J Korean Soc Appl Biol Chem (2015) 58(6):901–908
DOI 10.1007/s13765-015-0121-z

Online ISSN 2234-344X
Print ISSN 1738-2203

ARTICLE

Efficient secretory expression of gene encoding a broad pH-stable maltose-forming amylase from *Lactobacillus plantarum* S21 in food-grade lactobacilli host

Apinun Kanpiengjai[1] · Saisamorn Lumyong[2] · Pairote Wongputtisin[3] · Dietmar Haltrich[4] · Thu-Ha Nguyen[4] · Chartchai Khanongnuch[1]

Received: 11 June 2015 / Accepted: 2 September 2015 / Published online: 15 September 2015

Abstract The native and the *N*-terminal signal peptide sequence deleted gene encoding for α-amylase from *Lactobacillus plantarum* S21 were cloned into the inducible lactobacilli expression vectors pSIP409 and pSIP609 and expressed in *L. plantarum* WCFS1 and food-grade *L. plantarum* TGL02, respectively. Only the native amylase gene was expressed and secreted extracellular amylase at a level of approximately 2000 U/L with 90 % secretion efficiency from both hosts. The purified extracellular amylase from the *L. plantarum* TGL02 retained unique properties of the wild-type enzyme, particularly the broad pH stability (4.0–8.0) and maltose-forming activity. The results indicate high compatibility of *L. plantarum* S21 signal peptide sequence to both recombinant lactobacilli hosts. The recombinant lactobacilli exhibited high efficiency for direct lactic acid production from starch as found with *L. plantarum* S21. The efficient compatible signal peptide is also expected to be applied in secretory expression for production of valuable proteins in food-grade lactobacilli host.

✉ Chartchai Khanongnuch
chartchai.k@cmu.ac.th

[1] Division of Biotechnology, Faculty of Agro-Industry, School of Agro-Industry, Chiang Mai University, Chiang Mai 50100, Thailand

[2] Microbiology Section, Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai 50200, Thailand

[3] Program in Biotechnology, Faculty of Science, Maejo University, Chiang Mai 50290, Thailand

[4] Food Biotechnology Laboratory, Department of Food Science and Technology, BOKU University of Natural Resources and Life Science, Vienna, Austria

Keywords Amylase · Expression · *Lactobacillus plantarum* S21 · Secretion · Signal peptide

Introduction

Lactic acid bacteria are widely used in a variety of fermented food and feed products (e.g., dairy products, meat, and sausages) because of their specific metabolic activities (Cho et al. 2007). *Lactobacillus* spp. represent the members of the largest genus of lactic acid bacteria and play an important role for lactic acid production (Abdel-Rahman et al. 2010). Recently, the direct bioconversion of starch to lactic acid by amylolytic lactic acid bacteria (ALAB) was proposed to be a superior alternative strategy for the production of lactic acid. However, with the exception of *L. amylophilus* GV6, few potential ALAB have accomplished this goal. The efficacy of this bacterium in lactic acid production was affected by enzyme properties, but the amylase-encoding gene has not been isolated, cloned, or expressed in other lactobacilli hosts (Vishnu et al. 2006). One benefit of achieving the secretory expression of the amylase-encoding gene in *Lactobacillus* sp. and other lactic acid bacteria is an acquirement of new recombinant lactic acid bacteria capable of producing extracellular amylases which is able to be utilized for efficient lactic acid production from starch. Another application is the use as model for secretion and production of homologous and heterologous proteins in Lactobacilli. *L. plantarum* WCFS1 is a homofermentative lactic acid bacterium (Kleerebezem et al. 2003), widely used as an expression host for a number of intracellular and extracellular proteins (Karlskås et al. 2014); however, the mechanism that contributes to efficient secretory expression in this host is unknown. High-level

secretion is dependent on signal peptide-encoding sequences and the target genes (Mathiesen et al. 2008). Therefore, a well-functioning signal peptide is a key that leads high opportunity in secretion of proteins. *L. plantarum* S21 is a highly efficient ALAB that is capable of converting high concentrations of starch into lactic acid and producing a novel extracellular maltose-forming α-amylase with broad pH stability (Kanpiengjai et al. 2014b). Its amylase-encoding gene was isolated, sequenced, and found that first 108 nucleotides deduced 36 amino acids responsible for a signal peptide (Kanpiengjai et al. 2015). This signal peptide sequence challenges our research interest to enhance the secretion of target proteins in *Lactobacillus* sp. Therefore, this research aims to produce the maltose-forming α-amylase for further applications and to investigate feasibility for construction of recombinant ALAB with efficient amylase secretion for direct bioconversion of starch to lactic acid by expression of the amylase-encoding gene from *L. plantarum* S21 both with and without its native signal peptide sequence in *L. plantarum* WCFS1 and the food-grade bacterium *L. plantarum* TGL02.

Materials and methods

Bacterial strains and chemicals

Lactobacillus plantarum S21 (Kanpiengjai et al. 2014b) and *L. plantarum* WCFS1 were grown in de Man Rogosa Sharpe (MRS) medium (Kleerebezem et al. 2003). *L. plantarum* TGL02 was grown in MRS medium supplemented with D-alanine (Sigma, USA) at a concentration of 200 μg/mL (Nguyen et al. 2011). *E. coli* MB2159 was grown in Luria–Bertani (LB) medium supplemented with D-alanine to a final concentration of 200 μg/mL (Nguyen et al. 2011). *E. coli* NEB5α (New England Biolabs, USA) was grown in LB medium. Other substrates, reagents, and solvents were of analytical grade.

Construction of recombinant plasmids

Lactobacillus plantarum S21 genomic DNA was isolated using the method described by Kanpiengjai et al. (2014b) and used as a template. Different amylase genes were amplified using Phusion High-Fidelity PCR Kit (New England Biolabs, UK), and the primers AmyL7F-GGTCTCCATGAAAAAAAAGAAAAGTTTCTG and AmyLR- AGTAGTCTCGAGCGA-AGTGCTTGATGTGCT or AmyL9F-GGCGGACCATGGATAGTTATACGACATCA-ACTG and AmyLR (VBC Biotech, Austria). The first primer set was used to obtain the complete α-amylase gene (*AmyL7*) (accession no. KJ440080), and the second primer set was used to obtain the gene without the *N*-terminal signal peptide sequence (*AmyL9*). The amplified products were separated by agarose-gel electrophoresis and purified using the Illustra GFX PCR DNA and Gel Band Purification kit (GE Healthcare, Sweden). *AmyL7* was digested with *Bsa*I and *Xho*I (underlined) and ligated into the *Nco*I-*Xho*I fragment of the expression plasmids pSIP409 and pSIP609, resulting in plasmids pAmyL7 and pAmyL7.1, respectively; *AmyL9* was digested with *Nco*I and *Xho*I and ligated into the same expression plasmids, resulting in the plasmids pAmyL9 and pAmyL9.1, respectively. pAmyL7 and pAmyL9 were chemically transformed into *E. coli* NEB5α according to the manufacturer's instructions and plated on LB agar supplemented with 200 μg/mL erythromycin. pAmyL7.1 and pAmyL9.1 were transformed into electrocompetent *E. coli* MB2159 according to method of Inoue et al. (1990) and then plated on LB agar. Positive colonies were detected using restriction analysis, and the sequences were confirmed by a commercial sequencing service (LGC Genomics, Germany).

Expression of the α-amylase-encoding gene

The pAmyL7 and pAmyL9 plasmids were isolated from *E. coli* NEB5α and then transformed into electrocompetent *L. plantarum* WCFS1 according to the method of Aukrust and Blom (1992); the pAmyL7.1 and pAmyL9.1 plasmids were isolated from *E. coli* MB2159 and then transformed into electrocompetent food-grade *L. plantarum* TGL02. Cultures were prepared at 37 °C in MRS medium supplemented with 200 μg/mL erythromycin for *L. plantarum* WCFS1 harboring pAmyL7 and pAmyL9 but without any of antibiotics for *L. plantarum* TGL02 harboring pAmyL7.1 and pAmyL9.1. The induction was performed when the density at OD_{600} reached approximately 0.3 by the addition of the induction peptide (IP) into the culture media to a final concentration of 25 ng/mL under an induction temperature of 30 °C; these conditions were maintained until the OD_{600} reached approximately 6.0. The cultures were centrifuged at 16,100×*g* at 4 °C for 15 min. The supernatant was designated as the crude extracellular amylases. The cell pellet was washed twice with 50 mM Na-phosphate buffer (pH 6.5) and resuspended in the same buffer prior to disruption for 2 min using the Precellys 24 homogenizer in a bead beater (Germany). The resulting cell-free extract was designated as the crude intracellular enzyme. The secretion efficiency is expressed as the percentage of total amylase activity present in the supernatant.

Enzyme production and purification

Enzyme production was performed by the expression of recombinant *L. plantarum* TGL02 harboring pAmyL7.1 as

described above. The culture supernatant was harvested and used as the crude extracellular enzyme. Solid ammonium sulfate was gradually added to the crude extracellular enzyme with stirring at 4 °C until a saturation of 80 % was achieved. The precipitated protein was dissolved with 20 mM Na-phosphate buffer (pH 6.5) and dialyzed in the same buffer overnight. The equilibrium enzyme solution was loaded onto a 50-mL Q Sepharose™ Fast Flow column (GE Healthcare) equilibrated with 20 mM Na-phosphate buffer (pH 6.5). The elution was performed by a linear gradient of 0–500 mM NaCl in the same buffer with a flow rate of 5 mL/min. The active fractions were pooled and desalted using 10 kDa cut-off Amicon Ultra Centrifugal filter tubes (Millipore, USA) prior to applying them onto 20-mL Q-Sepharose High Performance columns (GE Healthcare) for polishing. The protein elution was performed as described for the Q-Sepharose Fast Flow, but with an elution flow rate of 0.1 mL/min. The active fractions were pooled, desalted, and stored at −80 °C for further characterization. Amylase activity and protein levels were determined using the method described by Kanpiengjai et al. (2015). One unit of amylase was defined as the amount of the enzyme that liberates 1 μmole of reducing sugars (as glucose equivalents) per min under the assay conditions.

Polyacrylamide gel electrophoresis

SDS–Polyacrylamide Gel Electrophoresis (SDS-PAGE) was performed using the procedures of Kanpiengjai et al. (2015). The precision Plus Protein™ standard (Bio-Rad, USA) was used as the protein molecular marker.

Enzyme characterization

The pH optimum of the amylase activity was determined using standard assay conditions with varying pH values from 3.0 to 10.0 using 100 mM of various buffer systems, including citrate–phosphate buffer (pH 3.0–6.0), Na-phosphate buffer (pH 6.0–8.0), and Tris–HCl buffer (pH 8.0–9.0). To determine the stability of the amylase activity at different pH values, purified amylase was incubated at 37 °C for 24 h in the aforementioned buffers at a concentration of 20 mM. The residual activity was determined under the standard assay condition. The temperature optimum of amylase activity was determined using the standard 10-min activity assay with varying temperature from 25 to 65 °C. To measure temperature stability, the purified enzyme was incubated in 100 mM Na-phosphate buffer (pH 6.5) at 25–65 °C for 1 h and then immediately placed in an ice bath. The residual amylase activity was determined under the standard assay condition.

For substrate specificity determination, amylase activity was assayed with 0.5 % (w/v) of different substrates including soluble starch, amylose, amylopectin, α-cyclodextrin, β-cyclodextrin, glycogen, dextran, and pullulan under the standard assay condition. The K_m and v_{max} values of amylase were determined for soluble starch, amylose, amylopectin, and glycogen, at concentrations in the range of 0.2–20 mg/mL. The assay conditions were otherwise identical to the standard assay. The experimental data were fitted to the Michaelis–Menten equation using SigmaPlot version 12.0 (Sysstat Software, USA). The k_{cat} value was defined as $v_{max}/[E]$, where $[E]$ is the enzyme concentration (μmol/mL) used.

Determination of hydrolysis products and starch hydrolysis

Purified α-amylase (1.0 U, corresponding to 0.2 U/mg substrate) was incubated with 0.5 % (w/v) of the substrates maltose (G2), maltotriose (G3), maltotetraose (G4), maltopentaose (G5), maltohexaose (G6), starch, amylose, amylopectin, or glycogen in 100 mM Na-phosphate buffer (pH 6.5) at 37 °C for 24 h. The enzymatic reaction was terminated by heating to 100 °C for 10 min prior to investigating the hydrolysis products by TLC using the method of Kanpiengjai et al. (2014b).

The crude extracellular α-amylase was harvested by centrifugation of a 2-L culture, partially purified using the ammonium sulfate precipitation technique, desalted, concentrated using the ultrafiltration technique, and filtered through 0.2 μm filter cartridges for further use. The excess amount of the partial purified enzyme (equivalent to 1000 U/g substrate) was used to hydrolyze 50, 100, and 150 g/L of soluble starch in 100 mM Na-phosphate buffer (pH 6.5) at 37 °C on a 180 rpm rotary shaker for 24 h. The reducing sugars liberated were determined using the DNS method. The degree of polymerization (DP) was calculated as the ratio of reducing sugars liberated compared to the initial soluble starch concentration.

Production of lactic acid from starch by food-grade recombinant *L. plantarum* TGL02

A single colony of *L. plantarum* TGL02 was inoculated into MRS medium and incubated at 37 °C until OD_{600} between 0.3 and 0.4 was obtained. Then, IP was added to the culture with a final concentration of 25 ng/mL, then incubated at 37 °C for 12 h and used as seed inoculum. The inoculum size of 5 % (v/v) was transferred to a modified MRS broth (consisted of (g/L); 10 g peptone, 10 g beef extract, 5 g yeast extract, 2 g K_2HPO_4, 5 $CH_3COONa{\cdot}3H_2O$, 2.0 g di-sodium hydrogen citrate, 1 mL Tween80, 0.2 g $MgSO_4{\cdot}7H_2O$, and 0.2 g $MnSO_4{\cdot}H_2O$)

containing 20 g/L starch as the sole carbon source supplemented with 25 ng/mL of IP. Cultivation of *L. plantarum* S21 in a modified MRS broth containing 20 g/L starch was used as a control treatment. The fermentation was carried out at 37 °C for 24 h. Samples were taken periodically to determine lactic acid, total carbohydrate, and amylase activity. Lactic acid was analyzed by high-performance liquid chromatography according to Kanpiengjai et al. (2014a). Total carbohydrate was determined by phenol–sulfuric method.

Results and discussion

Expression of *L. plantarum* S21 α-amylase-encoding genes

Various expression systems have been developed over the past two decades for the production and secretion of a number of heterologous proteins and the secretory expression of target genes in lactic acid bacteria (Karlskås et al. 2014). The pSIP vectors, which are inducible gene expression vectors that allow inducible protein expression in *L. sakei* and *L. plantarum*, were originally constructed by Sorvig et al. (2005). The gene of interest is driven by a strong promoter from the bacteriocin operons of *L. sakei* based on a quorum-sensing mechanism that is mediated by a secreted peptide-pheromone or induction peptide (IP). The pSIP409 vector is a food-grade pSIP609 vector constructed by Nguyen et al. (2011) in which the erythromycin resistant gene is replaced by an alanine racemase gene for use as a selectable marker.

An ORF of 2733 bp encoding a 910 amino acid protein was identified as a maltose-forming α-amylase. This enzyme possesses unique properties compared with the other amylases reported in *Lactobacillus* sp. (Kanpiengjai et al. 2015). The native gene (accession number KJ440080) includes a signal peptide sequence of 108 bp encoding 36 amino acids located at the *N*-terminus. Two different genes were amplified in this study, including the native α-amylase-encoding gene of *L. plantarum* S21 with its signal peptides sequence (*AmyL7*) and a gene lacking the original signal peptide sequence (*AmyL9*). Both *AmyL7* and *AmyL9* were inserted into the pSIP409 and pSIP609 expression vector by replacing the reporter glucuronidase-encoding gene (*GusA*); then, they were expressed in *L. plantarum* WCFS1 and food-grade *L. plantarum* TGL02. *L. plantarum* WCFS1 harboring both the pAmyL7 vector and *L. plantarum* TGL02 harboring the pAmyL7.1 vector exhibited efficient secretion of α-amylase into the MRS medium (90.6 and 90.1 % production efficiency, respectively). The SDS-PAGE analysis of the extracellular proteins secreted from both recombinants (Fig. 1) was in accordance with

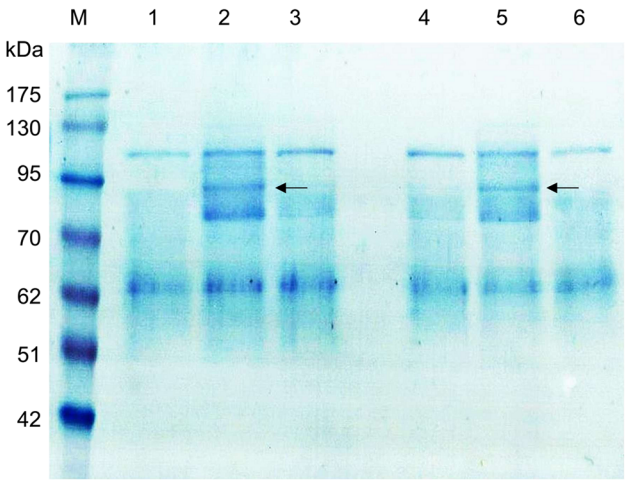

Fig. 1 SDS-PAGE analysis of cell-free supernatant of *L. plantarum* WCFS1 (*lane 1*), *L. plantarum* WCFS1 with (*lane 2*) and without signal peptide construct (*lane 3*), *L. plantarum* TGL02 (*lane 4*), *L. plantarum* TGL02 with (*lane 5*) and without signal peptide construct (*lane 6*) compared with molecular weight marker (*lane M*). *Arrow symbol* indicates the expected size of α-amylase

the quantitative data as shown in Table 1, indicating that the deduced signal peptide of the maltose-forming α-amylase from *L. plantarum* S21 (MKKKKSFWLVSFL VIVASVFFISFGLSNHSNQVAQA) effectively determines the functionality of the amylase secretion and enhances secretion efficiency. The performance of pSIP expression vectors strongly depends on the gene being expressed (Nguyen et al. 2012). The pSIP vectors construct with two signal peptides derived from *L. plantarum* WCFS1 for production and secretion of *Bacillus subtilis* oxalate decarboxylase gene in *L. plantarum* WCFS1 showed 30 and 50 % secretion efficiency for Lp_3050s and Lp_0373s signal peptide, respectively (Sasikumar et al. 2013). No extracellular chitinase was detected from *L. plantarum* WCFS1 with the construct containing complete chitinase-encoding gene of *B. licheniformis* which includes *N*-terminal signal peptide, similar to any of the constructs with three signal peptides derived from the secreted proteins of *L. plantarum* (Lp_2145s, Lp_3050s and Lp_0373s) (Nguyen et al. 2012). In addition, the pSIP vectors constructs with α-amylase-encoding gene of *L. amylovorus* and its signal peptide displayed approximately 69 % secretion efficiency (Mathiesen et al. 2008). Nevertheless, secretory expression of the α-amylase from *L. plantarum* S21 constructs with its signal peptide showed higher secretion efficiency than the mentioned reports. This indicates the high compatibility between the construct of signal peptide and α-amylase gene from *L. plantarum* S21 and the expression host, *L. plantarum* WCFS1. Bacteria have various mechanisms for secretion of proteins. Many protein secretions are Sec-dependent system which consists of a

Table 1 Expression of the amylase-encoding gene in *L. plantarum*

Expression vector	Gene	Extracellular fraction		Intracellular fraction		Secretion efficiency
		Activity (U/L)	Protein (mg/L)	Activity (U/L)	Protein (mg/L)	%
pSIP409	*AmyL7*	2120 ± 31.8	117.7 ± 1.1	221.0 ± 1.1	422.0 ± 18.2	91.6 ± 0.12
	AmyL9	0.0	158.5 ± 7.4	91.9 ± 4.8	464.4 ± 8.6	–
pSIP609	*AmyL7.1*	1984.1 ± 103.6	80.8 ± 6.4	218.2 ± 0.8	364.2 ± 6.5	90.1 ± 0.61
	AmyL9.1	0.0	148.6 ± 3.2	180.3 ± 9.4	537.9 ± 24.7	–
L. plantarum WCFS1		0.0	126.95 ± 8.2	0.0	391.3 ± 10.4	–
L. plantarum TGL02		0.0	105.3 ± 10.7	0.0	376.8 ± 15.6	–

Mean values are presented with standard deviation (SD)

characteristic *N*-terminal signal peptide with specific properties. For Gram-positive bacteria, it was found that secretion efficiency of proteins depends on an optimal combination between signal peptide and secretion target (Mathiesen et al. 2008). Based on the result from this study, the signal peptide sequence obtained from *L. plantarum* S21 could provide an efficient secretory expression of α-amylase in different strain of *L. plantarum*. Hence, the signal peptide is valuable and may be applicable for secretory expression of the amylase-encoding gene in other *Lactobacillus* sp. for effective direct lactic acid production from starch.

The secretion level of the recombinant *L. plantarum* WCFS1 and *L. plantarum* TGL02 was approximately 2000 U/L, which was similar to the result previously reported for *L. plantarum* S21 (Kanpiengjai et al. 2015). This result is in contrast to the result obtained for *L. plantarum* harboring the pAmyL9 vector, where extracellular α-amylase was undetectable; this finding was in agreement with the result of the food-grade *L. plantarum* TGL02 harboring the pAmyL9.1 vector. The pSIP expression vectors have been widely used for the overproduction of intracellular homologous and heterologous proteins. However, in this case, we observed lower than expected intracellular amylase activity (between 90 and 180 U/L) despite our optimization of the expression levels, including the induction time point and induction strength (data not shown). Gene expression and the effectiveness are difficult to predict because there are many influencing factors (e.g., protein folding efficiency and mRNA stability). The effectiveness is moreover limited by the combination of the promoter, the genes of interest, and the expression hosts (Nguyen et al. 2012). In this case, it is suggested that the signal peptide of the amylase derived from *L. plantarum* S21 may involve the expression level of the amylase-encoding gene.

Enzyme purification

In order to confirm the identical properties of the recombinant amylase, a food-grade *L. plantarum* harboring pAmyL7.1 was used for all subsequent studies, including the production, purification, and characterization of α-amylase. The initial extracellular α-amylase activity was 3728 U, and the specific activity was 21.1 U/mg. The enzyme was purified to electrophoretic homogeneity using a 3 step procedure, including precipitation by ammonium sulfate, Q-Sepharose FF, and Q-Sepharose HP. The purified α-amylase with 320 U of activity was recovered and had a specific activity of 500 U/mg and an estimated molecular mass of approximately 100 kDa by SDS-PAGE (Fig. 2).

Properties of recombinant α-amylase

The purified α-amylase was active at pH 6.0 and exhibited more than 80 % relative activity at pH values ranging from 5.0 to 6.5 (Fig. 3A). The enzyme had broad pH stability in the range from 4.0 to 8.0 when incubated at 37 °C for 24 h (Fig. 3B). The optimum temperature of the purified

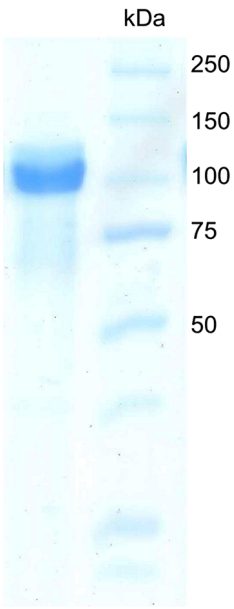

Fig. 2 SDS-PAGE of purified recombinant α-amylase from food-grade *L. plantarum* TGL02

enzyme ranged from 37 to 60 °C, with more than 90 % relative activity under the 10-min standard assay condition (Fig. 3C). Measuring the enzyme's thermostability for 1 h at pH 6.5 indicated that the enzyme retained 100 % of its initial activity at 25–45 °C, 90 % at 50 °C, and 53 % at 55 °C (Fig. 3D). The purified α-amylase exhibited high activity with amylose, soluble starch, amylopectin, and glycogen (Table 2). However, the activity towards raw starch, pullulan, and α- and β-cyclodextrin was undetectable. The K_m values for the aforementioned active substrates were 12.6, 12.1, 12.1, and 35.5 mg/mL, respectively, at 37 °C and pH 6.5 for 10 min. The highest v_{max}, k_{cat}, and k_{cat}/K_m values were obtained from amylose, followed by soluble starch, amylopectin, and glycogen (Table 2). The main hydrolysis products of the α-amylase obtained from the defined oligosaccharides (G3–G6) and amylaceous substrates, including soluble starch, amylose, amylopectin, and glycogen, were maltose as the major product and glucose as the minor product (Fig. 4). In contrast, maltotriose (G3) was partially hydrolyzed to maltose and glucose. Maltose was not cleaved by this enzyme under this condition. The biochemical and catalytic properties of the recombinant α-amylase are in accordance with the properties reported for the *L. plantarum* S21 α-amylase (Kanpiengjai et al. 2015). Therefore, the cloning and expression were successful.

Applications of food-grade *L. plantarum* TGL02 with maltose-forming activity

One of the most important enzyme properties for bioconversion of starch into lactic acid by ALAB is the enzyme efficiency on starch degradation. Although *L. plantarum* S21 is capable of producing lactic acid from high concentration of starch, the active amylase has not been determined for degradation of high concentration of starch. The partially purified α-amylase from *L. plantarum* TGL02 degraded 50, 100, and 150 g/L of soluble starch to liberate 39.7, 81.2, and 115.8 g/L of reducing sugars, respectively (Fig. 5). These values corresponded to approximately 80 % starch hydrolysis. The DP values of the hydrolyzed mixtures decreased within 6 h to a final DP value of 1.3, which was close to the theoretical DP of complete starch hydrolysis (DP = 1.11) (Huang et al. 2005). This finding

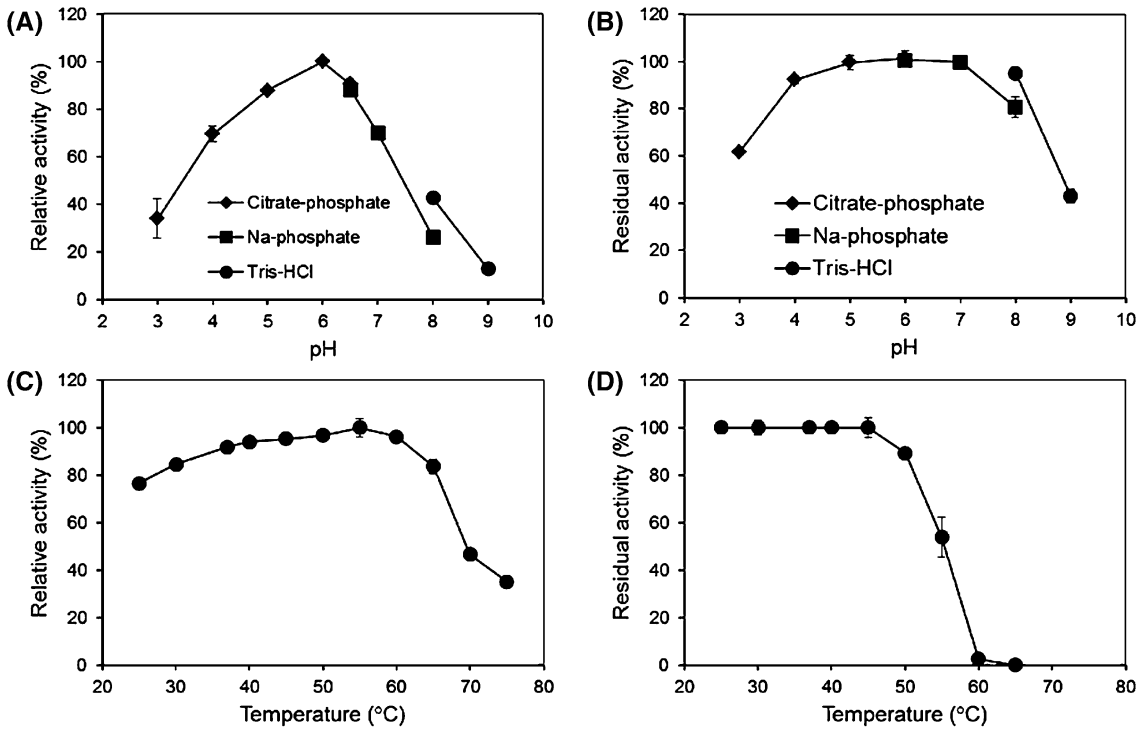

Fig. 3 Characterization of purified recombinant α-amylase. The optimal pH of α-amylase determined at 37 °C in various buffer systems for 10 min with the activity at pH 6.5 set as 100 %; the buffers used included citrate–phosphate buffer (3.0–6.0), Na-phosphate buffer (6.0–8.0), and Tris–HCl (8.0–9.0) (**A**). The pH stability of α-amylase; the enzyme activity was determined at pH 6.5 and 37 °C for 10 min after incubation of the enzyme solution at different pH values (3.0–9.0) at 37 °C for 24 h. The activity at pH 6.5 without incubation was set to 100 % (**B**). The optimum temperature of α-amylase; the enzyme assay was conducted at different temperatures (25–65 °C) at pH 6.5 for 10 min, with the activity at 37 °C set as 100 % (**C**). Thermostability of α-amylase; the enzyme activity was determined at pH 6.5 and 37 °C for 10 min after incubation at different temperatures for 1 h. The activity without incubation was set to 100 % (**D**)

Table 2 Kinetic parameters of purified α-amylase

Substrates	K_m (mg/mL)	v_{max} (μmol/mL min)	k_{cat} (s^{-1})	k_{cat}/K_m (mL/mg s)
Starch	12.1 ± 1.1	369.2 ± 16.2	1826	151
Amylose	12.6 ± 1.5	499 ± 37.6	2468	196
Amylopectin	12.1 ± 1.1	291.2 ± 12.7	1440	119
Glycogen	35.5 ± 3	144.6 ± 8.7	715	20

Mean values are presented with standard deviation (SD)

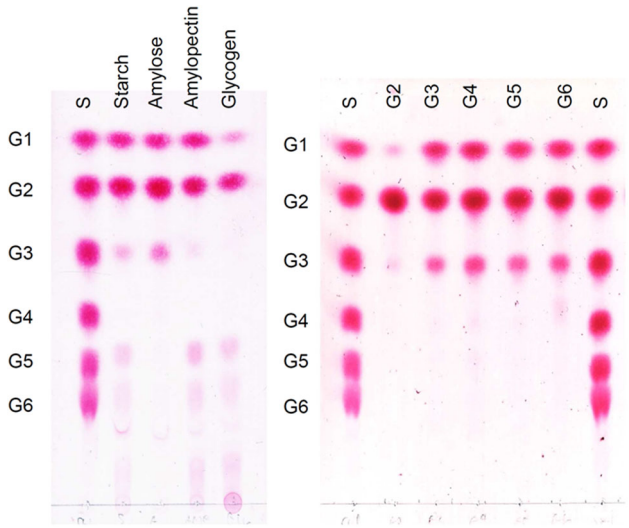

Fig. 4 Thin-layer chromatography (TLC) of hydrolysis products obtained from various amylaceous substrates with purified α-amylase. The enzyme (1.0 U) was incubated with 0.5 % (w/v) of the following substrates at 37 °C for 24 h: starch, amylose, amylopectin, glycogen, maltose (G2), maltotriose (G3), maltotetraose (G4), maltopentaose (G5), and maltohexaose (G6). *Lane S* is the standard glucose (G1) and malto-oligosaccharides (G2–G6)

indicates that this enzyme exhibited an efficiency equivalent to both α-amylase and glucoamylase and it is able to produce large amount of reducing sugars from high concentration of starch. The pH stability and hydrolysis products for this recombinant enzyme indicate the appropriate characteristic for industrial purposes, such as the manufacture of high maltose syrup from starch, brewing, and baking (Sharma and Satyanarayana 2013).

Lactobacillus plantarum WCFS1 is classified to be a homofermentative lactic acid bacterium as it exhibited homolactic acid fermentation pattern when cultivated in glucose (Kleerebezem et al. 2003). Therefore, *L. plantarum* TGL02 harboring pSIPAmyL7.1 was presumably assumed to be a homofermentative ALAB which could convert starch directly to lactic acid similar to our efficient ALAB, *L. plantarum* S21 (Kanpiengjai et al. 2014b). Direct lactic acid production was performed using food-grade *L. plantarum* TGL02 in comparable with *L. plantarum* S21 to evaluate efficient secretion of the maltose-forming α-amylase for lactic acid production from starch (Fig. 6). At level of 20 g/L starch, the recombinant *L. plantarum* TGL02 produced amylase rapidly within 12 h of the fermentation. The starch was converted to 17.3 ± 0.9 g/L lactic acid after cultivated for 24 h, equivalent to a yield of 0.98 g lactic acid/g carbohydrate consumed and 86.5 % production efficiency. These values were not significantly different comparing with those from *L. plantarum* S21

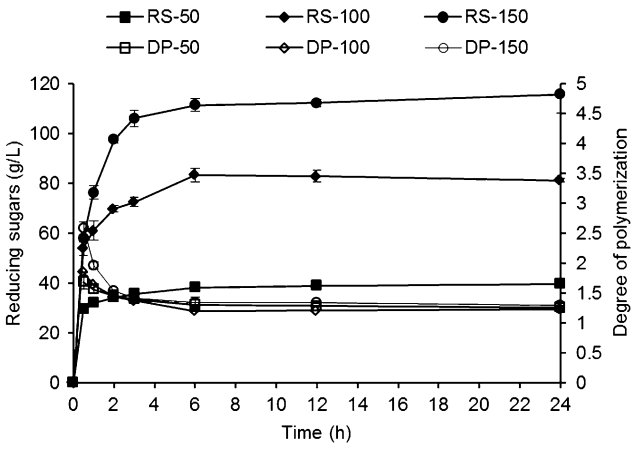

Fig. 5 Profile of reducing sugar (RS) liberated from the hydrolysis of 50, 100, and 150 g/L of soluble starch at 37 °C for 24 h and the degree of polymerization (DP)

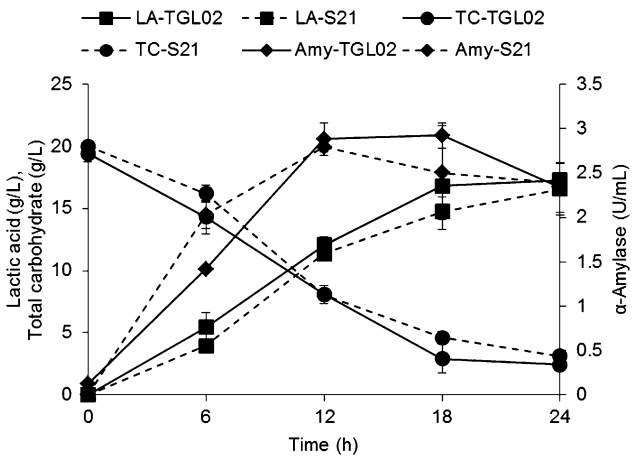

Fig. 6 Profile of lactic acid (LA), total carbohydrate (TC) and amylase activity (Amy) during direct lactic acid production from starch by food-grade *L. plantarum* TGL02 and *L. plantarum* S21 using modified MRS medium containing 20 g/L starch as the sole carbon source at 37 °C for 24 h

(16.5 ± 0.6 g/L lactic acid, 0.98 g/g yield and 82.5 % production efficiency). As the results, the functional signal peptide from *L. plantarum* S21 is sufficient to drive the amylase secretion in *L. plantarum* TLG02, leading to feasible direct lactic acid production from starch. Maltose-forming α-amylase is rarely found in lactic acid bacteria; therefore, success in the cloning, expression of a maltose-forming α-amylase gene in *L. plantarum* with a functional signal peptide sequence, and efficient secretion will serve as a guide for improving the efficient bioconversion of starch to lactic acid in lactic acid bacteria. This study is useful for further secretion of homologous and heterologous proteins in *L. plantarum* WCFS1 and the food-grade derivative as well as other lactobacilli strains that are compatible with pSIP vectors. In addition, a food-grade recombinant *Lactobacillus* sp. capable of producing maltose-forming α-amylase would be very useful for enhancing the nutritional values and increasing the sensory properties of bakery products (Muhialdin et al. 2013).

A maltose-forming α-amylase gene was successfully cloned and expressed in *L. plantarum* WCFS1 and a food-grade *L. plantarum* TGL02 using pSIP vectors. Its amylase signal peptide sequence influences on the enzyme secretion by giving 90 % secretion efficiency in both systems. The recombinant enzyme was confirmed to have identical properties to that of which the wild-type strain produced and also exhibited a good reaction towards soluble starch to high capacity of starch hydrolysis. We also obtained a good model of a new recombinant ALAB for efficient secretion of amylase for direct bioconversion of starch to lactic acid. A food-grade *L. plantarum* harboring amylase gene is expected to be useful for various food and feed applications.

Acknowledgments The authors are grateful to the ASEAN-European Academic University Network (ASEA Uninet) funded by the Austrian Federal Ministry of Science, Research and Economy (BMWFW). This work was also supported by Postdoctoral fellowship granted by Chiang Mai University. We also acknowledge the Thailand Research Fund (RTA 5880006).

Compliance with ethical standards

Conflict of interest No conflict of interest declared.

References

Abdel-Rahman MA, Tashiro Y, Sonomoto K (2010) Lactic acid production from lignocellulose-derived sugars using lactic acid bacteria: overview and limits. J Biotechnol 156:286–301

Aukrust T, Blom H (1992) Transformation of *Lactobacillus* strains used in meat and vegetable fermentations. Food Res Int 25:253–261

Cho MH, Park SE, Lee MH, Ha SJ, Kim HY, Kim MJ, Lee SJ, Madsen SM, Park CS (2007) Extracellular secretion of a maltogenic amylase from *Lactobacillus gasseri* ATCC33323 in *Lactococcus lactis* MG1363 and its application on the production of branched maltooligosaccharides. J Microbiol Biotechnol 17:1521–1526

Huang LP, Jin B, Lant P, Zhou J (2005) Simultaneous saccharification and fermentation of potato starch wastewater to lactic acid by *Rhizopus oryzae* and *Rhizopus arrhizus*. Biochem Eng J 23:265–276

Inoue H, Nojima H, Okayama H (1990) High efficiency transformation of *Escherichia coli* with plasmids. Gene 96:23–28

Kanpiengjai A, Lumyong S, Pathom-aree W, Khanongnuch C (2014a) Starchy effluent from rice noodle manufacturing process as feasible substrate for direct lactic acid production by *Lactobacillus plantarum* S21. J Korean Soc Appl Biol Chem 57:217–220

Kanpiengjai A, Rieantrakoonchai W, Pratanaphon R, Pathom-aree W, Lumyong S, Khanongnuch C (2014b) High efficacy bioconversion of starch to lactic acid using an amylolytic lactic acid bacterium isolated from Thai indigenous fermented rice noodles. Food Sci Biotechnol 23:1541–1550

Kanpiengjai A, Haltrich D, Nguyen T-H, Pathom-aree W, Lumyong S, Khanongnuch C (2015) Characterization of a maltose-forming α-amylase from an amylolytic lactic acid bacterium, *Lactobacillus plantarum* S21. J Mol Catal B Enzym 120:1–8

Karlskås IL, Maudal K, Axelsson L, Rud I, Eijsink VGH, Mathiesen G (2014) Heterologous protein secretion in lactobacilli with modified pSIP vectors. PLoS One 9:e91125

Kleerebezem M, Boekhorst J, van Kranenburg R, Molenaar D, Kuipers OP, Leer R, Tarchini R, Peters SA, Sandbrink HM, Fiers MW (2003) Complete genome sequence of *Lactobacillus plantarum* WCFS1. Proc Natl Acad Sci USA 100:1990–1995

Mathiesen G, Sveen A, Piard JC, Axelsson L, Eijsink VGH (2008) Heterologous protein secretion by *Lactobacillus plantarum* using homologous signal peptides. J Appl Microbiol 105:215–226

Muhialdin BJ, Hassan Z, Saari N (2013) Lactic acid bacteria in biopreservation and the enhancement of the functional quality of bread. In: Marcelino K (ed) Lactic acid bacteria R & D for food, health and livestock purposes. InTech, Rijeka, pp 155–172

Nguyen T-T, Mathiesen G, Fredriksen L, Kittl R, Nguyen T-H, Eijsink VG, Haltrich D, Peterbauer CK (2011) A food-grade system for inducible gene expression in *Lactobacillus plantarum* using an alanine racemase-encoding selection marker. J Agric Food Chem 59:5617–5624

Nguyen HA, Nguyen T-H, Nguyen T-T, Peterbauer CK, Mathiesen G, Haltrich D (2012) Chitinase from *Bacillus licheniformis* DSM13: expression in *Lactobacillus plantarum* WCFS1 and biochemical characterisation. Protein Expr Purif 81:166–174

Sasikumar P, Gomathi S, Anbazhagan K, Selvam GS (2013) Secretion of biologically active heterologous oxalate decarboxylase (OxdC) in *Lactobacillus plantarum* WCFS1 using homologous signal peptides. Biomed Res Int 2013:280432

Sharma A, Satyanarayana T (2013) Microbial acid-stable α-amylases: characteristics, genetic engineering and applications. Process Biochem 48:201–211

Sorvig E, Mathiesen G, Naterstad K, Eijsink VG, Axelsson L (2005) High-level, inducible gene expression in *Lactobacillus sakei* and *Lactobacillus plantarum* using versatile expression vectors. Microbiology (Reading, England) 151:2439–2449

Vishnu C, Naveena BJ, Altaf M, Venkateshwar M, Reddy G (2006) Amylopullulanase—a novel enzyme of *Lactobacillus amylophilus* GV6 in direct fermentation of starch to L(+) lactic acid. Enzym Microb Technol 38:545–550