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**RESEARCH ARTICLE**

# Molecular Cloning and Characterization of a Thermostable α-Amylase Exhibiting an Unusually High Activity

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**Abstract** An α-amylase gene was cloned from the thermophilic bacterium *Bacillus subtilis* isolated from Indonesian oil palm shell waste. The gene expressed an extracellular enzyme. Optimal hydrolysis conditions for the enzyme were 70ºC and pH 6.0. The specific activity of the enzyme was 16.0 kU per mg of protein, which was higher than for other thermostable amylases. Hydrolytic products of the enzyme using starch and glycogen were mainly maltohexaose and maltopentaose. The enzyme had a $K_m$ value of 0.099 mg/mL for amylopectin, more than 10 times lower than for amylose. The catalytic efficiency of the enzyme using amylopectin was 39,200 mL/mg·s and was 3,270 mL/mg·s using amylose. The enzyme liquefied corn starch at pH 5.0, which was successfully converted to glucose using commercial glucoamylase and pullulanase without pH adjustment. The enzyme has advantages for industrial applications.

**Keywords:** thermostable α-amylase, kinetic parameter, starch liquefaction, molecular cloning, *Geobacillus stearothermophilus*

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## Introduction

α-Amylase (EC 3.2.1.1) is the representative enzyme in the glycoside hydrolase family 13 (GH 13) (1) and is an amylolytic enzyme that hydrolyzes starch and related polysaccharides by hydrolyzing internal α-1,4-glycosidic linkages. The enzyme is widely distributed in various species from bacteria to mammals.

Starch is the most abundant carbon energy source on earth and consists of amylose (glucose polymers mostly linked by α-1,4-linkages) and amylopectin (glucose polymers linked by approximately 95% α-1,4-linkages, and 5% α-1,6-branch linkages). A huge amount of starch from plants is processed for use in the textiles, paper, and food industries. Sugar conversion from corn starch is one of the most important processes in the starch industry (2).

α-Amylase has been used for starch liquefaction for decades, and the properties of the enzyme have been improved for industrial applications. In the conventional method, a mixture of starch slurry and enzyme is jet-cooked at 105ºC-110ºC to partially breakdown starch molecules and reduce viscosity, and then cooled to 95ºC to continue α-amylase hydrolysis (2-4). Many commercial enzymes that are stable at a temperature of 95ºC or above have been developed by the enzyme industry from the α-amylase of *Bacillus licheniformis* (BLA). The wet-milling industry, however, is looking for further improvements in the properties of α-amylase, including thermostablity at low pH values with no calcium ion. Thermostable α-amylases isolated from *B. stearothermophilus* (BSTA), *B. amyloliquefacience* (BAA), and from fungi are widely used for starch hydrolysis in the food industry (2).

To develop new enzymes for industrial application, α-amylases from thermophilic archaea and bacteria have been screened and investigated worldwide. Several enzymes from *Thermococcus* and *Bacillus* species, which are thermophilic bacteria, have been reported in the past decades (5-10). Lee *et al.* (1996) reported an extracellular α-amylase from *T. profunus* possessing thermostability at 80°C (11). Ben Ali *et al.* (6,12) reported characterization of a hyper-thermostable α-amylase from *B. stearothermophilus* US100, (the enzyme is hereafter referred to as US100), which produced maltohexaose.

Crude palm oil and palm kernel oil are produced by squeezing oil from the oil palm mesocarp or kernels, leaving behind the non-oil substance consisting mainly of the non-starch polysaccharides cellulose, hemicellulose, and mannan. In most cases, these materials are discarded as "organic waste" in large piles that slowly decompose and generate heat. Inside the pile, the temperature can reach up to 80°C and, if enough flammable gas (hydrogen and methane) is produced during the "hot-composting", the pile can ignite and burn spontaneously. Therefore, this heated pile is an ideal ecological niche from which to isolate mannanolytic thermophilic microbes. In this study, a thermostable α-amylase was cloned from a bacterium isolated from decomposed Indonesian oil palm shell waste (13). The properties of the thermostable α-amylase were investigated using biochemical studies, reaction product analysis, and kinetics. Results were compared with commercial thermostable α-amylases.

## Materials and Methods

**Chemicals and enzymes** Chemicals used in this study were of reagent grade and purchased from Sigma-Aldrich (St. Louis, MO, USA) and Fisher Scientifics (Pittsburgh, PA, USA). Enzymes for DNA manipulation were purchased from Promega (Madison, WI, USA) and Stratagene (La Jolla, CA, USA).

**Cloning of the α-amylase gene** An α-amylase gene was isolated from the thermophilic bacterium *Geobacillus stearothermophilus* L07 (13,14). *Escherichia coli* DH5α was used as a host for transformation. *E. coli* was cultured in Luria-Bertani medium (10 g/L of tryptone, 5 g/L of yeast extract, 5 g/L of sodium chloride) supplemented with ampicillin at 100 μg/mL and kanamycin at 50 μg/mL. The genomic DNA of *G. stearothermophilus* L07 carrying the target DNA was extracted using the CTAB-method (15). The gene encoding α-amylase was amplified using the primers BsteamyF (*Nde*I, 5'-CATATGGTGCTAACGTTTC ACCGCATC-3'; restriction site is underlined) and BsteamyR (*Bgl*II, 5'-AGATCTTCAAGGCCATGCCACC AACC-3'; restriction site is underlined). These primers were designed based on the *G. stearothermophilus* TISTR 1517 α-amylase sequence (GenBank accession no. DQ852663). The amplified PCR product was digested using restriction enzymes and ligated into the T&A cloning kit vector (Real Biotech Corporation, Taipei, Taiwan). The inserted DNA was sequenced using an ABI PRISM 3100-Avant Genetic Analyzer (Applied Bioscience, Foster City, CA, USA). The *G. stearothermophilus* L07 amylase gene was subcloned into pET15b(+).

To express and purify the *G. stearothermophilus* L07 α-amylase enzyme (hereafter referred to as L07), the gene encoding L07 was subcloned into the *Bacillus-E. coli* shuttle vector pLip (Takara Korea Biomedical, Seoul, Korea) (16) The pET15b(+)-L07 and pLip vectors were digested using *Nde*I and *Hind*III restriction enzymes and isolated using agarose gel electrophoresis. The insert and vector were mixed at a molar ratio of 1 to 3 and ligated at 25°C for 3 h. The ligation mixture was transformed in *Escherichia coli* BL21(DE3) (*fhuA2* [*lon*] *ompT gal* (*λ DE3*) [*dcm*] Δ*hsdS λ DE3=λ sBamHIo* Δ*EcoRI-B int::* (*lacI::PlacUV5::T7 gene1*) *i21* Δ*nin5*) and the transformant was screened using a solid medium containing ampicillin (100 μg/mL). The recombinant plasmid, designated as pLip-L07, was extracted and sequenced.

**Protein expression and purification** pLip-L07 was transformed in *B. subtilis* LKS87 and cultured in a medium composed of 3.3% Bacto tryptone, 2% Bacto yeast extract, 0.74% NaCl, 0.8% $Na_2HPO_4$, 0.4% $KH_2PO_4$, 2% casamino acids, and 0.06 mM $MnCl_2$ supplemented with kanamycin (80 μg/mL) for 32 h at 37°C. Cells were removed by centrifugation (4°C and 10,000×*g*), and the supernatant was supplemented with $CaCl_2$ (5 mM) and incubated at 75°C for 40 min. Denatured proteins were removed by centrifugation (4°C and 10,000×*g*), and the supernatant was fractionated using 70% ammonium sulfate. Precipitated proteins were recovered by centrifugation (4°C and 8,000×*g*) and redissolved in a Tris-HCl buffer solution (pH 7.5, 25 mM). The soluble fraction was dialyzed using Tris-HCl buffer (pH 7.5, 25 mM) to more than 100,000× the volume of the enzyme solution, which was then applied to a diethylaminoethyl (DEAE)-cellulose (Sigma-Aldrich, St. Louis, MO, USA) column to remove pigments that originated from the culture medium.

**Determination of molecular mass** The molecular mass of the purified enzyme was determined using matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF MS, Voyager DE-PRO; Perceptive Biosystems, Framingham, MA, USA). Amounts of 1 μL of purified L07 (0.2-0.25 mg/mL) and 1 μL of the prepared matrix [1% sinapinic acid in 50%(v/v) acetonitrile]

were mixed and applied to a sample plate. The plate was then loaded into the MS instrument and the molecular mass of the sample was analyzed under conditions of a grid voltage of 87, a grid wire voltage of 0.3, delayed extraction for 300 ns, and a laser intensity of 2,000.

**α-Amylase activity assay** The activity of α-amylase was determined using a soluble-starch solution (1%, w/v, 50 mM sodium acetate, pH 6.0) as the substrate at 70°C for 10 min in the presence of 5 mM $CaCl_2$ and 0.2 mg/mL of bovine serum albumin (BSA). The reducing sugar amount was measured using the 3,5-dinitrosalicylic acid (DNS) method (17), and a standard curve was constructed using maltose solutions of different concentrations. An amount of 1 unit was defined as the amount of enzyme that produced 1 μmol of reducing sugar in 1 min.

For kinetic studies, enzyme solutions were diluted using a MOPS buffer (pH 7.5, 50 mM) containing 5 mM $CaCl_2$ and 0.2 mg/mL of BSA. Either amylose or amylopectin (400 mg) was dissolved in 20 mL of 90%(v/v) dimethyl sulfoxide with continuous stirring and heating at 100°C for 1 h. A reaction mixture of amylose was prepared at a final concentration of 0.2-1.5 mg/mL. The concentration of amylopectin was 0.075-0.5 mg/mL. The L07 enzyme solution (0.132 U) was added to the preheated reaction mixture (1,000 μL) at 70°C. During a reaction time of 2 min and 40 s, 100 μL of the reaction mixture was withdrawn at intervals of 20 s and mixed with the same volume of ice-cold 0.1 N NaOH to stop the reaction. The amount of reducing sugars in the reaction aliquot was measured using a copper-bicinchoninate reducing-value method with a microplate reader (BioTek Instrument, Inc., Winooski, VT, USA) (18). Kinetic parameters were determined using a Lineweaver-Burk plot.

**Starch liquefaction and glucose production** Liquefaction of starch (pH 5.0) was conducted using 5%(w/v) normal corn starch (Cargill, Minneapolis, MN, USA). The L07 α-amylase (0.3 U/mg of substrate) was added to a starch slurry containing 5 mM $CaCl_2$ at room temperature. The reaction mixture was heated to 75°C using a shaking water bath (120 stroke/min). After liquefaction, AMG and Promozyme (Novozyme A/S, Bagsvaerd, Denmark) were added for saccharification at 60°C.

**Reaction product analysis** Reaction patterns of L07 with different substrates were analyzed using linear maltododextrins (from maltotriose to maltoheptaose), polysaccharides (amylose, amylopectin, and glycogen), and cyclodextrins (α-, β-, and γ-CD). The L07 enzyme at 0.01 U of enzyme/mg of substrate and 0.025 U of enzyme/mg of substrate for amylose, amylopectin, and glycogen was used and incubated with substrates at pH 6.0 and 70°C for 18 h.

TLC was used to analyze reaction products with a Whatman silica-gel TLC plates (K5F; Whatman, Clifton, NJ, USA). After spotting the samples, the TLC plate was placed in a TLC-developing chamber containing a developing solvent mixture (acetonitrile:ethyl acetate:1-propanol:water =85:20:50:50, by volume). Separation of the samples on a TLC plate was carried out once and detected by dipping the plate in a methanol solution containing 3 g/L of *N*-(1-naphthyl)-ethylenediamine and 50 mL/L of concentrated $H_2SO_4$, then the plate was heated in an oven at 120°C for 15 min.

The composition of the L07 hydrolysis end products was determined using high performance anion exchange chromatography (HPAEC; Dionex, Sunnyvale, CA, USA). The instrument was equipped with CarboPac-PA1 and stabilized with a 0.1 N NaOH solution. An elution buffer (0.1 N NaOH and 300 mM sodium acetate) was applied from 0% to 60% over 30 min. Sugar peaks were compared with respective standard reference compounds, and peak areas were converted to a concentration (w/v). Relative concentrations of each component were calculated as a percentage of the sum of all components.

## Results and Discussion

**Cloning, sequence analysis, protein expression, and purification of the L07 α-amylase** The thermophilic bacterium *Geobacillus stearothermophilus* L07 that exhibited high mannanase and amylase activities was isolated from decomposed Indonesian old palm shell waste (13). 16S rRNA gene analysis indicated that this isolated bacterium was closely retalated to *Geobacillus stearothermophilus* (14). An approximately 1.6 kb α-amylase gene was amplified from the L07 genome, and subsequently cloned into an *E. coli* vector to generate recombinant plasmid pTN201 that was used as a template for further subcloning experiments.

The full sequence of the L07 α-amylase gene was deposited in GenBank (acession no. KC763474). Using the Basic Local Alignment Search Tool (BLAST; http://blast.ncbi.nlm.nih.gov/Blast.cgi) and a literature search, the BSTA (19-22) and US100 enzyme sequences (6,12,23,24) had high sequence similarities (approximately 99%) to the L07 sequence (data not shown). The amino acid sequence of L07, however, showed 24 amino acids missing at the C-terminal. The L07 sequence had a C-domain that was similar in length to the C-domain of the BLA, BAA, and *Bacillus* sp. 707 α-amylase sequences. BSTA, with an optimal temperature of 70°C and pH of 6.0, has been studied extensively and used for industrial starch processes for decades (20). US100 was first reported by Ben Ali *et al.* in 1999 (6) and produces G5 and G6 from starch. The

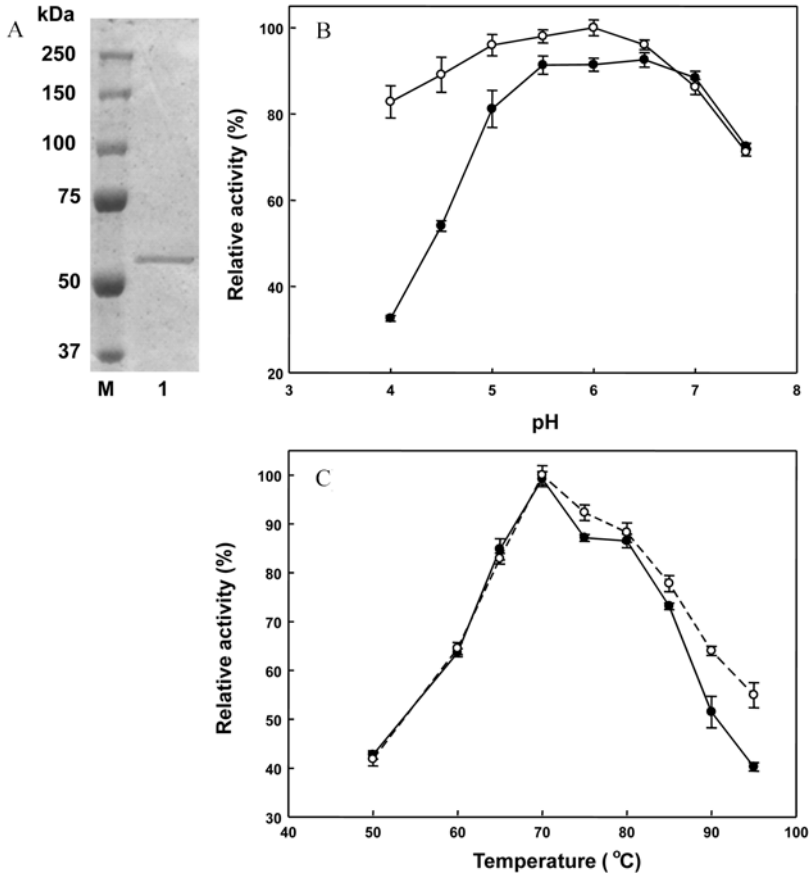


**Fig. 1. (A) SDS-PAGE analysis of purified L07.** Lane M, a size marker; lane 1, purified L07 after DEAE-cellulose chromatography. pH (B) and temperature (C) profiles of L07. Buffers with 50 mM sodium acetate (pH 4.0-6.0) and 50 mM MOPS (pH 6.5-7.5) were used for the assay in the presence (open circle) and absence (closed circle) of 5 mM $CaCl_2$. Error bars indicate standard deviations.

optimal reaction conditions for the US100 enzyme are similar to the conditions for BSTA, and both enzymes need $CaCl_2$ for thermostability.

The L07 α-amylase was secreted into the culture medium by *B. subtilis* LKS87. After heat treatment of the cell-free culture medium to denature other proteins, the activity of the L07 α-amylase in the culture medium was determined to be 37.8 U/mL.

After purification, the L07 enzyme solution was concentrated and analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and showed a single band in the gel (Fig. 1A). The molecular weight of the purified L07 enzyme, determined using MALDI-TOF-MS, was 55.7 kDa, which was the same as the molecular mass calculated from the primary sequence. In the absence of BSA, the specific activity of the L07 enzyme using soluble starch as a substrate was 9,278 U/mg. With BSA, however, the enzyme activity was 15,950 U/mg, more than 1.5× greater than without BSA. Results showed that addition of 0.2 mg/mL of BSA stabilized the enzyme during reaction at 70°C. Ben Ali *et al.* (12) reported the activity of the US100 α-amylase to be 1.8 U/mg of protein, and the activity of α-amylases from *Thermococcus* were 1,102-1,143 U/mg of protein (11,25). There is no report in the literature about the specific activity of wild type BSTA. Most bacterial α-amylases; however, have specific activities below 5,000 U/mg of protein. The L07 enzyme had an unusually high specific activity, compared with other reported α-amylases.

**pH and temperature profiles of the L07 enzyme** The calcium dependency of the L07 enzyme for thermostability was evaluated before determination of the biochemical properties. Results showed that the minimum calcium ion concentration for thermostability of L07 was 5 mM (data not shown). Figure 1B and 1C show that the optimal reaction conditions were pH 5.5-6.5 and 70°C-80°C without $CaCl_2$. The enzyme showed starch hydrolysis activity in broad ranges of pH and temperature. Maximal activity was observed at pH 6.0 and 70°C. In the presence of 5 mM $CaCl_2$, L07 had an activity similar to the activity without $CaCl_2$ at temperatures 70°C. However, the activity was greater than without $CaCl_2$ at temperatures above 70°C (Fig. 1C). L07 retained approximately 80% of the maximum activity at pH 4.0 in the presence of 5 mM $CaCl_2$. Without calcium, the enzyme had only 35% of the maximum

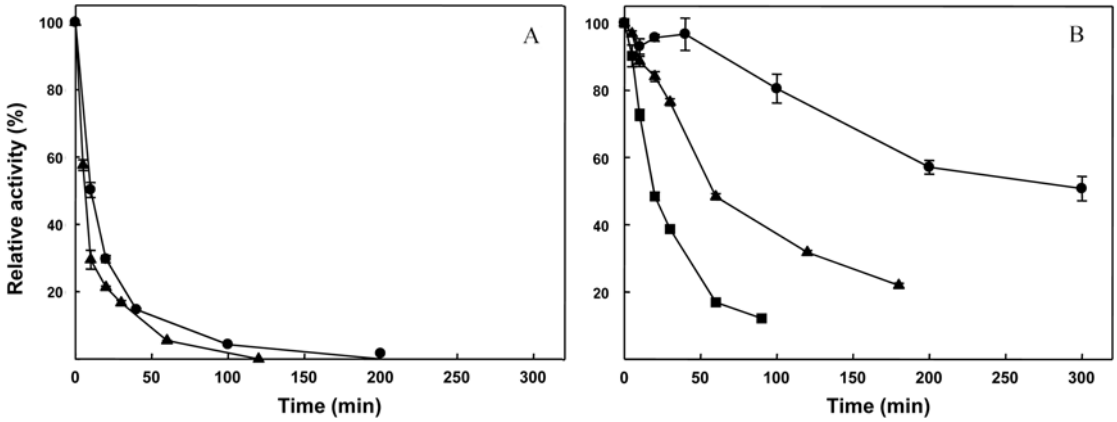


**Fig. 2. Thermostability of L07.** Thermal inactivation of the enzyme at different pH values (pH 5.0, A; pH 6.0, B) was evaluated in the presence of 5 mM $CaCl_2$. Temperatures were 80°C (circle), 90°C (triangle), and 95°C (square). The residual activity of L07 was determined as a percentage of the initial activity. Error bars indicate standard deviations.

activity at the same pH. The calcium ion is a common ligand that is essential for stabilizing the thermostable enzymes BLA, BSTA, and BAA (4,20,26). Amino acid sequence homology between these 3 enzymes is 65%-81%, and the optimal pH values of the enzymes are between 6.0 and 7.0 (20). Despite the significant homology in the amino acid sequences, the optimal temperatures of the enzymes differ (BSTA, 70°C-80°C; BLA, 90°C-100°C; and BAA, 60°C) (27,28). Results indicated that the optimal reaction conditions of L07 were similar to the conditions for BSTA.

**Thermostability of L07** The thermostability of L07 at higher than the optimal temperatures was determined at pH 5.0 and 6.0 with 5 mM $CaCl_2$. At 80°C and pH 5.0, the enzyme rapidly denatured and had a lower activity, compared with the activity at pH 6.0 (Fig. 2). The half life of the enzyme was approximately 10 min at pH 5.0. At pH 6.0; however, the enzyme retained more than 50% of the initial (initial activity is proper term for this case) activity after 5 h of incubation at 80°C. These results showed that pH is critical to the thermostability of the enzyme.

At 90°C, the enzyme quickly lost activity (Fig. 2). At pH 5.0, L07 had less than 10% of the initial activity after 60 min of incubation. The half life of the enzyme at pH 6.0 greatly decreased from 5 h at 80°C, to 1 h at 90°C. At 95°C, the enzyme had a half life of 20 min (Fig. 2B). The enzyme was hardly detectable after 10 min of incubation at pH 5.0 and 95°C because of rapid denaturation (data not shown).

The thermostable enzymes BLA, BAA, BSTA, and US100 all require $CaCl_2$ for thermostability (2,4,21,23,26,29-31). These four enzymes have optimal pH values between 5.6-7.0 for starch hydrolysis. Industrial starch processes use thermostable and low pH-stable α-amylases. To date, most known α-amylases, including industrial liquefying enzymes, are unstable at pH values as low as 5.0 (3). L07 showed tendencies of thermostability similar to BLA, BAA, BSTA, and US100. The thermostability of L07 was comparable to the thermostability of BSTA and US100, but much lower than the value for BLA. Comparison of the thermostablility of L07 at pH 5.0 with other enzymes was difficult because of limited available information. Khemakhem *et al.* (32) reported that US100 retains more than 50% of the initial enzyme activity after 80 min of incubation at the optimal pH and 90°C. At the same temperature, the half-lives of BSTA and BLA are 40 min and 270 min, respectively (33). Protein engineering has been used to improve the thermostability of α-amylases (2-4,34). To improve the thermostability of L07, site-directed mutagenesis can be used in future experiments.

**Reaction patterns of the L07 enzyme using various substrates** L07 did not hydrolyze short maltodextrins [i.e., maltotriose (G3) to maltopentaose (G5)] (Fig. 3A) and the enzyme barely hydrolyzed maltohexaose (G6). Results indicated that maltoheptaose (G7) was the minimum length maltodextrin that was usable as a substrate for the enzyme. G7 was hydrolyzed mainly to G5 and maltose (G2). The large polysaccharides amylose, amylopectin, and glycogen were hydrolyzed to produce G2-G7. Amylose was almost completely hydrolyzed after 18 h at pH 6.0 and 70°C. Branched polysaccharides; however, were partially hydrolyzed to the small G2 and G7 maltodextrins with limit dextrins remaining at the origin. The limit dextrins contained α-1,6-branch linkages that could not be hydrolyzed by L07.

When cyclodextrins (CDs) were used as a substrate (Fig. 3B), the enzyme did not hydrolyze α-, β-, and γ-CD to a linear maltodextrin. Most bacterial α-amylases do not hydrolyze CDs (35). Maltogenic amylase, one of the GH13 family members; however, does efficiently hydrolyze CDs

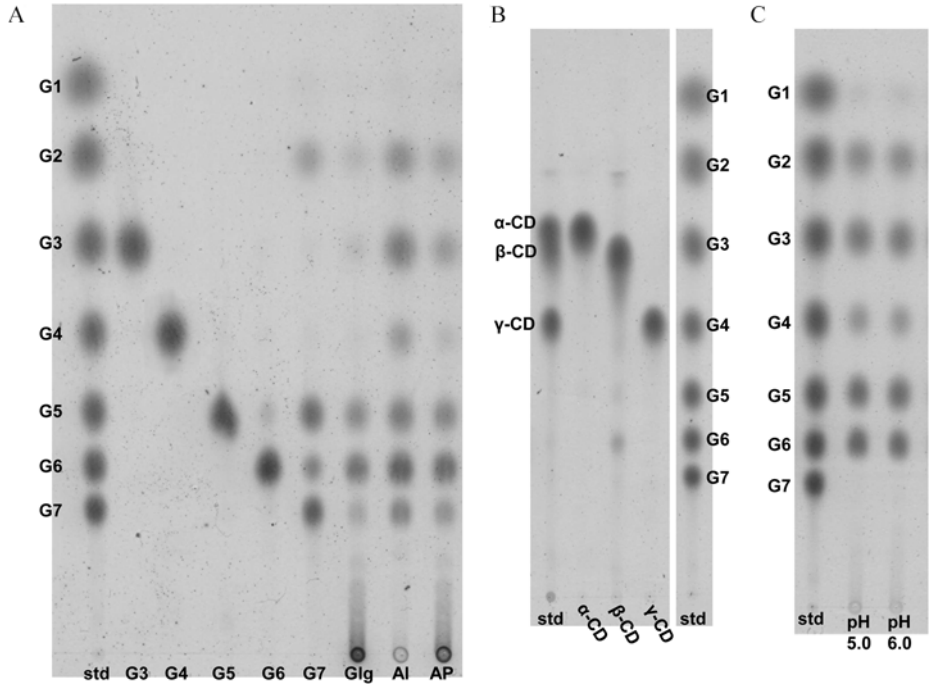


**Fig. 3. Reaction products hydrolyzed by L07 using different substrates.** (A) The linear maltodextrin reaction products from G3 to G7, glycogen (Glg), amylose (Al), and amylopectin (AP) at pH 6.0 and 70°C for 18 h. (B) Cyclodextrins used as a substrate under the same conditions as for (A) and (C) with amylose as a substrate. Hydrolysis was performed at 60°C for 72 h at different pH values.

to linear maltodextrins (35). These results showed that L07 exhibited the characteristics of a typical α-amylase.

To analyze the final products of amylose hydrolysis by L07, the reaction was extended to 72 h at 60°C and the reaction products were analyzed using TLC (Fig. 3C). Results showed that G7 completely disappeared at both pH 5.0 and 6.0 after 72 h. G6, G5, G3, and G2 were the main products found using TLC, along with a small amount of G4. The composition of the end products was also determined using HPAEC (Table 1). Results showed that there was a slightly higher degree of total hydrolysis at pH 6.0, compared with pH 5.0, perhaps due to the greater thermostability of the enzyme at pH 6.0. L07 had similar end product compositions at pH values of 6.0 and 5.0 (Table 1). It was clear that the pH value did not change the hydrolysis pattern of the enzyme. Extensive hydrolysis using L07 produced G5, G6, and G3 as major products (Table 1), similar to the products of other liquefying enzymes, especially US100 (6,12,23). US100 produces G6 and G5 as major products from amylose, but BLA, BSTA, and BAA all produce smaller end products than L07 (23,36).

**Kinetics of the L07 enzyme using amylose and amylopectin as substrates** To understand the kinetic behavior of L07 on different types of substrates, kinetic studies were conducted using amylose and amylopectin as substrates. Results revealed that the enzyme $K_m$ value for amylose was more than 10x higher than for amylopectin (Table 2). L07 showed similar turnover numbers ($k_{cat}$) for amylose and amylopectin. The catalytic efficiency ($k_{cat}/K_m$) values of the enzyme using amylose and amylopectin were 3,270 mL/mg·s and 39,200 mL/mg·s, respectively. According to differences in substrate affinity ($K_m$) values, L07 used amylopectin more efficiently than amylose, perhaps due to the highly branched structure of amylopectin in which branch chains are readily available to the enzyme for hydrolysis. This selectivity could be significant when the substrate concentration is close to the $K_m$ value. Kinetic parameters of thermostable α-amylases have been reported

**Table 1. End product analysis of L07 using amylose as a substrate[1)]**

| | pH | Product composition (%, w/w) | | | | | |
|---|---|---|---|---|---|---|---|
| | | G1 | G2 | G3 | G4 | G5 | G6 |
| L07 | 5.0 | 2.49 | 15.9 | 19.9 | 8.75 | 24.4 | 28.6 |
| | 6.0 | 2.62 | 16.2 | 20.7 | 9.10 | 28.0 | 23.5 |

[1)]Reaction products were analyzed using HPAEC.

**Table 2. Kinetic parameters of L07 using amylose and amylopectin as substrates**

| Substrate | L07[1)] | | Data from previous studies[2)] | | |
|---|---|---|---|---|---|
| | | | BLA | | BAA |
| | Amylose | Amylopectin | Soluble starch[a] | Soluble starch[b] | Amylose[c] |
| $K_m$ (mg/mL) | 1.07±0.09 | 0.099±0.016 | 0.073 | 0.68 | 2.34 |
| $k_{cat}$ ($s^{-1}$) | 3,490±430 | 3,870±230 | 185 | - | - |
| $k_{cat}/K_m$ (mL/mg·s) | 3,270±160 | 39,200±3900 | 2,540 | - | - |

[1)]Mean±standard deviation
[2)]Reactions were carried out at 30°C and pH 6.0 (28)[a], 25°C and pH 6.0 (37)[b], or 50°C and pH 5.0 (9)[c].

**Table 3. Liquefaction of corn starch using L07 and subsequent saccharification of glucose production using AMG and Promozyme**

| | Liquefaction reaction time (h) | | | |
|---|---|---|---|---|
| | 1.5 | 2.0 | 2.5 | 3.0 |
| Liquefaction using L07: DE value (%) | 19.9±0.6 | 20.5±0.1 | 21.5±0.5 | 22.2±0.9 |
| After saccharification: Glucose yield (%, w/w) | 94.3±1.3 | 94.1±1.0 | 91.6±1.9 | 92.7±0.3 |

(9,28,37). The reported reaction conditions were; however, different from the present study and between the reported studies. Therefore, direct comparisons of kinetic parameters between L07 and other enzymes were not possible (Table 2). L07 showed a relatively lower $K_m$ value for amylose than for BAA. The turnover number of L07 was significantly higher than for BLA, even though soluble starch was used for BLA. The unusually high specific activity of L07 might be due to a much greater turnover number for the enzyme than for other thermostable α-amylases.

**Application of the L07 enzyme for starch liquefaction**
To improve the conventional starch conversion process, starch liquefaction analysis using the L07 α-amylase at pH 5.0 was performed. After liquefaction, saccharification was followed without pH adjustment to pH 4.2-4.5, the optimal conditions for glucoamylase and pullulanase. Results showed that L07 liquefied a starch slurry, and the dextrose equivalent (DE) value of the liquefied starch was 20-22 (Table 3). Addition of glucoamylase and pullulanase without pH adjustment achieved a glucose yield of approximately 94.3%.

Commercial α-amylases are unstable at pH values less than 6.0 (2). Therefore, conventional liquefaction is conducted at pH 6.0 using BLA and/or BSTA (3,20). After liquefaction, saccharification is performed at pH 4.2-4.5 by adding acids to decrease the pH because glucoamylase and pullulanase are inactivated at pH 6.0. Addition of acids results in a considerable concentration of salts in the reaction products. In the wet-milling industry, salts in the product must be removed using an additional process that increases the production cost (3). In conclusion, a thermostable α-amylase was cloned from the thermophilic bacterium *G. stearothermophilus* L07 isolated from decomposed Indonesian oil palm shell waste. The enzyme had an unusually high specific activity and biochemical properties similar to commercial α-amylases. Results indicated that the L07 enzyme is superior to recently cloned thermostable α-amylases for use industrial starch processes.

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