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Heterologous expression, characterization, and application of recombinant thermostable α-amylase from *Geobacillus* sp. DS3 for porous starch production

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ABSTRACT

Novel *Geobacillus* sp. DS3, isolated from the Sikidang Crater in Dieng, exhibits promising characteristics for industrial applications, particularly in thermostable α-amylase production. Recombinant technology was used to express thermostable α-amylase in *E. coli* BL21(DE3) to overcome high-temperature production challenges. The study aimed to express, purify, characterize, and explore potential applications of this novel enzyme. The enzyme was successfully expressed in *E. coli* BL21(DE3) at 18 °C for 20 h with 0.5 mM IPTG induction. Purification with Ni-NTA column yielded 69.23 % from the initial crude enzyme, with a 3.6-fold increase in specific activity. The enzyme has a molecular weight of ±70 kDa (±58 kDa enzyme+11 kDa SUMO protein). It exhibited activity over a wide temperature range (30–90 °C) and pH range (6–8), with optimal activity at 70 °C and pH 6 with great stability at 60 °C. Kinetic analysis revealed Km and Vmax values of 324.03 mg/ml and 36.5 U/mg, respectively, with dextrin as the preferred substrate without cofactor addition. As a metalloenzyme, it showed the best activity in the presence of Ca^{2+}. The enzyme was used for porous starch production and successfully immobilized with chitosan, exhibiting improved thermal stability. After the fourth reuse, the immobilized enzyme maintained 62 % activity compared to the initial immobilization.

1. Introduction

α-amylase enzymes are of significant interest in the biotechnology industry due to their potential applications in various industrial processes. α-amylase is an enzyme that catalyzes the hydrolysis of starch into smaller carbohydrates, such as glucose, maltose, and dextrin by hydrolyzing the glycosidic bonds present in starch molecules [1], which are widely used in the production of biofuels, food, and pharmaceuticals.

Novel sources of enzymes are continuously explored to discover certain enzymes with distinguished characteristics for appropriate enzymatic processes such as thermostability, halophilicity, and pH-stable enzymes. Enzymes can be obtained from several sources whether from animal cells, plants, or even microorganisms. Microbial enzymes are preferred as they have higher yields and are easier to produce. Indonesia, located in the Ring of Fire volcanic belt, possesses about 40 % of the world's geothermal reserves, including volcanoes and hot springs [2]. This unique geological setting provides a significant opportunity to explore novel sources of thermostable enzymes.

For instance, Ardhiansyah [3] discovered a novel thermophilic bacteria, *Geobacillus* sp., isolated from Sikidang Crater in Dieng, Indonesia. Witasari et al. [4] found that *Geobacillus* sp. DS3 thrives optimally at temperatures between 50 and 70 °C. Further studies revealed that *Geobacillus* sp. DS3 produces thermostable enzymes: Phon et al. [5] identified a thermostable protease with optimum activity at 70 °C and pH 9.6, and Widiana et al. [6] discovered a thermostable α-amylase with optimum activity at 50 °C and pH 7. These findings highlight the immense potential of *Geobacillus* sp. DS3 from Sikidang Crater in Dieng, Indonesia, as a novel source of thermostable enzymes.

Producing thermostable enzymes at high temperatures with native thermophilic microorganisms can be energy-intensive. To address this, recombinant DNA technology is used to produce these enzymes at

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mesophilic temperatures, which require less energy. In this study, the thermostable α-amylase gene of *Geobacillus* sp. DS3 from Sikidang Crater in Dieng, Indonesia is cloned and expressed in *E. coli* BL21(DE3) expression host, followed to be purified and characterized to give a deeper understanding of the characteristics of recombinant thermostable α-amylase from novel *Geobacillus* sp. DS3 for future application in industry.

One promising application of the recombinant thermostable α-amylase is in the production of porous starch. Porous starch contains numerous pores from the surface to the center of the granules, making it an effective adsorbent or encapsulant. Porous starch has significant potential in the food, pharmaceutical, and environmental industries. It can be used as an adsorbent, a drug delivery system, an encapsulant for microspheres and nanoparticles, and a flavor carrier in food products [7]. Enzymatic methods are preferred for producing porous starch due to better process control and higher yields compared to other methods, such as freeze-thaw or solvent exchange [7,8]. This makes the recombinant thermostable α-amylase an excellent candidate for porous starch production.

For industrial applications, the reusability of biocatalysts is essential for cost-effectiveness. To enhance reusability, the expressed recombinant enzyme will be immobilized. Chitosan, a natural polymer derived from the deacetylation of chitin, is ideal for immobilization due to its renewability and low cost[9]. Chitosan could be used for immobilization with the activation step such as the addition of glutaraldehyde as the hydroxyl and amino groups of chitosan can make chemical bonds with the carboxyl group of the enzyme [10,11] thus, making chitosan has a high affinity with protein.

Therefore, this research aimed to give a comprehensive study starting from the heterologous expression of the thermostable α-amylase, characterization of the enzyme, and followed by the potential application of the recombinant α-amylase for porous starch production and its potential for immobilization with chitosan matrix.

2. Materials and methods

2.1. Transformation of plasmid

One tube of 50 μl of competent cells was thawed at room temperature, 5 μl vector DNA pET-SUMO was added and mixed by flicking the tube. The tube was then incubated for 30 min on ice. Heat shock was performed by incubating the tube for 45 s to 1 min in a 42 °C water bath and put immediately on ice for 2 min. After that, 950 μL of SOC medium (2 % tryptone 0.5 % yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM D-glucose) was added and then incubated at 37 °C in a shaking incubator (100 rpm) for 1 h. Then, 100 μL of the transformation reaction were plated on LB agar supplemented with 50 μg/mL kanamycin using a sterile spreader and then incubated for 15–18 h at 37 °C.

2.2. Expression of recombinant thermostable α-amylase

A single colony of *E. coli* BL21(DE3) carrying pET-SUMO-amy on the selection plates was picked and inoculated to a 10 ml tube of LB broth supplemented with 50 μg/mL kanamycin and incubated at 37 °C overnight with shaking at 100 rpm. The following day, 1 ml of the overnight culture was inoculated into 50 ml flask of LB broth supplemented with 50 μg/mL kanamycin and incubated at 37 °C with shaking (100 rpm) until reached OD600 = 0.5. Then, IPTG was added with the final concentration of 0.5 mM to induce expression. After that, the flask was incubated at 18 °C for 20 h with shaking at 100 rpm. The cells were then harvested by centrifugation (2500×*g* for 10 min at 4 °C).

2.3. Purification of recombinant thermostable α-amylase

The purification of the recombinant thermostable α-amylase initially started with the lysis of the cells. Pellet was resuspended with lysis buffer and then incubated on ice and heated at 42 °C repeated 3 times, each for 5 min. After that, the resuspended pellet was sonicated for 5 min to destroy the cell wall and free the protein (enzyme). The sample was then centrifuged at 13000×*g* for 20 min at 4 °C. The supernatant was transferred to a new tube and stored at −20 °C. The purification of recombinant α-amylase is done by using the HisPur Ni-NTA (Thermo Fisher Scientific) column with his-tag. Clarified lysate from amy-His cells (Amy-His) were allowed to flow through Ni–NTA matrix and washed extensively with washing buffer pH 7.4 containing 25 mM imidazole. Finally, proteins were eluted in an elution buffer (pH 7.4) containing 250 mM imidazole.

2.4. Molecular weight identification with SDS-PAGE

The molecular weight of the purified recombinant thermostable α-amylase was determined using the SDS-PAGE method. The composition of the gel is 12 % (w/v) resolving gel and 5 % (w/v) stacking gel. Samples were prepared by mixing purified enzyme and loading dye with a ratio of 4:1 with a total volume of 25 μL. The sample and standard are loaded into the gel well and run at 120 V constantly for 180 min. Then the gel was stained with a staining solution containing Coomassie blue dye overnight and the protein bands were observed the following day after being destained using a destaining solution.

2.5. Characterization of recombinant thermostable α-amylase

2.5.1. The optimum temperature, thermal stability, and optimum pH of the enzyme

The optimum temperature for optimum enzyme activity was tested at 30, 40, 50, 60, 70, 80 and 90 °C for 20 min at pH 5 (acetate buffer) using 1 % soluble starch substrate [6]. The activity was measured with DNS assay method. The thermal stability of the enzyme was tested at 60, 70, and 80 °C for 1 h. Every 15 min, 0.01 ml of samples are withdrawn and mixed with 1 % soluble starch and incubated at optimal temperature for 20 min to test its residual activity. Then the enzyme activity was measured with DNS assay method. The optimum pH for optimum enzyme activity was tested at different pH using acetate buffer (pH 4.0–5.0), phosphate buffer (pH 5.0–7.0), and Tris-HCl buffer (pH 8.0–9.0) for 20 min at optimal temperature [12]. Then the enzyme activity was measured with DNS assay method.

2.5.2. The cofactors and inhibitors on enzyme activity

The effect of salt as a source of ions (cofactors) on enzyme activity was analyzed using several salts such as $CaCl_2$, $MgCl_2$, and KCl with concentrations of 1 and 5 mM at optimum temperature and pH from the previous steps. The activity was compared to the control (without salt) [12]. The effect of several inhibitors on enzyme activity was analyzed using chemicals such as EDTA, 2-mercaptoethanol, and $HgCl_2$ (1 and 5 mM) [13]. The enzyme activity was measured with DNS assay method.

2.5.3. The substrate specificity profile

The substrate specificity profile is analyzed using several types of starch such as soluble starch, amylose, CMC, and dextrin [14]. The mixture was incubated at optimum temperature and pH from previous steps. The enzyme activity was measured with DNS assay method.

2.5.4. Enzyme kinetic assay

The kinetic assay was conducted using pure enzyme with various dextrin concentrations of 2.5–15 mg/mL The assay was performed at 70 °C and pH 6 for 20 min. The values of Km and Vmax were determined with the Lineweaver-Burke equation.

2.6. Porous starch production

The production of porous starch was conducted according to

Purwitasari et al. [15] method with modification. Canna edulis starch was added into phosphate buffer 0.1 M pH 6 with the ratio of 1:4 (w/v) and added with purified recombinant thermostable α-amylase. The mixture was then incubated at 60 °C with shaking at 80 rpm for 18 h. After that, the mixture was added with 0.06 V 95 % ethanol and centrifuged 1500×*g* for 10 min. The pellet was dried in the oven at 40 °C for 24 h.

2.7. Immobilization with chitosan

Enzyme immobilization with chitosan was conducted according to Kaushal et al. [16] method with modification. Chitosan solution (1.5 %) was prepared in 2 % acetic acid dissolved with heating at 50 °C and continuously stirred at 750 rpm for 1 h. After the bubble formed was gone, the solution was then dropped in NaOH 1 M to form the beads. After 30 min, washed with distilled water and immersed in a cross-linking solution of 40 mL of 2 % glutaraldehyde and kept at room temperature overnight. The reinforced beads were then collected and dried in an oven at 50 °C for 20 h. The beads are stored at 8 °C if not used. Before the immobilization, the beads were activated with immersion at 2 % glutaraldehyde for 20 min, washed with distilled water, and collected. The immobilization of the enzyme was done by immersing the chitosan beads into purified enzyme solution with a ratio of 1:1 (v/v) for 24 h with shaking at 135 rpm. The beads were then washed with distilled water and stored at 8 °C for further analysis.

2.8. HPLC analysis of the enzymatic reaction products

Enzymatic reaction products was analyzed using Reverse Phase HPLC with Aminex HPX 87H column based on its polarity and size exclusion. The stationary phase is non-polar and the mobile phase is polar using 100 mM perchloric acid. The flowrate was 0.6 mL/min with injection volume of 20 μL (Bio-Rad). Molecules with higher polarity and larger size will be eluted first.

3. Results and discussion

3.1. Purification of recombinant thermostable α-amylase

Recombinant thermostable α-amylase was expressed and purified with one-step purification using a Ni-NTA column. The column would only bind protein with several successive histidine sequences which the recombinant enzyme has in the form of His-tag. The specific activity of the enzyme after purification was observed to be 3.6 times higher than crude extract with the yield of purification about 69.23 % (Table 1). Other studies, revealed that after one-step purification with Ni-NTA column, recombinant α-amylase from *Nesterenkonia* sp. strain F showed an increase in specific activity up to 6.3 times [17]. While Gandhi et al. [18] purified recombinant α-amylase from *Geobacillus stearothermophilus* SR74 with immobilized metal affinity chromatography (Ni-Sepharose) resulted in 52.6 % of product yield and specific activity increase of 1.9 folds. Overall after purification, the specific activity increases. This was because the impurities were filtered out by the column through the affinity of Ni-NTA column with successive histidine sequences of the protein.

Table 1
Purification step and yield.

Purification steps	Volume (mL)	Total protein (mg)	Total activity (Units)	Specific activity (U/mg)	Yield (%)
Crude enzyme	3	3.43 ± 0.04	0.33 ± 0.06	0.09 ± 0.02	100
Ni-NTA column (E2)	1.50	0.64 ± 0.03	0.23 ± 0.06	0.35 ± 0.09	69.24

3.2. Characterization of recombinant thermostable α-amylase

The molecular weight of the recombinant enzyme was predicted based on the sequence using aatbio calculator which showed around ±58 kDa and with the addition of SUMO protein (±11 kDa) from the pET SUMO plasmid, it showed that the protein molecular weight is around ±70 kDa (Fig. 1). The result shown on the SDS-PAGE gels is consistent with the predicted molecular weight of the recombinant thermostable α-amylase. Other α-amylase from different species exhibited different protein molecular weights, novel recombinant α-amylase (Amy13A) has a protein size of 55 kDa on 12 % SDS-PAGE gel, while recombinant α-amylase from *Nesterenkonia* sp. strain F has a protein size of 52 kDa, and recombinant α-amylase from *Geobacillus stearothermophilus* SR74 has a protein size of 59 kDa [17–19]. However, compared with other studies, it could be inferred that the molecular weight of the recombinant thermostable α-amylase is similar to some species.

3.2.1. Optimum temperature and pH

The optimum pH was observed using different buffers such as acetate buffer (pH 4–5), phosphate buffer (pH 5–7), and tris-HCl buffer (pH 8–9). The recombinant thermostable α-amylase exhibits its highest activity at pH 6, with a slight decrease noted at pH 7, followed by a gradual decline in activity until pH 9, where a sharp plummet occurs (Fig. 2). At a pH of 5, the enzyme's activity was at its lowest, possibly due to changes in the protein's solubility. According to calculations from the sequence

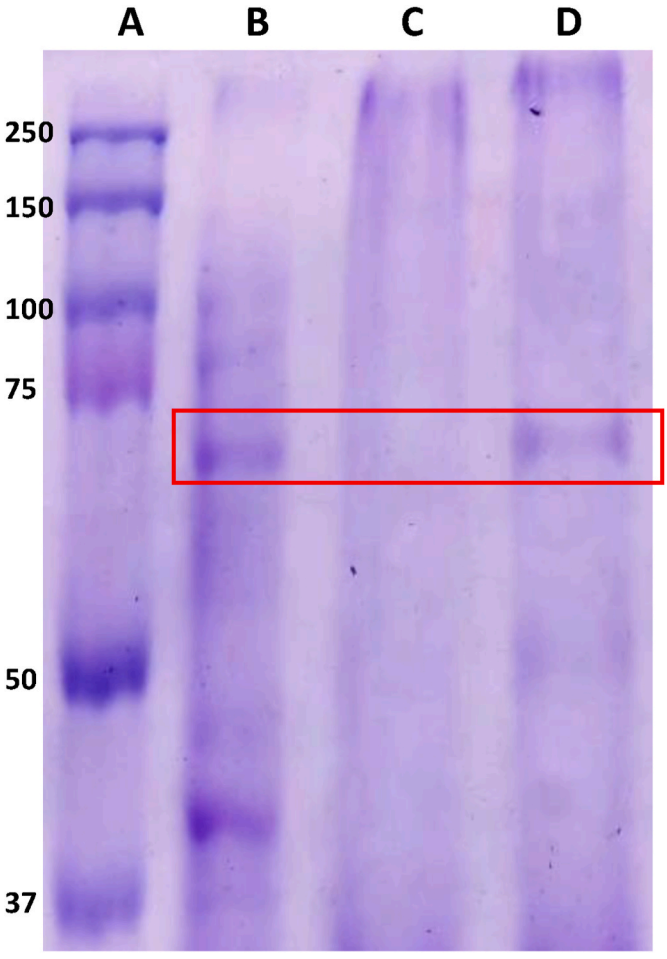

Fig. 1. SDS-PAGE analysis of recombinant thermostable α-amylase (A: Protein ruler, Precision Plus Dual Color™ Bio-Rad®; B: Crude enzyme; C: First elution; D: Second elution). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

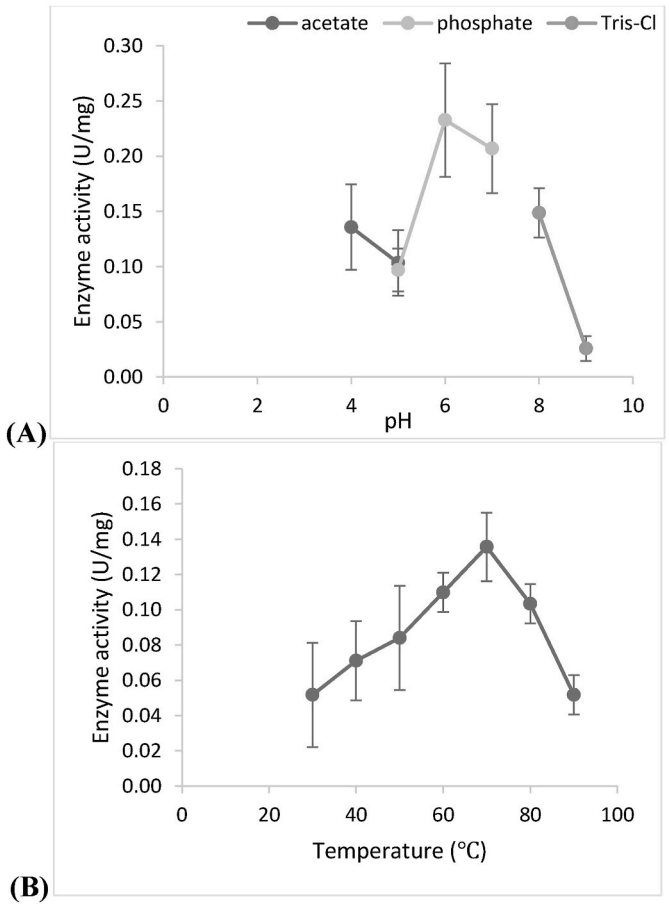

Fig. 2. Effect of different pH (A) and temperature (B) on enzyme activity.

using Expasy, the enzyme's isoelectric point (pI) is approximately 5.27, which is the pH where the protein carries no net charge. Below the pI, the protein surface predominately by positively charged, while above it, it predominately by negatively charged. At pH 5, which is close to the pI, the balance between positive and negative charges reduces electrostatic repulsion, leading to decreased solubility approaching precipitation [20]. At pH 6, the enzyme exhibited the highest activity, as the protein began to develop a negative charge on its surface, promoting dissolution due to repulsive electrostatic forces [20]. With increasing pH, the protein's net negative charge increases, enhancing repulsive electrostatic forces until strong enough to destabilize its structure and impact folding [21].

Analysis of the graph suggests that the enzyme's optimal activity occurs at 70 °C, with activity increasing steadily from 30 °C until reaching its peak at 70 °C. A decline in activity is observed at 80 °C, akin to that seen at 60 °C, yet the enzyme remains active even at 90 °C, indicating remarkable thermostability (Fig. 2). The differences in enzyme activity between low and high temperatures of thermostable enzymes can be related to the rigidity of the protein conformation [22]. As the thermophilic enzyme has higher rigidity and lower permeability, the specific activity at room temperature is usually less than that of mesophilic enzymes [22,23]. This means an appropriate level of protein mobility is required for catalysis [22]. At high temperatures, the rate of collisions per unit of time increases making the mobility of the enzyme increase loosening up weak bonds and making some regions of the molecules while still maintaining the overall 3D structure, especially maintaining the stability of the active site for catalytic activity [23,24].

Recombinant α-amylase from *Massila timonae* has an optimal pH of 6 and optimal temperature of 60 °C but this enzyme is still active at pH range of 5–8 and temperature of 40–70 °C [25]. While recombinant α-amylase from *Bacillus velezensis* has an optimal pH of 6 and optimal temperature of 70 °C, this enzyme is still active at pH range of 5–7 and temperature of 40–75 °C [26,27]. Another recombinant α-amylase from *Geobacillus stearothermophilus* SR74 has the optimal temperature of 65 °C but is still active in the range of 55–70 °C with an optimal pH of 7 and still highly active in pH 6–8 [18]. Compared to other findings, it showed that this recombinant enzyme had a similar pH range of activity (6–8) and similar optimum pH conditions. However, the recombinant enzyme has the advantage in thermostability as it was active in a wide range of temperatures (30–90 °C) and similar optimum temperature conditions at 70 °C.

3.2.2. *Thermal stability*

At 60 °C, the enzyme displayed better stability in activity because, after 45 min of incubation, its residual activity was 62 %. While at 70 °C, the activity declined to 70 % after 30 min of incubation and 34 % after 45 min. At 80 °C, the activity dropped similarly after 30 min around 67 %, and after 45 min its residual activity was 23 % (Fig. 3). After 60 min at 60 °C, 70 °C, and 80 °C, relative activity levels were measured at 32 %, 26 %, and 13 % respectively. Therefore, this enzyme is suitable for thermal processes that do not exceed 30 min of incubation at 70 and 80 °C or before 45 min at 60 °C without cofactor addition.

Other recombinant α-amylase from *Geobacillus stearothermophilus* SR74 displayed a great thermostability, with half-life of 85 min at 60 °C, 55 min at 65 °C, and 40 min at 70 °C [18]. Recombinant α-amylase from *Massilia timonae* showed that at 60 °C and 70 °C after 24 h of incubation still retained 86.1 % and 44.3 % of its activity, respectively. While at 80 °C at 30 min of incubation, its activity is less than 50 % [25]. On the other hand [26,27], recombinant α-amylase showed great thermostability at 40 °C and 50 where its activity was stable around >60 % after 60 min. However, after 10 min at 60 °C, 70 °C, and 80 °C its residual activity is at 59.83 %, 27.19 %, and 6.01 %, respectively. It could be observed that each recombinant α-amylase from different species has varied thermal stability. This could be caused by the variation in the structure of the enzyme especially the primary structure of the protein [28]. The structural features such as the arrangement of catalytic residues and the presence of specific domains influence the enzyme's substrate binding and catalytic efficiency. In addition, the structural stabilization of the enzyme under specific conditions is crucial for maintaining its active conformation, and consequently, its activity such as the presence of calcium ions and sodium chloride can enhance the thermostability of the enzyme and protect the enzyme against acidic -induced denaturation [29,30]. All interactions including hydrogen bonds, hydrophobic interaction, ion and metal bindings that affect the thermostability of the enzyme are affected by temperature to different degrees [22].

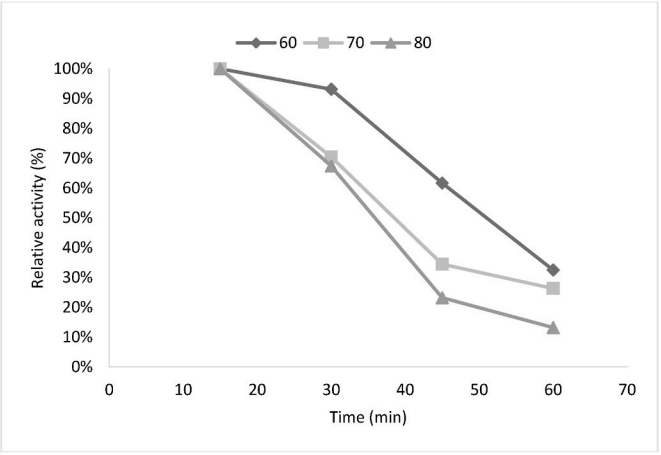

Fig. 3. Thermal stability profile of recombinant thermostable α-amylase.

3.2.3. Substrate specificity

Various substrate was tested to determine the most suitable substrate for this recombinant thermostable α-amylase. From the profile, it showed that the highest activity was observed with dextrin as its substrate followed by amylose and soluble starch (Fig. 4). While the enzyme activity with CMC as substrate was almost negligible. This result was to be expected as α-amylase cut α-1,4 glycosidic bonds between glucose which dextrin, amylose, and soluble starch have. CMC consists of β-1,4 glycosidic bonds between glucose, therefore not suitable as a substrate for α-amylase. The differences in activity between different substrates could be caused by the variation of the amylose/amylopectin ratio, the organization of glucose in the structure of dextrin, the length of the starch chain, and the degree of branching [31].

Starch comprises amylose (25–30 %) and amylopectin (70–75 %). Amylose consists mainly of glucose monomers linked by α-1,4-glycosidic bonds, with a small percentage (0.2–0.8 %) branching via α-1,6-glycosidic bonds [32]. Its molecular weight ranges from $1x10^5$ to $1x10^6$ Da. Amylopectin, on the other hand, is primarily composed of glucose monomers linked by α-1,4-glycosidic bonds, with branching via α-1,6-glycosidic bonds, and has a molecular weight ranging from $1x10^7$ to 1×10^9 Da [33]. According to Park and Rolling [34], amylose adopts an α-helix conformation in water-DMSO solutions, while amylopectin forms a random coil. This structural difference makes it easier for enzymes to bind to the exposed linear fragments of the more flexible amylopectin compared to the rigid amylose molecules [24]. Consequently, during hydrolysis, the initial cleavage primarily targets the linear fragments, generating clusters containing branches. Subsequently, due to the hindrance posed by these branches, access to glucose molecules within the clusters is restricted, resulting in faster hydrolysis of amylopectin into clusters compared to cleaving the shorter chains within the clusters [35,36].

Although the enzyme exhibits a greater affinity for amylopectin, the hydrolysis rate of amylose is generally faster [37]. This phenomenon may be attributed to the less branched structure of amylose. The presence of branching and clustered branches tends to decelerate the initially rapid hydrolysis rate of amylopectin [24]. Consequently, this explains why amylose demonstrates higher enzyme activity compared to starch.

Dextrin, also known as maltodextrin, is a polymer composed of saccharides, predominantly D-glucose units linked by α-1,4-glycosidic bonds, alongside some α-1,6 glycosidic bonds [38–41]. Its average DE values fall within the range of approximately 5–18 [42], equals to molecular weights of around 1000–3600 Da. This similarity in structure to amylose, with minimal branching and smaller molecular weight, explains why dextrin exhibits higher enzyme activity compared to amylose, albeit with no significant difference in enzyme activity.

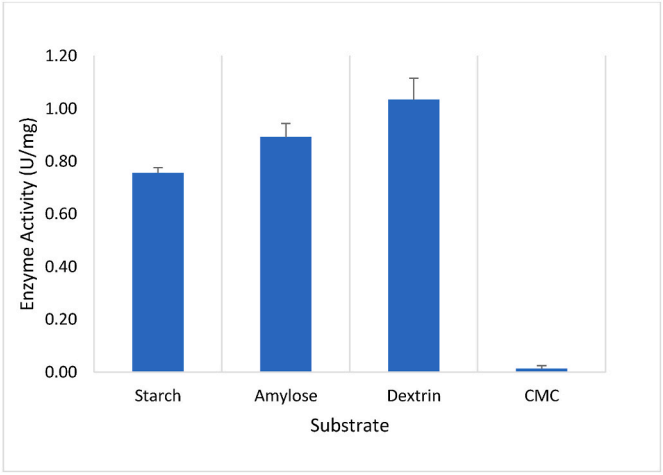

Fig. 4. Substrate specificity profile of recombinant thermostable α-amylase.

3.2.4. Enzyme kinetic

The enzyme kinetics revealed a Km value of 324.03 mg/ml and a Vmax value of 36.5 U/mg with dextrin as substrate without cofactor addition (Fig. 5). A high Km value indicates lower affinity between the enzyme and substrate, requiring more substrate to achieve Vmax. Various α-amylases from different species exhibit distinct Km and Vmax values. For instance, Xian et al. [43] reported that α-amylase from *Talaromyces pinophilus* exhibits a Km of 2.34 mg/mL and a Vmax of 877.19 U/mg with dextrin as substrate, while α-amylase from *Malbranchea cinnamomea* S168 exhibits a Km of 6.4 mg/mL and a Vmax of 175.4 U/mg with dextrin as substrate [44]. The notably higher Km value in this study suggests that dextrin has low affinity for the enzyme, requiring a higher substrate concentration to reach half of the maximum velocity. Dextrin has a reduced molecular weight and some branches caused by dextrinization (SCOGS Reports No.75). The presence of these branches creates a steric obstacle for the enzyme which affects the hydrolysis of α-(1,4)-glycosidic bond [24]. Another possible cause for this result is the enzyme structure that may not be efficient enough as the enzyme needs cofactors for optimal activity. Therefore, making the affinity between dextrin and the enzyme low.

3.2.5. Cofactors and inhibitors

The addition of salts such as $CaCl_2$ and $MgCl_2$ gave better activity, especially with the concentration of 5 mM. While with $CaCl_2$ even with 1 mM of concentration, it is already showed a positive impact on the enzyme activity. KCl salts also had a positive effect on enzyme activity at 5 mM concentration and a slight decrease at 1 mM concentration (Table 2). These results are similar to other studies, with the addition of Ca^{2+}, Mg^{2+}, and K^+ the activity of the enzyme also increases [12,17,45,46]. Generally, α-amylases are metalloenzymes that typically needs at least one calcium ion, essential for their activity [47] although, there are also reports of Ca^{2+} independent α-amylase (Sudan et al., 2018; [43]). Calcium ions are known to significantly boost enzyme activity by facilitating substrate binding [48]. Calcium ions role in α-amylase enzyme is mainly stabilizing of the enzyme structure to maintain native conformation with increased temperatures [29,49]. While calcium ions are preferred over magnesium ions, the presence of metal ion activators is crucial for maximizing catalytic activity and overall stability of the enzyme [50]. Addition of salt such as KCl can help to stabilize the enzyme by neutralizing the opposite ions charge thus decrease the electrostatic repulsive forces [30].

Inhibitors such as EDTA, β-ME, and $HgCl_2$ showed an inhibition effect on recombinant thermostable α-amylase activity. Higher

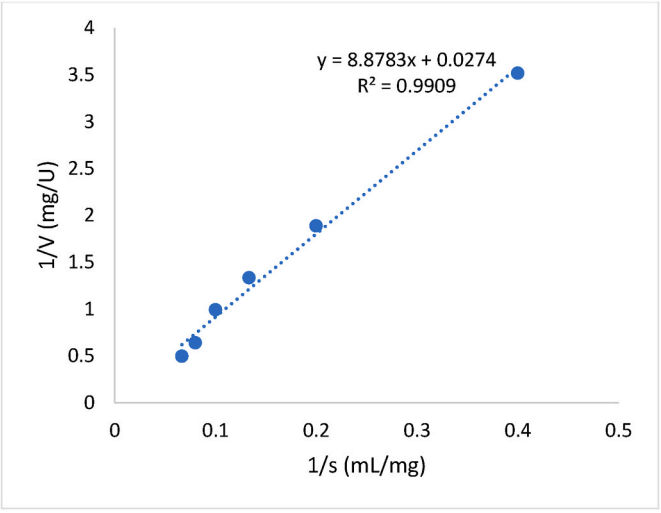

Fig. 5. Lineweaver-Burk plot between recombinant thermostable α-amylase velocity to dextrin as substrate concentration.

Table 2
Effect of different cofactors, salt, and inhibitors on enzyme activity.

Cofactors	Concentration (mM)	α-amylase activity (U/mg)	Relative activity (%)
Control	–	0.77 ± 0.07	100
$CaCl_2.2H_2O$	1	1.32 ± 0.28	172
	5	3.00 ± 0.36	390
$MgCl_2.6H_2O$	1	0.77 ± 0.16	100
	5	2.88 ± 0.16	374
Salt	Concentration (mM)	α-amylase activity (U/mg)	Relative activity (%)
Control	–	0.77 ± 0.07	100
KCl	1	0.76 ± 0.16	99
	5	0.96 ± 0.33	125
Inhibitors	Concentration (mM)	α-amylase activity (U/mg)	Relative activity (%)
Control	–	0.37 ± 0.07	100
EDTA	1	0.26 ± 0.05	70
	5	0.12 ± 0.03	33
β-ME	1	0.18 ± 0.06	49
	5	0.12 ± 0.01	33
$HgCl_2$	1	0.11 ± 0.05	30
	5	0.07 ± 0.01	19

concentrations of inhibitors also showed worse enzyme activity. These results are similar to other studies, where the addition of Hg^{2+} caused the enzyme activity to be severely inhibited. This inhibitory effect is possibly due to the competition for calcium-binding sites on the α-amylase [31] or could be affecting the enzyme structure-function relationship because of its thiol-depriving action that prevents the forming of a disulfide bond between two cysteine residues [14]. The addition of EDTA also inhibits the enzyme activity by noncompetitive inhibition by acting as a metal chelator, thus indicating that this recombinant thermostable α-amylase is a metalloenzyme [6,51]. As for β-mercaptoethanol, its inhibitory activity could be caused by forming β-mercaptoethanol adduct with cysteine side chain thus increasing the molecular weight and may disturb the catalytic reaction (Klarskov et al., 1994).

3.2.6. *The profile of enzymatic reaction product*

Fig. 6 shows the results of HPLC analysis with green, blue, and red lines are dextrin as substrate, maltotetraose standard, and glucose standard, respectively. The black and grey lines on Fig. 6 are the enzymatic reaction products. The findings from HPLC analysis confirmed the functionality of the recombinant thermostable α-amylase, demonstrating its ability to degrade dextrin substrate into maltotetraose (peak number 1) and glucose (peak number 2) as depicted on Fig. 6. This observation aligns with previous research indicating that α-amylase effectively hydrolyzes α-1,4-glycosidic linkages to produce various low molecular weight products, including glucose, maltose, maltotriose, maltotetraose, and limited dextrins [52–54].

3.3. *The application of recombinant α-amylase*

Alpha-amylase has a lot of applications in industries such as starch saccharification, beer and juice clarification, being incorporated in detergent to remove a starch stain, or even in textile desizing, and papermaking processes. Another novel application of α-amylase is for porous starch production. Porous starch has immense potential for application in food, pharmaceutical, and environmental industries. It could be used as an adsorbent, a drug delivery system, to encapsulate and produce microspheres and nanoparticles, and even as a flavor carrier in food products [7].

3.3.1. *Production of porous starch*

3.3.1.1. SEM analysis. The granular morphology analysis showed that after 18 h of incubation at 60 °C and pH 6, the α-amylase was successful in forming a large pore in the starch granule of *Canna edulis* Kerr. (Ganyong) (Fig. 7). The hydrolysis of porous starch with α-amylase was caused by attacking the natural pores, cracks, and holes in the granule thus making a larger and deeper pore on the starch granule surface [55]. This result is similar to the previous study by Purwitasari et al. [15] and Witasari et al. [56] where the pore formed in a wide and big pore on the surface of the canna starch granules.

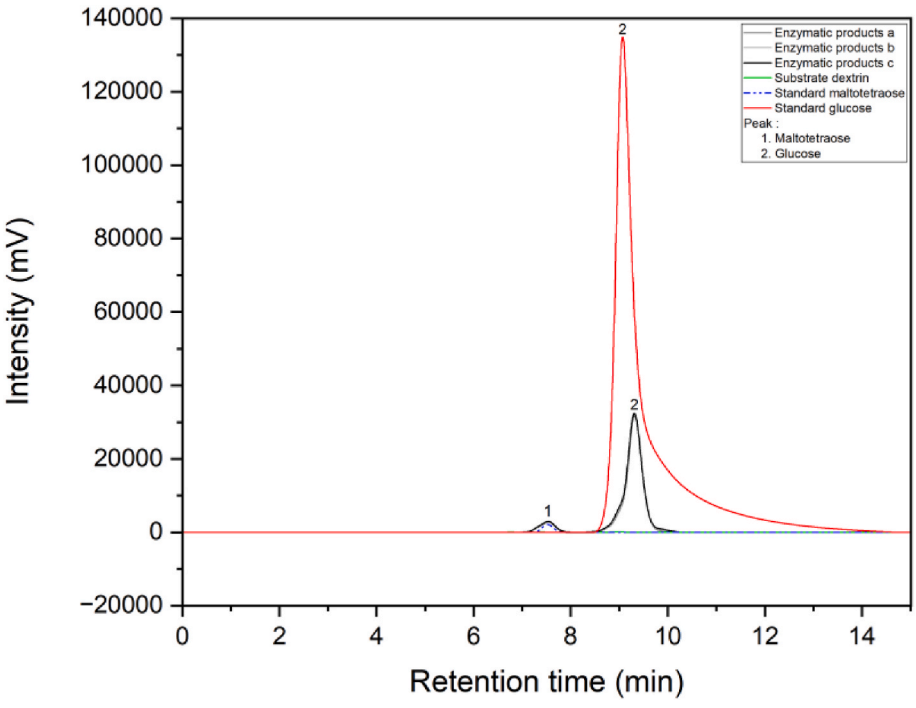

Fig. 6. Enzymatic reaction product profiling with HPLC.

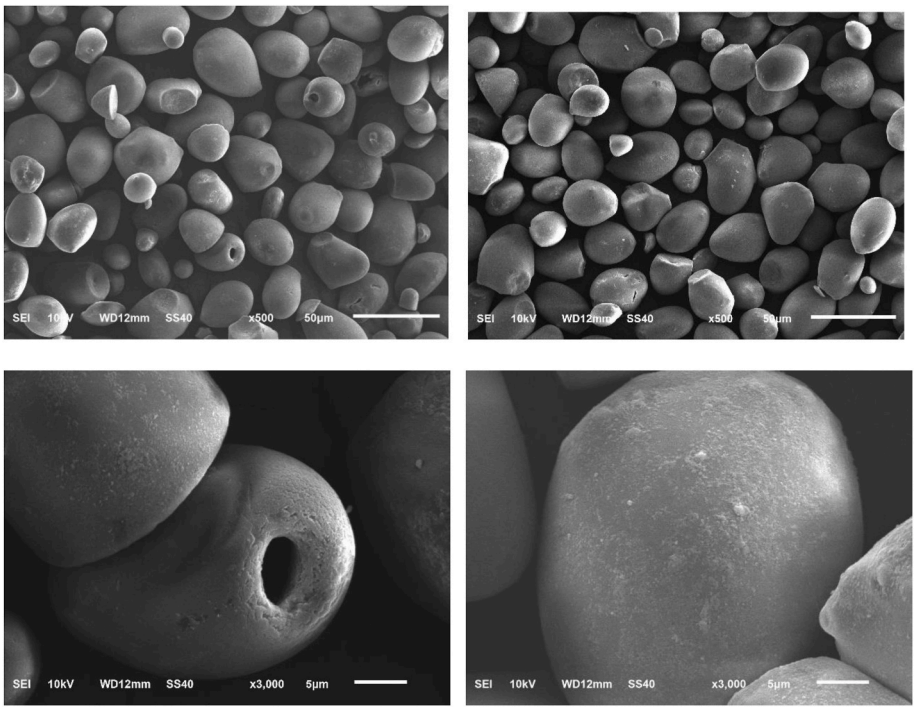

Fig. 7. Production of porous starch with recombinant α-amylase. Left: canna porous starch; right: canna starch granule.

From the previous study, it is indicated that canna porous starch has a type A crystal structure [15]. While several studies indicated that C. *edulis* Kerr. has a type B structure [26,27,57]. Type A crystal structure has a higher ratio of short-chain amylopectin with a less regular structure ratio, and some of the branches are on the crystalline side making this type of starch easier to hydrolyze with enzyme. Type B crystalline structure has a more compact structure with a higher ratio of long-chain amylopectin thus making a stable helix structure and a smooth surface. Therefore, the type B structure is harder to hydrolyze with enzymes. However, the result revealed that the pores formed by α-amylase on the surface of the canna granule are few and large, unlike a honeycomb with a lot of pores evenly distributed across the surface, similar to the previous study where sago starch granules formed a single large pore due to the smooth shape of the natural sago granules or without natural pores [58]. The inside of the granule, usually the amorphous region rich in amylose, is more susceptible to the attack of the enzyme than the surface therefore the holes made inside will enlarge in the lateral direction, making circular holes [58,59,60]. Then the enzyme will attack the deeper layer of the granule making the pore deeper [58].

3.3.2. *Immobilized α-amylase*

3.3.2.1. SEM analysis. SEM analysis was done to determine the surface morphology of chitosan beads immobilized with and without enzyme. The SEM results indicated that chitosan beads with and without enzymes have differences in shape, size, and texture. The control beads (without enzyme) shape were full of cracks and irregular. The enzyme-immobilized chitosan beads were also irregular with cracks but had a rougher and more porous surface texture (Fig. 8). As for the size, the control beads were smaller than the enzyme-immobilized beads. Changes in surface morphology were also observed by Singh et al. [61], Gür et al. [11], and Gilani et al. [62] making the occurrence of change in morphologies after activation with glutaraldehyde generally found. The increase in size after enzyme immobilization is possibly due to swelling because of the immersion of the beads in enzyme solution in the immobilization process.

3.3.2.2. Thermal stability. The immobilized thermostable α-amylase thermal stability was tested at 60 °C, 70 °C, and 80 °C for 1 h. At 60 °C the activity still slightly increased even at 60 min of incubation and at 70 °C showed a similar trend that after 45 min the activity slightly decreased. However, at 80 °C, after 30 min the activity slightly decreased and kept on declining until 60 min of incubation (Fig. 9). Compared to the free enzyme thermal stability, the immobilized enzyme showed better thermal stability, as the decline of activity is not as steep as the free enzyme. However, the overall activity of the immobilized enzyme is lower than the free enzyme. This could be caused by several reasons where the immobilization process can cause conformational changes in the enzyme leading to a decrease in its activity, the limitation of mass transfer to the chitosan matrix, and the steric interaction can also affect the enzyme activity [63–65].

3.3.2.3. Reusability. The activity of the immobilized thermostable α-amylase was observed for their stability by reusing the immobilized enzyme to convert starch substrate. Each use of the immobilized enzyme showed a decline in its activity. After four reuses, the activity decreased to 62 % of the initial immobilized enzyme activity. The decrease in activity in repeated uses could be caused by the escape of the enzyme from the matrix which in turn reduces the total amount of enzyme bound in the chitosan beads matrix. This phenomenon is also in line with Zusfahair et al. [10] findings where immobilized amylase on chitosan beads also showed a decrease in activity to 43.3 % at the fifth use.

4. Conclusion

The recombinant thermostable α-amylase from *Geobacillus* sp. DS3 was successfully expressed at 18 °C for 20 h with 0.5 mM of IPTG and successfully purified. The enzyme has great thermal stability at 60 °C with optimum activity at 70 °C at pH 6. The addition of metal ions such as Ca^{2+} increases the enzyme activity. This enzyme is functional and able to be used for porous starch production at 60 °C for 18 h. Immobilization of the enzyme with the chitosan matrix can increase the thermal stability and make the enzyme reusable. However, the right matrix still needs to be explored to maintain the high activity.

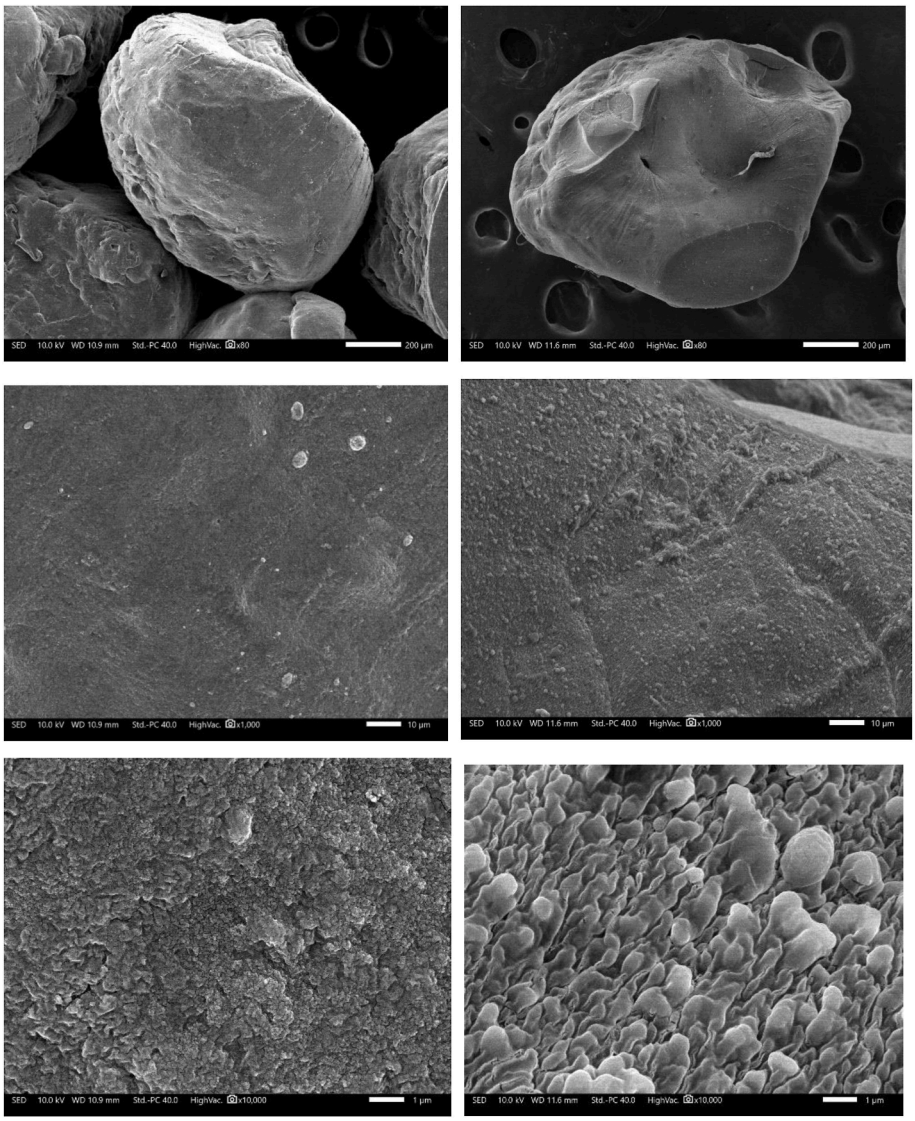

Fig. 8. Surface morphology of control chitosan beads (left) and immobilized enzyme-chitosan beads (right) using SEM analysis.

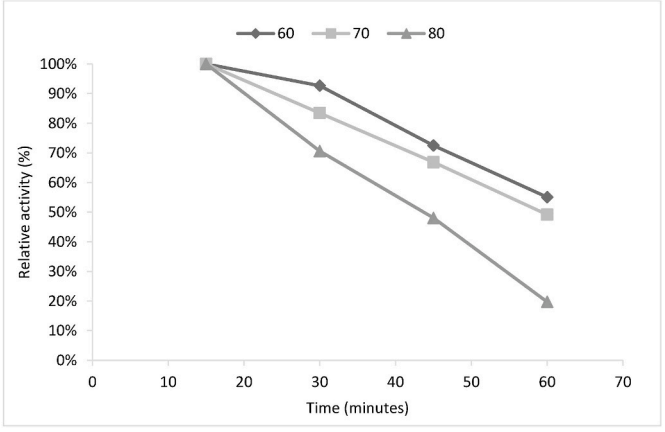

Fig. 9. Thermal stability profile of immobilized recombinant thermostable α-amylase to chitosan beads.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Consent to participate

All authors participated in the study either performing experiments or writing the manuscript.

Consent to publish

All authors agree to publish the manuscript.

CRediT authorship contribution statement

Dina Clarissa Kurniawan: Writing – original draft, Methodology, Investigation, Formal analysis. **Muhammad Saifur Rohman:** Writing – review & editing, Methodology, Data curation. **Lucia Dhiantika Witasari:** Writing – review & editing, Supervision, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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