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Research Article

A Novel α-Amylase from Haloalkalphlic Marine *Nocardiopsis* sp. Strain B2: Purification and Characterization

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Abstract

In this present study, a halopalkaliphilic marine Nocardiopsis sp. stain B2 with an ability to produce surfactants, oxidant and detergent stable α-amylase was isolated from marine sediments collected from west coast of India. The characterization of microorganism was performed by biochemical tests, and 16S rDNA sequencing. The α- amylase from strain B2 was purified to homogeneity by ammonium sulfate precipitation and gel filtration chromatography by using Sephadex G-75, insoluble corn starch and Sephacryl S-100 column, with a 43.92-fold increase in specific activity. Enzyme was found to be stable in presence of wide range of NaCl concentrations with maximum activity found at 11% (w/v) of NaCl. The α-amylase activity was stimulated by Ca^{+2} and inhibited by ethylenediamine tetraacetic acid (EDTA), suggesting that this enzyme is a metalloenzyme. Enzyme showed remarkable stability towards laboratory surfactants, detergents and oxidants. Glucose, maltose and maltotriose were the main end product of starch hydrolysis, indicating it is an α-amylase. Considering its promising properties, it can be concluded that this enzyme can find potential applications in various industrial sectors such as food, detergent, pharmaceutical where high salt concentrations inhibit enzymatic conversations.

Keywords: Haloalkaliphilic, *Nocardiopsis* sp., α-Amylase, surfactant, Detergent, Oxidant stable.

Introduction

Compared to organic synthesis, biocatalysts often have far better chemical precision, which can lead to more efficient production of single stereoisomer, fewer side reactions and a lower environmental burden. Up to now, more than 3,000 different enzymes have been identified and many of them have been applied in biotechnology and industry. But the current enzyme toolbox is still not sufficient to meet all demands. A major reason is that many available enzymes do not withstand industrial reaction conditions. Therefore, researchers are now trying to exploit extremophiles which are the valuable source of novel enzymes [1-3]. Alpha-amylases (EC 3.2.1.1, 1,4-α-D-glucan glucanohydrolase) were classified in family 13 of glycosyl hydrolases and hydrolyze starch, glycogen and related polysaccharides by randomly cleaving internal α-1,4-glucosidic linkages to produce different sizes of oligosaccharides . They have diverse applications in a wide variety of industries such as food, fermentation, textile, paper, detergent, pharmaceutical and sugar industries [4,5]. Over the past few decades, considerable researches have been undertaken with the extracellular α -amylase being produced by a wide variety of microorganisms. The major advantages of using microorganisms for the production of amylases are the economical bulk production capacity of microbes and their easier manipulation to obtain enzymes with desired characteristics [3]. The major concern in an enzymatic process is the instability of the enzyme under repetitive or prolonged use and inhibition by high substrate and product concentration.

It is also desirable that α-amylases should be active at the high temperatures of gelatinization (100–110^{0}C) and liquefaction (80–90^{0}C) to economize processes. Therefore, there has been a need and continual search for more thermophile and thermostable α-amylases [6]. The advantages of using thermostable amylases in industrial processes include the decreased risk of contamination, cost of external cooling, a better solubility of substrates and lower viscosity allowing accelerated mixing as well as pumping [7]. Each application of α-amylase requires unique properties with respect to specificity, stability, temperature and pH dependence. Since many of the commercially available amylases do not withstand industrial reaction conditions and also they do not meet a large industrial demand of this enzyme, therefore, isolation and characterization of novel amylases with desirable properties such as thermostability, alkaline stability and halophilicity are very important to meet the industrial demands [8]. Therefore, Screening of microorganisms with higher and novel α-amylase activities could therefore, facilitate the discovery of novel amylases suitable to new industrial applications [4,9,10]. Marine microorganisms have a diverse range of enzymatic activity and capable of catalyzing various biochemical reaction with novel enzymes such as amylase, lipase, deoxyribonuclease and protease [11].

Actinomycetes are one of the most investigated groups because they constitute a potential source of biotechnologically interesting substances [12-13]. *Nocardiopsis* strains are distributed ubiquitously in the environment. They are frequently isolated from habitats with moderate to high salt concentrations such as saline soil or marine sediments and salterns [14-15]. In the present study, we first report the purification and characterization of thermostable, oxidant, detergent and surfactant stable α-amylase secreted by a haloalkaliphilic *Nocardiopsis* sp. strain B2 that was isolated from west coast of India.

Materials and methods

Materials

Starch, peptone, malt extract, glycerol, yeast extract, glucose, asparagine, maltose, casein, dintrosalicylic acid were purchased from Hi-media laboratory, India. Maltotriose was purchased from Sigma, USA. Commercial detergents such as Rin, Surf and Tide were

purchased from Hindustan Unilever Limited, Mumbai, India and Ariel was purchased from Procter and Gamble, Mumbai, India All chemicals, and reagents used were of analytical grade.

Strain isolation and identification

Our research group already isolated α-amylase from haloalkaliphilic *Nocardiopsis* sp. strain B2 and employed an simple ion tropic gelation technique for entrapment of isolated α -amylase into gellan gum microspheres [16]. Sediment samples were collected from Goa, Alibagh and Mumbai coastal region of India at the time of low tide. Heat pretreatment at 40^{0}C for 30 to 60 days was employed for isolation of marine actinomycetes [17,18]. Marine soil samples were suspended in sterile sea water and thoroughly mixed on rotary shaker at 150 rpm for 20 min. Marine actinomycetes were isolated by using different selective media such as glycerol yeast extract agar, glucose aspargine agar, starch casein agar, maltose yeast extract and yeast extract malt extract agar. They were screened for amylase production by using starch agar medium consisted of following (g/l): starch 20g; beef extract 5g, peptone 3g prepared in artificial sea water (ASW). The artificial sea water contains (g/l): NaCl 23.37g; Na_2SO_4 3.91g; $NaHCO_3$ 0.19g; KCl 0.66g; KBr 0.09g; $MgCl_2$ 4.98g; $CaCl_2$ 1.10g, $SrCl_2$0.02g, and H_3BO_3 0.02g. The maximum amylase producing marine actinomycetes species B2 was maintained on maltose yeast extract agar medium containing (g/l): maltose 10g; yeast extract 4g; and K_2HPO_4 0.5g prepared in ASW at 4°C. Identification of strain was done by scanning electron microscopy (SEM), 16S r-DNA sequencing, and biochemical tests.

Inoculum preparation and culture condition

The maltose yeast extract broth 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 3.5-4.0$\times10^6$ CFU per ml and cultivated under agitation at 200 rpm at 45^{0}C for 4 days. Then 50 ml of seed culture was inoculated in the 500 ml of fermentation medium containing (w/v): 2% starch; 0.4% peptone; 0.2% of malt extract; 11% of NaCl and 10mM Ca^{+2} prepared in artificial sea water. The pH of the medium was adjusted to 9 and fermentation was carried for 12 days at the same conditions as pre-culture medium. Cell-free supernatant containing extracellular amylase was harvested by centrifugation at 5000 × g for 45 min and used for amylase purification and characterization.

Purification of amylase

The enzyme was precipitated by bringing the culture filtrate to 90% saturation with solid ammonium sulfate and kept at 40C for overnight. The precipitate was centrifuged at 12000 × g for 30 min. The precipitate was dissolved in glycine-NaOH buffer (pH 9) and dialyzed for 48 h against the same buffer. The dialyzed sample was assayed for α–amylase activity and protein content. Enzyme obtained (2ml) from the above step was loaded onto a Sephadex G-75 column (1.2×135 cm), pre-equilibrated with 50mM glycine-NaOH buffer (pH 9) at flow rate of 10ml/h. Fractions (3.5ml) were collected and those having specific activities more than 200 U/mg in the void volume were pooled for next step of purification. The enzyme obtained from Sephadex G-75 was loaded onto an equilibrated DEAE-Cellulose column (2×10 cm). The fractions with specific activities more than 500U/mg were pooled and loaded onto insoluble corn starch column [19]. The enzyme obtained from the above step was loaded onto an insoluble corn starch column (2.5×10cm), pre-equilibrated with 50mM glycine-NaOH buffer (pH 9). The column was washed with cold water and then the bound enzyme was eluted by incubation in 50mM of glycine-NaOH Buffer of pH 9 at 45°C. Fractions with high amylase activities (above 1200 U/mg) were pooled and dialyzed against 50mM of glycine-NaOH buffer (pH 9). Enzyme (2ml) from starch column was loaded onto a Sephacryl S-400 column (1.2×130 cm). The fractions (3.5ml) were collected and checked for the enzyme activities and protein concentrations. [20].

Assay of amylase

The activity of α-amylase was estimated by determining the amount of reducing sugar released from starch. 1 ml of enzyme solution was added to 1ml of starch solution (1% w/v). The mixture was incubated at 45^{0}C for 60 min. The reaction was stopped by the addition 2 ml of 3, 5- dinitrosalicylic acid and A_{540nm} was measured in Jasco V-530 spectrophotometer. One unit (U) of enzyme activity is defined as the amount of enzyme required for the liberation of 1μmol of reducing sugar as glucose per minute under assay condition [21].

Effect of metal ions

To study the effect of various metal ions on amylase activity, enzyme was dialyzed against glycine-NaOH buffer (pH 9). The dialyzed enzyme was then pre-incubated with various metal ions (5mM and 10mM) for 30 min at 45^{0}C and then the remaining activities were determined using the standard enzyme assay. All metal ions were used in salt form such as $CaCl_2.2H_2O$, $MgSO_4.7H2O$, $HgCl_2$, $ZnSO_4.7H_2O$, $NiCl_2$, $FeCl_3$, $CoCl_2$ $CuSO_4$, KCl and $MnSO_4$. $4H_2O$. The Enzyme activity was determined as percentage relative activity as compared to control (without metal ions), considered to have 100% of enzyme activity.

Effect of enzyme inhibitors

The effects of enzyme inhibitors on α-amylase activity were studied using phenylmethylsulfonyl fluoride (PMSF), urea, β-mercaptoethanol and ethylenediamine tetraacetic acid (EDTA) at a final concentration of 5 mM. The purified enzyme was pre-incubated with inhibitors at 45°C for 30 min and then the remaining enzyme activity was measured under standard assay conditions. The enzyme activity without inhibitors was taken as 100%.

Temperature stability of amylase

The enzyme was incubated at different temperatures (55, 65, 75, 85, 95 and 100 ^{0}C) for 6 to 48h in glycine-NaOH buffer (pH 9) containing 11% (w/v) NaCl in absence and presence of Ca^{+2} (10mM) for determination of temperature stability of amylase. Residual amylase activity was expressed as percentage relative activity as compared to control considered as having 100% of activity. Enzyme activity after pre-incubation at 45^{0}C for 60 min in glycine-NaOH buffer (pH 9) containing 11% (w/v) of NaCl and Ca^{+2}(10 mM) was considered as control.

pH stability of amylase

The pH stability of amylase was determined by incubating the enzyme in presence of different buffer systems in presence of 11% NaCl and 10 mM Ca^{+2} at 45^{0}C for different time period (6-48h). Buffers used were as same as that used to determine influence of pH on amylase activity. Residual activity of enzyme was determined as mentioned above.

Salt tolerability study of amylase

Salt tolerability of amylase was determined by incubating the enzyme in presence of different percentages of NaCl (3.5%, 7% 11% 14% and 17% w/v) in glycine- NaOH buffer (pH 9) containing10mM Ca^{+2} for 6-48h at 45^{0}C . Residual activity was determined as described above.

Surfactants stability study of amylase

Surfactants stability of amylase was determined by incubating the enzyme in presence of anionic and nonionic surfactants [0.5% (w/v)] in glycine -NaOH buffer (pH 9) containing 11% (w/v) of NaCl and10mM Ca^{+2} at 45^{0}C. The residual activity was measured as described above.

Stability study of amylase toward commercial detergents

Amylase enzyme was incubated in presence of commercial detergents [0.5% (w/v)] such as rin, surf, ariel and tide in glycine-NaOH buffer (pH 9) containing 11% (w/v) of NaCl and 10mM Ca^{+2} for

7 to 90 days 45^{0}C to investigate the stability of amylase toward detergents . The residual activity was measured as described above.

Stability study of amylase towards oxidants

For the determination of stability of amylase towards oxidant, amylase was incubated for 2h in presence of H_2O_2 and $NaClO_3$ in glycine-NaOH buffer (pH 9) containing 11% (w/v) of NaCl and 10mM Ca^{+2} at 45^{0}C. The residual activity of enzyme was determined as described above.

HPTLC analysis of amylase

Amylase (test sample) was incubated with 1% (w/v) starch in glycine-NaOH buffer at pH 9 containing 11% (w/v) of NaCl and 10mM Ca^{+2} at 45^{0}C for 6 h. Standards (glucose, maltose and maltotriose) and test sample were spotted as bands (width, 6 mm) with a CAMAG microlitre syringe on precoated silica gel aluminium plate 60F-254 (20 cm x 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^{0}C for 20 min. Densitometric scanning was carried out at 370 nm for detection of hydrolyzed products.

Statistical analysis of data

All the data are expressed as mean ± standard error mean (SEM). The experimental data were analysed using two-way analysis of variance (ANOVA) followed by Bonferroni post test. Statistical difference yielding $P < 0.001$ were considered significant. The analysis was performed by using Graph Pad Prism software (version 5.01).

Results and discussion

Identification of strain B2

The strain shows good growth at 45^{0}C in 7 days on maltose yeast extract medium. Aerial and substrate mycelium was observed and both the mycelia fragmented into short rods. Spore chain was found to be short and spiral; and it was observed on aerial mycelium. Meso-diaminopimelic acid (meso-DAP), arabinose and galactose were found to be major constituents of cell wall. 16S rDNA sequencing showed that strain B2 was closely related to the genus Nocardiopsis with highest level of similarity to Nocardiopsis antarctica DSM 43884^T(X97885). In our laboratory, we have isolated forty actinomycetes strains from marine sediments collected from west coast of India by pre- heat treatment at 40°C. Isolate B2 was selected for further studies because it was appeared to be the one of the best producers of extracellular amylase in both liquid and solid media.

Growth kinetics and amylase production

Actinomycetes species are slow growing in nature. Enzyme production started in early log phase but there was drastic increase in production of enzyme at late logarithmic growth phase and early stationary phase; and amylase production continued up to late stationary phase, after that it declined (Figure 1). It indicates that the kinetics of enzyme production is more of the growth associated rather than non-growth associated type. Effective induction may not occur until the stationary phase has been reached and the readily available carbon source was depleted.

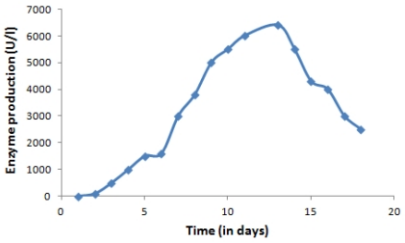

Figure 1. Amylase production of strain B2

Purification of amylase

The enzyme was precipitated by ammonium sulphate for concentration and followed by gel filtration (Sephadex G-75) to remove low molecular weight proteins and desalting. Ion exchange chromatography and starch column were used to remove contaminated proteins and induction of specific activity up to 1457.14 U/mg. Finally, the enzyme was subjected to Sephacryl S-400 column to obtain a homogeneous amylase with specific activity of 1754.9 U/mg. It corresponds to 43.92 – fold purification with 20.27 % of yield (Table 1).

Table 1. Summary of purification of amylase from strain B2

Steps	Total activity (U)	Total protein (mg)	Specific activity (U/mg protein)	Yield (%)	Production fold
Cell free extract	35210.5	881.25	39.75	100.0	1
Ammonium Sulphate fractionation	31241.6	573.27	54.49	92.5	1.388
Sephadex G-75	2645	37.54	704.74	75.13	17.64
DEAE-Sephadex	203466	22.68	897.08	57.78	22.45
Starch column	14178	9.73	1457.14	40.26	36.47
Sephacryl S- 400	7139	4.06	1754.9	20.27	43.92

Effect of metal ions on amylase activity

Amylase from *Nocardiopsis* sp. strain B2 was stimulated by calcium ion as it showed 130% and 163% of relative activities in presence of 5mM and 10mM calcium ion respectively. Results suggest that α-amylase did not require any ions for catalytic activity except Ca^{+2} (Table 2). Alpha-amylases contain at least one Ca^{+2} ion and affinity for calcium is much stronger than that of other ions. Slight activity inhibition was observed by Mg^{+2}, Ni^{+2}, Fe^{+3}, Mn^{+2}, and Zn^{+2}; since the relative activity was higher than 75%. A stronger inhibitory effect was observed in case of Co^{+2}, Cu^{+2}, and Hg_{+2}. The inhibition by Hg^{+2} may indicate the importance of indole amino acid residues in enzyme function as have been demonstrated for other microbial α-amylases [1]. The inhibition of α-amylase by Co^{+2}, Cu^{+2}, and Ba^{+2} ions could be due to competition between the exogenous cations and the protein-associated cations, resulting in decreased metalloenzyme activity (Table 2).

Effect of enzyme inhibitors on amylase activity

EDTA was found to cause significant decrease in the activity of amylase, indicating calcium ion dependent nature of amylase. This is due to the fact that the EDTA forms complex with the Ca^{2+} which is required for activity and stability of enzyme, therefore causing decrease in enzyme activity. Halophilic α-amylases from N. halobius [22], *Bacillus* sp. TSCVKK [23], Archaeon Haloarcula hispanica [24] and Streptomyces sp. D1 [25] also show similar behavior. Urea has strong inhibitory effect on amylase activity as it is a strong denaturant, therefore unfolds the proteins. PMSF and β-mercaptoethanol had no significant effect on the enzyme activity. This

Table 2. Effect of metal ions and enzyme inhibitors on amylase activity

Metal ions and enzyme inhibitors	% Relative activity	Metal ions and enzyme inhibitors	% Relative activity
Ca^{+2} (5 mM)	129.98	Hg^{+2} (5 mM)	31.45
Ca^{+2} (10 mM)	163.23	Hg^{+2} (10 mM)	23.78
Mn^{+2} (5 mM)	84.37	Fe^{+3} (5 mM)	78.67
Mn^{+2} (10 mM)	78.23	Fe^{+3} (10 mM)	75.85
Co^{+2} (5 mM)	44.78	Zn^{+2} (5 mM)	84.78
Co^{+2} (10 mM)	35.9	Zn^{+2} (10 mM)	79.61
Cu^{+2} (5 mM)	34.61	EDTA	15.23
Cu^{+2} (10 mM)	23.59	Urea	16.45
Ni^{+2} (5 mM)	82.14	PMSF	99.90
Ni^{+2} (10 mM)	76.42	Beta-mercaptoethanol	99.79
Mg^{+2} (5 mM)	87.38	Control	100.0
Mg^{+2} (10 mM)	83.19		

means that serine residues and disulfide bonds are not essential for the enzyme activity.

Temperature stability of amylase

Amylase from B2 retained 54% of its activity at 85^{0}C after 6hr of incubation. This clearly indicates thermostable nature of enzyme (Figure 2a). Although, there was decrease in activity after 48 hr of incubation but the thermostability significantly increases in presence of calcium ion (Figure 2b). This data also provides an insight calcium dependent nature of enzyme and role of calcium ion in structural integrity, stability and activity of enzyme. The amylase from strain B2 revealed that it can also be commercially exploited for starch liquefaction, food processing and baking.

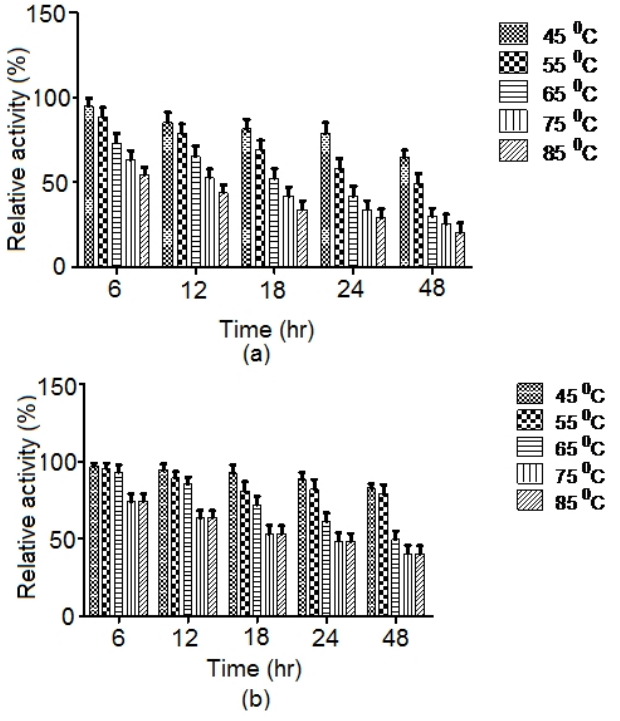

Figure 2. Temperature stability of amylase (a) in absence and (b) presence of calcium ion.

pH stability of amylase

Enzyme drastically lost its activity in buffers of acidic pH range, whereas it was found to be significantly stable in various buffers of neutral or alkaline pH. Amylase retained almost 43% and 45% of its activity in disodium hydrogen phosphate buffer (pH 12) and phosphate buffer (pH 7) respectively after 48 h of incubation (Figure 3a). Enzyme was found to be most stable in glycine- NaOH buffer of pH 9 as it retained almost 98% of its activity after 48 h of incubation. These data clearly indicate alkaline nature of enzyme. A growing new area of application of α-amylases is in the fields of laundry and dish-washing detergents. A modern trend among consumers is to use colder temperatures for doing the laundry or dishwashing. At these lower temperatures, the removal of starch from cloth and porcelain becomes more problematic. Detergents with α-amylases optimally working at moderate temperatures and alkaline pH can help to solve this problem. Nowadays, 90% of all liquid detergents contain α-amylase. Therefore, demand for α-amylases for automatic dishwashing detergents is growing day by day [4, 5, 26].

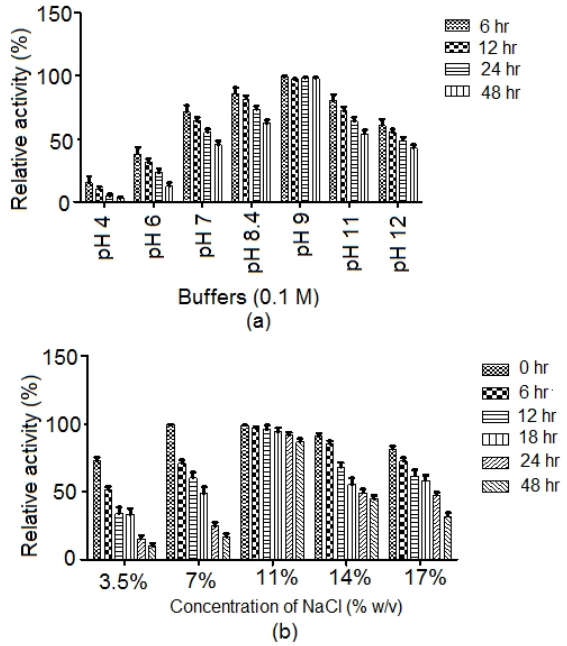

Figure 3. pH stability (a) and salt tolerability (b) of amylase.

Salt tolerability study of amylase

Enzyme activity was drastically decreased in presence of 3.5% (w/v) of NaCl after 48 h incubation. Enzyme retained almost 87% of its activity in presence of 11% (w/v) of NaCl (Figure 3b) after 48 h of incubation; whereas, enzyme retained almost 30% of its activity in presence of 17% (w/v) of NaCl after 48 h incubation. This result suggest that amylase from strain B2 was found to have more salt tolerability than that of *Bacillus* sp. TSCVKK [23] and marine Streptomyces sp. D1 [25]. Therefore, it can be concluded that enzyme can find favorable applications in detergent, food and other industrial process containing high salt concentrations.

Surfactants stability study of amylase

Amylase was found to be very stable towards laboratory surfactants such as Tween 40, Tween 60, Tween 80 and cholic acid. It retained almost 84% to 97% of its activity; when incubated in presence of surfactants (0.5% w/v) for 24 and 48 h. Though enzyme production decreased in presence of triton X-100 (data not shown) but reported amylase retained almost 73 % of its activity in presence of Triton X-100 after 48 h incubation (Figure 4a).

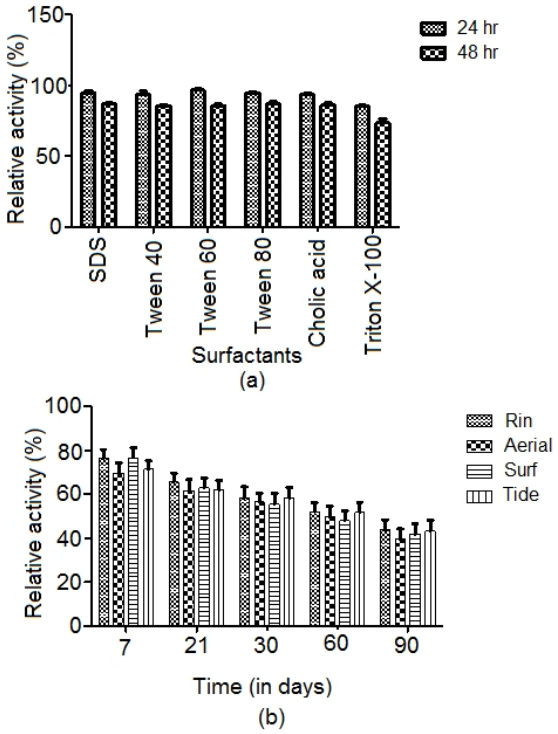

Figure 4. Stability of amylase in presence of (a) Surfactants and (b) detergent

3.10. Stability study of amylase toward commercial detergents

Enzyme also retained almost 70 to 40% of its activity in presence of commercial detergents, when incubated for 7 to 90 days (Figure 4b).

3.11. Stability study of amylase toward oxidants

Enzyme retained almost 80 to 100% of its activity when incubated in presence of different concentrations of oxidizing agents such as sodium hypochlorite and H_2O_2 (Figure 5). 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 enzyme with better oxidation and heat stability. However, the reported amylase already possessed good oxidation stability.

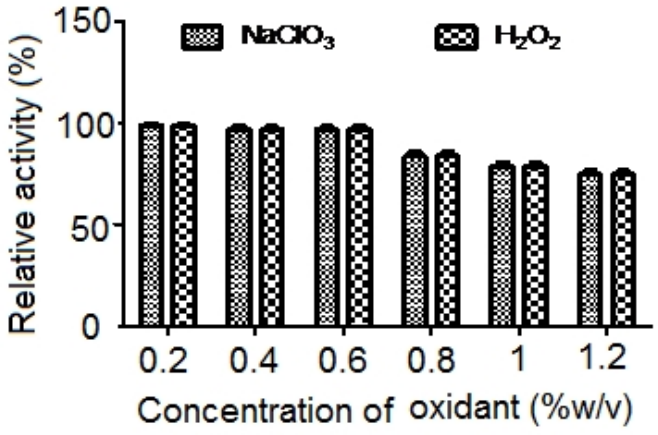

Figure 5. Effect of oxidants on stability of amylase

HPTLC analysis of amylase

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 Rf to that of standard glucose, maltose and maltotriose (Figure 6). 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. The hydrolysis pattern presented by amylase from strain B2 showed similarity with that of H. *meridiana* [27] producing glucose, maltose, and maltooligosaccharides as the main products. Hence, it is an alpha-amylase.

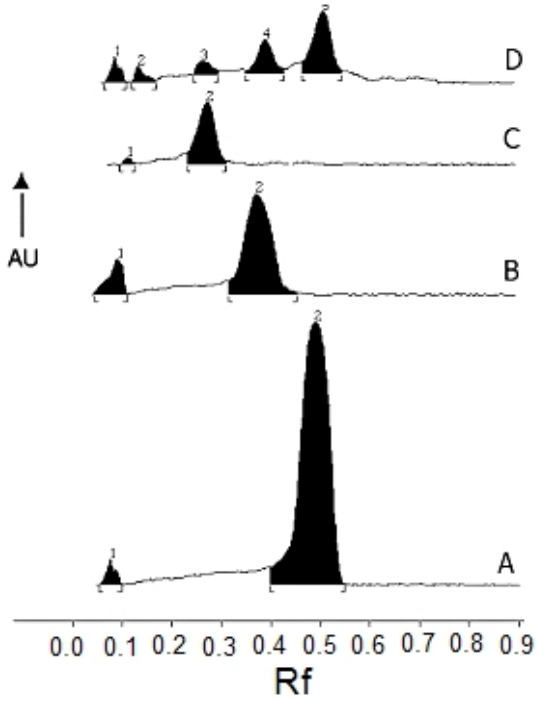

Figure 6. HPTLC analysis of hydrolytic products of starch digested by isolated enzyme.(A) Peak of standard glucose, (B) Peak of standard maltose, (C) Peak of standard maltotriose, (D) Peak of hydrolysis product of starch consisting of peaks corresponding to glucose, maltose and maltotriose generated by isolated amylase enzyme.

Conclusion

Considering most of the available literatures on amylase, we can conclude that thermostable, halophilic, commercial detergent, surfactants and oxidant stable α–amylase enzyme from marine *Nocardiopsis* sp. has not been reported so far. On the other hand, several researchers have isolated novel amylases of microbial origin but none of them posses such as remarkable stability characteristics all together for a long period as that of amylase secreted by *Nocardiopsis* strain B2. The strain shows good growth at 45^{0}C in 7 days on maltose yeast extract medium. 16S rDNA sequencing showed that strain B2 was closely related to the genus *Nocardiopsis* with highest level of similarity to *Nocardiopsis* antarctica DSM 43884^T(X97885). The amylase reported in the present study was found to be more stable and exhibited exhibited specific activity of 1754.9 U/mg. Finally, we can infer that amylase from marine *Nocardiopsis* sp. strain B2 can find potential applications in detergent, food and pharmaceutical industries where higher salt concentrations, surfactants and detergents inhibit enzymatic conversions. In order to understand the mechanisms of biochemical properties of the α-amylase, determination of the encoding gene sequences are now under investigation.

Conflicts of interest

The author declares no competing interests.

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