CHAPTER 5

General discussion and conclusion

Lactobacillus sp. S21 has been considerably identified by species specific primers in order to differentiate the bacterium from *L. pentosus* and *L. paraplantarum*. The positive result concludes species identification of *Lactobacillus* sp. S21 belongs to *Lactobacillus plantarum* S21 which is amylolytic and homofermenative lactic acid bacterium. The extracellular enzyme is mainly produced by the bacterium and is consequently purified and characterized for properties determination and elucidation of starch degrading system involving direct conversion of starch to lactic acid. Accomplishment of purification and characterization reveals excellent properties of the enzyme and amino acid sequence that are very useful for enzyme classification.

The properties of the purified enzyme are certainly different to other lactobacilli α-amylase particularly the pH stability. The outstanding pH stability at 37^{o}C under pH ranging from 3.5-8.0 is the merit of this enzyme. Moreover, it is stable at the mentioned temperature at pH ranging from 4.0-8.0 for 72 h. The patterns of hydrolysis products towards maltooligosaccharides (G2-G7), starch, amylose, amylopectin and glycogen are certainly the same. Maltose is found to be the main product with approximately 60% of total oligosaccharide released from starch, amylose and amylopectin, the other 30-38% is found to be glucose. The amino acid sequence of purified amylase has high similarity to lactobacilli α-amylase particularly α-amylases from *L. plantarum* A6, *L. manihotivorans* LMG18010 and *L. amylovorus* NRRL B4540. Therefore, the extracellular amylase from *L. plantarum* S21 is classified to be a member of α-amylase. Pattern of hydrolysis product moreover indicates that the enzyme is maltose forming α-amylase. Mentioned above, amino acid sequence of *L. palntarum* S21 deduced from amylase gene (*AmyW*) consists of 910 amino acids which are in the agreement with amino acids obtained from mass spectrometry. The 874 amino acids is found to be mature α-amylase coupling with first 36 amino acids are predicted to be signal peptide sequence by signal peptide prediction software. The N-terminus is jointed with

C-terminus at first serine (S)-threonine (T) rich region encoded from *Bam*HI restriction site. The C-terminus is assumed to be a key of pH stability of the extracellular α-amylase. It consists of 4 repeat units of 91 amino acids jointing with 3 intermediary regions (IR) which have rich of S and T. The pattern of the mentioned amino acid sequence has been reported to have significant impact to raw starch degradation of α-amylases from *L. plantarum* A6, *L. manihotivorans* LMG18010 and *L. amylovorus* NRRL B4540 (Giraud and Cunny 1997, Rodriguez Sanoja *et al*. 2000, Rodriguez Sanoja *et al.* 2005) in contrast to the case of *L. plantarum* S21α-amylase. The *L. amylovorus* amylase lacking of C-terminus are proved that it has the same pH and temperature stability as the complete form of α-amylase but it is certainly unable to hydrolyze raw starch (Giraud and Cunny 1997). It is believed that the flanking regions and intermediary regions (IRs) are involved in maintenance of protein structure against stress, adsorption onto raw starch granule and secretion (Rodriguez-Sanoja *et al.* 2005). Kim *et al.* (2009) moreover, expected that the repeat units located at C-terminus of *L. plantarum* L137 amylopullulanase relates to the adhesion to saccharides and the stability of the enzyme structure. Therefore, it is inferred that the C-terminus of *L. plantarum* S21 α-amylase probably exhibits important roles not only on pH stability of the enzyme but also on substrate hydrolysis of which maltose is main hydrolysis product. The distinction has *L. plantarum* S21 α-amylase more exotic than α-amylase from *L. plantarum* A6, *L. amylovorus* NRRL B4540 and *L. manihotivorans* LMG18010 and other lactobacilli α-amylases in term of hydrolysis products and pH stability. Certainly, maltose forming α-amylase from *L. plantarum* S21 could be utilized in many applications such as bakery, foods and particular simultaneous liquefaction, saccharification and lactic acid fermentation (SLSF) in order to discard addition of glucoamylase.

Starch degrading system in *L. plantarum* S21 has been proposed and explained that starch is regularly hydrolyzed by extracellular maltose forming α-amylase to liberate maltose and glucose that are consequently assimilated by the organism. Maltose is hydrolyzed to glucose for lactic acid fermentation by activity of α-glucosidase. Typically, homofermenative lactic acid bacteria fermented fermentable sugar through Embden-Meyerhof-Parnas pathway in which glucose is the main substrate to produce

equivalent mass of lactic acid. The given mechanism best describes rapid production of lactic acid in *L. plantarum* S21.

According to valuable and useful α-amylase, overproduction of α-amylase is required for further applications. The α-amylase gene (*AmyW*) is successfully expressed in *E. coli* especially the mature α-amylase (*AmyM*) that is overexpressed in *E. coli*. In case of expression of the two genes in *L. plantarum* WCFS1 and *L. plantaum* TGO02, *AmyW* is expressed the same level as *L. plantarum* S21 while *AmyM* did at very low level. It is inferred that signal peptide of *L. plantarum* S21 is required in order to express in *L. plantarum* WCFS1. However, the enzymes expressed from *AmyW* obtained from recombinant *E. coli* and *L. plantarum* have identical properties to wild type enzyme. Lactic acid produced from starch by *L. plantarum* S21are considerably evaluated to racemic D- or L-lactic acid in ratio of 1:1. The successful α-amylase gene expression in *Lactobacillus* sp. system exhibits feasibility in manipulation of the gene in other non-amylolytic *Lactobacillus* sp. for direct conversion of starch to high optical purity D-or L-lactic acid. Intensive study of α-amylase can be performed practically since it is easy to overproduce and purify the enzyme for using in further characterization and utilization.

L. plantarum S21 produces lactic acid efficiently in 50% diluted mMRS medium in both 1 and 10 liter fermentation scale according to the growth kinetics are similar to that from the medium without dilution. Therefore, the production cost regarding production medium is truly reduced for 50%. The medium is practically utilized as production medium for direct conversion of high concentration of starch to lactic acid since high production efficiencies of lactic acid are obtained when starch concentrations are initiated in range of 10-100 g/L. Operating batch system, *L. plantarum* S21 is a high efficient amylolytic lactic acid bacterium that produces high concentration of lactic acid up to 94 g/L from 100 g/L of starch. Starchy wastewater is feasible to use as a carbon source for lactic acid fermentation in order to produce value adding products like lactic acid. At high level of starch of starchy wastewater, *L. plantarum* S21 has potentiality in starch conversion to lactic acid considerably. As mentioned, direct conversion of starch to lactic acid by *L. plantarum* S21 is feasible and efficient in term of applicability in large scale fermentation.