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RESEARCH ARTICLE

Purification and characterization of α-amylase from *Paenibacillus* sp. D9 and *Escherichia coli* recombinants

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ABSTRACT

The aim of this study was to purify and characterize α-amylase of *Paenibacillus* species D9 and cloned α-amylase in *Escherichia coli* transformants. Optimal growth and production of D9 α-amylase were obtained after the 24-h incubation period, at pH 7, 30 °C and 200 rpm, using starch as the sole carbon source. Sodium nitrate stimulated α-amylase production by 3 folds while urea had an inhibitory effect comparing with yeast extract as the control. Characterization results of the crude and purified enzyme were similar. Purified D9 α-amylase possessed 97% residual activity after incubation at 30 °C for 120 min while 50% at 45 °C and 27% at 60 °C, respectively, under the same condition. The presence of Ca^{2+} ions enhanced the activity, while EDTA and Fe^{3+} had an inhibitory effect. Kinetic parameters were obtained having values of 37.3 mM, 19.8 U/mg and 4.84 s^{-1} for K_m, V_{max} and k_{cat}, respectively. The α-amylase gene (*amyS*) of *Paenibacillus* sp. D9 was amplified using polymerase chain reaction (1482 bp), cloned into PSF-OXB20 vector and expressed in *E. coli* BL21 DE3 (pLysS) after transformation. The α-amylase purified from the transformant using a Hispur Cobalt column possessed 1.4 folds (450.8 U/mg) higher specific activity than the purified α-amylase from the wild type. Cloned α-amylase was more thermostable at 45 °C and at 60 °C, but less pH stable at pH 5 and 6. All other biochemical properties of the native and recombinant α-amylase show no significant differences. In conclusion, α-amylase of *Paenibacillus* sp. D9 was purified, cloned and characterized. The potential biotechnological application(s) of α-amylase may be explored.

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KEYWORDS
Paenibacillus sp. D9; α-amylase; purification; characterization; cloning

1. Introduction

Starch is an important and prevalent food constituent, known as a common substrate exploited for the production of numerous industrial products (Božić et al. 2017). It is found in abundance in an array of plant sources (wheat and maize) and vegetables (potato and cassava) among others (Sundarram and Murthy 2014; Sachdev et al. 2016). Among the wide variety of enzymes, the exploitation of starch degrading enzymes known as amylases account for ∼25% of the enzyme market in comparison to other enzymes (Ikram-Ul-Haq et al. 2012; Avcı et al. 2016; Sachdev et al. 2016; Flory et al. 2017). These amylases have commercial value in several industrial applications which mainly include processes such as starch liquefaction, brewing, textiles, pharmaceuticals, paper and detergents (Abdel-Fattah et al. 2013; Abd-Elhalem et al. 2015; Wu et al. 2018).

Microorganisms are significant in producing these industrially amylolytic enzymes due to their high levels of flexibility (Hussain et al. 2013; Dey et al. 2016), for their simplicity and cost-effectiveness (Gupta et al. 2003; Tanyildizi et al. 2005; Rigoldi et al. 2018). Hence, microbial amylases have almost completely replaced the chemical hydrolysis in the starch processing industry (El-Fallal et al. 2012; Sachdev et al. 2016; Flory et al. 2017). In addition, overproduction of enzymes can be achieved by genetic engineering (Abdel-Fattah et al. 2013).

Paenibacillus sp. D9 was isolated from diesel-contaminated soil (Ganesh and Lin 2009). Sequencing results (GenBank Accession number JZEJ00000000) show that this microorganism possesses many polysaccharide degradation genes of amylases, chitinases, inulinase (Jeza et al. 2018), proteases, pectinases and xylanases. Literature indicated that the *Paenibacillus* genus is capable of secreting a variety of hydrolytic enzymes (Mihajlovski et al. 2018). A preliminary study demonstrated that *Paenibacillus* sp. D9 is a good starch degrader. Currently, there are only a few reports

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on α-amylases purified from *Paenibacillus* species (Rajesh et al. 2013; Xu et al. 2018). Since there is great importance of α-amylase in the commercial sector (Gopinath et al. 2017), the objective of this study was to purify and characterize D9 α-amylase. Furthermore, D9 α-amylase gene (*amy*) was cloned into an *Escherichia coli* transformant whereby the recombinant was purified and compared with the purified D9 α-amylase and with the enzymes from other sources.

2. Materials and methods

2.1. Growth and maintenance of Paenibacillus sp. D9

Paenibacillus sp. D9 was collected from the Discipline of Microbiology at the School of Life Sciences, University of KwaZulu-Natal, Westville campus. The strain was cultivated at 37 °C for 24 h in Luria-Bertani (LB) (10 g/L of tryptone, 5 g/L of yeast extract and 5 g/L NaCl) medium and stored in the bio-freezer at −80 °C, after suspending with an equal volume of 80% glycerol. Nutrient agar plates of *Paenibacillus* sp. D9 were prepared every two weeks for maintenance and preservation. All reagents used in this study were purchased from Sigma Aldrich, South Africa unless stated otherwise.

2.2. Optimization of α-amylase production of Paenibacillus sp. D9

The inoculum was prepared by transferring a freshly grown D9 colony from nutrient agar into 5 mL sterile nutrient broth, which was incubated at 200 rpm at 30 °C overnight. Production of D9 α-amylase was conducted using submerged fermentation conditions. One millilitre of the overnight *Paenibacillus* sp. D9 culture (OD_{600nm}=1) was inoculated into 100 mL of Minimal salt medium (MSM) (g/L: 3 g yeast extract, 2 g peptone, 0.5 g NaCl, 1 g K_2HPO_4, 0.15 g $CaCl_2 \bullet 2H_2O$, 0.5 g $MgSO_4 \bullet 7H_2O$, pH 7.0) (Annamalai et al. 2011) containing 1% soluble starch substrate (MSM-S medium) in 250 mL plugged Erlenmeyer flasks. The inoculated medium was incubated at 200 rpm at 30 °C for a period of 4 days. Aliquots (5 mL) of the cultured production medium were removed daily for the determination of cell growth, α-amylase activity and protein profile as described below.

The optimal production of D9 α-amylase was subsequently assessed at selected incubation temperatures (25–37 °C), pH (5–9) and incubation intervals. Different nitrogen sources (0.2% of sodium nitrate, ammonium nitrate or urea) were used to replace yeast extract (control) for its effect on α-amylase production. The sample collected under optimal conditions was used for D9 α-amylase purification and biochemical characterization.

2.3. Purification of α-amylase of Paenibacillus sp. D9

The cell-free supernatant after 1-day incubation was obtained by centrifugation at 10,000× *g*, 4 °C for 15 min and used for the purification of the D9 α-amylase. The use of ammonium sulphate precipitation to purify D9 α-amylase, resulted in a loss of enzyme activity with numerous attempts. Therefore, the ammonium sulphate precipitation step was not included.

A volume of 50 mL of the cell-free supernatant was dialyzed against 500 mL of MSM at 4 °C overnight. The dialyzed sample (50 mL) containing α-amylase was filtered through 0.2 μm membrane filters (Whatman) and then loaded onto a 5.0 mL Hitrap QFF column (GE Health Care, Sweden) within an AKTA purifier 100-950 system (GE Healthcare, Sweden). The column was washed with 5 × column volumes of 0.1 M citrate buffer (pH 6) containing 1 mM $CaCl_2$ to wash out unbound proteins. Bound proteins were eluted out with 0–1 M NaCl gradient in the same buffer at a flow rate of 0.4 mL/min and the protein concentration of the eluent was monitored by the absorbance at 280 nm wavelength. The fractions containing α-amylase activity were pooled and concentrated using Ultra spin column with 10 kDa cut-off membrane (Merck) to make up a final volume of 2 mL. The concentrated sample was further purified with size exclusion chromatography using a Hiprep 16/60 Sephacryl S-200 HR 120 mL column (GE Healthcare, Sweden). Proteins were eluted out at a flow rate of 0.4 mL/min using 0.1 M citrate buffer (pH 6) containing 1 mM $CaCl_2$. The eluates containing α-amylase activity were pooled and concentrated using an Ultra spin column with 10 kDa cut-off membrane (Merck) to make up a final volume of 5 mML. All the crude and purified D9 α-amylase (as well as cloned α-amylase) were stored at 4 °C until analysis. The α-amylase activity, protein concentration and protein profile of the samples at each purification step were examined as described below.

2.4. Characterization of α-amylase of Paenibacillus sp. D9

2.4.1. α-Amylase activity assay

The α-amylase activity of the sample was determined using the DNS method (Miller 1959). Briefly, the

sample was centrifuged at 10,000× *g* for 10 min. Supernatant aliquots of 100 µL were mixed with 900 µL of 0.1 M citrate buffer (pH 6) with 1 mM $CaCl_2$ and 1% soluble starch. The mixture was incubated at 60 °C for 20 min. A volume of 1.5 mL of DNS reagent (10 g/L, 3,5-dinitrosalicylic acid; 0.2% [v/v], phenol; 0.5 g/L, sodium sulphite; 10 g/L, NaOH and 200 g/L, sodium tartrate) was added to the mixture to stop the reaction and then boiled for 15 min. After cooling to room temperature, the enzyme activity was determined based on the absorbance reading at 540 nm. Adding 1.5 mL of DNS reagent at time 0 with the same procedures were used as the blank. The amount of glucose liberated from starch was measured spectrophotometrically using a Varian UV spectrometer. The enzyme activity was calculated based on the standard curve of known glucose concentration (0–11 mM). One unit of α-amylase activity is defined as one micromole of glucose generated per minute under standard assay conditions. All experiments were conducted in triplicates.

2.4.2. Determination of protein concentration using the Bradford assay

Protein concentration of the sample was determined by the Bradford method using bovine serum albumin (BSA) as a standard (Bradford 1976). The experiment was conducted on 96 wells microtitre plate. A ratio of 1:50 for sample to Bradford reagent was kept. Following 5 min of incubation at room temperature in the dark, the absorbance of samples was measured spectrophotometrically at 595 nm using a plate reader. Protein concentration (mg/mL) was estimated by interpolation from the BSA standard curve (0–2 mg/mL). All experiments were conducted in triplicates.

2.4.3. Determination of the protein profile and molecular weights using SDS polyacrylamide gel electrophoresis

The protein profile and molecular weights of the samples were subjected to the SDS polyacrylamide gel electrophoresis (PAGE) analysis according to the method of Laemmli (1970). The protein profile of samples and the enzyme purity were obtained by a 10% SDS PAGE. Protein samples (25 µg) were subjected to gel electrophoresis. The gel was electrophoresed at 80 V in 1× SDS running buffer (25 mM TrisHCl, 192 mM glycine, 0.1% SDS). The gel was stained using Coomassie blue R250 for 2 h and de-stained using 40% (v/v) methanol and 10% acetic acid (v/v) mixture overnight. The protein profile was captured using Syngene Gel documentation system (BioGenie System 2). The molecular weight of α-amylase was determined based on the standard protein markers (BioRad).

2.5. Optimum pH, temperature, thermostability and pH stability

The optimal temperature of D9 α-amylase was determined as described by Aguilar and colleagues (Aguilar et al. 2000). α-Amylase activity of samples was assayed at temperatures over a range of 25 °C–60 °C with 1% (w/v) soluble starch substrate at pH 6. The thermostability of D9 α-amylase was investigated by incubating the samples (10 U/mL) at temperatures 30, 45 and 60 °C in 50 mM citrate buffer with 1.0 mM $CaCl_2$ for 120 min. Aliquots of samples were removed at 30-min intervals for the α-amylase activity assay as described above.

The pH optimum of D9 α-amylase activity was determined using the standard assay conditions under the optimal temperature at a range of pH from 5 to 9 (50 mM citrate buffer – pH 5 and 6 and 50 mM Tris-HCl buffer – pH 7–9) (Aguilar et al. 2000). For the determination of pH stability of α-amylase, samples containing 10 U of α-amylase activity were mixed with 10× volumes of 1% starch solution with the desired pH on ice (Aguilar et al. 2000). The residual activities of samples were determined at the 30 min intervals for a period of 120 min. All experiments were done in triplicates.

2.6. The effects of metal ions and EDTA on Paenibacillus sp. D9 α-amylase activity

α-Amylase assays were performed for respective samples in the presence of different metal ions i.e. $MgCl_2$, $CaCl_2$, $FeCl_3$ and $CuSO_4$ at 1 mM final concentration (Aguilar et al. 2000). The relative activity of α-amylase was compared using the activity under standard conditions without heavy metal as 100%. Addition of 1 mM EDTA was also used to determine the effects on α-amylase activity. All experiments were conducted in triplicates.

2.7. Kinetic study of Paenibacillus sp. D9 α-amylase

The α-amylase activity of D9 α-amylase was assayed with increasing substrate concentrations (0–1000 µM) under the optimal conditions. The Michaelis constant (K_m), maximum reaction velocity (V_{max}), turn over number (k_{cat}) and catalytic efficiency (k_{cat}/K_m) of the

purified α-amylase were determined using Lineweaver and Burk plot (Lineweaver and Burk 1934).

2.8. Cloning and expression of α-amylase of Paenibacillus sp. D9 into E. coli

A loopful colony of *Paenibacillus* sp. D9 was inoculated into 5 ml LB broth and subsequently incubated at 30 °C at a speed of 200 rpm overnight. Genomic DNA from *Paenibacillus* sp. D9 was purified using the GeneJET genomic DNA purification kit as per manufacturer's instruction (Thermo Scientific). The DNA concentration was measured using a Nanodrop 2000 UV – Vis Spectrophotometer (Thermo Scientific) and purity was measured using OD_{260}/OD_{280} ratio.

2.8.1. Amplification of α-amylase of Paenibacillus sp. D9 using polymerase chain reaction (PCR)

Primers used for α-amylase gene (*amyS*) amplification were designed according to the total genomic sequences of *Paenibacillus* sp. D9 (GenBank Accession number JZEJ00000000) using Snap Gene software. The α-amylase gene was amplified using forward primer 5′–ATG GAG CGC AAC CAT ACG ATG ATG CAG TTT TT -3′ containing HindIII restriction site and reverse primer 5′-CTC GAG GCC GCC CTC AGG CTC TA-3′ containing XhoI restriction site. PCR reactions were performed according to iProof high fidelity DNA polymerase kit (Bio-Rad) using T100™ Thermal Cycler (BIO-RAD). The PCR mixture contained 0.5 μM of the forward primer and 0.5 μM of the reverse primer, 2 μL of the DNA, 1 μL of 5× iProof HF buffer, 0.2 U/μL iProof DNA polymerase and 2.4 μL of double distilled nuclease-free water. PCR conditions were as follows: initial denaturation at 98 °C for 10 s, denaturation at 98 °C for 1 s, annealing at 62.9 °C for 30 s and extension at 72 °C for 30 s. Denaturation, annealing and extension steps were repeated for 35 cycles. Subsequently, the final extension step occurred at 72 °C for 5 min. The PCR products of α-amylase (expected 1482 bp) were confirmed using DNA electrophoretic analysis on an agarose gel (1%) (Sambrook et al. 1989). The image of the gel was visualized and captured under ultraviolet light using a UV transilluminator (SYNGENE BioSys).

2.8.2. Cloning of α-amylase gene (amy) into PSF-OXB20 cloning vector and transformation of E. coli BL21 (DE3) pLysS expression system

The *amyS* PCR amplicon from the agarose gel was excised under UV light and purified using GeneJet Gel Extraction Kit as per manufacturer's instruction (Thermo Fisher Scientific) and the PCR product was eluted by the use of 50 μL sterile dH_2O. Both PCR amplicon (insert) and PSF-OXB20 plasmid (GE Health care, USA) were restricted with FastDigest endonucleases, 1 μL of 10 U HindIII and of XhoI each (Thermo Fisher Scientific) for directional cloning as per the manual. The double digested PCR product and PSF-OXB20 vector were purified using a DNA Clean & Concentrator™ kit (Zymo Research) as per the manufacturer's instruction. A ratio of 2:5 of the digested vector and plasmid were added to reaction mixtures. Ligation reaction was performed as per standard conditions using a Rapid DNA Ligation kit (Thermo Fisher Scientific). A successfully cloned plasmid (designated as PSF-*AmyS*) containing the *amy* gene with 5299 bp in size was obtained. *Escherichia coli* DH5α (Novagen, USA) was used for the mini-preparation of the recombinant plasmid while *E. coli* BL21 (DE3) pLysS was used for the expression of α-amylase.

The PSF-*amyS* plasmid was transformed into *E. coli* BL21 (DE3) pLysS expression system using the heat shock procedure (Sambrook et al. 1989). The positive clones grown on an LB plate containing chloramphenicol and kanamycin (34 mg/mL and 50 mg/mL, respectively) were confirmed for the presence of α-amylase gene using colony PCR.

2.8.3. Expression of the recombinant α-amylase in E. coli BL21 (DE3) pLysS expression system

One millilitre of the overnight culture containing PSF-*AmyS* plasmid was transferred into 100 mL of LB broth containing 34 mg/mL chloramphenicol and 50 mg/mL kanamycin. The cells were allowed to grow for 24 h at 37 °C, 160 rpm. Aliquots (1 mL) of samples were removed after 6 and 12 h of growth, respectively, for the enzyme activity assays. A negative control was also set up using the PSF-OXB20 vector, following the same protocol. The cells containing α-amylase activity were harvested by centrifugation at 8000× *g* at 4 °C. The cell pellet was re-suspended in 50 mM Tris buffer, pH 8.0 containing 1 mM PMSF, DNase I (final concentration of 1 U/mL) and 0.1 M $MgCl_2$ followed by sonication (Omni International Sonic Ruptor 400 Ultrasonic homogenizer) with an operating frequency of 20 kHz. The homogenized sample was subjected to 30 s on/30s off pulses for 10 min at 20% amplitude. Thermal effect was minimized by placing the sonicated sample on ice. The supernatant obtained after centrifugation at 10,000× *g* for 20 min was used for analysis.

2.9. Purification and characterization of α-amylase expressed by E. coli transformant

The clear supernatant (25 mL) was loaded into a Hispur Cobalt column (1 × 5 mL HiTrap Chelating HP, Thermo Scientific) using the AKTA purifier 100-950 system. The column was equilibrated with 5× column volumes of sodium phosphate buffer (20 mM NaH_2PO_4, 300 mM NaCl and 20 mM imidazole). After loading, unbound proteins were washed with the same buffer. Bound samples were eluted from the column using a linear gradient of 20 mM to 250 mM imidazole at a flow rate of 0.4 mL/min. Active fractions (2 mL) were collected and dialyzed against citrate buffer, pH 6.0, overnight at 4 °C. The purified α-amylase was characterized as described in Sections 2.4–2.7.

3. Results

3.1. Optimization of α-amylase production of Paenibacillus sp. D9

The cell growth and production of α-amylase by *Paenibacillus* sp. D9 were monitored daily for a duration of 4 days. There was a considerable increase in α-amylase production (2.54 U/mL) at Day 1 (Figure 1) corresponding with the cell growth. After Day 1, the D9 α-amylase activity subsequently decreased daily to 1.42 U/mL at Day 4. SDS-PAGE analysis was conducted and showed that the protein band of 54 kDa was induced at Day 1 and the induced band subsequently decreased with an increase of incubation time, which corresponded to the enzyme activity pattern shown in Figure 1. The molecular weight of the induced protein band also matched with the expected molecular weight of D9 α-amylase based on its gene sequence. The results from SDS-PAGE analysis and Figure 1 confirmed that optimal production of α-amylase from *Paenibacillus* sp. D9 was at its peak at Day 1of incubation.

The effect of varying pH and temperature of the medium as independent variables showed that the optimal α-amylase production was observed at pH 7 at 30 °C with an enzyme activity of 4.68 U/mL (data not shown). Yeast extract in MSM medium was replaced by different nitrogen sources, show that sodium nitrate replacement enhanced D9 α-amylase production (9.51 U/mL) by more than 3 folds compared to using yeast extract (3.03 U/mL) as a nitrogen source (Figure 2). Ammonium nitrate and urea, however, caused a decline in the yield of α-amylase having amylase activities of 1.30 U/mL and 0.37 U/mL, respectively.

3.2. Purification of α-amylase of Paenibacillus sp. D9

Alpha-amylase from *Paenibacillus* sp. D9 was purified to its homogenous form (Figure 3). The purification was achieved by 2 major steps i.e. anion-exchange (QFF) and size-exclusion chromatographies. Addition of 1 mM $CaCl_2$ during the purification was essential to retain α-amylase activity. In addition, the ammonium sulphate precipitation was also omitted due to the loss of α-amylase activity. Prior to column chromatography, the specific activity of the crude extract was increased by dialysis (12 kDa cut-off) with 118% recovery (Table 1). The results indicate that small molecule(s) in the crude extract acted as the α-amylase inhibitor.

The enzyme activity and protein concentration of the samples in each purification step are summarized in Table 1. D9 α-amylase of 54 kDa was purified to homogeneity using HiTrap QFF chromatography and

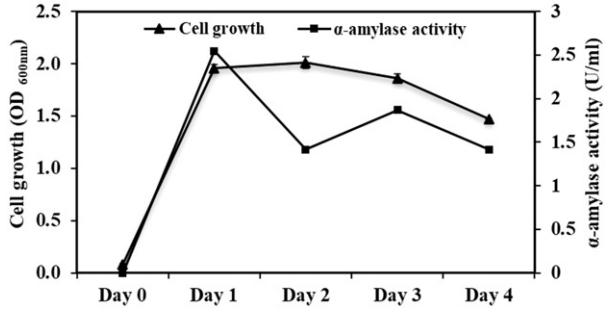

Figure 1. Growth pattern and α-amylase production by *Paenibacillus* sp. D9 during submerged fermentation at a 4-day incubation time, grown in 1% starch supplemented MSM medium at 200 rpm, 30 °C. Error bars are indicative of the mean ± SD of experiments in triplicate.

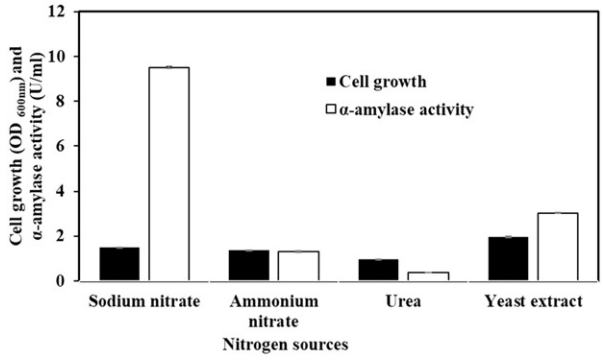

Figure 2. Effect of nitrogen sources supplemented to the MSM medium on cell growth and α-amylase production at the incubator shaker (200 rpm) after 24 h incubation at 30 °C grown at pH 7 using yeast extract as a control. Error bars are indicative of the mean ± SD of experiments in triplicate.

size-exclusion chromatography with a specific activity of 323 U/mg and an 11.7-fold increase in purity. All forms of D9 α-amylase (crude, purified and cloned), stored at 4 °C, were found to be stable up to 6 months.

3.3. Characterization of the crude and purified α-amylase of Paenibacillus sp.D9

3.3.1. Optimal pH and temperature of D9 α-amylase activity

The effect of pH on D9 α-amylase activity was investigated using a selected range of pH. As represented in Figure 4(A), the crude α-amylase showed optimal activity at pH 7. As shown in Figure 4(A), the purified enzyme possessed a similar pattern with an optimal activity (11.5 U/mL) at pH 7.

The effect of temperature on D9 α-amylase activity was also investigated using selected temperatures 25 °C, 30 °C, 37 °C, 42 °C and 60 °C. As expected, the optimal enzyme activity for the crude and purified α-amylase was attained at 30 °C (Figure 4(B)).

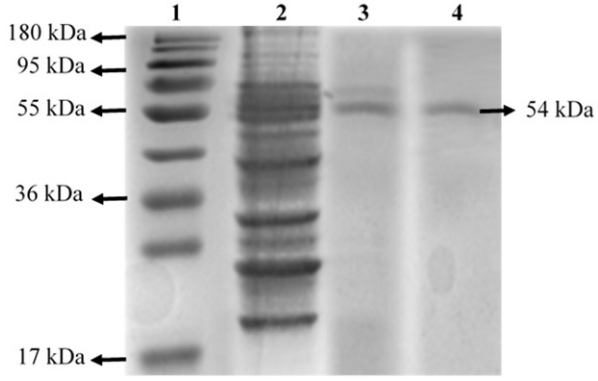

Figure 3. SDS PAGE (10%) of the purified α-amylase from *Paenibacillus* sp. D9 Lane 1: molecular weight marker (10–180 kDa), Lane 2: crude extract, Lane 3: QFF eluate, Lane 4: size exclusion eluate.

3.3.2. Thermostability and pH stability of crude and purified D9 α-amylase

Thermostability of the crude and purified enzymes were evaluated using temperatures 30 °C, 45 °C and 60 °C for a period of 120 min monitored in 30-min intervals. Figure 5(A) demonstrates that purified D9 α-amylases retained 95% or more of their full activity at 30 °C after 120 min. Higher temperatures caused a major decline in activity, whereby the enzyme lost 73% of its activity at 60 °C after 2 h of incubation. D9 α-amylase in the crude samples showed a similar pattern.

The pH stability study also shows that both crude and purified D9 α-amylase were stable at pH 7, retaining at least 95% of its relative activity after 120 min (Figure 5(C)), followed by pH 8, at which the enzyme being able to retain 60–70% of relative activity after 120 min. As pH decreased (pH 5 or pH 6), there was a faster decline in activity as incubation time increased.

Table 1. Summary of major steps in purification of α-amylase from *Paenibacillus* sp. D9.

Purification steps	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification fold
Crude extract	19.80	545	27.5	100	1.0
Dialysis	15.10	644	42.6	118	1.5
Hitrap QFF	1.95	168	86.0	31.0	3.1
Size exclusion	0.16	52	323	9.0	11.7

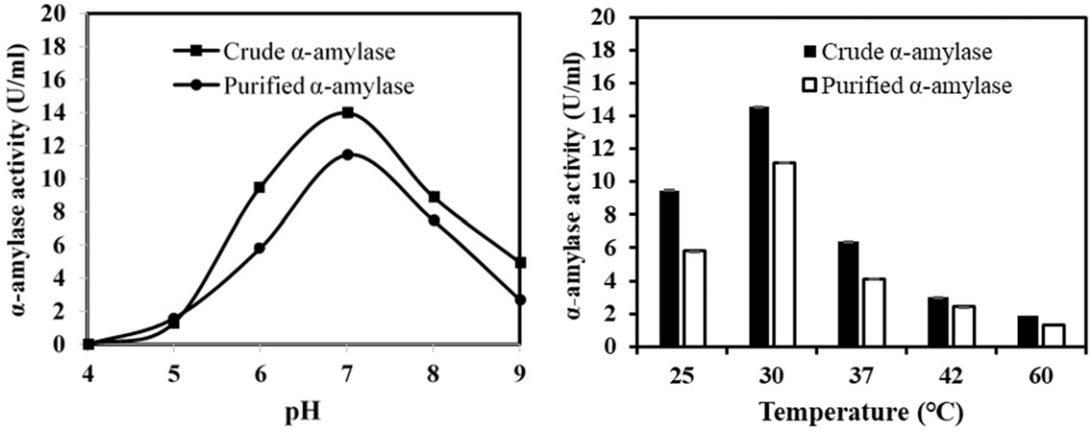

Figure 4. Optima profile of crude and purified α-amylase from *Paenibacillus* sp. D9. (A) pH; (B) temperature.

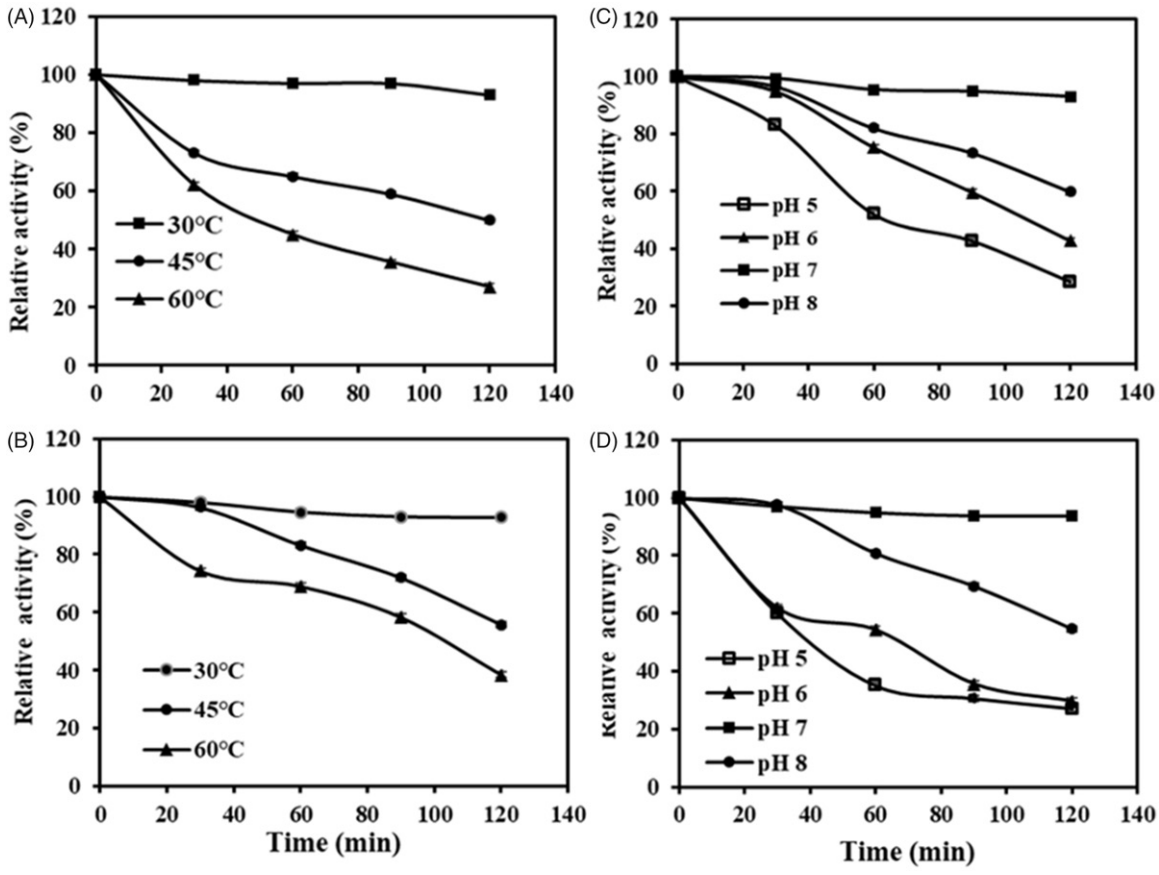

Figure 5. Thermostability (A,B) and pH stability (C,D) of α-amylase from *Paenibacillus* sp D9 expressed as residual percent of activity versus time (minutes). (A,C) Purified D9 α-amylase; (B,D) recombinant D9 α-amylase.

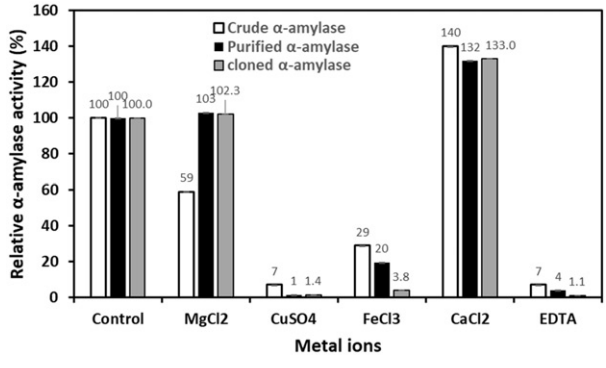

Figure 6. The effect of metal ions on α-amylase activity of the crude α-amylase and purified α-amylase from *Paenibacillus* sp. D9 expressed as residual activity in comparison to the control without the incorporation of a heavy metal.

3.3.3. Effect of metal ions on D9 α-amylase

The effect of metal ions was also considered in the study and incorporated in the standard assay mix whereby untreated samples were taken as a control (100%). As shown in Figure 6, 1 mM $CaCl_2$ is a stimulant to both the crude and purified D9 α-amylase as it increased activity by more than 40% for the crude amylase and more than 30% for the purified amylase. Evidently, $CuSO_4$ and EDTA inhibited more than 90% of activity while $FeCl_3$ inhibited more than 70% for both crude and purified amylase.

Metal ions such as $CuSO_4$, $FeCl_3$ and metal chelator, EDTA, inhibited enzyme activity markedly.

3.3.4. Kinetic parameters of the crude, purified D9 α-amylase and recombinant α-amylase

The purified D9 α-amylase possessed a K_m of 37.3 mM and V_{max} of 19.8 U/mg and k_{cat} of 4.84 s^{-1} with a catalytic efficiency of 0.13 $s^{-1}mM^{-1}$ (Table 2).

3.4. Cloning and expression of α-amylase of Paenibacillus sp. D9 into E. coli

The PCR amplicon of D9 α-amylase (1482 bp) was obtained, double digested using HindIII and XhoI and inserted into the PSF-OXB20 plasmid. A successfully cloned plasmid (designated as PSF-*amyS*) containing the *amy* gene was then transformed into *E. coli* BL21 (DE3) pLysS. One millilitre of the overnight culture containing PSF-*amyS* plasmid was grown in 100 mL of LB broth containing 34 mg/mL chloramphenicol and 50 mg/mL kanamycin for 24 h at 37 °C at a speed of

Table 2. Summary of kinetic properties exhibited by wild type and recombinant α-amylase based on the Lineweaver–Burk plot.

Sample	V_{max} (U/mg)	K_m (mM)	k_{cat} (s^{-1})	Catalytic efficiency ($s^{-1}.mM^{-1}$)
Purified D9 α-amylase	19.8	37.34	4.84	0.13
Purified recombinant D9 α-amylase	18.7	36.10	4.20	0.12

160 rpm. Production of D9 α-amylase was clearly visible after 6 h of incubation and increased as the time increased. The cells containing α-amylase activity were harvested by centrifugation and the re-suspended cell pellet was sonicated. The supernatant obtained after centrifugation at 10,000× *g* was used for the purification of D9 α-amylase.

3.5. Purification and characterization of cloned D9 α-amylase

Cloned D9 α-amylase was purified to homogeneity using a Hispur Cobalt column, to a molecular weight of 54 kDa as expected (data not shown). Active fractions (2 mL) were dialyzed against citrate buffer, pH 6.0, overnight at 4 °C. Cloned D9 α-amylase possessed the same optimal temperature (30 °C) and optimal pH (pH 7) values of the purified α-amylase from *Paenibacillus* sp. D9. The cloned D9 α-amylase also had similar kinetic parameters (K_m, V_{max}, k_{cat} and k_{cat}/K_m values of 36.10 mM, 18.7 U/mg, 4.20 s^{-1} and 0.12 s^{-1} mM^{-1}, respectively) compared to those of the purified D9 α-amylase (Table 2). The effect of metal ions on α-amylase activity was also similar (Figure 6).

Surprisingly, the cloned D9 α-amylase retained α-amylase activity without the presence of calcium ions even after the dialysis. The recombinant D9 α-amylase also possessed a higher specific activity of 451 U/mg, which was 1.4-folds higher than that of the purified D9 α-amylase (323 U/mg). Furthermore, the thermo-stability pattern of the recombinant D9 α-amylase shows the recombinant D9 α-amylase was more stable than the purified D9 α-amylase (Figure 5(B)). The recombinant D9 α-amylase lost only 4% of its relative activity in the first 30 min, 44% after 2 h of incubation at 45 °C and retained 74% and 38% in 30 min and 2 h, respectively at 60 °C (Figure 5(B)). On the other hand, the cloned D9 α-amylase demonstrated similar pH stability at pH 7 and pH 8 but was less stable at pH 5 and 6 compared to the purified D9 α-amylase (Figure 5(D)).

4. Discussion

In this study, α-amylase from *Paenibacillus* sp. D9 has been purified and characterized. The maximal α-amylase production by *Paenibacillus* sp. D9 was noted after 24-h incubation time, pH 7 at 30 °C (Figure 1), using soluble starch as the sole carbon source. The SDS-PAGE results showed that the extra protein band (54 kDa) was induced by soluble starch since Day 1 which corresponded with the encoded α-amylase of *amyS* gene of *Paenibacillus* sp. D9. The results also show that *Paenibacillus* sp. D9 preferred sodium nitrate as the nitrogen source for α-amylase production (Figure 2).

Alpha-amylase of *Paenibacillus* sp. D9 was purified to electrophoretic homogeneity (Figure 3) with an 11.7-fold increase in purity (Table 1). During the protein purification, $CaCl_2$ supplementation was essential to retain α-amylase activity. According to literature, some α-amylases are described as metalloenzymes. The presence of Ca^{2+} is important in the stability and integrity of these enzymes (Li et al. 2017; Wu et al. 2018; Yin et al. 2018). In addition, dialysis prior to column chromatography, increased the specific activity of the crude extract, indicating that small molecule(s) (<12 kDa) in the crude extract acted as α-amylase inhibitors.

The specific activity of the purified D9 α-amylase was found to be 323 U/mg (Table 1), which is higher than α-amylase (52.9 U/mg) purified from *Paenibacillus* PP70 (Tsusaki et al. 2012) and cloned α-amylase (114 U/mg) from *Paenibacillus* sp. SSG-1 (Xu et al. 2018). The results were comparable with other *Bacillus* spp. (Xie et al. 2014). Several marine microorganisms possess α-amylases with high specific activities such as *Streptomyces* strain A3 (1755 U/mg) (Chakraborty et al. 2012), *Nocardiopsis* sp. B2 (1755 U/mg) (Chakraborty et al. 2015) and *Zunongwangia profunda* (AmyZ2) (1663 U/mg) (Wu et al. 2014). Other studies also reported that *Bacillus* species possess α-amylases with high specific activities (Das et al. 2004; Liu and Xu 2008). The specific activity of D9 α-amylase is comparable or higher than most of the reports (Gangadharan et al. 2009; Asoodeh et al. 2010; Annamalai et al. 2011; Jana et al. 2013). The results of optimal temperature, pH and stability show that D9 α-amylase is most active in the neutral range (pH 6–8) and at 30 °C, which is in accordance to a *Paenibacillus* species study conducted by Tsusaki et al. (2012). The properties of D9 α-amylase will limit its industrial applications as most starch processing industries usually require α-amylases that are functional at high temperatures

(Sharma and Satyanarayana 2013). Thermostable α-amylases extracted from *B. stearothermophilus* and *B. amyloliquefaciens*, are extensively used for starch hydrolysis in the food sector (Park et al. 2014). However, literature has reported that there are various ways to improve the efficiency of α-amylases which are mainly achieved using genetic engineering strategies (Sindhu et al. 2017). These characteristics can be modified by site-directed mutagenesis or by directed-evolution (Dey et al. 2016).

In this study, the α-amylase gene (*amyS*) of *Paenibacillus* sp. D9 was successfully cloned into the PSF-OXB20 vector and expressed in *E. coli* BL21 (DE3) pLysS after transformation. The molecular weight (54 kDa) of the purified recombinant α-amylase was in accordance with the purified α-amylase from *Paenibacillus* sp. D9. Interestingly, the specific activity (451 U/mg) of the recombinant α-amylase was 1.4-fold higher than the purified D9 enzyme. Sharma and Satyanarayana (2012) reported that a cloned α-amylase (lacking 37 amino acids from N- and 34 amino acids from C-terminal) from *B. acidicola* possessed a 15-fold higher specific activity than the native strain. In this study, the recombinant D9 α-amylase contains an additional 10× histidine residues and a TEV cleavage tag, following the C-terminal of D9 α-amylase sequence. These extra amino acid sequences might induce some conformational changes of D9 α-amylase to an active form. In addition, the recombinant α-amylase was able to retain the enzyme activity without Ca^{+2} and was more thermostable and less pH stable at lower pH values compared to the wild strain, which is calcium-dependent. In the starch industry, α-amylases that are Ca^{+2}-independent are in higher demand, as Ca^{+2} ions inhibit glucose isomerase (Antranikian 1992; Mehta and Satyanarayana 2016).

The presumed structural differences of the recombinant D9 α-amylase does not affect the physical and kinetic properties of the wild type enzyme. The optimal pH, temperature, effect of metal ions as well as the K_m, V_{max} and k_{cat}, remain similar between the recombinant D9 α-amylase and the purified one. Additional Ca^{+2} concentration enhanced the catalytic ability up to 130%–140% (Figure 6), in accordance with a study of *Paenibacillus* sp. SSG-1 by Xu et al. (2018) analyzed the protein sequence of *Bacillus methylotrophicus* strain P11-2 and propose a Ca^{+2} binding site in α-amylase. Calcium ions can facilitate the binding of the substrate to the active site, thus increases in activity of the α-amylase and/or induce the conformational changes to stabilize the structure of α-amylase that corresponds to the higher activity of amylase (Sachdev et al. 2016; Xu et al. 2018). Khajeh et al. suggested that Ca^{+2} not only enhances the activity but also the thermo-stability of the α-amylase (Khajeh et al. 2001). The presence of EDTA, Fe^{+3} and Cu^{+2} strongly inhibited the D9 α-amylase activity.

Enzyme kinetics inclusive of K_m and V_{max} are significant coefficients that facilitate scientific research and engineering development (Singh et al. 2018). The variation in kinetic parameters appeared to be expected as literature evidently indicated that the removal of undesired proteins from the crude extract allows for better efficacy of the enzyme (Cavalcante Braga et al. 2013; Bashir et al. 2014).

5. Conclusion

In conclusion, the characterization of α-amylase is a crucial factor, as it signifies its potential use in the industrial sector for most commercial industries. In this study, the wild type and recombinant α-amylase from *Paenibacillus* sp. D9 were purified to homogeneity and characterized. Both D9 α-amylases exhibited similar biochemical properties in terms of its functionality in pH and temperature conditions (pH 7 and 30 °C) and stable at 30 °C for 120 min. Both D9 amylases possessed comparable kinetic parameters for K_m, V_{max} and k_{cat}.

Interestingly, unlike the wild type enzyme, the cloned D9 α-amylase retained α-amylase activity without the presence of calcium ions. In addition, the recombinant α-amylase possessed an increased specific activity of 4501 U/mg, which is 1.4-folds higher than the wild-type enzyme. The recombinant α-amylase is more thermostable at higher temperature and less stable at acidic conditions than the wild type enzyme. The findings led to a proposal that the presence of a poly-histidine tag and TEV cleavage site in the recombinant α-amylase may have played an integral role in inducing a conformational change to stabilize the enzyme into correct conformation. This theory additionally supports the variation in the results of thermal and pH-stability. However, since there is no evidential support, future studies will focus on elucidating structural differences between the wild type and recombinant α-amylase, concluding valid reasoning for these findings.

Disclosure statement

No potential conflict of interest was reported by the authors.

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