteria (ALAB) have been screened and a number of novel strains have been being discovered during decades.

Amylolytic lactic acid bacteria (ALAB) are groups of lactic acid bacteria capable of producing amylolytic enzymes constitutively along with producing lactic acid efficiently. They can be either homo- or heterofermentative LAB. With their exclusive characters, lactic acid can be produced directly from starch. Hence, starch pretreament steps or saccharification and liquefaction are probably discarded from the fermentation steps of lactic acid production. Up to now, there are a few ALAB, have been discovered and utilized for example *L. plantarum* A6 (Pintado *et al.* 1999), *L. plantarum* MTCC 1407 (Panda *et al.* 2008), *L. amylphilus* GV6 (Vishnu *et al.* 2000), *L. amylovorus* ATCC 33620 (Xiaodong *et al.* 1997), *L. paracasei* B41 (Petrova and Petrov 2012), *L. manihotivorans* (Ohkouchi *et al.* 2006), *Lactococcus lactis* subsp. *Lactis* B84 (Petrov *et al.* 2008), *Enterococcus faecium* (Shibata et al. 2007) and *Streptococcus bovis* 148 (Narita *et al.* 2004).

4.2.6 Lactic acid production from starch

Productions of lactic acid from starch are typically divided into two strategies on criteria of preparation of refine sugar and lactic acid fermentation.

Two steps of lactic acid fermentation (TSF)

Two step of lactic acid fermentation is conventional method for lactic acid production from starch. In fact, most lactic acid bacteria have naturally inability in converting starch to lactic acid. Therefore, direct conversion of starch to lactic acid is unavailable. Starch liquefaction and saccharification step are required to provide refine sugars for further lactic acid production. Either acid/alkaline hydrolysis or amylolytic enzymes is performed as typically performed in lactic acid fermentation from agricultural residues. The enzymatic method is in fact used preferably and practically used in industrial scale of lactic acid production. It is therefore conducted to two steps of lactic acid production. First, starch is gelatinized by heating, liquefied by α-amylase and saccharified by glucoamylase and refined sugars are subsequently generated.

Second, the fermentation of lactic acid is performed using refine sugars from the first procedure. For example, Lu *et al.* (2009) accomplished in lactic acid production from acorn starch by TSF. Acorn starch was liquefied and saccharified by α-amylase and amyloglucosidase, respectively to provide refined sugars in the first step. The proper concentration of refine sugar was used as a carbon source for production of lactic acid by *L. rhamnosus* HG 09. Wee *et al.* (2008) also used two steps of lactic acid production by *E. faecalis* RKY1 using starch hydrozate from enzymatic method.

However, there were other methods to provide refined sugars before initiating of the fermentation regarding many reports published in the past decades. It was called simultaneous saccharification and fermentation (SSF). This strategy, starch containing materials was primarily liquefied by α-amylase to provide oligosaccharides and limit dextrin. Subsequently, additional α-amylase and amyloglucosidase were added in inoculum prior to starting the fermentation using liquefied starch as carbon source. Hofvendahl *et al.* (1999) liquefied starch by using Termamyl 120 L and heating at 95^{o}C to prepare starch suspension. Then simultaneous saccharification by Fungamyl and AMG and lactic acid fermentation was performed. This strategy was also reported in case of lactic acid production from starch by *L. delbrueckii* NCIM 2365 (Anuradha *et al.* 1999); in addition to lactic acid production from protease-treated wheat bran by a combination culture of *L. casei* NCIMB 3254 and *L. delbrueckii* NCIM 2025 (John *et al.* 2006).

Single step of lactic acid fermentation

Production of lactic acid can be manufactured in only single step by discarding starch pretreament step, known as simultaneous and direct lactic acid fermentation. The processes are based on the lactic acid bacteria utilized.

- **Simultaneous liquefaction, saccharification and lactic acid fermentation (SLSF)**

According to the limitation in starch degradation of lactic acid bacteria, either additional starch degrading enzymes or amylolytic producing

microorganisms are applied to liquefy and saccharify starch for lactic acid fermentation by lactic acid bacteria. Therefore, lactic acid production from starch becomes only single step. Linko *et al.* (1996) satisfactorily attained lactic acid yield from direct conversion of starch to lactic acid by *L. casei* NRRL B-441 through SLSF. The enzymes applied during the fermentation for simultaneous liquefaction and saccharification, were α-amylase and glucoamylase. Wang *et al.* (2010) accomplished the highest lactic acid productivity of 1.6 g/L h from SLSF by *L. rhamnosus* CALS compared to different strategies of lactic acid production from starch including two steps fermentation (TSF) and simultaneous saccharification and fermentation (SSF). The lactic acid yields obtained from TSF, SSF and SLSF were summarized in Table 4.1.

Table 4.1 Comparison of lactic acid volume from starch by different lactic acid bacteria operated by different fermentation strategies

Microorganisms	Strategy	Starchy substrate (g/L)	lactic acid (g/L)	LA/substrate (g/g)	References
Enterococcus faecalis RKY1	Two step	125	129.9	1.04	Wee *et al*. (2008)
L. rhamnosus HG 09	Two step	100	57.61	0.58	Lu *et al.* (2009)
L. casei NRRL B-441	SLSF	130-170	140-162	0.95-1.07	Linko *et al*. (1996)
L. coryniformis ATCC 25600	SLSF	100	97.13	0.97	Nguyen *et al*. (2013)
L. paracasei LA104	SLSF	100	91.61	0.92	Nguyen *et al*. (2013)
L. rhamnosus CASL	SLSF	275	187.2	0.68	Wang *et al*. (2010)
L. delbrueckii NCIM 2356	SSF	100	79	0.79	Anuradha *et al.* (1999)
L. casei NCIMB 3254 *L. delbrueckii* NCIM 2025	SSF (co-culture)	130	123	0.95	John *et al.* (2006)
Lactococcus lactis ATCC 19435	SSF	180	179	0.99	Hofvendahl *et al*. (1999)

- **Direct lactic acid fermentation**

Depending on lactic acid bacteria used for lactic acid production from starch, direct lactic acid production from single lactic acid bacteria had many great advantages. Particularly, starch degrading enzymes were not necessary for either starch liquefaction or starch saccharification. Single step was easy to handle. The fermentation was performed under specialty of lactic acid bacteria called "amylolytic lactic acid bacteria (ALAB)". The currently publications about direct lactic acid production from starch by ALAB was concluded in Table 4.2. This strategy had however no current reports available in industrial scale (Miller *et al.* 2011).

4.2.7 Fermentation strategies for direct lactic acid fermentation by amylolytic lactic acid bacteria

Not many strategies of lactic acid production from starch have been reported in several past decades. Most fermentation strategies of lactic acid production operated by batch fermentation system rather than repeated batch, fed batch and continuous fermentation as reported above. Advantages and disadvantages of different fermentation modes described by Abdel-Rahman *et al.* (2013) are shown in Table 4.3. Although the description mentioned the limitation of batch fermentation, lactic acid fermentation from starch still operates by batch system since the limitation from high substrate concentration which leads to high viscosity of gelatinized starch and is subsequently difficult to manufacture. Moreover, growth of lactic acid bacteria is inhibited at high starch concentration according to substrate inhibition and poor mass and energy transfer. There are some papers reported other fermentation modes for direct conversion of starch to lactic acid. Son and Kwon (2013) produced lactic acid directly from starch by *L. manihotivorans* LMG18010 using fed-batch fermentation system. The lactic acid yield of 71.40 g/L with 0.60 g/L·h was obtained from 100 g/L starch feeding. Bomrungnok *et al.* (2012) attained highest productivity of lactic acid of 4.53 g/L·h from 20 g/L of cassava starch by using high cell density of *L. plantarum* SW14 operated by continuous fermentation system. As mentioned, it is too difficult to apply high concentration of starch to fermentation system for bioconversion of starch to lactic acid

by lactic acid bacteria. Therefore, enzymatic hydrolysis is pretreated onto starch prior to fermentation of lactic acid for example Nolasco-Hipolito *et al*. (2012) performed repeated batch system for lactic acid production from liquefied sago starch by *Enterococcus faecium* No. 78. The average lactic acid yield of 19.2 g/L and 36.3 g/L was obtained from first 5 and last 6 cycles, respectively.

4.2.8 Amylolytic enzymes from amylolytic lactic acid bacteria

Typically, starch degrading enzyme from amylolytic lactic acid bacteria are in group of amylases. Most of them are α-amylase followed by a few cases of amylopullulanase and maltogenic α-amylase as concluded in Table 4.4. Most of them are monomeric enzymes and generally have high molecular weight compared to other source of amylase producing bacteria particularly *Bacillus* sp.. The amylases from lactic acid bacteria are not stable as high as 70^{o}c or higher but they are seemed to be acid stable amylases and are therefore very suitable for direct lactic acid production from starch

Table 4.2 Comparison of lactic acid and lactic acid production efficiency obtained from different ALAB

Amylolytic lactic acid bacteria	Substrate	Lactic acid*	LA/initial substrate	References
L. plantarum A6	10.33 g/L total sugar	8.1 g/L	0.81 g/g	Pintado *et al*. (1999)
	45 g/L raw starch	41.0 g/L	0.91 g/g	Giraud *et al*. (1994)
L. plantarum MTCC 1407	55 g/L raw starch	23.7 g/L	0.43 g/g	Panda *et al*. (2008)
L. amylophilus GV6	100 g/L soluble starch	75.7 g/L	0.76 g/g	Vishnu *et al*. (2002)
	70 g/L corn starch	49.1 g/L	0.70 g/g	Vishnu *et al*. (2002)
	40 g/L potato starch	32.0 g/L	0.80 g/g	Vishnu *et al*. (2002)
	90 g/L starch	72.6 g/L	0.84 g/g	Vishnu *et al*. (2000)
	60 g/L corn starch	49.0 g/L	0.81 g/g	Vishnu *et al*. (2000)
L. paracasei B41	40 g/L starch	37.3 g/L	0.93 g/g	Petrova and Petrov (2012)
L. manihotivorans	50 g/L starch	40.7 g/L	0.81 g/g	Ohkouchi *et al*. (2006)
Lactococcus. lactis subsp. *Lactis* B84	18 g/L starch	18.0 g/L	0.31 g/g	Petrov *et al*. (2008)
Enterococcus faecium	20 g/L sago starch	16.6 g/L	0.83 g/g	Shibata *et al*. (2007)
Streptococcus bovis 148	20 g/L raw starch	14.7 g/L	0.74 g/g	Narita et al. (2004)

Table 4.3 Advantages and disadvantages of different fermentation strategies

Fermentation mode	Advantages	Disadvantages
Batch	Simple operation	Low productivity
	High production concentration	Substrate and/or end product inhibition
	Reduced risk of contamination	
Fed-batch	Overcome substrate inhibition problem	End production inhibition
	High production concentration	Difficult to conduct optimal design
Repeated batch	Time-saving process	Requirement of special devices (e.g., hollow fiber-module)
	Labor-saving	or special connection lines used for cell concentration
	Omission of seed preparation time	
	Short main culture and high growth rates	
Continuous	High productivity	Incomplete utilization of the carbon source
	Control growth rate	
	Less frequency shut down process	

Source: Abdel-Rahman *et al.* (2013)

Table 4.4 Amylases from ALAB

Lactobacilli	strain	Amylase	MW (kDa)	Structure	References
L. amylophilus	GV6	Amylopullulanase	90	monomeric	Vishnu *et al*. (2006)
L. manihotivorans	LMG18010	α-amylase	135	monomeric	Aguilar *et al*. (2000)
L. amylovorus	NRRL B4540	α-amylase	140	monomeric	Cassler and Imam (1991)
L. plantarum	A6	α-amylase	150, 50	monomeric	Giraud *et al*. (1993)
Streptococcus bovis	JB1	α-amylase	77	monomeric	Freer (1993)
Streptococcus bovis	148	α-amylase (intr)	57	monomeric	Satoh (1997)
		α-amylase (extr)	-	-	Satoh (1993)
Lactococcus lactis	IBB	α-amylase	121	monomeric	Adam *et al*. (2010)
L. gasseri	ATCC33323	Maltogenic amylase	66	-	Oh *et al*. (2005)
L. plantarum	L137	Amylopullulanase	216	monomeric	Kim *et al*. (2008)

4.3 Materials and Methods

4.3.1 Media

De Man-Rogosa Sharpe medium (Appendix A)

Optimized medium (Chakkuruang, 2011)

4.3.2 Chemical reagents

HPLC grade of lactic acid and malic acid were purchased from Sigma Aldrich. Sulfuric acid (H_2SO_4) was purchased from Labscan Analytical Science. All chemicals used for preparation of systhetic wastewater were analytical grade. Cassava starch used as carbon source was purchased from Bangkok Inter Food Co. ltd, Thailand which guaranteed by the company to have no protein, fat, minerals and sugar content.

4.3.3 Equipment and instrument

Lactic acid was analyzed by Shimazu High Performance Liquid Chromatography (HPLC) using Aminex HPX-87H column (Bio-Rad Laboratories, Richmond, Calif., USA).

4.3.4 Media preparation and sterilization

The modified MRS (mMRS) medium was used for lactic acid fermentation consisted (g/L) of 10.0 g peptone, 10.0 g beef extract, 5.0 g yeast extract, 1.0 g tween80, 2.0 g K_2HPO_4, 5.0 g $CH_3COONa \cdot 3H_2O$, 2.0 g $C_6H_5O_7(NH_4)_2H$, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.2 g $MnSO_4 \cdot H_2O$ and 10.0 g cassava starch as a sole carbon source and adjusted to pH 6.8 prior to sterilization. Cassava starch was added up to desired concentrations for further direct conversion of high starch concentration to lactic acid. The mentioned mMRS composition was dissolved in starchy wastewater in order to directly ferment to lactic acid from starchy wastewater. Cassava starch was added up to the desired concentration for direct conversion of starchy wastewater containing high starch

concentration to lactic acid. The sterilization was performed by autoclaving at 121^{o}C for 15 min.

4.3.5 Preparation of seed inoculum and culture condition

A single colony of *L. plantarum* S21 was inoculated in mMRS broth containing 10 g/L cassava starch as a sole carbon source. The inoculated medium was static incubated at 37^{o}C for 16-18 h for using as seed inoculum.

4.3.6 Effect of different concentration of mMRS medium on direct lactic acid production

The mMRS medium was diluted with water to 25, 50 and 75% (v/v) dilution. Thereafter, cassava starch was added to the final concentration of 10 g/L and adjusted pH to 6.8. The mMRS medium was used as control treatment and 10 g/L cassava starch solution was also provided. A 1%(v/v) of seed inoculum was transferred to 200 mL of each medium in 250 mL laboratory bottle (Duran, Germany) and static incubated at 37°C for 48 h. Samples were taken out for 3 h intervals to determine viable cell, pH and lactic acid content. The best medium was selected and confirmed its capacity by determination of kinetic parameters in 1 liter fermentation.

4.3.7 Direct conversion of starch to lactic acid using diluted mMRS medium in 1 and 10 liter fermenter operated by batch system

The 10% of seed inoculums was transferred to 1 L fermenter containing 50% diluted mMRS broth and mMRS broth containing 10 g/L of cassava starch as a carbon source with 90% of working volume. The fermentation was static incubated at 37^{o}C and operated by batch system. The samples were collected 3 h intervals to determine lactic acid content, pH, viable cell and substrate consumption. Kinetic study of direct lactic acid fermentation in both media was compared. The specific growth rate (μ) was determined as (1/X)(dX/dt) where X represented dried cell weight, product yield ($Y_{p/s}$) was defined as lactic acid formation (ΔP) to substrate consumption (ΔS), biomass yield was defined as biomass formation (ΔX) to substrate consumption, specific substrate uptake rate (Q_s) was determined as (1/X)(dS/dt), specific rate of product formation (Q_p)

was determined as (1/X)(dP/dt) and lactic acid production efficiency was determined as ΔP/ΔS of overall fermentation.

4.3.8 Direct conversion of starch to lactic acid using diluted mMRS medium in 1 liter fermenter operated by repeated batch system

Repeated batch of direct conversion of starch to lactic acid was performed in 1 liter fermenter (90% working) volume containing 50% diluted mMRS medium containing 10 g/L cassava starch as a carbon source. A 10%(v/v) of seed inoculum was transferred to the fermenter and static incubated at 37^{o}C for 48 h. Every 48 h, the 90%(v/v) of the fermentation culture was replaced with the same volume of the fresh mMRS medium. The fermentation was continued by the same procedure until 10 batch cycles were attained. Lactic acid and viable cell was monitored during the medium replacement.

4.3.9 Efficiency evaluation on direct conversion of starch to lactic acid

To increase the lactic acid yield using diluted medium and to evaluate efficiency and feasibility in direct conversion of cassava starch to lactic acid, various concentrations of cassava starch of 10, 25, 50, 75 and 100 g/L were used as a carbon source for the above selected medium. Seed culture of *L. plantarum* S21 was transferred to each medium to final concentration of 10%(v/v) and mixed thoroughly before static incubating at 37^{o}C. The pH of the cultures was maintained at pH 6.0 during the fermentation by adding 10 N NaOH.

4.3.10 Direct conversion of starch to lactic acid using starchy wastewater

Analysis of starchy wastewater

Starchy effluent (SE) derived from a rice noodle factory in Chiang Mai, Thailand, was investigated. The factory processed rice at approximately 10–15 tons per day in order to distribute rice noodle products to Thai consumers in the upper northern region. The starchy wastewater was discharged at approximately 60 cubic meters per day. A part of the starchy wastewater from the milling process was collected and used for lactic

acid production. The basic composition of the starchy wastewater, including total solids, ash, total carbohydrates, starch, reducing sugar, total proteins, and moisture content, were analyzed prior to preparing the medium. Quantification of starch was determined by measuring the light absorption of the iodine–starch complex color at a wavelength of 620 nm (Xiao *et al*., 2006). Reducing sugar was determined by DNS method (Miller *et al*., 1959). Total protein was determined according to Kjeldahl method (1883)

Medium preparation, sterilization, seed inoculum preparation and culture condition

The medium was provided based on modified MRS (mMRS) medium that consisted of (g/L) 5.0 g peptone, 5.0 g beef extract, 2.5 g yeast extract, 1.0 g K_2HPO_4, 2.5 $CH_3COONa{\cdot}3H_2O$, 1.0 g di-ammonium hydrogen citrate, 0.5 mL Tween80, 0.1 g $MgSO_4{\cdot}7H_2O$, and 0.1 g $MnSO_4{\cdot}H_2O$. The composition described above was dissolved in SE to a final concentration of 10 g/L total carbohydrate. For a high starch concentration level, the super quality grade of rice starch (RS) was purchased from Bangkok Inter Food Co. ltd, Thailand, which was guaranteed by the company to have no protein, fat, minerals, and sugar contents, and was further adjusted to the desired concentration levels of total carbohydrates. Sterilization was performed by autoclaving at 121^{o}C for 15 min. Seed inoculum was prepared by spiking a single colony of *L. plantarum* S21 in 50 mL of mMRS containing 10 g/L RS as a carbon source and was statically incubated at 37℃ for 16–18 h. Culture condition was carried out statically at 37^{o}C.

Determination of feasibility to direct convert starch to lactic acid using starchy effluent from rice noodle manufacturing process

The growth study of *L. plantarum* S21 was investigated by transferring 10% (v/v) inoculum to mMRS medium containing either SE or RS as the sole carbon source. Lactic acid and viable cells were monitored every 6 for 48 h. Total carbohydrates was also investigated in order to calculate the lactic acid yield.

Determination of direct conversion of high concentration of starch to lactic acid using starchy effluent from rice noodle manufacturing process

To observe the possibility of direct conversion of high starch concentrations from starchy wastewater, 10% of the inoculum size was transferred to the mMRS medium containing a starchy carbon source at total carbohydrate levels of 10, 20, 40, 60, and 80 g/L. During the fermentation stage, the pH of the culture was maintained at 7.0 by the addition of 10 N NaOH. Lactic acid, total carbohydrates, and amylase activity were determined.

Direct lactic acid fermentation using starchy wastewater containing high starch concentration

A 10% of inoculum size was transferred to mMRS medium containing starchy carbon source at level of starch concentration of 10, 20, 40, 60 and 80 g/L. During the fermentations, culture condition was maintained at pH 7.0 by addition of 10 N NaOH. Lactic acid, total carbohydrate, amylase activity and reducing sugar were determined.

4.3.11 Analytical methods

Lactic acid productivity was determined by titration method and confirmed by HPLC using an Aminex HPX-87H column (Bio-Rad Laboratories, Richmond, Calif., USA) with 0.005 M H_2SO_4 as a mobile phase at flow rate of 0.8 mL/min. Column temperature was carried out at 65°C and separated sample was detected with a UV detector at 210 nm. The pH was measured by pH meter (Eutech, Sigapore). Viable cell was determined by pour plate on MRS agar. Total sugar was determined by phenol-sulfuric method (Dubois *et al*. 1956). Viable cell was determined by pour plate method. Amylase activity was determined as method previously described. Lactic acid yield was defined as formation of lactic acid to total carbohydrate consumption ($\Delta P/\Delta S$). The productivity defined as rate of lactic acid formation ($\Delta P/\Delta T$).

4.4 Results

4.4.1 Effect of different concentrations of mMRS medium on lactic acid production

To investigate feasibility of direct lactic acid fermentation from economical starchy medium, *L. plantarum* S21 was cultured in various concentrations of mMRS medium containing 10 g/L starch as an initial concentration. It was found that lactic acid was produced rapidly within the first 15 h of fermentation in 0-75% dilution of mMRS and thereafter it was constant at around 9 g/L until 48 h of cultivation. Here, it seemed like the strain entered to log phase in this condition more rapidly than in other dilutions since lactic acid was growth associated product. However, there was no significant difference of lactic acid yield among mMRS without and with 50 and 75% dilution as shown in Figure 4.1. The maximum lactic acid of approximately 9 g/L was also obtained from the indicated conditions at the end of fermentation. On the other hands, small amount of lactic acid was found when cultured the bacterium in 10 g/L of cassava starch solution.

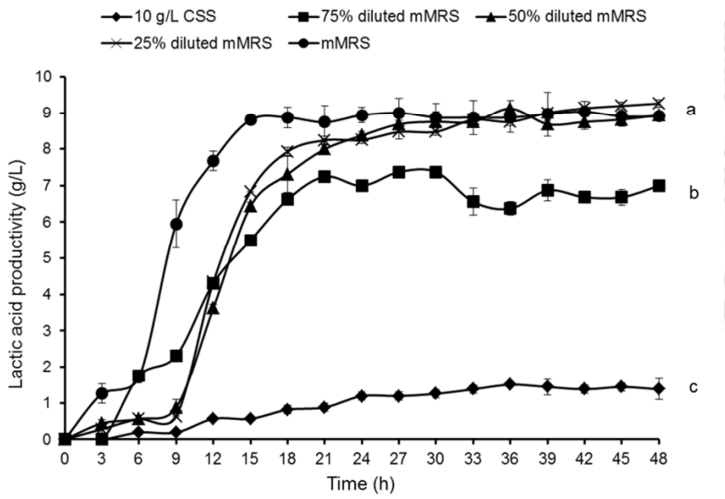

Figure 4.1 Profiles of lactic acid production from different concentration of mMRS medium containing 10 g/L cassava starch as the sole carbon source

4.4.2 Direct conversion of starch to lactic acid using mMRS and optimal medium operated by batch system

To evaluated capability and efficiency of *L. plantarum* S21 in direct conversion of cassava starch to lactic acid using economic medium including diluted mMRS medium and optimal medium designed by Chakkuruang (2011), Kinetic values were determined

and considered for further used. The mMRS medium was used as a control medium according its plentiful compositions. The profiles of lactic acid production in 1 liter fermenter were presented in Figure 4.2a-d and the kinetic parameters were calculated and concluded in Table 4.5. Considering the diluted mMRS medium, all kinetic parameter presented in Table 4.5 were closed to that from mMRS medium but the lactic acid production efficiency was slightly lower than that without dilution. However, the lactic acid of 89% production efficiency satisfied us and this data clarified the efficiency of the bacterium in production of lactic acid. In the agreement with 1 liter production, the scaling up to 10 liter fermenter was also successful when using the diluted medium. Either kinetic values or the production efficiency were surprisingly similar to that obtained from 1 liter fermentation. The cheapest medium as described by Chakkuruang (2011) was also applied for lactic acid production in both 1 and 10 liter fermentation. The result revealed that the specific growth rate of *L. plantarum* in optimal medium was still not different to that in mMRS. However, the specific rate of production formation and the specific uptake rate were decreased to 50% of these values obtained from mMRS medium. This could be seen in Figure 4.3d. The lactic acid was not produced exponentially as it was in mMRS medium. In contrast, it was slightly increased until the maximum at 24 h of fermentation. However, the yield of lactic acid was considerably acceptable regarding to 82% production efficiency was attained at the end of fermentation. When the fermentation was scaled up to 10 liter fermenter, the production efficiency was still maintained at 84%.

Table 4.5 Kinetic values of direct conversion of cassava starch to lactic acid using mMRS and optimal medium

Parameters	μ (h^{-1})	Q_p ($g\ g^{-1}\ l^{-1}$)	Q_s ($g\ g^{-1}\ l^{-1}$)	$Y_{p/s}$ ($g\ g^{-1}\ h^{-1}$)	$Y_{x/s}$ ($g\ g^{-1}\ h^{-1}$)	Production efficiency (%)
mMRS	0.24	1.76	1.64	0.93	0.13	95
Diluted mMRS (1L)	0.26	1.66	1.51	0.91	0.16	89
Diluted mMRS (10 L)	0.24	1.90	1.78	0.94	0.12	88
Optimal medium (1L)	0.27	0.73	0.76	0.97	0.35	82
Optimal medium (10L)	0.23	0.70	0.85	0.82	0.27	84

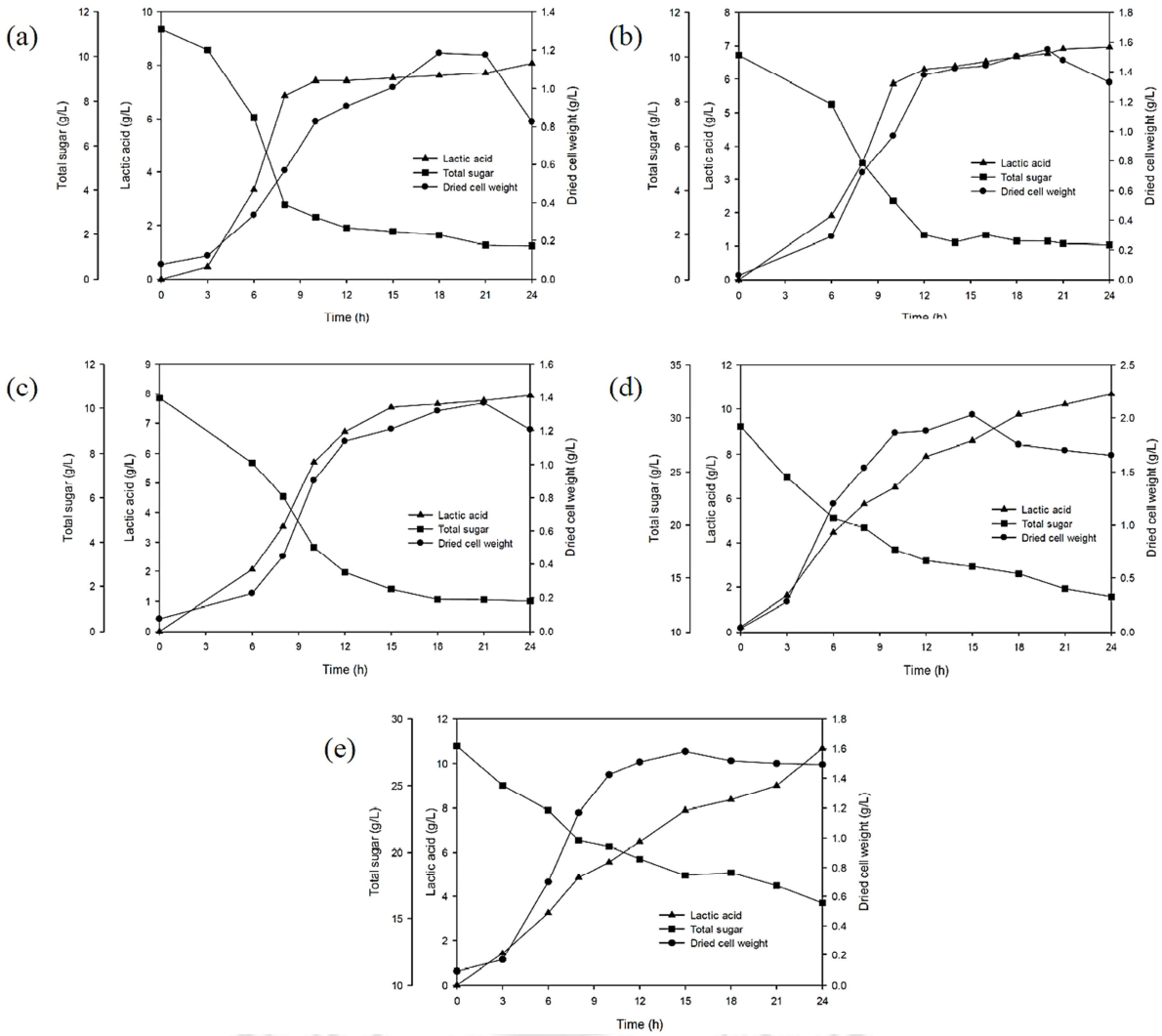

Figure 4.2 Profiles of direct lactic acid production from cassava starch using mMRS medium in 1 liter fermenter (a), 50% diluted mMRS medium in 1 and 10 liter fermenter (b and c), optimal medium in 1 and 10 liter fermenter (d and e)

4.4.3 Direct conversion of cassava starch to lactic acid operated by repeated batch system

Lactic acid production from starch operated by repeated batch system revealed efficiency of *L. plantarum* S21 obviously since the production efficiency did not definitely changed within 10 cycles of fermentation. Each cycle was operated for 48 h to attain the maximum lactic acid yield of approximately 9 g/L. When carefully

observed at 24 h of fermentation, approximately 8 g/L of lactic acid was perfectly obtained for all 10 cycles (Figure 4.3).

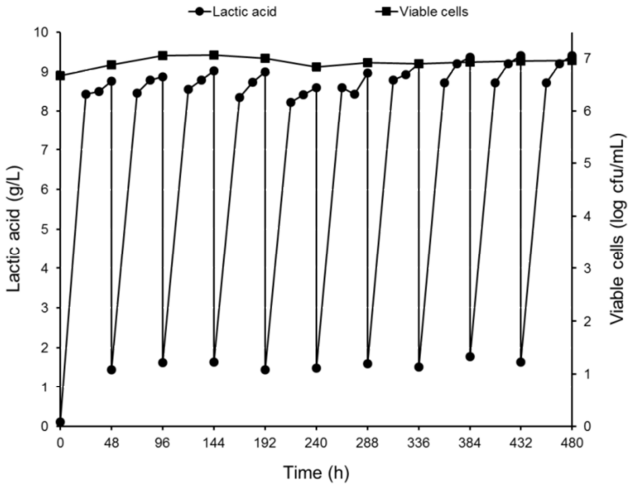

Figure 4.3 Profile of direct conversion of cassava starch to lactic acid using mMRS medium operated by repeated batch system

4.4.4 Feasibility in direct conversion of high starch concentration to lactic acid

Starch was converted to lactic acid remarkably at initial cassava starch concentration ranging from 10-100 g/L with more than 90% conversion. The extraordinary lactic acid concentration of 9.41, 24.48, 46.50, 74.33 and 94.04 g/L was produced perfectly from 10, 25, 50, 75 and 100 g/L starch at 48, 48, 72, 120 and 120 h, respectively (Figure 4.4a). Detection of amylase was gone along with the determination of lactic acid and the extracellular amylase activity produced during the fermentation was demonstrated in Figure 4.4b. Of cassava starch concentration ranging from 10-75 g/L, the maximum amylase was obtained in range of 13.5-23.5 U/mL in the respect of cassava concentration at 24 h of cultivation except for at 100 g/L of cassava starch; the amylase remained constantly of 13.5 U/mL until 72 h of cultivation.

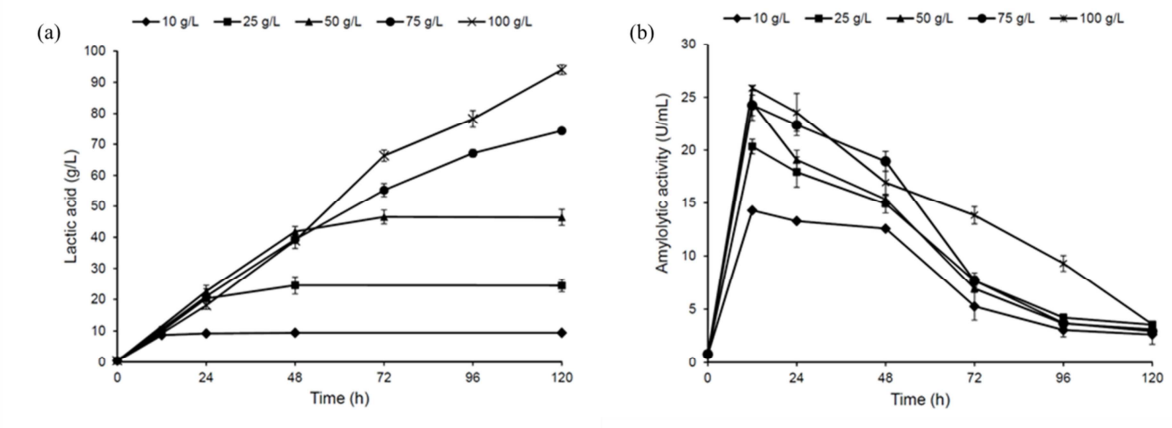

Figure 4.4 Effect of different concentration of cassava starch on production of lactic acid (a) and α-amylase (b)

4.4.5 Direct conversion of starch to lactic acid using starchy effluent from rice noodle manufacturing process

Analysis and selection of starchy wastewater

The starchy effluent as a by-product of the rice noodle manufacturing process used in this study was derived from the broken rice caused by washing and milling processes, from which 60 m^3 of effluent was discarded per day.

Table 4.6 Content of starchy effluent and gelatinized starchy waste based on wet weight

Components	Starchy wastewater % (g/100 g)*	Gelatinized starchy waste % (g/100 g)*
Total solid	2.55±0.12	10.37±0.01
-Ash	0.72±0.21	0.21±0.01
-Total protein	0.06±0.00	0.07±0.00
-Total carbohydrate	1.78±0.40	10.10±0.26
-Starch	1.43±0.02	9.58±0.30
-Reducing sugar	0.17±0.07	0.48±0.01
Moisture	97.45±0.12	89.63±0.01
pH	3.39±0.10	ND**

*Mean values are presented with standard deviation (SD)

**ND= Not determined

It basically contained 18.0±0.9 total carbohydrates, 14.4±0.2 starch content, 1.8±0.1 reducing sugar, and 0.6±0.02 g/L total protein with a pH value of 3.39±0.10 (Table 4.6). The gelatinized starchy waste achieved from this steaming process had 10.10±0.26 g/100 g gelatinized starchy waste with 9.58±0.30 g starch/100 g calculated to approximately 100 g/L of total carbohydrates, as well as other components.

Direct conversion of starch to lactic acid using starchy effluent from rice noodle manufacturing process

The growth study of *L. plantarum* S21 in SE was determined based on 50% diluted mMRS medium in comparison with mMRS medium alone with 10 g/L of total carbohydrates. Results showed that it was possible to grow *L. plantarum* S21 in mMRS containing SE, which remarkably produced more lactic acid than RS (Fig. 4.5), indicating that SE did not affect the bacterial growth and was consumed equally to that of RS. The lactic acid amounts obtained from both media were insignificantly different at 24 and 48 h of fermentation (9.0±0.3, 9.4±0.4 g/L from RS and 8.9±0.2, 9.2±0.1 g/L from SE), in agreement with the lactic acid yields. This demonstrated insignificant differences between the two media at the above-mentioned stages. The results indicated that *L. plantarum* S21 displays clear feasibility for direct conversion of SE to lactic acid.

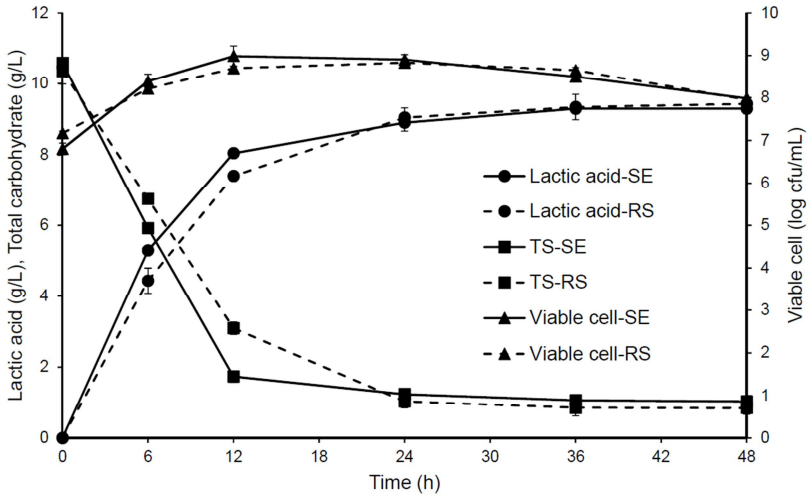

Figure 4.5 Profile of lactic acid production, total carbohydrate consumption and viable cells during lactic acid fermentation by *L. plantarum* S21 at 37^{o}C in mMRS broth containing 10 g/L rice starch (RS) and starchy effluent (SE).

Direct conversion of high concentration of starch to lactic acid using starchy effluent from rice noodle manufacturing process

Before creating the pilot scale of lactic acid production, the SE was used as a part of the carbon source in mMRS medium by supplementation of RS to initiate the final total carbohydrate content of 10–80 g/L. Considering the amylase activity during lactic acid fermentation, we found that the higher concentrations of the starchy carbon source induced *L. plantarum* S21 to produce higher amount of amylase (Figure 4.6A). The highest amylase activity of 24 U/mL came from the fermentation of 60 and 80 g/L of total carbohydrates. Amylase activity was increased to the maximum at 6, 12, 18, 24, and 36 h of lactic acid fermentation from 10, 20, 40, 60, and 80 g/L total carbohydrate, respectively, and then decreased thereafter. The maximum lactic acid of 11.2±1.1 and 19.0±0.3 g/L with lactic acid productivity of 0.94±0.13 and 1.58±0.03 g/ L·h, which were obtained at 12 h of fermentation of medium containing 10 and 20 g/L of starch, respectively. The result revealed a rapid consumption of the starchy carbon source of *L. plantarum* S21 and apparently produced lactic acid, as shown in Figure 4.6B and 4.6C. In addition, lactic acid of 37.8±2.1, 47.3±0.2, and 59.4±2.5 g/L were obtained from the use of 40, 60, and 80 g/L of total carbohydrates at 48 h of fermentation. At higher concentration levels, total carbohydrate readings of 60 and 80 g/L seemed to limit lactic acid production since lactic acid yield was decreased significantly to the range of 0.90 g/g (Table 4.7**)**, however, the total lactic acid content (59.4±2.5 g/L) and productivity (1.23±0.07 g/ L·h) obtained from 80 g/L initial total carbohydrates were the highest when compared to those of 40 and 60 g/L.

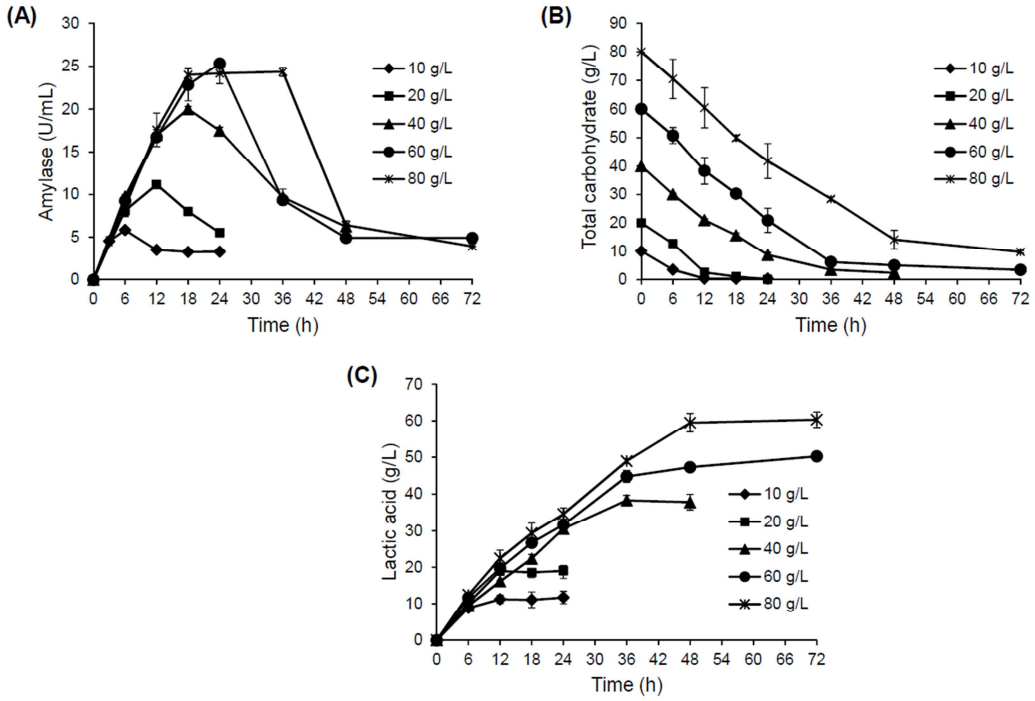

Figure 4.6 Profile of amylase activity (A), total carbohydrate (B) and lactic acid (C) during direct conversion of rice noodle factory effluent containing various concentrations of the starchy carbon source to lactic acid by *L. plantarum* S21 at 37^{o}C

Table 4.7 Comparison of lactic acid, lactic acid yield and lactic acid productivity of *L. plantarum* S21 from starchy effluent with various concentrations of rice starch. The presented values were obtained from the steady state of lactic acid formation in each substrate concentration.

Total carbohydrate (g/L)	Time (h)*	Lactic acid (g/L)*	Lactic acid yield (g/g)*	Productivity (g/L·h)*
10	12	11.23±1.11^{e}	1.17±0.14^{a}	0.94±0.13^{cd}
20	12	18.96±0.28^{d}	1.09±0.02^{ab}	1.58±0.03^{a}
40	48	37.82±2.12^{c}	1.00±0.06^{bc}	0.79±0.06^{d}
60	48	47.30±0.18b^{b}	0.86±0.03^{d}	0.98±0.00^{c}
80	48	59.44±2.54^{a}	0.90±0.07^{cd}	1.23±0.07^{b}

4.5 Discussion

A large number of publications reported lactic acid production by LAB particularly lactobacilli using MRS medium for reason; LAB were fastidious microorganisms and required nutrient rich media regarding to the limitation of vitamin B synthesis (Hofvendahl and Hahn-Hagerdal 2000). Therefore still growth factors, amino acids, vitamins, fatty acids, purines and pyrimidines were necessary and typically occurred in the growth medium (Kwon *et al.* 2000). The MRS medium was excellent for the growth of lactobacilli (Leroy and Vuyst 2001) and had rich compositions so it was widely used for lactic acid production. However, it contained high concentration of some expensive components especially peptone, yeast and beef extract or meat extract. The replacement of this organic nitrogen typically reduced the cost from production medium that related to 15-20% of lactic acid production cost (Matthew and Fu 2001). In this study, different diluted mMRS broths were examined in order to find out feasibility of using non-complex medium for direct conversion of starch to lactic acid and also tried to use mMRS medium effectively. The results wondered us since *L. plantarum* S21 still remained the capability of direct conversion of starch to lactic acid using 50% diluted mMRS containing 10 g/L cassava starch without any pH control agents but the highest production yield had been delayed for 6 h compared to the that without dilution. It was suggested that the bacterium may require some components affected to its lag phase. However, the lactic acid yield was not different to that from undiluted mMRS medium at the end of fermentation. We assumed that *L. plantarum* S21 probably needed non-complex medium regarding its origin, Thai fermented rice noodle where rice flour was fermented in water for a few days, washed, milled until the fermented flour became slurry. The slurry was fermented again in tank before it was pre-gelatinized and extruded in hot water to form noodle (Keatkrai and Jirapakkul 2010). So, the condition was certainly under low pH and lack of nutrients. Direct lactic acid production from starch by *L. plantarum* S21 using 50% diluted medium was repeatedly performed and evaluated its feasibility and efficiency by determination of kinetic parameters. It was found that μ, Q_p, Q_s, $Y_{p/s}$, $Y_{x/s}$ and lactic acid production efficiency were certainly similar to that obtained from the use of mMRS without dilution. Same kinetic values not only obtained which 1 liter cultivation was applied but also in 10 liter fermentation.

The result verified efficiency and feasibility in term of applicability of *L. plantarum* S21 in diluted starchy medium.

Considering cheaper medium for lactic acid production, optimal medium consisting of inexpensive organic nitrogen source was also evaluated for kinetic values. Chakkuruang (2011) designed cheap medium for production of lactic acid from cassava starch based on MRS medium by *L. plantarum* S21 using response surface methodology. Expensive compositions including peptone, beef extract and yeast extract were replaced by cheap materials capable of promoting growth of *L. plantarum* S21 and production of lactic acid. To evaluate potentiality of the medium in direct lactic acid production, kinetic study was determined. From the result, *L. plantarum* S21 had the same specific growth rate but the Q_p and Q_s were lower than that obtained from mMRS medium. The result indicated that either yeast extract or beef extract or peptone was still necessary for the bacterium. This caused slowly production of lactic acid. This phenomenon was similar to the case of Yan *et al*. (2001) when yeast extract increased fermentation rate of lactic acid by *L. amylovorus*. However, lactic acid production efficiency was still acceptable in term of lactic acid yield and production efficiency. Scaling up to 10 liter fermentation did not cause of low lactic acid yield, growth and rate of substrate consumption since similar μ, Q_p, Q_s, $Y_{p/s}$ and $Y_{x/s}$ was satisfactorily obtained. *L. plantarum* A6 had rather similar kinetic values to *L. plantarum* S21 when it was culture in MRS broth containing different carbon source at level of 10 g/L including glucose, starch, glycogen and mussel processing waste (Pintado *et al.* 1999). As well as *L. manihotivarans* LMG18010, it produced lactic acid at different concentration of starch including 20, 50, and 100 g/L. The highest specific growth rate obtained when the 50 g/L of starch was applied (Son *et al*. 2013). Repeated batch operation system was one of the simplest fermentation systems. It was time saving and labor saving process. No seed preparation step was required because high cell density from the rest of batch fermentation was used as seed inoculum for further fermentation. Therefore, the fermentation became continuously for several batch fermentation (Abdel-Rahman *et al*. 2013). As described, *L. plantarum* S21 showed its high efficiency and capability in direct lactic acid production. Lactic acid was produced steadily for at least 10 cycles in every cycle. Only Nolasco-Hiplito *et al*. (2012) reported direct lactic acid

production from liquefied sago starch operated by repeated batch system. Up to now there was no report of direct lactic acid production operated by repeated batch system. This may be due to difficulty of substrate preparation. High viscosity of gelatinized starch generally occurred while production of lactic acid using high concentration of starch.

In previous experiment, direct lactic acid productions were performed using 10 g/L which was low starch concentration. For industrial application, lactic acid production typically preferred lactic acid yield as high as 100 g/L in order to reduce cost from downstream processes (Miller *et al.* 2011). Therefore, attempt directly converted high concentration of cassava starch to lactic acid in single step. Potentiality of direct conversion of high concentration of starch to lactic acid by *L. plantarum* S21 using diluted mMRS medium was investigated at 37^{o}C under pH control condition at pH 6.0 in order to utilize the diluted medium efficiently for high concentration of lactic acid production. The result revealed the excellent character of *L. plantarum* S21 in production of lactic acid from high concentration of cassava starch. High yield of lactic acid of 94 g was produced from 100 g initial cassava starch in which similar to other lower concentrations of cassava starch. The perfect conversion rate and yield of lactic acid using mMRS medium was such higher than that from other papers of direct lactic acid production by ALAB reported so far. The comparison of lactic acid and initial substrate concentration were concluded in Table 4.8. Recently, Petrova and Petrov (2012) presented a novel strain of amylolytic *L. paracasei*. It was capable of direct conversion of 50 g/L of starch to 37.3 g/L of lactic acid. Not many publications reported the production of lactic acid using as high concentration as 100 g/L. Vishnu *et al*. (2000) isolated *L. amylophilus* GV6 that capable of best producing lactic acid of 72.6 g/L and 49 g/L from 90 g/L and 60 g/L of soluble starch and corn starch, respectively. However at high concentration of 100 g/L of soluble starch, corn starch and potato starch, 75.7 g/L and only 39.2 and 38.1 g/L of lactic acid was produced in the respect substrates used as a sole carbon source. To our knowledge, the 50% diluted mMRS medium was used effectively throughout the experiment and *L. plantarum* S21 exhibited high efficiency and feasibility in high production yield from high substrate concentration as starch. At the present time, lactic acid production was often trialed at

low concentration of starch owing to the fact that viscosity was ordinarily found at high concentration of starch after gelatinization or sterilization step and this restricted the mass and energy transfer during the fermentations. Therefore, direct production of high concentration of lactic acid was unavailable and impractical for application in industrial scale. In contrast to the case of *L. plantarum* S21, it was capable of secreting effective extracellular enzyme. Considering at 100 g/L of cassava starch, the medium became semi-solid after seed inoculation. Interestingly, the bacterium was still able to grow and the effective amylolytic enzyme was capable of hydrolysis cassava starch substrate astonishingly. The entire semi-solid medium became liquid within 48 h and the fermentation was fermented straightforward. As previous discussion, the amylolytic enzyme was such stable under low pH condition; therefore, the decreasing of enzyme activity did not much affect the starch degradation. In addition, the enzyme was able to degrade starch to maltose rapidly according to the evidence from starch hydrolysis as results in Chapter 2. Decreasing of amylolytic activity was probably because of the high ionic strength from NaOH titration. Ionic strength was an important factor affecting enzyme activity. The movement of binding charges of substrate to enzyme and the movement of charges within the catalytic site was influenced by ionic composition in the medium (Chaplin and Bucke 1990). The *Haloferax* sp. and *Halorubrum* sp. α-amylase were influenced by Na^{+}/Mg^{2+} ratio. The increasing of NaCl concentration negatively affected to the enzyme activity and decreased the hydrolysis of starch (Enache *et al.* 2009)

Table 4.8 Comparison of lactic acid contents obtained from different ALAB

Amylolytic lactic acid bacteria	Substrate	Lactic acid	Production efficiency	Incubation time	pH controlling agent	References
L. plantarum S21	10 g/L cassava starch	9.2 g/L	0.92 g/g	24	NaOH	This study
	25 g/L cassava starch	24.5 g/L	0.92 g/g	48		
	50 g/L cassava starch	46.5 g/L	0.98 g/g	72		
	75 g/L cassava starch	74.3 g/L	0.99 g/g	120		
	100 g/L cassava starch	94.0 g/L	0.94 g/g	120		
L. plantarum A6	10.33 g/L total sugar	8.1 g/L	0.81 g/g	48	NaOH	Pintado *et al.* (1999)
	45 g/L raw starch	41.0 g/L	0.91 g/g	72	NaOH	Giraud *et al.* (1994)
L. amylophilus GV6	100 g/L soluble starch	75.7 g/L	0.76 g/g	96	$CaCO_3$	Vishnu *et al*. (2002)
	70 g/L corn starch	49.1 g/L	0.70 g/g	96	$CaCO_3$	Vishnu *et al*. (2002)
	40 g/L potato starch	32.0 g/L	0.80 g/g	72	$CaCO_3$	Vishnu *et al*. (2002)
	90 g/L starch	72.6 g/L	0.84 g/g	96	$CaCO_3$	Vishnu *et al*. (2000)
	60 g/L corn starch	49.0 g/L	0.81 g/g	96	$CaCO_3$	Vishnu *et al.* (2000)
L. paracesei B41	40 g/L starch	37.3 g/L	0.93 g/g	48	NaOH	Petrova and Petrov (2011)
L. manihotivorans LMG18010	50 g/L starch	40.7 g/L	0.81 g/g	140	NaOH	Ohkouchi *et al.* (2006)
Lactocuccus lactis subsp. *Lactis* B84	18 g/L starch	5.5 g/L	0.31 g/g	144	NaOH	Petrov *et al.* (2008)
Enterococcus faecium	20 g/L sago starch	16.6 g/L	0.83 g/g	15	NaOH	Shibata *et al.* (2007)
Streptococcus bovis 148	20 g/L raw starch	14.7 g/L	0.74 g/g	96	NaOH	Narita *et al.* (2004)

The starchy effluent as a by-product of the rice noodle manufacturing process used in this study was derived from the broken rice caused by washing and milling processes, from which 60 m^3 of effluent was discarded per day. The starch content of this SE is close to that of the food waste collected from cafeterias (Ohkouchi and Inoue, 2006) and mussel processing waste (Pintado *et al*., 1999), and was used for the investigation of direct lactic acid production. Probst *et al*. (2013) mentioned that bio-waste with high acidic and high organic content is the preferred habitat of lactic acid bacteria, thus, it could be an alternative substrate for lactic acid production. Few attempts have been made to utilize starchy waste for lactic acid production (Pintado *et al*., 1999; Kim *et al.*, 2003; Ohkouchi and Inoue, 2006). The result revealed that SE did not affect the bacterial growth and was consumed equally to that of RS. The results indicated that *L. plantarum* S21 displays clear feasibility for direct conversion of SE to lactic acid. However, not only have the soaking and milling processes generated starchy effluent, but the steaming process has also been found to discard a great deal of high concentrations of gelatinized flour. At high level of starchy substrate, out result was of great value and meaningful in the research of direct lactic acid production using food waste by amylolytic lactic acid bacterium, since high concentrations of lactic acid, as well as greater yields and higher productivity were certainly obtained from the experiment. So far, Pintado *et al*. (1999) used amylolytic lactic acid bacteria, including *L. plantarum* A6, *L. manihotivorans* LMG18010, *L. plantarum* R10101/2 and *Pediococcus* sp. VA403, for direct conversion of mussel processing wastes for lactic acid production, but the study reported that the conversion rate of food waste contained low concentrations of either starch or total carbohydrates. High concentration levels of food waste were perfectly converted into lactic acid by *L. manihotivorans* LMG18011 (Ohkouchi and Inoue, 2006), with the highest yield of 1.11 g/g and similar production efficiency (81.2%) to that of our report, but the concentration levels of the carbon source and the achieved lactic acid were obviously lower. Few papers have assessed the direct conversion of a high concentration of starch into lactic acid since substrate inhibition has typically been found (Son and Kwon, 2013). Hence, this study is of particular interest, because the capabilities of *L. plantarum* S21 are distinct when compared other previously mentioned studies. The results revealed that the starchy effluent from the rice noodle manufacturing process is feasible for using as a substrate

in the direct conversion of starch to lactic acid by *Lactobacillus plantarum* S21. We therefore expect to combine the starchy effluent and gelatinize starchy waste for the direct conversion of starchy substrate to lactic acid in the next study in order to utilize starch effluent most efficiently.

4.6 Conclusion

This chapter demonstrated high potentiality of *L. plantarum* S21 in direct conversion of starch to lactic acid. The success in chapter was concluded as followed;

1. Direct conversion of cassava starch to lactic acid using mMRS medium was satisfactorily accomplished at level of starch concentration of 10 g/L.

2. The 50% diluted mMRS medium could be used to replace mMRS as *L. plantarum* S21 still maintained its potentiality in production of lactic acid. Not only operated by batch fermentation but this medium was also feasible for the production operated by repeated batch system.

3. *L. plantarum* S21 was capable of producing lactic acid from synthetic and starchy wastewater. It moreover fermented starchy wastewater to produce the highest lactic acid yield of 0.96 g/g with the maximum lactic acid of 9.3 g/L from initial starch concentration of 10 g/L

4. *L. plantarum* S21 showed its great merit of producing lactic acid from cassava starch up to 100 g/L with high conversion rate of 0.9 g/g.

5. Starchy wastewater could be used as substrate for lactic acid production.

6. To the best of our knowledge, *L. plantarum* S21 have such high potentiality of direct lactic acid production from starch and it is feasible to produce industrially lactic acid according to excellent properties of amylolytic enzyme and its capability in production of lactic acid.