



Low molecular weight alkaline thermostable α -amylase from *Geobacillus* sp. nov.



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ABSTRACT

Industrial demands for enzymes that are stable in a broad range of conditions are increasing. Such enzymes, one of which is α -amylase, could be produced by extremophiles. This study reports a thermostable α -amylase produced by a newly isolated *Geobacillus* sp. nov. from a geothermal area. The phylogenetic analysis of the 16S rRNA gene showed that the isolate formed a separate branch with 95% homology to *Geobacillus* sp. After precipitation using ammonium sulphate followed by ion-exchange chromatography, the enzyme produced a specific activity of 25.1 (U/mg) with a purity of 6.5-fold of the crude extract. The molecular weight of the enzyme was approximately 12.2 kDa. The optimum activity was observed at 75 °C and pH 8. The activity increased in the presence of Ba²⁺ and Fe²⁺ but decreased in the presence of K⁺ and Mg²⁺. Ca²⁺ and Mn²⁺ increased the activity slightly. The activity completely diminished with the addition of Cu²⁺. EDTA and PMSF also sharply reduced enzyme activity. Although the stability was moderate, the low molecular weight could be an important feature for its future applications.

1. Introduction

α -amylase (α -1-4 D-glucan glucanohydrolase, EC 3.2.1.1) is an enzyme with the ability to catalyse the hydrolysis of the α -1-4 glycosidic bonds in starch molecules, producing simple molecules containing glucose units (Zhang et al., 2017). The enzyme has been a great interest in food, detergent, pharmacy, textile, paper and bioethanol industries (Souza and Magalhaes, 2010). Those that can retain the activities over a broad range of pH and temperature, as well as in the presence of denaturant are increasingly used for industrial applications because it reduces costs, increases substrate solubility, and reduces contamination from mesophilic microorganisms. Such enzymes can be produced by extremophiles, i.e. microorganisms that live in extreme conditions (Elleuche et al., 2014).

Increasing industrial demand for thermostable α -amylase has become a driving force for the steady growth of studies to discover new variants of the enzyme with better thermal and pH stability, as well as low molecular weight (Jeon et al., 2014; Jung et al., 2014; Sindhu et al., 2017; Sudan et al., 2018). Small enzymes are easy to modify, thus give advantages in the immobilisation process or genetic manipulation, and subsequently could increase enzyme selectivity (Cowana and

Fernandez-Lafuente, 2011). Most α -amylases are produced by *Bacillus* (Zhang et al., 2017), while those showing thermostability are also often produced by *Bacillus* or *Geobacillus* (Xie et al., 2014; Fincan et al., 2014; Jiang et al., 2015; Sudan et al., 2018; Du et al., 2018; Wu et al., 2018). Most microorganisms produce thermostable α -amylase with a molecular weight in the range of 21–160 kDa and most work well on acidic pH. α -amylase with low molecular weight and active on alkaline pH is rarely reported (Mehta and Satyanarayana, 2016).

With regards to the need of novel α -amylases to meet the industrial demands, this study reported the purification and characterization results of α -amylase produced by a newly isolated microorganism from a geothermal hot spring in the area of Jaboi Sabang, Aceh Province, Indonesia. The microorganism was identified by 16s rRNA gene sequence alignment. The enzyme purification was done using ammonium sulphate precipitation and anion exchange chromatography. The molecular weight was determined using SDS PAGE, while the characters of the enzyme were measured at various temperatures, pH and in the presence of metal ions and small organic molecules. The substrate preference of the enzyme was also studied. The enzyme is expected to have a better performance at extreme environmental conditions.

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2. Materials and methods

2.1. Regeneration of microorganism

The microorganism was isolated from a geothermal hot spring in Jaboi area, Sabang, Aceh Province, Indonesia and hereinafter referred to as strain Jaboi Sabang Isolate (JSI). Strain JSI was initially screened on a ½ LB medium. The microorganism has been one of the culture collections of the Biochemistry Laboratory Syiah Kuala University. For this study, strain JSI from glycerol stock was initially incubated on a solid medium containing 0.8% polypeptone, 0.4% yeast extract, 0.2% NaCl and 3% agar, at 70 °C for 18 hours.

2.2. Biochemical characterization and genotypic identification of JSI

Biochemical characterization of strain JSI was done by aseptically inoculating a single colony from the solid medium as in Section 2.1 to the API 50 CHB medium. After proper mixing, the suspension was transferred into the API 50 CHB ampules, then incubated at 70 °C for 24 hours. The change in colours from red to yellow indicated positive results. The results were analysed by Apiweb™ software.

A single colony from the solid medium as in Section 2.1 was transferred to a liquid medium containing 0.8% polypeptone, 0.4% yeast extract and 0.2% NaCl, incubated at 70 °C and 150 rpm for 24 hours. The culture was then centrifuged at 10000×g for 10 minutes. The bacterial genome was isolated from the pellets using isolation kit (Genetika Science) and used as the template to amplify the 16S rRNA gene. PCR was performed using a pair of primers, i.e. Bac27F [5'-AGA GTT TGA TCC TGG CTC AG-3'] and Uni1492R [5'-GGT TAC CTT GTT ACG ACT T-3']. The PCR consisted of 30 cycles of denaturation at 95 °C for 5 minutes, annealing at 55 °C for 1 minute and a final extension at 72 °C for 2 minutes. The amplicon was sequenced by direct sequencing method (Macrogen Inc.). The 16S rRNA gene sequence was compared with GenBank entries by BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The alignment was performed using the CLUSTAL W program (<http://www.ebi.ac.uk/Tools/msa/clustalw2>). Phylogenetic analysis was conducted using the Molecular Evolutionary Genetics Analysis (MEGA) version 6.06 (Tamura et al., 2013). The gene sequence of 1464 bp was deposited in the NCBI with an accession number of KC599211.

2.3. Production of α-amylase

A single colony from the solid medium as in Section 2.1 was transferred to a liquid medium containing 2% soluble starch, 4% tryptone, 0.5% KH₂PO₄, 0.3% MgSO₄·7H₂O, 0.2% CaCl₂ and 0.1% FeSO₄·7H₂O. The medium was then incubated at 70 °C and 150 rpm for 24 hours. The crude enzyme was obtained in the supernatant after centrifugation at 10000×g and 4 °C for 15 minutes.

2.4. Purification of α-amylase

2.4.1. Ammonium sulphate precipitation

The crude enzyme was initially precipitated by ammonium sulphate with saturation levels of 0–20%, 20–40%, 40–60%, 60–80%, and 80–100% at a cold temperature (Scopes, 1987). For each saturation level, the solution was stirred for 10 minutes and settled overnight at 4 °C. The precipitate was isolated by centrifugation at 10000×g, 4 °C for 10 minutes and dialyzed overnight in cellophane membrane using 20 mM tris-HCl buffer pH 8. The buffer was changed several times until no indication of sulphate in the solution.

2.4.2. Anion exchange chromatography

Anion exchange column chromatography (DEAE Sepharose Fast Flow) was employed to further purify the ammonium sulphate fraction showing the highest specific activity. The column was initially calibrated with 0.01M Tris-HCl buffer pH 8.0. The enzyme (5 mL) was then eluted

using the same buffer containing NaCl gradient 0.15, 0.3, 0.6, and 1.0 M (25 mL each) at an elution rate of 1.5 mL/min. Every 2 mL of eluent was collected as a fraction, subjected to α-amylase activity and protein concentration assays.

2.5. Determination of α-amylase activity and protein concentration

The α-amylase activity was determined by measuring the amount of reducing sugar released by Dinitrosalysilic Acid method, referring to the standard procedure of Miller (1959), using a standard curve of glucose (1–10 µg/mL) measured at 540 nm. One enzyme unit (U) is defined as the amount of enzyme required to release 1 µmol reducing sugar per minute under the assay conditions. All measurements were done in triplicate. The enzyme activity was calculated using the following formula.

$$\text{Activity} \left(\frac{\text{U}}{\text{mL}} \right) = \frac{[\text{reducing sugar}] \mu\text{mol}}{L} \times \text{dilution} \times \frac{1 L}{1000 \text{ mL}} \times \frac{1}{t \text{ incubation}}$$

Protein concentration was determined by the Bradford method (Bradford, 1976), using a standard curve of Bovine Serum Albumin (125–1500 µg/mL) measured at 595 nm. All measurements were done in triplicate.

2.6. Determination of the molecular weight

The molecular weight of the α-amylase was determined by SDS-PAGE using ThermoScientific Spectra™ Multicolour Broad Range Protein Ladder 10–250 kDa, referring to the procedure of Laemmli (1970). The concentration of stacking and separating gel was 4% and 12%, respectively. Electrophoresis was run in 0.05M phosphate buffer pH 7 at 20 mA for 2 hours. The gel was stained using the Pierce® Silver Staining Kit.

2.7. Characterization of α-amylase

The activity of the purified α-amylase was characterized by varying the temperature (55, 60, 65, 70, 75 and 80 °C) and pH (6, 7, 8, and 9). The buffers for pH study on protease activity were made using 0.1M sodium acetate (pH 5–6), 0.1M dipotassium hydrogen phosphate (pH 7) and 0.1M glycine-NaOH (pH 9.0). Substrate specificity study was done at optimum temperature and pH by using 1% soluble starch, amylose and amylopectin. The results were presented as a relative activity, which is the ratio of the activities on any substrates to that on soluble starch. The effect of metal ions was observed by adding Ba²⁺, Fe²⁺, Mn²⁺, Ca²⁺, Mg²⁺, K⁺, Cu²⁺ to a final concentration of 10 mM. The effect of 10 mM EDTA and PSMF on enzyme activity was also studied. All treatments were subjected to amylase activity and protein concentration assays, as described in Section 2.5. All results were compared to that of the control (without the addition) and presented as a relative activity.

2.8. Statistical analysis

Student's t-test, using SPSS version 18, was performed to determine the significant difference of enzyme activity data in the temperature and pH study.

3. Results and discussion

3.1. Identification of strain JSI

Strain JSI was an aerobic microorganism, Gram-positive, and rod-shaped with a size of 0.5–1.0 × 3.5–6.0 µm. The colonies had circular cream-coloured with smooth edges, 3–5 mm in diameter. It grew optimally at 70 °C and pH 7.0 using yeast extract as the carbon source.

The biochemical test was conducted to evaluate the ability of strain

JSI in fermenting 49 types of carbohydrate. The results show that it produced acids using various sizes of carbohydrate monomers, i.e. 3C (Glycerol), 5C (L-Arabinose, D-Ribose, D-Xylose), and 6C molecules (D-Glucose, D-Fructose, D-Mannose D-mannitol). The fermentation also occurred on disaccharides (D-Maltose) and polysaccharides (starch and glycogen). The analysis indicated that strain JSI has the closest relationship to *Bacillus subtilis* with a score of 77.9%. The value is well below the threshold of 95–96%, indicating that strain JSI could be a distinct species (Kim et al., 2014). The biochemical profile was also compared with biochemical tests of six *Bacillus* and *Geobacillus* strains (Table 1) that were used in constructing the phylogenetic tree (Fig. 1). Strain JSI performed many different biochemical reactions than the other strains.

Homological analysis of 16S rRNA gene showed that strain JSI related

Table 1

Characteristics that differentiate strain JSI from closely related species of *Bacillus* and *Geobacillus*.

Characteristic	1	2	3	4	5	6	7
Control	-	ND	ND	ND	ND	ND	ND
Glycerol	+	+	+	+	+	-	-
Erythritol	-	-	-	ND	ND	ND	ND
D-Arabinose	-	-	-	ND	ND	ND	ND
L-Arabinose	+	+	+	-	W	-	-
D-Ribose	+	+	+	-	ND	ND	ND
D-Xylose	+	+	+	+	W	V	+
L-Xylose	-	-	-	ND	ND	ND	ND
Adonitol	-	-	-	-	ND	V	+
Methyl-D Xylopyranoside	-	-	-	ND	ND	ND	ND
D-Galactose	-	+	+	-	-	+	V
D-Glucose	+	+	+	ND	ND	+	+
D-Fructose	+	+	+	ND	ND	+	+
D-Mannose	+	+	+	ND	ND	ND	ND
L-Sarbose	-	W	-	ND	ND	ND	ND
L-Rhamnose	-	W	-	+	-	-	-
Dulcitol	-	-	-	ND	ND	ND	ND
Inositol	-	+	+	-	-	-	+
D-Mannitol	+	+	+	+	ND	+	+
D-Sorbitol	-	+	+	+	-	-	-
Methy- α -D-Mannopyranoside	-	-	-	ND	ND	ND	ND
Methyl- α -D-Glucopyranoside	-	+	+	ND	ND	ND	ND
N-Acetylglucosamine	-	+	+	ND	ND	ND	ND
Amygdalin	-	+	+	ND	ND	ND	ND
Arbutin	-	+	+	ND	ND	ND	ND
Esculine Ferric Citrate	+	+	+	+	+	ND	ND
Salicine	+	+	+	ND	ND	ND	ND
D-Cellobiose	-	+	+	+	-	+	+
D-Maltose	+	+	+	ND	ND	ND	ND
D-Lactose (Bovine)	-	+	+	-	-	-	-
D-Melibiose	+	+	+	ND	ND	ND	ND
D-Sucrose	-	+	+	ND	ND	ND	ND
D-Trehalose	-	+	+	ND	ND	ND	ND
Inulin	-	+	+	ND	ND	ND	ND
D-Melezitose	-	-	-	ND	ND	ND	ND
D-Raffinose	-	+	+	ND	ND	ND	ND
Starch	+	+	+	+	+	+	+
Glycogen	+	+	+	ND	ND	ND	ND
Xylitol	-	-	-	ND	ND	ND	ND
Gentiobiose	-	+	+	ND	ND	ND	ND
D-Turanose	-	-	+	ND	ND	ND	ND
D-Lycose	-	-	-	ND	ND	ND	ND
D-Tagatose	-	-	-	ND	ND	ND	ND
D-Fucose	-	-	-	ND	ND	ND	ND
L-Fucose	-	-	-	ND	ND	ND	ND
D-Arabitol	-	-	-	ND	ND	ND	ND
L-Arabitol	-	-	-	ND	ND	ND	ND
Potassium Gluconate	-	+	-	ND	ND	ND	ND
Potassium 2-Ketogluconate	-	-	-	ND	ND	ND	ND
Potassium 5-Ketogluconate	-	+	-	ND	ND	ND	ND

Strains: 1. JSI; 2. *Bacillus subtilis* (Logan and Berkeley, 1984); 3. *Bacillus amylo-liquefaciens* (Logan and Berkeley, 1984); 4. *Geobacillus thermoglucosidasius* DSM 2542^T (Nazina et al., 2004); 5. *Geobacillus stearothermophilus* (Sung et al., 2002); 6. *Geobacillus thermoleovorans* (Rahman et al., 2007); 7. *Geobacillus kaustophilus* (Rahman et al., 2007); (+) Positive; (-) Negative; (W) Weakly positive; (ND) Not determined. (V) Variable within the group.

to *Geobacillus* with the highest homology of only 95% (Table 2). The phylogenetic tree showed that strain JSI formed a new branch (Fig. 1). Microorganisms with the closest similarity to JSI are (1) Uncultured organism clone ELU0028-TI75-S 000004 (HQ749614.1), (2) *Geobacillus* sp-SBS-4S (AB3056519.1), and (3) *Bacillus* sp. SP93 (JQ808140.1). The similarity of 99% indicates that the microorganism could be of the same species, while 97%–99% similarity suggests that the species could be in the same genus. The low similarity is a strong indication that the species is theoretically new (Drancourt et al., 2000). It is thus suggested that strain JSI is a novel species of the genus *Geobacillus*, for which the name *Geobacillus jabois* sp. nov. is proposed.

3.2. Purification of α -amylase

Precipitation of the crude extract with ammonium sulphate (60–80%) gave the highest purification level, with a specific activity of 13.7 U/mg and a purity of 3.6-fold of the crude enzyme. Further purification using DEAE Sepharose Fast Flow chromatography increased the specific activity to 25.1 U/mg with a purity of 6.5-fold (Table 3). However, the yield in each purification step was low, which is unexpected as a study using similar methods to purify α -amylase from *Bacillus methylotrophicus* strain P11-2 give 4.2-fold purity and 39.1% yield (Xie et al., 2014).

Diethylaminoethyl (DEAE), cross-linked to agarose beads, is a weak anion exchanger. Other studies using DEAE Cellulose Fast Flow, instead of DEAE Sepharose, also produce a considerably high yield. For example, ammonium sulphate precipitation followed by DEAE Cellulose Fast Flow chromatography of α -amylase from *B. amyloliquefaciens* BH072 give 4.2-fold purity and 39.1% yield (Du et al., 2018) while α -amylase from *B. megaterium* VUMB109 shows 6.97-fold purity and 85.6 % yield (Jana et al., 2013). Ion exchange chromatography is typically followed by size exclusion chromatography to increase the purity (Jana et al., 2013; Xie et al., 2014; Du et al., 2018), although it was not conducted in this study.

SDS-PAGE result shows that the molecular weight of the purified enzyme was about 12.2 kDa (Fig. 2). The weight is substantially lower than other microbial α -amylases, which generally have molecular weight well above 20 kDa (Mehta and Satyanarayana, 2016). The one produced by *B. megaterium* VUMB109 even has a molecular weight of 150 kDa (Jana et al., 2013). The molecular weight of some α -amylases from *Geobacillus* is also high. For example, the molecular weight of α -amylase from *Geobacillus thermoleovorans* varies from 26 kDa (Rao and Satyanarayana, 2007) to 51 kDa (Sudan et al., 2018), while *Geobacillus* sp. LH8 produced α -amylase of 52 kDa (Mollania et al., 2010).

α -amylases are part of glycoside hydrolases (GH-H) superfamily and mostly belong to the GH13 family. Based on the similarity of the amino acid sequence of the catalytic domains, α -amylase may also be part of the GH57, GH119 and GH126 families (Zhang et al., 2017). Those in the GH13 family have a single polypeptide chain of about 500 residues. The structure comprises three domains (A, B, and C). Domain A forms a (β / α) 8 TIM barrel containing the catalytic residues, which is positioned in the centre of the protein structure. Domains B and C are adjacent to Domain A in the opposite direction. Most of the known α -amylases contain calcium-binding site within Domain B, which is projected towards the surface between Domains A and B (Offen et al., 2015). The function of Domain C is unknown, although it is associated with the role of raw starch binding (Mehta and Satyanarayana, 2014).

Considering the low molecular weight of α -amylase from strain JSI, it seems that only small conserved regions in the structure define the catalytic activity. As long as they are intact, the enzyme remains active. It is thus speculated that the intact structure in the enzyme was predominantly in Domains A, while Domain B was probably truncated as there is no increased activity with the addition of Ca²⁺ ion (see explanation in Section 3.4). Domain C is thought to be non-existence. The enzymes in GH57 family support this assumption, as they are smaller α -amylases due to an incomplete TIM barrel but still showing the enzyme activity (Blesak and Janecek, 2012). Interestingly, GH57 family is predominantly produced by extremophiles (Li et al., 2013). However, further experiments

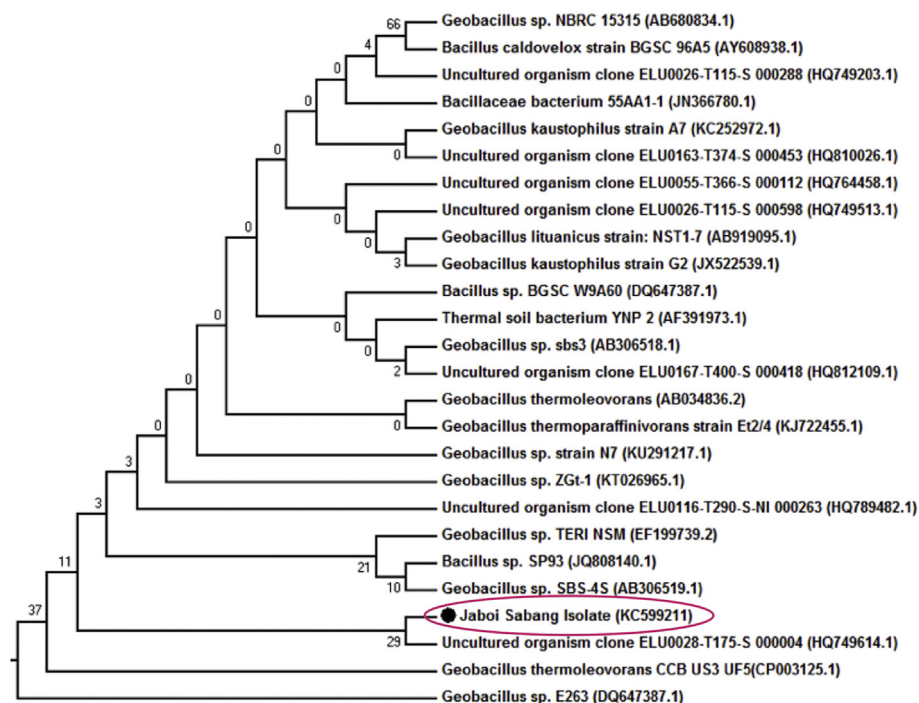


Fig. 1. Phylogenetic relationships of the 16S rRNA gene sequence of the JSI using the neighbour-joining method of the MEGA6.06 with 1000 bootstrap replications.

Table 2

Twenty-five highest homologous with 16S rRNA gene sequence of strain JSI.

Accession Number	Description	Similarity
CP003125.1	<i>Geobacillus thermoleovorans</i> CCB US3 UF5	95 %
DG647387.1	<i>Geobacillus</i> sp. E 263	95%
HQ749614.1	Uncultured organism clone ELU0028-T175-S	95%
AB306519.1	<i>Geobacillus</i> sp.-SBC-4S	95%
JQ808140.1	<i>Bacillus</i> sp. sp93	95%
EF199739.2	<i>Geobacillus</i> sp. TERINSM	95%
HQ789482.1	Uncultured organism clone ELU0116T290	95%
KT026965.1	<i>Geobacillus</i> sp. ZGt-1	95%
KU291217.1	<i>Geobacillus</i> sp. strain N7	95%
KJ722455.1	<i>Geobacillus</i> sp. thermo paraffinivorans strain tt2/4	95%
AB034836.1	<i>Geobacillus thermoleovorans</i>	95%
HQ812109.1	Uncultured organism clone ELU0167-T400.S	95%
AB306518.1	<i>Geobacillus</i> sp. SGS3	95%
AF391973.1	Thermal soil bacterium YNP 2	95%
DQ647387.1	<i>Bacillus</i> sp. BGSC W9A60	95%
JX522539.1	<i>Geobacillus kaustophilus</i> strain G2	95%
AB919095.1	<i>Geobacillus lituanicus</i> strain NST1-7	95%
HQ749513.1	Uncultured organism clone ELU0026-T115-S	95%
HQ764458.1	Uncultured organism clone ELU0055-T366.S	95%
HQ810026.1	Uncultured organism clone ELU0163-T374.S	95%
KC252972.1	<i>Geobacillus kaustophilus</i> strain A7	95%
JN366780.1	<i>Bacillaceae</i> bacterium 55AA1.1	95%
HQ749203.1	Uncultured organism clone ELU0026-T115.S	95%
AY608938.1	<i>Bacillus caldovelox</i> strain BGSC96A5	95%
AB680834.1	<i>Geobacillus</i> sp. NBRC15315	95%

are needed to confirm if α -amylase from strain JSI belongs to other GH family or it is a novel family by its own.

3.3. The effect of temperature and pH on the α -amylase activity

The activity of purified amylase was measured in a temperature range of 55–85 °C (in 0.1M phosphate buffer, pH 7.0). The results show that the enzyme has a short range of thermostability. The highest α -amylase activity was observed at 75 °C (25.5 U/mL). The activity was still considerably high at 60–70 °C (17.8–23.5 U/mg). It dropped to 14.9 U/mg at 80 °C and almost diminished at 55 °C (Fig. 3A). The result is comparable

Table 3

Purification of α -amylase from strain JSI.

Steps	Total activity (Units)	Total protein (mg)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude enzyme	6708	1738	3.86	1.00	100
Ammonium sulphate fraction 60–80%	10.1	0.73	13.7	3.6	0.15
DEAE Sepharose Fast Flow	28.3	1.13	25.1	6.5	0.42

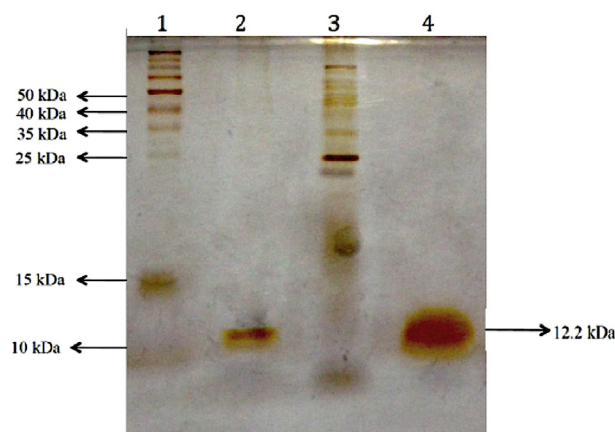


Fig. 2. SDS PAGE results of α -amylase from JSI (1) Protein Marker, (2) Crude enzyme, (3) Ammonium sulphate fraction 60–80%, (4) DEAE Sepharose Fast Flow chromatography.

to those from *G. thermoleovorans* at 80 °C (Sudan et al., 2018), from *Geobacillus* sp. LH8 at 80 °C (Mollania et al., 2010) and from *B. methylotrophicus* strain P11-2 at 70 °C (Xie et al., 2014). A very high

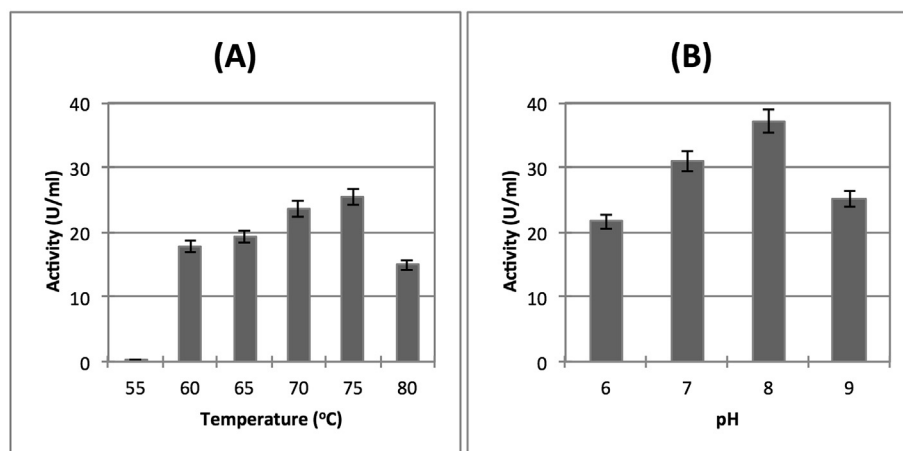


Fig. 3. The activity of α -amylase from JSI purified by DEAE Sepharose Fast Flow chromatography. (A) Effect of temperature (at pH 7.0), and (B) Effect of pH (at 75 °C). The assay was conducted using 1% soluble starch and 10 minutes of incubation time. The values were average of triplicates (error bars = \pm SD). There is a difference for all temperature and pH for a confidence level of 95% ($p < 0.05$).

optimum temperature (100 °C) is reported for α -amylase from *B. licheniformis* B4-423 (Wu et al., 2018) isolated from hot springs. The optimum temperature for enzyme action is generally correlated to the temperature of the area from which the microorganisms are isolated. The strain JSI was isolated from an area with an onset temperature of about 80 °C, which is in agreement with the optimum temperature for its α -amylase activity. However, this is not always the case, as sometimes enzyme from mesophiles also shows thermostability (Ribera et al., 2017).

The pH profiles of amylase were measured at a pH range of 6–8 at 75 °C. The highest specific activity of 37.2 U/ml was observed at pH 8 (Fig. 3B), while the activity at pH 7.0 and 9.0 was 83% and 67% of the optimum value, respectively. Thermostable α -amylases are mostly reported to have an optimum activity in the range of neutral to acidic pH (Mollania et al., 2010; Fincan et al., 2014; Jiang et al., 2015; Sudan et al., 2018). Some studies show that some amylase may work well at pH above 10 (Chakraborty et al., 2011; Murakami et al., 2008). However, those that work well at alkaline pH and temperature at least as high as that in this study are rare. Several examples of the enzyme with these characters include α -amylase from *Bacillus* sp. that shows an optimum activity at 110 °C and pH 8 (Pancha et al., 2010) and from *Bacillus* sp. ANT-6, which is optimum at 80 °C and pH 10.4 (Burhan et al., 2003).

3.4. The effect of metal ion and organic compounds

The addition of monovalent and divalent metal ions shows that the presence of Ba^{2+} and Fe^{2+} increased the activity to 158% and 123% of the control, respectively, while Ca^{2+} , Mn^{2+} only slightly increased the activity. In contrast, the addition of Mg^{2+} and K^+ reduced the activity significantly while the activity was diminished in the presence of Cu^{2+} (Fig. 4).

Most α -amylases require metal ions as cofactors to stabilize the structure or mediate the catalytic process and, in general, have a high affinity for Ca^{2+} (Mehta and Satyanarayana, 2014; Li et al., 2016). Ca^{2+} may increase thermal stability in some α -amylases by binding to the non-catalytic sites, as the numbers of bound Ca are more prevalent in thermophiles than mesophiles (Hiteshi and Gupta, 2014). However, some α -amylases are Ca-independent and, in some cases, inhibited by Ca^{2+} (Sudan et al., 2018; Du et al., 2018; Mehta and Satyanarayana, 2016; Xie et al., 2014). The presence of Ca^{2+} ions is also able to increase stability across a long incubation period (Pan et al., 2019).

Although affected by Ca^{2+} only slightly, α -amylase from strain JSI could be still a Ca-dependent enzyme. The addition of EDTA and PSMF significantly reduced the α -amylase activity, with residual activity of 56% and 77%, respectively (Fig. 4). Both compounds are known as

α -amylase inhibitors. EDTA acts by removing Ca^{2+} from the catalytic sites and is thought to be a non-competitive inhibitor (Burhan et al., 2003). Meanwhile, PSMF inhibits the enzyme by disrupting the seryl hydroxyl groups in the structure (Jiang et al., 2015).

3.5. Substrate preference

The result of substrate preference study shows that the enzyme preferred soluble starch to amylose or amylopectin. The use of amylose and amylopectin decreased the activity to 55% and 43%, respectively, relative to that of soluble starch (Table 4). Similar results have been reported for α -amylase from *Halorubrum xingjiangense* that show better activity when using soluble starch than amylose, amylopectin, dextrin or glycogen (Moshfegh et al., 2013). Its activity disappears when using α -cyclodextrin, β -cyclodextrin and pullulan. This indicates that α -amylase from strain JSI may be an endolytic enzyme which hydrolysed the α -1, 4-glycosidic bonds in the interior of polysaccharide molecules (Shafiei et al., 2010).

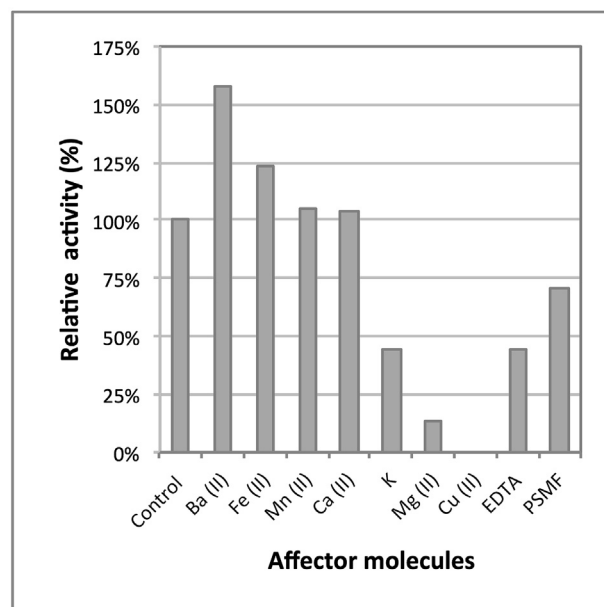


Fig. 4. Effect of metal ions and inhibitors on α -amylase activity from JSI. The assay was conducted using 1% soluble starch with the addition of 10 mM metal ions at pH 8 and 75 °C.

Table 4
Substrate preference for α -amylase from strain JSI.

Substrate	Relative Activity (%)
Soluble starch	100
Amylose	55
Amylopectin	43

Starch is a complex molecule, and its physicochemical properties are defined by the ratio of amylose and amylopectin, degree of polymerization, as well as the ratio of crystalline and amorphous structures. Therefore, the enzyme activity on starch from various sources may give different results. The activity of starch hydrolysis is highly determined by the particle size, molecular branching, crystalline structure, and interior pore of the starch (Dhitala et al., 2015; Guo, 2018).

4. Conclusion

Strain JSI was a new microorganism within the genera of *Geobacillus*. The name of *Geobacillus jabois* sp. nov. is proposed. The α -amylase produced by strain JSI had a low molecular weight of 12.2 kDa, which is rarely reported. Small molecules enzyme provides advantages, as its gene would be more easily manipulated. Small genes are also easier to handle, particularly in recombinant techniques. The enzyme could be more easily immobilised, particularly on nanomaterials, to increase enzyme selectivity and reusability.

A combination of optimum activity at 75 °C and pH 8.0 makes the enzyme unique because such a combination is seldom reported. This, combined with small molecular weight, could be utilized for specific industrial needs. It may be a suitable enzyme for detergent industries to enhance the detergents ability to remove starchy food residues.

Declarations

Author contribution statement

Febriani, Teuku M. Iqbalsyah: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Rayyana, Mildatul Ulya: Performed the experiments.

Frida Oesman, Akhmaloka: Analyzed and interpreted the data.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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