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THESIS

UTILIZATION OF HIGH SUGAR SAP SQUEEZED FROM OIL PALM TRUNK FOR LACTIC ACID FERMENTATION

NISA SAELEE

A Thesis Submitted in Partial Fulfillment of
the Requirements for the Degree of
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Oil palm trunk (OPT) juice is considered as a potential substrate for lactic acid fermentation because it is cheap, abundant, renewable and contains high sugar contents. This study attempted to investigate the use OPT juice as a substrate for lactic acid production by *Lactobacillus rhamnosus* TISTR 108. The fermentation processes were optimized in order to achieve the highest lactic acid yield and productivity. OPT had an average moisture content of 67.1±4.5% and yielded squeezed juice of 24.6% w/w. Undiluted OPT squeezed juice contained total sugars of 104.61±2.36 g l^{-1} which was composed of glucose (56.10±1.46 g l^{-1}), fructose (40.43±4.68 g l^{-1}), sucrose (3.23±0.69 g l^{-1}), raffinose (2.44 ±1.28 g l^{-1}) and cellubiose (2.41±0.71 g l^{-1}). Without nutrient supplementation, fermentation at 10% inoculum, 40^0C and pH of 6.5 achieved the highest lactic acid concentration, yield and productivity of 44.86 g l^{-1}, 0.78 g g^{-1} and 1.36 g l^{-1} h^{-1}, respectively. The addition of 10 g l^{-1} yeast extract, 5 g l^{-1} peptone and salts as nutrient supplementations in undiluted OPT juice could shorten the fermentation time and complete sugar consumption. The highest lactic acid yields and productivities were 0.82 - 0.85 g g^{-1} and 2.47 - 3.83 g l^{-1} h^{-1}, respectively. The use of Box Behkhen Design to optimize the fermentation process suggested that the addition of 10 g l^{-1} peptone and mineral salts (per liter), containing 0.4 g $MgSO_4.7H_2O$, 0.1 g $MnSO_4.4H_2O$, 3 g K_2HPO_4, 3 g KH_2PO_4 and 3 g $CH_3COONa.3H_2O$ was the appropriate nutrients for lactic acid production by *L. rhamnosus* TISTR 108. Under the optimized condition with peptone and salt supplementation, the maximum concentration, yield and productivity of 78.46 g l^{-1}, 1.01 g g^{-1} and 3.27 g l^{-1} h^{-1}, respectively were obtained in a 2 l fermenter.

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Nisa Saelee

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LIST OF ABBREVIATIONS

BBD	=	Box Behnken Design
CaL_2	=	calcium lactate
cal	=	calory
cm	=	centimetre
cfu	=	colony forming unit
^0C	=	degree Celsius
MRS	=	de Man Rogosa and Sharpe
K_a	=	dissociation constant
g	=	gram
h	=	hour
kg	=	kilogram
LA	=	lactic acid
LAB	=	lactic acid bacteria
L.	=	*Lactobacillus*
Lc.	=	*Lactococcus*
l	=	liter
µg	=	microgram
µl	=	microliter
µmol	=	micromolar
m	=	metre
m^2	=	metre square
mg	=	milligram
ml	=	milliliter
mm Hg	=	millimeter of mercury
mM	=	millimolar
min	=	minute
M	=	molar
mol	=	mole
nm	=	nanometer

LIST OF ABBREVIATIONS (Continued)

N	=	normality
OPT	=	oil palm trunk
OD	=	optical density
%	=	percentage
rpm	=	rotation per minute
SSF	=	simultaneous saccharification and fermentation
UV	=	ultraviolet
v	=	volume
vvm	=	volume per volume per minute
w	=	weight
YE	=	yeast extract

UTILIZATION OF HIGH SUGAR SAP SQUEEZED FROM OIL PALM TRUNK FOR LACTIC ACID FERMENTATION

INTRODUCTION

Lactic acid and its derivatives are widely used in many industrial applications like food, pharmaceutical, leather, textile and chemical industries. It also can contribute to a cleaner environment for example polylactic acid (PLA) production. Lactic acid produced by chemical synthesis or microbial fermentation. Specific stereo isomeric form obtained by microbial fermentation, while chemical synthesis results in racemic mixture of lactic acid. Currently, approximately 90 % of lactic acid is produced lactic acid bacteria fermentation because of the significant advantage over chemical synthesis which could use cheap raw material such as molasses, starchy waste, cellulosic and other carbohydrate rich materials (Miura *et al.*, 2004; Xu *et al.*, 2007; Wee *et al.*, 2009). Oil palm trunks (OPT) are the agricultural wastes left in the field on replanting and pruning. It contains fibrous materials which can be used in many industries such as wood, gypsum board, wood-cement composites, pulp and paper making, animal feed and other related useful products. The sap squeezed from old OPT contained high glucose and low concentration of other sugars such as sucrose, fructose, galactose, xylose and rhamnose. In addition, oil palm sap contained amino acids, organic acids, minerals and vitamins (Lim *et al.*, 1997; Kosugi *et al.*, 2010). The parameters for efficient conversion of lactic acid included the fermentation processes (Gao *et al*., 2009; Zhao *et al.*, 2010a; Abdel-Rahman *et al.*, 2013), microorganisms (Milcent *et al*., 2001; John *et al*., 2006; Lu *et al*., 2010), the complex nutritional requirements such as carbon sources, nitrogen sources, vitamins and minerals (Gao *et al.*, 2008; Li *et al*., 2012). Furthermore lactic acid fermentation always needs optimal conditions. The optimal environment for growth such as temperature, incubation period, pH, osmotic pressure, oxygen and other high stress factors (Kwon *et al*., 2000; Nancib *et al*., 2005) have been considered for the improvement of the lactic acid production with efficient yield and productivity. Although OPT juice is rich in carbohydrates, their utilization may limit due to low

protein content. Different nitrogen sources studied for lactic acid production and the increase of yeast extract nearly linearly lactic acid production. Most have been pointed at yeast extract supplementation even though its high cost but can lead to the highest lactic acid concentration among varieties of nitrogen sources due to a wide range of growth factors including amino acids, vitamins, specific minerals, fatty acids, purines, and pyrimidines (Nancib *et al*., 2005; Yu *et al*., 2008a). Furthermore lactic acid bacteria have limit ability to synthesize B-vitamin and amino acids (Chopin, 1993).

There are some constraints for lactic acid fermentation such as lack of strains for high yield efficiency, for high substrate utilization efficiency and for abundantly use inexpensive crude wastes for the cost effective process. Many lactic acid fermentation methods were studied to bear these criteria such as simultaneous saccharification and fermentation (SSF) (Sreenath, *et al*., 2001; Miura *et al*., 2004; John *et al*., 2007), submerged fermentation (SmF) (Oh *et al*., 2005; Lu *et al*., 2010), fed-batch fermentation (Ding and Tan, 2006), the cell recycle continuous fermentation (Wee *et al.*, 2009), the two-stage continuous cultures (Bouguettoucha *et al*., 2009), open repeated batch fermentation (Zhao *et al*., 2010b), Electrodialysis fermentation (EDF) (Min-tian *et al*., 2004), solid state fermentation (SSF) (Altaf *et al*., 2006; John *et al*, 2006) and immobilized cell method (Schepers *et al*., 2006).

The past studies, the use of the low concentration of sap squeezed from old OPT as nutrients sources for lactic acid production were performed (Lim *et al.*, 1997; Kosugi *et al.*, 2010). In this study, the fermentation of lactic acid using OPT juice as a cheap raw material substrate with concentration as high as possible was emphasized. The optimal conditions, nutrients requirement such as yeast extract, peptone and inorganic salts, and fermentation techniques were studied in order to enhance high conversion efficiency and the lactic acid yield and productivity by *L. rhamnosus* TISTR 108.

OBJECTIVES

1. To investigate the properties of juice extracted from oil palm trunk as a function of trunk part**.**

2. To investigate the effects of fermentation conditions including oil palm trunk (OPT) juice concentration, amount of inoculum, temperature and pH for lactic acid fermentation from OPT juice without nutrient supplementation.

3. To investigate the effect of nutrient supplementation, i.e. nitrogen source including yeast extract and peptone and salt on the lactic acid fermentation of oil palm trunk juice.

4. To investigate the optimization of nutrient supplementation for lactic acid fermentation from oil palm trunk juice using Box Behkhen Design.

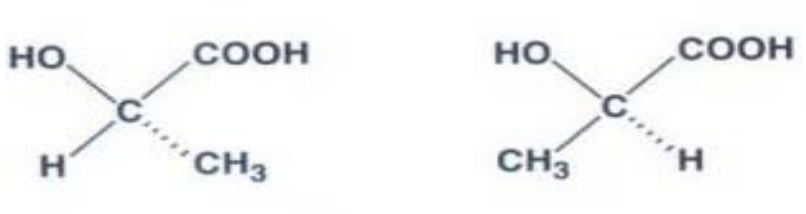
LITERATURE REVIEW

Lactic acid

1. Properties, uses and application

Lactic acid (LA) and its salts are natural fermentation products occurring in sour milk, cheese and other fermented foods. LA is known as milk acid. It was first isolated in 1780 by the Swedish chemist Carl Wilhelm Scheele. It is an organic acid named 2-hydroxypropionic acid or 2-hydroxypropanoic acid with the chemical formula $C_3H_6O_3$. It is a three carbon organic acid, one terminal carbon atom is part of an acid or carboxyl group, the other terminal carbon atom is part of a methyl or hydrocarbon group and a central carbon atom having an alcohol carbon group (Narayanan *et al*., 2004; Wikipedia, 2013a). LA has two optical isomers, L(+)-lactic acid or (S)-lactic acid and D(-)-lactic acid or (R)-lactic acid (Figure 1). L-lactic acid is an important isomer in biological. Wee *et al*. (2006b) reported that D(–)-lactic acid is at times harmful to human metabolism and can result in acidosis and decalcification.

LA is miscible with water or ethanol but insoluble in other organic solvents. It is hygroscopic and exhibits low volatility. Other identification and physical-chemical properties of LA are summarized in Table 1.The thermodynamic characteristics of lactic acid are listed in Table 2



L (+) Lactic acid D (-) Lactic acid

Figure 1 L (+) lactic acid and D (-) lactic acid.

Source: Narayanan *et al*. (2004)

Table 1 Identification and physical-chemical properties of lactic acid.

Identification		**Physical and chemical properties**	
CAS number	D/L: [50-21-5]	Melting point	L: 53^0C
	L: [79-33-4]		D: 53^0C
	D: [10326-41-7]		D/L: 16.8^0C
Einecs No.	200-018-0	Boiling point	122^0C(12 mmHg)
H.S. Code	2918.11	Specific gravity	1.2 g/ml
Formular	$CH_3CH(OH)COOH$	Molar mass	90.08 g/mol

Source: Ren (2010)

Table 2 Thermodynamic characteristics of lactic acid.

Items	Characteristics
Dissociation constant (K_a)	0.000137 (at 25^0C)
Heat of dissociation (ΔH)	-63 cal mol^{-1} (at 25^0C)
Free energy of dissociation (ΔF)	5000 cal mol^{-1}
Heat of solution	1868 cal mol^{-1}
	(for crystalline L(+) lactic acid at 25^0C)
Heat of dilution	-1000 cal mol^{-1}
	(for dilution with a large volume of water)
Heat of fusion	2710 cal mol^{-1} (for racemic lactic acid)
	4030 cal mol^{-1} (for L(+) lactic acid)
Entropy of solution	6.2 cal mol^{-1} $^0C^{-1}$
Entropy of dilution	-3.6 cal mol^{-1} $^0C^{-1}$
Entropy of fusion	9.4 cal mol^{-1} $^0C^{-1}$ (for racemic lactic acid)
	12.2 cal mol^{-1} $^0C^{-1}$ (for L(+) lactic acid)
Heat of combustion (ΔH_{co})	-321 220 cal mol^{-1} (for crystalline L(+) lactic acid at 25^0C)
	-325 600 cal mol^{-1} (for liquid racemic lactic acid at 25^0C)
Heat of formation (ΔH_{fo})	-165 890 cal mol^{-1} (for crystalline L(+) lactic acid at 25^0C)
	-163 000 cal mol^{-1} (for liquid lactic acid)
	-164 020 cal mol^{-1} (for lactic acid in dilute solution)
	-164 080 cal mol^{-1} (for dissociated and diluted lactic acid)
Heat capacity (C_p)	0.338 cal g^{-1} $^0C^{-1}$ (for crystalline lactic acid at 25^0C)
	0.559 cal g^{-1} $^0C^{-1}$ (for liquid lactic acid at 25^0C)
Absolute entropy (S_0)	34.0 cal mol^{-1} $^0C^{-1}$ (for crystalline L(+) lactic acid at 25^0C)
	45.9 cal mol^{-1} $^0C^{-1}$ (for liquid racemic lactic acid at 25^0C)
Entropy of formation (ΔS_{fo})	-137.2 cal mol^{-1} $^0C^{-1}$ (for crystalline L(+) lactic acid at 25^0C)
	-125.3 cal mol^{-1} $^0C^{-1}$ (for liquid lactic acid at 25^0C)
Free energy of formation (ΔF_{fo})	-124 980 cal mol^{-1} (for crystalline L(+) lactic acid at 25^0C)
	-126 500 cal mol^{-1} (for liquid racemic lactic acid at 25^0C)

Source: Ren (2010)

2. Chemical synthesis

Lactic acid was first produced commercially by Charles E. Avery at Littleton, Massachusetts, USA in 1881 (Narayanan *et al*., 2004). It can be produced either chemical synthesis or microbial fermentation. The commercial process for chemical synthesis is mainly based on the hydrolysis of lactonitrile by strong acids and this process yields a racemic mixture of the two forms D (-) and L (+) lactic acid (Gao *et al*., 2011; Lasprilla *et al*., 2012).

Chemical process is represented by the following reactions (Narayanan *et al*., 2004).

(a) Addition of hydrogen cyanide

$$\underset{\text{acetaldehyde}}{CH_3CHO} + \underset{\text{hydrogen cyanide}}{HCN} \xrightarrow{\text{catalyst}} \underset{\text{lactonitrile}}{CH_3CHOHCN}$$

(b) Hydrolysis by H_2SO_4

$$\underset{\text{lactonitrile}}{CH_3CHOHCN} + H_2O + \tfrac{1}{2}\underset{\text{sulphuric acid}}{H_2SO_4} \longrightarrow \underset{\text{lactic acid}}{CH_3CHOHCOOH} + \tfrac{1}{2}\underset{\text{ammonium salt}}{(NH_4)_2SO_4}$$

(c) Esterification

$$\underset{\text{lactic acid}}{CH_3CHOHCOOH} + \underset{\text{methanol}}{CH_3OH} \longrightarrow \underset{\text{methyl lactate}}{CH_3CHOHCOOCH_3} + H_2O$$

(d) Hydrolysis by H_2O

$$\underset{\text{methyl lactate}}{CH_3CHOHCOOCH_3} + H_2O \longrightarrow \underset{\text{lactic acid}}{CH_3CHOHCOOH} + \underset{\text{methanol}}{CH_3OH}$$

Hydrogen cyanide is added to acetaldehyde in the presence of a base to produce lactonitrile. This reaction occurs in liquid phase at high atmospheric pressures. The crude lactonitrile is recovered and purified by distillation. It is then hydrolyzed to lactic acid, either by concentrated HCl or by H_2SO_4 to produce the corresponding ammonium salt and lactic acid. Lactic acid is then esterifies with methanol to produce methyl lactate, which is removed and purified by distillation and hydrolyzed by water under acid catalyst to produce lactic acid and the methanol, which is recycled.

The other chemical processes for LA synthesis have been reported such as alkaline degradation of glucose (Onda *et al.*, 2008), catalyzed triose-sugars, glyceraldehydes and dihydroxyacetone directly methyl lactate and lactic acid by zeolite (West *et al.*, 2010), oxidation of glycol to lactic acid (Lakshmanan *et al*., 2013), hydrolysis of methyl lactate to lactic acid (Edreder *et al.*, 2011), hydrothermal treatment (Sanchez *et al*., 2012), hydrolysis of chloropropionic acid; and nitric acid oxidation of propylene, are not technically and economically feasible processes for lactic acid (Gao *et.al*., 2011).

3. Fermentation

Through chemical synthesis, racemic DL-lactic acid is always produced. The stereo specific acid, D(-) or L(+)-lactic acid can be obtained by microbial fermentation (Narayanan *et al*., 2004). The broth containing calcium lactate is filtered to remove cells, carbon treated, evaporated and acidified with sulphuric acid to get lactic acid and calcium sulphate. The insoluble calcium sulphate is removed by filtration, lactic acid is obtained by hydrolysis, esterification, distillation and hydrolysis. This process is represented by the following reactions.

(a) Fermentation and neutralization

$$\underset{\text{carbohydrate}}{C_6H_{12}O_6} + \underset{\text{calcium hydroxide}}{Ca(OH)_2} \xrightarrow{\text{fermentation}} \underset{\text{calcium lactate}}{2(CH_3CHOHCOO^-)Ca^{2+}} + H_2O$$

(b) Hydrolysis by H_2SO_4

$$2(CH_3CHOHCOO^-)Ca^{2+} + H_2SO_4 \longrightarrow 2CH_3CHOHCOOH + CaSO_4$$

calcium lactate sulphuric acid lactic acid calcium sulphate

(c) Esterification

$$CH_3CHOHCOOH + CH_3OH \longrightarrow CH_3CHOHCOOCH_3 + H_2O$$

lactic acid methanol methyl lactate

(d) Hydrolysis by H_2O

$$CH_3CHOHCOOCH_3 + H_2O \longrightarrow CH_3CHOHCOOH + CH_3OH$$

methyl lactate lactic acid methanol

Both chemical and biotechnological methods are available for manufacturing lactic acid, but the biotechnological production offers several advantages compared to chemical synthesis like low substrate costs, mild temperature, and low energy consumption and environmental friendliness in petrochemicals (Narayanan *et al*., 2004; Mussatto *et al*., 2008; Gao *et al*., 2011). Moreover, biotechnological production of lactic acid can use renewable raw material such as molasses, starchy waste, cellulosic and other carbohydrate rich materials (John *et al*., 2009) and specific stereo isomeric forms D- or L-lactic acid can be obtained depending on the microorganism used.

4. Application of lactic acid

Lactic acid has received a significant amount of attention as a chemical with many potential applications. The annual world market for LA production is expected to reach 367,300 metric tons by the year 2017 and the demand for LA has been estimated to grow yearly at 5 - 8% (Abdel-Rahman *et al*., 2013).

LA and its derivatives are widely used in food, pharmaceutical, leather, textile industries and chemical industries. L (+) LA is the preferred isomer in food and pharmaceutical industries as human have only L-lactate dehydrogenase that metabolizes L (+) LA (Akerberg *et al*., 1998; Reddy *et al*., 2008).

Food and food related applications account for approximately 85% of the demand for LA, whereas the nonfood industrial applications account for only 15% of the demand (John *et al*., 2009). Since LA is classified as GRAS for use as food additive, it is approved by the EU, USA, and Australia and New Zealand (Wikipedia, 2013a). It has been used as a preservative and acidulant, flavoring or buffering agent or inhibitor of bacterial spoilage in a wide variety of process food such as candy, breads and bakery products, soft drinks, beverages, meat products, soups, salads, dressings, mayonnaise, sherbets, dairy products, beer, jam and jellies, confectionery, pickled vegetables and processed food. Calcium lactate is a good dough conditioner, whereas sodium lactate acts both as conditioner and as emulsifiers.

In pharmaceutical, LA has been used as formulations in ointments, lotions, and parenteral solutions (Wee *et al*., 2006b). LA employed water soluble lactates from other insoluble active ingredients. It is used as a supplement in the synthesis of dermatologic drugs and against osteoporosis.

In the cosmetic industry, LA derivatives such as lactate esters are widely used in the manufacture of hygiene and esthetic products, owning to moisturizing, antimicrobial and rejuvenating effects on the skin, as well as of oral hygiene products (Martinez *et al.*, 2013).

The potential use of LA as a raw material in the production of biodegradable plastics, polylactic acid (PLA) has made LA more valuable. This is a good option for plastic produced from petrochemical. However, the biodegradable plastics have not been spreaded due to its high cost relative to those derived from petroleum (Gao *et al*., 2008). Lactide, a cyclic lactone can polymerize to polylactide, a biodegradeable polyesters with much valuable. LA can be potentially used for the manufacturing of

large-volume oxygenated chemicals such as propylene glycol, propylene oxide, acrylic acid, and acrylate esters, and other chemical intermediates such as lactate ester plasticizers (John *et al*., 2009). It was reported that an equimolar blend of poly-L-lactic acid and poly-D-lactic acid generated a racemic crystal called stereo-complex poly (lactic acid) which was more heat-resistant than the poly-L-lactic acid homo-polymer due to the high melting temperature. This finding make D-lactic acid more and more important (Lu *et al*., 2009b). In detergent industry, LA is often used in decaling properties such as household cleaning products, soap-scum remover and natural antibacterial agent in disinfecting products. LA is used as additive in animal nutrition to promoted health and enhanced animal growth. The controlled LA fermentation increases the shelf life, palatability and nutritive value of silage. Ammonium lactate is an excellent non-protein nitrogen source, which is preferred in cattle to urea and ammonium citrate because its results in milk with higher nutritive value and does not require any expensive purification (Martinez *et al.*, 2013).

The potential applications of lactic acid are illustrated in Figure 2.

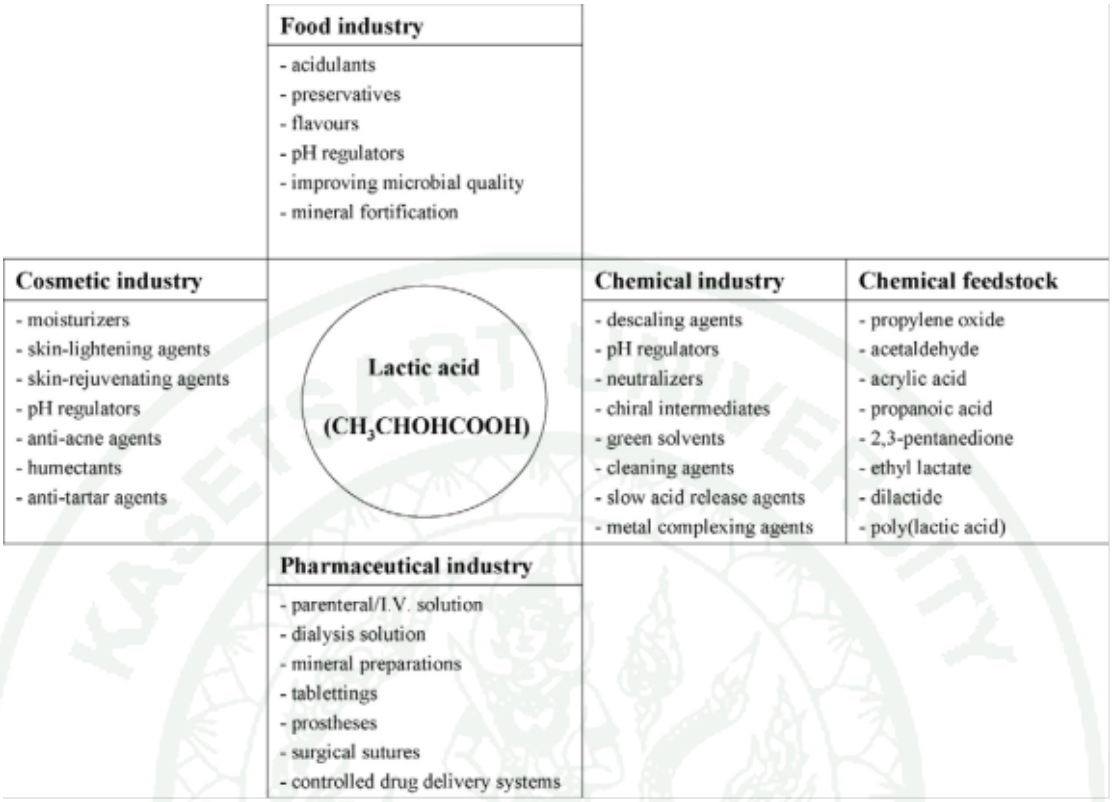


Figure 2 Diagram of the commercial uses and applications of lactic acid and its salts.

Source: Wee *et al*. (2006b)

Microorganisms used for lactic acid production

Nowsday, different fermentation processes and difference microorganism used for LA production and resulted in different LA yield and conversion efficiency. Various microorganisms such as bacteria, fungi, yeast, cyanobacteria and algae have been reported for LA production.

In starchy material, the homofermentative lactic acid bacteria, *Lactobacillus delbrueckii* (John *et al*, 2006), *L. rhamnosus* (Li *et al*., 2010; Lu *et al*., 2010), *Enterococcus faecalis* RKY1 (Oh *et al*., 2005; Nandasana and Kumar, 2008), *L. delbrueckii* ssp. *lactis* (Milcent and Carrere, 2001) were used. The others species of *Lactobacillus* such as *L. lactis* ssp. *lactis* B84 (Petrov *et al*., 2008), *L. casei* (Alonso *et al*., 2010), *L. amylophilus* GV6 (Altaf *et al*., 2007), *L. helveticus* (Schepers *et al*., 2006) and *Lactococcus lactis* ATCC 19435 (Kosugi *et al*., 2010) were studied for LA production. Some species of bacteria such as thermophilic *Bacillus* sp. (Zhao *et al*., 2010b) and *Bacillus coagulans* SIM-7 DSM 14043 (Michelson *et al*., 2006) and fungi such as *Rhizopus oryzae* (Bulut *et al*., 2004) and *Rhizopus arrhizus* (Zhang *et al*., 2009) were used for LA production.

For lignocellulosic materials, *R. oryzae* (Woiciechowski *et al*., 1999), *L. plantarum, L. delbrueckii* (Sreenath *et al*., 2001; Parajo *et al*., 1996), *Lactobacillus* sp. RKY2 (Wee *et al*., 2009), *L. rhamnosus* (Marques *et al*., 2008) *L.lactis* IO-1 (Laopaiboon *et al*., 2010), *B. coagulan* (Budhavaram and Fan., 2009) were studied for LA production.

Cellulose hydrolyzate also could be done by using mixed culture or pure culture under mild conditions relative to most chemical hydrolysis and enzymatic hydrolysis. Mixed culture or co-culture system for the production of LA was carried out using *Lactobacillus casei* NCIMB 3254 and *L. delbrueckii* and the maximum LA yield of 81 g l^{-1} was obtained when 15% (w/v) cassava bagasse treated with enzyme mixtures (John *et al*., 2007). Ge *et al*. (2009) reported the use of *Aspergillus niger* SL-09 and *Lactobacillus* sp G-02 as a mixed culture in a 7 l fermenter to directly

produced LA from Jerusalem artichoke tubers and fed-batch simultaneous saccharification and fermentation was used. The highest yield of LA yield was 120.5 g l^{-1} in 36 h. Lee (2005) also reported that the cell density and LA production by mixed type of five Lactobacilli were better than single culture. Even though nitrogen source consumption in mixed culture was lower than that of single culture. Increased LA production was observed, when mixed cultures of *Kluyveromyces marxianus* (IFO 288), *L. delbrueckii* ssp. *bulgaricus* (ATCC 11842) and *L. helveticus* (ATCC 15009) were used in comparison to individual ones (Plessas *et al*., 2008). Mixed culture of *L. pentosus and L brevis* used in LA production from wheat straw hemicelluloses hydrolysate (Garde *et al*., 2002).

LA producing bacteria include wild-type and engineered producers can be divided into 4 main producers, namely, lactic acid bacteria (LAB), *Bacillus* strains, *Escherichia coli*, and *Corynebacterium glutamicum*. In general, LAB 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 by-product formation (iii) use of nutritionally rich medium and (iv) high risk of bacteriophage infection that results in cell lysis and subsequent cessation of LA production. Various studies have investigated methods to overcome these problems in the field of metabolic engineering, i.e., (i) improvement of optical purity *via* the deletion of either D- or L-LDH genes (ii) increased LA yields through reduction of byproduct levels by the deletion of genes encoding pyruvate formate lyase (formic acid production), alcohol dehydrogenase (ethanol production), and/or acetate kinase (acetic acid production) (iii) development of bacterial strains, e.g., *E. coli*, producing LA on chemically defined media and (iv) strain improvements for blocking steps in phage life cycle. Usage of mixed strains and/or development of phage-resistant strains are sometimes necessary to prevent bacteriophage infection (Abdel-Rahman *et al*., 2013).

Lactic acid bacteria

Lactic acid bacteria (LAB) comprise a diverse group of gram-positive, non-spore-forming bacteria. They occur as cocci or rods and generally lack of catalase although pseudo-catalase can be found in rare case. They devoid cytochromes, anaerobic but aerotolerant, acid tolerant and produce lactic acid as the major end product, during sugar fermentation. They have received wide interest because of their high growth rate and product yield (Zhang *et al*., 2007). However, they are chemo-organotrophic and grow only in complex media (Wood and Holzapfel, 1995).

LAB can be classified as homofermentative or heterofermentative based on the fermentation end product of hexoses and pentose as described by Abdel-Rahman *et al*. (2013) in Table 3. Homofermentative LAB follows the familiar Embden-Meyerhof-Parnas (EMP) pathway for glycolysis. They posses aldolase enzymes and produce more than 85% LA from glucose as the major end product. They ferment 1 mol of glucose to 2 mol of LA, generating a net yield of 2 mol of ATP per molecule of glucose metabolized. They are of interest for commercial scale LA production.

Heterofermentative LAB use the alternate pentose monophosphate pathway, converting 6-carbon sugars (hexoses) to 5-carbon sugars (pentoses) and carbon dioxide catalyzed by several enzymes (Figure 3). Then, the resulting pentose is cleaved to glyceraldehydes 3-phosphate and acetyl phosphate by phosphoketolase. Most heterofermentative LAB strains convert the pentose sugars to LA and by-products (e.g., acetic acid) through phosphoketolase pathways with a maximum LA yield at 0.6 g lactic acid per gram of pentoses. These resulted in less growth per mole of glucose metabolized (Reddy *et al*., 2008: Abdel-Rahman *et al*., 2013).

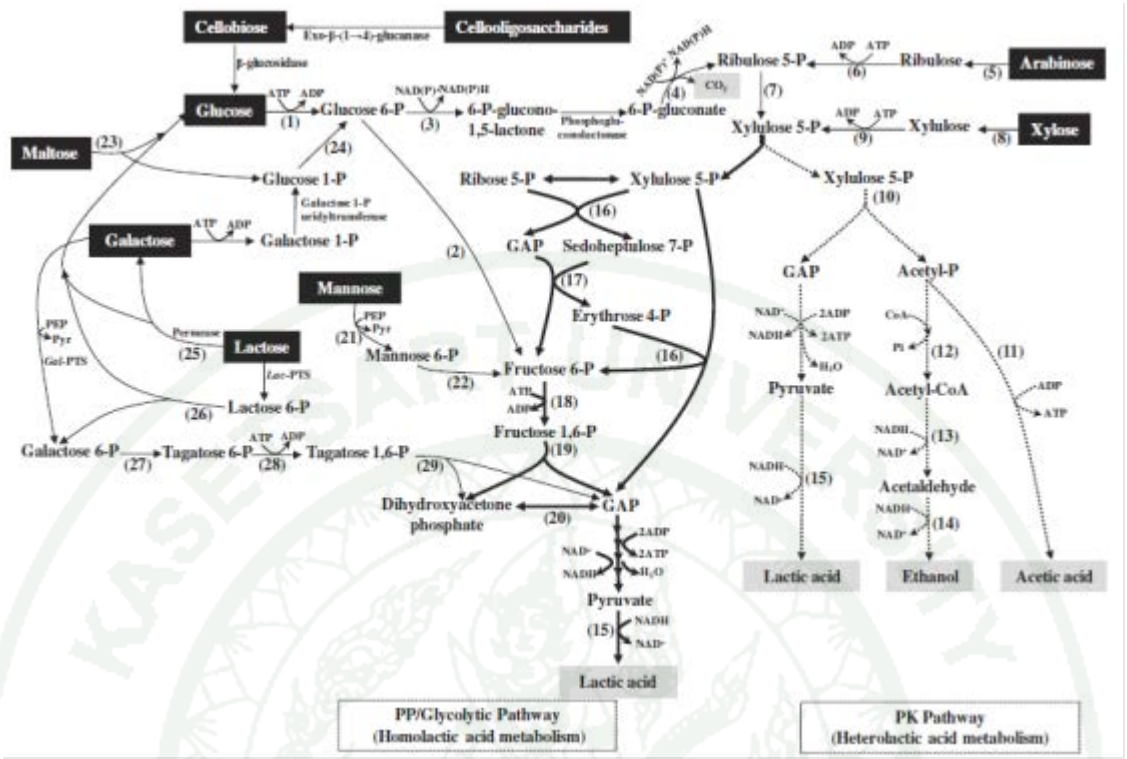


Figure 3 Metabolic pathways for lactic acid production from various sugars by lactic acid bacteria. Enzymes: (1) hexokinase; (2) glucose 6-phosphate isomerase; (3) glucose 6-phosphate dehydrogenase; (4) 6-phosphogluconate dehydrogenase; (5) arabinose isomerase; (6) ribulokinase; (7) ribulose 5-phosphate 3-epimerase; (8) xylose isomerase; (9) xylulokinase; (10) phosphoketolase; (11) acetate kinase; (12) phosphotransacetylase; (13) aldehyde dehydrogenase; (14) alcohol dehydrogenase; (15) lactate dehydrogenase; (16) transketolase; (17) transaldolase;(18) 6- phosphofructokinase; (19) fructose bisphosphate aldolase; (20) triosephosphate isomerase; (21) mannose phosphotransferase system; (22) phosphomannose isomerase (23) maltosephosphorylase; (24) phosphoglucomutase ; (25) β-galactosidase; (26) phospho-β-galactosidase; (27) galactose 6-phosphate isomerase; (28) tagatose 6-phosphate kinase; and (29) tagatose1,6-diphosphate aldolase. Solid lines indicate the homofermentative pathway. Thick-solid lines and dashed lines indicate PP/glycolytic pathway and PK pathway, respectively. Lac-PTS:phosphoenolpyruvate-lactose phosphotransferase system.

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

Table 3 Homoferementative and heterofermentative lactic acid bacteria.

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^{-1} (2.0 mol mol^{-1}) Pentose: 1.0 g g^{-1} (1.67 mol mol^{-1})	Hexose: 0.5 g g^{-1} (1.0 mol mol^{-1}) Pentose: 0.6 g g^{-1} (1.0 mol mol^{-1})
Genera	*Lactococcus*, *Streptococcus*, *Pediococcus*, *Enterococcus*, some *Lactobacillus*	*Leuconostoc, Oenococcus*, some *Lactobacillus species*
Availability of commercial lactic acid production	Available due to high selectivity	Not available due to high by-product formation

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

The obligatory homofermentative LAB include *L. acidphilus, L. amylophilus, L. bulgaricus, L. helveticus and L. salivarius*. The obligatory heterofermentative LAB include *L. brevis, L. fermentum, L. parabuchneri* and *L. reuteri* (Martinez *et al.,* 2013).

The major LAB including the following genera: *Lactobacillus, Lactococcus, Streptococcus, Pediococcus* have been used for LA production and used as inocula for many fermentation applications. Currently, LAB, such as *L. rhamnosus* and *Lc. lactis* are usually used in LA industrial production (Wang *et al.,* 2010). *L. rhamnosus* is a bacterium that was originally considered to be a subspecies of *L. casei*, but later genetic research found it to be a species of its own. Some strains of *L. rhamnosus* are being used as probiotics (Wikipedia, 2013b). *L. rhamnosus* ferments the acid from variety of carbon sources, nitrogen sources and different vitamin supplementation (Kwon *et al.,* 2000; Nancib *et al.,* 2001; Nancib *et al.,* 2005; Yu *et al.,*2008a; Li *et al.,* 2010; Wang *et al.,* 2010). Mel *et al.* (2008) optimized the main media component of *L. rhamnosus* in shake flask. The results indicated the glucose and peptone concentration were 9.8 and 9.98 g l^{-1} for optimum bacterial growth and LA production, respectively and the optimum productivity of LA was 0.630 g g^{-1}. Djukic´-Vukovic´ *et al.* (2012a) studied the *L. rhamnosus* cells immobilized onto zeolite and a maximal process productivity of 1.69 g l^{-1}, maximal LA concentration of 42.19 g l^{-1} and average yield coefficient of 0.96 g g^{-1} were achieved in repeated batch fermentation on the liquid stillage without mineral or nitrogen supplementation. Gao *et al.* (2009) indicated that the strain *L. rhamnosus* (NBRC 3863) was resistant to glucose inhibition and final calcium lactate concentration increased with initial glucose concentration increasing up to 190 g l^{-1}. Senthuran *et al.* (1997) reported that Lactobacilli was desirable to produce LA with concentrations of substrate as high as possible because it would lead to the increase of LA concentration and the reduction of downstream process costs. Researchers reported that *L. rhamnosus* was classified as facultative heterofermentative that performed both fermentations, consuming hexoses by the homolactic pathway and pentose by the hetero lactic one. *L. rhamnosus* can also metabolize disaccharide, cellubiose. It is LAB that produces L (+) lactic acid (Martinez *et al.,* 2013).

Attempts have used biotechnological tools to engineer LAB and other microorganisms to improve LA production. Yu *et al*. (2008b) have applied the genome shuffling to improve the glucose tolerance of *L. rhamnosus* ATCC 11443 which enhanced the L-lactic acid production. Kadam *et al*. (2006) used acclimatization and ultraviolet mutagenes to develop strains of *L. delbrueckii* (NCIM 2365) that produced increased LA concentrations. John *et al*. (2008) has developed a low pH tolerant, fast growing mutant of *L. delbueckii* by mutagenesis using nitrous acid and genome shuffling was done between this mutant and an amylase producing non-fastidious *B. amyloliquefaciens* to get a fusant capable of growing at low pH and utilizing starchy materials directly for L-lactic acid production with minimal addition of nutrients. The LA yield of 40 g l^{-1} was obtained with a productivity of 0.42 g $l^{-1}h^{-1}$ and 96% conversion of starch to LA was quite comparable and even better to the reported single step conversion of starch to LA. Gao *et al*. (2009) has investigated the new transgenic yeast, *Saccharomyces cerevisiae* OC-2T T165R to produce optically pure L(+)-lactic acid with a high performance extractive LA fermentation process. Moreover, the use of the immobilization technique involving photo-crosslinkable resin gels can improve the productivity of the metabolically engineered yeast in the fermentation with or without extraction, showing promise for using the immobilized engineered yeast for LA production.

Nevertheless, metabolic engineering was performed to enhanced L-lactate production or the ratio of L-/D-lactate. Jin *et al*. (2009) changed the pyruvate carbon flux in *L. citreum* from D-lactate into L-lactate by heterologous expression of L-lactate dehydrogenase (*ldhL*) gene. For this, *ldhL* from *L. plantarum* was cloned and introduced into *L. citreum* using a shuttle vector pLeuCM. In the transformant, ldhL was successfully transcribed and L-lactate dehydrogenase was expressed. A newly isolated species, *L. paracasei* LA104 (Nguyen *et al.*, 2012) to produce LA has reported.

In general, the desirable characteristics of industrial microorganisms depend on their ability to rapidly and completely ferment cheap raw materials, requiring minimal amount of nitrogenous substances, providing high yields of preferred stereo

specific LA under conditions of low pH and high temperature, production of low amounts of cell mass and negligible amounts of other by-products. The choice of an organism primarily depends on the carbohydrate to be fermented. *L. delbreuckii* subspecies *delbreuckii* are able to ferment sucrose. *L. delbreuckii* subspecies *bulgaricus* is able to use lactose. *L. helveticus* is able to use both lactose and galactose. *L. amylophylus* and *L. amylovirus* are able to ferment starch. *L. lactis* can ferment glucose, sucrose and galactose. *L. pentosus* have been used to ferment sulfite waste liquor (Narayanan *et al*., 2004).

Factors influencing lactic acid fermentation

Researches focused on the homofermentative LAB which ferment LA and lack the ability to produce a whole range of others fermentation products, grow substantially faster than other bacteria present in the same ecological niche. However the higher growth of LAB is a result of simple metabolism, but also adapted to rich environments. Most LAB have complex nutritional requirements, due to they have a limited biosynthetic capabilities and depend on their environment for supply of growth factors for growth. The influences of nutritional factors were reported on the LA production such as carbon sources, amino acids, peptides, nitrogen sources, vitamins and minerals. Nevertheless, other environmental factors such as temperature, incubation period, pH, osmotic pressure (NaCl), oxygen and high pressure have been considered for the improvement of the LA production.

1. Carbon sources

One of the most obstructions in the large scale fermentation of LA is the cost of raw material. Cheap raw materials are necessary for the feasible economic production of LA. Raw materials for LA 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 (John *et al*., 2007, 2009).

A number of different substrates have been used for biotechnological production of LA, including glucose, sucrose, lactose, maltose, mannose, xylose, and galactose. The most pure 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 LA is a relatively cheap product (Mussatto *et al*., 2008**).**

Conventional production of LA from starchy materials required two step process involving enzymatic saccharification to glucose and subsequent fermentation of glucose to LA. Several substrates like starch (Petrov *et al*., 2008, Zhang *et al*., 2009), acorn starch and hydrolyzed corn starch (Lu *et al*., 2010), wheat bran (Altaf *et al*., 2006), glucose, sucrose, beet molasses, carob pod (Bulut *et al*., 2004), wheat and barley (Oh *et al*., 2005), fresh cassava roots (FDEs) (Ghofar *et al*., 2005), beet molasses (Milcent and Carrere, 2001; Nandasana and Kumar, 2008) are the preferred carbon sources used in L(+)-lactic acid production at present.

Several alternative substrates including agro-industrial residues have been studied to reduce the cost of LA production. Various lignocellulosic materials consisted of wood *(Pinus taeda)* chips (Woiciechowski *et al*., 1999), corn cob (Miura *et al*., 2004; Sanchez *et al*., 2012), soy bean stalk hydrolysate (Xu *et al*., 2007), alfafa fibers (Sreenath, *et al*., 2001), Eucalyptus globules wood (Parajo *et al*., 1996), oat wood ships (Wee *et al*., 2009), recycle paper sludges (Marques *et al*., 2008), sugar cane bagasse (Laopaiboon *et al*., 2010), sap squeezed from oil palm trunks (Kosugi *et al*., 2010) and paper sludge (Budhavaram and Fan., 2009) were studied to use as a cheaper alternative sources to sugars for LA production.

For economic LA production, raw material cost is one of the major factors to be considered. However, the other problems associated with efficiency conversion of LA production by fermentation were the nutritional requirements, the end product inhibition, optically pure D-(-) or L-(+)-lactic acid.

Since the chemical formula of L(+)-lactic acid is $C_3H_6O_3$, carbon source is the main raw material in L(+)-lactic acid production. The selection of a specific raw material depends mainly on its availability, composition, and price. Thus the need of the carbon source is far more than that of the nitrogen source in L(+)-lactic acid biosynthesis. However, the distribution of C in cell growth or products biosynthesis may be partially determined by the carbon–nitrogen ratio during the fermentation. Effect of different carbon–nitrogen ratios on L(+)-lactic acid production by *L. rhamnosus* HG 09 was investigated. The cell growth showed different increasing

trend with the increase of carbon–nitrogen ratios, the growth rate of cell was higher when the carbon–nitrogen ratio was 40.12 than that of 60.05 (Lu *et al*, 2010). Carbon can be presented in the culture medium in the form of sugars, amino acids and organic acids that have high energy content. Most L-lactic acid fermentation has used the pure or easily fermentable substrates such as glucose, sucrose, fructose, mannose, galactose.

Utilization of cheap carbon sources was considered as an effective approach. Though many starchy materials from agriculture such as corn, cassava powder, rice and rice starch were used as carbon sources in many studies, the media costs were still high in relation to synthetic media (Lu *et al*, 2009; Wang *et al*., 2010). Thus, some cheap raw materials such as molasses, starchy and cellulosic materials have been used as the carbon sources in L(+)-lactic acid production (Hofvendahl and Hahn-Haerdal, 2000).

Oil palm, (*Elaeis guineensis*), is one of the most economical perennial oil crops for its valuable oil-producing fruits in tropical region such as Southeast Asia. Thailand with a relatively small share of world production, just 3%, compared to Malaysia and Indonesia, which together make about 90%, presents a different picture. Thai operation holds little more than 1,000 hectares of oil palm plantations. Thailand's palm oil production is increasing rapidly on the back of its promotion as a feedstock for biodiesel, mirroring a growing global demand over the last few decades that has seen its wide application in the food, chemical and bio energy industries.

Oil palm for palm oil production needs to be replanted at intervals of 20 to 25 years in order to maintain oil productivity (Kosugi *et al*., 2010). The large amount of oil palm biomass is generated by the palm oil industry. The palm trunk structure is not strong enough for use as lumber, and thus, only the outer part of the trunk, which is relatively strong, is partially utilized for plywood manufacturing. In the plywood production process, the inner part is discarded in large amounts due to its extremely weak physical properties. Meanwhile, it is known that palm sugar and palm wine are produced from sap obtained by tapping the inflorescence of varieties of palm species,

such as *Arenga pinnata, Borassus flabellifer, Cocos nucifera, Nypa fruticans* and oil palm.

In order to utilize the old palm trunks for replanting, especially the inner part, an attempt to produce bioethanol and LA, the material for bio-plastics, from felled trunks. Sugars in the sap of the felled trunk was focused and observed a large quantity of high glucose content sap in the trunk. Other components in the squeezed sap that may affect fermentation, namely, amino acids, organic acids, minerals and vitamins, were also assessed. The sap obtained was used for ethanol and lactic acid production using an industrial yeast strain and a LAB, respectively (Kosugi *et al*., 2010).

Oil palm trunks (OPT) are the agricultural wastes left in the field on replanting and pruning. It contains fibrous materials which can be used in many industries such as wood, gypsum board, wood-cement composites, pulp and paper making, animal feed and other related useful products. Chemical composition of OPT fibre consists of cellulose 29.2%, lignin 18.8%, hemicellulose 16.5%, pentosan 18.8%, ash 2%. OPT also contains enormous of N and K and very little of the N, P, Ca and Mg (Kee, 2004). The numerous considerable amount of starch is found in OPT (Noor *et al*., 1999). Henson *et al*. (1999) reported that the trunk might contain as much as starch (24%) and sucrose was the main form of soluble sugars and some of glucose, xylose and fructose were found in OPT.

The sap sqeezeed from old OPT was studied by Kosugi *et al*. (2010) which contained glucose up to 85.2 g l^{-1} of the sap from the inner part and other sugars such as sucrose, fructose, galactose, xylose and rhamnose were found in low concentrations. In addition, oil palm sap was found to be rich in amino acids, organic acids, minerals and vitamins (Lim *et al*., 1997; Kosugi *et al*., 2010). Acid hydrolysis of OPT was studied by Lim *et al.*, (1997). The palm trunks were hydrolyzed using different concentration of sulphuric acid. Results indicated that glucose yield began to decline at acid concentration higher than about 3%. The steam-exploded and post

hydrolysis by 1% w/v H_2SO_4 of oil palm trunk chips resulted the highest amounts of xylose and glucose of 8.15 and 2.46% by weight, respectively.

2. Nitrogen sources

Nitrogen is a key nutrient in microbial culture media even though it is reported that some microbes are capable of synthesizing all the primary metabolites by themselves. They can be stimulated to grow faster and reach higher cell densities in a rich complex medium containing more easily convertible nitrogen such as proteins with small molecular weight. The production costs of LA are greatly influenced by the price of nutritional factors. The increasing demand for microbial growth media by the biotechnological fermentation industry comes the increasing demand for nitrogen source, which makes nitrogen one of the most expensive components in microbial culture media. The high cost of commercially available defined media formulated with nitrogen from premium sources has limited their use in mass production processes. Therefore, there is an acute need for seeking an alternative, inexpensive nitrogen source in a timely manner (Yao *et al.*, 2010). Nitrogen implied either in anabolic or catabolic processes, is available in the form of amino acids, peptides and inorganic compounds that can be either added to the culture media as peptone, yeast extract, urea or ammonium sulfate (Nacib *et al.*, 2001; Martinez *et al.*, 2013).

Yeast extract (YE) is a usual nutrient for both microbial growth and lactic acid production. Among the nutritional parameters affecting the growth and lactic acid fermentation, YE leads to the highest LA concentrations in a variety of nitrogen sources due to a wide range of growth factors including amino acids, vitamins, specific minerals, fatty acids, purines, and pyrimidines (Yu *et al*., 2008a). However, the high cost of yeast extract, YE (about six times more expensive than ammonium sulfate) has a negative impact on the economics of its use in industrial scale processes because YE is estimated to account for about 38% of the total production (Nancib *et al*., 2005; Yu *et al*., 2008a). Chopin (1993) reported that LAB have complex nutrient requirements because of their limited ability to synthesize B-vitamins and amino acids, making supplementation of sufficient nutrients such as YE to media is

necessary. Michelson *et al.* (2006) has described that YE exhibited clearly the most significant effect on LA production of LAB, particularly in the beginning of growth, with LA concentration increasing nearly linearly with the increase of YE level. However amounts of YE added to the optimal concentration of YE did not improve characteristics of fermentation. Many studies have been pointed at YE supplement. Wang *et al.* (2010) studied the different YE concentrations (0, 2.5, 5, 7.5, and 10 g l^{-1}) on L-lactic acid production with the medium containing 275 g l^{-1} cassava powder. Result concluded that L-lactic acid concentration increased with the addition of YE, and 173.4 g l^{-1} of L-lactic acid was obtained with 5 g l^{-1} of YE. Further addition did not significantly improve the L-lactic acid production. Laopaiboon *et al*. (2010) revealed that 7 g l^{-1} of YE gave the highest yield of LA (0.26 g lactic per g sugar utilized), when *L. lactis* IO-1 (JCM 7638) cultured on the hydrolysate from sugarcane bagasse. Nancib *et al*. (2005) revealed that date juice supplemented with yeast extract increased in LA production compared with unsupplemented date juice and ten gram per liter of initial YE supplement was increased the final cell biomass and LA productions.

Mussatto *et al.*(2008) concluded that the LA production by *L. delbrueckii* UFV H2B20 in brewer's spent grain cellulosic hydrolysate could be performed with high yield (0.76 g g^{-1}) and productivity (0.79 g $l^{-1}h^{-1}$) when the hydrolysate was supplemented with nutrients. Furthermore, addition of 5 g l^{-1} YE enhanced the LA volumetric productivity that attained 0.53 g $l^{-1}h^{-1}$, value 18% higher than that obtained from non-supplemented hydrolysate.

The alternative, cheaper nitrogen sources from inorganic nitrogen sources and agricultural by-products have been used to achieve a partial and total replacement of YE, including, peptone, corn steep liquor (CSL) (Yu *et al*., 2008a), rice bran (Gao *et al*., 2008; Li *et al*., 2012), soybean hydrolysate, soytone (Kwon *et al*., 2000) and wheat bran (Li *et al*., 2010).

Very few of these low-cost nitrogen sources, even when some are combined with YE, yield LA concentrations as high as those obtained its YE alone. The media

that obtained the same concentrations of LA were achieved either at the expense of fermentation time or supplemented other expensive nutrients such as peptone and vitamins. However, use of commercial peptones is not economical due to their higher charges. Marine peptones from fish by-products (shark and thornback ray) could be a possible alternative commercial peptone (Vazquez *et al*., 2009). However, the maximal LA output needed inclusion of meat peptone and full exclusion of meat and yeast extracts that redirected the metabolic processes from LA fermentation to the accumulation of biomass when starch saccharification to produce LA by a natural strain *L. lactis* subsp. *lactis* B84 (Petrov *et al*., 2008). Yu *et al*. (2008a) supplemented with 5% malt coming nuts in the whey permeate containing lactose at a concentration of 55 g l^{-1} achieved similar LA yield to YE supplementation at around 55 h. Alonso *et al*. (2010) concluded that control of pH and YE supplementation in the preculture stage has shown a remarkable enhancement of fermentation performance due to the reduction in the toxicity and the increase in nitrogen content. Red lenti-RL and Baker'yeast cells could replace commercial peptone and YE (Altaf *et al*., 2007).

Corn steep liquors (CSL) which has a nitrogen content of 3.6% (1.44% belongs to amino nitrogen and the ratio of amino nitrogen to total nitrogen is 0.4) (Bustos *et al*., 2004; Gao *et al*., 2008; Vazquez and Murado, 2008; Yu *et al*., 2008a; Lu *et al*., 2009a; Vazquez *et al*., 2009; Yao *et al*., 2010) were also studies for LA production. Because of the low ratio of C/N could result in a high yield of LA as well as shortening the fermentation time. Yu *et al*. (2008a) concluded that CSL is an excellent source of nitrogen for most microorganisms because it is high in amino acids and polypeptides with considerable amounts of B-complex vitamins. It could not only replace YE as the sole nitrogen source in the optimized medium, but also help to enhance the LA production when cooperated with other beneficial medium components.

The unpolished rice saccharificate, wheat bran powder and YE are employed as carbon source, nitrogen source and growth factors, respectively. It provides not only carbon source but also other important nutrients such as amino acids and

B-vitamins (Lu *et al*., 2009b). Wheat bran (WB), rich in proteins, oil, nutrients, and calories, is one of the major by-products of wheat production. It is an inexpensive and light-colored nitrogen source (Lu *et al*., 2009b; Li *et al*., 2010). Because of wheat bran contains the total nitrogen 4.6 %, the use of 25 g l^{-1} wheat bran hydrolysate combined with 30 g l^{-1} corn steep liquor was performed. The L-lactic acid fermentation efficiency (yield 0.99 g g^{-1}, productivity 3.75 g $l^{-1}h^{-1}$) is even higher than that of the control with 15 g l^{-1} yeast extract (yield 0.95 g g^{-1},productivity 2.46 g $l^{-1}h^{-1}$) (Li *et al*., 2010). Gao *et al*. (2008) has investigated that the hydrolyzed rice bran at initial pH 1, 30 g l^{-1} rice bran could provide a productivity of 8 g l^{-1} YE on LA production for *L. rhamnosus* NRCC 3863. Dairy manure, the recycling nitrogen in agricultural wastes has been investigated as nitrogen sources in the culture medium for LA fermentation (Yao *et al.*, 2010).

The inorganic salts, such as ammonium sulfate, ammonium nitrate were also the most widely used in LA fermentation. Ammonium sulfate could be economically used to reduce YE supplement without a significant decrease in the LA production rate (Nancib *et al.*, 2001).

Since carbon–nitrogen ratio is important for cell growth. If the nitrogen supply is short, the cell cannot produce necessary enzymes for metabolism. If the nitrogen supply is too much, particularly in the form of ammonia, the cell growth will be inhibited because of the toxicities of some key enzymes (Fontenot *et al*., 2007). There is non-linear correlation between LA production and cell growth. The final concentration of L(+)-lactic acid could not increase by supplying much nitrogen source. Higher carbon–nitrogen ratio could not result higher final concentration of L-(+)-lactic acid. The productivity of L-(+)-lactic acid remarkably increased with the decrease of the carbon–nitrogen ratio. The results of the present work suggested that a lower carbon–nitrogen ratio could result a higher growth rate of cell and productivity of L-(+)-lactic acid for *L. rhamnosus* HG 09. Therefore, an appropriate carbon–nitrogen ratio of the medium must be considered to obtain a high productivity of L-(+)-lactic acid. However, the lower carbon–nitrogen ratio will increase the cost

of medium, because the nitrogen source is generally more expensive than the carbon source (Lu *et al*., 2010).

3. Temperature

Temperature is one of the key environmental parameters that affect the fermentation process and determine the optimum microbial growth rate and LA production (Yuwono and Kokugan, 2008). Different optimal temperatures for LA production have reported which vary depend on the fermentation conditions. In general, the optimal temperature growth of LAB is in the range of 5 to 45 ^{0}C (Abdel-Rahman *et al.*, 2013). In contrast to Hofvendahl and Hahn-Haggerdal (2000) reported the optimum temperature of LAB varies between the genera from 20 to 45^{0}C. For *Lc. Lactis* and *L. rhamnosus* exhibited the highest yield and productivities at 33 to 35^{0}C and 41 to 45^{0}C, respectively. For *L. casei* and *L.paracasei* the optimum temperature was reported between 37^{0}C and 44^{0}C. *Lc. Lactis* spp. *lactis* ATCC 19425 has optimum temperature between 33^{0}C and 35^{0}C (Akerberg and Zacchi, 2000). Akao *et al.*, (2007) reported the optimum growth temperature of *L. amylolyticus* is from 45 to 48^{0}C and it can grow up to 52^{0}C. Djukic-Vukovic *et al*. (2012b) reported that LA fermentation of the liquid stillage by *L. rhamnosus* ATCC 7469, the highest of biomass production was obtained at 41^{0}C and 48 h of fermentation. Tango and Ghaly (1999) has demonstrated that the effect of temperature on the growth of *L. helveticus* and production of LA from lactose. The increasing in temperature from 23^{0}C to 42^{0}C (with no pH control), enhanced the lactose utilization and LA production by 26.6% and 6.2 g l^{-1}, respectively.

Yuwono and Kokugan (2008) reported a study of the effects of temperature and pH on the kinetics of LA production from fresh cassava roots (FCR) by *Streptococcus bovis* in new media, tofu liquid waste (TLW); TLW with 2% by weight concentrated maguro waste (TLW+ CMW2) and in a standard trypto-soya broth (TSB) compared with a standard medium (glucose in TSB). The results showed that 39°C and pH 5.5 were optimal for fermentation properties (LA concentration, productivity and specific growth rate) in all media, including the standard.

At high temperature and high concentrations, LA is polymerized which is complicated to recover of LA by conventional techniques such as evaporation (Akerberg and Zacchi, 2000). When the temperature was increased, acetic and ethanol formation was increased in equimolar amounts. The amount of formic acid was decreased with the increasing fermentation time. Temperature above 30^0C and pH level below 6 enhanced the formation of by-products and D-lactic acid (Akerberg *et al*., 1998) which is essential for cost effective LA fermentation production.

Temperature may affect the isomer purity of LA production. Lower than optimum temperature under 45 and 50 ^{0}C at pH 5.0, *L. amylolyticus* produced racemic lactate (Akao *et al.,* 2007). Zhang *et al*. (2008) evaluated the interaction on L-(+) or D-(-)-lactic acid production during LA fermentation of the non starch kitchen wastes. When increasing the fermentation temperature from 35^0C to 45^0C at pH 7, the enhancement the isomer purity was increase from 60:40 to 83:17 and the total LA concentration declined by 60%. The optimum fermentation time for the purity of LA isomers was found to depend on the corresponding pH and temperature. However, most LAB produced only one isomeric form of LA, but sometimes there is slight production of the other isomer. *L. helveticus* and *L. plantarum* produce a racemix mixture where as *L. amylophilus, L. delbrueckii* and *L. rhamnosus* no produce D-isomer even when the pH is varied or the amount of nutrients was changed. On the other hand, only D-LA is form by *L. delbrueckii* spp. *bulgaricus* from glucose and lactose, and when the amount of nutrients is changed. The composition of the racemate formed by *L. plantarum* changed with aeration and amount of NaCl. The isomers composition of which varies depend on the isomeric form of lactate dehydrogenase (LDH) enzyme present in LAB (Hofvendahl and Hahn-Hagerdal, 2000).

4. pH

pH is one of the main factors influencing LA production by fermentation process because the catalytic activity of the enzymes and metabolic activity of the microorganisms depend on the extracellular pH (Mussatto *et al*, 2008). Hofvendahl and Hahn-Hagerdal (2000) elucidated that the optimal pH for LA production varies between 5.0 and 7.0. A pH below 5.7 was only optimal for *Lactobacillus* strains, which are known to tolerate lower pH than *Lactococci.* Fu and Mathews (1999) concluded the optimum pH for cell growth and acid production was found to be between 5 and 6 when conducted the fermentation of LA using *L. plantarum* under aerobic and anaerobic conditions (operation without aeration). pH 5.0 was optimum for specific growth rate and LA production by *L. fermentum* Ogi E1 (Santoyo *et al*., 2003).

Hetenyi *et al*. (2011) studied the pH dependence of *Lactobacilli* and the optimal pH value was between 6.8 and 7.0. Yuwono and Kokugan (2008) studied the effects of pH on productivity and specific growth rate of LA production from cassava root by *S. bovis* using Michaelis-Menten and Monod-type equations. For pH, the inhibition is competitive for both productivity and specific growth rate. By controlling the pH at 5.80, the inhibition for both productivity and specific growth rate were minimized in all media.

In case of LA fermentations pH regulation is crucial. The produced free LA inhibits both cell growth and product formation at even low concentration. Neutralizing it (putting the free acid into ionized form) with pH control can partly eliminate this problem. However, such a high concentration of ionized lactate also inhibits the growth and further production (Hetenyi *et al*., 2011). Mussatto *et al*. (2008) concluded that fermentation runs pH-controlled at 6.0 gave better results than those where the initial pH was not further controlled when LA fermentation by *L. delbruecki* and the best result, 35.54 g l^{-1} LA (0.99 g g^{-1} glucose consumed) was obtained during the pH-controlled fermentation of hydrolysate medium supplemented

with MRS components. The volumetric productivity at the end of the fermentation was 0.59 g $l^{-1}h^{-1}$, with a maximum of 0.82 g $l^{-1}h^{-1}$ during the first 12 h.

Thus, during LA fermentation, several neutralizing agents, such as sodium hydroxide, ammonium hydroxide, calcium carbonate, calcium hydroxide, sodium carbonate resulting in lactate formation were added to acidifying and pH control. Sodium hydroxide was the dominate raw material cost in the LA fermentation step. The LA production cost can be reduced by lowering the pH and/or by recycling the sodium hydroxide produced by electrodialysis to the fermenter (Pintado *et al*., 1999; Akerberg and Zacchi, 2000; Nancib *et al.*, 2001, 2005; Zhang *et al.*, 2008; Gao *et al.*, 2009; Kosugi *et al.*, 2010). Among of these agents, calcium carbonate has been widely used to maintain pH in laboratory shake flask and small scale experiments (Pintado *et al*., 1999; Kwon *et al.*, 2000; Altaf *et al.*, 2006; Gao *et al.*, 2009; Ge *et al.*, 2009; Lu *et al*., 2009b, 2010; Li *et al.*, 2010; Wang *et al.*, 2010; Karp *et al*., 2011; Li *et al*., 2012). In comparison with $CaCO_3$, NaOH is a simpler and cleaner alternative. When $CaCO_3$ is used, the calcium lactate produced has to be converted to LA by H_2SO_4, and the resulting by-product $CaSO_4$ is a solid waste that is produced in large amounts and has little utility. In contrast, when NaOH is used, the sodium lactate produced can be electrodialyzed to LA and NaOH is produced as a by-product (Zhao *et al.*, 2010a, 2010b). Using Calcium hydroxide to control pH also resulted in the production of large amount of solid calcium sulfate (Hofvendahl and Hahn-Hagerdal, 2000; Karp *et al*., 2011; Li *et al*., 2012). Ammonia or calcium carbonate can leads to production of fertilizer ammonium sulfate or gaseous carbon dioxide, respectively (Hofvendahl and Hahn-Hagerdal, 2000).

Five pH regulation agents were tested: addition of ammonium hydroxide, sodium hydroxide, dimethylamine and trimethylamine, and pH buffering using calcium carbonate. Among of them trimethylamine gave the best productivity result (3.13 g $l^{-1}h^{-1}$), but considering technological aspects (such as dilution) ammonium hydroxide is also recommended (Hetenyi *et al*., 2011).

Nakano *et al*. (2012) reported the effects of $Ca(OH)_2$, NH_4OH, and NaOH as neutralizing agents for efficient recovery of LA was produced from broken rice in a simultaneous saccharification and fermentation (SSF) process with *L. delbrueckii*. Results showed consumption of glucose (from broken rice) by the cells and the cell growth were the best with $Ca(OH)_2$ among the three neutralizing agents used. Maximum productivities of lactic acid reached with $Ca(OH)_2$, NH_4OH, and NaOH were 3.59, 1.51 and 1.40 g $l^{-1}h^{-1}$, respectively. Neutralization of LA fermentation with $Ca(OH)_2$ or $CaCO_3$ is applied in industrial plants, mainly to make the downstream processing easier and cheaper compared to lactates from other neutralizing agents (e.g., NH_4OH or NaOH). However, whether $Ca(OH)_2$ could improve LA productivity or not has not been clearly understood. Neutralization processes in L-(-)-lactic acid fermentations was either NaOH or NH_4OH.

Akerberg and Zacchi (2000) reported that the cost of sodium hydroxide dominates the operation cost in the fermentation step. The cost evaluations indicated that performing fermentation at a lower pH is favorable. Lowering the pH decreases the cost of sodium hydroxide in the fermentation step, as well as the cost of conversion of lactate in the electrodialysis step.

Gao *et al*., (2009) showed higher inhibitory effect on calcium-L-lactate (CaL_2) production than ammonium-L-lactate (NH_4) at lactate concentration lower than 100 g l^{-1}, but it showed lower inhibitory effect at higher lactate concentration. The strain *L. rhamnosus* (NBRC 3863) was resistant to glucose inhibition and final CaL_2 concentration increased with initial glucose concentration increasing up to 190 g l^{-1}. High-concentration CaL_2 induced flocculation of fermentation broth, causing incomplete fermentation. It was confirmed that stirring speed markedly influenced flocculation, an increase of the stirring speed leading to an earlier flocculation. Formation of colloidal CaL_2 during fermentation was considered to be the cause of flocculation in this study. Addition of YE raised fermentation efficiency and made complete fermentation feasible. In the fermentation with 25 g l^{-1} YE, glucose was completely consumed and final CaL_2 concentration of 220 g l^{-1} was obtained, when flocculation did not yet occur.

Nevertheless, the by-product formation increased with decreasing pH. Akerberg and Zacchi (2000) concluded that at pH levels below 6 also enhanced by-product formation in supplemented flour medium. The increase in acid resistance of LAB may be due to the restoration of the optimum intracellular pH through arginine utilization by arginine deiminase and NH_3 production. In addition, the use of strains able to tolerate acidic conditions would help to reduce the addition of buffering agents like calcium carbonate, thereby reducing the cost and pollution problems and making the recovery of free LA from the fermentation broth easier (Martinez *et al.,* 2013).

pH also affect the isomer purity of LA. Akerberg *et al*. (1998) reported the fermentation of LA from glucose by *Lc. lactis* ssp. *lactis* ATCC 19435 in whole-wheat and found that the LA productivity was highest between 33°C and 35°C and at pH 6. At temperatures above 30°C and pH levels below 6 enhanced the formation of by-products and D-lactic acid. Calabia and Tokiwa (2007) concluded that *L. delbrueckii* was grown on sugarcane molasses, sugarcane juice and sugar beet juice in batch fermentation at pH 6 and at 40 ^{0}C. After 72 h, the LA from 13% (w/v) sugarcane molasses (119 g total sugar per liter and sugarcane juice (133 g total sugar per liter) was 107 and 120 g l^{-1}, respectively. With 10% (weight by volume) sugar beet juice (105 g total sugar per liter), 84 g l^{-1} was produced. The optical purities of D-lactic acid from the feedstock ranged from 97.2 to 98.3%. Zhang *et al*. (2008) has elucidated that pH, temperature and fermentation time had an obvious influence on the variations of the isomer purity. The isomer purity was much higher at acidic or alkali pH (non-controlled pH, pH 5 and pH 8) than neutral pH (pH 6 and pH 7).

5. Other nutritional factors

Mineral salts such as PO_4, Mg, Mn, Zn and Fe, are provided in the medium in the form of salts KH_2PO_4, $MgSO_4$, $MnSO_4$ and $FeSO_4$. Normally, concentration of 0.03 - 2.5 g l^{-1} KH_2PO_4, 0.1 - 5 g l^{-1} $MgSO_4$, 0.03 - 0.2 g l^{-1} $MnSO_4$, 0.02 - 0.03 g l^{-1} $FeSO_4$ and 0.01 - 5 g l^{-1} NaCl have been used for LA fermentation (Kwon *et al.,* 2000; Nancib *et al.,* 2001, 2005; Gao *et al.,* 2008; Yuwono *et al.,* 2008; Gao *et al.,* 2009; Ge *et al*., 2009; Liu *et al.,* 2010; Li *et al*., 2012). The addition of vitamins enhanced

the LA production to some extent in all cases except for the nitrogen sources YE, urea and peptone (Nancib *et al.*, 2005). Among the vitamins present in YE, folic acid, niacin, panthothenic acid, pyridoxine and riboflavin are essential B vitamins for supporting the growth of *L. casei*. These vitamins are essential elements that act as cofactors in many enzymatic reactions (Martinez *et al.*, 2013).

However, the cost of nutrients is one of the main drawbacks for the commercial LA production. Thus, to find new low cost media that could employ in conventional processes is very interesting.

Lactic acid fermentation processes

Batch, fed-batch, repeated batch, and continuous fermentations are the most frequently used methods for LA production. Higher LA concentrations may be obtained in batch and fed-batch cultures than in continuous cultures, whereas higher productivity may be achieved by the use of continuous cultures. Another advantage of the continuous culture compared to the batch culture is the possibility to continue the process for a longer period of time. The cell-recycle system, together with repeated batch and continuous processes, enables the achievement of a higher cell concentration and product productivity in the process (Wee *et al.*, 2006c).

Selection of fermentation processes may vary with respect to the type and nature of substrate, microbial growth, and viscosity of fermentation broth. In the present section, fermentation processes and their developments for enhancing fermentative LA production are described. This includes batch, fed-batch, repeated, and continuous fermentations. The advantages and disadvantages of these fermentation modes are described in Table 4.

Table 4 Advantages and disadvantages of fermentation processes.

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

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

1. Batch fermentation and repeated batch fermentation

Batch culture is the most simple and commonly used fermentation process. It is considered as close system (except for aeration) containing a limit amount of medium in which all the substrate are added at the beginning of fermentation and the inoculated culture pass through a number of phases (Ward, 1992). This closed system has advantages in reducing the risk of contamination and obtaining high LA concentrations in comparison to other fermentation methods (Hofvendahl and Hahn-Hägerdal, 2000). On the other hand, batch fermentation suffers from low cell concentrations due to limited levels of nutrients and low productivity mainly because of either substrate and/or product inhibition. Kinetic studies on LA production by *L. paracasei* subsp. *paracasei* CB2121 showed that the final LA concentration increases up to 192 g l^{-1} with an increase in the initial glucose concentration as high as 200 g l^{-1} (Abdel-Rahman *et al.*, 2013). Ding and Tan (2006) carried out the batch fermentation of LAB. The best results obtained with glucose as the substrate are 150.2 g l^{-1} for the final L-lactic acid concentration and 1.34 g $l^{-1}h^{-1}$ for the productivity

Many studies have been carried out using the method of batch fermentation for industrially L-lactic acid production. Alonso *et al.* (2010) used yoghurts that have past their 'best before' date constituted as a source for LA production by *L. casei*. Results suggests that *L. casei* was able to metabolize most glucose and sucrose within 34 h to produce 25.9 g l^{-1} LA, but the lactose content left was 15.2 g l^{-1}. Li *et al.* (2012) studied the possibility to used white wheat bran as carbon and nutrient for LA fermentation by *L. rhamnosus* LA-04-1 with a productivity of 3.73 kg $m^{-3}h^{-1}$. The sugar solutions obtained from eucalyptus globules wood were supplemented with nutrients and used as fermentation media for LA production with *L. delbrueckii* NRRL B-445 (Parajo *et a*l., 1996). *L. plantarum* A6 was selected for LA production from mussel processing wastes (Pintado *et al.*, 1999). A new *Lc. lactis* subsp. *lactis* B84 is capable for utilizing starch as a sole carbon source and producing L(+)-lactate. In MRS-starch medium (with absence of yeast and meat extracts), at 33^0C, agitation 200 rpm and pH 6.0 for 6 days complete starch hydrolysis occurred and 5.5 g l^{-1} LA were produced from 18 g l^{-1} starch (Petrov *et al.*, 2008). To reduce the nutrient cost

of L-lactic acid production, wheat bran was chosen as a nutrient source. Pretreated wheat bran showed a better performance than that without treatment, especially for L-lactic acid yield (0.99 g g^{-1}) (Li *et al*., 2005). Among ten strains, *L. agilis* LPB 56 was selected for fermentation, due to its ability to metabolize the complex oligosaccharides. Fermentation was conducted without need for supplementary inorganic nitrogen sources or YE (Karp *et al*., 2011).

However, the major disadvantage of batch fermentation is that L-lactic acid concentration and productivity decreased due to inhibition by high substrate concentration, a conventional property of batch fermentation. Setlhaku *et al*. (2012) indicated the classical batch fermentation, when compared with fed-batch and continuous fermentation results in lower productivity and increased process downtimes.

In addition, selecting a suitable low-cost nutrient for the medium has usually been considered a major aspect for improvement and development of LA production. Increased undissociated LA in accordance with decreasing pH due to LA production is considered to inhibit the fermentation of several LA producers. Therefore, trapping the undissociated LA during fermentation as lactate salt by the addition of neutralizing agents, such as sodium hydroxide, potassium hydroxide, calcium hydroxide, calcium carbonate, or ammonium solution, would partially overcome such inhibition and improve fermentation efficacy as shown in Table 5 . Factors affecting batch fermentation efficiency were reported to improve lactic acid production efficacy including nitrogen sources, pH and neutralizing agents and aeration. These factors are briefly discussed in Table 5 (Abdel-Rahman *et al.,* 2013).

However, there are some constraints for LA fermentation such as lack of strains for high yield efficiency, high substrate utilization efficiency and abundantly use inexpensive crude wastes for the cost effective process. Different fermentation methods were applied in a batch fermentation mode to bear these criteria, including, simultaneous saccharification and fermentation (SSF) (Sreenath, *et al*., 2001; Miura *et al*., 2004; John *et al*., 2007; Marques *et al.,* 2008; Romani *et al.,* 2008; John *et al.,*

2009), separate hydrolysis and fermentation (SHF) , solid state fermentation (SSF) (Bustos *et al.*, 2004; Naveena *et al.*, 2005; Altaf *et al*., 2006; John *et al*, 2006), two step fermentation (TSE), simultaneous liquefaction, saccharification and fermentation (SLSF) (Wang *et al.*, 2010), submerged membrane fermentation process (Ramchandran *et al*., 2012), open repeated batch fermentation (Zhao *et al*., 2010b) were investigated. The SSF method offers various advantages over SHF, such as the use of a single-reaction vessel, rapid processing time, less enzyme loading, reduced end-product inhibition of hydrolysis, and increased productivity (Abdel-Rahman *et al.*, 2013).

Improvement of batch fermentation was also reported with a mixed culture**.** Use of mixed culture or open fermentation to improve lactic acid production from different substrates was shown in Table 5. Open or non sterilized condition strategy is favorable because (i) Millarge reaction and formation of furfural compounds during sterization can be avoid; (ii) equipment requirement and energy consumption can be lowered; and (iii) fermentation process can be simplified and labor can be saved (Zhao *et al*., 2010b).

Non sterile (open) fermentation has received more attention for LA production. Several studies have focused on enhancement of LA optical purity in open fermentation by complex natural microbial composition. Variations in conditions and methods of feeding substrate in open fermentation have been shown to affect the microbial community structure and consequently the optical purity of LA. Controlling pH in the fermentation broth has been reported to suppress the growth of indigenous bacteria for open D-lactic acid fermentation from rice bran powder. With the pH maintained at 5.0, *L. delbrueckii* IFO 3202 produced 28 g l^{-1} D-lactic acid from 100 g l^{-1} rice bran at a yield of 0.78 g g^{-1} and optical purity of 95 % (Abdel-Rahman *et al.*, 2013).

Repeated batch fermentation was performed to increase productivity because continuous culture is a well-known condition for high productivity. The advantage of repeated batch operation is simplifying the fermentation process, time and labor

saving, lowering equipment requirement and energy-efficient. These include less time required for washing and sterilizing the fermenter, omission of seed preparation time, high growth rates, and short main culture time due to the high initial inoculation volume. On the other hand, the strain used in this strategy must be highly robust to resist increased possibility of contamination during the repetition of open culture, especially for producing highly optically pure lactic (Zhao *et al*., 2010a).

Semicontinuous operation such as repeated batch culture was also combines the advantages in batch and continuous culture, such as possibility of high product concentration due to complete conversion of substrate. However the product concentration is usually lower than that in batch culture (Yin *et al.*, 1998). Multiple-stage repeated fermentation was reported to improve productivity in repeated fermentations by avoiding the inhibitory effects of high product concentrations on LA producing strains. Two-stage repeated batch fermentation exhibited a higher performance for D-lactic acid production than the 1 reactor system (Table 6) by *Sporolactobacillus* sp. CASD was increased the cell concentration at an early stage of fermentation (Zhao *et al*., 2010a). Repeated open fermentative production by *Bacillus* sp. was shown to be feasible for optically pure L-LA (Zhao *et al*., 2010b).

2. Fed-batch fermentation and repeated fed-batch fermentation

Fed-batch processes, substrate is added in increments throughout the process without the removal of fermentation broth. Culture feed could be continuously or sequentially with substrate. Fed-batch processes may be operate to remove the repressive effects of rapidly utilized carbon sources, to reduce the viscosity of the medium, to reduce the effect of toxic medium constituents or simple to extend the product formation stage of the process for as long as possible (Ward, 1992). During fed-batch cultivation, one or more nutrients are supplied to the fermenter while cells and products remain in the fermenter until the end of operation. Fed-batch is generally superior to batch processing and is especially beneficial when changing nutrient concentrations affect the productivity and yield of the desired product (Ding and Tan, 2006). Since both overfeeding and underfeeding of nutrient is detrimental to cell

growth and product formation, development of a suitable feeding strategy is critical in fed-batch cultivation (Lee *et al.*, 1999).

Several innovative fed-batch fermentation methods have been developed for LA production as indicated in Table 7 (Abdel-Rahman *et al.*, 2013). Different fed-batch methods such as pulse fed-batch, constant feed rate fed-batch, constant residual glucose concentration fed-batch and exponential fed-batch were studied on the LA fermentation by *L. casei*. Among of these, exponential fed-batch culture is an effective method for the fermentation of L-lactic acid. The maximum LA concentration (210 g l^{-1}) and L-lactic acid concentration (180 g l^{-1}) in exponential feeding glucose and YE culture was obtained, respectively, which is very useful to reduce the recovery cost of the product.

Studies of fed-batch process for LA production were perform in various microorganisms such as *B. coagulans* SIM-7 (Michelson *et al.*, 2006), *Streptococcus zooepidemicus* (Vazquez *et al.*, 2009), *R. oryzae* (Yamane and Tanaka, 2013). These studies were carried out using sugars or substrate feeding in order to improve production rate. Michelson *et al.*, (2006) elucidated that the maximum production rates of LA were different. In batch culture the maximum production rate of LA reached 9.7 g $l^{-1}h^{-1}$ by the 5^{th} hour of growth, while in fed-batch culture the maximum production rate of LA (11.2 g $l^{-1}h^{-1}$) was achieved already by the 3^{rd} hour. Higher production rate of LA in fed-batch mode was suppressed by end product inhibition thus the fermentations were performed for 21 and 23 h for fed-batch and batch modes, respectively. Ge *et al.* (2009) studies the fed-batch fermentation of Jerusalem artichoke tubers to improve LA production using mixed culture of *A. niger* and *Lactobacillus* sp. Results found that maximum L-lactic acid concentration of 120.5 g l^{-1} was obtained after 36 h in the fed-batch fermentation without additional nutrients, and the conversion efficiency of total sugar to L-lactic acid was 94.5% of the theoretical yield, which higher than batch fermentation.

Fed-batch fermentation is used to prevent or reduce substrate-associated growth inhibition by controlling nutrient supply because of substrate inhibition on

strains caused cell lysis and long lag phase, which would result in decreases in the fermentation rate and sugar utilization (Lee *et al.*, 1999; Ding and Tan, 2006). However, it still does not address severe product inhibition resulting from accumulating high LA product. To maximize the product concentration in fed-batch fermentation, several factors should be taken into consideration, including times and terms of feeding the substrate, the substrate concentration to be maintained in the fermentation broth, and how to feed the substrate (Abdel-Rahman *et al.*, 2013).

Fed-batch could produce high cell densities due to extension of working time, protect substrate inhibition especially glucose, control over the by-products production or catabolic repression effects. Various fermentation strategies were employed to achieving the high productivity and overcoming the problem of nutrient concentration within the optimal range, the high-cell-density fed-batch fermentation of *Chelatococcus* sp. starin MW10 was demonstrated (Ibrahim and Steinbuchel, 2010).

Repeated fed-batch or cyclic fed-batch culture was developed, the additional advantages of repeated fed-batch is prolonged fermentation stage of product synthesis. Different strategies of the repeated fed-batch were reported, the repeated fed-batch fermentation for citric acid production by *Yarrowia lipolytica* was carried out by Moeller *et al.* (2011). Other substrate feeding with repeated fed-batch fermentation such as phosphorous feeding is employed for industrial fermentation of penicillin G (Li *et al*., 2005). Coupling continuous reactor with repeated fed-batch reactor is studied to acetone-butanol-ethanol production (Setlhaku *et al*., 2012).

Table 5 Lactic acid production in batch fermentation with different methods.

Fermentation substrate	Strain	Lactic acid				Fermentation method
		C ($g\ l^{-1}$)	Y ($g\ g^{-1}$)	P ($g\ l^{-1}\ h^{-1}$)	Isomer and optical purity (%)	
Broken rice	*Lb. delbrueckii*	79.0	0.81[a]	3.58	D (96.1)	●SSF with glucoamylase ●Performed in 5 l fermenter with 2.5 l basal medium at 40°C, 150 rpm, pH controlled at 6.0 with $Ca(OH)_2$
Corn starch	*Lb. plantarum* NCIMB 8826 (engineered)	73.2	0.85	3.86[b]	D (99.6)	●Performed in a 2 l bioreactor with a 700 ml working volume, at 37°C,100 rpm, pH controlled at 5.5 by NH_3 solution.
Corn stover	*Lb. rhamnosus* and *Lb. brevis* (mixed culture)	21.0	0.70	0.58	ND	●SSF with cellulases ●Performed in 250 ml flasks containing 100 ml media at 37°C, shaking at 100 rpm, initial pH 5 with $CaCO_3$.
Glucose	*Lb. paracasei* subsp. *paracasei* CHB2121	192	0.96	3.99	L (96.6)	●Performed in a 2.5 l jar fermenter with a working volume of 1.5 l at 38°C and 200 rpm, pH controlled at 6.5 by addition of NaOH.
Glucose	*Bacillus sp*. Na-2	106	0.94	3.53	L (99.5)	●Two stage aeration method ●Open fermentation ●Performed in a 5 l bioreactor containing 4 l unsterilized fermentation medium at 50 °C, pH controlled at 6.0 by NaOH.
Glucose	*Rhizopus oryzae* GY18	115	0.81	1.6	L (98.5)	●Performed in 500 ml flask at 35 °C and $CaCO_3$ as a neutralizing agent.

Table 5 (Continued)

Fermentation substrate	Strain	Lactic acid				Fermentation method
		C ($g\ l^{-1}$)	Y ($g\ g^{-1}$)	P ($g\ l^{-1}\ h^{-1}$)	Isomer and optical purity (%)	
Jerusalem artichoke tuber extract	*Lb. paracasei* KCTC13169	92.5	0.98	1.2	L (93.2)	●Performed in 5 l jar fermenter containing 2 l medium at 37°C, at 150 rpm, pH controlled at 6.0 with NaOH.
Liquid distillery stillage	*Lb. rhamnosus* ATCC 7469	18.6	0.73	-	L (ND)	●No supplementation with nitrogen or mineral salts ●Performed in 500 ml flasks with 200 ml of liquid stillage under anaerobic conditions in a gas pack system for 72 h at 41 °C, shaking (90 rpm), $CaCO_3$ (1%) as a neutralizing agent.
Sucrose	*H. halophilus* JCM 21694	65.8	0.83	1.1	L (98.8)	●Performed in a 5 l jar fermenter with 2.5 l fermentation medium at 30°C, 250 rpm, pH controlled at 9.0 by NaOH.
Sucrose	*Escherichia coli* (engineered)	85	85	1.0	D (98.3)	●Performed in 15 l fermentor with 10 l medium at 37°C, 200 rpm, and pH 7.0 controlled by a 3.5 M $Ca(OH)_2$ slurry.
Sucrose	*R. oryzae* GY18	80.1	0.89	1.67	L (98.5)	●Performed in a 500 ml flask at 35°C and $CaCO_3$ as a neutralizing agent.
Xylose	*R. oryzae* GY18	68.5	0.85	0.57	L (98.5)	●Performed in 500 ml flask at 35°C and $CaCO_3$ as a neutralizing agent.

Table 5 (Continued)

Fermentation substrate	Strain	Lactic acid				Fermentation method
		C ($g\ l^{-1}$)	Y ($g\ g^{-1}$)	P ($g\ l^{-1}\ h^{-1}$)	Isomer and optical purity (%)	
Xylose	*Candida utilis* (engineered)	93.9	0.91	2.18	L (99.9)	●Performed in100 ml spherical flat bottom flasks containing 30 ml of medium at 35°C, 100 rpm, and initial pH 6.8. Calcium carbonate was used as a neutralizing agent.
Xylose	*R. oryzae* NBRC 5378	14.4	-	0.56	L (ND)	●Performed in 1 l fermenter vessel with 600 ml medium at 30°C, 300 rpm, pH controlled at 3.5 by calcium hydroxide slurry. ●Aeration started at 1 vvm of air with changing agitation to 750 rpm 12 h after inoculation.
White rice bran hydrolysate	*Lb. Rhamnosus* LA-04-1	82.0	0.81	3.73	L (ND)	●SHF with amylase and glucoamylase ●Performed in a 5 l jar fermenter with 2 l working volume at 42°C, the rotation speed was 2.5 Hz, pH controlled at 6.25 by $Ca(OH)_2$ solution.

C, concentration; Y, yield; P, productivity; H., Halolactobacillus; Lb., Lactobacillus; SHF, separate hydrolysis and fermentation; SSF, simultaneous saccharification and fermentation; ND, not determined. [a] Based on starch content of rice. [b] Maximum lactic acid productivity.

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

Table 6 Different methods used for lactic acid production in repeated batch fermentation processes by bacteria and fungi.

Cell recycle methods	Strain	Fermentation substrate	Lactic acid				Fermentation method
			C ($g\ l^{-1}$)	Y ($g\ g^{-1}$)	P ($g\ l^{-1}\ h^{-1}$)	Isomer and optical purity (%)	
For bacteria							
1. Centrifugation	*Bacillus* sp.2-6	Glucose	107	0.95	2.9	L-(ND)	●Open fermentation ●Performed in 5 l fermenter with a working volume of 3 l at 50°C, at 200 rpm, pH controlled at 6.5 by NaOH ●At the end of each batch, the broth was centrifuged and all recycled cells were used as the seed for the next batch.
	E. faecium No. 78	Liquefied sago starch	36.3	0.57	1.96	L-(ND)	●Performed in 3 l fermenter with a working volume of 3 l at 30°C, at 200 rpm, pH controlled at 6.5 by 10 M NaOH ●At the end of each batch, the cells were harvested by centrifugation and used as the seed for the next batch.
2. Hollow-fiber filtration module	*E. faecalis* RKY1	Glucose	92-94	-	6.03-6.2	L-(ND)	●Performed in 2.5 l jar fermenter containing 1 l of medium at 38°C, 200 rpm, pH 7.0 controlled by 10 N NaOH ●Cell recycled was obtained using a hollow-fiber filtration module..

Table 6 (Continued)

Cell recycle methods	Strain	Fermentation substrate	Lactic acid C ($g\ l^{-1}$)	Y ($g\ g^{-1}$)	P ($g\ l^{-1}\ h^{-1}$)	Isomer and optical purity (%)	Fermentation method
	E. faecalis RKY1	Wood hydrolyzate	48.0	0.92	4.0	L-(ND)	●Performed in 2.5 l jar fermenter containing 1 l of medium at 38°C, 200 rpm, pH 7.0 controlled by10 N NaOH ●Cell recycle was obtained using a hollow-fiber filtration module.
3. Using part of the culture	*Sporolacto bacillus* sp. strain CASD	Glucose	82.8[a]	0.93[a]	1.72[a]	D-(98.9)	●Performed in one reactor system in 500 ml flasks with 200 ml working volume, at 42°C, 50 rpm, pH is self regulated by $CaCO_3$ at 5.6–5.8
			87.3[a]	0.93[a]	1.81[a]		●Performed in two reactor system at same conditions as above
For fungi							
1. Filtration	*R. oryzae* ATCC 9363	Glucose				L-(ND)	●Performed in 5 l stirred tank bioreactor containing 3 l medium at 35°C, agitation rate 300 rpm, aeration rate 2 vvm, and pH controlled at 6.0 using 40% (w/w) $CaCO_3$ slurry ●The broth was filtered out using a glass tube and

Table 6 (Continued)

Cell recycle methods	Strain	Fermentation substrate	Lactic acid				Fermentation method
			C ($g\ l^{-1}$)	Y ($g\ g^{-1}$)	P ($g\ l^{-1}\ h^{-1}$)	Isomer and optical purity (%)	
							cells used for next runs.
2. Mycelial pellet precipitation	*R. oryzae* NRRL 395	Corn starch				L-(ND)	●Performed in 3 l stirred tank bioreactor containing 2 l medium at 35°C, agitation rate 300 rpm, aeration rate 0.5 vvm, and pH controlled at 6.0 using 30% (w/w) $CaCO_3$ ●After batch culture and stopping aeration, mycelia pellets were precipitated and used for next run

C, concentration; Y, yield; P, productivity; *E.*, *Enterococcus*; *R.*, *Rhizopus*. [a] Data of the 2nd batch. [b] Data of the first 6 batches.

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

Table 7 Different methods used for lactic acid fermentation in fed-batch fermentation process.

Feeding method	Fermentation substrate	Strain	Lactic acid C ($g\ l^{-1}$)	Y ($g\ g^{-1}$)	P ($g\ l^{-1}\ h^{-1}$)	Isomer and optical purity (%)	Fermentation method
Continuous feeding	Glucose	*Lb. lactis* BME5-18M	210 161	0.97	2.2 2.02	L (ND)	● Performed in 5 l fermentoe at 37°C, pH controlled at 6.2 by 50% (w/w) $CaCO_3$ slurry ●pH-controlled feeding with continuous feeding of glucose ●pH controlled at 7.0 by 5 M NH_4OH.
Constant feed rate	Glucose	*Lb. casei* LA-04-1	135	0.88	1.61	L (ND)	●Performed in a 5 l jar fermenter with an initial broth volume of 2.2 l at 42°C ●pH controlled at 6.25 by 25% (w/w) NH_4OH.
Constant residual substrate concentration			153	0.93	1.82		
Exponential feeding			158[a] 180[b]	0.91 0.90	1.88 2.14		●The nutrient feeding rate is determined by a specific equation, which is derived from a mass balance with the assumption of a constant cell yield on substrate and constant maintenance coefficient throughout the fermentation.

Table 7 (Continued)

Feeding method	Fermentation substrate	Strain	Lactic acid C ($g\ l^{-1}$)	Y ($g\ g^{-1}$)	P ($g\ l^{-1}\ h^{-1}$)	Isomer and optical purity (%)	Fermentation method
pH feedback-controlled substrate feeding	Glucose	*Lb. lactis*-11	96.3	0.99	1.9	L-(ND)	●Performed in 5 l fermenter at 42°C, pH controlled at 6.0 by 6 M NH_4OH/glucose solution.
		Lb. rhamnosus LA-04-1	170	-	2.6	L-(ND)	●Feeding by 770 g l^{-1} glucose using glucose controller ●Performed in 5 l fermenter at 42°C, pH controlled at 6.25 by 33% (w/w) calcium hydroxide solution or ammonia.
	Lime-treated wheat straw	*B. coagulans* DSM 2314	40.0	0.43	-	L-(97.2)	●SSF-fed-batch with cellulose, cellobiase and xylanase ●Performed in 20 l fermenter at 50°C and pH 6.0 ●Neutralization of acid by fed-batch addition of alkaline substrate
Pulse feeding	Glucose	*Lb. casei* LA-04-1	130	0.89	1.55	L-(ND)	●Multi-pulse feeding
		R. oryzae NRRL 395	92.0	0.60	0.7	L-(ND)	●Multi-pulse feeding ● Performed in7 l fermentor at 27°C, pH controlled at 7.0 by 20% $Ca(OH)_2$

Table 7 (Continued)

Feeding method	Fermentation substrate	Strain	Lactic acid C ($g\ l^{-1}$)	Y ($g\ g^{-1}$)	P ($g\ l^{-1}\ h^{-1}$)	Isomer and optical purity (%)	Fermentation method
		Corynebacterium glutamicum	120	0.87	4.0	D-(99.9)	●Multi-pulse feeding ●Performed in 100 ml bottles under oxygen deprivation conditions at 45°C, pH-controlled at 7.0 by ammonia
	Jerusalem Artichoke tubers	*Aspergillus niger* SL-09 and *Lactobacillus* sp. G-02 (mixed culture)	121	0.95	3.3	L-(95)	●Multi-pulse feeding ●SSF fed-batch with inulinase and invertase produced by *A. niger* ●Multi-pulse feeding ●Performed in 7 l fermenter containing 4 l medium at 30°C, aeration rate was 4.5 l min^{-1}, and the agitation speed was controlled at 140 rpm, initial pH was 7 (2% $CaCO_3$).
		Lactobacillus sp. G-02	142	0.94	4.7	L-(95)	●Multi-pulse ●SSF-fed-batch with inulinase ●Performed in 7 l fermenter containing 4 l of a medium with enzyme and sodium citrate 10 g l^{-1}, at 30°C, initial pH of 6.5, 5% $CaCO_3$
	Peanut meal and glucose	*Sporolactobacillus* sp. strain CASD	207	0.93	3.8	D-(99.3)	●Pulse fed method ●Performed in 30 l bioreacter containing 24 l working volume at 42°C, pH is self regulated at 5.0–6.0 with $CaCO_3$ inside the fermenter
			226	0.84	4.4		

Table 7 (Continued)

Feeding method	Fermentation substrate	Strain	Lactic acid C ($g\ l^{-1}$)	Y ($g\ g^{-1}$)	P ($g\ l^{-1}\ h^{-1}$)	Isomer and optical purity (%)	Fermentation method
		Bacillus sp. WL-S20	225	0.99	1.04	L-(100)	●Multi-pulse fed method ●Multi-pulse feeding ●Performed in a 1.5 l bioreacter with a working volume of 700 ml at 45°C, 200 rpm, pH-controlled at 9.0 by NaOH.
			180	0.98	1.61		●Pulse feeding

C, concentration; Y, yield; P, productivity; *Lb*., *Lactobacillus*; *B*., *Bacillus*. [a] Feeding glucose solution (850 $g\ l^{-1}$). [b] Feeding glucose solution (850 $g\ l^{-1}$) and yeast extract (1%).

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

3. Continuous fermentation

Continuous fermentation may be considered as open systems in which medium is continuously added to the bioreactor and an equal volume of fermented medium is simultaneously removed. Continuous system, high output can much more efficient in terms of fermenter productivity (product output per unit volume per unit time, kg m-^{3}) for certain applications (Ward, 1992). Continuous fermentations have an advantage of shorter down time, automatic operation tends to be simpler than in batch fermentations and usually higher productivity is achieved (Setlhaku *et al*., 2012). Because of production of LA is strictly associated with cell growth, which allows the cells to obtain the necessary energy for growth from LA producing pathways. Continuous LA fermentation is attractive in terms of avoiding the end-product inhibition that occurs in batch/fed-batch fermentation by diluting the product in the fermentation broth with fresh medium (Abdel-Rahman *et al*, 2013).

Chemostat fermentation is a typical continuous fermentation system in which feeding of fresh medium to the fermenter and withdrawing fermentation broth are performed at the same rate to provide constant control of the concentration of a component in the fermentation broth (Abdel-Rahman *et al*, 2013). In chemostat fermentation, the concentrations of cells, products, and substrates in the fermentation broth can be stably maintained at constant levels during certain periods. The specific growth rate can be adjusted by the dilution rate because the specific growth rate would equal the dilution rate under steady state conditions in chemostat fermentation (Bustos *et al*., 2007). In a chemostat, the cells can be maintained at a constant physiological state and growth rate. The growth rate can be adjusted by changing the feed flow rate and consequently it is easier to optimize the global productivity. Moreover, a biological phenomenon observed in batch cultures is the lag phase, which occurs at the beginning of the fermentation and represents a physiological adaptation of the culture to the new environment. Growth during this phase is very slow and the lag phase represents a period of very low productivity.

When referring to continuous culture systems, the terms lag phase, exponential phase, stationary phase and death phase have no meaning. This is because the system is operating continuously and growth cannot be segregated into phases. Besides, continuous cultures are shut down with less frequency as compared to batch reactors (where the reactor must be emptied, cleaned, sterilize and re-filled) and there is less loss of productivity during lag phases. Finally, during the continuous fermentation the complex nutritional requirements found in several *Lactobacillus* species can be reduced (Bustos *et al*., 2007). *L. pentosus* can be used to produce LA and biosurfactants in a chemostat system. The highest volumetric productivity (QP = 3.100 g $l^{-1}h^{-1}$) was achieved under high dilution rates (0.200 h^{-1}) (Bustos *et al*., 2007).

However, the efflux of unutilized carbon sources and cells from the fermenter and the decrease in LA concentration with an increase in the dilution rate are problematic points during lactic acid production with continuous fermentation (Abdel-Rahman *et al,* 2013). Several attempts have been made to replace fermentation with continuous to reduce end-product inhibition and improve lactate productivity. To improve lactate productivity utilizing of higher cell density, different fermentation feedstocks, different lactate producing strain (Nolasco-Hipolito *et al*., 2002), the cell recycle continuous fermentation (Wee *et al*., 2009), the two-stage continuous cultures (Bouguettoucha *et al*., 2009), Electrodialysis fermentation (EDF) (Min-tian *et al*., 2004) were studied.

MATERIALS AND METHODS

Materials

1. Oil palm trunk (OPT)
2. Fermenter Model BIOFLO 2000, 2 L, pH 2000 Control Module, DO 2000 control Module, New Brunswick, U.S.A.
3. High Performance Liquid Chromatography (HPLC) (System controller: Water 2690: Alliance, Software MLN32, RI detector: WATER (U.S.A.),
4. Chromatographic Column: Animex HPX-87H Column, 300 x 7.8 mm, BioRad, U.S.A.
5. Chromatographic column: Shodex SUGAR SP0810, Showa DHOWA DENKO EUROPE GmbH, Japan
6. Ultracentrifuge (Sigma, Germany)
7. UV-visible spectrophotometer (Shimazu, Japan)
8. Water bath
9. pH meter
10. Incubator
11. Laminar flow
12. Autoclave
13. Glass wares and chemicals

Methods

1. Raw materials preparation and chemical compositions

1.1 Extraction of OPT juice

Oil palm trunks (OPT) of approximately 20 years old grown in the southern province of Thailand were collected in August, 2009. The trunks were cut vertically into 3 logs (top (T), middle (M), and base (B)) and each log was cut horizontally into inner (1), middle (2) and outer (3) regions with machine saw. The

sap was extracted from each piece, using sugar cane press machine. The sap squeezed from OPT was subsequently centrifuged at 4,000 rpm for 40 min at 4^0C and the OPT juice was stored at -18^0C for further analysis.

1.2 Chemical composition of OPT juice

The moisture content of OPT juice was determined by drying at 105^0C in an oven until a constant weight (Ehrman, 1994). The content of crude protein was estimated as total nitrogen content by Kjeldahl method and multiplied by 6.25. The total ash content was examined by incineration at 550^0C for 3 h. Other proximate compositions including starch and crude fat were estimated as per the standard ‘‘A.O.A.C.” procedure (A.O.A.C., 1980). The total soluble protein contents were determined by Lowry method (Lowry *et al*., 1951).

The sugars compositions of OPT juice were analyzed by High Pressure Liquid Chromatography (HPLC), using Shodex Sugar SP0810 column (8.0 x 300 mm, Showa Denko, Japan) at 80^0C. Distilled water was used as an eluent with a flow rate of 1 ml min^{-1}. The Animex HPX 87H column was also used to determine sugar compositions of OPT juice, using 5 mM H_2SO_4 as an eluent with a flow rate of 0.6 ml min^{-1}. The compounds of interest were detected with a refractive index detector (Waters 2414, Waters, USA).

2. OPT juice fermentation

2.1 Microorganisms and inoculum preparations

2.1.1 *Lactobacillus casei* subsp. *rhamnosus* TISTR 108

The lactic acid bacteria, *Lactobacillus casei* subsp. *rhamnosus* TISTR 108 was used for lactic acid fermentation. The strain was maintained on MRS medium containing 30% glycerol.

2.1.2 Seed preparation

The 250 µl of *L. rhamnosus* TISTR 108 was transferred from a stock culture to 15 ml test tubes containing 5 ml MRS broth. The tubes were incubated at 40^0C, 48 h in a static culture. One percentage of this inoculum was transferred to preculture medium in 250 ml Erlenmeyer flask with 100 ml MRS medium containing 20 g glucose, 10 g bactotryptone, 10 g yeast extract, 2 g K_2HPO_4, 5 g $CH_3COONa.3H_2O$, 0.2 g $MgSO_4.7H_2O$ and 5 mg $MnSO_4.4H_2O$ per liter (Kosugi *et al*., 2010). The pH was adjusted to 7.0 with 6 N NaOH or conc. HCl. The medium was sterilized by autoclaving at 121^0C for 15 min. The fermentation was performed under the controlled temperature at 40^0C for 18 h and the rotation speed was 150 rpm. The number of cell or OD 620 nm was adjusted to constant values before use as seed inoculum.

2.2 Optimization fermentation of OPT juice without nutrient supplementation

Batch fermentations of LA were performed aseptically in a 2 l fermenter that contained 1.6 l OPT juice as basal medium. After autoclaving at 110^0C, 10 min, seed culture (10% v/v) was inoculated into the fermentation medium. pH was automatic controlled at 6.0 with 6 N $Ca(OH)_2$ solution. The culture was carried out without aeration. The temperature and agitator speed were maintained at 40^0 C and 200 rpm, respectively. The fermentation parameters including OPT juice dilutions (OPT juice: water = 4:0, 3:1, 2:2, 1:3), inoculums (5, 10, 15%v/v), temperature (30, 35, 37, 40, 43^0C) and pHs (5.0, 5.5, 6.0, 6.5, 7.0) were optimized for LA production. During fermentation, the samples were taken at 0, 6, 12, 18, 24, 30, 36, 42, 48, 54, 60 and 72 h. Cell growth, LA and sugars concentrations were examined as described in Section 3. The fermentation time was selected based on no lactic acid produced.

The performance of LA production was described as lactic acid yield ($Y_{P/S}$) and productivity at the fermentation time that no lactic acid was produced, i.e. (dy/dt = 0); dt, the increase of lactic acid concentration at defined time interval; dt =3 h for the first 36 h and 6 h for the rest of fermentation i.e. upto 72 h, unless specified

as others. The lactic acid yield, $Y_{P/S}$, was expressed as gram of lactic acid produced per gram of total sugars consumed (g g^{-1}). The lactic acid productivity (Q_P, g $l^{-1}h^{-1}$) was determined as the ratio of lactic acid concentration (g l^{-1}) to the fermentation time (h). The total amount of sugars consumed (g l^{-1}) was calculated as the difference between the total initial sugars (g l^{-1}) and the residual sugars (g l^{-1}). The total initial sugars were calculated as the total amount of available glucose, fructose, and sucrose present in the sample while the residual sugars (g l^{-1}) were referred to the total sugars in fermentation broth at that fermentation time.

The lactic acid yield and lactic acid productivity were calculated by the following equation:

$$Y_{P/S}\ (g\,g^{-1}) = \frac{\text{Lactic acid produced (g)}}{\text{Total sugars consumed (g)}}$$

$$Q_P\ (g\,l^{-1}h^{-1}) = \frac{\text{Lactic acid concentration}(g\,l^{-1})}{\text{Fermentation time (h)}}$$

2.3 Enhanced OPT juice utilization by nutrient supplementation of undiluted OPT juice for lactic acid production

Undiluted OPT juice was used as a basal medium for lactic acid fermentation with nutrient supplementation. The modified medium was based on the study by Timbuntam (2008). The medium contained the following compositions (per liter), which were undiluted OPT juice, 10 g yeast extract (YE), 5 g peptone and salts (1.5 g $CH_3COONa.3H_2O$, 1.5 g KH_2PO_4, 1.5 g K_2HPO_4, 0.2 g $MgSO_4.7H_2O$ and 0.05 g $MnSO_4.4H_2O$). The medium was autoclaved at 110^0C for 10 min. The fermentation without salts addition and the fermentation without nitrogen supplemented were also examined. The control fermentation was carried out by using 100 g glucose, 10 g YE, 5 g peptone and salts as above. Ten percentage of inoculum volume was used. All experiments were performed under optimized conditions obtained in section 2.2.

2.4 Optimization of the dosage of nitrogen sources and salts for lactic acid fermentation of undiluted OPT juice using the Box Behkhen Design

In order to obtain the high lactic acid in undiluted OPT juice fermentation, the experimental conditions were optimized through the Box Behkhen Design (BBD). Three factors, namely peptone, yeast extract, and salts in three levels, were evaluated. The variables used for the BBD was summarized as shown in Table 8. According to this design, 15 experiments were conducted. The experiments were performed in 250 Erlenmeyer flasks containing 100 ml fermentation mediums, depending on the experiment design. One gram of $CaCO_3$ was added to maintain pH at 6.5. The fermentation experiments were conducted on the rotary shaker at 150 rpm for 48 h. All shake flasks were performed in duplicate. The fermentation broth was heated at 70^0C for 30 min and centrifuged at 1,000 rpm for 3 min to remove $CaCO_3$ pellet. Ten ml of the supernatant was heated at 70^0C for 30 min and 16 ml of 2M H_2SO_4 was added to neutralize. The reaction tubes were left for complete precipitation and centrifuged at 10,000 rpm for 5 min. The clear supernatant was diluted before determination of sugars and lactic acid by HPLC (Section 3).

The statistical analysis of the data was analyzed using the MINITAB statistical package. A full quadratic model was used to predict the optimal point, as the following equation:

$$Y = b_0 + b_1A + b_2B + b_3C + b_{12}AB + b_{13}AC + b_{23}BC + b_{11}A^2 + b_{22}B^2 + b_{33}C^2$$

Where Y is the predicted response (lactic acid concentration (g l^{-1})), b_0 is intercept; b_1, b_2 and b_3 are linear coefficients, b_{12}, b_{13} and b_{23} are interaction coefficients, b_{11}, b_{22} and b_{33} are squared coefficients, A, B and C are the coded level of the independent variables. The response surface curves and corresponding contour plots were drawn. The maximum LA production was obtained by using response optimization. The confirmation of the predicted value and the maximum LA production was confirmed.

2.5 Batch fermentation in 2 l fermenter

The lactic acid fermentation under the optimized medium obtained by response optimization in section 2.4 was conducted in 2 l fermenter containing 1.2 l OPT juice based medium. The medium was autoclaved at 110^0C for 10 min. The amount of 10% of *L. rhamnosus* TISTR 108 was then inoculated. The temperature and agitation speed were controlled at 40^0C, 200 rpm, respectively. The pH was controlled by the automatic addition of 6 N $Ca(OH)_2$ at pH 6.5. The samples were taken every 6 h intervals for 72 h to determine viable cells, lactic acid and sugar concentration. Lactic acid and sugar contents in fermentation broth were analyzed by HPLC as stated in Section 3. Lactic acid yield ($Y_{P/S}$) and lactic acid productivity (Q_P) were calculated as follow:

$$Y_{P/S} = \frac{\Delta P_{la}}{\Delta S_{total}} = \frac{P_{la}^{f} - P_{la}^{i}}{S_{total}^{f} - S_{total}^{i}}$$

$$Q_P = \frac{\Delta P_{la}}{t} = \frac{P_{la}^{f} - P_{la}^{i}}{t}$$

Where P_{la}^{f} = the lactic concentration at the end of fermentation (g l^{-1})

P_{la}^{i} = the lactic concentration at the beginning of fermentation (g l^{-1})

S_{total}^{i} = total sugar concentration at the beginning of fermentation (g l^{-1})

S_{total}^{f} = total sugar concentration at the end of fermentation (g l^{-1})

t = the fermentation time (h)

3. Other analytical methods

Lactic acid and total sugar concentrations of fermentation broth were analyzed using HPLC system with Animex HPX-87H column, 300x7.8 mm (Bio-Rad) with a refractive index detector (Waters, USA). The 0.005 M H_2SO_4 was used as an eluent with a flow rate 0.6 ml min^{-1} and the column temperature was controlled at 50^0C. The

number of viable cells was estimated using a spreading plate technique on MRS agar after incubation at 40^0C in an anaerobic jar.

Table 8 Experimental design variables used in the Box Behkhen Design.

Factors	**Coded units**		
	-1	0	1
Peptone (g l^{-1})	0	5	10
Yeast extract (g l^{-1})	0	10	20
Salts concentrations*	1	3	5

*Salts concentration (per liter) (1) without salt addition, (3) 0.2 g $MgSO_4.7H_2O$, 0.05 g $MnSO_4.4H_2O$, 1.5 g K_2HPO_4, 1.5 g KH_2PO_4 and 1.5 g $CH_3COONa.3H_2O$ and (5) 0.4 g $MgSO_4.7H_2O$, 0.1 g $MnSO_4.4H_2O$, 3.0 g K_2HPO_4, 3.0 g KH_2PO_4 and 3.0 g $CH_3COONa.3H_2O$.

RESULTS AND DISCUSSION

Part I: Oil palm trunk characterization and oil palm trunk juice properties

Oil palm trunks, after harvested, were immediately cut into 3 logs, i.e. top (T), middle (M), and base (B). Each log, 1.9 m in length was then cut in cross section into inner (1), middle (2) and outer (3) regions and sliced into disks of 1.5-2 cm thick and 10 cm wide, as illustrated in Figure 4. OPT disks were then mechanically squeezed using a conventional sugar cane pressing machine to obtain sap or oil palm trunk juice (OPT juice), which was collected and further centrifuged to remove some precipitates.

The material balance for the OPT juice extraction was shown in Figure 5. OPT was found to contain the highest amount of fiber and peel (69.5% w/w). A total of 134.3 kg of clear, undiluted OPT juice was obtained after centrifugation from a trunk of 545 kg, which accounted for 24.6% w/w of OPT fresh weight with the precipitate of 16 kg (2.98%w/w). The loss during this process was approximately 2.89%.

The total weights of OPT juice obtained from different parts of harvested trunk were summarized in Table 9. The yields of squeezed juice obtained from each log were 23.1, 45.2 and 31.7% for the top, middle and basal parts, respectively. The lowest yield of juice from basal part might be due to a large amount of hard fiber, being difficult to be disintegrated by mechanical force.

The results of moisture content (MC) of each part of OPT are presented in Table 10. The highest moisture content was found in the basal parts of OPT. The MCs of top, middle and basal parts were 64.0±2.5, 66.3±5.9 and 70.9±5.3%, respectively. The average MC of OPT in this study was 67.1±4.5%. The inner OPT region of all parts contained the highest MC (72±4.8%) that radially decreased along the middle (66.1± 4.4%) and outer (63.2±2.1%) regions, respectively. These results were similar to the study of Yamada *et al.* (2010), who reported that the trunk was consisted of fibrous vascular bundles and powdery parenchyma. The parenchyma

seems to hold more moisture than the vascular bundles. The difference in MC between these sections might be attributed to the weight ratio of parenchyma and vascular bundles. The trunk of oil palm contained high moisture contents which implied high amounts of squeezed juice from OPT.

The ash and crude protein contents in different parts of OPT are shown in Table 11 and Table 12. The total amounts of ash and crude protein of OPT were 2.39±0.1 and 1.75± 0.11% dry weight, respectively. The total amounts of soluble protein of juice obtained from different parts of OPT were measured by a Lowry method. The results were shown in Table 13. The soluble protein contents of 1.87±0.31, 2.02±0.27 and 2.64±0.84 g l^{-1} were observed in juice extracted from the inner, middle and outer region of OPT, respectively. Komonkiat and Cheirsilp (2013) reported the total soluble nitrogen in OPT sap of 3.5±0.10 g l^{-1}. Kosugi *et al*. (2010) reported that total amount of amino acids in the sap was 198.3 µg g^{-1} and the major amino acids were serine, alanine, glutamic acid and aspartic acid.

The sugar contents of OPT juice squeezed from different trunk parts are shown in Table 14. The sugar concentrations of the inner, middle and outer regions of OPT juice were approximately 119.4, 100 and 84.8 g l^{-1}, respectively. The highest total sugar concentration was observed in inner region of trunk and decreased radially in the outer region of the trunk. These results were similarly to those reported by Kosugi *et al*. (2010) and Yamada *et al.* (2010). In this study, an increase in the amount of observed sugar concentrations was similar to an increase in the observed moisture content of OPT.

The sugar compositions were summarized in Table 15. The high value of total free sugar content of 104.61±2.36 g l^{-1} was higher than that reported by Kosugi *et al*. (2010), Noparat *et al*.(2011) and Komonkiat and Cheirsilp (2013). The top of OPT, reported by Noparat *et al.* (2011), contained 18.06±1.6 g l^{-1} of total sugars. The average total amount of sugar of 76.09±2.85 g l^{-1} was reported for oil palm frond (OPF) juice by Zahari *et al.* (2012). Juice of all trunk parts from the top, middle and basal and from the inner, middle and outer regions was then mixed as undiluted OPT

juice for lactic acid fermentation and their sugar compositions was characterized (Table 15). Glucose and fructose were the main sugars found at the amount of 56.10±1.46 and 40.43±4.68 g l^{-1}, accounting for 53.63% and 38.95% of the total free sugars, respectively. In addition, minor components of sucrose (3.23± 0.69 g l^{-1}), raffinose (2.44±1.28 g l^{-1}), cellobiose (2.41±0.71g l^{-1}) and trehalose were found in undiluted, mixed OPT juice. A similar result of main sugar component was found in a study of Komonkiat and Cheirsilp (2013) and Noparat *et al.* (2011), who reported that glucose was the highest sugar composition. The compositions of glucose, fructose and sucrose in OPT juice were also reported by Yamada *et al.* (2010). In contrast, sucrose was the main dominant sugar from OPT sap at the beginning but decreased rapidly at a very early stage of the storage (Yamada *et al.*, 2010). The decrease of sucrose in the OPT sap was caused by decomposition of sucrose to glucose and fructose with corresponding to an increase of these two sugars. The sugar compositions of OPT juice might be varied with the variety of oil palm, soil, climate, locality, processing method and storage time.

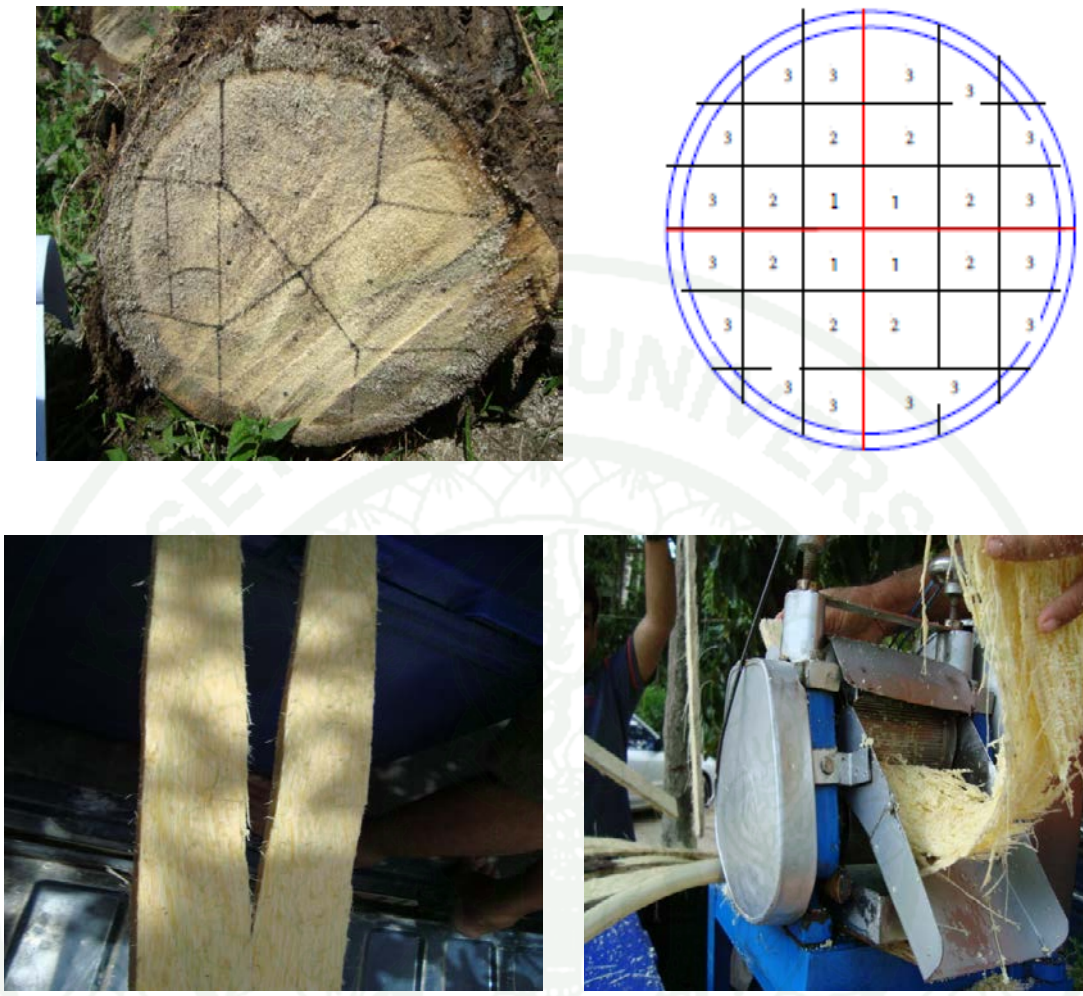


Figure 4 Preparation processes of oil palm trunk juice. Each oil palm trunk log was cut into inner region (1), middle region (2) and outer region (3) and juice was squeezed using conventional sugar cane pressing machine.

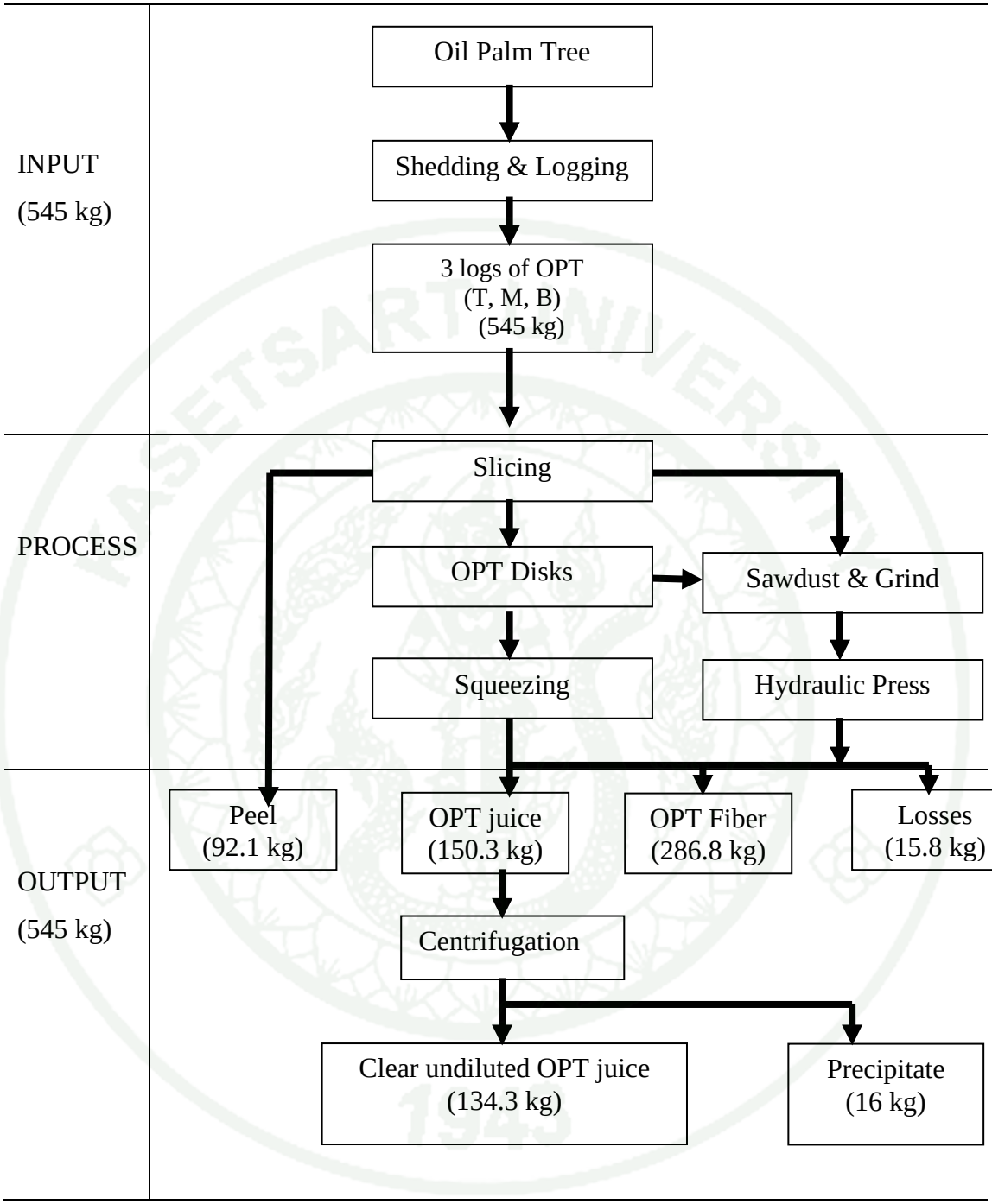


Figure 5 Mass balance of oil palm trunk juice extraction.

Note. OPT = oil palm trunk, T, M and B = the top, middle and basal parts of palm tree

Table 9 Total juice weight obtained from different parts of oil palm trunk.

Sample parts	Juice weight (kg)	% by weight
Top		
T1 (Inner)	8.5	5.7
T2 (Middle)	11.6	7.7
T3 (Outer)	14.6	9.7
Total of top part	34.7	23.1
Middle		
M1(Inner)	16.2	10.8
M2 (Middle)	33.0	22.0
M3 (Outer)	18.8	12.5
Total of middle part	68.0	45.2
Basal		
B1(Inner)	9.6	6.4
B2 (Middle)	17.7	11.8
B3 (Outer)	20.3	13.5
Total of basal part	47.6	31.7
Total weight	**150.3**	100.0

Note. OPT = oil palm trunk; Top, Middle and Basal = the top, middle and basal parts of oil palm trunk log; Inner, Middle and Outer = the inner, middle and outer regions of oil palm trunk.

Table 10 Moisture contents in juice extracted from different parts of oil palm trunk.

Sample parts	**Moisture content (%)**						***Mean***	***SD***
	Inner(1)	**SD**	**Middle (2)**	**SD**	**Outer (3)**	**SD**		
Top	66.7	0.7	63.4	0.2	61.8	0.7	**64.0**	**2.5**
Middle	73.1	0.3	63.7	0.2	62.2	0.2	**66.3**	**5.9**
Basal	76.1	0.3	71.1	0.1	65.6	0.4	**70.9**	**5.3**
Mean	**72.0**	**4.8**	**66.1**	**4.4**	**63.2**	**2.1**	**67.1**	**4.5**

Note. OPT = oil palm trunk; Top, Middle and Basal = the top, middle and basal parts of oil palm trunk log; Inner, Middle and Outer = the inner, middle and outer regions of oil palm trunk.

Table 11 Ash contents in juice extracted from different parts of oil palm trunk.

Sample parts	**Ash content (% dry weight)**						***Mean***	***SD***
	Inner (1)	**SD**	**Middle (2)**	**SD**	**Outer (3)**	**SD**		
Top	2.24	0.16	2.41	0.23	2.11	0.21	2.25	0.15
Middle	3.02	0.19	1.82	0.13	2.56	0.02	2.47	0.61
Basal	2.21	0.46	2.93	0.09	2.22	0.03	2.45	0.41
Mean	**2.49**	**0.46**	**2.39**	**0.55**	**2.30**	**0.24**	**2.39**	**0.10**

Note. OPT = oil palm trunk; Top, Middle and Basal = the top, middle and basal parts of oil palm trunk log; Inner, Middle and Outer = the inner, middle and outer regions of oil palm trunk.

Table 12 Crude protein contents in juice extracted from different parts of oil palm trunk.

Sample parts	Crude protein content (% dry weight)						*Mean*	*SD*
	Inner (1)	SD	Middle (2)	SD	Outer (3)	SD		
Top	2.81	0.44	2.34	0.21	2.72	0.65	**2.62**	**0.25**
Middle	1.29	0.00	1.30	0.00	1.18	0.00	**1.26**	**0.07**
Basal	1.22	0.00	1.27	0.24	1.66	0.02	**1.38**	**0.24**
Mean	**1.77**	**0.90**	**1.63**	**0.61**	**1.86**	**0.79**	**1.75**	**0.11**

Note. OPT = oil palm trunk; Top, Middle and Basal = the top, middle and basal parts of oil palm trunk log; Inner, Middle and Outer = the inner, middle and outer regions of oil palm trunk.

Table 13 Total soluble protein concentrations in juice extracted from different parts of oil palm trunk.

Sample parts	Total soluble protein (g l^{-1})						*Mean*	*SD*
	Inner (1)	SD	Middle (2)	SD	Outer (3)	SD		
Top	2.12	0.04	2.23	0.05	3.61	0.02	2.65	0.83
Middle	1.96	0.02	2.11	0.09	2.17	0.01	2.08	0.11
Basal	1.52	0.10	1.71	0.05	2.15	0.00	1.79	0.33
Mean	1.87	0.31	2.02	0.27	2.64	0.84	2.18	0.41

Note. OPT = oil palm trunk; Top, Middle and Basal = the top, middle and basal parts of oil palm trunk log; Inner, Middle and Outer = the inner, middle and outer regions of oil palm trunk.

Table 14 Sugar concentrations in juice extracted from different parts of oil palm trunk juice.

Sample parts	**Free sugars concentration (g l^{-1})**			***Mean***
	Inner	**Middle**	**Outer**	
Top	132.1	85.9	98.4	105.4
Middle	123.0	114.5	84.6	107.4
Basal	103.2	99.5	71.4	91.4
Mean	119.4	100.0	84.8	101.4

Note. OPT = oil palm trunk; Top, Middle and Basal = the top, middle and basal parts of oil palm trunk log; Inner, Middle and Outer = the inner, middle and outer regions of oil palm trunk.

Table 15 Sugar compositions in undiluted, mixed juice from all parts of oil palm trunk.

Sugar	**Concentration (g l^{-1})**
Glucose	56.10±1.46
Fructose	40.43±1.68
Sucrose	3.23±0.69
Raffinose	2.44±1.28
Cellobiose	2.41±0.71
Total	**104.61±2.36**

Note. Values are reported as Mean ± S.D. from two replicates.

Part II: Batch optimization of lactic acid fermentation from undiluted oil palm trunk juice by *Lactobacillus rhamnosus* TISTR 108 without nutrient supplementation

It was found that oil palm trunk (OPT) juice contained a high concentration of sugars which was sufficient for using as a substrate for lactic acid fermentation. The main sugars presented in mixed OPT juice were glucose, fructose and sucrose. OPT juice also contained some soluble protein which was a potential nutrient source for lactic acid production. To enhance the low cost production of lactic acid, undiluted OPT juice was investigated for its potential use as a cheap and renewable raw material.

1. Effects of initial OPT juice concentration on lactic acid fermentation

Lactic acid fermentation of OPT juice by *L. rhamnosus* TISTR 108 was performed without nutrient supplementation in order to evaluate the effect of OPT juice concentration as the carbon and nitrogen sources for lactic acid production. OPT juice was used as undiluted and diluted samples. The juice was diluted with water with the ratio of OPT juice to water at 4:0 (undiluted), 3:1, 2:2 and 1:3 of which the initial sugar levels were 101.8, 78.2, 53.2 and 26.9 g l^{-1}, respectively.

The results of lactic acid fermentation using different sugar concentrations are summarized in Table 16 and Figure 6. The amounts of lactic acid produced were found to be dependent on OPT juice concentrations. When the dilution ratio between OPT juice and water increased, the maximum lactic acid production decreased accordingly. During batch fermentation of *L. rhamnosus* TISTR 108, lactic acid contents were obtained at 39.19, 35.61, 27.01 and 16.99 g l^{-1} at 33, 36, 36 and 33 h.

The lactic acid yields of 0.75, 0.73, 0.69 and 0.85 g g^{-1} (g lactic per g sugars utilized) and the productivity of 1.19, 0.99, 0.75 and 0.51 g $l^{-1}h^{-1}$ were obtained when the initial sugar levels of OPT juice were approximately 101.8, 78.2, 53.2 and 26.9 g l^{-1}, respectively. When the lowest initial sugar concentration in OPT juice (26.9

g l^{-1}) was used, the lowest sugar content remained at the end of fermentation was obtained at 6.93 g l^{-1} with the highest yield of 0.85 g g^{-1} at 33 h of fermentation. The highest productivity of 1.19 g $l^{-1}h^{-1}$ was observed when the initial sugar levels of OPT juice was 101.8 g l^{-1}. Karp *et al.* (2011) indicated that increasing in sugar concentration in the vinasse was considered as a possibility to reach higher lactic acid accumulation at the end of fermentation by *Lactobacillus agilis* LPB 56.

The cell growth in term of viable cells was reached to the values of $1.38x10^{11}$, $4.98x10^{10}$, $6.93x10^{9}$ and $9.85x10^{8}$ cfu ml^{-1} at 33, 36, 36 and 33 h cultivation time for the initial OPT juice concentrations of 101.8, 78.2, 53.2 and 26.9 g l^{-1}, respectively. The higher the initial sugar levels in OPT juice were the better the growth of *L. rhamnosus* TISTR 108 to reach the stationary phase.

It was clear that sugars were continuously consumed in all OPT juice concentrations without nutrient supplementation but it was not completely utilized during fermentation by *L. rhamnosus* TISTR 108. At the beginning of fermentation, sugars were rapidly consumed during the log phase of cell growth in corresponding with the decrease in sugar levels and the increase in cell growth. Gao *et al.* (2009) showed that the strain of *L. rhamnosus* NBRC 3863 was resistant to glucose inhibition and the final calcium lactate concentration was increased with the increase of initial glucose concentrations up to 190 g l^{-1}. This experiment demonstrated that the higher the initial OPT juice concentration used for lactic acid fermentation, the more the residual sugars left in fermentation medium during 72 h of fermentation time. The high level of residual sugars remaining during fermentation might be due to the growth limitation according to the insufficiency of some nutrients, e.g. nitrogen and minerals the unbalance of nutrient composition or the poor buffering capacity of the medium (Nancib *et al.*, 2001; Pintado *et al.*, 1999). In contrast, sugarcane juice, sugarcane molasses and sugar beet juice with the high initial sugar concentrations of 133, 119 and 105 g l^{-1} were efficiently fermented to lactic acid by *Lactobacillus delbruskii*, resulting in the lactic acid production of 120, 107 and 84 g l^{-1}, respectively (Calabia and Tokiwa, 2007). This was supported by a kinetic model of the fermentative production of lactic acid from glucose by *Lactobacillus lactis* ssp. *lactis*

ATTC 19435 from whole-wheat flour developed by Akerberg *et al.* (1998), who showed that in the concentration interval of glucose up to 180 g l^{-1} and lactic acid of 89 g l^{-1}, the effect of glucose inhibition was small compared to the inhibition effect due to lactic acid.

In this study, calcium hydroxide was used as a neutralizing agent in the amount that did not exceed the maximum tolerance at the end of fermentation. However, Wang *et al.* (2010) reported that at glucose concentrations of 100 and 150 g l^{-1}, the yields of L-lactic acid was 0.86 g g^{-1} whereas in the condition of higher glucose concentration (200 g l^{-1}), the lower L-lactic acid yield (0.67 g g^{-1}) from *L. rhamnosus* was obtained. When sugar concentrations were increased by 1, 5, 10, 15 and 20% w/w, the time required for obtaining the maximum L-lactic acid production became longer and the L-lactic acid concentrations were decreased at the sugar concentration greater than 10% w/w, presumably due to the substrate inhibition by *Streptococcus bovis* (Ghofar *et al.*, 2005).

Several researches have been conducted to evaluate the use of oil palm trunk juice for different fermentation production. A study of Kosugi *et al*. (2010) for lactic acid fermentation by *L. lactis* ATCC 194355 was performed by using OPT juice having low initial sugar concentrations of 16.7, 1.28, 0.79 and 0.18 g l^{-1}. A research of Komonkiat and Cheirsilp (2013) demonstrated the use of oil palm sap with sugar concentration of 30 g l^{-1} for butanol production by *Clostridium* spp. A work done by Zahari *et al*. (2012) was the use of oil palm frond (OPF) juice with the concentration ranging of 5.19 - 20.84 g l^{-1} for the production of parahydroxy butyrate. Our study exhibited that without supplementation, the fermentation of undiluted OPT juice resulted in the highest lactic acid concentration compared to the lower OPT juice concentration, although the residual sugars were higher. Therefore, for economic feasibility of lactic acid production, the undiluted OPT juice was chosen for further study.

Table 16 Effects of initial oil palm trunk juice concentration on lactic acid fermentation by *Lactobacillus rhamnosus* TISTR 108.

OPT juice : water	Sugars levels ($g\ l^{-1}$)	Lactic acid[a] ($g\ l^{-1}$)	$Y_{P/S}$ ($g\ g^{-1}$)	Q_P ($g\ l^{-1}h^{-1}$)	Residual sugars[a] ($g\ l^{-1}$)	Fermentation Time[b] (h)	Viable cells[a] ($cfu\ ml^{-1}$)
4:0	101.8	39.19	0.75	1.19	49.83	33	$1.38x10^{11}$
3:1	78.2	35.61	0.73	0.99	29.42	36	$4.98x10^{10}$
2:2	53.2	27.01	0.69	0.75	14.25	36	$6.93x10^{9}$
1:3	26.9	16.99	0.85	0.51	6.93	33	$9.85x10^{8}$

Note. The fermentation was conducted in 2 l fermenter at 40°C, pH 6.0, using 10% inoculum. [a] Values were obtained at a defined fermentation time. [b] The fermentation time was chosen when no lactic acid was produced.

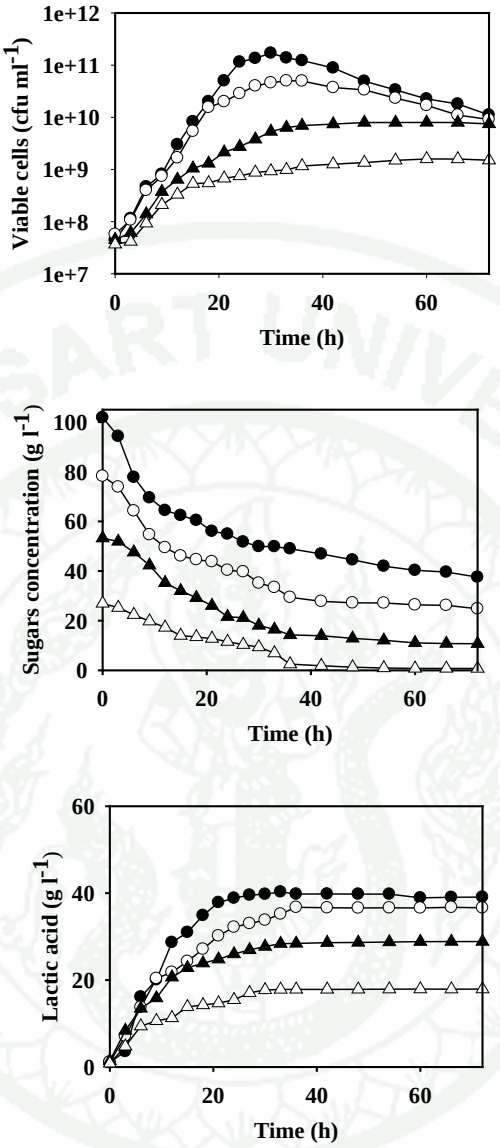


Figure 6 Profiles of cell growth, sugar and lactic acid concentrations during lactic acid fermentation of oil palm trunk juice with various dilution by *Lactobacillus rhamnosus* TISTR 108 without nutrient supplementation. The batch fermentation was performed in 2 l fermenter with 1.6 l working volume at 40°C, pH 6.0, using the agitation speed of 200 rpm and 10% inoculum. The ratios between OPT juice to water were 4:0 (●), 3:1 (○), 2:2 (▲) and 1:3 (Δ).

2. Effects of inoculum sizes on lactic acid fermentation

The increase in inoculum sizes was found to accelerate growth and lactic acid production by *L. rhamnosus* TISTR 108 from undiluted OPT juice fermentation without nutrient supplementation, as shown in Figure 7. The cell growths were reached $5.25x10^{10}$, $1.70x10^{11}$ and $4.50x10^{11}$ cfu ml^{-1} at 30, 30 and 27 h cultivation with 5, 10 and 15 % of inoculum sizes, respectively. In corresponding to rapid increasing of sugar consumed, the large amount of inoculums used was found to accelerate fermentation efficiency in terms of organism growth and lactic acid production. The higher the inoculums used, the shorter the time for the maximum lactic acid produced. The lactic acid yields and productivities were found to be 0.77, 0.74, 0.73 g g^{-1} and 1.19, 1.29, 1.47 g $l^{-1}h^{-1}$ with 5, 10, 15% inoculums, respectively (Table 17). Increasing in inoculum size could promote lactic acid productivity and lactic acid concentration. The lactic acid concentrations were 35.73, 38.66 and 39.81 g l^{-1} at 5, 10 and 15% inoculums at 30, 30 and 27 h, respectively. However, at 15% inoculum, a slightly decrease in lactic acid yield was observed in comparison to 10% inoculum. Similar patterns were demonstrated by a work done by Karp *et al.* (2011). When the large inoculum sizes (10, 25 and 50%) of *Lactobacillus agillis* LPB 56 were applied in the medium being composed of 30^0Brix vinasse and molasses, the highest productivity and the maximum lactic acid yield were achieved at 50 and 25% inoculums used, respectively and the sugar consumption was decreased with 50% inoculum used. However, Dujukic-Vukovic *et al.* (2012a, 2012b) reported that no significant difference of lactic acid yield was observed between the use of 2 and 10% inoculum on liquid stillage and at 10% inoculums, a decrease in cell number was observed after 35 h. This suggested that the decline in the cell numbers could be induced by the lack of fermentable sugars and an inhibition by non-dissociated form of lactic acid. In this study, residual sugars were still left in fermentation medium of 55.42, 49.89 and 47.00 g l^{-1} at 30, 30 and 27 h cultivation time when 5, 10 and 15% of inoculums were used, respectively. In consequence, a constant lactic acid production was found which might be a result of insufficiency of nutrients and product accumulation. The maximum cell was also observed when large inoculum sizes were used. For this reason, 10% inoculum was chosen in order to enhance an increase of

lactic acid yield and productivity, and minimize residual sugars at the end of fermentation.

Table 17 Influences of inoculum sizes on lactic acid production by *Lactobacillus rhamnosus* TISTR 108 from undiluted oil palm trunk juice without nutrient supplementation**.**

Inoculum size (%)	**Lactic acid[a] ($g\ l^{-1}$)**	**$Y_{P/S}$ ($g\ g^{-1}$)**	**Q_P ($g\ l^{-1}h^{-1}$)**	**Residual sugars[a] ($g\ l^{-1}$)**	**Fermentation time[b] (h)**	**Viables cells[a] ($cfu\ ml^{-1}$)**
5	35.73	0.77	1.19	55.42	30	$5.25x10^{10}$
10	38.66	0.74	1.29	49.89	30	$1.70x10^{11}$
15	39.81	0.73	1.47	47.00	27	$4.50x10^{11}$

Note. The fermentation was conducted in 2 l fermenter using undiluted OPT juice with the initial sugar concentration ≈ 101.8 g l^{-}, at 40^0C and pH 6.0. [a] Values were obtained at a defined fermentation time. [b] The fermentation time was chosen when no lactic acid was produced.

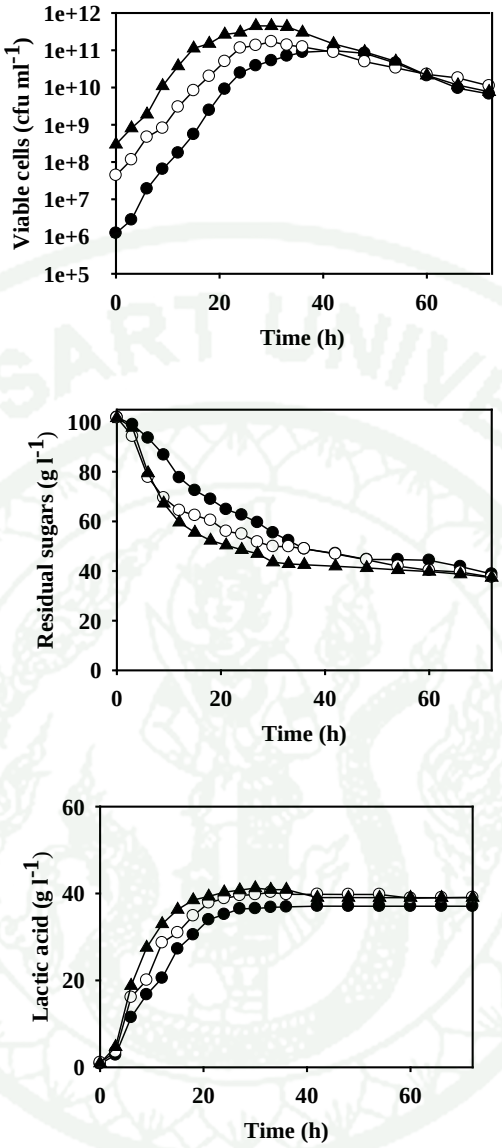


Figure 7 Effects of inoculum sizes on cell growth, sugar and lactic acid concentrations during lactic acid fermentation of undiluted oil palm trunk juice by *Lactobacillus rhamnosus* TISTR 108 without nutrient supplementation. The batch fermentation was performed in 2 l fermenter with 1.6 l working volume at 40°C, pH 6.0, using the agitation speed of 200 rpm with inoculum size of 5% (●), 10% (○) and 15% (▲).

3. Effects of temperature on lactic acid fermentation

Several studies suggest that *L. rhamnosus* provides the highest lactic acid yield and productivity at 38 to 45^{0}C (Hofvendahl and Hahn-Hagerdal, 2000; Kwon *et al.*, 2000; Nancib *et al.*, 2005; Dujukic-Vukovic *et al.*, 2012a). The differences in such optimum temperatures may be because of substrate heterogeneity, variations in carbon and nitrogen sources and overall fermentation kinetics (Dujukic-Vukovic *et al.*, 2012b). However, in industrial fermentation processes, the operating temperature is practically raised to the optimum level to increase microbial activity. The effects of temperature on lactic acid production by *L. rhamnosus* TISTR 108 on undiluted OPT juice without nutrient supplementations are shown in Figure 8 and Table 18. The lactic acid concentrations were 30.76, 37.44, 40.06 and 38.07 g l^{-1} when the temperatures were carried out at 30, 37, 40 and 43^{0}C at 42, 33, 33 and 30 h cultivation, respectively. The highest lactic acid yield of 0.78 g g^{-1} was achieved at 30^{0}C with the lowest productivity of 0.73 g $l^{-1}h^{-1}$. However, lactic acid concentration was quite low when the fermentation temperature was performed at 30^{0}C and 42 h cultivation. At 43^{0}C the lowest lactic acid yield (0.74 g g^{-1}) and concentration (38.08 g l^{-1}) was obtained compared to that at 40°C (0.75 g g^{-1} and 40.06 g l^{-1}), respectively. This might be because at higher temperatures and high concentrations, lactic acid could be polymerized (Akerberg and Zacchi, 2000). The residual sugar concentrations of 62.33, 50.13, 48.78 and 39.39 g l^{-1} were found at the tested temperature of 30, 37, 40 and 43^{0}C at the cultivation time of 42, 33, 33 and 30 h, respectively, in which the maximum lactic acid concentration was achieved. However, the residual sugars still remained in culture medium at the end of fermentation time, i.e. 72 h. The inability of sugar consumption might be caused by the nutrient deficiency. Dujukic-Vukovic *et al.* (2012b) explained that the residual glucose concentration remained at the end of the fermentation indicated that a certain amount of reducing sugars was hardly converted to lactic acid by the *Lactobacillus rhamnosus* ATCC 7469. Bacterial growth was also affected by temperature. When the temperature increased, the viable cell numbers were $8.70x10^9$, $3.95x10^{10}$, $1.72x10^{11}$ and $7.92x10^{10}$ cfu ml^{-1} at fermentation time of 42, 33, 33 and 30 h at temperature of 30, 37, 40 and 43^{0}C, respectively.

Table 18 Influences of temperature on lactic acid production by *Lactobacillus rhamnosus* TISTR 108 from undiluted oil palm trunk juice without nutrient supplementation.

Temperature (^{0}C)	Lactic acid[a] (g l^{-1})	$Y_{P/S}$ (g g^{-1})	Q_P (g l^{-1} h^{-1})	Residual sugars[a] (g l^{-1})	Fermentation Time[b] (h)	Viable cells[a] (cfu ml^{-1})
30	30.76	0.78	0.73	62.33	42	$8.70x10^9$
37	37.44	0.72	1.13	50.13	33	$3.95x10^{10}$
40	40.06	0.75	1.21	48.78	33	$1.72x10^{11}$
43	38.07	0.74	1.27	39.39	30	$7.92x10^{10}$

Note. The fermentation was conducted in 2 l fermenter using undiluted OPT juice with the initial sugar concentration ≈ 102 g l^{-1}, at pH 6.0 using 10% inoculum. [a] Values were obtained at a defined fermentation time. [b] The fermentation time was chosen when no lactic acid was produced.

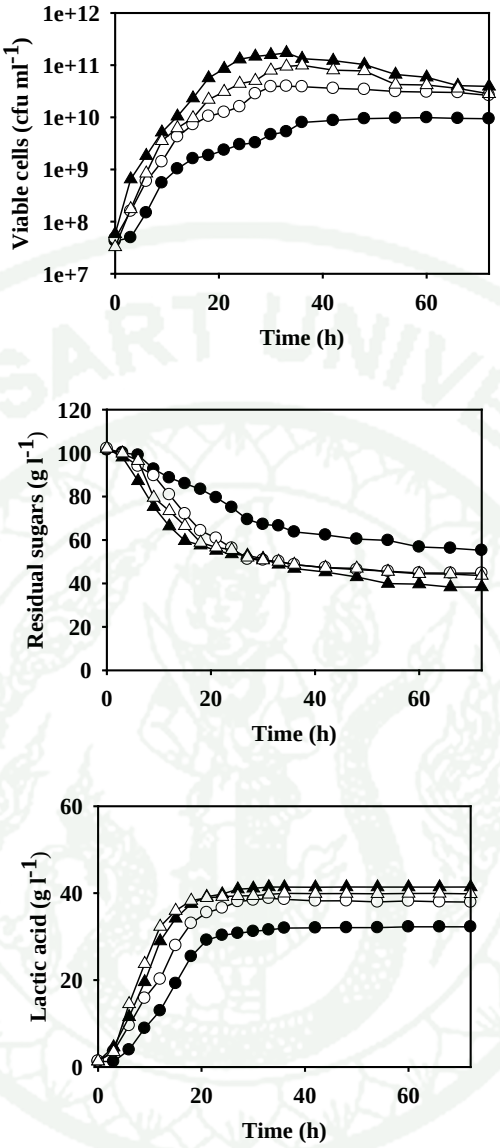


Figure 8 Effects of temperature on cell growth, sugar and lactic acid concentrations producing during lactic acid fermentation of undiluted oil palm trunk juice by *Lactobacillus rhamnosus* TISTR 108 without nutrient supplementation. The batch fermentation was performed in 2 l fermenter with 1.6 l working volume at pH 6.0, using 10% inoculum and the agitation speed of 200 rpm at the temperature of 30^0C (●), 37^0C (o), 40^0C (▲) and 43^0C (Δ).

4. Effects of pH on lactic acid fermentation

The pH was varied from 5.0 to 7.0 at 0.5 intervals. The profiles and kinetic parameters of lactic acid fermentation by *L. rhamnosus* TISTR 108 using undiluted OPT juice without nutrient supplementation are summarized in Table 19 and Figure 9. At pH 5.0, the lactic acid concentration was 34.81 g l^{-1} at 27 h cultivation, with the yield of 0.90 g g^{-1} and 1.29 g l^{-1} h^{-1} volumetric productivity. At pH lower or higher than 6.5, the decrease in lactic acid production, growth of *L. rhamnosus* TISTR 108, lactic acid yields and productivities were observed. The lactic acid concentrations were 34.81, 36.80, 40.06, 44.86 and 37.67 g l^{-1} at 27, 30, 33, 33, and 36 h cultivation time at pH 5.0, 5.5, 6.0, 6.5 and 7.0, respectively. The decrease in lactic acid production might be because of the by-product formation. Akerberg *et al.* (1998) concluded that a slight increase in acetic acid and ethanol was observed with decreasing pH. The highest viable cell of 3.64 x 10^{11} cfu ml^{-1} was achieved at pH 6.5. Decreasing pH below 6.5 reduced the cells of 1.72x10^{11}, 1.82x10^{10} and 7.10x10^{9} cfu ml^{-1} at the tested pH were 6.0, 5.5 and 5.0, respectively. At pH 7.0, the maximum cell was achieved at 1.78x10^{10} cfu ml^{-1}, which was lower than that of pH 6.0. Mussatto *et al.* (2008) elucidated that weak acids inhibited bacterial growth because as the external pH declined, the acid was protonized as soon as it was exported out of the bacteria. Uncharged acid diffused back into the cell and dissociated due to the higher intracellular pH. The cell then had to use ATP to pump out protons, and the energy eventually was depleted, causing the growth stop and the death of bacteria. However, in this study, the use of $Ca(OH)_2$ as a neutralizing agent, might help reduce the lactate molarity of the fermentation broth, and result in the highest lactic acid productivity. Nakano *et al.* (2012) reported that the divalent cation (Ca^{2+}) was more effective in neutralizing the cultures compared to the monovalent (Na^+ and NH_3^+) cations. Nakano *et al.* (2012) also emphasized that a low concentration of calcium lactate did not reduce the growth of *L. delbrueckii* (a gram positive bacteria) until the concentration of lactic acid increased considerably (about 79 g l^{-1}). However, the solubility of calcium lactate increased rapidly with the temperature, which could enhance lactic acid productivity and recovery when cultured the broth at high temperature. Gao *et al.* (2009) showed that additional yeast extract (YE) raised the

fermentation efficiency and made complete fermentation feasible in the presence of colloidal calcium lactate.

Table 19 Influences of pH on lactic acid production by *Lactobacillus rhamnosus* TISTR 108 from undiluted oil palm trunk juice without nutrient supplementation.

pH	Lactic acid[a] ($g\ l^{-1}$)	$Y_{P/S}$ ($g\ g^{-1}$)	Q_P ($g\ l^{-1}h^{-1}$)	Residual sugars[a] ($g\ l^{-1}$)	Fermentation Time[b] (h)	Viable cells[a] ($cfu\ ml^{-1}$)
5.0	34.81	0.90	1.29	62.91	27	$7.10x10^{9}$
5.5	36.80	0.76	1.23	53.18	30	$1.82x10^{10}$
6.0	40.06	0.75	1.21	48.78	33	$1.72x10^{11}$
6.5	44.86	0.78	1.36	44.57	33	$3.55x10^{11}$
7.0	37.67	0.73	1.05	50.75	36	$1.78x10^{10}$

Note. The fermentation was conducted in 2 l fermenter using undiluted OPT juice with the initial sugar concentration ≈ 102 $g\ l^{-1}$, at 40 ^{0}C, using 10% inoculum. [a] Values were obtained at a defined fermentation time. [b] The fermentation time was chosen when no lactic acid was produced.

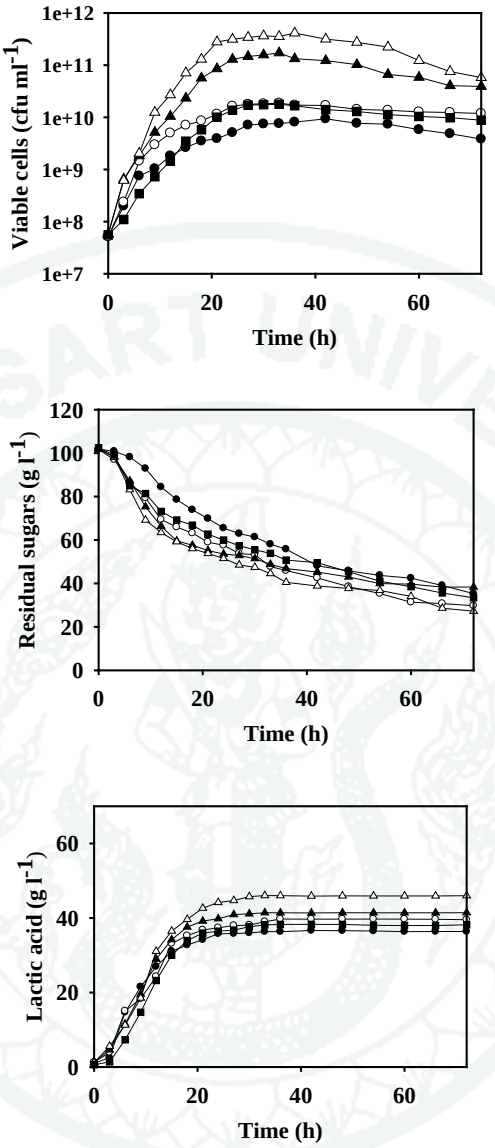


Figure 9 Effects of pH on cell growth, sugar and lactic acid concentration during lactic acid fermentation of undiluted oil palm trunk juice by *Lactobacillus rhamnosus* TISTR 108 without nutrient supplementation. The batch fermentation was performed in 2 l fermenter with 1.6 l working volume at 40^0C, using 10% inoculum and the agitation speed of 200 rpm with the medium pH of 5.0 (●), 5.5 (o), 6.0 (▲), 6.5 (Δ) and 7.0 (■).

5. Nitrogen and salt supplementation for lactic acid fermentation of undiluted oil palm trunk juice

The effects of nitrogen and mineral salts supplementation on lactic acid production from undiluted OPT juice by *L. rhamnosus* TISTR 108 were evaluated by the addition of 10 g l^{-1} yeast extract and 5 g l^{-1} peptone (YE+P), the addition of 10 g l^{-1} yeast extract, 5 g l^{-1} peptone and salts (YE+P+T) and the addition of salts (T). The control experiment was performed by using glucose with the addition of nitrogen and salts as mentioned in materials and methods, section 2.3.

The results are shown in Table 20 and Figure 10. The lactic acid yields were 0.85, 0.82 and 0.70 g g^{-1} and productivities were 2.47, 3.86 and 1.41 g l^{-1} h^{-1} when YE+P, YE+P+T and T were added, respectively. The maximum lactic acid concentration was obtained at 82.69 and 82.10 g l^{-1} when supplemented with YE+P and YE+P+T, respectively. The maximum time for the highest lactic acid was shortened when nitrogen and salts were supplemented. The sugars were almost completely consumed at 33, 21 and 42 h when YE+P, YE+P+T and T were supplemented to undiluted OPT juice, respectively. Some residual sugars were still found, which might be because they were difficult to be utilized by *L. rhamnosus* TISTR 108. The maximum yield for YE+P+T was obtained at 21 h whereas the fermentation with YE+P supplementation reached the highest yield at 33 h, thus the higher productivity was obtained in YE+P+T supplemented fermentation. This suggests that the addition of YE+P and YE+P+T not only shortened the lag time, but also increased the lactic acid production. Furthermore, the addition of salts was found to increase lactic acid concentration and productivity in comparison with non-supplemented undiluted OPT juice but still prolonged the lag phase and fermentation time. This might confirm the deficiency of some nutrients such as nitrogen sources in undiluted OPT juice for lactic acid production. Concerning the bacterium growth, viable cells were found to increase with all supplementations. The viable cells were 1.86×10^{12}, 2.68×10^{12} and 5.76×10^{11} cfu ml^{-1} at 30, 21 and 42 h with YE+P, YE+P+T and T supplementation in undiluted OPT juice, respectively.

This study showed that yeast extract, peptone and salts supplementation were essential for lactic acid fermentation by *L. rhamnosus* TISTR 108 when undiluted OPT juice was used as a substrate under the sterilization condition. In comparison, the control, i.e. 100 g l^{-1} glucose supplemented with nitrogen and salts yieled 94.09 g l^{-1} lactic acid with the productivity of 4.44 g l^{-1} h^{-1} at 21 h cultivation and sugars were completely consumed during fermentation. Although the undiluted OPT juice contained 2.18±0.41 g l^{-1} total soluble proteins, it was much lower than the content used in the control which contained 10 g l^{-1} yeast extract and 5 g l^{-1} peptone. Thus, to achieve the highest yield and productivity of lactic acid by *L. rhamnosus* TISTR 108, the supplementation of nitrogen and salts were necessary. In comparison with the control, i.e. glucose, the lower yield and productivity of lactic acid obtained by OPT juice fermentation were obtained. This might be because the inhibition of some products or other components in OPT juice occurred during fermentation. In further study, the suitable amounts of these nutrients should be optimized in order to minimize cost and obtain the highest lactic acid yield and productivity.

The additions of nutrients and high nutrient concentrations generally have a positive effect on the lactic acid production (Hofvendahl and Hahn-Hagerdal, 2000). Gao *et al.* (2008) reported that with the initial YE concentrations of 3 to 15 g l^{-1}, the productivity of lactic acid increased with increasing YE concentrations and a combination of YE and rice bran shortened the lag phase and increased the fermentation efficiency. Nancib *et al.* (2001) described that yeast extract was the best supplement for efficient lactic acid production due to the amino acid and vitamin required for the growth of *L. casei* subsp. *Rhamnosus*. The addition of YE above 20 g l^{-1} did not significantly increase the microbial growth. Lu *et al.* (2010) found that cell growth showed different increasing trend with the increase of carbon-nitrogen ratios. If the nitrogen supply is short, the cell cannot produce necessary enzymes for metabolism. If the nitrogen supply is too much, the cell growth can be inhibited because of toxicosis of some key enzymes.

Table 20 Influences of nutrient supplementation on lactic acid fermentation of undiluted oil palm trunk juice by *Lactobacillus rhamnosus* TISTR 108.

Nutrient supplements	Lactic acid[a] ($g\ l^{-1}$)	$Y_{P/S}$ ($g\ g^{-1}$)	Q_P ($g\ l^{-1}h^{-1}$)	Residual sugars[a] ($g\ l^{-1}$)	Fermentation Time[b] (h)	Viable cells[a] ($cfu\ ml^{-1}$)
YE*+P*	82.69	0.85	2.47	5.43	33	$1.86x10^{12}$
YE*+P*+T*	82.10	0.82	3.83	2.65	21	$2.68x10^{12}$
T*	59.38	0.70	1.41	16.51	42	$5.76x10^{11}$
Control	94.09	0.93	4.44	0.59	21	$1.21x10^{12}$

Note. The fermentation was conducted in 2 l fermenter using undiluted OPT juice with the initial sugar concentration ≈ 101 $g\ l^{-1}$, at 40 ^{0}C, pH 6.5, 200 rpm with 10% inoculum.

[a] Values were obtained at a defined fermentation time. [b] The fermentation time was chosen when no lactic acid was produced. YE, P and T represent yeast extract, peptone and salts, respectively. The control experiment was performed by using 100 $g\ l^{-1}$ glucose supplemented with yeast extract, peptone and salts as indicated in materials and methods.

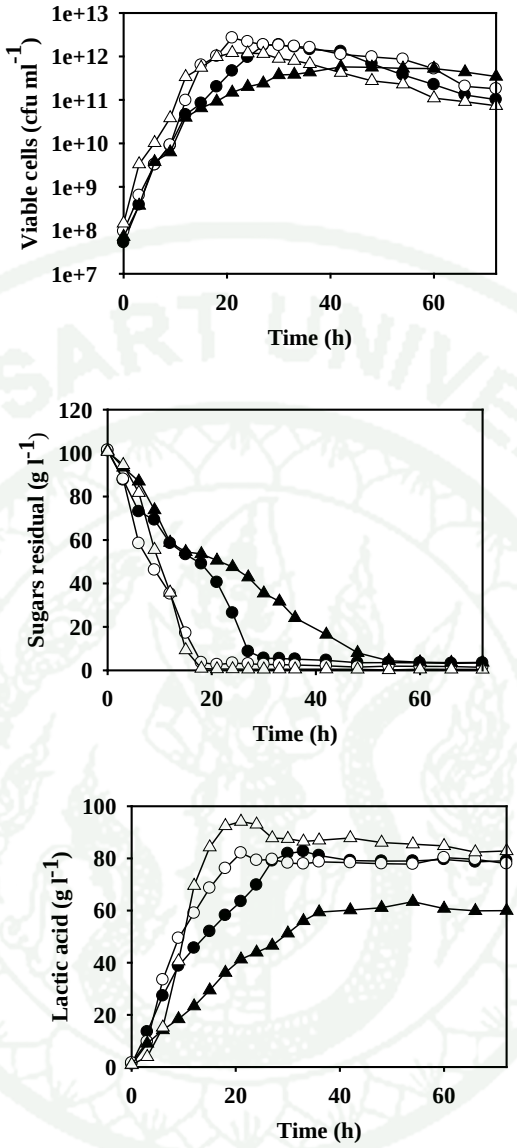


Figure 10 Effects of nutrient supplementation on cell growth, sugar and lactic acid concentration during lactic acid fermentation of undiluted oil palm trunk juice by *Lactobacillus rhamnosus* TISTR 108 with nutrient supplementation. The batch fermentation was performed in 2 l fermenter with 1.6 l working volume at 40^0C, pH 6.5 and the agitation speed of 200 rpm, using 10% inoculum and the medium supplemented with yeast extract + peptone (YE+P) (●), yeast extract +peptone + salts (YE+P+T) (o), salts (T) (▲) and control (Δ).

6. Box Behkhen Design for optimizing nutrient supplementation in lactic acid production from undiluted oil palm trunk juice

It is very clear that the addition of yeast extract, peptone and mineral salts gave the better cell growth and lactic acid production from undiluted OPT juice by *L. rhamnosus* TISTR 108, comparing to non-supplemented medium. The Response surface methodology (RSM) involving Box Behkhen Design (BBD) was applied to evaluate the optimum use of nutrients and predict the maximum lactic acid concentration during fermentation of undiluted OPT juice by *L. rhamnosus* TISTR 108. The design matrix of the variables in coded units and responses of lactic acid concentration are shown in Table 21. The analysis of variance (ANOVA) for the quadratic model of lactic acid production is shown in Table 22 and the multiple regression analysis for lactic acid concentration is exhibited by the following equation.

$$Y = 42.1364 + 6.2201A + 1.1515B + 4.9509C - 2.6415A^2 + 6.3119B^2 + 2.9386C^2 - 0.2891AB + 2.8991AC - 2.1057BC$$

Where Y is the lactic acid concentration (g l^{-1}), A, B, C are the coded levels for peptone, yeast extract and mineral salt concentrations (g l^{-1}), respectively.

The full quadratic correlation for the estimated regression equation model had R^2 of 94.17% and the predicted R^2 for lactic acid production was 84.06%. The R^2 value of 94.17% (close to 100%) indicated that the variables including peptone, yeast extract and mineral salts contributed to a highly positive response. The adjusted R^2 of 91.11% indicated that the model was highly accurate. The linear and quadratic effects of variables were significant at level of $p \leq 0.01$. The linear effect of yeast extract was significant at the level of $p \leq 0.01$, whereas the linear effect of peptone and mineral salts was not significant. The square terms of peptone (A^2), yeast extract (B^2) and mineral salts (C^2) were highly significant ($p \leq 0.01$). The interaction terms (A*C, B*C), were significant ($p \leq 0.05$) while the interaction terms between A and B was not significant. Lack of fit of this equation was not significant when $p > 0.05$ which

indicated the good predictability of the model. The significant linear and quadratic terms of parameters suggests that peptone, yeast extract and mineral salts can act as limiting nutrients for lactic acid production. In this study, only one nitrogen source, i.e. peptone was selected to investigate the optimization of nutrient supplementation in order to reduce the cost. The 3D plots for the interactions between peptone (*A*) and mineral salts (*C*) of lactic acid fermentation are shown in Figure 12. It can be noticed that by increasing the peptone and mineral salts, the lactic acid production from undiluted OPT juice was increased.

The response optimization condition of 3 variables was 1, -1, 1 for peptone, yeast extract and mineral salts as indicated in Figure 13. The highest lactic acid concentration of 64.05 g l^{-1} was predicted from the model at the optimum condition of peptone 10 g l^{-1} and mineral salts, containing 0.4 g l^{-1} $MgSO_4.7H_2O$, 0.1 g l^{-1} $MnSO_4.4H_2O$, 3 g l^{-1} K_2HPO_4, 3 g l^{-1} KH_2PO_4 and 3 g l^{-1} $CH_3COONa.3H_2O$.

Furthermore, the fermentation of undiluted OPT juice under the optimized condition of nutrient supplementation obtained by BBD were performed to validate the model. The maximum lactic acid concentration of 63.2±3.45 g l^{-1} was obtained experimentally and this was close to the predicted valve of 64.05 g l^{-1}. However, it is important to note that this experiment was performed in 250 ml Erlenmeyer flask which $CaCO_3$ was used as a titrant. This optimized nutrient condition was then further evaluated in a 2 l fermenter (with 1.2 l working volume), using $Ca(OH)_2$ as a neutralizing agent.

The profiles of cell growth, sugar and lactic acid concentration during lactic acid production from undiluted OPT juice with optimized nutrient supplementation in a fermenter are presented in Figure 13 and some kinetic parameters are summarized in Table 23. The high lactic acid concentration of 78.46 g l^{-1} was obtained with the yield of 1.01 g g^{-1} and productivity of 3.27 g $l^{-1}h^{-1}$ at 24 h fermentation. However, some residual sugars were present at the end of fermentation time. The incomplete sugar consumption might be caused by some inhibitory effect as indicated by lower viable cells ($< 10^{11}$ cfu ml^{-1}), compared to the condition with YE+P and YE+P+T

(1.86×10^{12} and 2.68×10^{12} cfu ml^{-1}). The nutrient optimization by using yeast extract and others should be further investigated. Nevertheless, the use of peptone and salts could improve lactic acid production by *L. rhamnosus* TISTR 108. Even though, OPT juice was rich in carbon source but the amount of nitrogen and salts were low and not enough for efficient lactic acid production. In this study, the statistical approaches were successfully applied and overcame the limitation of empirical method. Many studies reported high lactic acid yields and productivities by statistical optimization (Payot *et al.*, 1999; Bustos *et al.*, 2004; Gullon *et al.*, 2008; Lu *et al.*, 2009, 2010; Sun *et al.*, 2012). John *et al.* (2007) used a Box Behnken Design to optimize the production of L(+)–lactic acid by *Lactobacillus casei* and *Lactobacillus delbrueckii*; the obtained lactic acid yield was 81 g l^{-1}. RSM was applied to evaluate the effect of corn steep liquor along with glucose, molasses, Tween 80 and $MnSO_4$ on L-(+)-lactic acid fermentation by *Lactobacillus rhamnosus* GCMCC 1466 (Yu *et al.*, 2008a).

Table 21 Observed and predicted lactic acid concentrations obtained from undiluted oil palm trunk juice fermentation with various nutrient supplementations by Box Behkhen Design.

Run	Coded Units			Lactic acid (g l^{-1})	
	A (Peptone, g l^{-1})	B (Yeast extract, g l^{-1})	C (Mineral salts)	Observed	Predicted
1	-1	0	1	37.75	38.27
2	-1	0	-1	37.23	34.16
3	0	-1	-1	41.27	43.18
4	1	0	1	53.52	56.50
5	-1	-1	0	36.65	38.15
6	-1	1	0	38.63	41.03
7	0	1	-1	47.75	49.69
8	0	0	0	43.72	42.14
9	1	-1	0	53.03	51.16
10	0	-1	1	58.47	57.29
11	1	0	-1	39.90	40.80
12	0	0	0	42.04	42.14
13	0	1	1	55.92	55.38
14	1	1	0	54.18	52.89
15	0	0	0	40.12	42.14
16	-1	0	1	38.94	38.27
17	-1	0	-1	37.54	34.16
18	0	-1	-1	42.15	43.18
19	1	0	1	53.05	56.50
20	-1	-1	0	36.14	38.15
21	-1	1	0	40.33	41.03
22	0	1	-1	48.29	49.69
23	0	0	0	42.82	42.14
24	1	-1	0	52.39	51.16
25	0	-1	1	59.46	57.29
26	1	0	-1	41.55	40.80

Table 21 (Continued)

Run	Coded Units			Lactic acid (g l^{-1})	
	A (Peptone, g l^{-1})	B (Yeast extract, g l^{-1})	C (Mineral salts)	Observed	Predicted
27	0	0	0	43.05	42.14
28	0	1	1	57.79	55.38
29	1	1	0	55.10	52.89
30	0	0	0	41.07	42.14

Table 22 ANOVA results for nutrient optimization in lactic acid production from undiluted oil palm trunk juice by Box Behkhen Design.

Effect	DF	Seq SS	Adj SS	Adj MS	F value	P value
Blocks	1	3.01	3.01	3.010	0.59	0.451[c]
Regression	9	1556.14	1556.14	172.904	34.06	0.000[a]
Linear	3	1032.43	285.82	95.272	18.77	0.000 [a]
A	1	619.04	7.60	7.602	1.50	0.236 [c]
B	1	21.21	244.71	244.713	48.20	0.000 [a]
C	1	392.18	12.45	12.481	2.16	0.133 [c]
Square	3	420.33	420.33	140.109	27.60	0.000 [a]
*A*A*	1	81.42	51.52	51.525	10.15	0.005 [a]
*B*B*	1	275.14	294.20	294.203	57.95	0.000 [a]
*C*C*	1	63.77	63.77	63.769	12.56	0.002 [a]
Interaction	3	103.38	103.38	34.459	6.79	0.003 [a]
*A*B*	1	0.67	0.67	0.668	0.13	0.721 [c]
*A*C*	1	67.24	67.24	67.238	13.24	0.002 [a]
*B*C*	1	35.47	35.47	35.472	6.99	0.016[b]
Residual error	19	96.46	96.46	5.077		
Lack-of fit	15	87.62	87.62	5.842	2.64	0.179 [c]
Pure Error	4	8.83	8.83	2.209		
Total	29	1655.61				
R^2 = 94.17, adj. R^2 = 91.11, pred. R^2 = 84.06						

Note. The analysis was done using the coded units; [a] significant at $p \leq 0.01$; [b] significant at $p \leq 0.05$; [c] non significant; SS, sum of square; MS, mean square; DF, degree of freedom; *A*, *B* and *C* represent yeast extract, peptone and salt concentration, respectively.

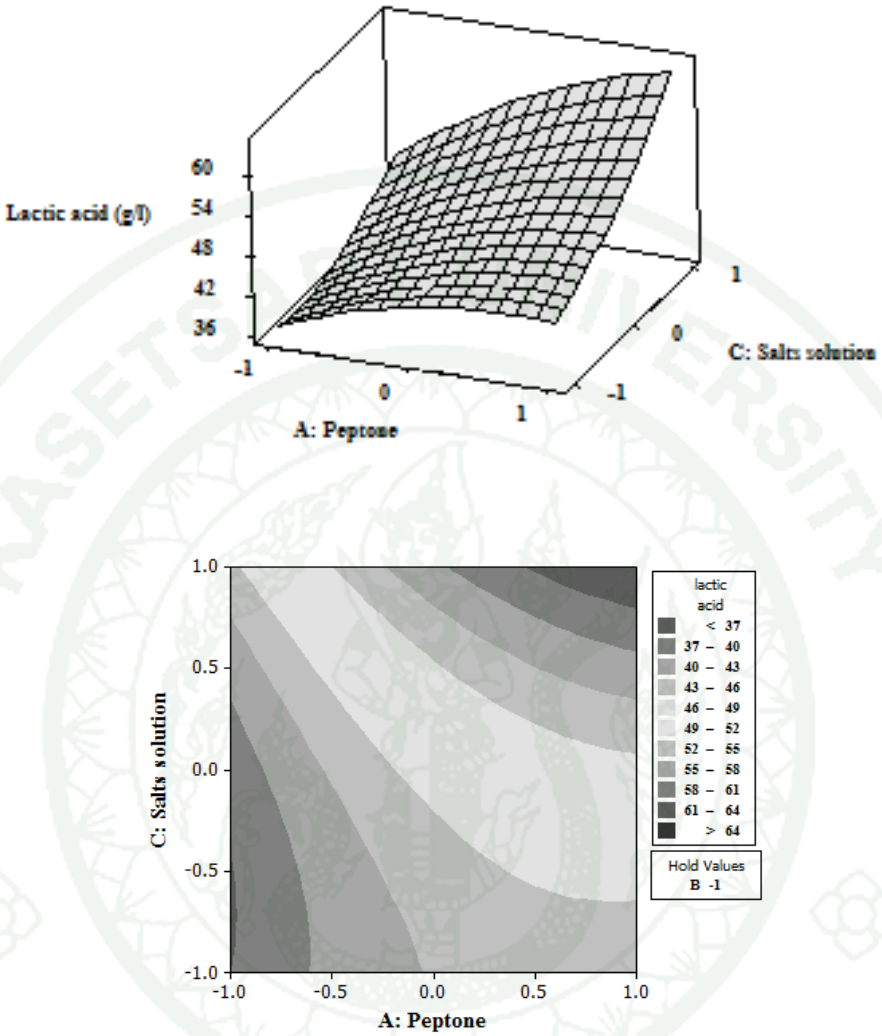


Figure 11 Response surface and contour plots of lactic acid concentration during fermentation of undiluted oil palm trunk juice by *Lactobacillus rhamnosus* TISTR 108, showing the interaction between peptone (*A*) and mineral salts (*C*).

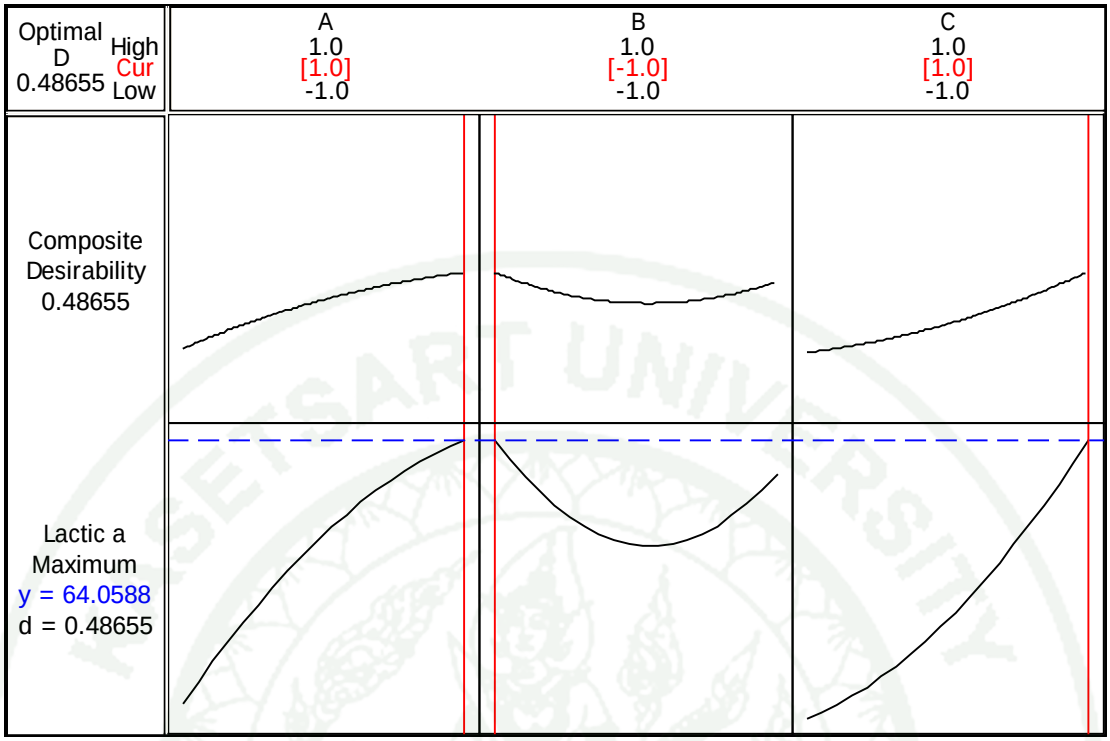


Figure 12 Optimized condition of nutrient supplementation in lactic acid production from undiluted oil palm trunk juice and the predicted values of lactic acid concentration. *A*, *B* and *C* represent peptone, yeast extract and mineral salt concentration, respectively.

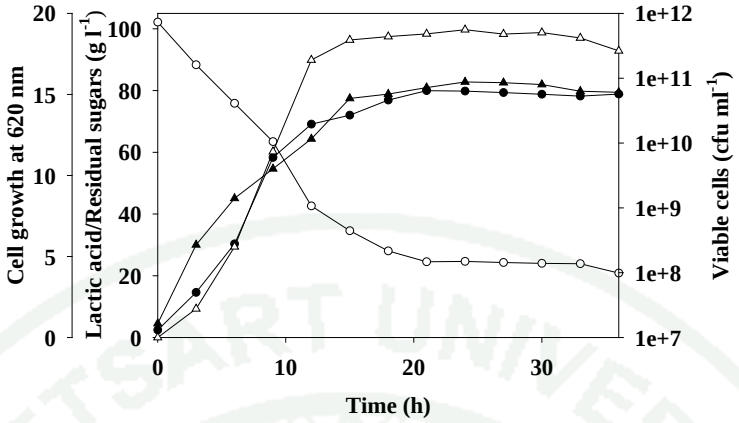


Figure 13 Profiles of cell growth, sugar and lactic acid concentrations during lactic acid fermentation of undiluted oil palm trunk juice with statistically optimized nutrient supplementation by *Lactobacillus rhamnosus* TISTR 108. The batch fermentation was performed in 2 l fermenter with 1.2 l working volume at 40^0C, pH 6.5 and the agitation speed of 200 rpm, using 10% inoculum; lactic acid (●), residual sugars (o), viable cells (▲) and cell growth at 620 nm (Δ).

Table 23 Kinetic parameters of lactic acid fermentation on undiluted oil palm trunk juice by *Lactobacillus rhamnosus* TISTR 108 with nutrient supplementation, optimized by Box Behkhen Design.

Parameters	**Values**
Fermentation time (h)	24
Lactic acid (g l^{-1})	78.46
$Y_{P/S}$ (g g^{-1})	1.01
Q_P (g $l^{-1}h^{--1}$)	3.27
Residual sugars (g l^{-1})	24.64

Note. The fermentation was conducted in 2 l fermenter using undiluted OPT juice, at 40 ^{0}C, pH 6.5 with 10% inoculums and agitation speed of 200 rpm.

CONCLUSION

The conventional process of sugar cane juice extraction was applied to squeeze the sap from harvested oil palm trunk (OPT), yielding around 24.6% w/w of clear undiluted OPT juice. The undiluted OPT juice contained high amount of sugar (104.61±2.36 g l^{-1}) and protein (2.18±0.41 g l^{-1}) contents which could be used as carbon and nitrogen sources for lactic acid production. Glucose and fructose were the main sugar components in OPT juice. Squeezed juice from different parts of OPT contained different amounts of sugars. The inner region contained the highest amount of sugars and gradually decreased radially to the outer region of the trunk.

The optimization of lactic acid fermentation from undiluted OPT juice without nutrient supplementation was preliminary performed in 2 l bioreactor by *L. rhamnosus* TISTR 108. The initial concentration of OPT juice was found to affect the lactic acid yield and productivity. The use of undiluted OPT juice resulted in higher lactic acid production, however incomplete sugar consumption was observed. Hence, the undiluted OPT juiced was used for further process optimization. Inoculum sizes, temperatures and pH were found to significantly affect the growth of *L. rhamnosus* TISTR 108 and its lactic acid production. Under the optimized process, i.e. 10% inoculum, 40^0C and pH 6.5, undiluted OPT juice fermentation without nutrient supplementation yielded the highest lactic acid concentration of 44.86 g l^{-1} with the yield and productivity of 0.78 g g^{-1} and 1.36 g $l^{-1}h^{-1}$, respectively. However, the remaining sugars in fermentation broth indicated insufficiency of nutrient sources for bacterium growth during lactic acid fermentation.

The addition of 10 g l^{-1} yeast extract (YE) and 5 g l^{-1}peptone (P) as nitrogen sources together with salts (T) (1.5 g $CH_3COONa.3H_2O$, 1.5 g KH_2PO_4, 1.5 g K_2HPO_4, 0.2 g $MgSO_4.7H_2O$ and 0.05 g $MnSO_4.4H_2O$) in undiluted OPT juice exhibited an acceleration of fermentation processes by shortening the fermentation time (from 42 h without nutrient supplementation to 21 h with YE+P+T supplementation) and sugars were efficiently consumed. The highest lactic acid concentration of 82.69 and 82.1 g l^{-1}, yield of 0.85 and 0.82 and productivity of 2.47

and 3.83 g l^{-1} h^{-1} were obtained with YE+P and YE+P+T supplementation, respectively.

The response surface methodology with Box Behkhen Design (BBD) was carried out to optimize the amount of added nutrients, i.e. peptone, yeast extract and salts. The predicted model confirmed that the proper amounts of peptone and salt supplementation were necessary for lactic acid fermentation from undiluted OPT juice by *L. rhamnosus* TISTR 108. The fermentation of undiluted OPT juice with optimized nutrients, i.e. peptone and salt in a 2 l batch fermenter at an optimum condition, i.e. 40°C, pH 6.5 using 10% inoculum demonstrated improved lactic acid concentration, yield and productivity (78.46 g l^{-1}, 1.01 g g^{-1} and 3.27 g l^{-1} h^{-1}, respectively) compared to that without nutrient supplementation under the same condition (38.66 - 77.86 g l^{-1}, 0.74 - 0.78 g g^{-1} and 1.19 - 1.36 g l^{-1} h^{-1}, respectively. The findings in this study demonstrate the potential use of undiluted OPT juice as a cheap carbon source for lactic acid production.

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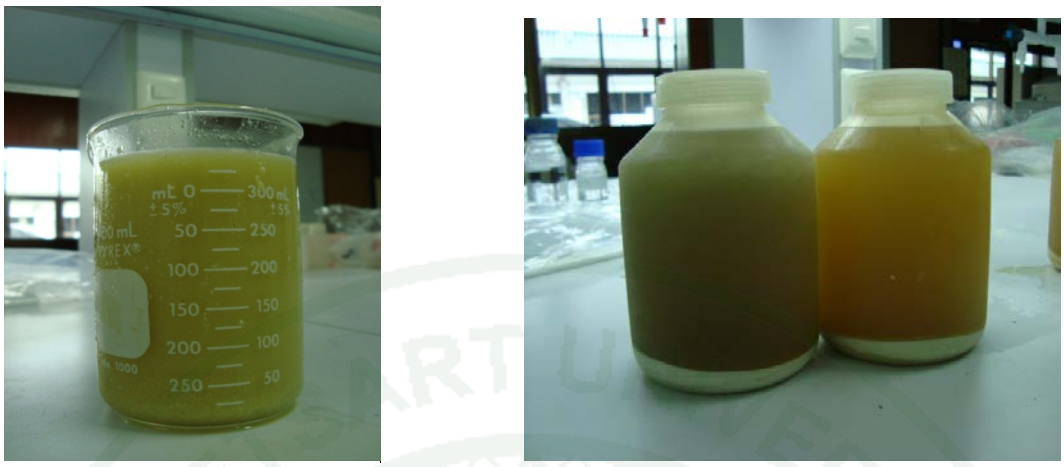
APPENDIX

Appendix Figure 1 Oil palm tree, oil palm tree logs and oil palm trunk disks preparation



Appendix Figure 2 Oil palm trunk juice before and after centrifugation

Appendix Figure 3 Clear undiluted oil palm trunk juice

Appendix Figure 4 Precipitate of oil palm trunk juice obtained from centrifugation

Appendix Figure 5 Oil palm trunk juice before and after autoclaving

Appendix Figure 6 Oil palm trunk juice fermentation

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