CHAPTER 3

Homologous and heterologous expression of α-amylase gene

3.1 Introduction

The α-amylase (EC 3.1.1.4) has long been known and widely used in many industry including foods, detergent, textile and pharmaceutical industry. The first production of amylase for commercial purpose known as Taka diastase was established by Takamine (Sivaramakrisnan *et al*. 2006). The enzyme is produced universally by either plants or animal or microorganisms. However, the most attempts have been obviously focused on microbial α-amylases particularly bacterial and fungal amylases according to their wide varieties, economic advantages and easy manipulations (Gupta *et al*. 2003). The main purpose of utilization of α-amylase are considered in starch liquefaction and saccharification procedures for preparation of various kind of refine sugars and dextrin to serve other applications such as production of glucose, fructose and maltose syrup (van der Maarel *et al*. 2002). On the other hand, it is used to prepare starch substrate for production of lactic acid, one of the most important organic acids, plays roles in food and biodegradable plastic industry. Many reports propose different procedures for production of lactic acid from starch by biological method. Lactic acid bacteria are concerned to be the most important group of lactic acid producers used for lactic acid production. Considering, lactic acid production from starch by lactic acid bacteria, it is however necessary to pretreat starch by either acid or alkaline hydrolysis or starch liquefaction and saccharification by enzymatic method owing to the organisms are unable to consume starch directly. As mentioned, the α-amylase demonstrates a significant impact on starch degradation. Up to now, attempts convert starch to lactic acid in both two and single step. Lu *et al*. (1999) and Wee *et al*. (2008) applied amylase enzyme to prepare starch prior to mixing with glucoamylase enzyme to prepare starch prior to mixing with glucoamylase and the lactic acid fermentation was then initiated by

lactic acid bacteria. Attempts produce lactic acid from starch within single step called simultaneous liquefaction, saccharification and lactic acid fermentation (SLSF) but the two enzymes require the same optimum pH condition (Linko *et al*. 1996; Wang *et al.* 2010; Nguyen *et al*. 2013). Therefore, α-amylase is key enzyme to degrade starch to dextrin for subsequent degrading to glucose, a substrate of lactic acid fermentation by glucoamylase. Among α-amylase producing bacteria, *Bacillus* sp. is typically used as the enzyme producer (Sivaramakrishnan *et al*. 2006) and it is also practical to serve industrial demands. However, few *Lactobacillus* sp. α-amylases are less studied and applied regarding its properties and availability. So far, α-amylase genes from *Lactobacillus* sp. were manipulated and expressed in various systems such as *E. coli* (Oh *et al*. 2005), *Leuconostoc citreum* (Eom *et al*. 2009) but most of them did not succeed in overexpression.

The α-amylase from *L. plantarum* S21 have been intensively studied and is found that it has high stability in broad pH range and produce 60 and 35% of maltose and glucose from starch, respectively. A great merit of this enzyme interests us to isolate the α-amylase gene and express it in homologous and heterologous system for overproduction of the enzyme for reasons; large amount of the recombinant enzyme could be used for lactic acid production by SLSF in addition to the production of maltose and glucose, great achievement of homologous expression may conduct to feasibility in other lactobacilli hosts. Fitzsimons *et al.* (1994) succeeded in heterologous expression of amylase gene of *L. plantarum* in *L. amylovorus* with higher amylolytic activity yield. However, lactic acid produced by *L. plantarum* S21 is typically occurred to be racemic D- and L-lactic acid. Therefore, feasibility in cloning and expression of α-amylase gene in other *Lactobacillus* sp. was investigated to use as a model for expression of the gene in other *Lactobacillus* sp. for direct conversion of starch to high optical purity of D- or L-lactic acid production.

This chapter described the isolation of α-amylase gene, homologous and heterologous expression of α-amylase genes in *E. coli* and *L. plantarum* and purification and characterization of recombinant enzymes from these two recombinant strains. Overproduction of α-amylase was also expected.

3.2 Literature review

3.2.1 Production of recombinant enzyme by microbes

It is basically known that enzymes can be produced by different sources of organisms including animals, plants and microorganisms. However, the microorganisms are definitely exhibited high potentiality in term of high productivity but lower cost, simpler and faster. Over the past several decades, recombinant DNA technology had been attempted for using as high feasible and applicable sources of enzymes for industrial production for reasons; plant and microbial enzymes could be produced by microbial fermentation, it was easier to produce the enzyme from organisms that were difficult to grow and also hard to handle, the enzyme productivity was increased easily by using the overexpressed recombinant microorganisms, production of enzymes from pathogenic or toxin producing microorganisms in safe hosts was feasible, and improvement of enzyme properties could also be done (Demain and Vaishnav 2009). Regarding a number of profits from production of recombinant enzymes, recombinant DNA technology is such interesting and expected.

In this literature reviews, basic principle of gene cloning and expression is briefly described. The main purpose is to introduce basic understanding of gene cloning and expression of α-amylase gene from *L. plantarum* S21 in *E. coli* and particular *L. plantarum* for homologous and heterologous expression.

3.2.2 Basic of gene cloning

Gene cloning is generally defined as the insertion of a fragment of DNA into a cloning vector and subsequent propagation of the recombinant DNA molecule in a host organism (Sheikh and Hornby, 2013). The basic of cloning is demonstrated in Figure 3.1, The steps include isolation and purification of target DNA to be clone such as from genomic DNA, cDNA or PCR amplified DNA, isolation and purification of the expression vector, enzymatic restriction digestion of the vector and target DNA to prepare them for ligation. Depending on the expression hosts, many expression systems currently are available. Then, transformation step is performed to introduce recombinant plasmids to cell culture of bacteria, yeasts, molds, mammals, plants, insect or transgenic

plants and animals and screening to identify colonies that are positive for the recombinant DNA (Demain and Vaishnav 2009; Sheikh and Hornby, 2013).

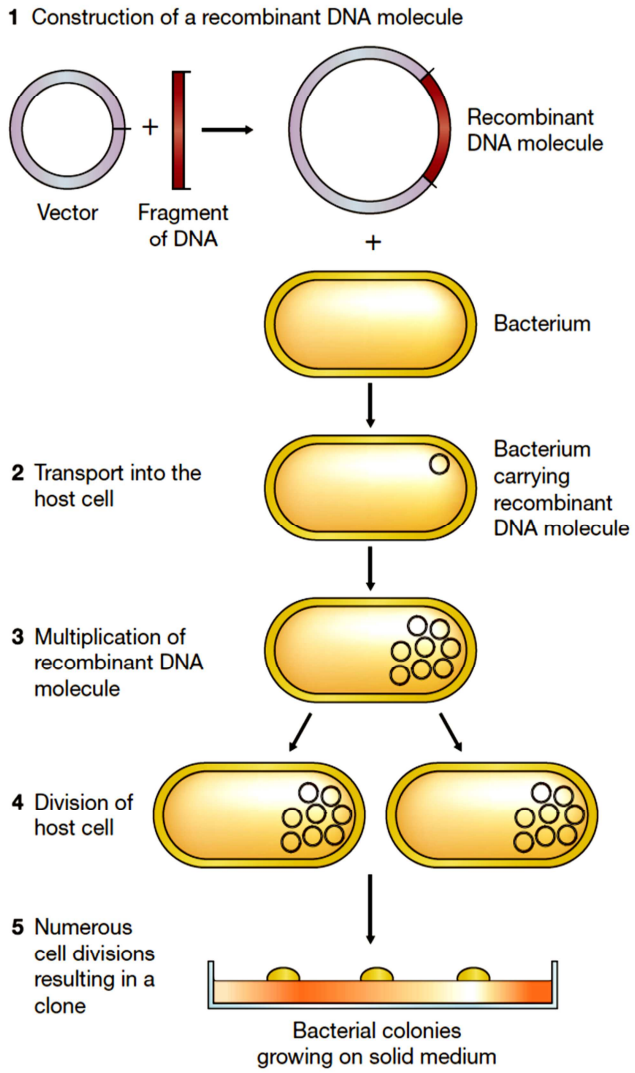

Figure 3.1 Schematic of basic of cloning (Brown, 2010)

In this literature review, the author emphasizes on cloning and expression of α-amylase gene in *E. coli* and *L. platarum* for homologous and heterologous expression system. The main tools for cloning are described in this section.

3.2.3 Expression hosts

A number of expression hosts has been being used for production of recombinant protein including bacteria, yeast, mold, insect cells, mammalian cells, transgenic animals and transgenic plants. Depending on the desire protein in different organisms, the expression hosts are selected based on where the desired gene obtained according to functionality and protein quality. Non-glycosylated proteins are usually handle in *E. coli*, yeasts but glycosylated protein are usually made in mammalian cells. However, most recombinant enzymes produced commercially are obtained from recombinant bacteria (Demain and Vaishnav 2009).

Bacterial expression hosts

E. coli

The most well-known expression host for expression of gene in bacteria is *E. coli*. It is suitable for expression of proteins or enzymes that do not require post-translational modification of the proteins (Rai and Padh 2001). Advantages and disadvantages are included in Table 3.1 (Demaain and Vaishnav 2009). *E. coli* BL21 is the most common host and has proven outstanding in standard recombinant expression applications (Sorensen and Mortensen 2005). Even it is the nice expression system; *E. coli* is considered to produce extracellular proteins. Similar to the secretion ability, the development of alternative markers is also recognized to replace the use of antibiotic and antibiotic resistance markers (Chen *et al.* 2012).

Table 3.1 Advantages and disadvantages of *E. coli* expression systems

Advantages	Disadvantages
Rapid expression	Proteins with disulfide bonds difficult to express
High yields	Produce unglycosylated proteins
Ease of culture and genome modification	Proteins produced with endotoxins
Inexpensive	Acetate formation resulting in cell toxicity
Mass production fast and cost effective	Protein produced as inclusion bodies, are inactive; require refolding

Source: Demain and Vaishnav 2009

***Bacillus* sp.**

Gram positive expression hosts mainly used for homologous expression, *Bacillus* sp. has many advantages and some disadvantages as presented in Table 3.2. It is widely utilized for industrial application rather than academic researches. The recombinant proteins produced by *Bacillus* sp. can be secreted in the medium so the harvest becomes easily and no need cell disruption process. The most typically used strains are *B. subtilis*, *B. licheniformis*, *B. meaterium* and *B. brevis*. However, there are some limitations of using *B. subtilis* as expression caused by protease activity during the expression that sometimes destroys the recombinant proteins. Extracellular proteases including subtilisin (a major alkaline serine protease) and neutral protease, (major metalloprotease contained Zn) shared 96-98% of extracellular protease activity (Demain and Vaishnav 2009).

***Pseudomonas* sp.**

Pseudomonas sp. has advantage in rapid growth and is an alternative gram positive expression host. So far, *P. fluorescens, P. aeruginosa* and *P. putida* have been used for protein expression according to their higher yields than *E. coli* (Chen 2012).

Table 3.2 Advantages of *Bacillus* sp. expression host

Strong secretion with no involvement of intracellular inclusion bodies
Ease of manipulation
Genetically well characterized systems
Highly developed transformed and gene replacement technologies
Superior growth characteristics
Metabolically characteristics
Generally recognized as safe (GRAS status) by US FDA
Efficient and cost effective recovery

Source: Demain and Vaishnav 2009

***Lactococcus* sp.**

Lactococcus lactis has been recognized as GRAS which promotes its advantage for food applications. NICE (nisin-inducible controlled gene expression) is the most widely used inducible system in this microorganism (Chen 2012). NICE expression system is driven by nisin, an antimicrobial peptide with 34 amino acids and a food grade is now available, capable of activating nisin operon at nisin A promotor (P_{nisA}) from *Lactococcus lactis* strain and it therefore regulates gene involving nisin biosynthesis or a gene of interest (Maischberger *et al*. 2010).

***Lactobacillus* sp.**

A group of gram positive bacteria, *Lactobacillus* sp. is also GRAS lactic acid bacteria and it presents in gastro intestinal (GI) tract. It is therefore considered attractive for therapeutic applications. The lactococcal nisin system (NICE) is successfully developed and it is used for expression in lactococci, lactobacilli and other lactic acid bacteria and also attract as food-grade delivery vehicles. However, the use of NICE in lactobacilli is preferably avoided since it is non-self-gene. For therapeutic applications of lactobacilli, *Lactobacillus* sp. has relatively high resistance against bile acid and low pH and they are able to colonize the GI-tract. Immunization through mucosal surfaces of the GI-tract may be an idea of immunizing human by recombinant lactic acid bacteria as antigen-delivery lactic acid bacteria (Diep *et al*. 2009).

3.2.4 Expression of amylase gene in *E. coli* for heterologous expression

In this chapter, amylase gene was isolated from *L. plantarum* S21. The gene was amplified and cloned to pET21 as an expression vector and *E. coli* BL21(DE3) was selected as an expression host.

- **Expression host: *E. coli* BL21(DE3)**

As described Sorensen and Mortensen (2005), *E. coli* BL21 is the most common host and has proven outstanding in standard recombinant expression applications. It is a robust *E. coli* B strain that able to grow vigorously in minimal

medium media but however non-pathogenic and unlikely to survive in host tissues and cause disease. The BL21 is deficient in ompT (outer membrane protein T) and lon, two proteases that may interfere with isolation of intact recombinant protein. The most common promoter system for BL21 is T7 expression system such as pET vector since this strain has been modified to carry the T7 RNA polymerase gene which itself has been modified by addition of a lactose (IPTG, isopropyl-β-d-thiogalactopyranoside) inducible genetic element and therefore drives the promoter system for the over-expression of the encode recombinant protein (Sheikh and Hornby 2013).

- **Expression vector: pET21a and pET21d**

The pET expression system was first described by Studier and colleagues and it was developed for using as various expression systems. Varieties of pET expression systems are commercially available and more than 40 different expression systems include hybrid promoters, multiple cloning site for the incorporation of different fusion partners and protease cleavage sites and a high number of genetic backgrounds modified for various expression purposes. Expression requires a host strain lysogenized by a DE3 phage fragment, encoding the T7 RNA polymerase (bacteriophage T7 gene 1) under the control of the IPTG inducible *lacUV5* promoter as shown in Figure 3.2. The mechanism is described as followed. *LacI* represses the *lacUV5* promoter and the T7/*lac* hybrid promoter encodes by the expression plasmid. A copy of the *lacI* gene presents on the *E. coli* genome and on the plasmid in a number of pET configurations. *LacI* is a weakly expressed gene and a 10-fold enhancement of the repression is achieved when the overexpressing promoter mutant $LacI^{q}$ is employed. T7 RNA polymerase is transcribed when IPTG binds and triggers the release of tetrameric *LacI* from the *lac* operator is subsequently initiated by T7 RNA polymerase (Sorensen and Mortensen 2005).

The pET expression system is categorized into 2 types; transcription vectors and translation vectors. The transcription vectors are designed for expression of target genes that carrying their own prokaryotic ribosome binding site and ATG start codon while

translation vectors carries high efficient ribosome binding site from the phage T7 major capsid protein (Novagen).

The pET21-a-d(+) are in group of translation vectors. The suffix letter such as a and d in pET21-a(+) or pET21-d(+) distinguishes the translation vectors from the transcription. All vectors with the suffix “a” express from the GGA triplet of the *BamH*I recognition sequence. Vectors with “b” express from the ATC triplet of the *BamH*I recognition sequence and also contained an upstream *Nco*I cloning site in place of the *Nde*I site of pET21-a-(+) for insertion of target genes directly into the ATG start codon. Both pET-21a-(+) and pET-21-d-(+) contain fusion tag of hexahistidine (6xHis) protein located at C-terminus of target proteins. It however should be noted that the expression of desired C-terminal fusion required lack of a stop codon in the insert as well as the proper reading frame at the cloning junction (Figure 3.2).

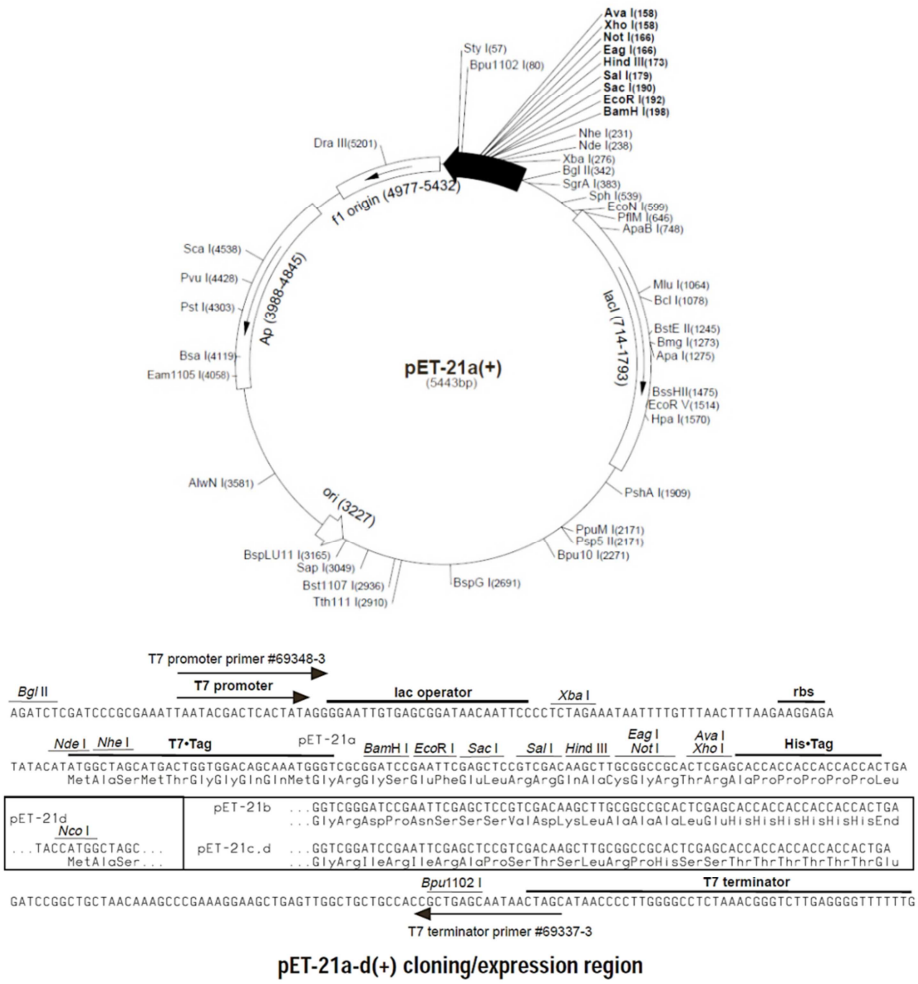

Figure 3.2 pET21-a-d (+) vector

The f1 origin is oriented so that infection with helper phage will produce virions containing single-stranded DNA that corresponds to the coding strand. Therefore, single-stranded sequencing should be performed using the T7 terminator primer.

3.2.5 Expression of amylase gene in *L. plantarum* for homologous expression

The amylase gene was cloned in pSIP409 (*erm*) and *L. plantarum* WCFS1 was acted as an expression host.

- **Expression host: *L. plantarum* WCFS1, *L. plantrum* TGL02**

A complete genome of *L. plantarum* WSCF1 has been found and reported by Kleerebezem *et al*. (2003) and tools for genetic engineering are available. *L. plantarum* WCFS1 is capable of surviving in extreme condition of gastro-intestinal tract and exhibits potential probiotic effects and it also has high survival rate which made them a promising candidate as a vehicle for *In situ* delivery of therapeutically interesting proteins (Mathiesen *et al*. 2009). Recently, it was used as an expression system of some enzymes such as β-galactosidase (Nguyen *et al.* 2011a, 2011b), chitinase (Nguyen *et al*. 2012b), malolactic enzyme (Schumann *et al.* 2012). A food grade system of *L. plantarum* WCFS1 was attained to avoid the antibiotic as selective marker. Nguyen *et al*. (2011a) did alanine racemase gene knock out of *L. plantarum* WCFS1 to construct food grade expression host, *L. plantarum* TGL02. This host was compatible to the vector harboring alanine racemase gene to grow on the medium without antibiotics.

- **Expression Vector: pSIP409 (*erm*) GusA, pSIP609 (*alr*) GusA**

The pSIP vectors are inducible expression vectors that have been developed for gene expression in *L. sakei* and *L. plantarum*. The genes are expressed via strong promoter of bacteriocin operons of *L. sakei* strains; promoter of SakacinP (*spp*) and SakacinA (*sap*). The promoters can be induced by peptide pheromone (Induction peptide, IP) for which the promoters are regulated by a two-component signal transduction system. The great advantages of pSIP vector are permitting easy exchange of all essential elements including inducible promoter, the cognate

regulatory system, the gene of interest, the antibiotic resistance marker and the replicon (Sorvig *et al*. 2005). There are many different kinds of pSIP vectors. The pSIP400 series (Figure 3.3), first constructed by Sorvig *et al*. (2003), are *spp*-based expression vectors. The sppK and sppR gene encode the proteins in the two-component regulatory system. The gene of interest is under control of one of the strictly regulated bacteriocin promoters, P_{sppA} (sakacin P) for pSIP401 with *GusA* as a reporter gene or P_{sppQ} (sakacin Q)/or P_{orfX} for pSIP409 with *GusA* and is translationally coupled to the promoter via an *Nco*I restriction site that incorporated the ATG start codon. Unique restriction sites for easy replacement of different modules are indicated at multiple cloning sites (MCS). For pSIP403 and pSIP409, *GusA* from Gram-negative *E. coli* is used as reporter gene for several homologous and heterologous expressions. The replicon region consists of two determinants; pUC (pGEM)-ori for *E. coli* and 256 rep for *L. sakei* and *L. plantarum*. The genes of interest are expressed by induction of peptide pheromone, also known as induction peptide (IP) in the principles. A great property of probiotics is production of bacteriocins to inhibit the growth of pathogen. It was known that the bacteriocins are produced strictly under the regulation of quorum-sensing based mechanism mediated by a secreted peptide-pheromone, IP, a membrane-located sensor (histidine protein kinase, HPK) or sppK as presented in Figure 3.3 and a cytoplasmic response regulator (RR) or sppR.. The interaction between an IP and its sensors are very highly specific and conduct to activation of the cognate RR which subsequently binds to regulated promoter (SppA) and eventually activates the gene expression. The regulation of sppK and SppR by IP is then called *KR* operon. This operon is driven by read-through from the upstream resistance marker gene *(ermB*) and by the original inducible promoter (P_{sppIP}) in pSIP403 and pSIP409. Normally, the structural gene for the IP is inactivated by deletion (Diep *et al*. 2009; Sorvig *et al*. 2003, Sorvig *et al*. 2005).

The pSIP609 vector, constructed by Nguyen *et al*. (2011a), is a food grade expression vector derived from pSIP409. The gene encoded erythromycin resistant marker has been replaced by alanine racemase gene in which corresponds to the synthesis of D-alanine, the amino acid that important for cell wall synthesis.

This expression vector is compatible with *L. plantarum* TGL02, a mutant of *L. plantarum* WCFS1 which alanine racemase gene (*alr*) was deleted as well as *E. coli* MB2159, the alanine auxotroph strains for construction of recombinant plasmids.

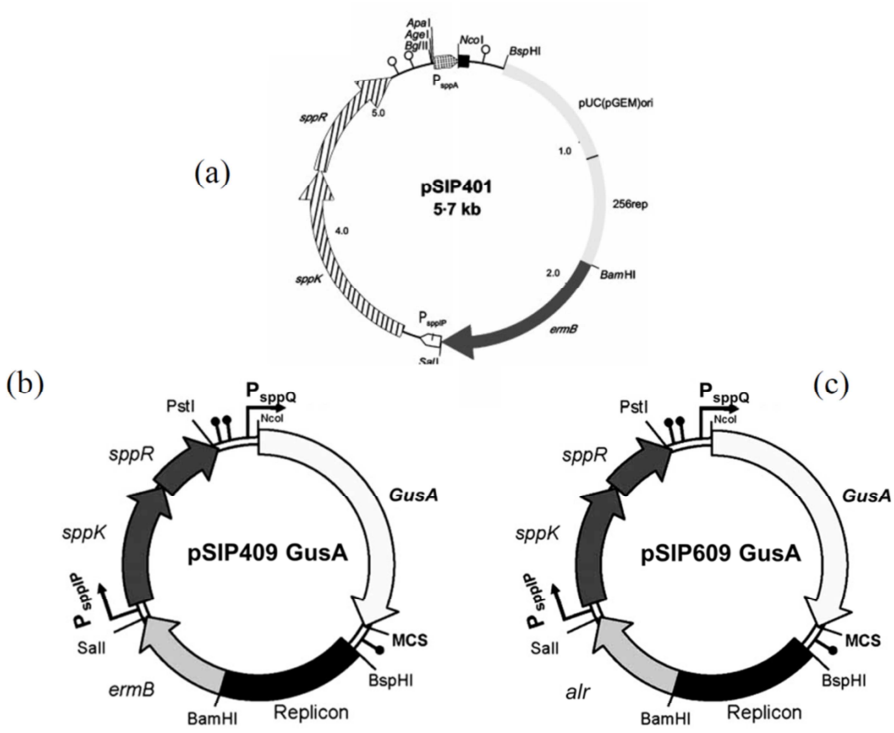

Figure 3.3 pSIP401 expression vector (a), pSIP409 vector carring *GusA* as reporter gene (b) and pSIP609 carrying *GusA*, a food grade expression vector (c)

3.3 Materials and methods

3.3.1 Microorganisms

Lactobacillus plantarum S21 isolated from indigenous Thai fermented foods, *L. plantarum* WCFS1 (Kleerebezem *et al*. 2003 and Nguyen *et al*. 2011a), *L. plantarum* TGL02 (Nguyen *et al.* 2011a), *E. coli* BL21(DE3) (New England Biolabs), *E. coli* NEB5α (New England Biolabs), *E. coli* MB2159 (Nguyen *et al*. 2011a) were maintained in 15% glycerol and stored at -80^{o}C. Vectors including pET21a (Novagen, Germany), pET21d (Novagen, Germany), pSip409 *GusA* (Nguyen *et al.* 2011a), pSip609 *GusA* (Nguyen *et al*. 2011a) were maintained in *E. coli* NEB5α system in 15% glycerol and stored at -80^{o}C.

3.3.2 Materials

All plasmids were extracted using PureYield^{TM} Plasmid Miniprep System (Promega Corp., Madison, WI). DNA fragments from reaction and agarose gel were purified by illustraTM GFX^{TM} PCR DNA and Gel Band Purification Kit (GE Healthcare, UK). All restriction enzymes and T4 ligase were purchased from Thermo Fisher Scientific (Walthman, MA). All primers used in this study were ordered from providers and included in Table 3.3.

3.3.3 Equipment and instrument

Gene Pulser XcellTM Electroporation System (Bio-Rad, CA) were used as a tool for electroporation in both *E. coli* and *L. plantarum*

Table 3.3 Primers used in this study

Primer	Sequence (5'→3')
P1	GTGAAAAAAAAGAAAAGTTTCTGG
P2	AAGAACATCGATTTTCCATGGCAG
P3	ATTAGGCTGGGCTGTTGTTG
P4	GACGCACATATGAAAAAAAAGAAAAGTTTCTGG (*Nde*I)
P5	AGTAGTCTCGAGCGAAGTGCTTGATGTGCT (*Xho*I)

Table 3.3 Primer used in this study

P6	GGCGGACCATGGATAGTTATACGACATCAACTG	(*Nco*I)
P7	AGTAGTCTCGAGCGAAGTGCTTGATGTGCT	(*Xho*I)
P8	GGTCTCCCATGAAAAAAAAGAAAAGTTTCTG	(*Bsa*I)
P9	AGCATCTCGAGTTACGAAGTGCTTGAT	(*Xho*I)

3.3.4 Isolation of maltose forming α-amylase gene from *L. plantarum* S21

The primer P1 and P2 were used to isolate the amylase gene using colony PCR technique using condition as followed a pre-denaturation step at 98^{o}C for 2 min, 35 cycles of 10 s at 98^{o}C, 20 s at 8 temperatures gradient between 55-65^{o}C and 60 s at 72^{o}C and final cycle at 72^{o}C for 7 min. The PCR product was applied on agarose gel to observe the expected band. The blunt end fragment of amylase gene was purified and inserted into pJET/1.2 kb using CloneJET PCR cloning kit (Thermo Fisher Scientific Inc., EU) and transformed to *E. coli* NEB5α. A set of primer P1-P3 was used to obtain the entire amylase gene of *L. plantarum* S21 by sequencing. Nucleotide sequences were assembled and analyzed by GENETYX program ver. 5.0 (Genetyx Corp., Tokyo, Japan) and signal peptide sequence was also predicted by the same software. Multiple alignments were performed using nucleotide BLAST program of the National Center for Biotechnology Information (NCBI) available at http://blast.ncbi.nlm.nih.gov/Blast.cgi.

3.3.5 Construction of recombinant plasmid

Construction of recombinant plasmids for heterologous expression

The primer P4-P5 and P6-P7 were used to isolate amylase gene by colony PCR. The DNA fragment obtained from primer P4-P5 was whole nucleotide sequence of amylase gene (*AmyW*) with restriction site of *Nde*I and *Xho*I at 5' and 3'-flanking while that obtained from primer P6-P7 was mature nucleotide sequence of amylase (*AmyM*) with restriction site of *Nco*I and *Xho*I at 5' and 3'-flanking. The DNA fragments were purified, digested with restriction enzymes according to their flanking sites and used as insert fragments for cloning. Two vectors of pET21a and pET21d were extracted from

E. coli NEB5α and digested by *Nde*I-*Xho*I and *Nco*I–*Xho*I, respectively. The *AmyW* was inserted in pET21a while *AmyM* was inserted in pET21d.

Construction of recombinant plasmids for homologous expression

The primer P6-P7 and primer P8-P9 were flanked with *Bsa*I-*Xho*I and *Nco*I-*Xho*I for cloning of whole (*AmyW-1*) and mature (*AmyM-1*) amylase gene, respectively. The expression vector used in this study was pSIP409 *Gus*A containing erythromycin resistant gene (*erm*) as a selective marker and pSIP609GusA which carried alanine racemase gene (*alr*). All vectors were digested with *Nco*I-*Xho*I to discard *gusA* gene. The DNA fragments were inserted in pSIP409 and pSIP609.

3.3.6 Ligation and transformation

The ligation was performed using T4 ligase under 4^{o}C for 16-18 h prior to purifying. The recombinant plasmids were transformed into *E. coli* by Gene Pulser XcellTM Electroporation System (Bio-Rad, CA). The gene pulser was set at 1.8 kV, 200 Ω, 25 µF. Vector pET21a and pET21d harboring inserts were transformed in to *E. coli* BL21 (DE3). While pSIP409 vector carrying the inserts were transformed into *E. coli* NEB5α and pSIP609 vector carrying the inserts were transformed into *E. coli* MB2159, an alanine racemase auxotroph strain. For cloning in pET, pSIP409 and pSIP609 vector, the recombinant plasmids were plated on LB agar containing 100 µg/mL ampicillin, 200 µg/mL erythromycin and only LB agar, respectively and incubated at 37^{o}C for 24 h. Positive transformants were screened by restriction analysis and confirmed by nucleotide sequencing. The recombinant *E. coli* BL21 carrying pET21a+*AmyW* and pET21d+*AmyM* was named pAmyW and pAmyM, respectively and *E. coli* NEB5α carrying pSIP409+*AmyW-1* and pSIP409+*AmyM-1* was named pAmyW41 and pAmyM41, respectively. Moreover, the recombinant *E. coli* MB2159 carrying pSIP609+*AmyW-1* and pSIP609+*AmyM-1* were named as pAmyW61 and pAmyM61, respectively.

For homologous expression, recombinant carrying pSIP409 and pSIP609 vector were extracted and then transformed to electrocompetent cell of *L. plantarum* WCFS1 and *L. plantarum* TGL02, respectively using the condition as followed; 1.5 kV, 400 Ω,

25 µF. Then, recombinant *L. plantarum* WCFS1 was plated on MRS agar supplemented with 5 µg/mL erythromycin while *L. plantarum* TGL02 was plated on MRS agar and incubated at 37^{o}C for 48 h. The positive transformants were screened and selected by colony PCR technique.

3.3.7 Expression of α-amylase gene

Heterologous expression

Recombinant *E. coli* BL21 harboring pAmyW and pAmyM were cultured in LB broth supplemented with 200 µg/mL ampilicin on 180 rpm rotary shaker at 37^{o}C. The induction was performed when OD_{600} became approximately 0.60-0.80 by adding 0.1, 0.5 and 1.0 mM IPTG. The induction was carried out on 180 rpm rotary shaker at 25^{o}C for 12, 18 and 24 h.

Homologous expression

Recombinant *L. plantarum* harboring pAmyW41 and pAmyM41 were cultured in MRS broth supplemented with 5 µg/mL erythromycin while the recombinant harboring pAmyW61, pAmyM61 were cultured in MRS broth alone and static incubated at 37^{o}C. The induction was performed when OD_{600} became ~0.30 by adding 25 ng/mL peptide pheromone or induction peptide (IP). The induction was performed at 25^{o}C until OD_{600} became ~6.00.

3.3.8 Preparation of recombinant α-amylase from *E. coli*

Biomass was harvested by centrifugation at 6000 rpm for 20 min. The cell was washed twice with 20 mM Na-phosphate buffer pH 6.5 and resuspended with the same buffer prior to disruption by French pressure cell press. Cell debris was removed by centrifugation at 25000 rpm at 4^{o}C. The clear supernatant was designated as crude intracellular enzyme.

3.3.9 Purification and characterization of recombinant α-amylase from *E. coli*

Cell extract of *E. coli* was used as crude enzyme for purification. The enzyme was purified to homogeneity within two steps including immobilized-metal affinity chromatography (IMAC) and Q-sepharose anion exchange chromatography. Crude enzyme was applied into HiPrep IMAC FF 16/10 column (GE Healthcare, Freiburg, Germany) equilibrated with binding buffer containing 0.5 M NaCl, 20 mM $Na_2HPO_4 \cdot 2H_2O$ and 5 mM imidazole pH 6.5. After binding step, the enzyme was eluted with linear gradient of 5-500 mM imidazole in 0.5 M NaCl and 20 mM $Na_2HPO_4 \cdot 2H_2O$ pH 6.5 with flow rate of 1 mL/min. The active fractions were pooled, desalted and equilibrated with 20 mM Na-phosphate buffer pH 6.5 for further applying in to Q-sepharose polishing column that equilibrated with 20 mM Na-phosphate buffer pH 6.5. The enzyme was eluted by linear gradient of 0-0.5 M NaCl in the same buffer with flow rate of 0.2 mL/min. The active fractions were pooled, desalted and stored at 4^oC prior to characterization.

To confirm the identical properties of native and recombinant α-amylase, the pH and temperature optimum and stability, substrate specificity and kinetics were performed according to the method as previously described in Chapter 2. The hydrolysis products of recombinant α-amylase from *E. coli* were determined by TLC as described previously in Chapter 2.

3.3.10 Preparation of recombinant α-amylase from *L. plantarum*

After induction by IP, biomass of recombinant *L. plantarum* carrying pAmyW61 was harvested out of culture medium by centrifugation at 6000 rpm, 4^oC for 20 min. The supernatant was used for enzyme purification.

3.3.11 Purification and characterization of recombinant α-amylase from *L. plantarum*

According to higher enzyme quantity and higher specific activity, the extracellular α-amylase was used for purification. Culture supernatant was precipitated by 80% of ammonium sulfate and the pellet was resuspended in 20 mM Na-phosphate buffer pH 6.5. The solution was dialyzed against the same buffer overnight at 4^oC. The enzyme was homogenously purified by Q-sepharose fast flow column equilibrated with 20 mM

Na-phosphate buffer pH 6.5. The elution was performed by linear gradient of 0-0.5 M NaCl in the same buffer with flow rate of 15 mL/min. The active fractions were pooled, desalted and equilibrated with 20 mM Na-phosphate buffer pH 6.5 for applying in DEAE-sepharose fast flow column equilibrated with the same buffer. The elution step was performed as described above with the flow rate or 5 mL/min. The active fractions were pooled, desalted and equilibrated with 20 mM Na-phosphate buffer pH 6.5 for applying in Q-sepharose polishing column equilibrated with the same buffer. The elution step was carefully performed as described above with the flow rate of 0.2 mL/min. The active fractions were pooled, desalted and stored at 4^{o}C for further used.

To confirm the identical properties of native and recombinant α-amylase, the pH and temperature optimum and stability, substrate specificity and kinetics were performed according to the method as previously described in Chapter 2. The hydrolysis products of recombinant α-amylase from *L. plantarum* were determined by TLC as described previously in Chapter 2.

3.4 Results

3.4.1 Isolation of α-amylase gene

According to the result from enzyme purification and characterization, we found that α-amylase from *L. plantarum* S21 had molecular weight of approximately 100 kDa as estimated by SDS-PAGE and confirmed by mass spectrometry. The data were very useful to find out some *Lactobacillus* sp. α-amylase genes from genebank of which the amylases had similar molecular weight to that from *L. plantarum* S21. The α-amylases from *L. plantarum* A6 (U62095), *L. amylovorus* NRRL B4540 (U62096), *L. manihotivorans* LMG18010 (AF12605) had high similarity to each other and were chosen for primer design. The nucleotides of 3000 bp were completely obtained by colony PCR method. The gene was inserted in pJET/1.2 vector for further nucleotide sequencing. The sequencing result were analyzed and blasted to other similarity. It was found that *L. plantarum* S21 α-amylase gene consisted of 2730 bp, encoded to 910 amino acids, in which initiated by GTG and terminated by TAA as shown in Figure 3.4. First 108 nucleotides, encoded to 36 amino acids, were predicted to be signal DNA

sequence of the amylase gene. The restriction site of *BamH*I was found at position 1422 in which identified to be the joining region of the N and C-terminus of the enzyme as previous described in Chapter 2. The repeated nucleotide sequence also found corresponding to the deduced amino acids.

3.4.2 Expression of α-amylase gene in *E. coli*

The entire (*AmyW*) and mature gene (*AmyM*) of α-amylase were amplified and cloned into pET21a and pET21d resulting in pAmyW and pAmyM for pET21a harboring *AmyW* and pET21d harboring *AmyM*, respectively. The recombinant plasmids were transformed to *E. coli* BL21 (DE3) and the positive clones were selected and confirmed. The expressions of these positive clones were performed under the induction of IPTG at different concentrations and different induction temperatures. The result was demonstrated in Table 3.4 and 3.5. It was found that the recombinant *E. coli* produced the highest α-amylase when it was induced with 0.1 mM IPTG. At higher concentration of IPTG conducted to lower enzyme production and the temperature of 25^{o}C was found to be the best temperature for induction of the amylase. The highest amylase activity and specific activity of 21700 U/L and 49 U/mg was attained after induction with 0.1 mM IPTG for 18 h, respectively. This was approximately 5 times higher production yield compared to the wild type.

Figure 3.4 The α-amylase gene of *L. plantarum* S21; nucleotides sequence encoded signal peptide was underlined; *Bam*HI restriction site represented in box; start and stop codon represented in triangle

Table 3.4 Effect of IPTG concentration on recombinant α-amylase induction

[IPTG] (mM)	Induction (h)	OD_{600}	Intracellular enzyme		
			U/L medium	mg/L medium	U/mg
0	12	4.0905	2253.09	556.13	4.05
	18	4.199	2728.71	503.65	5.42
	24	4.315	1075.27	420.10	2.56
0.1	12	2.8955	18228.85	404.75	45.04
	18	3.5265	21665.62	442.85	48.92
	24	3.725	13704.11	418.40	32.75
0.5	12	2.8787	18650.43	412.47	45.22
	18	3.5212	17774.72	428.98	41.43
	24	3.7467	10102.29	421.78	23.95
1	12	2.8165	16666.24	392.09	42.51
	18	3.496	15508.29	396.30	39.13
	24	3.671	9110.03	394.70	23.08

Table 3.5 Effect of temperature on recombinant α-amylase production

Temperature	OD_{600}	Intracellular enzyme		
		U/L medium	mg/L medium	U/mg
18	2.2782	13583.74	325.18	41.77
25	3.5212	17774.72	428.98	41.43
30	2.901	1425.88	390.43	3.65

3.4.3 Purification and characterization of recombinant α-amylase from *E. coli*

The recombinant α-amylase was obtained by harvesting the cell biomass of recombinant *E. coli*. The α-amylase activity was found in cell extract fraction. The enzyme was purified within two steps including affinity chromatography and Q-sepharose chromatography. The enzyme was homogenously purified to 1060 U/mg with

15.7% recovery as confirmed by SDS-PAGE (Figure). All purification steps were concluded in Table 3.6.

Table 3.6 Purification of recombinant α-amylase from *E. coli*

Purification steps	Protein (mg)	Activity (Unit)	Specific activity (U/mg)	Purification (folds)	Recovery (%)
Crude extract	212	14305	67.5	1.0	100
Ni-NTA Affinity chromatography	7.5	2356	314.1	4.7	16
Q-sepharose	1.3	1378	1060.0	15.7	10

The molecular mass of recombinant α-amylase was estimated by SDS-PAGE under reduced condition. It was found that the recombinant enzyme had the similar molecular weight to that from wild type (Figure 3.5). The result indicated that the recombinant enzyme had identical molecular weight to the wild type.

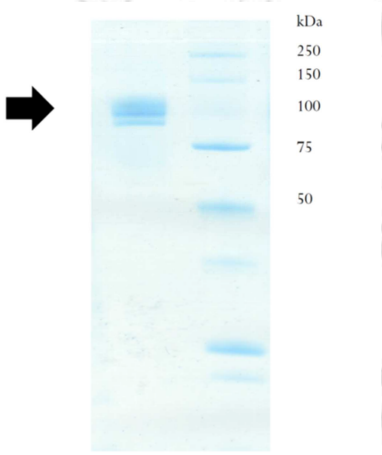

Figure 3.5 SDS-PAGE of recombinant α-amylase from *E. coli*

pH optimum and stability of recombinant α-amylase

Optimum pH for amylase activity was found in range of 4.0-6.5 as shown in Figure. Surprisingly, the enzyme also had broad pH stability from pH 4.0-8.0 at 37^{o}C as shown in Figure 3.6a and 3.6b.

Temperature optimum and stability of recombinant α-amylase

Optimum temperature of α-amylase was in range of 37-55°C which similar to that from the wild type. The recombinant enzyme seemed to have slightly higher temperature optimum as shown in Figure 3.6c and 3.6d. Stability for 1 h against various temperature revealed that the enzyme was stable at temperature from 25-50°C. The enzyme retained approximately 50% of initial activity at 55°C but it lost most activity at higher temperature for 1 h.

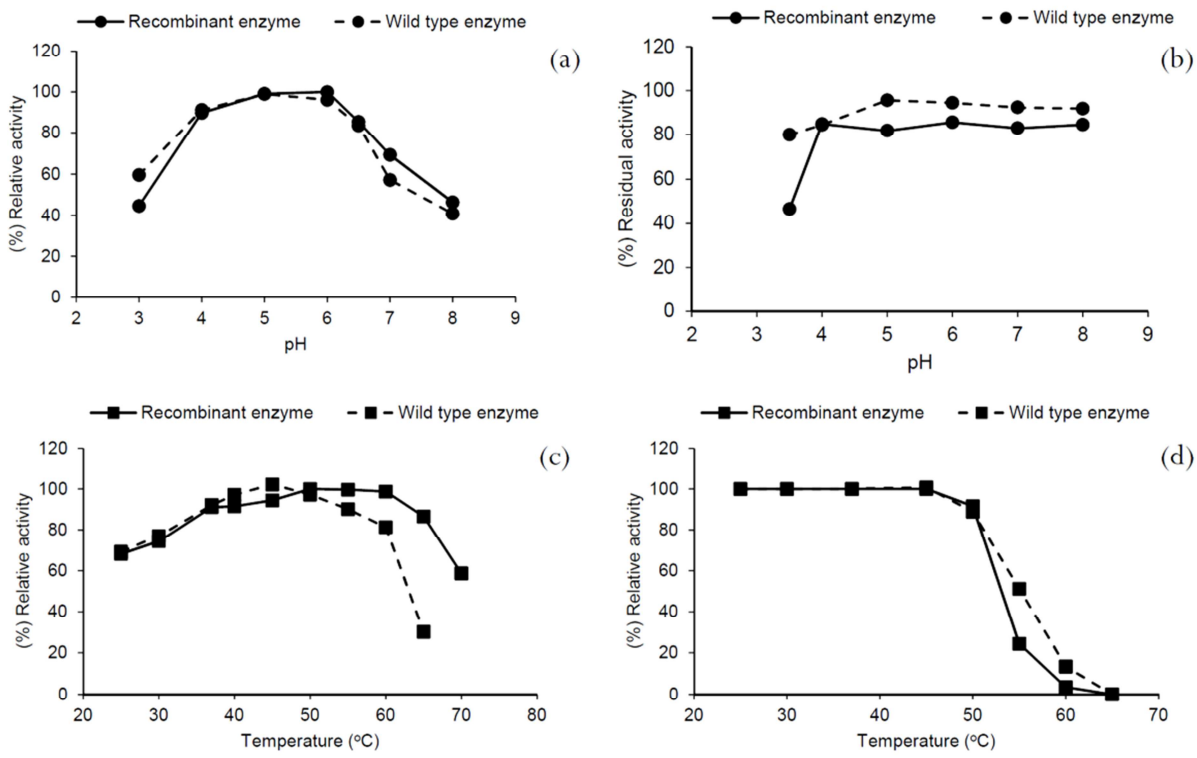

Figure 3.6 pH optimum (a) and stability (b); temperature optimum (c) and stability (d) of recombinant α-amylase from *E. coli*

Substrate specificity

The substrate specificity indicated that the recombinant enzyme was identical to wild type enzyme as presented in Table 3.7. Starch and amylose were the best substrate for the enzyme.

Table 3.7 Substrate specificity of recombinant α-amylase from *E. coli*

Substrate	Relative activity (%)	
	Wild type enzyme	Recombinant enzyme
Starch	100.0	100.0
Amylose	105.5	117.5
Amylopectin	89.0	72.6
Glycogen	49.5	47.3

Kinetic parameters

The recombinant enzyme best reacted with starch amylose and amylopectin insignificantly regarding the K_m values as demonstrated in Table 3.8. The V_{max} values were corresponded to the substrate specificity and did not have significant difference to the wild type enzyme.

Table 3.8 Comparison of K_m and V_{max} values of recombinant α-amylase from *E. coli* to the wild type enzyme

Substrate	Wild type enzyme		Recombinant enzyme	
	K_m (mg/mL)	V_{max} (U/mL·min)	K_m (mg/mL)	V_{max} (U/mL·min)
Starch	8.43±0.74	1167.27±42.67	7.60±0.63	482.54±15.79
Amylose	9.78±0.96	1995.12±115.36	8.28±1.08	858.22±62.32
Amylopectin	9.10±0.99	879.69±41.18	9.07±1.12	396.39±21.00
Glycogen	12.74±1.03	322.98±13.06	15.18±1.15	225.06±25.36

As well as the k_{cat} *and* k_{cat}/K_m values of recombinant enzyme, it was high affinity to amylose, starch, amylose and glycogen respectively as presented in Table 3.9. The maximum reducing sugar of 7.25×10^2 mg/mL·sec obtained from amylose that was not differed to the wild type enzyme.

Table 3.9 Comparison of k_{cat} *and* k_{cat}/K_m values of recombinant α-amylase from *E. coli* to the wild type enzyme

Substrate	Wild type enzyme		Recombinant enzyme	
	k_{cat} (per sec)	k_{cat}/K_m (mg/mL·sec)$^{-1}$	k_{cat} (per sec)	k_{cat}/K_m (mg/mL·sec)$^{-1}$
Starch	4.13×10^3	4.90×10^2	3.38×10^3	4.45×10^2
Amylose	7.05×10^3	7.21×10^2	6.01×10^3	7.25×10^2
Amylopectin	3.11×10^3	3.42×10^2	2.78×10^3	3.06×10^2
Glycogen	2.21×10^3	1.46×10^2	2.26×10^3	1.78×10^2

Hydrolysis products

Maltose and glucose were still the main hydrolysis products from the substrates used as same as the wild type (Figure 3.7).

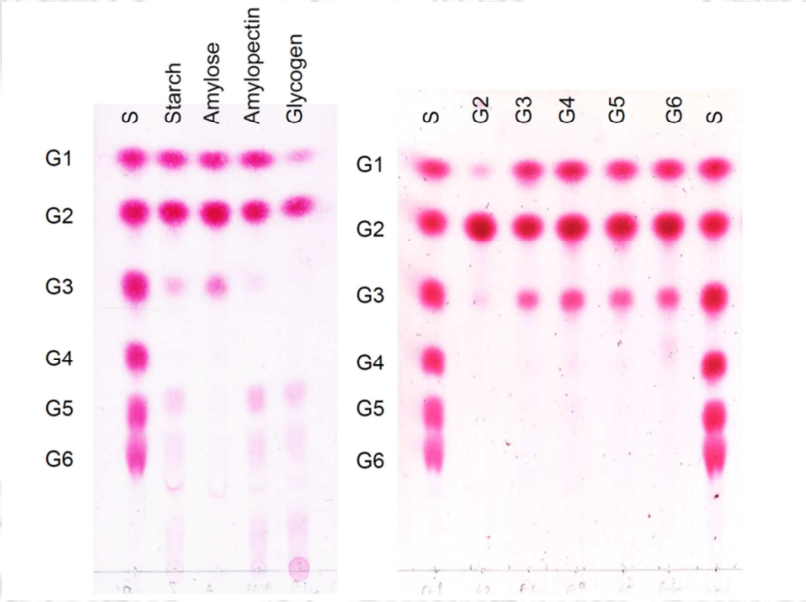

Figure 3.7 Pattern of hydrolysis products from recombinant α-amylase from *E. coli*

3.4.4 Overexpression of recombinant α-amylase from *E. coli*

Overexpression of α-amylase (*AmyM*) was attained when signal peptide sequence of the gene was discarded. The enzyme yield and enzyme specificity were higher than that from wild type and the recombinant *E. coli* harboring whole sequence of α-amylase

gene. The maximum α-amylase activity of 80400 U/L was attained by induction of 0.5 mM IPTG at 25°C for 18 h. The specific activity was considerably higher than the previous expression with 166 U/mg but highest specific activity of 198 U/mg was found when induction was performed with 0.5 mM IPTG for 18 h (Table 3.10).

Table 3.10 Overexpression of α-amylase gene in *E. coli*

Induction	[IPTG] (mM)	OD_{600}	Intracellular enzyme		
			U/L	mg/L	U/mg
12 h	0.1	3.184	69550.61	376.90	184.53
	0.5	3.089	68314.23	344.78	198.14
	1	3.046	68370.01	380.03	179.91
18 h	0.1	3.184	76090.62	419.19	181.52
	0.5	3.089	80397.83	482.58	166.60
	1	3.046	72485.79	474.11	152.89
24 h	0.1	3.313	73057.71	476.41	153.35
	0.5	3.257	71094.37	431.91	164.61
	1	3.231	71562.89	413.06	173.25

3.4.5 Expression of α-amylase gene in *L. plantarum*

Homologous expression of amylase gene in *L. plantarum* was performed in both conventional and food grade system. The results were demonstrated in Table 3.11 which revealed that recombinant *L. plantarum* expressed the gene in form of extracellular enzyme rather than intracellular enzyme. The main activity of 3243 and 3567 U/L was obtained from *L. plantarum* WCFS1, a conventional host and *L. plantarum* TGL02, a food grade host. The equal amount of the enzyme and their specific activity obtained from these systems were also closed to each other at 55 and 60 U/mg. However, only recombinant *L. plantarum* TGL02 was selected for enzyme production in order to purify the enzyme and for further utilization.

Table 3.11 Expression of α-amylase genes in *L. plantarum* TGL02

Plasmids	Extracellular enzyme			Intracellular enzyme		
	U/L	mg/L	U/mg	U/L	mg/L	U/mg
pAmyW61	3242.91	59.05	54.92	320.15	224.69	1.42
pAmyW41	3567.15	59.90	59.55	718.22	295.05	2.43

Effect of IP concentration on α-amylase production

The IP (Induction peptide) concentration between 25-50 ng/mL had positive effect on induction of extracellular α-amylase in term of recovery yields in contrast to intracellular enzyme. However, the yields of extracellular enzyme had no significant difference among IP concentration among 50-100 ng/mL (Table 3.12). To attain high enzyme specific activity, the IP concentration of 75 ng/mL should have been selected.

Table 3.12 Effect of IP concentration on induction of recombinant α-amylase from *L. plantarum* TGL02 harboring pAmyW61

[IP]	Extracellular enzyme			Intracellular enzyme			Secretion efficiency
(ng/mL)	U/L	mg/L	U/mg	U/L	mg/L	U/mg	(%)
25	2590.03^{a}	80.75	32.07	698.41^{a}	582.72	1.20	78.76
50	3134.99^{b}	82.25	38.12	1076.48^{b}	568.96	1.89	74.44
75	3324.46^{b}	71.95	46.21	1260.40^{bc}	533.44	2.36	72.51
100	3199.45^{b}	68.70	46.57	1428.85^{c}	571.12	2.50	69.13

3.4.6 Purification and characterization of recombinant α-amylase from *L. plantarum* TGL02 harboring pAmyW61

To confirm the identical properties of the recombinant enzyme obtained from *L. plantarum* harboring pAmyW61. The α-amylase enzyme obtained from food grade *L. plantarum* was harvested and precipitated by 80% ammonium sulfate as similar step as purification of wild type enzyme. The enzyme was further purified until homogeneity by cation exchange chromatography. The recovery yield was obtained by only 8.6% but

the enzyme was purified to 41 folds with 1007 U/mg (Table 3.13). The purity of the enzyme could also observed by a single protein band on SDS-PAGE as shown in Figure 3.8.

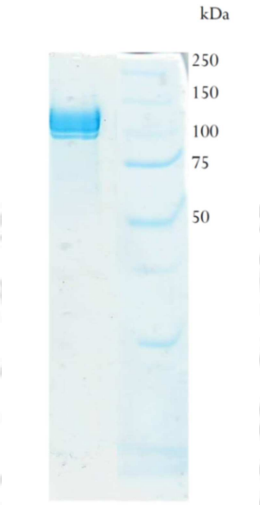

Figure 3.8 SDS-PAGE of recombinant α-amylase from *L. plantarum* TGL02 harboring pAmyW61

Table 3.13 Purification of recombinant α-amylase from *L. plantarum* TGL02 harboring pAmyW61

Steps	Protein (mg)	Activity (Units)	Specific activity (Units/mg)	Purity (folds)	Recovery (%)
Crude	303.65	7456.30	24.56	1.00	100.00
Precipitation	302.48	3458.00	11.43	0.47	46.38
Q-sepharose	17.46	3020.40	172.99	7.04	40.51
DEAE-sepharose	3.85	1178.67	306.15	12.47	15.81
Q-sepharose (polishing step)	0.64	639.97	1007.51	41.03	8.58

pH and temperature optimum and stability of α-amylase from *L. plantarum*

As described previously, the enzyme obviously had identical pH and temperature optimum to enzyme from wild type as well as pH and temperature stability as shown in Figure 3.9

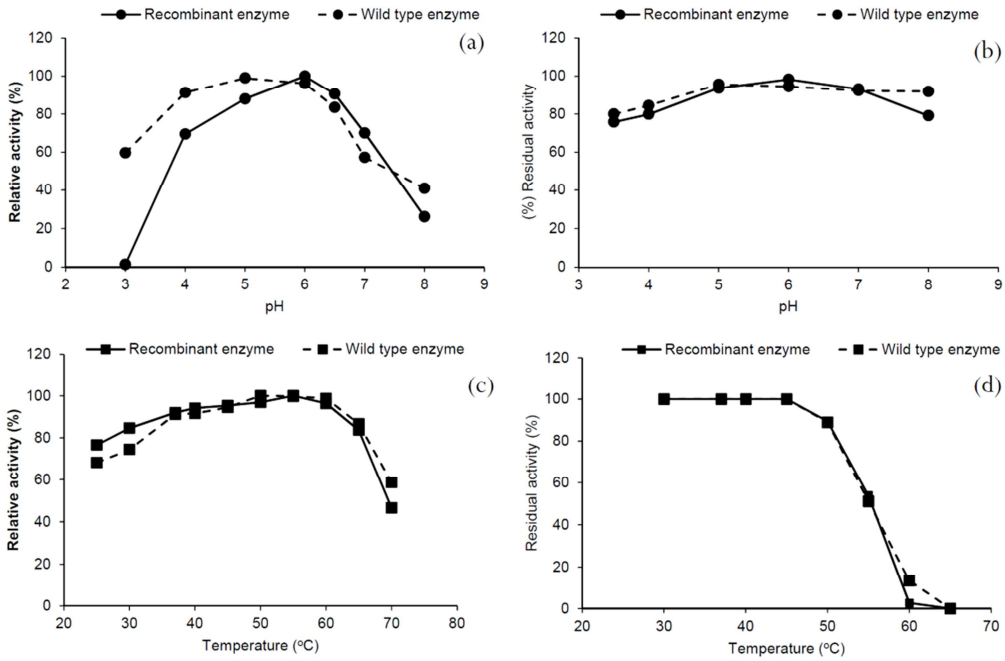

Figure 3.9 pH optimum (a) and stability (b); temperature optimum (c) and stability (d) of recombinant α-amylase from *L. plantarum* TGL02 harboring pAmyW61

Substrate specificity

The recombinant enzyme had certainly the same substrate specificity to wild type enzyme as concluded in Table 3.14.

Table 3.14 Substrate specificity of recombinant α-amylase from *L. plantarum* TGL02 harboring pAmyW61

Substrate	(%) Relative activity	
	Wild type enzyme	Recombinant enzyme
Starch	100.0	100
Amylose	105.5	127
Amylopectin	89.0	92
Glycogen	49.5	55
Raw starch	0	0
Pullulan	0	0
α-cyclodextrin	0	0
β-cyclodextrin	0	0

Kinetic parameters

The K_m and V_{max} of purified recombinant α-amylase was slightly different to the values from wild type (Table 3.15). The recombinant enzyme had somewhat higher K_m values but lower V_{max} values towards starch amylose, amylopectin and glycogen than that from wild type as well as the k_{cat} and k_{cat}/K_m values (Table 3.16). However, all kinetic values from the recombinant enzyme had obviously the same trends as wild type enzyme and recombinant enzyme from *E. coli*. Here, the recombinant enzyme still had high affinity to amylose as the best substrate and released $7.21x10^2$ μmole per min.

Table 3.15 Comparison of K_m and V_{max} values of recombinant α-amylase (*AmyW*) from *L. plantarum* TGL02 to the wild type enzyme

Substrate	Wild type enzyme		Recombinant enzyme	
	K_m (mg/mL)	V_{max} (U/mL·min)	K_m (mg/mL)	V_{max} (U/mL·min)
Starch	8.43±0.74	1167.27±42.67	12.09±1.08	738.39±32.38
Amylose	9.78±0.96	1995.12±115.36	12.56±1.47	998.01±75.12
Amylopectin	9.10±0.99	879.69±41.18	12.12±1.08	582.36±25.48
Glycogen	12.74±1.03	322.98±13.06	35.49±3.00	289.21±17.43

Table 3.16 Comparison of k_{cat} *and* k_{cat}/K_m values of recombinant α-amylase (*AmyW*) from *L. plantarum* TGL02 to the wild type enzyme

Substrate	Wild type		Recombinant enzyme	
	k_{cat} (per sec)	k_{cat}/K_m (mg/mL·sec)$^{-1}$	k_{cat} (per sec)	k_{cat}/K_m (mg/mL·sec)$^{-1}$
Starch	$4.13x10^3$	$3.87x10^3$	$4.90x10^2$	$3.20x10^2$
Amylose	$7.05x10^3$	$5.24x10^3$	$7.21x10^2$	$4.17x10^2$
Amylopectin	$3.11x10^3$	$3.06x10^3$	$3.42x10^2$	$2.52x10^2$
Glycogen	$2.21x10^3$	$1.52x10^3$	$1.46x10^2$	$4.28x10^1$

Hydrolysis products

Perfect expression of recombinant enzyme from *L. plantarum* was considerably confirmed by pattern of hydrolysis products. The result revealed that the recombinant enzyme still conserved the same properties in *L. plantarum* as homologous expression host. The main hydrolysis products regarding all substrates for hydrolysis were found to be maltose and glucose (Figure 3.10).

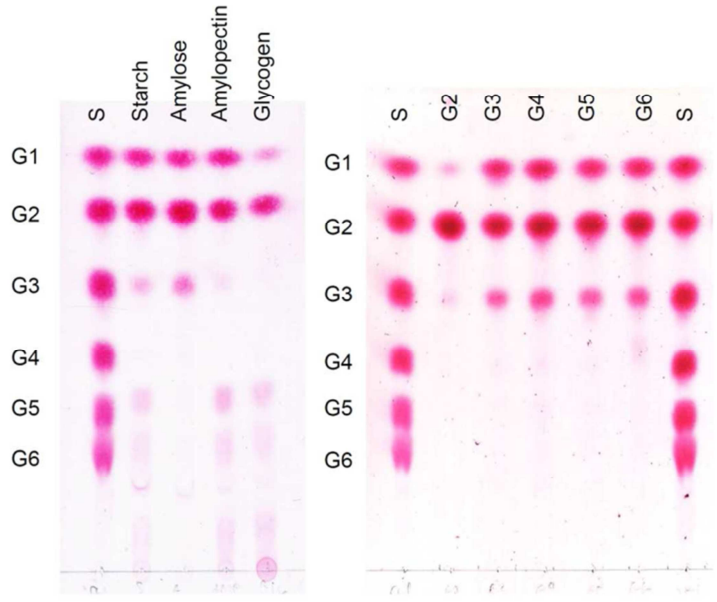

Figure 3.10 Pattern of hydrolysis products from recombinant α-amylase from food grade *L. plantarum* TGL02 harboring pAmyW61

3.4.7 Expression of mature α-amylase gene in *L. plantarum*

The mature α-amylase gene was trialed expectantly to express through pSIP409 and pSIP609 for overproduction of enzyme as found in *E. coli*. The result was definitely different to that in *E. coli* since very low amount of intracellular enzyme and the specific activity were attained from both expression systems (Table 3.17).

Table 3.17 Expression of mature α-amylase gene (*AmyM*) in *L. plantarum*

Plasmids	Extracellular enzyme			Intracellular enzyme		
	U/L	mg/L	U/mg	U/L	mg/L	U/mg
pAmyM41	0	88.03	0.00	174.95	564.61	0.31
pAmyM42	0	82.53	0.00	362.32	737.89	0.49

3.5 Discussion

The α-amylase gene was isolated from genomic DNA of *L. plantarum* S21 in order to clone and express in *E. coli* and *L. plantarum* WCFS1 to investigate overproduction of α-amylase. Production of α-amylase was important since the enzyme had excellent properties as described in Chapter 2. The entire amylase gene of 2733 nucleotides was isolated and sequenced. We found GTG as start codon and TAA as terminated codon and found that the first 108 nucleotides were corresponded to signal peptide sequence as predicted by Genetyx software. However, this sequence was considered and definitely related to the peptide sequence as presented in Chapter 2. The N-terminus and C-terminus joined at *BamH*I site. This gene shared high similarity with α-amylase genes of *Lactobacillus* spp., particularly *L. plantarum* A6 and *L. manihotivorans* LMG18010. Two insert fragments were provided in order to clone entire and mature α-amylase gene for homologous and heterologous expression.

The pET21a and pET21d was used as expression vector for expression in *E. coli* BL21 (DE3). The result showed that the gene was perfectly expressed in *E. coli* particularly when the mature α-amylase gene used as insert fragment. Recombinant α-amylase was produced after induction of *E. coli* by IPTG. The enzyme was accumulated inform of intracellular enzyme. It was found that entire α-amylase was expressed successfully with amylase yield of 21665 U/L which was 5 times higher yield than production in *L. plantarum* S21, wild type. Amylase specific activity was moreover high up to 49 U/mg which was higher than that obtained from wild type (35 U/mg). Surprised expression was found when mature amylase gene was expressed. Amylase activity of 80400 U/L was produced with the highest specific activity of 166 U/mg. The result indicated successful overexpression of α-amylase gene in *E. coli* since the production yield was not only 20 times higher than wild type but the specific activity was also increased to 4.7 times. *E. coli*, the most well know expression host, typically used for expression of gene from prokaryotes. For further properties determination of purified enzymes and utilization, many α-amylase genes from lactic acid bacteria were isolated and expressed using this system according to the overproduction and easy purification steps could be approached. A matogenic amylase from *L. gasseri* expressed in *E. coli* was performed in order to determine the notable properties of the recombinant

enzymes (Oh *et al*. 2005). Two α-amylase genes of *S. bovis* 148 were expressed in *E. coli* for the same purpose (Satoh *et al*. 1993).

Homologous expression was performed using *L. plantarum* WCFS1 and its derivative, food grade *L. plantarum* as host strains. Entire amylase gene was expressed in both *L. plantarum* in form of extracellular enzyme in contrast to case of mature gene. Previous discussion revealed the discovery of signal peptide sequence of amylase gene. In addition, α-amylase activity was found in cell extract fraction when mature α-amylase gene was expressed in pSIP-vector. This result indicated that *L. plantarum* WCFS1 required complete gene for higher expression which indicated that the nucleotide sequence encoded signal peptide was so important for *L. plantarum* in order to express the gene. A graceful result advantaged expression of other genes in *L. plantarum* if extracellular proteins were required. Up to now, there was few paper reported production of extracellular enzyme in *L. plantarum* using pSIP409 and pSIP609 expression vector. *L. plantarum* WCFS1 and the food grade system had been proved to be probiotics for which it was used as vehicle for drug delivery (Mathiesen *et al.* 2009). *L. plantarum* WCFS1 had been so far used as expression host to accumulate proteins or enzymes in the cell and most papers reported the production of intracellular enzymes. Therefore, those enzymes came from mature gene. Nguyen *et al.* (2011b) succeeded in overproduction of *Lactobacillus* spp. β-galactosidase in *L. plantarum* WCFS1 carrying pSIP409 recombinant vector. Then, this vector and the compatible host were manipulated to food grade system under induction of IP for overproduction the enzyme (Nguyen *et al.* 2011a). The evidence revealed the successful of overexpression of the gene for intracellular enzyme production. Nguyen *et al.* (2011b) expressed *B. licheniformis* chitinase gene with its complete chitinase gene in *L. plantarum* WCFS1 but they did not success in production of extracellular enzyme with the complete chitinase gene because the signal peptide sequence was not recognized by the host. This result was in contrast to our result since we found that the level of entire α-amylase gene expression was really similar to wild type in addition to the specific activity that was slightly increased to 55 U/mg. When the mature α-amylase gene was expressed in *L. platarum* WCFS1, the adverse result was furthermore found. No extracellular enzyme was detected and less intracellular enzyme was moreover

obtained. We suggested that the nucleotide sequence encoded signal peptide was so important to either transcription or translation of α-amylase gene.

Recombinant α-amylases from *E. coli* and food grade *L. plantarum* harboring entire α-amylase gene were purified for further characterization. Cell extract of recombinant *E. coli* were purified within two steps. According to great advantage of pET vector, amylase protein was fused with 6xhistidines tag for purification by immobilized metal ion affinity chromatography (IMAC). The specific activity of 314 U/mg was obtained with 16% recovery; however Q-sepharose chromatography was still required to polish interfere proteins. The recombinant enzyme was purified until homogeneity with the specific activity of 1060 U/mg as confirmed by SDS-PAGE. Properties of the purified enzyme including optimum and stability of pH and temperature, substrate specificity, Kinetic parameters (K_m, V_{max}, k_{cat}, k_{cat}/K_m) and pattern of hydrolysis products were identical to the purified enzyme from wild type. As well as recombinant α-amylase from wild type, properties of extracellular α-amylase from food grade *L. plantarum* were definitely similar to α-amylases from wild type and recombinant *E. coli*. The results in this chapter were valuable and meant to production of α-amylase for further application particularly conversion of starch to lactic acid.

3.6 Conclusion

This chapter described the homologuous and heterologous expression of α-amylase gene isolated from *L. plantarum* S21. The accomplishment was concluded as followed;

1. Isolation of α-amylase gene from *L. plantarum* S21 was successful by colony PCR technique using primers designed based on α-amylases from *L. plantarum* A6, *L. manihotivorans* LMG18010 and *L. amylovorus* NRRL B4540. The complete sequence consisted of 2733 nucleotides was obtained. The 108 nucleotides encoded for 36 amino acid sequence identified to signal peptide sequence.

2. Overproduction of α-amylase was successful when the mature gene was expressed in *E. coli* in contrast to the result of expression in *L. plantarum*. The α-

amylase yield of 20 times was increased with specific activity of 166 U/mg compared to *L. plantarum* S21.

3. Signal peptide of *L. plantarum* S21 is functioned by *L. plantarum* WCFS1 and its derivative according to the occurrence of α-amylase activity in the production medium.

4. Both intracellular enzyme from recombinant *E. coli* and extracellular enzyme from recombinant *L. plantarum* are identical to wild type enzyme regarding the same biochemical characteristics, molecular mass and pattern of hydrolysis products.

5. The overproduction of recombinant enzyme is useful for direct conversion of starch to lactic acid. By the way, the signal peptide of *L. plantarum* S21 could be used as alternative sequence for production of extracellular enzyme from recombinant *L. plantarum* WCFS1 and its derivative.