CHAPTER 2

Enzymes in starch degrading system in *Lactobacillus* sp. S21

2.1 Introduction

Amylolytic enzymes, starch degrading enzymes or amylases are classified into group of glycoside hydrolase family (GH) based on substrate specificity (Bijttebier *et al.* 2008). They belong to 3 families including GH13 (α-amylase, pullulanase, isoamylase, etc.), GH14 (β-amylase) and GH15 (glucoamylase) (Sharma and Satyanarayana 2013; Taniguchi and Honda 2009). The GH13 is typically known as α-amylase (EC. 3.2.1.4) which has long historically been discovered since 1811 (Gupta *et al*. 2003). It has been utilizing in various applications up to now and shares approximately 30% of world enzyme production (Sivaramakrishnan *et al.* 2006). Around 5.1 billion dollars was supplied for global enzyme market in 2009 (Sharma and Satyanarayana 2013). At the present, industrial applications of α-amylase are served in food and non-food industry in fact the main purpose is directly particularized in food-industry. The addition of α-amylase in sour dough improves volume and texture of bread regarding the reduction of viscosity in dough (Gupta *et al*. 2003; Souza and Magalhaes 2010). Maltogenic α-amylases release maltose and glucose in fermented dough also improve color and taste of bakery products and acts as antistaling agent (Taniguchi and Honda 2009). One of the most important applications of α-amylase in food industry is to produce commercial refined sugars obtained from starch liquefaction and saccharification like maltose, glucose and fructose for using as sweeteners. In non-food industry, α-amylase is used to desize textiles and papers, enzyme mixture in detergents (Taniguchi and Honda 2009; Souza and Magalhaes 2010) and it is moreover developed and used as oligosaccharides detector in medicinal application (Gupta *et al*. 2003). Recently, it is applied in biofuel production from starch through microbial fermentation of sugars obtained from starch liquefaction and saccharification enzymatic methods (Linko and Javaninen 1996; Souza and Magalhaes 2010)

As important as biofuel production, production of lactic acid from starch is also attended for using as substrate for synthesis of polylactic acid (PLA), biodegradable plastic. Consequently the demand of lactic acid for application in food and non-food industry is increasing annually. To achieve large amount of lactic acid with low production cost, cheap materials are offered to encounter the best materials for lactic acid production. Starch is an alternative material for reason; abundance, cheapness and availability. Very few lactic acid bacteria have capability of direct conversion of starch to lactic acid. Hence, liquefaction and saccharification and fermentation strategies by high efficient non amylolytic lactic acid producers are still attempted conventionally. The α-amylase and glucoamylase hydrolyze starch to refine sugars under high temperature condition and additional pH adjustment step is required since these two enzymes work well at different pH condition (Crabb and Mitchinson 1997). The easiest and simplest strategy is simultaneous liquefaction, saccharification and fermentation (SLSF) that is the way to convert starch to lactic acid in single step. This strategy requires broad pH stable enzymes in spite the fact that α-amylase liquefied starch at pH 5.8-6.5 and glucoamylase is applied in saccharification step at pH 4.2-4.5 (Crabb and Mitchinson 1997) and pH variation during the fermentation and pH adjustment step may cost of enzyme instability. As mentioned, either acid stable starch degrading enzymes or broad pH stable enzymes certainly plays important role in direct lactic acid fermentation from starch.

In this chapter, *Lactobacillus* sp. S21 was repeatedly identified by species specific primers to confirm its species. Production of amylase from this high efficient lactic acid bacterium was studied for preparation of enzyme for purification, characterization and classification. Moreover, elucidation of enzymes in starch degrading system in *Lactobacillus* sp. S21 was determined for well understanding of direct conversion of starch to lactic acid.

2.2 Literature review

2.2.1 Starch

Starch, one of macromolecules, is polymer of glucose made up from biosynthesis of plants. It is very important as it serves as carbon for using as energy source for creature and living microorganisms on earth. The α-glucosidic linkages between glucose make up two forms of polymers in starch: i) amylose ii) amylopectin. Most component of 72-82% is amylopectin while the rest is amylose (Buleon *et al.* 1998).

2.2.2 Amylose

Amylose, a linear structure of glucose, is polymerized by covelent bond of C1 oxygen in glucose moleculeand C4 hydrogen in another. The α, (1-4) glucosidic linkages are generated with the degree of polymerization (DP) of 700-6000 depend upon its origin (Taniguchi and Honda 2009 and van der Maarel *et al*. 2002). It was moreover found that few of α, 1-6 glucosidic linkages are also occurred in the molecule (Buleon *et al.* 1998). The molecular weight of approximately $1x10^5$ to $1x10^6$g/mol is estimated with 6000 glucose units (Tester *et al.* 2004). A sample of linear molecule of glucose is demonstrated in Figure 2.1 where the most left hands glucose generates non-reducing end.

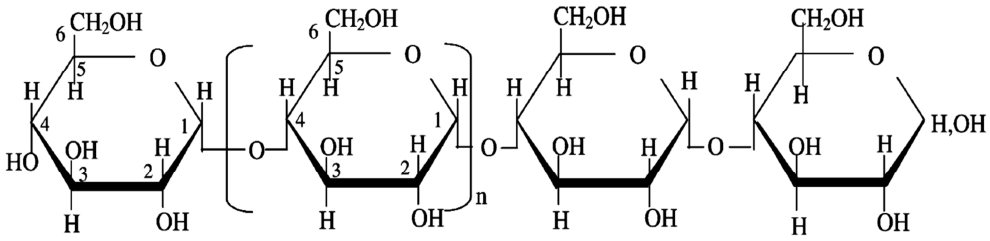

Figure 2.1 Structure of amylose (Tester *et al.* 2004)

2.2.3 Amylopectin

Amylopectin, a branched structure of amylose, is polymerized by α, 1-6 glucosidic linkages between amylose molecules. It has molecular weight of $1x10^7$-$1x10^9$g/mol that is much higher than amylase (Tester *et al*. 2004). The 95% of its

structure contains α, 1-4 glucosidic linkage with 10-60 glucose units and 5% is α, 1-6 linkage with 15-45% glucose units. Amylopectin is short compared to amylose but the complete molecule contains glucose up to 2,000,000 glucose units averagely (van der Maarel *et al*. 2002). A sample of amylopectin structure is given in Figure 2.2 where branch point is generated by α, 1-6 glucosidic linkage.

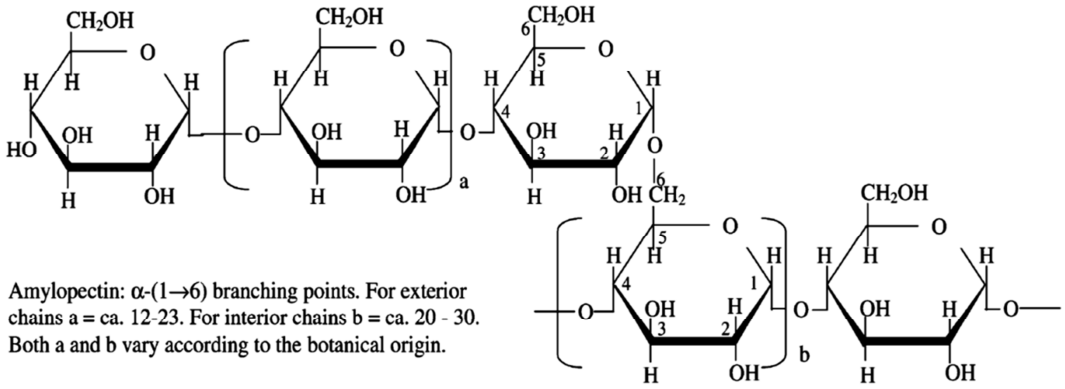

Figure 2.2 Structure of amylopectin (Tester *et al.* 2004)

2.2.4 Starch degrading enzymes

The above described starch structure briefly and it is used for well understanding how enzymes convert starch to products. The classification of amylolytic enzymes has long been studying for several decades. Amylases are most well-known enzymes capable of hydrolysis glucosidic bonds occurred in starch molecules. Marshall (1975) divided starch degrading enzymes into two groups: i) the enzymes specify to α, 1-6 glucosidic linkage (debranching enzymes) ii) the enzymes specify to α, 1-4 glucosidic linkage in which subdivided into two groups including those randomly hydrolyze starch typically known as endo-enzymes and those hydrolyze starch from the non-reducing end and release glucose typically known as exo-enzymes. van der Maarel *et al*. (2004) reviewed the starch converting enzymes in which divided into four groups including i) endo-amylases ii) exo-amylases iii) debranching enzymes and iv) transferases.

Taniguchi and Honda (2009) concluded the classification of amylases in different criterions

i) Base on IUBMB Enzyme Nomenclature, the amylase enzymes are classified by their catalytic properties such as substrate and product specificities. There are 3 EC classes of amylases: i) transferases (EC 2.x.x.x) ii) hydrolase (EC 3.x.x.x) iii) isomerase (EC 5.x.x.x) and the most of them are belonged to hydrolase EC 3.

ii) Base on mode of action of enzymes, the amylase enzymes are classified into two groups including retaining and inverting enzymes. Almost of all members are retaining enzymes except for β-amylase and glucoamylase is inverting enzyme.

iii) Base on the action specificity towards α-glucan substrate, the amylases are classified to endo- and exo-enzymes.

iv) Base on bioinformatics data according to primary and tertiary structure of the enzyme for instances amino acid sequence similarities or mechanistic characteristics of enzymes. The enzyme can be classified using database available at www.cazy.org. The carbohydrate active enzymes are divided into 5 families including Glycoside Hydrolase (GH family), Glycosyltransferase (GT family), Polysaccharide Lyase (PL family), Carbohydrate Esterase (CE family) and Carbohydrate-Binding Module Family (CBM family). Starch degrading enzymes are belonged to glycoside hydrolase family that described the enzymes capable of hydrolysis or rearrangement of glycosidic bonds.

In several past publications, the classification of starch degrading and related enzymes was categorized in 7 glycoside families (Svensson *et al*. 2002) but 5 families included microbial amylase (Taniguchi and Honda 2009). Regarding Table 2.1., of 5 families consisting of GH 13, 14, 15, 31 and 77, the GH 13 is the largest families of which have 35 subfamilies based on their sequence similarity and structural differences. The modes of action of amylases are also included in Figure 2.3.

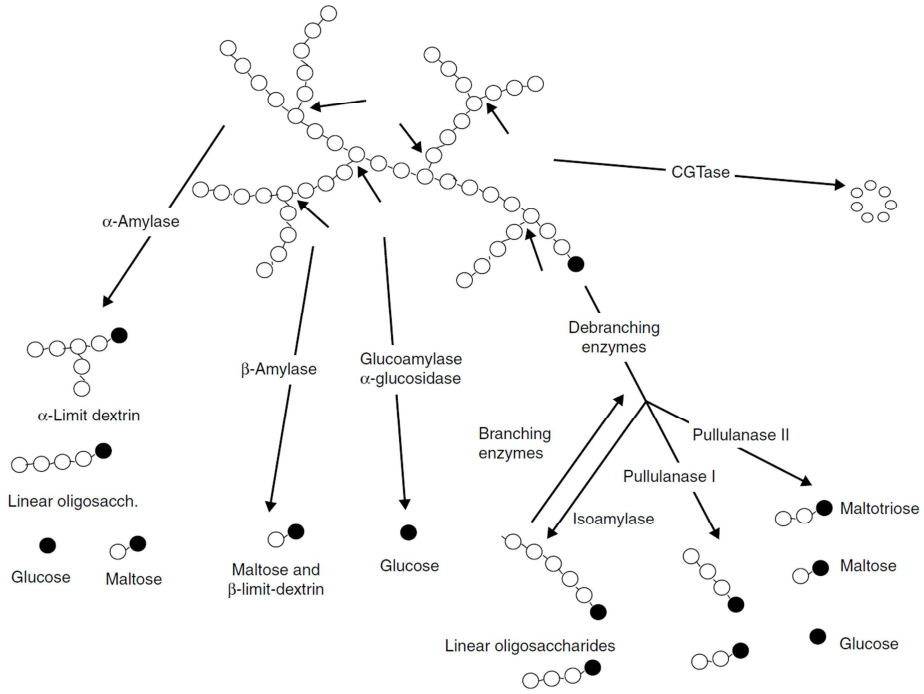

Figure 2.3 Modes of action of amylases (Bertoldo and Antranikian 2002)

The classification of amylolytic enzyme has been developed from time to time since Bijttebier *et al.* (2008) referred in the review of action pattern of amylases towards starch polymers. Because some enzymes may not be able to hydrolyze only a substrate leading to difficulties in classification of amylases EC classes system. The utilization of bioinformatics data are the most powerful as it is able to consider on enzyme sequences, structures and mechanisms and function of gene coding enzymes. As mentioned above, starch degrading enzymes are belonged to 5 GH families. In this literature review, the enzymes act on α, 1-4 and α, 1-6 glucosidic linkages and only some important enzymes are discussed.

Table 2.1 Classification of amylases

Enzyme	EC number	GH family	Mechanism	Mode
α-Amylase	3.2.1.1	13	Retaining	Endo
β-Amylase	3.2.1.2	14	Inversion	Exo
Glucoamylase	3.2.1.3	15	Inversion	Exo
Oligo-1,6-glucosidase	3.2.1.10	13	Retaining	Endo
α-Glucosidase	3.2.1.20	13, 31	Retaining	Exo
Amylo-1,6-glucosidase	3.2.1.33	13	Retaining	Exo
Pullulanase	3.2.1.41	13	Retaining	Endo
Cyclomaltodextrinase	3.2.1.54	13	Retaining	Endo
Glucan-1,4-α-maltotetraohydrolase	3.2.1.60	13	Retaining	Exo
Isoamylase	3.2.1.68	13	Retaining	Endo
Glucan-1,4- α-maltohexaohydrolase	3.2.1.98	13	Retaining	Exo
Glucan-1,4- α-maltotriohydrolase	3.2.1.116	NC	Retaining	Endo
Neopullulanase	3.2.1.135	13	Retaining	Endo
Cyclomaltodextrin glucanotransferase	2.4.1.19	13	Retaining	Endo
4- α-glucanotransferase	2.4.1.25	77	Retaining	Endo
4- α-glucan-1 α-D-glucosylmutase	5.4.99.15	13	Retaining	Exo

NC= not classified

Source: Taniguchi and Honda (2009)

2.2.5 Family 13 Glycoside hydrolases

The enzymes in GH 13 family are typically known as α-amylase as an important enzyme but it is actually contained some other important enzyme. The enzymes in this family are able to hydrolyze/or transglycosylate α, 1-4 or α, 1-6 glucosidic linkages on

starch, amylose, amylopectin and glycogen or combination of both reactions. They have $(\alpha/\beta)_8$ barrel structure in catalytic domains in which consist of domain A, B and C. The N-terminal amino acid sequences typically contain conserved regions in which catalytic and major substrate binding sites are located. Aspatate and glutamate residue are conserved in catalytic sites. Moreover, some amylases have other domains in C-terminus which involves thermostability (Kim *et al.* 2009) and raw starch degrading ability (Rodriguez-Sanoja *et al.* 2005)

α-amylases

The α-amylases play many important roles in particular industrial applications. The α-amylases, EC 3.2.1.1, are endo-acting enzymes catalyze the hydrolysis of α, 1-4 glucosidic linkages of starch polymers internally. It is impossible to catalyze the hydrolysis of α, 1-6 glucosidic linkages. The released products from hydrolysis are various oligosaccharides and different α-limit dextrins containing α, 1-6 bonds.

Glucan-1,4-α-maltohydrolases

The EC 3.2.1.133 known as maltogenicα-amylases or maltose forming enzymes, catalyze the hydrolysis of starch polymers to α-maltose as main product. As referred by Diderichsen and Christiansen (1988), it is in groups of exo-acting enzymes. So far, maltogenicα-amylases have been studied and they are found to have many hydrolytic activities. Some publications reported that they were capable of hydrolysis both α, 1-4 and α, 1-6 glucosidic linkages of starch polymers and they were also performed glycosylation activity of α, 1-3, α, 1-4, α,1-6 glucosidic linkages (Mabrouk *et al*. 2007 and Kolcuoglu *et al.* 2010).

Maltooligosaccharides forming enzymes

The enzymes form maltooligosaccharides with specific DPs are also classified in GH 13 family. These enzymes are Glucan-1,4- α-maltotriohydrolases (EC 3.2.1.116), Glucan-1,4- α-maltotetraohydrolases (EC 3.2.1.60) and Glucan-1,4- α-maltohexaohydrolases (EC 3.2.1.98). They catalyze the hydrolysis of α, 1-

4glucosidic linkages in starch polymers from non-reducing end and release maltotriose (G3), maltotetraose (G4) and maltohexaose (G6) in α-configuration, respectively. The G5 forming enzymes have not been given the EC number because the mode of action is still unclear (Taniguchi and Honda 2009).

Iso-amylases

The exo acting enzymes, EC 3.2.1.68, catalyze the hydrolysis of α, 1-6 glucosidic linkages of starch polymers and release maltooligosaccharides with degree of polymerization (DP) at least 3 (Bijttebier *et al.* 2008). Iso-amylases are in groups of debranching enzymes. They are impossible to hydrolyze α, 1-4 glucosidic linkages in starch polymers

α-glucosidases

The exo-acting enzymes, EC 3.2.1.20, catalyze the hydrolysis of α, 1-4 glucosidic linkages of various types of saccharides including disaccharides, oligosaccharides and aryl- and alkyl-α-glucopyranosides (Zdzieblo and Synowiecki, 2002) from non-reducing end of substrates and produce β-glucose as main product . Moreover, they are capable of hydrolysis of α, 1-2, α, 1-3 and α, 1-6 glucosidic linkages (Taniguchi and Honda 2009). They are typically occurred as intracellular enzymes in microorganisms. The α-glucosidasesare belonged to two families including GH 13 and GH 31 family. The difference between these two families has been discussed in Nishimoto *et al.* (2007). However, the X-ray crystallographic analysis has revealed the tertiary structure of two enzyme families and concluded that they have distinct active sites in which lead to differences in catalytic reactions (Nishimoto *et al.* 2007).

Cyclomaltodextrinases

Preferably cyclomaltodextrins, cyclic oligomers of α, 1-4 glucosidic linkages of glucose, rather than starch, the enzymes classified to EC 3.2.1.54 catalyze the hydrolysis of α, 1-4 glucosidic linkages in cyclomaltodextrins to linear open chain of maltooligosaccharides. Actually they possess mode of action similar to α-

amylases but difference in affinity while attacking cyclomaltodextrins and because of some reasons as revealed by Lee *et al.* (2005). As a reason, they are in the same subfamily as maltogenic α-amylases and neopullulanses.

Neopullulanases

The one of 4 types of pullulan hydrolyzing enzymes, neopullulanases (EC 3.2.1.135) has been found as new enzymes based on their action pattern by Kuriki and Imanaka since 1989. Neopullulanases catalyze the hydrolysis of α, 1-4 glucosidic linkages in pullulan and release panose (α-glucosyl-maltose). They are capable of hydrolysis starch, cyclodextrins and maltooligosaccharides and produce α-maltose and glucose (Taniguchi and Honda 2009). Takata *et al.* (1992) have introduced action of these enzymes that they catalyze both hydrolysis and transglycosylation of α, 1-4 and α, 1-6 glucosidic linkages.

2.2.6 Family 14 Glycoside hydrolases

β-amylases

The enzymes catalyze the hydrolysis of starch to maltose possesses β-configuration. The β-amylases, EC 3.2.1.2, are exo-acting enzymes typically found in most plants (Taniguchi and Honda 2009).However, there have been some publication produced the enzymes from microbial resources such as *Clostridium thermosulphurogenes* (Shen *et al*. 1988), *Bacillus polymyxa* (Friedberg and Rhodes, 1986) and *Halobacillus* sp. LY9 (Li and Yu 2011). The β-amylases catalyze the hydrolysis of α, 1-4 glucosidic linkages in starch from non-reducing end to β-maltose, β-limit dextrins and very small amounts of β-glucose in contrast to α, 1-6 glucosidic linkages in which the enzymes are unable to break down the bonds. The action pattern is similar to maltogenic α-amylases but different in product configuration. It is said that α-amylases are liquefied enzymes but β-amylases are saccharified enzymes (Friedberg and Rhodes 1986).

2.2.7 Family 15 Glycoside hydrolases

Glucoamylases

The third amylase enzymes discovered after α- and β-amylases, glucoamylases or γ-amylases (EC 3.2.1.3) as the exo-acting enzymes, catalyze the hydrolysis of both α, 1-4 and α, 1-6 glucosidic linkages in starch from non-reducing end and produce a sole product of β-glucose. The β-glucoamylases are able to catalyze the hydrolysis of α, 1-4 glucosidic linkages more rapidly and better than that of the α, 1-6 glucosidic linkages so they produce completely β-glucose as a sole hydrolysis products (Taniguchi and Honda 2009).

The starch degrading enzymes in GH family 77 as 4-α-glucanotransferase (EC 2.4.1.25) are not reviewed in this introduction because they involve in modification of saccharides rather than degrading starch polymers.

2.2.8 Amylolytic enzymes from amylolytic lactic acid bacteria

Typically, starch degrading enzymes from amylolytic lactic acid bacteria are in group of amylases. Most of them are α-amylase followed by a few cases of amylopullulanases and maltogenic α-amylase as concluded in Table 2.2. Most of them are monomeric enzymes and generally have high molecular weight compared to other source of amylase producing bacteria particularly *Bacillus* sp.. The amylases from lactic acid bacteria are not stable as high as 70^{o}C or higher but they are seemed to be acid stable amylases and are therefore very suitable for direct lactic acid production from starch

Table 2.2 Amylases from amylolytic lactic acid bacteria

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

2.2.9 *Lactobacillus* sp. S21

The *Lactobacillus* sp. S21 has been screened by Rientragoonchai (2009) from Thai noodle, a traditional northern Thai fermented food. It forms white smooth colony and produced yellow clear zone around colony on MRS agar containing starch as a sole carbon source supplemented with 125 ppm bromocresol purple after incubation at 37°C for 24 h. It is Gram positive, rod shaped (approximately 0.2 µm width and 2 µm length) and nonspore-forming bacterium as shown in Figure 2.4. The temperature and pH range for growth is between 15-40°C and pH 4-10, respectively. The catalase is negative but positive in oxidase test, acid is produced from glucose and approximately 90% of total acid is lactic acid. Indole is not produced and nitrate is not reduced to nitrite. The intensive study revealed that the 1467 bp of 16S rRNA gene shares high similarity with the genus *Lactobacillus* as its nearest neighbor of 99.86% identity with *L. pentosus*, 99.80% with *L. plantarum* and 99.60% identity with *L. paraplantarum* (Figure 2.5) (Rientragoonchai, 2010). Therefore, differentiation of *Lactobacillus* sp. S21 to the mentioned species is necessary. Lactic acid is produced as main organic acid from the utilization of carbon source and amylolytic enzymes are produced extracellularly. This strain has feasibility in direct lactic acid production with 90% conversion of starch to lactic acid initiated at 10 g/L of starch. The partial purified enzyme is able to hydrolyze starch to release glucose and maltose predominantly.

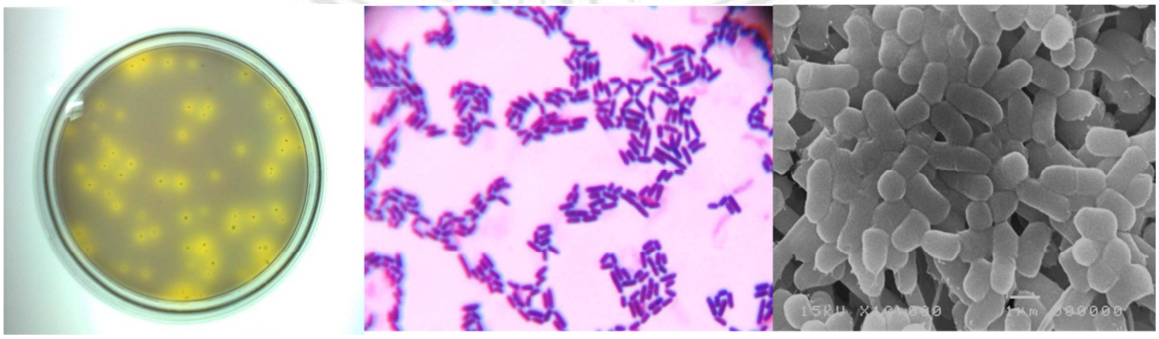

Figure 2.4 Cell morphology of *L. plantarum* S21

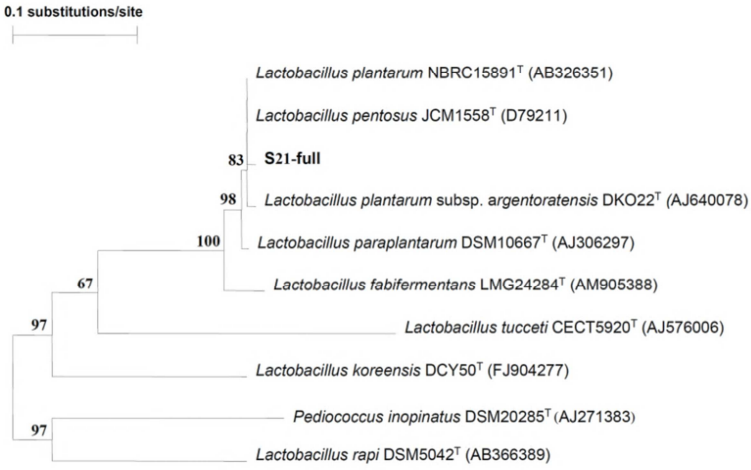

Figure 2.5 Phylogenetic tree of *L. plantarum* S21 and its related species

2.3 Materials and methods

2.3.1 Media

Modified de Man-Rogosa Sharpe medium, mMRS (Appendix A)

2.3.2 Chemical reagents

Mono-, di, trisacchariedes for production of amylase and maltooligosacharides for Thin layer chromatography analysis were purchased from Sigma Aldrich. Materials for enzyme purification (Q-sepharose) were purchased from GE, Amersham. Soluble starch, amylose, amylopectin, glycogen, pullulan, maltodextrin, dextran, α-cyclodextrin, β-cyclodextrin for determination of substrate specificity were all analytical grade and were purchased from Sigma Aldrich and Fluka. Chemicals used for preparation of different buffer systems were purchase from Sigma Aldrich. Chemicals and reagents for SDS-PAGE were purchased from Bio-Rad and Sigma Aldrich. Substrate for α-glucosidase assay and activity staining were purchased from Sigma Aldrich.

2.3.3 Equipment and instrument

Arkta Prime Plus fast protein liquid chromatography system was purchased from GE, Amersham. High performance liquid chromatography system was purchased from Shimazu, Japan. Carbo Pac PA100 anion exchange column was purchased from Dionex, Sunnyvale, CA.

2.3.4 Species identification of *Lactobacillus* sp. S21

According to result from Rientragoonchai (2010), isolate S21 was identified based on morphological and biochemical characteristics and analysis of 16S rDNA sequence. Therefore, it was identified as *Lactobacillus* sp. but the 16S rDNA sequence had high similarity with that of *L. plantarum*, *L. pentosus* and *L. paraplantarum* with 99.6, 99.86 and 99.8. To differentiate *Lactobacillus* sp. S21 from these species, species specific primers were designed for amplification of *recA* of each species. The para F; 5'-GTCACAGGCATTACGAAAAC-3', pent F; 5'-CAG TGG CGC GGT TGA TAT C-3' and plant F; 5'-CCGTTTATGCGGAACACCTA-3' was used as forward primer for amplification of *rec A* from *L. paraplantarum*, *L. pentosus* and *L. plantarum*, respectively and pREV; 5'-TCG GGATTACCAAACATCAC-3' was used as reverse primer. The procedure was performed as described by Torriani *et al*. (2001). Genomic DNA of *Lactobacillus* sp. S21 was extracted by method of Nishiguchi *et al.* (2002) and was used as a template.

2.3.5 Enzyme production

Preparation of *L. platarum* S21 culture

L. plantarum S21 stored in 15% (v/v) glycerol stock was transferred to mMRS broth and incubated at 37^{o}C for 12 h. The culture was purified by streaking on MRS agar containing 10 g/L of soluble starch as a sole carbon source with the supplementation of 125 ppm of bromocresol purple as indicator. The single colony gave clear zone with yellow area on dark purple background according to the starchy hydrolysis and lactic acid production was selected for seed inoculums preparation.

Preparation of seed inoculums

The single colony was inoculated to 5 mL of mMRS broth and static incubated at 37^{o}C for 12 h. Only 1 mL of the culture was then transferred to 100 mL of mMRS broth containing 10 g/L of soluble starch and carried out at the same condition for 24 h.

Production of *L. plantarum* S21 amylase

Seed inoculum prepared as described above was centrifuged at 12000 rpm for 10 min. The supernatant was discarded and cells were washed twice with sterilized 0.85%(w/v) and resuspended in the same solution. The resuspended cells of 1%(v/v) was transferred to MRS broth containing 10 g/L of various carbon sources as a sole carbon source including some representatives of monosaccharides, disaccharides, trisaccharides and polysaccharides. Culture was taken 12 h intervals to determine protein content and amylase activity.

Preparation of extracellular amylase

The crude enzyme taken from the culture medium was prepared by centrifugation at 12000 rpm under 4^{o}C for 10 min.

Assay of amylase activity

The reaction mixture was performed by adding 0.25 mL of 0.5% (w/v) of soluble starch substrate in 0.1 M Na-phosphate buffer pH 6.5 and 0.25 mL of proper dilution of enzyme. The condition was carried out at 37^{o}C on water bath or dry bath. After 10 min of incubation, 0.5 mL of DNS solution was added to stop the reaction and to measure the reducing sugar liberated from the starch (Miller 1959). The solution was placed on boiling bath for 10 min and leave until cooling. The total 1 mL of reaction was added 4 mL of distilled water prior to measuring absorbance at 540 nm. One unit of enzyme was defined as enzyme that liberated 1 µmole of reducing sugar under assay condition.

Assay of protein content

The 15 µL of enzyme was added in 600 µL of Bradford reagent. The reaction was mixed well and stood at room temperature for 10 min prior to measuring absorbance at 595 nm.

2.3.6 Enzyme purification

Production of amylase for enzyme purification

Seed inoculums prepared as described above was transferred to 900 mL of MRS broth containing 10 g/L of soluble starch as a sole carbon source and static incubated at 37^{o}C until 36-48 h of cultivation

Purification of *L. platarum* S21 amylase

Crude enzyme was precipitated by 0-80% saturation of ammonium sulfate. The precipitated protein was dissolved with small amount of 20 mM sodium phosphate buffer pH 6.5. This solution was dialyzed against the same buffer to remove ammonium salt in the solution until equilibrium. The solution was clarified by centrifugation to get rid of some insoluble particles before applying into Q-sepharose Fast Flow column (strong anion exchange chromatography) with condition as described in appendix. The unbound protein was washed out with 20 mM sodium phosphate buffer pH 6.5. The bound protein was eluted by 0-0.5 M NaCl linear gradient in 10 mL fraction tube. The active fractions were pooled, desalted and polished by the same material to obtain the highest homogenized enzyme.

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was performed according to method of Laemmli (1970) in 10% resolving gel and 4% stacking gel with and without 0.1% of SDS as SDS and Native-PAGE, respectively. Denaturing condition was carried out for SDS-PAGE by heating the protein under the presence of β-mercaptoethanol at 100^{o}C for 8 min. Protein was stained with Coomassie blue R250.

Staining of amylase activity

Protein containing 1 unit activity of amylase was prepared and heated at 70^{o}C for 4 min before carried out on SDS-PAGE. The protein was renatured by washing the gel in cold solution of 20 mM Na-phosphate buffer pH 6.5 for 30 min and immersed in 0.1%(w/v) of soluble starch in 20 mM Na-phosphate buffer pH 6.5 for 5 min. The gel

was rinsed by water and flooded with 0.3% I_2, 3% KI to visualize the clear zone under dark blue background as amylase activity (Lack and Springhorn 1980).

2.3.7 Characterization of purified amylase

pH optimum and stability

To study on pH optimum, purified amylase was assayed activity under various pH conditions ranging from 3.0-10.0 using 20 mM Britton-Robinson universal buffer (Britton and Robinson, 1931) (20 mM phosphoric acid, 20 mM acetic acid and 20 mM boric acid, 1 M NaOH was used to adjusted to the desired pH units). The relative activity was calculated compared to that of the highest activity. For pH stability, purified amylase was prepared under various pH conditions as described and incubated at 4 and 37^{o}C for 24 h prior to determination of the residue amylase activity.

Temperature optimum and stability

The purified amylase was assayed activity under various temperatures ranging from 25-65^{o}C. The relative activity was calculated compared to that of the highest activity. For temperature stability, purified amylase was incubated at 25, 30, 37, 45, 50, 55, 60 and 65^{o}C for 1 h prior to determination of residue amylase activity.

Synergistic effect of pH and temperature

The central composite design (CCD) as a statistical experimental design was used to evaluate the synergistic effect of pH and temperature on amylase activity. The 9 replicates at the center points, 4 replicates at factorial point and axial star point of pH and temperature within 41 runs. All treatments consisted of 5 levels of pH and temperature as shown in Table 2.3 and were run compared with control in which the enzyme was reacted with soluble starch under pH 6.5 at 37^{o}C. The relative activity was calculated and the quadratic equation of optimal temperature and pH for this amylase was generated by multiple regression analysis as followed;

$$Y = b_0 + \sum b_i x_i + \sum b_{ii} x_i^2 + \sum b_{ij}\, x_i x_j$$

Where Y is predicted value (Relative activity); b_0 is the offset term; b_i is the linear effect; b_{ii} is the squared effect; b_{ij} is the interaction effect; x_i and x_j is the independent variable (pH and temperature).

Table 2.3 Level of coded and actual values of temperature and pH for CCD

Variables	Symbols	Level				
		-2	-1	0	+1	+2
Temperature (^oC)	X_1	20	34	48	61	75
pH	X_2	3.5	4.4	5.2	6.1	7

Substrate specificity

Amylase activity was assayed in 0.5% (w/v) of different substrates including soluble starch, amylase, amylopectin, α-cyclodextrin, β-cyclodextrin, glycogen, dextrin, maltodextrin, dextran and pullulan. The relative activity was calculated compared to that obtained from soluble starch.

Effect of metal ion and other chemicals

Amylase activity was assayed in the presence of 5 mM of cations including K^+, Na^+, Ag^+, Ca^{2+}, Mn^{2+}, Zn^{2+}, Cu^{2+}, Co^{2+}, Ni^{2+}, Fe^{2+}, Sr^{2+}, Mg^{2+} and Fe^{3+}, 0.25% SDS and 15 mM EDTA. The relative activity was calculated compared to that without any metal ion and chemical.

Determination of kinetic parameters

The Michaelis constant was determined at various substrate concentrations ranging from 0.2-20 g/L of 4 substrates including soluble starch, amylose, amylopectin and glycogen. The condition was carried out at pH 6.5 and 37^{o}C. Lineweaver-Burk plot was generated and K_m and $V_{max}$was calculated by SigmaPlot 12.0 (Sysstat Software, Inc., San Jose, CA, USA). The k_{cat} and k_{cat}/K_m were also calculated.

Analysis of hydrolysis products from purified amylase by thin layer chromatography (TLC)

The hydrolysis was performed towards the following substrates; maltose (G2), maltotriose (G3), maltotetraose (G4), maltopentaose (G5), maltohexaose (G6), starch, amylose, amylopectin and glycogenusing 0.4 unit/mg substrate to make final concentration of starch to 5 mg/mL in 1 mL reaction mixture. The condition was carried out at 37^{o}C for 24 h. Moreover, starch hydrolysis was performed until 96 h of incubation at 37^{o}C. To terminate the reaction, heating at 100^{o}C was performed. The hydrolyzed products were examined by TLC. The 1.00 µL of each sample was spotted on Merck classical silica TLC plate and dried by dryer. The plate was developed in mobile phase system containing n-butanol: ethanol: water in a ratio of 5:3:2 by volume at ambient temperature for 4 h. To stain the hydrolysis products, the plate was sprayed with 0.5%(w/v) of thymol in 5%(v/v) of sulfuric acid in ethanol and heat at 105^{o}C for 5 min to visualize spots in pink color.

Analysis of hydrolysis products by high performance liquid chromatography (HPLC)

The hydrolysis products from starch, amylose and amylopectin were quantified by HPLC using Carbo Pac PA100 anion exchange column (Dionex, Sunnyvale, CA) equilibrated with 150 mM NaOH. The separation was performed under 30^{o}C by linear gradient of 500 mM Na-acetate. Maltooligosaccharides were dectected by ED40 electrochemical detector

Amino acid sequence analysis

The purified enzyme was analyzed by Liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) according to the procedures provided by Division of Biochemistry, Department of Chemistry, University of Natural Resources and Applied Life Science (BOKU), Vienna, Austria. The similarity of amino acid sequence was compared and calculated the statistical significant of matches using protein BLAST program from National Center for Biotechnology Information (NCBI) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Amino acid composition, theoretical isoelectric

point and calculated molecular weight were determined using tools from ExPASY Bioinformatics Resource Portal (www.expasy.org).

2.3.8 Elucidation of starch degrading system in *L. plantarum* S21

Extraction of intracellular enzyme

A single colony of *L. plantarum*S21 was inoculated in MRS broth containing 10 g/L of maltose as a sole carbon source and incubated at 37^{o}C for 24 h. This culture medium was served as seed inoculums which was subsequently transferred to 900 mL of the same medium and incubated at the same condition for 24 h. The cell was harvested by centrifugation at 6000 rpm for 30 min and washed twice with 20 mM Na-phosphate buffer pH 6.5 and resuspended the cell with 30-50 mL of the same buffer prior to extraction of the intracellular enzyme by French press (Aminco, Maryland, USA).

Assay of α-glucosidase activity

The α-glucosidase activity assay was performed by preparing the mixture of 50 µL of proper dilution of enzyme and 50 µL of 4 mM of 4-Nitrophenyl-α-D-glucopyranoside and incubated at 37^{o}C. After 30 min, the reaction was terminated by adding 200 µL of 1 M Sodium carbonate. The 4-Nitrophenol released from the substrate was measured at absorbance 400 nm. One Unit (IU) of enzymatic activity was defined as the amount of the enzyme liberated glucose or 4-Nitrophenol in a minute under assay condition.

Staining of α-glucosidase activity

Native-PAGE was performed as described previously. The gel was washed twice with 20 mM Na-phosphate buffer pH 6.5 and immersed in 4 mM of 4-Methylelumberiferyl-α-D-glucopyranoside in 20 mM Na-phosphate buffer pH 6.5. The α-glucosidase was visualized under UV light in a few minutes.

2.4 Results

2.4.1 Species identification

The *recA* gene of *Lactobacillus* sp. S21 was successfully amplified by Plant F species specific primer with the estimated sized of 300 bp. This size was in accordance with the PCR products obtained from the amplification of genomic DNA of *L. plantarum* JCM 8341 and *L. plantarum* TISTR 951 by Plant F as shown in Figure 2.6. The other two primers including pent F and para F were definitely unable to amplified recA gene of *Lactobacillus* sp. S21.

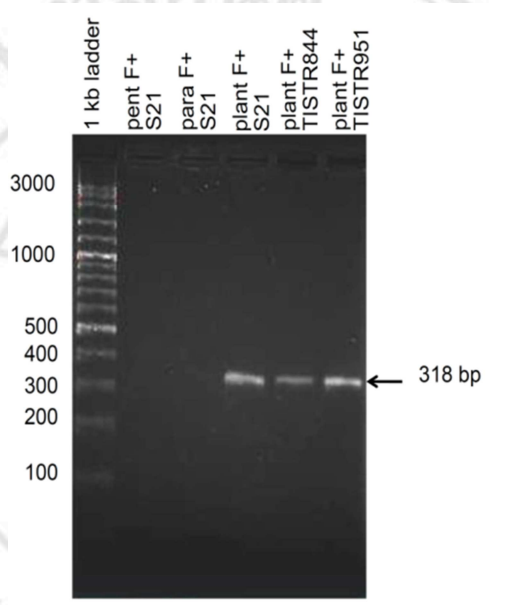

Figure 2.6 The PCR products from *recA* amplification between genomic DNA of *Lactobacillus* sp. S21 and *L. pentosus* species specific primer (pent F+S21), *L. paraplantarum* species specific primer (para F+S21), *L. plantarum* species specific primer (plant F+S21) compared with products from amplification between *L. plantarum* species specific primer and *L. plantarum* TISTR844 (plant F+TISR844), *L. plantarum* TISTR951 (plant F+TISTR951)

2.4.2 Enzyme production

Various carbon sources were used as carbon source for amylase production in order to find proper carbon source for amylase production and induction. The result was reported in form of profiles of amylase production within 72 h. It was found that galactose as representative of monosaccharides, lactose and cellobiose as

representatives of disaccharides obviously showed positive effects on amylase production compared to starch while other mono, di and trisaccharides did not enhance the production of amylase (Figure 2.7a, 2.7b). Moreover, other polysaccharides including cellulose, xylan, gum from locust bean and pullulan did not induce the enzyme production because very few growths of *L. plantarum* S21 was observed (data not shown).

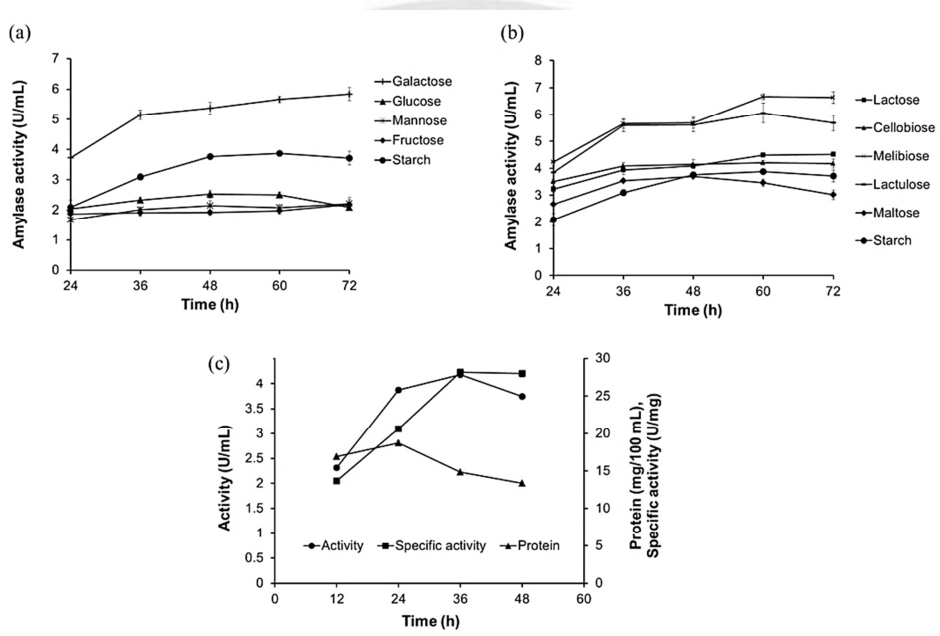

Figure 2.7 Effect of monosaccharides (a), disaccharides (b) and starch (c) on amylase production

To produce amylase for enzyme purification, starch was considerably proper carbon source since it was cheap, abundant, and available and it was not reducing sugar which probably interfered while enzyme activity assay. Profile of amylase production by using soluble starch was performed to investigate the maximum amylase specificity. It was found that *L. plantarum* S21 produced the highest amylase activity of 4.2 U/mL at 36 h of cultivation (Figure 2.7c). The soluble protein was produced increasingly within 24 h of cultivation; then it was decreased till the end of cultivation. In view of specific activity, it was increasing until the maximum at 36 h to the highest amylase specific activity of 28 U/mg. Therefore, the amylase was harvested for enzyme purification.

2.4.3 Enzyme purification

The enzyme was initially purified by ammonium sulfate precipitation in order to reduce volume, to concentrate the enzyme and to exclude other proteins. The enzyme was however lost the activity by this step and decreased the specificity to 0.9 U/mg but the purification was improved satisfactorily to 10 times by Q-sepharose material with 355 U/mg. The partial purified enzyme was polished by high resolution Q-sepharose material until homogeneity with certain specific activity of 936 U/mg and the purification was 27 times higher than crude enzyme. Within this technique, the amylase was purified homogeneously with 23% recovery (Table 2.4) and was used for further characterizations.

Table 2.4 Purification of extracellular amylase from *L. plantarum* S21

Step	Total activity (Units)	Total protein (mg)	Specific activity (U/mg)	Purification (folds)	Recovery (%)
Crude enzyme	10620	298.2	35	1	100
Precipitation	6656	215.4	31	0.9	63
Q-sepharose	3628	10.2	355	10	34
Q-sepharose (polishing column)	2424	2.6	936	27	23

The SDS-PAGE gel revealed that the enzyme was purified homogeneously with a single faint protein band that had estimated molecular weight of 100 kDa corresponded to active protein band under dark blue background from zymogram (Figure 2.8a) caused by starch degradation. The native PAGE moreover revealed the estimated molecular mass of 107 kDa (Figure 2.8b) in corresponded to that from SDS-PAGE gel and also confirmed that the enzyme has monomeric structure.

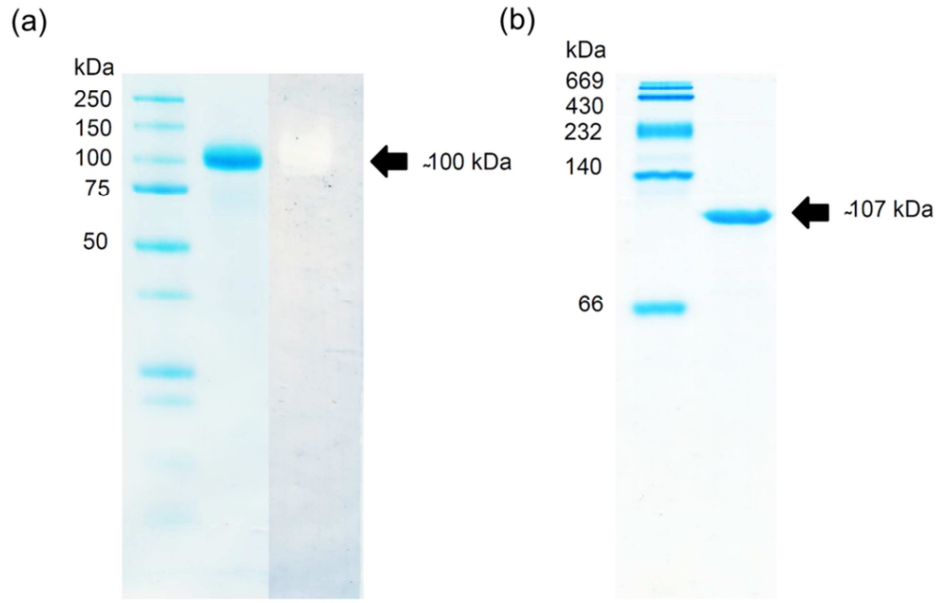

Figure 2.8 SDS-PAGE, zymogram (a) and native PAGE of purified amylase (b)

2.4.4 Properties of purified amylase

pH optimum and stability

The purified amylase gave more than 80% relative activity at wide ranges of pH from 4.0-6.0 with optimum at pH 5.0 (Figure 2.9a). More than 80% residual activity was found when incubated the enzyme in 10 mM buffer pH ranging from 3.5-8.0 for 24 h at 37^{o}C as shown in Figure 2.9b. It was moreover found that the enzyme was lost more than 30% of its initial activity under pH 3.0 and 8.5 and definitely inactivated when the pH of out 3.0-8.5 in contrast to at 4^{o}C, the purified enzyme showed excellent broad pH stability in pH ranging from 3.0-10.0 with residual activity of 100%. The pH stability was moreover performed at pH 3.5-8.0 at 37^{o}C to evaluate the capability of the enzyme on long term stability. The result revealed that the enzyme was stable at pH 4.0-8.0 up to 72 h but at 3.5 for 24 h as shown in Figure 2.10.

Temperature optimum and stability

The optimum temperature for purified amylase activity was found to be in range of 37-55^{o}C with the maximum activity at 45^{o}C as shown in Figure 2.9c. The 100% residual activity was retained for 1 h at temperature ranging from 25-45^{o}C and stable up

to 50°C (Figure 2.9d). When the purified enzyme was incubated at temperature higher than 55°C, less than 50% of residual activity was obtained.

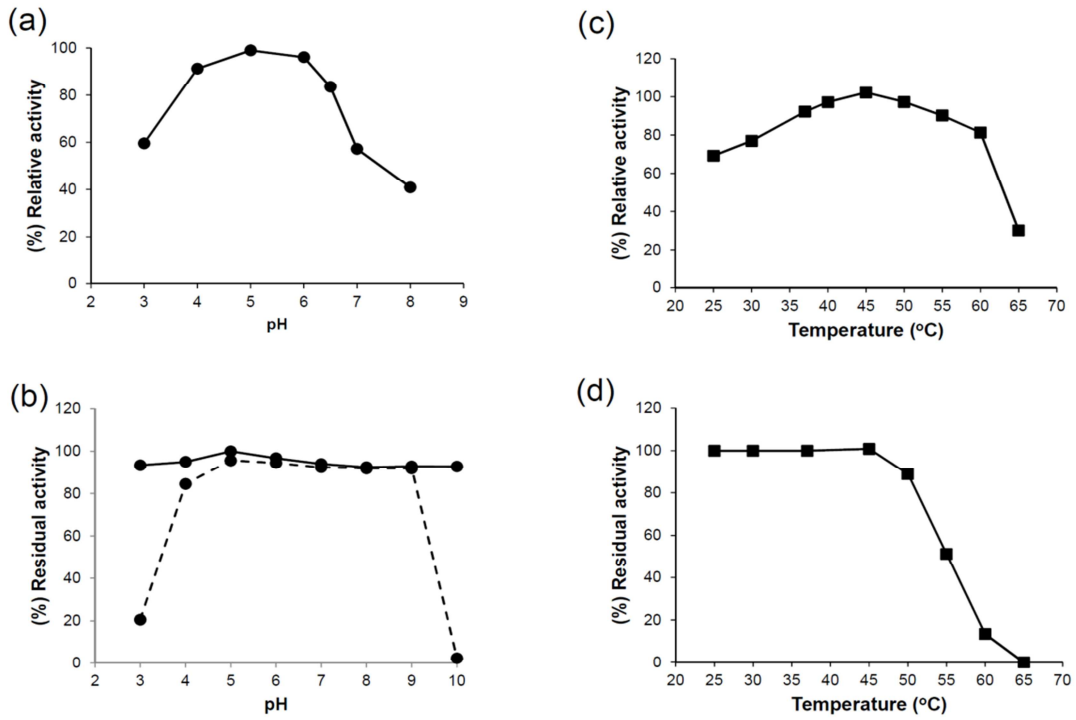

Figure 2.9 pH optimum (a) and stability for 24 h at 4°C (solid line) and 37°C (dotted line) (b) of amylase and temperature optimum (c) and stability for 1 h (d) of amylase from *L. plantarum* S21

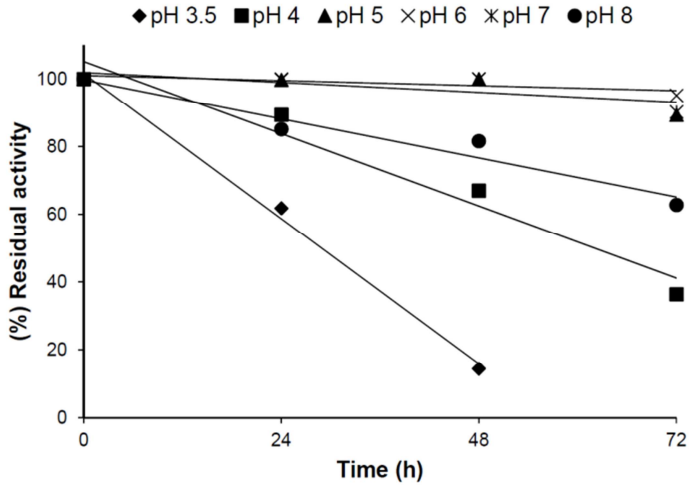

Figure 2.10 pH stability of amylase from *L. plantarum* S21 at 37°C

Synergistic effect of pH and temperature

The synergistic effect between pH and temperature on amylase activity was statistically determined by response surface methodology. Statistical model was established according to quadratic equation in addition to optimum pH and temperature as optimum condition for purified α-amylase was evaluated. The result revealed that there was collaboration effect between pH and temperature significantly at *p*-value <0.05 which identified to be positive effect by quadratic model via response surface plot as shown in Figure 2.11. The quadratic model predicted the optimum condition and the experiment data verified the maximum relative activity of 150% when the condition was carried out at pH 4.8 and 50^{o}C compared to the control condition at pH 6.5, and 37^{o}C.

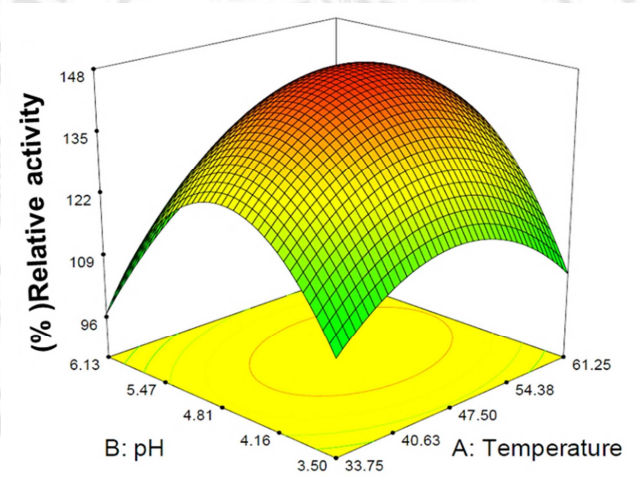

Figure 2.11 Quardratic response surface represented the optimum pH and temperature of amylase from *L. plantarum* S21

Effect of cations on enzyme

In the presence of 5 mm metal ions in the reaction mixture, it was found that Ag^{2+} inhibited enzyme by reducing 80% relative activity in contrast to Mn^{2+}, Co^{2+} and Fe^{3+} that obviously enhanced the enzyme activity with 139.48±0.47, 135.51±4.54 and 124±0.35% relative activity. Concentration ranging from 10 to 30 mM of these cations however did not certainly increase the relative enzyme activity (data not shown). Other cations on the other hand, had no effect on enzyme activity but EDTA and SDS slightly inhibited the enzyme activity (Table 2.5).

Table 2.5 Effect of cations and chemical reagents on enzyme activity

Metal ion	(%) Relative activity	Metal ion	(%) Relative activity
KCl	101	$FeCl_2$	102
NaCl	103	$SrCl_2$	101
$AgNO_3$	24	$MgSO_4$	96
$CaCl_2$	107	$FeCl_3$	124
$MnCl_2$	140	$CoCl_2$	139
$ZnCl_2$	103	15 mM EDTA	89
$CuSO_4$	91	0.25% (w/v) SDS	91
$NiCl_2$	98		

Substrate specificity

The amylase from *L. plantarum* S21 best specified to amylose, soluble starch, amylopectin, dextrin and glycogen, respectively with 105, 100, 89, 100, 50% relative activity, respectively (Table 2.6). The enzyme was unable to hydrolyze raw starch, pullulan, α- and β-cyclodextrin.

Table 2.6 Substrate specificity

Substrate	(%) Relative activity	Substrate	(%) Relative activity
Soluble starch	100	Dextrin	73
Amylose	105	α-cyclodextrin	0
Amylopectin	89	β-cyclodextrin	0
Glycogen	50	Pullulan	0
Maltodextrin	100	Dextran	0

Kinetics of enzyme

The K_m values towards starch, amylose, amylopectin and glycogen under 37°C, pH 6.5 was 8.42±0.74, 9.78±0.96, 9.10±0.99 and 15.18±1.15 mg/mL, respectively. The highest V_{max}, k_{cat} and k_{cat}/K_m vaule was obtained from amylose as shown in Table 2.7.

Table 2.7 Kinetics constants of amylase from *L. plantarum* S21

Substrate	K_m (mg/mL)	V_{max} (U/mL)	k_{cat} (s^{-1})	k_{cat}/K_m $(mg{\cdot}s/mL)^{-1}$
Starch	8.42±0.74	1167.26±42.67	$4.13x10^3$	$3.38x10^3$
Amylose	9.78±0.96	1995.12±115.36	$7.05x10^3$	$6.04x10^3$
Amylopectin	9.10±0.99	879.69±41.18	$3.11x10^3$	$2.78x10^3$
Glycogen	15.18±1.15	625.06±25.36	$2.21x10^3$	$2.26x10^3$

Amino acid sequence

The purified extracellular amylase of *L. plantarum* S21 was homogenously purified and sequenced to determine the amino acid sequence which was useful to intensively classify the enzyme. The LC-ESI-MS/MS confirmed 874 amino acids as the mature extracellular amylase containing N- and C-terminus as shown in Figure 2.12. The whole amino acid sequence shared 97, 96 and 91% similarity to α-amylases from *L. plantarum* A6, *L. manihotivorans* LMG18010, and *L. amylovorus* NRRL B4540, respectively in contrast to *Bacillus* sp. α-amylase that shared only 50% similarity. It was moreover noticed that catalytic domains was found within the N-terminus and it had high similarity with typical α-amylases reported in genbank such as α-amylases from *Lactobacillus* sp. (97-98%), *Bacillus* sp. (66-68%), *Streptococcus* sp. (52-63%). Analysis by InterProScan software clearly confirmed the result above. It was revealed that the *L. plantarum* S21 amylase belonged to glycosyl hydrolase family 13 as it structure contained 8 stranded α/β-barrel and 4 catalytic domains were found to be conserved within N-terminus. Other C-terminus of *L. plantarum* S21 α-amylase had high similarity with only α-amylases from *L. manihotivorans* LMG18010 (96%), *L. plantarum* A6 (94%) and *L. amylovorus* NRRL B4540 (93%). Moreover, repeat units of *L. plantarum* S21 α-amylase were certainly found as similar as that reported by the other *Lacbacillus* sp. α-amylases mentioned above. The N- and C-terminus was linked together with flanking region of TSSSSSSTTTET called linker. Of 4 tandem repeat units were connected with 3 intermediary regions (IRs) where rich in serine (S) and threonine (T). IR1 (KTSDSSSSSSTTTET) was similar to IR3 but IR2 had a serine

missing (KTSDSSSSSSSTTTET). The repeat unit was terminated by the conserved flanking region of KTSDSSS.

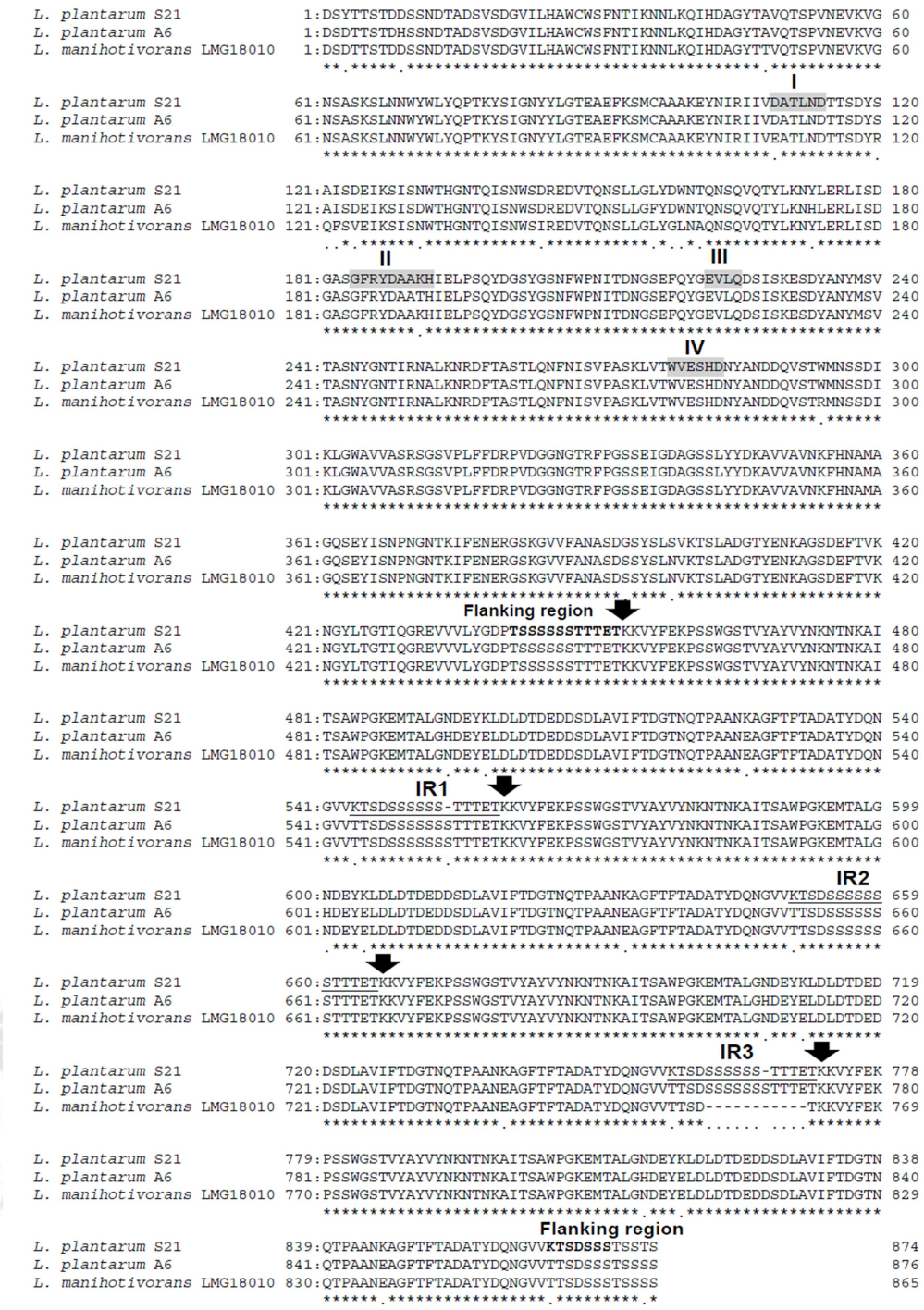

Figure 2.12 Alignment of amino acid sequence of α-amylase from *L. plantarum* S21. Catalytic conserved domains were shaded; repeat amino acid started at arrow; intermediary regions (IR) were underline; flanking regions were bold

Hydrolysis products

The pattern of hydrolysis products according to the substrate degradation was investigated by thin layer chromatography (TLC) and determined the definite amount by high performance liquid chromatography (HPLC). Within 24 h of substrate hydrolysis under 37^{o}C, pH 6.5, purified amylase degraded starch, amylose, amylopectin, glycogen and maltooligosacharides among G3-G6 to liberate G2 as main hydrolysis product, G1 as the minor and some G3 (Figure 2.13a, 2.13b). In long term of hydrolysis, the G3 was hydrolyzed to G2 and G1 eventually as shown in Figure 2.13.

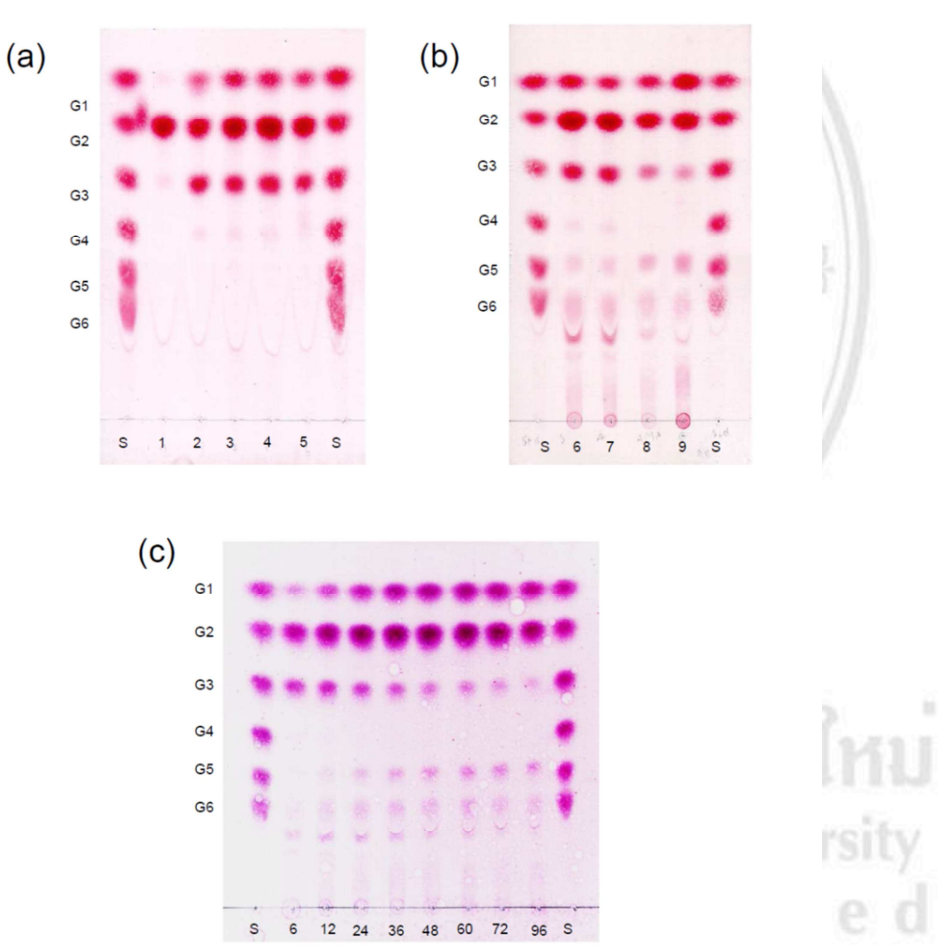

Figure 2.13 TLC plates of hydrolysis products from G2-G6 (Lane 1-5) (a) and products from starch (Lane 6) amylose (Lane 7) amylopectin (Lane 8) and glycogen (Lane 9) (b) and time course of starch hydrolysis at 6-96 h of incubation at 37^{o}C (c)

The HPLC result indicated the amount of maltooligosaccharides liberated from starch, amylose and amylopectin regarding the degradation for which clearly explained mechanisms of substrates hydrolysis (Table 2.8). The HPLC chromatograms revealed that the amylase rapidly catalyzed the hydrolysis of starch, amylose and amylopectin and released G1 and certain amount of G2. The G3 was also released from the hydrolysis and was moreover found to be an intermediate of G2 and G1. Approximately 55, 34 and 8% of G2, G1 and G3 was released from starch, amylose and amylopectin within 3 h of degradation. The %G2 was gradually increased from 3 h until the end of hydrolysis (72 h) with around 58-60% yield in contrast to G1 that was increased exponentially corresponding to the decreasing of G3. Ultimately, 60% of G2 was produced from starch, amylose and amylopectin while G1 was produced up to 37, 29 and 38% from these substrates, respectively.

Table 2.8 Percentage of maltooligosaccharides liberated from starch, amylose and amylopectin by amylase from *L. plantarum* S21

Time (h)	% Oligosaccharides											
	Starch				Amylose				Amylopectin			
	G1	G2	G3	G4	G1	G2	G3	G4	G1	G2	G3	G4
0	0	0	0	0	0	0	0	0	0	0	0	0
3	9.55	55.77	33.48	1.2	7.89	52.87	35.9	3.01	9.42	55.99	33.77	1.22
6	12.99	59.64	26.75	0.62	9.86	55.7	33.71	0.73	15.19	56.03	28.12	0.66
12	17.95	61.57	20.02	0.47	12.77	57.12	29.51	0.6	17.83	59.22	22.5	0.45
24	25.91	62.12	11.65	0.3	17.22	59.26	23.03	0.49	26.02	59.7	14.28	0
48	33.98	61.08	4.83	0.11	24.12	60.8	14.85	0.23	34.71	58.67	6.62	0
72	37.25	60.72	1.92	0.09	28.99	60.55	10.46	0	38.72	57.97	3.31	0

2.4.5 Starch degrading system in *L. platarum* S21

Other amylases of *L. plantarum* S21 were investigated in order to understand the mechanism of starch degrading system in the bacterium. Crude extracellular enzyme (CEE), crude intracellular enzyme (CIE) and purified enzyme (PE) were provided for starch hydrolysis. The patterns of hydrolysis products from various enzyme sources were visualized and found that CEE had the same hydrolysis product pattern as PE as shown in Figure 2.14. In the agreement with the enzyme activity found in CIE and CEE but the later gave the main activity (Table 2.9). CIE hydrolyzed starch and liberated glucose rather than maltose which was in accordance with the occurrence of α-glucosidase activity in intracellular fraction. Moreover, the mentioned enzyme was capable of hydrolysis of maltose to glucose as shown in Figure 2.13.

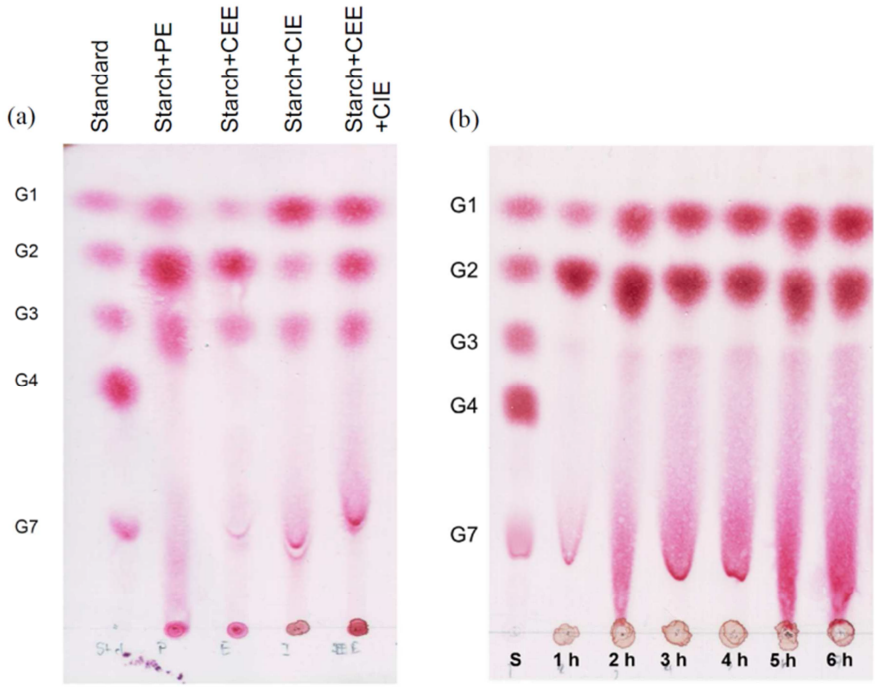

Figure 2.14 Hydrolysis products from starch of purified enzyme (PE), crude extracellular enzyme (CEE), crude intracellular enzyme (CIE) and combination of (CEE+CIE) compared to standard (a) and hydrolysis products of maltose of crude α-glucosidase from *L. plantarum* S21 (b)

Table 2.9 Amylases found in culture supernatant and cell extract of *L. plantarum* S21

Enzyme activity	Crude extracellular enzyme	Crude intracellular enzyme
α-amylase (Units/L)	2394	80
α-glucosidase (Units/L)	0.65	0

2.5 Discussion

The problem of differentiation of *Lactobacillus* sp. S21 was occurred during 16S rDNA sequence analysis since high similarity was found between *L. plantarum, L. pentosus* and *L. paraplantarum*. Therefore, it was unable to classify *L. plantarum* group species by using phylogenetic distances. High specific and reliable methods have been developed for further simple differentiation of three species mentioned. RecA is a small protein implicated in homologous DNA recombination, SOS induction, and DNA damage-induced mutagenesis (Eisen 1995). Regarding great important roles, *recA* gene is presented everywhere and its gene products have been proposed as a phylogenetic marker and resulted in accomplishment of identification for many bacterial genera such as biofidobacteria. Moreover, short *recA* gene sequence has been successfully used to differentiate *L. plantarum* out of *L. pentosus* and *L. paraplantarum* by Torriani (2001). Like this research, short *recA* gene was isolated from genomic DNA of *Lactobacillus* sp. S21 in order to confirm its 16S rDNA sequence in which closed to other related species. The estimated *recA* gene size of 300 bp amplified from plan F primer revealed its nearest neighbor to *L. plantarum*.

L. plantarum S21 is a high efficient amylolytic lactic acid bacterium capable of direct conversion of starch to lactic acid. The ability of production of amylolytic enzyme probably conducted it to rapid lactic acid production. A few members of this species were reported to have high potentiality in production of amylolytic enzymes and used for direct lactic acid production from starch. There were only *L. plantarum* A6 (Giraud *et al*. 1994 and Pintado *et al*. 1999) and *L. plantarum* MTCC 1407 (Panda et al. 2008) and *L. plantarum* L137 (Kim et al. 2008 and Kim *et al*. 2009) that were reported to produce amylolytic enzyme but only *L. plantarum* A6 and *L. plantarum* MTCC1407

that were used them for direct conversion of starch to lactic acid. For well understanding about enzymes in starch degrading system of *L. plantarum* S21, purification and characterization of particular extracellular amylolytic enzymes were required.

To produce amylase for enzyme purification, time course of amylase production was performed to evaluate suitable incubation time for harvest of amylase at the maximum yield. The amylase was harvested at the maximum amylase activity and specificity. In addition, soluble protein was also evaluated to avoid proteolytic activity from cell lysis. From the result, higher incubation time conducted to the higher specific activity according to lower protein concentration. It was explained that *L. plantarum* S21 approached to death phase where cell was lysed and probably released proteolytic enzymes (Maurizi 1992 and Florencio *et al*. 2000) or instability of the enzyme under acidic condition from lactic acid during cultivation (Martinez *et al.* 2013). The enzyme purification was satisfactorily accomplished within two techniques including precipitation and anion exchange chromatography. It was observed that the amylase purification was always successful with precipitation by ammonium sulfate and anion chromatography (Sharma *et al*. 2013). The SDS-PAGE was used to evaluate the purity of the enzyme and also used to estimate molecular weight of the protein. A single faint band at position was located at approximately 100 kDa and this result was also in the agreement with the active band on zymogram. A single faint protein band was found to be the same protein as it was from the purified enzyme after confirmation by amino acid sequencing using mass spectrometry. The mentioned result was probably took place from instability of protein structure of purified enzyme according to SDS and heating under reduced condition in which similar to *L. plantarum* L137 amylopullulanase (Kim *et al*. 2008). The native PAGE gel demonstrated only a single protein band with estimated molecular mass of 107 kDa. Therefore, *L. plantarum* S21 amylase was designated to monomeric enzyme. Most lactobacilli α-amylases exhibited in past several decades for direct conversion of starch to lactic acid were often monomeric enzymes with high molecular weight of 135, 140 and 150 kDa (Aguilar *et al*. 2000; Burgess-Cassler and Imam 1991 and Giraud *et al.* 1993) as well as α-amylase from *Lactococcus lactis* IBB500 (121 kDa) (Adam *et al*. 2010) and amylopullulanase from *L. plantarum*

L137 (216 kDa) (Kim *et al*. 2008) while smaller molecular weight of 57 and 77 kDa was found from *Streptococcus lactis* α-amylases (Freer 1993 and Satoh *et al*. 1997).

One of the greatest merits of amylase from *L. plantarum* S21 was broad pH stability. The amylase from *L. plantarum* S21 had optimum pH similar to other amylases from *Lactobacillus* sp. and other lactic acid bacteria. The purified *L. plantarum* A6 α-amylase had optimum pH ranging from 4.5-6.0 with the maximum activity at pH 5.5 (Giraud *et al*. 1993) while that of *L. mantihotivorans* had optimum pH at 4.0-5.5 (Aguilar *et al*. 2000). Similar to *L. paracasei* amylase, the enzyme currently studied for direct lactic acid production which had optimum pH of 5.0 (Petrova *et al*. 2012). The *L. plantarum* L137 amylopullulanase had optimum pH at 4.0-4.5 (Kim *et al*. 2009). Other lactic acid bacteria, *Lactococcus lactis* IBB500 α-amylase had optimum pH at 4.5 (Adam *et al*. 2010) and *Streptococcus bovis* JB1 α-amylase had optimum pH at 4.0-5.0 (Freer 1993). Stability of purified α-amylase from *L. plantarum* S21 against pH was better than other α-amylases described in pH optimum. It was considerably stable broad pH range from pH 3.5-8.0 at 37^{o}C for 24 h. The pH stability at 4^{o}C was moreover surprising since the purified enzyme was stable in broader pH range than at 37^{o}C. It was suggested that this enzyme probably proper for simultaneous liquefaction, saccharification and fermentation in order to avoid pH adjustment step according to the increasing of lactic acid and neutralization step in lactic acid production. The excellent pH stability of the amylase had not been reported so far in lactobacilli amylases.

The purified amylase had optimum temperature similar to other α-amylases and amylopullulanases from lactic acid bacteria. The optimum temperature was found in range of 35 up to 65^{o}C. Most of them were however not thermostable enzymes that were typically found in *Bacillus* sp.. The α-amylase from *L. manihotivorans* was stable at 40^{o}C for 1 h however it was lost 50% of original activity at 60^{o}C within 10 min (Aguilar *et al*. 2000) while *L. amylovorus* α-amylase lost 50% of activity when it was incubated for 1 h at 55^{o}C as well as *S. bovis* α-amylase (Freer 1993) and within 20 min at higher temperature (Rodriguez-Sanoja *et al*. 2000). *Lactococcus lactis* α-amylase was stable at 35^{o}C for only 10 min (Adam *et al.* 2010). In addition, it was unnecessary to use thermostable enzyme in simultaneous liquefaction, saccharification and fermentation (SLSF) according to the fact that the enzymes had to be mixed with the seed inoculum

and fermentation was carried out at low temperature corresponding the optimum temperatures for growth of the lactic acid bacteria (Linko *et al*. 1996; Wang *et al*. 2010 and Nguyen *et al*. 2013).

Few publications studied on the interaction of pH and temperature to enzyme activity. The response surface plot analyzed by quadratic model demonstrated the condition related to the maximum activity of amylase. In some cases of enzymes, interaction between pH and temperature had significant impact to enzyme activity. This study revealed that there was a significant positive effect between these two variables (p>0.20). As a reason, the enzyme activity was increased up to 1.5 times.

Amylase activity was assayed in the presence of 5 mM of mono-, di- and trivalent ions. Monovalent ions as Ag^{+} certainly had high impact to purified amylase unlike Co^{2+} and Mn^{2+} which significantly promote the amylase activity. Co^{2+} bound on surface of *B. amyloliquefaciens* α-amylase contributed to the increasing of enzyme activity and was concluded as activator by Saboury (2002). *L. plantarum* L137 amylopullulanase was also activated by Co^{2+} with 50% higher of relative activity (Kim et al. 2008). Co^{2+} and Mn^{2+} of 1 mM moreover activated α-amylase from soya bean seeds up to 350 and 250% relative activity (Prakash and Jaiswal 2010) as well as Al-Quadan (2011) who found that α-amylase from *Bacillus* sp. strain HUTBS62 was activated by Co^{2+} and Mn^{2+} to 135 and 129% relative activity in respect. Lily *et al*. (2012) discussed that Fe^{3+} probably stabilized conformation of α-amylase through interaction effect of positive charges and negative charges of amino acids of the enzyme structure resulting in enhancement of enzyme activity. The amylase was moreover able to work independently without Ca^{2+}. It had long been known that some α-amylase required Ca^{2+} as an essential cofactor for enzyme stabilization and activation (Tanaka and Hoshino 2002) particularly a number of bacilli α-amylases in contrast to amylases from lactobacilli (Giraud *et al*. 1993 and Aguilar *et al*. 2000;) and some other lactic acid bacteria (Freer 1993 and Adam *et al*. 2010).

Regularly found in nature, amylase from *L. plantarum* S21 best specified to amylose, starch, amylopectin and glycogen since the best reacted with α-1, 4 glucosidic linkage of glucose. The result implied that there was no cross activity of pullulanase found from the purified enzyme. Therefore, starch degrading enzyme from *L. plantarum*

should not have pullulanase activity. The K_m, V_{max}, k_{cat} and k_{cat}/K_m value also had trended similar to result of substrate specificity. The purified enzyme had high affinity to starch insignificantly and the result was similar to amylose and amylopectin but definitely different to glycogen according to the k_{cat}/K_m values. The highest reducing sugar of $7.05x10^3$ µmole/s was liberated from amylose and it was also the best substrate for *L. plantarum* S21 amylase. Rodriguez-Sanoja *et al*. (2005) reported *L. amylovorus* NRRL B4540 α-amylase had K_m values of 1.97, 8.00 and 2.87 mg/mL towards soluble potato starch, amylose and amylopectin and 1.92, 5.00, 5.10 mg/mL for *L. plantarum* A6 α-amylase. *L. manihotivorans* α-amylase had K_m value of 3.44 mg/mL towards soluble starch (Aguilar *et al*. 2000). The distinction among starch substrates used for determination of kinetics and the assayed condition were difficulties to compare kinetic values (Aguilar *et al*. 2000). In this experiment, *L. plantarum* S21 amylase gave higher K_m values towards starch, amylose, amylopectin and glycogen than others but it exhibited satisfying k_{cat} values towards the three substrates that were higher than that from *L. amylovorus* and *L. plantarum* reported by Rodriguez-Sanoja *et al*. (2005).

The *Lactobacillus plantarum* S21 was classified to α-amylase according to the fact that 8 stranded α/β-barrel occurred in the N-terminus as well as conserved regions typically found at catalytic domains of α-amylases. The enzyme had theoretical molecular mass of 95 kDa and estimated pI of 4.41 which was in the agreement of result from SDS and native-PAGE. The amino acid of *L. plantarum* S21 α-amylase significantly shared high similarity to α-amylase from *L. plantarum* A6, *L. manihotivorans* LMG18010 and *L. amylovorus* NRRL B4540 which had been reported to have 4 domains according to Svensson *et al*. (2002). Domain A, domain B, domain C were included within the N-terminus while the last domain, domain E or starch binding domain covered whole C-terminus and influenced on raw starch degradation and it was connected by the linker. Although *L. plantarum* S21 α-amylase had high similarity to these α-amylases and also had tandem repeat units, capability of raw starch degradation was unavailable. It was explained that the catalytic domains of the enzyme located in N-terminus was able to catalyze the starch hydrolysis independently as reported in *L. amylovorus* NRRL B4045 (Santiago *et al*. 2005) and also shared 98% similarity to the *L. amylovorus* α-amylase. Unfortunately, starch binding domain (SBD) of the enzyme was not functioned according to some distinctions from either linker or IRs or RUs. The

linker of *L. plantarum* S21 α-amylase was the same as that from *L. plantarum* A6 and *L. manihotivorans* LMG18010. However, the IRs and RUs were slightly different. Threonine of IR1-IR3 (Thr544, Thr640, Thr757) was replaced by lysine as well as that located at the termination of repeat unit (Thr863) and moreover, a serine of IR1 and IR3 was missed. The RU of *L. plantarum* S21 α-amylase had few distinctions from *L. plantarum* A6 and *L. manihotivorans* LMG18010 α-amylase particularly Glu498 and Glu526, Glu604 and Glu632, Glu711 and 739, Glu817 and Glu845 of each RU of the mentioned α-amylase were replaced by lysine. This reason may cause of inability in raw starch degradation. However, either linker or IR or RU did not only served capability of degrading raw starch but also stabilized the enzyme structure against stress, adsorption onto raw starch and secretion (Rodriguez-Sanoja *et al*. 2005; Santiago *et al*. 2005). Moreover, direct RU regions affected to enzyme stability against pH and temperature since it maintained the correct conformation of the enzyme (Santiago *et al*. 2005). Rodriguez-Sanoja *et al*. (2000) found that RUs truncated α-amylase from *L. amylovorus* had lower temperature stability than the whole enzyme. Considering to the pH stability of *L. plantarum* S21 α-amylase, it could be suggested that α-amylase was able to work independently within N-terminus but the non-function starch binding domain at C-terminus supported the enzyme on pH stability.

Pattern of hydrolysis products from various carbohydrate substrates of the α-amylase was obviously maltose and glucose. It was revealed that the enzyme was an endo-acting enzyme rapidly randomly catalyzed the hydrolysis and liberated mono-, di- and trisaccharides as final end products. The result promoted and confirmed the analysis of amino acid sequence previously discussed and could be concluded that the enzyme was classified to maltose forming α-amylase. Over the past few decades, a number of papers reported starch degrading enzymes from lactic acid bacteria and most of them were classified to α-amylase, amylopullulanase and pullulanase. The α-amylase from *L. amylovorus* was found several decades ago following by the enzyme from *L. plantarum* and *L. manihotivorans*, respectively. Recently, Petrova *et al*. (2012) found starch degrading enzyme from *L. paracasei* B41. Even there were many papers reported about the discovery of lactobacilli starch degrading enzyme, a few were purified, characterized and identified the hydrolysis products regarding the enzymes. Talamond *et al*. (2002 and 2006) studied on action mechanism of *L. fermentum* α-amylase and

found that G2, G3 and G4 was released from the hydrolysis of raw starch and G2 and G3 was released from maltoligosaccharides. Oh *et al*. (2005) offered the mode of action of *L. gasseri* α-amylase that had high specificity with β-cyclodextrin and pullulan and released G2 and G2 from this substrate. The main hydrolysis products obtained from raw starch regarding α-amylase from *L. plantarum* were mixture of G3 and G4; that of *L. manihotivorans* were mixture of G2-G4 and that of *L. amylovorus* was mixture of G2-G7 (Talamond *et al*. 2002). Outstanding properties of the enzyme particularly broad pH stability and its hydrolysis product pattern obviously demonstrated that the enzyme could be used as single enzyme in single step bioconversion of starch to lactic acid and was definitely useful and applicable for lactic acid production from starch particularly SSF and SLSF.

Starch degrading mechanism of *L. plantarum* S21 had been elucidated after investigation of hydrolysis products from intra- and extracellular enzyme and purified enzyme towards starch and maltose. The mechanism exactly involved maltose forming α-amylase since the enzyme was found to be the main activity in cultivated medium in order to hydrolyze starch and liberated maltose and glucose. Amylase activity was found in both intra- and extracellular fraction. Maltose was then degraded feasibly by α-glucosidase activity found only in the cell of *L. plantarum* S21. Therefore, starch degrading system of the organism could be proposed and explained by extracellular maltose forming α-amylase and intracellular α-glucosidase in term of hydrolysis products detected by TLC as shown in Figure 2.15. Starch was degraded by the extracellular enzyme and liberated main production, maltose that could be facilitated to the cells by permease activity and degraded to liberate glucose by α-glucosidase. During the hydrolysis of starch to maltose, glucose was found to be a minor product and could be consumed directly by the bacterium. Regarding the mentioned mechanism, large amount of glucose could be generated and consumed by *L. plantarum* S21 for lactic acid production through Embden-Meyerhof-Parnas pathway, a main pathway for lactic acid production by homofermentative lactic acid bacteria. This proposed mechanism was corresponded to the mechanism of starch degradation of *L. fermentum* Ogi E1 described by Calderon-Santoyo *et al*. (2003). However there was another pathway of maltose assimilation by lactic acid bacteria that proposed by Andersson and Radstrom (2002) where maltose phosphorylase and β-phosphoglucomutase played important roles

in maltose assimilation and the final product was glucose-6-phosphate, an intermediate in glycolysis pathway.

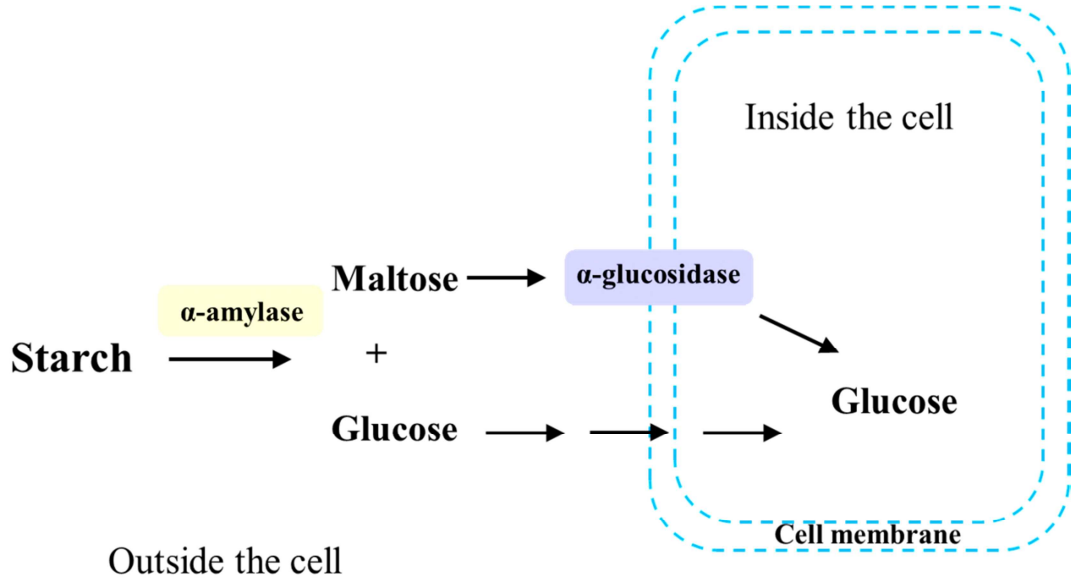

Figure 2.15 Proposed starch degrading mechanism of *L. plantarum* S21 for lactic acid fermentation

2.6 Conclusion

In this chapter, starch degrading system in *L. plantarum* S21, could be concluded that;

1. *Lactobacillus* sp. S21 is clearly designated to *L. plantarum* according to the size of *recA* amplicon of 318 bp in accordance with other reference strains of *L. plantarum*.

2. The extracellular amylase was purified until homogeneity with 936 U/mg. The purified enzyme is monomeric enzyme which has molecular weight of approximately 100 kDa estimated from SDS-PAGE, 107 kDa estimated from native PAGE and 95.3 kDa estimated from amino acid sequence. It has theoretical pI of 4.44.

3. Optimum pH and temperature of the enzyme is pH 4.8 at 50^{o}C as determined by quadratic model of response surface methodology.

4. The purified enzyme has excellent broad pH stability ranging from 3.0-8.0.

5. The substrate specificity of purified enzyme is in the agreement with k_{cat}/K_m values. The purified enzyme has high affinity to amylose, starch, amylopectin and glycogen, respectively

6. The amino acid sequence of purified enzyme consists of 874 amino acids. The N-terminus has high similarity with most α-amylase particularly that of *Lactobacillus* spp. It is suggested that four repeat units located at C-terminus promoted pH stability of the purified enzyme.

7. The extracellular amylase produced by this strain is classified to maltose forming α-amylase based on amino acid sequence analysis and pattern of hydrolysis products in which maltose was found to be the main product with 60% and glucose of 30% maltooligosaccharides during hydrolysis of starch, amylose and amylopectin.

8. Elucidation of starch degrading system in *L. plantarum* S21 is clearly revealed that maltose forming α-amylase is secreted to the medium to degrade starch to maltose and glucose. Maltose is consequently transferred into the cells and is hydrolyzed to glucose according to the α-glucosidase activity in the cell. The final product from starch is glucose, an important substrate for lactic acid production. However, there may be other starch degrading mechanisms in *L. plantarum* S21 but the main mechanism is probably the route mentioned.