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ORIGINAL ARTICLE

Molecular cloning and characterization of a halotolerant α-amylase from marine metagenomic library derived from Arabian Sea sediments

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Abstract Functional screening of a metagenomic library of marine sediment revealed an amylolytic clone BTM109. This report states the purification and characterization of a moderately halotolerant α-amylase, with more than 51% activity in 2.5 M NaCl. The molecular mass of purified protein was determined to be 55.7 kDa by MALDI-TOF MS. The optimum pH for enzyme activity was pH 7 and temperature for maximal activity was 40 °C. At 5 mM concentration, Ca^{2+} enhanced the enzyme activity indicating that the enzyme is a Ca^{2+} dependent α-amylase which was confirmed by the starch hydrolysis pattern using TLC. These physico-chemical properties support the suitability of this enzyme for various industrial applications.

Keywords Marine · Metagenome · Amylase · Halotolerant · Arabian Sea

Introduction

Microbes are omnipresent in nature. Soil microorganisms represent a huge reservoir of genes with potential for application in health and other industries. The search for biomolecules among cultivable microbiota represented by only a small fraction of the microbes in nature is mainly due to information deficit about nutrient requirements of the 'uncultured' ones. Standard microbiological methods account for less than 1% of the total microbes from the environment (Handelsman 2004). This impediment to culturing can be bypassed by metagenomic methods, wherein the total community DNA is used to construct libraries, which can be screened for novel biomolecules. Several enzymes like esterase, protease, amylase and others with biotechnological application have been identified from metagenomic libraries (Jin et al. 2012; Neveu et al. 2011; Vidya et al. 2011).

Among industrial enzymes, amylases represent one of the largest group and account for 30% of the overall worldwide sale of enzymes. Although amylases can be derived from several sources, including animals, plants and microorganisms, microbial enzymes generally meet industrial demands. Amylases find wide applications in the preparation of sugar syrups, cyclodextin as well as detergent and feed additives. They are also useful for biopharmaceutical, medicinal and clinical applications (Pandey et al. 2000). α-amylases (E.C. 3.2.1.1.) catalyze the hydrolysis of α-1,4-glucosidic linkages between adjacent glucose units in starch, and belong to family 13 of the glycoside hydrolase group of enzymes (GH-13) (Bordbar et al. 2005). Most are metalloenzymes requiring calcium ions (Ca^{2+}) for their stability and activity.

The characterization of an α-amylase obtained through metagenomic approach from Arabian Sea sediments is reported, wherein the physico-chemical properties of the amylase were also investigated.

Materials and methods

Bacterial strains, plasmids and growth conditions

Electro-competent *E. coli* DH10B (Invitrogen, CA, USA) as host and pUC 19 (Thermo Scientific, MA, USA) were used as vector. *E. coli* transformants were grown at 37 °C

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in Luria–Bertani (LB) (HiMedia, Mumbai, India) medium supplemented with 60 mg/mL ampicillin unless otherwise mentioned.

Sediment sampling and DNA manipulation

Marine sediments from 96 m depth were collected from the Arabian sea (9°59′10.9968″N, 75°39′26.4564″E) using grab onboard the research vessel FORV Sagar Sampada (Cruise No: 305). Samples were transferred to sterile containers and were frozen at −20 °C until brought on shore for further analysis.

All DNA manipulations were done according to the standard techniques (Sambrook and Russell 2001). Metgenomic DNA was isolated using Ultra clean soil DNA isolation kit (Mo Bio Laboratories Inc., CA, USA) and partially digested using *Sau*3A1 (Thermo). DNA fragments isolated from agarose gels employing gel extraction kit (GeNei, India) were ligated using Rapid DNA ligation kit (Promega, Madison, USA). Ligated vectors were transformed to *E. coli* DH10B by electroporation using Micropulser II (BioRad, CA, USA).

Construction of genomic libraries and screening for amylase activity

Gel eluted, partially digested metagenomic DNA (∼2–10 kb) was ligated into the *Bam*HI digested and dephosphorylated pUC19 vector. Transformed cells were plated onto Luria–Bertani (LB) agar plates containing ampicillin (60 mg/mL), X-Gal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside) (20 mg/mL) and IPTG (isopropyl-thiogalactopyranoside) (100 mM). Clones patched on LB plate with ampicillin were maintained as a metagenomic library. Library was screened for amylase activity by patching onto 1% starch agar plates and amylolytic activity was visualized by flooding the plates with iodine–potassium iodide solution (0.1:1%) (Schwartz 1971).

Amylase assay

Amylase activity was determined by measuring the liberation of maltose employing dinitrosalicylic acid method (Miller 1959). 500 µL of appropriately diluted enzyme was reacted with 500 µL of 1% rice starch (w/v) prepared in 0.1 M phosphate buffer (pH 7) and the mixtures were incubated at 40 °C for 15 min. The reaction was terminated by adding 2 mL dinitrosalicylic acid solution followed by boiling for 5 min. Absorbance was measured at 540 nm using spectrophotometer (Shimadzu, Japan). One unit of enzymatic activity was defined as the amount of enzyme liberating 1 µmol of reducing sugar as maltose per min under assay conditions. Unless otherwise mentioned, α-amylase activity was assayed at the conditions mentioned above.

Purification of amylase and zymogram analysis

The culture supernatant after bacterial cell separation was precipitated with 1 volume of chilled acetone, vortexed well and kept at −20 °C for 1 h. The precipitated protein was collected by centrifugation at 15,000 rpm for 15 min (Sigma, 2–16 K, Germany) at 4 °C and dissolved in 0.1 M phosphate buffer (pH 7). Acetone precipitated protein was concentrated by 30 kDa cutoff Amicon Ultra filter (Millipore, Germany) and applied onto Sephadex-G75 gel column equilibrated with 0.1 M of phosphate buffer (pH 7.0), followed by elution with the same buffer at a rate of 0.3 mL/min. Fractions were assayed to determine enzyme activity. Active fractions were analyzed by SDS-PAGE to determine purity, pooled, concentrated and used as purified enzyme and designated as P109. Protein was determined according to Bradford method using bovine serum albumin as standard (Bradford 1976).

SDS-PAGE used 10% resolving gel and 5% stacking gel (Laemmli 1970). One portion of the gel was stained using Coomassie Brilliant Blue to visualize protein bands, while the other was incubated in 1% rice starch solution for 1 h at room temperature followed by staining with iodine–potassium iodide solution for zymography. Intact molecular mass of protein was determined by MALDI-TOF mass spectrometer (Bruker Daltonics, Germany).

Physico-chemical characterization of P109

The pH optimum for enzyme activity was determined by varying the pH of the reaction mixture using different buffer solutions. pH stability was also determined by pre-incubating the enzyme in different buffers of pH 1–13 for 30 min and estimating residual enzyme activity. Similarly, optimum temperature for enzyme activity was determined by varying incubation temperature 4–100 °C. Thermostability was determined by pre-incubating purified enzyme at temperatures from 4–100 °C for 30 min and estimating residual activity.

Effect of various metal compounds ($BaCl_2$, $FeCl_3$, $AlCl_3$, $CdSO_4$, $CuSO_4$, $CaCl_2$, $CoCl_2$, $ZnSO_4$, Na_2CO_3, $Pb(NO_3)_2$, $MgSO_4$, and $MnCl_2$) and protein inhibitors such as dithiothreitol (DTT), Ethylenediaminetetraacetic acid (EDTA), N-bromosuccinimide (NBS), Phytic acid, Phenyl methyl sulfonyl fluoride (PMSF), and Urea was tested by adding to the enzyme reaction mixture at 5 mM concentration.

The substrate specificity of P109 was studied using substrates including rice starch, potato starch, wheat starch,

corn starch, amylose from potato, amylopectin from maize (Sigma-Aldrich, MO, USA). The reducing sugars produced were determined by DNS method and relative activity was calculated. The kinetic properties of the enzyme were determined by conducting enzyme assay at varying substrate concentrations from 0.5 to 20 mg/mL. The K_m and V_{max} were estimated from Lineweaver–Burk plot.

Halotolerance of amylase was analyzed in the presence of 0.5–2.5 M NaCl. The enzyme reaction was performed in buffer with different salt gradient and residual activity was calculated.

End product analysis by thin-layer chromatography (TLC)

End products of starch hydrolysis were analyzed after incubating with enzyme for 2 h, followed by spotting on a TLC silica gel 60 F plate (Merck, Germany). The plates were developed in a saturated chromatographic chamber with 2-propanol: *n*-butanol: water (12:3:4) solvent system (Vester et al. 2015), and the spots of sugars were visualized by spraying aniline-diphenylamine reagent followed by incubation in a hot air oven at 110 °C.

Determination of starch grain degradation by scanning electron microscopy (SEM)

The efficiency in digesting starch grains (rice starch and potato starch) was analyzed by incubating 1% starch grains with P109 for 2 and 4 h. After hydrolysis, grains were pelleted, rinsed with pure ethanol, air dried and examined under SEM (JSM-6390).

Detergent compatibility testing and wash performance analysis

The compatibility and stability of P109 in the presence of commercial detergents were determined using 7 commercial detergents such as Ariel®, Surf Excel®, Sunlight®, Tide®, Wheel® and Ujala® at 1% (w/v) each. The enzymes in the detergent were first heat inactivated by boiling for 15 min, followed by enzyme assay and residual activity was calculated.

To determine the suitability of P109 as detergent additive, wash performance analysis was done with chocolate stained cotton fabric (5 cm × 5 cm). The cloth pieces were stained with 200 µL of liquefied chocolate and dried using a hot air oven. The stained pieces of cloth were taken in separate flasks and subjected to wash treatment with appropriate controls and the reaction setup is as follows. Flask 1: 100 mL distilled water + stained cloth piece, Flask 2: 100 mL detergent solution + stained cloth piece, Flask 3: 100 mL detergent solution + stained cloth piece + 1 mL P109, Flask 4: 100 mL distilled water + -stained cloth piece + 1 mL P109. After 30 min incubation at room temperature with shaking at 200 rpm (Orbitek, Scigenics India), the cloth pieces were taken out, rinsed with tap water, oven dried and visually examined to check the effectiveness of stain removal.

Results and discussion

Clone library construction

The pUC19 clone library ($n = 1113$) screened for enzyme production yielded one amylase positive clone designated as BTM109 indicated by zone of clearance on starch agar plate and was selected for further study. Sequence analysis of insert DNA showed the presence of an open-reading frame consisting of 1554 nucleotides, encoding a protein with 517 amino acids (unpublished data). Metagenomic analysis of acid mine drainage reported the characterization of two non-homologous endo-acting amylases from metagenomic library constructed in plasmid vector (Delavat et al. 2012).

Purification and mass determination of P109

While SDS-PAGE followed by Coomassie staining identified multiple protein bands in the crude acetone fraction of P109, a single band was noted in the gel filtered purified fraction. The apparent molecular weight of P109 was about ~55 kDa (Fig. 1). Zymogram analysis revealed a single amylolytic band corresponding to this molecular weight. The intact mass by MALDI-TOF MS was 55.7 kDa (Fig. 2); this molecular weight concurred with that deduced from the amino acid sequences and that determined by PAGE. Other studies on cloning α-amylases genes and deduced amino acid sequences suggested that the molecular mass of microbial α-amylases usually ranges from 50 to 60 kDa (Vihinen and Mantsala 1989).

Effect of pH and temperature on activity and stability

P109 amylase showed higher activity as pH increased from the acidic range, peaking at pH 7, maintaining >80% relative activity at pH 8 and 9, and declining thereafter, with complete loss of activity at higher alkaline pH (Fig. 3a). Similarly, maximal stability was at pH 7, with more than 75% activity between pH 6 and 11. At the same time, P109 lost approximately 80% of its activity at pH 1, 2, 3, 4 and 13 (Fig. 3b). Even though P109 is highly active at neutral pH, it maintained more than 75% relative

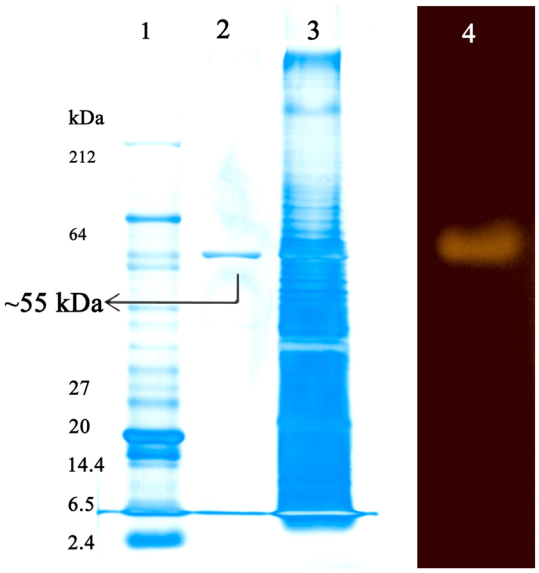


Fig. 1 SDS-PAGE and zymogram of P109. *Lane 1* protein marker (NEB), *Lane 2* purified P109, *Lane 3* acetone precipitated fraction, *Lane 4* zone of clearance obtained by zymogram analysis

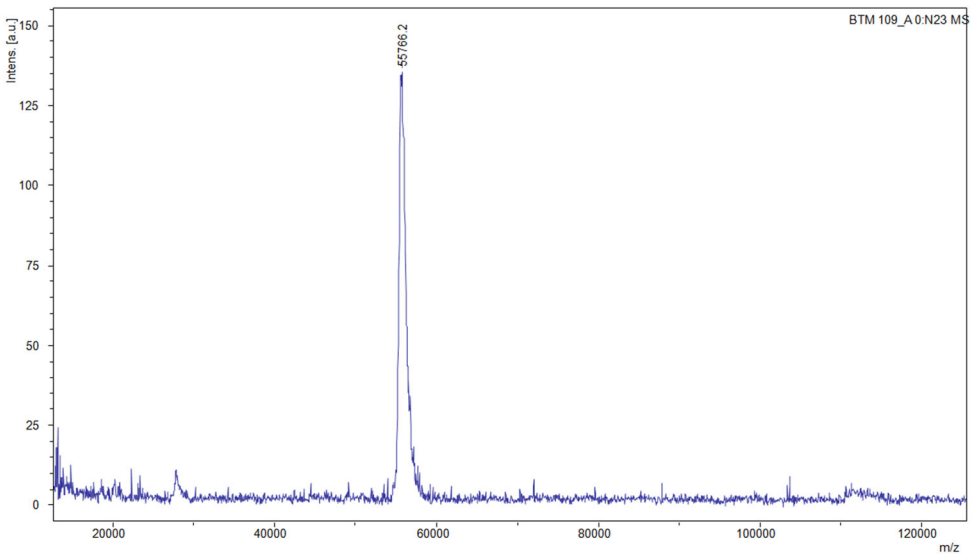
activity at near alkaline pH, suggesting its potential as a detergent additive for stain removal. The optimal reaction temperature for P109 was 60 °C (Fig. 3c), and amylase activity decreased greatly at temperatures >80 °C. It was revealed that P109 retained only 50% activity at 60 °C after 1 h (Fig. 3d). For this reason, the amylase assay was conducted at 40 °C for maximal activity. The influence of pH on the enzyme derived from Northwestern Himalayas soil metagenomic library showed activity from pH 5.5–7.5, with maximum activity at 6.5 and enzyme stability between pH 5.5 and 7.0, while the temperature optima was 35 °C and stability at 10 to 50 °C (Sharma et al. 2000).

Effect of metal ions and inhibitors on activity of P109

Metal ions stabilize or inhibit enzyme activity. In this study interaction with Ca^{2+} ions enhanced the activity of P109 to 110% of its initial activity, while other ions did not have any stimulatory effect. This activity enhancement in the presence of Ca^{2+} supports the finding that P109 is an α-amylase. On the other hand, the presence of Cu^{2+} and Zn^{2+} decreased residual activity of P109 to 69 and 67%, respectively. P109 maintained around 75% residual activity in the presence of other metal ions tested (Table 1).

Among the six enzyme inhibitors tested, NBS treatment caused 96% inhibition of amylase activity. NBS acts by oxidizing tryptophan resides, which have a major role not only in active site of amylases, but also in maintaining the secondary structure, thereby coordinating amylase activity (Uma Maheswar Rao and Satyanarayana 2008). This was further confirmed by the conserved domain search of deduced amino acid sequence that tryptophan was present as an active site residue in P109 (data not shown). EDTA and phytic acid caused 35 and 61% reduction of the initial enzyme activity. As both are metal ion chelators, the reduction in activity suggests that P109 may be a metal-dependent amylase, which was also established. Similarly, PMSF caused 50% inhibition of amylase activity, implying a role for serine in enzyme catalysis. DTT acts as inhibitor by either acting on the thiol groups present in the proteins or can possibly alkylate lysine residues. Inhibitory action of urea is ascribed to its denaturing character that unfolds the amylases by acting on the hydrophobic amino acids in the enzyme polypeptide chain (Chakraborty et al. 2011). The loss of activity of P109 due to DTT and urea was 49 and 35%, respectively (Table 2).

Fig. 2 Mass spectrum of P109 obtained by MALDI-TOF MS



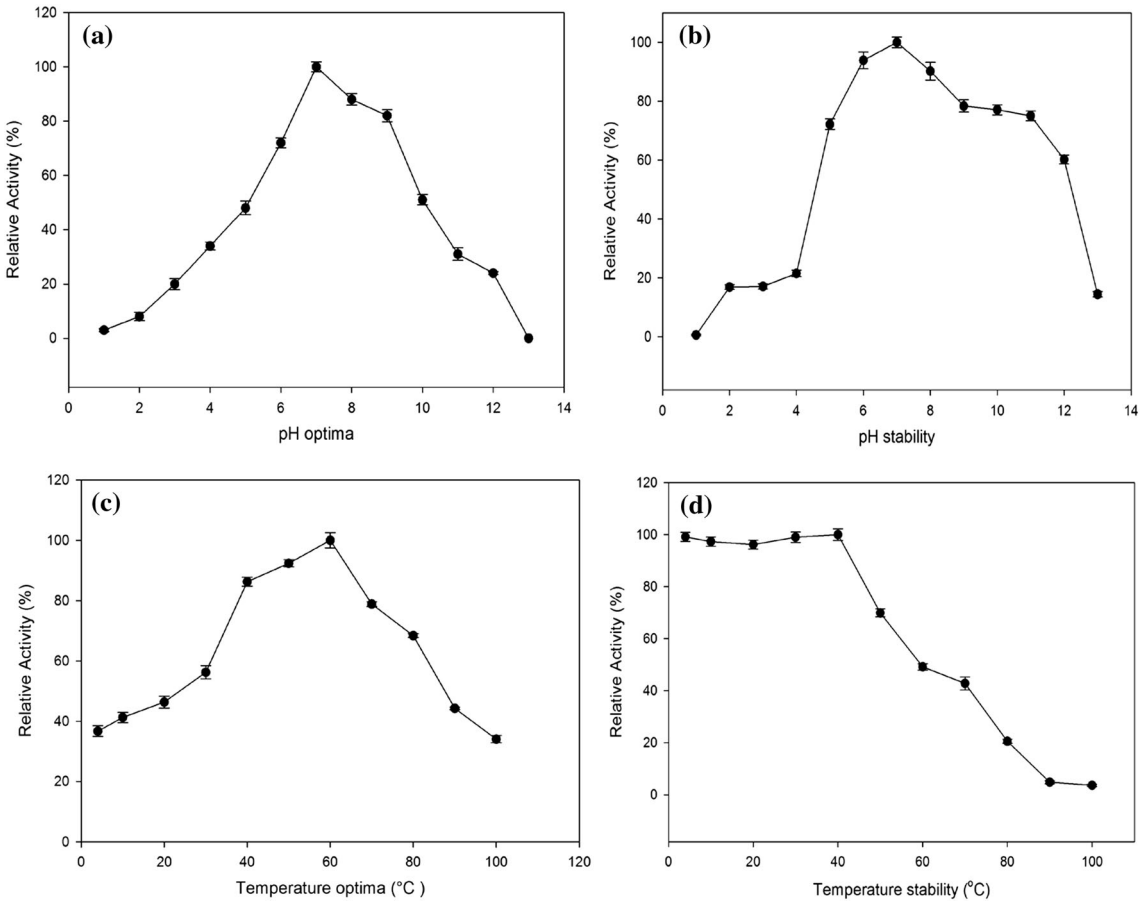


Fig. 3 Effect of pH and temperature on the activity and stability of P109, **a** pH optima, **b** pH stability, **c** temperature optima, **d** temperature stability

Table 1 Effect of metal ions on enzyme activity of P109

Metal ions (5 mM)	Residual activity (%)
Ba^{2+}	78.44 ± 1.7
Fe^{3+}	82.13 ± 1.9
Al^{3+}	89.25 ± 2.6
Cd^{2+}	74.97 ± 2.1
Cu^{2+}	30.49 ± 3.1
Ca^{2+}	110.21 ± 2.2
Co^{2+}	77.08 ± 1.4
Zn^{2+}	32.14 ± 1.2
Na^{+}	77.23 ± 1.2
Pb^{2+}	85 ± 3.1
Mg^{2+}	85.49 ± 2.2
Mn^{2+}	85.75 ± 1.8

Table 2 Effect of inhibitors on activity of P109

Inhibitors (5 mM)	Residual activity (%)
DTT	51.62 ± 0.8
EDTA	35.01 ± 1.8
NBS	4.33 ± 0.7
Urea	65.34 ± 2.1
PMSF	50.90 ± 2.8
Phytic acid	61.01 ± 3.1

Substrate specificity and kinetic constants of P109

Studies suggested that amylases display the highest activity towards starch followed by amylose (Gupta et al. 2003). Rice starch was identified as the best substrate for P109, followed by potato starch and amylopectin from maize, giving 97 and 94% relative activity, respectively. Action of P109 on corn starch resulted in 73% relative activity, while the lowest activity was with amylose from potato (23% relative activity) (Fig. 4).

The kinetic values, K_m and V_{max}, were calculated from the Lineweaver–Burk plot to be 2.7 mg/mL and 454 U/mL, respectively. The K_m of the most detergent compatible amylases was identified to be in the range 0.1–5.0 mg/mL using soluble starch as substrate (Niyonzima and More 2014). A K_m value of 3.28 mg/mL has been reported for a α-amylase from metagenomic using amylopectin as substrate (Vester et al. 2015).

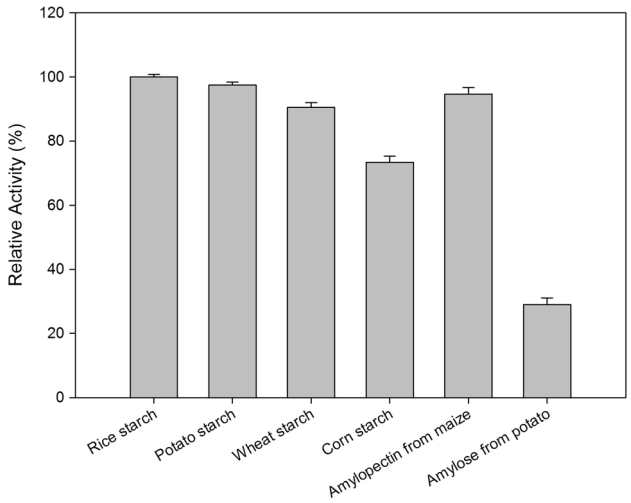


Fig. 4 Substrate specificity of P109

Table 3 Halotolerence of P109

NaCl	Residual activity (%)
0.5 M	66.42 ± 1.9
1 M	62.65 ± 2.1
1.5 M	61.12 ± 3.3
2 M	53.04 ± 0.9
2.5 M	51.12 ± 1.1

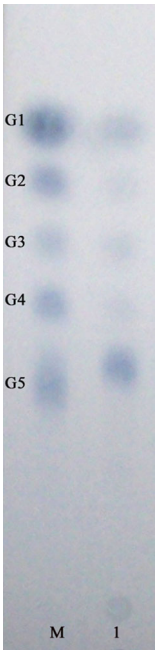
Halotolerance studies

As P109 is from a marine metagenomic clone library, salt tolerance was examined. P109 retained 63% activity at 1 M NaCl and almost 67% at 0.5 M NaCl, but residual activity was 51% at 2.5 M NaCl. At 1.5 M NaCl, only 37% inhibition in P109 activity was noted (Table 3). This indicates that enzyme P109 was halotolerant at the NaCl concentrations tested, further suggesting the marine nature of the product. Extracellular enzymes produced by halophilic microorganisms are adapted to high salinity. Halotolerant amylases retaining >80% activity at different NaCl concentrations have been reported (Aygan et al. 2008; Srimathi et al. 2007), including metagenomics-based amylases. A metagenomic α-amylase exhibited 90% activity in 1 M NaCl, and 20% of its initial activity at 2 M NaCl (Vidya et al. 2011).

Chromatographic analysis of hydrolysis products

The nature of enzyme hydrolysis was characterized by TLC based on the degradation products obtained after starch hydrolysis (Fig. 5). Starch hydrolysis pattern by P109 indicated that the main hydrolysis products post 2 h of incubation were glucose (G1), maltose (G2) and malto

Fig. 5 Thin-layer chromatographic analysis of hydrolysis products. *M* represents standards [glucose (G1), maltose (G2), maltotriose (G3), maltotetrose (G4) and maltopentose (G5)], *1* represents end products formed after 2 h of hydrolysis



oligosaccharides such as maltotriose (G3) and maltopentose (G5). The degradation pattern of P109 was typical of an α-amylase.

Determination of starch grain degradation by scanning electron microscopy (SEM)

The enzyme showed substrate specificity, with rice starch as the best substrate. Hence, the effect of P109 on rice starch after 2 and 4 h of enzyme treatment and that on potato starch after 2 h of enzyme treatment was studied and is as in Fig. 6. Rice starch was hydrolyzed, to a greater extent, than the potato starch which supported the substrate specificity studies. Compared to the control, distortions and holes were observed in P109 treated starch granules after 4 h treatment suggesting the hydrolyzing property of P109. Prior reports suggest that starch hydrolysis is due to the interplay of many other factors like granule shape, amylose–lipid complexes, amylose to amylopectin ratio, amylose chain length, phosphorus content, degree of crystallinity, botanical origin and cultivar/variety (Tester et al. 2006). The method of adsorption of enzymes on starch granules is still unclear, but binding probably occurs through a C-terminal binding domain (Jespersen et al. 1991).

Commercial detergent compatibility and wash performance studies

Suitability of an enzyme preparation for detergents use depends on its compatibility. As shown in Fig. 7, P109 showed excellent stability and compatibility in the

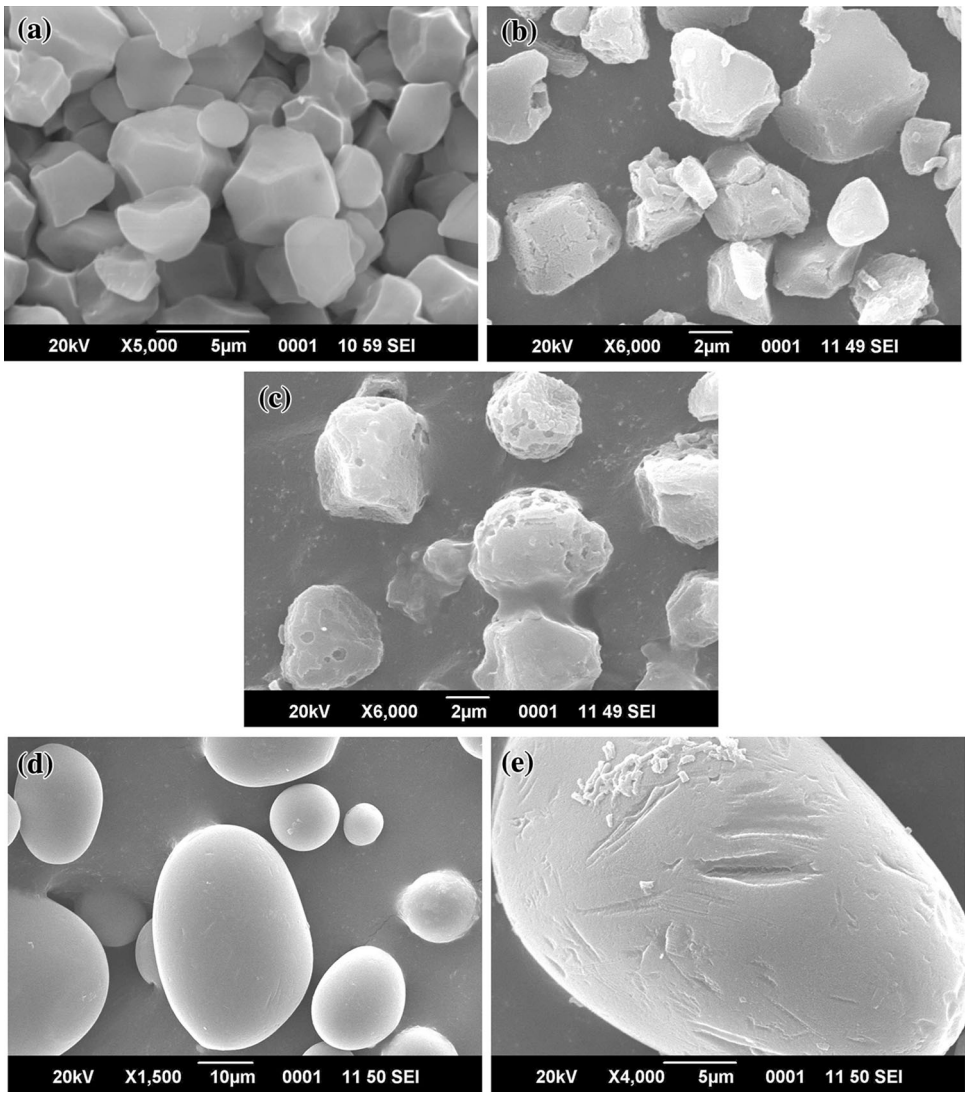


Fig. 6 SEM images of starch granules before and after hydrolysis. **a** Untreated rice starch, **b** 2 h treated rice starch, **c** 4 h treated rice starch, **d** Untreated potato starch, **e** 4 h treated potato starch

presence of Sunlight®, while with all other detergents tested the enzyme was maintaining less than 60% residual activity. Therefore, the effectiveness of P109 along with Sunlight® detergent in starchy stain removal was tested. The partial loss of α-amylase activity in the presence of some of the laundry detergents may be attributed to the inhibitory effect of other components of these detergents like bleaching agents, anionic surfactants, water softening agents, etc., which may influence the stability of enzyme in detergent (Mukherjee et al. 2009).

Wash performance studies identified Sunlight® supplemented with P109 was better at stain removal from cotton fabrics than detergent alone (Fig. 7c, e) Similarly, P109 could also remove stain even in the absence of the detergent, as is visible from Fig. 7d. Similarly, previous reports on wash performance in chocolate-stained cloth piece recognized that the enzyme-detergent combination resulted in better stain removal from cotton fabrics as compared to that of detergent alone (Roy and Mukherjee 2013). Likewise, the alkaline amylase of *B. cereus* also improved the washing capacity of a detergent by removing food gravy stain from white cloth (Roohi et al. 2013).

Conclusion

In conclusion, an amylolytic clone BTM109 was identified from metagenomic library derived from Arabian Sea sediments. The physico-chemical characterization revealed its halotolerant nature, with Ca^{2+} acting to enhance enzyme activity. The study revealed the potential of marine microbial community of Arabian Sea sediments suggesting that metagenomic library screening is a valuable tool to explore novel biomolecules with

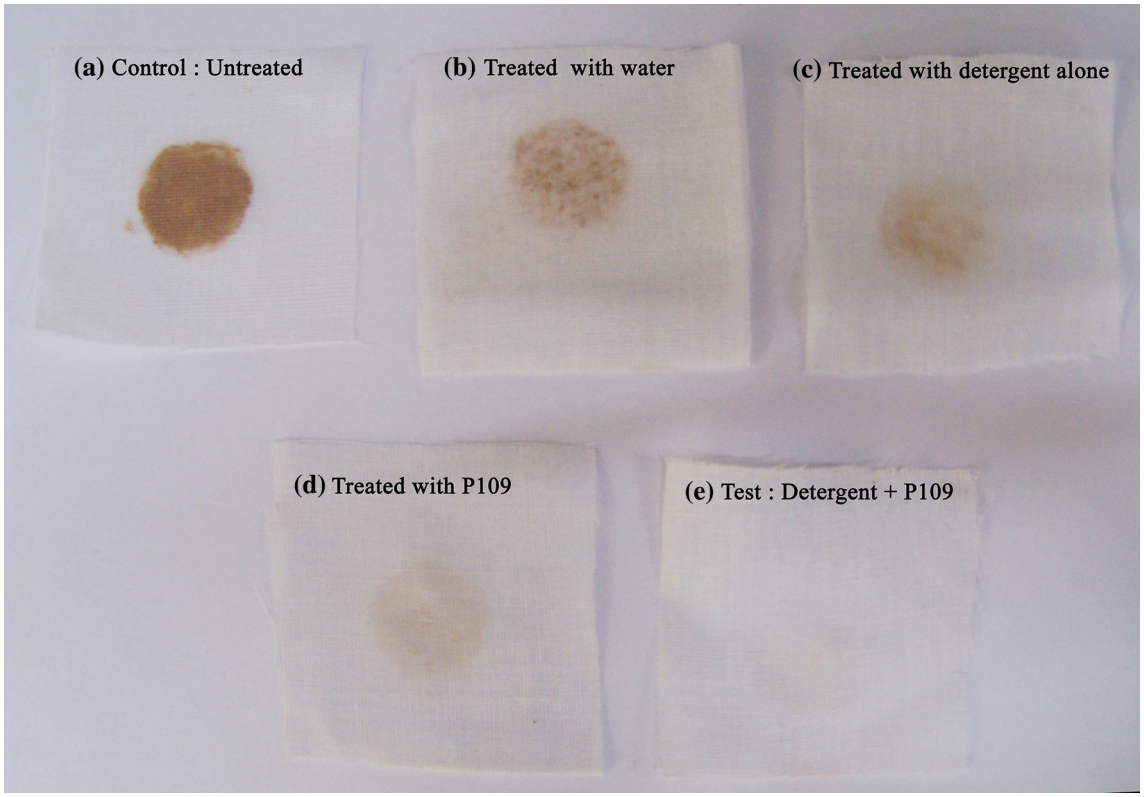


Fig. 7 Wash performance studies of P109

improved properties for suitability in various industrial purposes.

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Compliance with ethical standards

Conflict of interest All the authors declare that they have no conflict of interest.

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