Journal of Molecular Catalysis B: Enzymatic 58 (2009) 17–23

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

# Isolation and characterization of novel α-amylase from marine *Streptomyces* sp. D1

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ARTICLE INFO

*Article history:*
Received 18 August 2008
Received in revised form
19 September 2008
Accepted 20 October 2008
Available online 11 November 2008

*Keywords:*
Marine
Streptomyces
Halophilic
Surfactant
Detergent stable

ABSTRACT

In this study, we have reported novel α-amylase enzyme from less extensively studied marine *Streptomyces* sp. D1. Enzyme production was determined by using media containing 2% sucrose, 0.35% peptone and 0.15% of malt extract. Optimum temperature for enzyme production and activity was found to be 45 °C and enzyme retained almost 50% of its activity at 85 °C. Enzyme activity was also retained in presence of commercially available detergent and oxidizing agents. The partially purified enzyme from strain D1 exhibited specific activity of 113.64 U/mg protein that corresponds to 2.8-fold purification. SDS-PAGE and zymogram activity staining showed a single band equal to molecular mass of 66 kDa. The reported enzyme may have wide spread application for detergent and pharmaceutical industry.



## 1. Introduction

Enzymes have attracted attention from researchers all over the world because of wide range of physiological, analytical and industrial application; especially from microorganisms because of their broad biochemical diversity, feasibility of mass culture and ease of genetic manipulation [1]. More than 3000 different enzymes have been identified and many of them found their way into biotechnological and industrial applications [2]. The present enzyme toolbox is not sufficient to meet many industrial demands. Therefore, researchers are now trying to exploit extremophiles which are the valuable source of novel enzymes [3,4]. Among the extremophiles, halophiles are microorganisms that live, grow, and multiply in highly saline environments. Moderately halophilic bacteria are able to grow over a wide range of salt concentrations from 0.4 to 3.5 M with optimum growth at 0.5–2.0 M [5]. Exoenzymes from these organisms with polymer-degrading ability at low water activity are of interest in many harsh industrial processes where concentrated salt solutions would inhibit enzymatic conversions [6].

Amylase (endo-1, 4-α-D-glucanohydrolases EC 3.2.1.1) constitutes 25% of the sales in enzyme market [7,8]. This enzyme plays a vital role in many industrial processes such as sugar, textile, paper, brewing, baking and distilling industries. It is also used in food industry and in pharmaceutical industry [9] as a digestive aid. The demand for amylase is increasing day by day because of its tremendous potentiality in above mentioned industrial sectors.

Marine microorganisms are capable for catalyzing various biochemical reactions with novel enzymes such as amylase, deoxyribonuclease, lipase and protease [10,11]. *Streptomyces* exhibit remarkable capacity for the synthesis of secondary metabolites and use of numerous extracellular hydrolytic enzymes to degrade organic material in their natural habitat [12].

There are many reports on thermostable α-amylase production from bacteria belonging to genus *Bacillus* such as *Bacillus coagulans, Bacillus stearothermophilus* and *Bacillus candolyticus* [13,14]. Amylase production has been reported from eubacterial moderate halophiles such as *Acinetobacter* [15], *Micrococcus halobius* [16], yeast such as *Cryptococcus flavus* [17] and *Halomonas meridiana* [18]. But very limited research has been directed towards α-amylase production from marine *Streptomyces* species [19]. The present study was carried out to select a *Streptomyces* strain that produces moderately halophilic, detergent and surfactant stable α-amylase and to investigate its properties and stabilities.

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doi:10.1016/j.molcatb.2008.10.011

## 2. Materials and methods

### 2.1. Source for actinomycetes strain

Marine Sediment samples were collected from Goa, Alibagh and Mumbai coastal region of India at the time of low tide. Heat pretreatment at 40 °C for 30–60 days was used for isolation of marine actinomycetes [20,21]. Soil samples were suspended in sterile water and mixed on rotary shaker at 150 rpm for 20 min. Different marine actinomycetes were isolated by using selective media such as glycerol yeast extract agar, starch casein agar, maltose yeast extract agar and glucose aspargine agar. The isolated strains were screened for amylase production by using starch agar medium prepared in artificial sea water. The artificial sea water (ASW) contains (g/l) NaCl, 23.37; $Na_2SO_4$, 3.91; $NaHCO_3$, 0.19; KCl, 0.66; KBr, 0.09; $MgCl_2$, 4.98; $CaCl_2$, 1.10; $SrCl_2$, 0.02 and $H_3B0_3$ 0.02. Maximum amylase producing marine *Streptomyces* sp. D1 was maintained on glycerol yeast extract agar medium.

### 2.2. Identification of strain D1

Identification of strain was done by scanning electron microscopy (SEM), biochemical and cultural characterization. The preparation of culture for SEM was done as described by Williams and Davies [21].

### 2.3. Inoculum preparation and culture condition

The glycerol yeast extract medium containing starch 1% (w/v) prepared in artificial sea water (ASW) was used for development of inoculum. The seed culture was prepared in 100 ml of conical flasks containing 50 ml of medium by inoculating 2.0 ml of spore suspension containing 2.5 to $3.0 \times 10^6$ CFU $ml^{-1}$ and cultivated under agitation (200 rpm) at 45 °C for 4 days. The seed culture (50 ml) was inoculated in the 500 ml of fermentation medium containing; 2% sucrose, 0.5% peptone, 3% malt extract prepared in artificial sea water and fermentation was carried out 12 days under agitation at 200 rpm at 45 °C. The cell free supernatant containing α-amylase was harvested by centrifugation at $1000 \times g$ for 15 min and subjected to partial purification and characterization.

### 2.4. Effect of pH, temperature and NaCl on α-amylase enzyme production and activity

Effect of pH on production of enzyme was studied by adjusting the pH of the basal medium to different levels. Similarly, effect of temperature was studied in basal medium at different temperatures. Effect of NaCl on enzyme production was studied by varying the concentrations of NaCl % (w/v) added to the basal medium.

Effect of pH, temperature and NaCl on enzyme activity was determined by carrying out the enzyme substrate reaction in various buffer systems, at different temperatures and in presence of different concentrations of NaCl % (w/v) respectively. Enzyme activity was expressed [17] as percentage relative activity.

### 2.5. Effect of metal ions, EDTA and various chemicals on enzyme activity

Effect of metal ions, EDTA and various chemicals on enzyme activity was determined by carrying out the enzyme substrate reaction in presence of above mentioned additives. Enzyme activity was determined as percentage relative activity as compared to control (without additives) considered as having 100% of relative activity.

### 2.6. Repression study

Repression of enzyme secretion by glucose was studied by incorporating different concentration of glucose to the production medium. Effect of glucose on amylase activity was determined by carrying out the enzyme substrate reaction in presence of glucose. Amylase activity was expressed as percentage relative activity.

### 2.7. Partial purification of α-amylase enzyme

The enzyme was precipitated by bringing the culture filtrate to 90% saturation with solid ammonium sulfate and kept at 4 °C for overnight. The precipitate was centrifuged at $12{,}000 \times g$ for 30 min. The precipitate was dissolved in glycine–NaOH buffer of pH 9 and dialyzed for 48 h against the same buffer. The dialyzed sample was assayed for α-amylase activity and protein content [22]. The partially purified enzyme was used for investigating the effect of NaCl, temperature, pH and metal ions on enzyme activity.

### 2.8. Assay of α-amylase enzyme

The activity of α-amylase was estimated by determining the amount of reducing sugar released from starch. The liberated reducing sugars were quantified by the method of 3,5-dinitrosalicylic acid (DNSA) according to Miller [23]. One unit (U) of enzyme activity was defined as the amount of enzyme required for the liberation of 1 mmol of reducing sugar as glucose per minute under assay condition.

### 2.9. Stability studies of amylase

The pH stability of amylase was determined by incubating the enzyme in presence of different buffer systems. Residual amylase activity was expressed as % relative activity as compared to control (100% relative activity). Amylase enzyme was incubated at different temperatures (6–48 h) for determination of temperature stability. Amylase was incubated in presence of different percentage (w/v) of NaCl for different time intervals (6–48 h) and residual activity of amylase was determined.

Surfactant and detergent stability of amylase was determined by incubating the enzyme in presence of anionic detergents, nonionic detergents and commercial detergents such as rin, surf, aerial and tide [24]. Role of bleaching agent on enzyme activity was determined by incubating the enzyme in presence of different bleaching agents and enzyme activity was expressed as percentage relative activity determined under standard assay condition.

### 2.10. Electrophoretic method

SDS-PAGE was used to determine protein purity and the molecular mass of the purified enzyme using a 10% acrylamide gel [25]. After electrophoresis, gel was stained with Coomassie R-250. Phosphorylase b (97 kDa), bovine serum albumin (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa) and soyabean trypsin inhibitor (20.1 kDa) were used as molecular mass markers. For activity staining, gel was suspended in 20% (v/v) isopropanol and incubated for 30 min, then transferred to 0.1 M glycine–NaOH buffer (pH 9) and incubated for 30 min. Amylolytic activity [24] was determined by placing the gel onto agarose gel containing 1% (w/v) starch and incubating at 45 °C for 3 h. The transparent band on the amylase containing agarose gel was observed after flooding with $I_2$-KI solution.

### 2.11. HPTLC analysis of enzyme

Amylase was incubated with 1% (w/v) starch in glycine–NaOH buffer at pH 10 and at 45 °C for 2 h. Standards (glucose, maltose and maltotriose) and test sample (comprising of 1% (w/v) starch and enzyme in glycine–NaOH buffer at pH 9) were spotted as bands (width, 6 mm) with a CAMAG microlitre syringe [24] on precoated silica gel aluminium Plate 60F-254 (20 cm × 10 cm with 250 μm thickness, E. Merck, Germany) using a CAMAG Linomat IV spotter (Switzerland). The plates were developed using n-butanol–absolute ethanol–water (5:3:2) mixture as mobile phase. Plates were dried and derivatized by spraying aniline-diphenylamine reagent [aniline 1% (v/v): diphenylamine 1% (w/v): phosphoric acid 1% (v/v)] in acetone followed by baking at 120 °C for 20 min [13]. Densitometric scanning was carried out at 370 nm for detection of hydrolyzed products.

## 3. Results and discussion

There are many reports on isolation of amylase enzymes from genus *Bacillus* [26], but very few reports on isolation of amylase enzyme from marine actinomycetes [27]. In our laboratory, we have isolated the strain of marine *Streptomyces* from sediments collected from west coast of India by pre-heat treatment at 40 °C.

### 3.1. Characterization of strain D1

The strain shows good growth in temperature range 37–55 °C in 7 days on glycerol yeast extract agar medium. Outer surface of colonies were perfectly round initially, but later they developed aerial mycelium that may appear velvety and spore formation started after 4th day of incubation. Spore chain was long (up to 16 spores) and sporulating hyphae were straight. Spores were oval and warty, seen like hairy and 1–2.5 μm in size (Fig. 1). Good growth was observed at neutral and alkaline pH. By morphological, biochemical, cultural characterization and SEM, the isolated strain was found to be a member of *Streptomyces* genus.

### 3.2. Growth characteristics and amylase production from strain D1

Most of the actinomycetes species are slow growing. Enzyme production started in early log phase but there was drastic increase in production of enzyme at late growth phase and early stationary phase; and amylase production continued up to late stationary phase and after that it declined (Fig. 2). It clearly indicates that amylase production was independent of growth phase [28,29]; and prominent role of amylase in primary metabolism and ecological sustenance of strain D1. Similar reports on amylase production were reported on bacteria belonging to genus *Bacillus* and other haloalkaliphilic organisms [5].

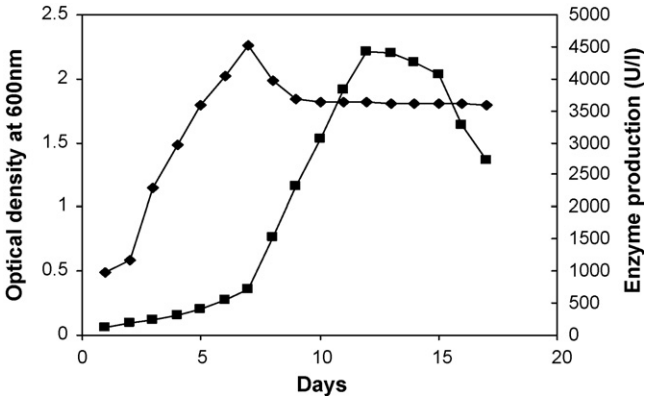


**Fig. 2.** Growth kinetics of strain A3 with reference to amylase production. Cell growth ($OD_{600}$) (♦) and amylase production (■).

**Table 1**
Partial purification of amylase enzyme from *Streptomyces* strain D1.

| Properties | Cell-free supernatant | Ammonium sulfate fractionation |
|---|---|---|
| Total protein (mg) | 789.05 | 240.08 |
| Total activity (unit) | 31070.5 | 27284.6 |
| Specific activity | 39.70 | 113.64 |
| Yield (%) | 100 | 87.81 |
| Purification (- fold) | 1.0 | 2.8 |

### 3.3. Partial purification of amylase

Partial purification of amylase was done by bringing the culture filtrate to 90% saturation with solid ammonium sulfate. The partially purified enzyme from strain D1 exhibited specific activity of 113.64 U/mg protein that corresponds to 2.8 fold purification (Table 1). The partially purified protein showed single band about 66 kDa (Fig. 3) and activity staining confirms the high enzyme purity.

### 3.4. Effect of NaCl, pH and temperature on amylase production and activity

The strain D1 was found to be moderately halophilic in nature as maximum enzyme production was obtained in presence of 7% (w/v) of NaCl and enzyme retained almost 80% of its activity in presence of 12% (w/v) of NaCl. There was no amylase production in absence of NaCl (Fig. 4). These results clearly indicate halophilic nature of the

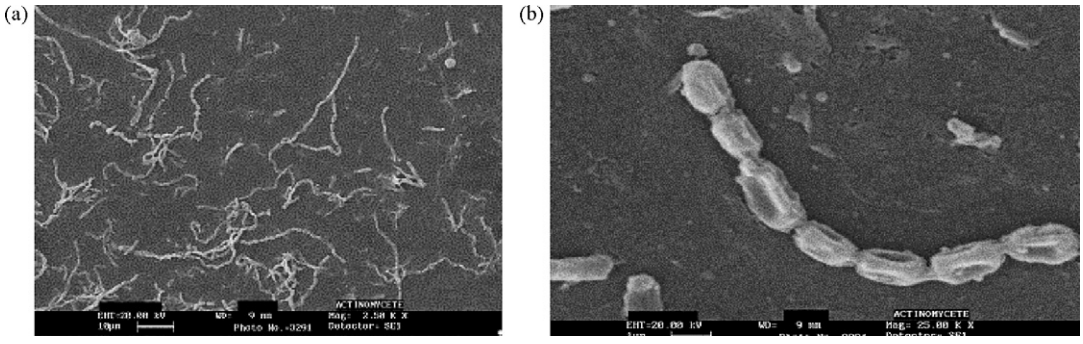


**Fig. 1.** Scanning electron microscopy of strain D1. (a) ×10,000 magnification. (b) ×25,000 magnification.

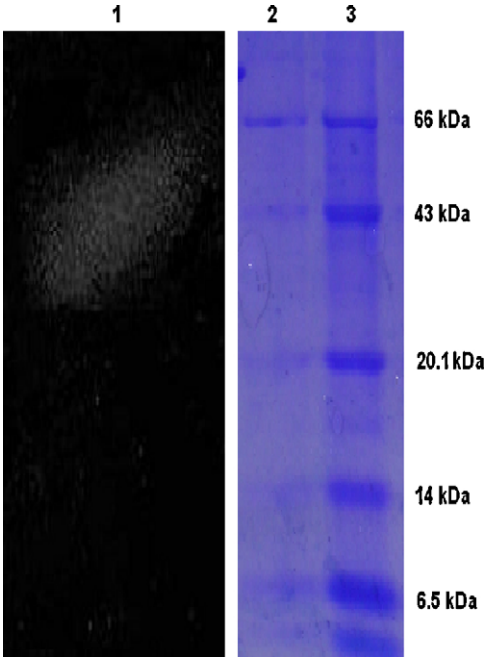


**Fig. 3.** SDS-PAGE and zymogram activity staining analysis of amylase enzyme. Lane 1, activity staining of enzyme; lane 2, amylase enzyme; lane 3, molecular mass standard.

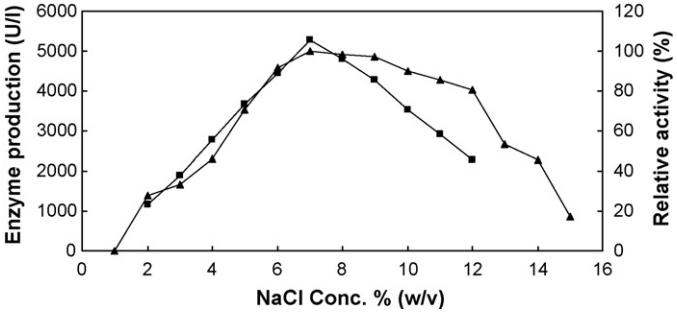


**Fig. 4.** Effect of NaCl on amylase production (■) and activity (▲).

enzyme, where salt appears to be prerequisite for amylase production and activity. Similar behavior for other halophiles producing amylase [30] and protease has been described [31].

Haloalkaliphilic microorganisms not only require high salt but alkaline pH for growth and enzyme production [5]. Good growth

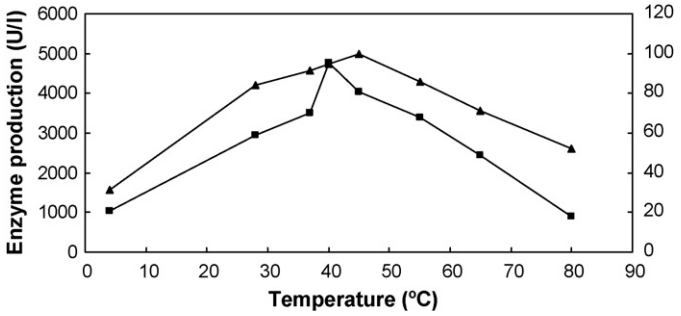


**Fig. 6.** Effect of temperature on amylase production (■) and activity (▲).

of strain was observed at a pH range of 8–11; however, growth at pH 8 and 9 was quite comparable (Fig. 5a). This indicates alkaliphilic nature of isolate D1. A similar response on growth was observed in some haloalkaliphilic archea such as *Natronuincul* [32], *Natronorubrum bangense* [33] and bacteria belonging to genus *Bacillus* [5]. The strain D1 showed gradual increase in amylase production and optimum pH for enzyme production was found to be 9. The enzyme was found to be stable in different buffer as it retained almost 80% in phosphate buffer (pH 7) and 60% of its activity in disodium hydrogen phosphate buffer (pH 12) but optimum amylase activity was obtained in glycine–NaOH buffer having pH 9 (Fig. 5b). Research was focused on the isolation of alkaline amylase enzyme from microbes because there is tremendous potentiality of alkaline enzymes in detergent industry [24].

The strain D1 showed good growth in the temperature range of 37–55 °C but optimum growth was observed at 45 °C. Maximum amylase production was obtained at 45 °C and enzyme retained almost 50% of its activity at 85 °C (Fig. 6). This clearly indicates moderately thermostable nature of enzyme. Until today, bacteria belonging to genus *Bacillus* have been exploited for commercial production of thermostable amylase enzymes [9,13]. There is no such report on isolation of moderately thermostable amylase enzymes from marine *Streptomyces* sp.

### 3.5. Effect of metal ions, EDTA and various chemicals on amylase activity

Amylase activity was stimulated in presence of calcium ion (5 and 10 mM). Other divalent cations such as $Mg^{2+}$, $Mn^{2+}$, $Co^{2+}$, $Cu^{2+}$ were also found to potentiate the enzyme activity but not to the extent of calcium ion. But amylase activity was inhibited by $Hg^{2+}$ and $Fe^{3+}$ ions. Metal ions such as $K^{+}$ have no effect on amylase

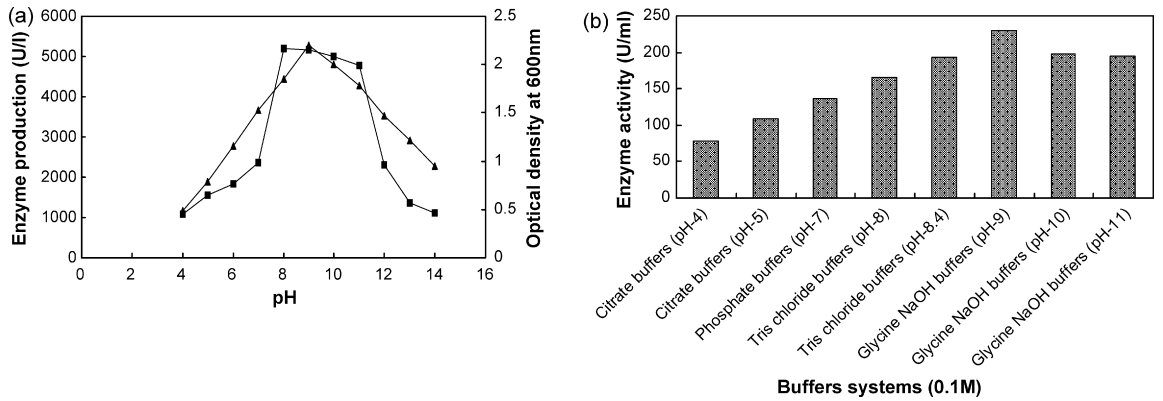


**Fig. 5.** Effect of pH on (a) production (▲) and growth (■); and (b) activity of amylase.

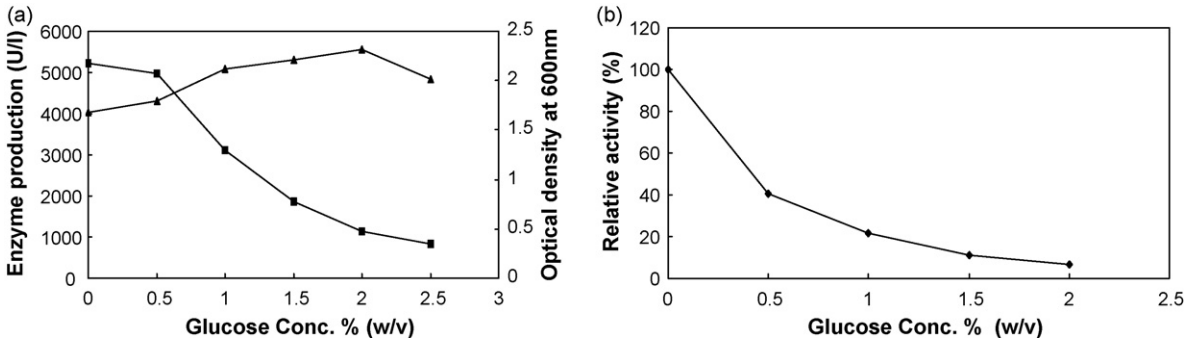


**Fig. 7.** Effect of glucose on growth (▲) and amylase (■) production; and activity.

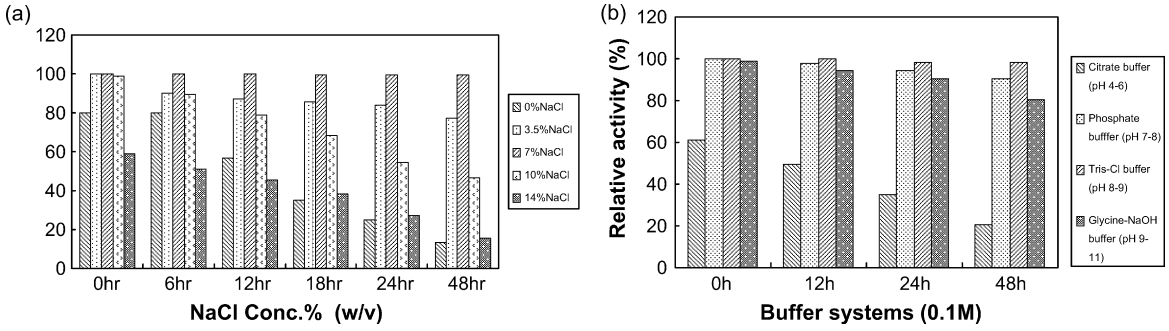


**Fig. 8.** (a) Salt tolerability and (b) pH stability of amylase.

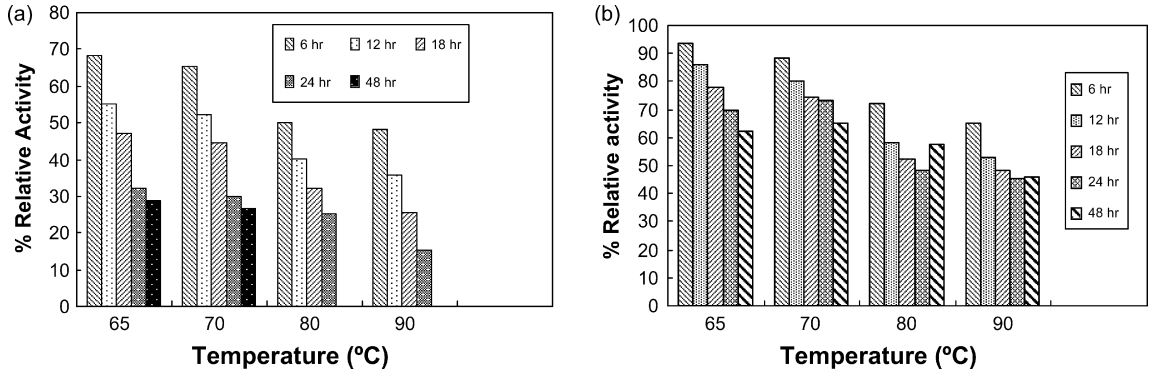


**Fig. 9.** Temperature stability of amylase (a) in absence (b) presence of calcium ion.

activity (Table 2). In previous reports, most of the amylase activity was inhibited in the presence of $Ni^{2+}$, $Cd^{2+}$, $Cu^{2+}$, $Ag^{+}$, $Pb^{2+}$, $Fe^{2+}$, and $Zn^{2+}$. The α-amylase from *Bacillus* sp. KSM-1378 [34] and *Bacillus firmus* [27] were strongly inhibited by $Ni^{2+}$, $Cd^{2+}$, $Zn^{2+}$ and $Hg^{2+}$. The α-amylase from *Thermus* sp. was strongly inhibited by $Cu^{2+}$ and $Fe^{2+}$ [35] and the α-amylase from *B. subtilis*, *B. amyloliquefaciens* I, and *B. amyloliquefaciens* II were strongly inhibited by $Zn^{2+}$, $Ag^{+}$, $Cu^{2+}$ and $Fe^{2+}$ [36]. Enzyme activity decreased drastically in presence of EDTA (5 and 10 mM) which provides evidence that this amylase is calcium ion dependent. EDTA forms complex

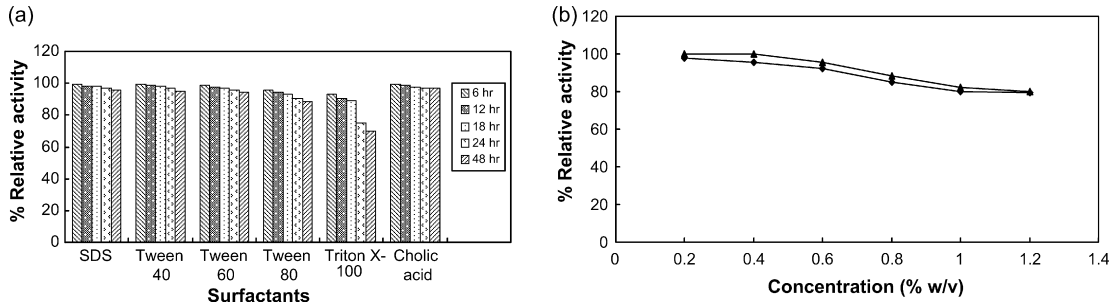


**Fig. 10.** Effect of (a) surfactants; and (b) bleaching agents, $H_2O_2$ (▲) and $NaClO_3$ (♦) on stability of amylase.

**Table 2**
Effect of various metal ions, EDTA and chemicals on amylase activity.

| Metal ions and Chemicals | % Relative activity |
|---|---|
| $Ca^{+2}$ (5 mM) | 129.98 |
| $Ca^{+2}$ (10 mM) | 143.23 |
| $Mn^{+2}$ (5 mM) | 102 |
| $Mn^{+2}$ (10 mM) | 107 |
| $Co^{+2}$ (5 mM) | 104 |
| $Co^{+2}$ (10 mM) | 118.21 |
| $Cu^{+2}$ (5 mM) | 103.79 |
| $Cu^{+2}$ (10 mM) | 109.47 |
| $K^{+}$ (5 mM) | 100 |
| $K^{+}$ (10 mM) | 99.96 |
| $Mg^{+2}$ (5 mM) | 104.12 |
| $Mg^{+2}$ (10 mM) | 112 |
| $Hg^{+2}$ (5 mM) | 64.23 |
| $Hg^{+2}$ (10 mM) | 46.78 |
| $Fe^{+3}$ (5 mM) | 56.67 |
| $Fe^{+3}$ (10 mM) | 42.12 |
| EDTA (5 mM) | 45.23 |
| Urea 1% | 13.45 |
| Glycerin 1% | 78.90 |
| Olive oil 1% | 84.23 |
| Control | 100 |

with the $Ca^{2+}$ which is required for activity and stability of enzyme, therefore causing decrease in enzyme production and activity. Effect of various chemicals on amylase activity was recorded in Table 2.

### 3.6. Repression study with glucose

Strong catabolic repression was observed with glucose as amylase production (Fig. 7a) and activity (Fig. 7b) decreased drastically with increase in glucose concentration from 0.5 to 2% (w/v). However, growth slightly increased up to 2% (w/v) of glucose. A similar behaviour was reported in case of alkaline protease from an alkaliphilic *Bacillus* sp. [5] and amylase production from marine yeast *Aerobasidium pullulans* [37].

### 3.7. Stability studies of amylase

Isolated amylase was found to be moderately salt tolerant. Enzyme activity drastically decreased in presence of 0% (w/v) of NaCl after 48 h incubation. Enzyme retained almost 100% and 50% of its activity in presence of 7% (w/v) of NaCl and 10% (w/v) of NaCl after 48 h incubation (Fig. 8a). Though, enzyme retained almost 50% of its activity in presence of 14% (w/v) of NaCl after 6 h incubation but there was drastic decrease in activity after 48 h incubation. Similar behaviour has been reported for bacteria belonging to genus *Bacillus* [5,24]. But this is probably the first report on moderately salt tolerant amylase from marine *Streptomyces* sp.

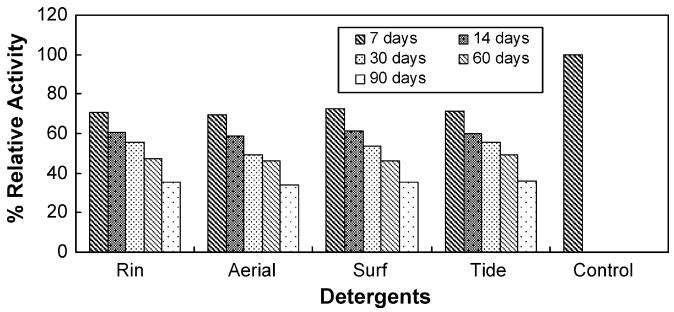


**Fig. 11.** Detergent stability of amylase.

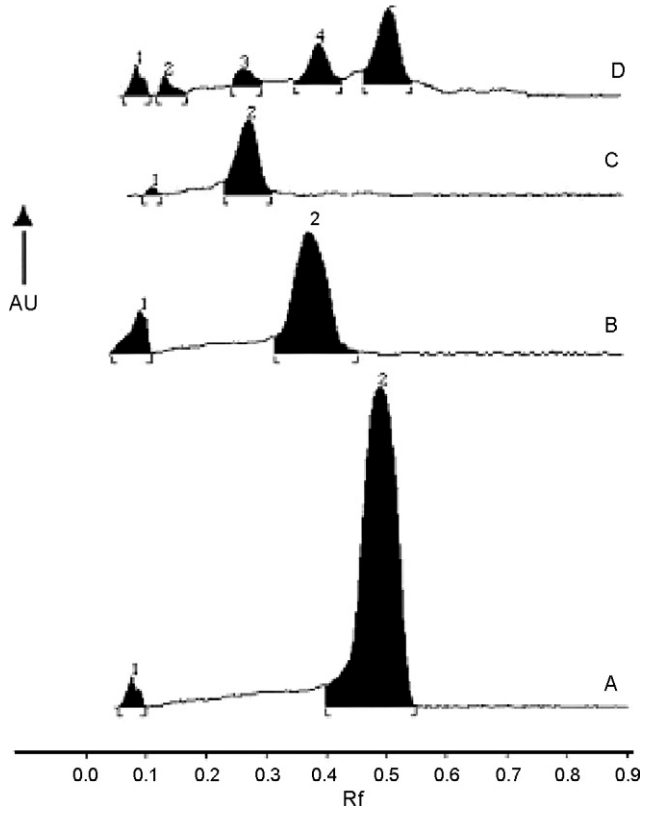


**Fig. 12.** HPTLC analysis for hydrolyzed products of starch digested by amylase from *Streptomyces* sp. D1. A. Peak of standard glucose (Himedia), B. Peak of standard maltose (Himedia), C. Peak of standard maltotriose (Sigma), D. Peak of hydrolysis product of starch consisting of peaks corresponding to glucose, maltose and matotriose generated by isolated amylase enzyme.

The amylase enzyme was found to be stable in buffer systems having pH 7–8 (phosphate buffer) to pH 9–11 (glycine–NaOH buffer) when incubated for 6–48 h. There are various papers dealing with amylase having stability over wide range of pH; but this is probably the first report of amylase enzyme from haloalkaliphilic marine *Streptomyces* strain having wide range of pH stability (Fig. 8b). Therefore, enzymes may have widespread application for detergent industry [24].

Amylase activity decreased drastically at higher temperature such as 80 and 90 °C (Fig. 9a) but there was drastic increase in stability (Fig. 9b) of enzyme in presence of calcium ion (10 mM) especially at higher temperature as mentioned above. This data provides an insight into calcium dependent nature of enzyme and role of calcium ion in structural integrity, stability and activity of enzyme [9,38].

Amylase enzyme was found to be very stable towards laboratory surfactants such as Tween 40, Tween 60, Tween 80 and cholic acid as enzyme retained above 95% of its activity; when incubated in presence of these surfactants [0.5% (w/v)] for 24–48 h. Though enzyme production decreased in presence of triton X-100 but reported amylase retained almost 70% of its activity in presence of Triton X-100 after 48 h incubation (Fig. 10a).

Enzyme retained almost 80–100% of its activity when incubated in presence of different concentrations of oxidizing agents such as sodium hypochlorite and $H_2O_2$ (Fig. 10b). Ideally, amylase used in detergent industry should tolerate oxidizing chemicals and surfactants. However, not all of currently used detergent enzymes are active in presence of bleaching agents. Hence, the latest trend in enzyme-based detergents is to introduce site directed mutagenesis and protein engineering techniques to produce enzymes with bet-

ter oxidation and heat stability [7]. However, the reported amylase already possessed good oxidation stability.

Amylase enzyme retained almost 70–35% of its activity in presence of commercial detergents, when incubated for 7–90 days (Fig. 11). There are reports on detergent stable amylase enzyme from bacteria belonging to genus *Bacillus* [24] but no such reports on detergent stable amylase enzyme from marine *Streptomyces* sp.

### 3.8. HPTLC analysis of enzyme

The hydrolysis pattern of soluble starch digested with partially purified amylase was studied by using HPTLC. The test sample (soluble starch and enzyme) showed three peaks having similar $R_f$ to that of standard glucose, maltose and maltotriose (Fig. 12). These results provide an insight on degradation mode of the amylase suggesting that it has α-1-4, α-1-6 (debranching) and α-glucosidase activity.

## 4. Conclusions

In the present investigation, we have isolated thermostable, moderately halophilic, commercial detergent, surfactant and oxidant stable α-amylase enzyme from marine *Streptomyces* sp. D1. Reported amylase enzyme exhibited specific activity of 113.64 U/mg and molecular mass of enzyme was found to be 66 kDa. Enzyme retained good activity in presence of commercially detergents such as rin, surf, aerial and tide; and oxidizing agents. Enzymes may have tremendous applications in detergent and pharmaceutical industries where higher salt concentration, surfactant and detergents inhibit enzymatic conversions.

## Acknowledgements

Chandrakant Kokare acknowledges All India Council for Technical Education (AICTE), HRD Ministry, New Delhi, Govt. of India for financial support to this research project under the scheme of 'Career Award for Young Teachers, 2005-06'.

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