ISSN 0026-2617, Microbiology, 2015, Vol. 84, No. 2, pp. 210–218. © Pleiades Publishing, Ltd., 2015.

EXPERIMENTAL ARTICLES

Insight on Biochemical Characteristics of Thermotolerant Amylase Isolated from Extremophile Bacteria *Bacillus vallismortis* TD6 (HQ992818)[1]

Chandrasekaran Suganthi, Anbazhagan Mageswari, Sivashanmugam Karthikeyan, and Kodiveri Muthukaliannan Gothandam[2]

School of Bio Sciences and Technology, VIT University, Vellore 632014, Tamil Nadu, India

Abstract—Halotolerant bacterium *Bacillus vallismortis* (HQ992818) was isolated from saltern sediments in India, and produced significantly high levels extracellular amylase. A detailed investigation on the culture conditions including period of incubation, media pH, and inoculum size in addition to different sources of carbon and nitrogen, metal ions, NaCl, and amino acids was carried out for optimized production. Maximum amylase production (62 U/mL) was attained after 26 h of incubation. The optimized conditions for maximal production of amylase were found to be 1% NaCl, pH 8, temp 37°C, 1% starch, 1% sodium nitrate, phenyl alanine (0.01%) and calcium chloride (10 mM). The biochemical characteristics of the extracellular amylase were studied with respect to change in temperature, pH and metal ions. The enzyme was found to be optimally active in the temperature range of 40–70°C and pH 8. Activation of the enzyme by Ca^{2+} (135%), Fe^{2+} (113%) and Mg^{2+} (109%) occurred at 5 mM concentration and strongly inhibited by Hg^{2+}, Zn^{2+} and Mn^{2+} occurred at 10 mM. Significant compatibility of the enzyme with the commercial laundry detergents and the results of washing performance test confirmed its effectiveness. Available data on the optimized culture conditions enables for easily adaptable setup of large scale production of the enzyme for use in detergent formulations.

Keywords: amylase, *Bacillus vallismortis*, halotolerant, optimization, thermotolerant

DOI: 10.1134/S0026261715020162

Amylases are a major class of hydrolases involved in starch catalysis. They cleave starch molecules to give distinct products including dextrin and progressively smaller polymers composed of glucose units. Based on their mode of action, amylase are classified into; endoamylases, exoamylases and debranching amylases. Amylase occupies 25–30% of the world enzyme market and it has great significance for biotechnological applications [1]. Amylase has a wide spectrum of biotechnological applications in industrial sectors such as textile, paper, sugar, brewing and baking. It is also used in the preparation of cakes, fruit juices, starch syrups and used as digestive aid in pharmaceutical industries [2]. Amylases obtained from mesophilic organisms possess extensive applications in industries. However their industrial application is often limited by factors such as salt solutions, high temperature, extremes of pH, organic solvents and heavy metals which inhibit the enzyme activity [3]. In the field of biotechnology, bacteria are considered as a source of diverse enzymes possessing features worthy of industrial utility, in particular enzymes from extremophilic bacteria are of greater important due to the production of stable and valuable enzymes [4]. Among the extremophiles, halophiles are microbes which can survive and multiply in hypersaline environments. They are found to inhabit salt marshes, hypersaline seas, salt evaporation pools, salt mines and salted meats [5]. Halophiles can be classified into three different groups based on the requirement of NaCl. (A) Slight halophiles (2–5%), (B) moderate halophiles (5–20%) and (C) extreme halophiles (20–30%) [6]. Amylases from halophilic bacteria would have the advantage of the enzymes having optimal activities at higher salt concentrations with prospective use in industries [7]. Very few genera of halophilic or halotolerant bacteria which include *Halobacillus* sp. strain MA-2 [8], *Bacillus* sp. strain TSCVKK [2], *Chromohalabacter* sp. TVSP101 [9], *Bacillus barbaricus* [3] have been reported to producer as amylase. The increasing urge from biotechnological industries for enzymes with specific properties necessitates the current investigations on microorganisms prevailing in extreme environments [10]. Hence, screening for microbes inhabiting the extreme environments for α-amylase activities could facilitate the sighting of novel amylases fitting industrial applications. Due to the fact that bacteria are greatly influenced by media components such as carbon and nitrogen sources,

[1] The article is published in the original.

[2] Corresponding author; e-mail: gothandam@yahoo.com

minerals and physical factors such as pH and inoculum density [11]. Media optimization for enhancing amylase production from the selected organism was performed in the present study. Hence this work aims to identify a halotolerant bacterium capable with high amylase activity, characterize the enzyme properties and optimize culture parameters for sustained industrial production.

MATERIALS AND METHODS

Strains and culture conditions. Sample collected from Tuticorin (Tamil Nadu, India) saltern sediments at 3–4 cm depth. Collected samples were serially diluted in sterile saline water and the dilutions were plated in nutrient agar medium with 5% NaCl. Plates were incubated at 37°C for 48 h. After incubation, morphologically varying colonies were picked and purified on the same medium. For further studies pure isolated colonies were maintained as glycerol stocks and stored at –80°C.

Screening for amylase activity and quantification. All the isolates were screened for amylase production in nutrient agar media supplemented with 1% starch and 5% NaCl. The plates were incubated at 37°C for 24 h. After incubation plates were flooded with iodine solution. Amylase producing strains were chosen based on the zone of clearance and were subjected for quantification. Amylase produced by isolated strains was quantitatively determined using the method of Miller [12]. One unit of enzyme activity was defined as the amount of enzyme releasing 1 μmol glucose per minute with glucose as standard.

Identification of bacteria. Morphological characteristics and Gram's staining were studied for the isolated bacteria as per the standard protocols. Genomic DNA of the microbes was isolated using the method of Sambrook et al. [13]. The genomic DNA isolated from the bacteria was amplified using the following universal 16S rRNA primers: forward primer 5' GAG TTT GAT CCT GGC TCA G 3' (*E. coli* positions 8–27) and reverse primer 5' ACG GCT ACC TTG TTA CGA CTT 3' (*E. coli* positions1494–1513). The amplified PCR product was carried out for 16S rRNA gene sequencing in Macrogen (Seoul, Korea). The obtained sequence was compared with NCBI database using BLAST search tool and the phylogenetic tree was constructed with the MEGA v. 5.04 using neighbour joining method with a bootstrap value of 1000. The 16S rRNA gene sequences of *Bacillus vallismortis* TD6 was submitted to GenBank database.

Effect of salt concentration on amylase production. To find out the optimal salt concentration for amylase production in the medium by varying 1–5% of NaCl concentration. Inoculated medium was incubated at 37°C for 24 h. After incubation enzyme activity was quantified.

Growth kinetics for amylase production. To determine the growth and incubation period for extracellular amylase production, the culture were inoculated in the nutrient broth medium with 1% NaCl and 1% starch. Inoculated medium was incubated for a period of 48 h at 37°C and 130 rpm. At every six hours interval culture was taken and amylase assay was quantified for cell free extracts. For growth kinetics cells were measured at 600 nm.

Effect of pH and inoculum. In order to investigate the influence of pH on amylase production, the strain was grown in 1% salt medium and 1% starch at different pH (3.0–11.0) and constant temperature at 37°C. Amylase production was studied for varying inoculum (1–5%) level. After 26 h incubation amylase activity was quantified.

Effect of various carbon and nitrogen sources. Various simple and complex carbon sources including glucose, fructose, galactose, maltose, lactose, sucrose, starch and pectin were used as a sole source of carbon (1%). Organic nitrogen sources peptone and yeast extract, inorganic nitrogen sources including ammonium chloride, ammonium nitrate and sodium nitrate (1%) were used. The pH was adjusted to 8. The enzyme activity was monitored after 26 h incubation at 37°C under shaking conditions of 130 rpm.

Effect of different amino acids and metal ions. The effect of amino acids on the medium was studied with (0.01%) of L-alanine, L-methionine, L-phenylalanine, L-lysine and glycine. The pH was adjusted to 8. The medium was incubated at 37°C at 130 rpm. After a period of 26 h the amylase activity was quantified. Similarly the effect of metals ions also was investigated with different metal ions (10 mM) such as calcium chloride, manganese chloride, cobalt chloride, ferric chloride, magnesium sulphate and sodium chloride.

Characterization of amylase enzyme. The extra cellular culture supernatant obtained from the optimized medium was used for determining the biochemical characteristics of the enzyme with respect to different temperature, pH and metal ions.

Effect of temperature and pH on amylase activity. The effect of pH on the activity of amylase was measured in the 3.0–10.0 range, using the appropriate buffers at a concentration of 50 mM (3, 4, citrate buffer; 5, acetate buffer; 6–8, phosphate buffer; 9–10, glycine–NaOH) under standard assay conditions. The effect of temperature on the activity of amylase enzymes was measured in the range of 20–100°C.

Effect of metal ions. Enzyme assays were performed in presence of different metal ions, at 5 and 10 mM final concentration. The chloride salts of Zn^{2+}, Ca^{2+}, K^{+}, Mn^{2+}, Hg^{2+}, Cu^{2+}, Na^{+}, Ba^{2+}, Fe^{2+}, Co^{2+} and Mg^{2+} were used.

Compatibility of amylase with commercial detergents. In order to validate the potential of *Bacillus vallismortis* TD6 crude amylase as a detergent composition and its compatibility towards commercial laundry detergent such as Rin®, Surf excel® (Hindustan Unilever), Ariel®, Tide Naturals® (Procter and Gamble),

Table 1. Morphological and biochemical characterization of strain TD6

Characteristics	Result
Gram staining	G + ve
Shape	Ellipsoidal
Motility	+
Growth in NaCl concentration	
3%	+
5%	+
8%	+
10%	+
Catalase	+
Oxidase	+
Sugar fermentation	
Glucose	+
Xylose	+
Mannitol	+
Sorbitol	+
Sucrose	+
Nitrate reduced to nitrite	+
Starch hydrolysis	+

+, positive reaction.

Ujala® (Jyothi Laboratories), Wheel®, Arasan®, Ponvandu® (Hindustan Unilever), Mr. White® and Henko® (Henkel India Ltd.) were used for detergent compatibility studies. The solid detergents (7 mg/mL was dissolved in tap water) were heated at 100°C for 90 minutes to inactivate the indigenous enzymes present in the commercially available detergents. Crude enzyme containing 50 U/mL was mixed with the detergent in the ratio of 1 : 1 (v/v) and incubated at 55°C for 1 h, followed by the relative activity was calculated. The enzyme activity of control sample was mixed with tap water and crude enzyme (1 : 1 v/v) without detergent was taken as control 100%.

Evaluation of wash performance of halotolerant amylase with detergent on different stained clothes was done. Clean cotton fabrics (7 cm × 7 cm) were soiled with 100 μL of five different stains which include: Ketchup, chocolate, blood, tea and coffee were applied on the cotton fabrics without any pre treatment and dried. All the stained cotton fabrics were subjected for washing performance in individual 100 mL conical flask containing 7 mg/mL of detergent and 7 mg/mL detergent with 50 U/mL of TD6 crude amylase. Then each flask was incubated for 1 h at 45°C. After 1 h incubation stained cotton fabrics were taken out, rinsed with tap water and dried. Stain removal was confirmed by visual examination.

RESULTS AND DISCUSSION

Isolation and screening of amylase producing bacterium. In the present study, we have isolated fifteen halotolerant bacteria from saltern sediments of Tuticorin. All the strains were screened for amylase production. Among them seven isolates capable of producing zone of clearance was considered as amylase positive organisms. Based on the level of amylase production isolate TD6 was found to produce maximum units of enzyme (20.32 U/mL) after 24 h of incubation period at 37°C. Therefore, TD6 was selected for further studies.

Identification of bacterium. The isolated strain TD6 was observed to be gram positive rods; smooth, circular, spore forming, motile and rod like structure under microscopic observation. The morphological and biochemical characteristics of the isolated strain TD6 are shown in (Table 1). The strain was capable of growing up to 10% NaCl and found to be catalase, oxidase and nitrate reductase positive. It was capable of fermenting glucose, xylose, mannitol, sorbitol and sucrose. On the basis of 16S rRNA gene sequence analysis, the strain was found to have 99% homology with *Bacillus vallismortis* (DV1-F3) isolated from desert soil in Death Valley, California [14]. Hence the isolate TD6 was identified as type strain of *Bacillus vallismortis* (Fig. 1). The 16S rRNA sequence of *Bacillus vallismortis* TD6 was submitted to GenBank (accession number HQ992818).

Effect of salt concentration on amylase production. Amylase production was initially observed for different concentration of NaCl from 1–5%. Maximum enzyme secretion was observed (Fig. 1b) at 1% NaCl (40.67 U/mL). With increase the salt concentration, enzyme production and growth was decreased. But organism was found to grow up to 10% NaCl concentration. Due to high salt concentration in the medium growth of bacteria was found to decrease which was found to directly affect the production of enzyme. Similarly were seen in *Bacillus* sp. [15]. Vijayabaskar et al. reported that the maximum amylase production was attained at 3% NaCl from moderately halophilic bacterium *Bacillus cereus* [16].

Kinetics of bacterial growth and amylase production. The bacterial growth and enzyme production was studied in the nutrient broth supplemented with 1% NaCl. The effect of incubation period on bacterial growth showed that the strain had a lag phase up to 2 h after which exponential phase was observed up to 12 h followed by stationary phase. The enzyme production was observed from log phase and extended up to the stationary phase (Fig. 1c). Maximum amylase production was achieved during stationary phase at 26 h (38.72 U/mL) of incubation period, after which both the enzyme production and growth was decreased as the culture entered into death phase and also due to depletion of nutrients in the medium [17]. In case of *Bacillus* sp. ANT-6 maximum amylase production was attained after 24 h of incubation [18]. Most of the

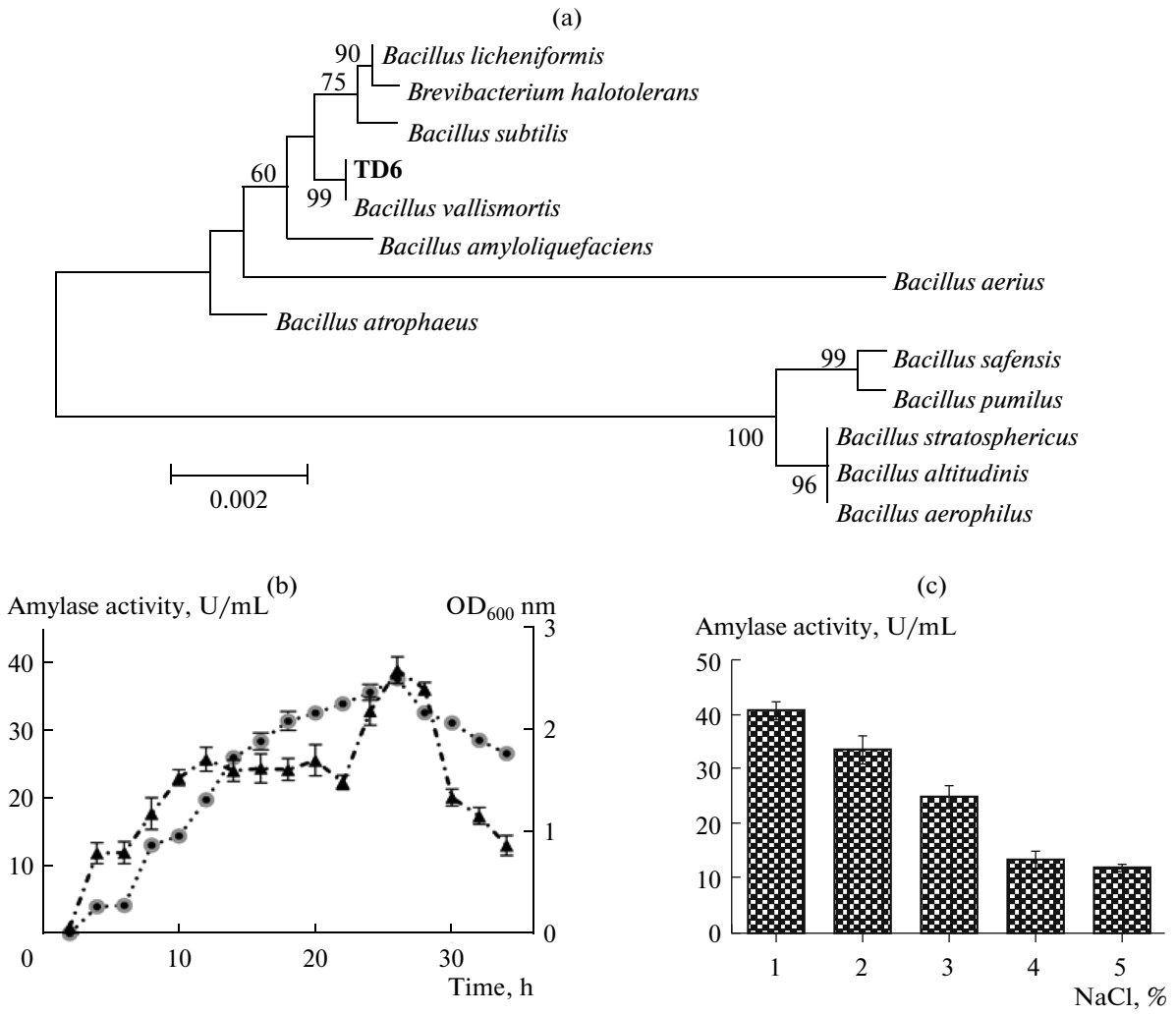

Fig. 1. (a) Phylogenetic position of strain TD6 and other closely related *Bacillus* species based on 16S rRNA gene sequencing analysis. The phylogenetic tree was constructed by neighbour joining method, strain TD6 showing the position of among phylogenetic neighbours. Bootstrap ($n = 1000$) values below 50 are not shown. (b) Effect of varying NaCl concentration on amylase production by *Bacillus vallismortis* TD6. (c) Effect of incubation period and growth on amylase production by *Bacillus vallismortis* TD6 OD at 600 nm (circle) and amylase activity (triangle). Results represent the means of three separate experiments, and bars indicate ± standard deviation. Absence of bars indicates that errors were smaller than symbols.

halophilic bacteria produce amylase during stationary phase [2, 9].

Effect of pH and inoculum on amylase production. Amongst the physical parameters, the pH of the growth medium plays vital role by inducing morphological change in the organism and in enzyme production. The pH also serves as a valuable indicator of the initiation and end of enzyme synthesis. Most of the *Bacillus* sp. produce α-amylases by SmF (Submerged Fermentation) have most favourable pH ranging between 6.0 and 9.0 for growth and enzyme production [19]. In the present study, there was moderate growth and amylase production was seen in acidic pH but maximum amylase production was observed at alkaline pH 8 (38 U/mL) (Fig. 2a). Similar findings have been reported on *Bacillus barbaricus* [3]. *Bacillus subtilis* JS2004 produced maximum amylase at pH 7 [20]. Inoculum percentage in the medium was investigated for amylase production; maximum amylase production was obtained in the presence of 2% inoculum (Fig. 2b). Further increase in inoculum size was found to decrease the enzyme production. Low level of inoculum results in a less number of cells in the production medium, which requires a longer time to grow an optimum number to use the substrate and form the desired product. High level of inoculum in the medium may secrete high amount of enzymes which rapidly depletes the nutrients required for growth and product synthesis. In the present study 2% inoculum (45 U/mL) was found to be produce maximum amylase production. An inoculum size of 5% enhanced amylase production by *Bacillus cereus* [21].

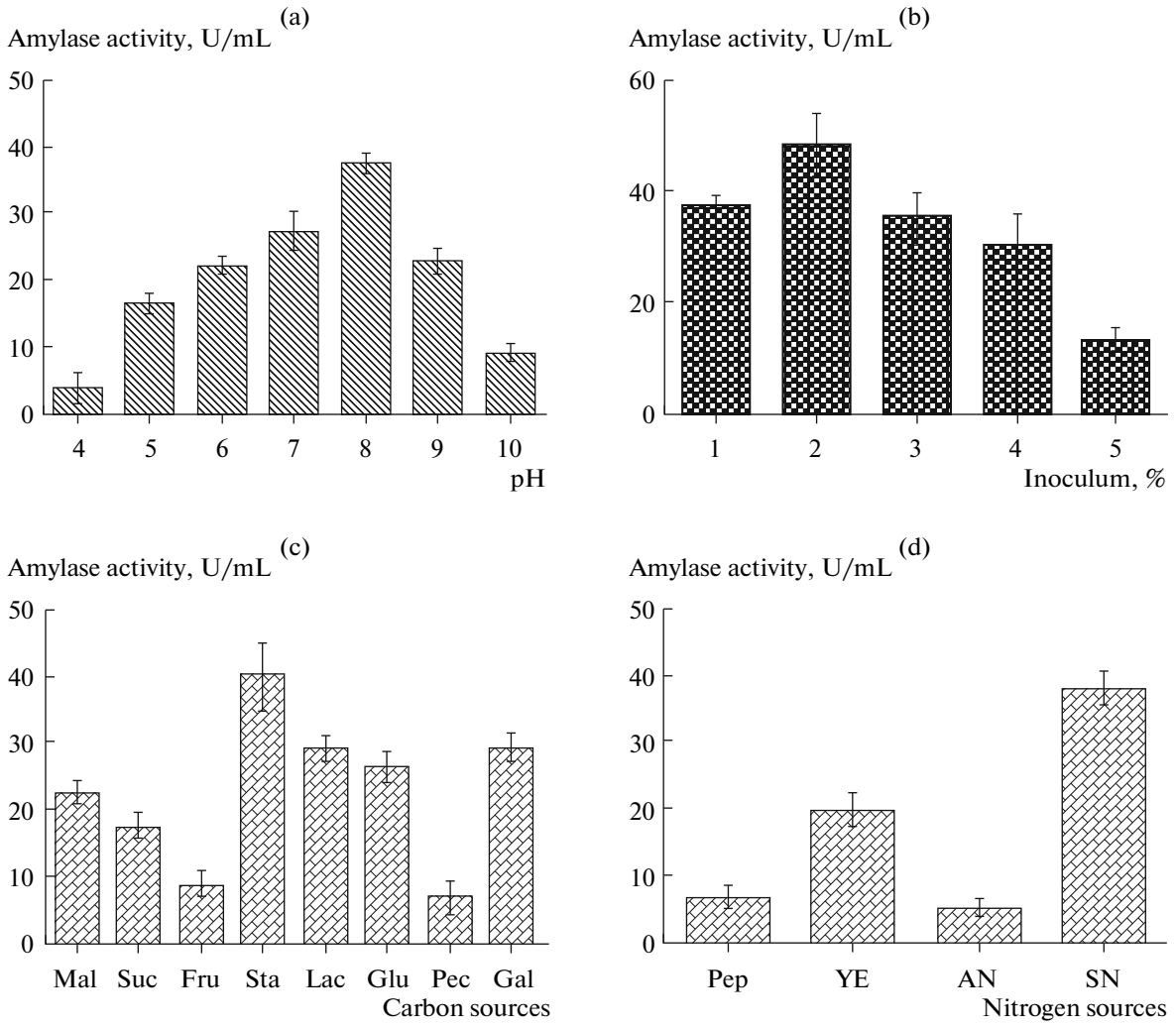

Fig. 2. (a) Effect of pH on amylase prodution in *Bacillus vallismortis* TD6. (b) Effect of inoculum level on amylase production by *Bacillus allismortis* TD6. (c) Influence of various carbon sources on amylase production by *Bacillus vallismortis* TD6. Mal—maltose, Suc—sucrose, Fru—fructose, Sta—starch, Lac—lactose, Glu—glucose, Pec—pectin, Gal—glactose. (d) Influence of various sources of nitrogen on amylase production by *Bacillus vallismortis* TD6. Pep—peptone, YE—yeast extract, AN—ammonium nitrate, SN—sodium nitrate.

Effect of carbon and nitrogen sources on amylase production. The addition of carbon source in the form of either monosaccharide or polysaccharides may influence the production of amylase enzyme. Among the different carbon sources tested (Fig. 2c), starch (40 U/mL), lactose (29 U/mL) and galactose (29 U/mL) were found to support maximum amylase production. In general the synthesis of carbohydrate degrading enzymes in most of the *Bacillus* sp. is subjected to catabolic repression by readily metabolizable substrates such as glucose and fructose [22]. Similar result was reported from *B. subtilis* strain AS-S01 [17] maximum amylase production was found when starch was used as carbon source. Use of dextrin showed maximum amylase production from *Halobacillus* sp. strain MA-2 [8]. The nitrogen sources also play significant function in the growth of the organism and enzyme production. In the present investigation, various nitrogen sources were tested (Fig. 2d), amylase production was highest when sodium nitrate was used (38 U/mL). Ammonium chloride was found to have a significant negative effect of both growth and enzyme production. *Bacillus* sp. HPE 10 [23] and *Bacillus* sp. [24] reported maximum amylase production on use of yeast extract.

Effect of amino acids and metal ions on amylase production. Different amino acids, metal ions were tested for amylase production (Figs. 3a and 3b). Moderate growth and amylase production was seen in L-alanine and glycine. L-Methionine was found to be inhibiting the growth and enzyme production. The addition of L-phenyl alanine resulted in maximum (58 U/mL) amylase production. L-Cysteine has been reported to stimulate amylase production [25]. Metal ions and trace elements are often essential for

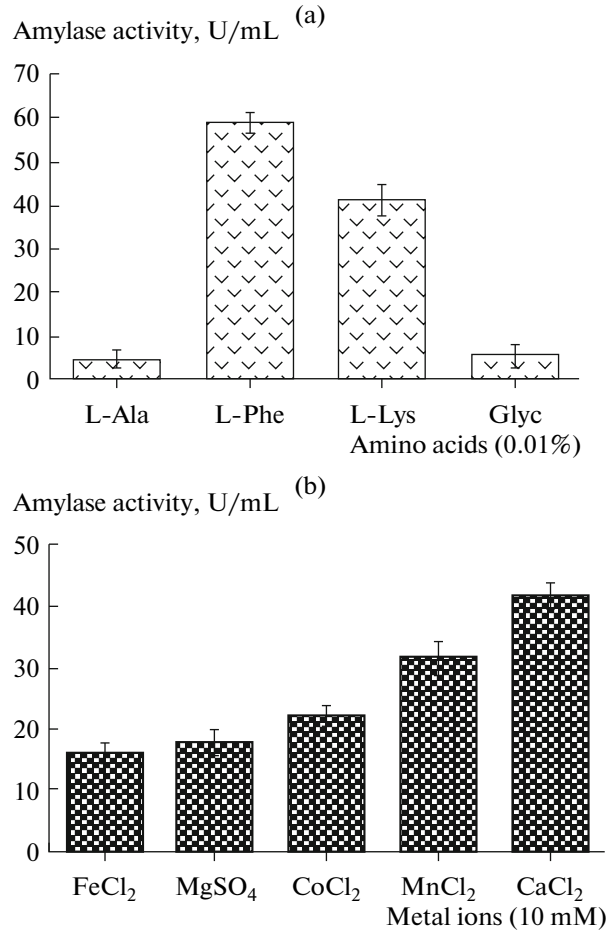

Fig. 3. (a) Effect of amino acid sources on the production of *Bacillus vallismortis* TD6 amylase. L-Ala—alanine, L-Phe—phenyl alanine, L-Lys—lysine, Gly—glycine. (b) Effect of different metal ions on amylase production from *Baciilus vallismortis* TD6.

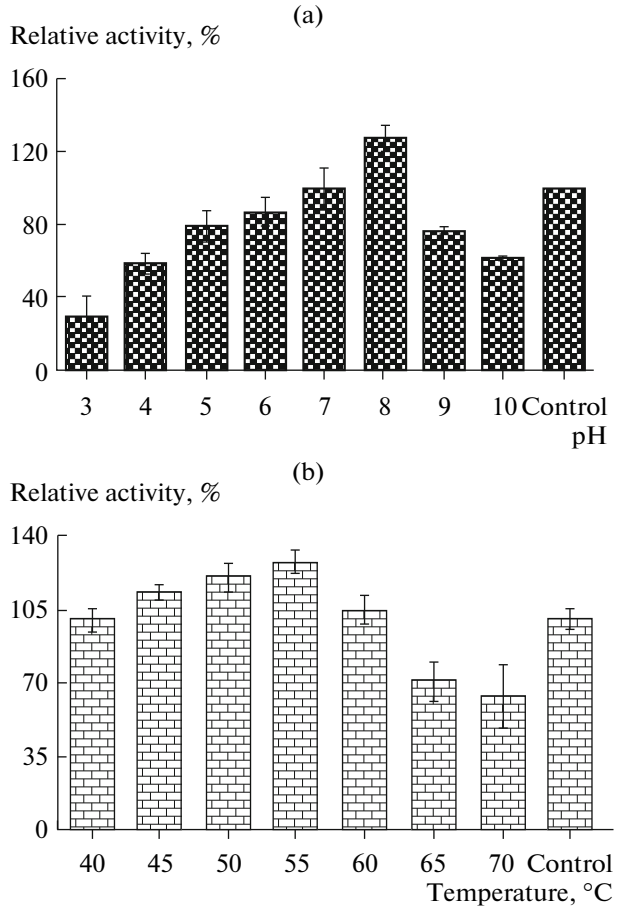

Fig. 4. (a) Effect of pH on amylase activity of *Bacillus vallismortis* TD6. (b) Effect of temperature on amylase activity of *Bacillus vallismortis* TD6.

enzyme production. The presence of calcium chloride in the medium showed a remarkable enhancement in amylase production (41 U/mL). Similar observations were observed in *Bacillus cereus* [16] and *Bacillus* sp. TSCVKK [2] where amylase production was enhanced by addition of calcium chloride in the medium.

Effect of pH and temperature on amylase activity. The influence of pH and temperature on the enzyme activity is shown in (Figs. 4a and 4b). The enzyme produced from strain TD6 was found to be active in a broad pH range of 6–8. The optimum pH was observed at pH 8 in alkaline condition and it also showed activity at pH 9 and 10. Similar results were reported on *Bacillus subtilis* [20]. Enzyme activity declined at a greater pace when the pH of the medium turned acidic compared to change in pH to alkaline conditions. These results are rather divergent from most of the bacterial α-amylase which are active at slight acidic to neutral pH [26]. The supernatant amylolytic activities were assayed at different temperatures ranging from 20 to 100°C. The enzyme was found to be optimally active at 55°C attained 127% of relative activity and it was found to be active in the range of 40 to 70°C confirming that the enzyme is thermotolerant. A reduction in enzyme activity was observed at temperature above 70°C. The optimum pH and temperature was reported for amylase activity as 7.5–8.5 and 50°C, respectively [8].

Effect of metal ions on amylase activity. The activity of various amylases is influenced by the presence of metal ions. Certain metal ions are required as cofactors and are thus called metalloproteins. The amylase activity was determined in the presence of different metal salts. Ca^{2+}, were found to stimulate amylase activity by 135% (Table 2). Other divalent metal ions such as Fe^{2+} (113%) and Mg^{2+} (109%) were also found to potentiate the amylase activity but not to the extent of calcium ion. Ca^{2+} was found to be involved in the salting out of hydrophobic residues in the enzyme, thereby causing adoption of compact structure [27]. The enzyme activity of amylase from strain TD6 was significantly enhanced in the presence of Fe^{2+} which is

Table 2. Effect of metal ions on activity of amylase produced by *Bacilllus vallismortis* TD6

Metal ions	Relative activity, %	
	5 mM	10 mM
Control	100	100
Ca^{2+}	135 ± 1	106 ± 1
Fe^{2+}	113 ± 5	105 ± 9
Mg^{2+}	109 ± 5	81 ± 6
K^{+}	96 ± 2	62 ± 1
Mn^{2+}	45 ± 6	34 ± 8
Hg^{2+}	43 ± 9	18 ± 3
Cu^{2+}	83 ± 1	51 ± 7
Na^{+}	88 ± 4	79 ± 9
Ba^{2+}	68 ± 2	57 ± 8
Zn^{2+}	51 ± 1	26 ± 3
Co^{2+}	57 ± 7	51 ± 5

Values are mean ±SD of three determinations.

Table 3. Compatibility of crude amylase from *Bacillus vallismortis* TD6 against commercial laundry detergents pre incubated at 35 and 45°C

Detergents	Relative activity, %	
	35°C	45°C
Control	100	100
Rin®	102 ± 1	112 ± 1
Surf Excel®	60 ± 5	64 ± 9
Ariel®	47 ± 5	50 ± 6
Tide naturals®	60 ± 2	65 ± 1
Ujala®	98 ± 6	97 ± 8
Wheel®	87 ± 9	93 ± 3
Arasan®	85 ± 1	92 ± 7
Ponvandu®	97 ± 4	100 ± 9
Mr. White®	73 ± 2	79 ± 8
Henko®	65 ± 1	75 ± 3

Values are mean ±SD of three determinations.

a rare characteristic of amylase. Majority of amylases are inhibited in the presence of Fe^{2+} where as only one report from thermostable amylase of *Bacillus* sp. I 3 reported by Goyal et al., was found to be enhanced in the presence of Fe^{2+} [16]. Enzyme activity was strongly inhibited by Hg^{2+}, Zn^{2+} and Mn^{2+} at 10 mM. However, metal ions such as Na^{+}, K^{+} and Cu^{2+} did not have any significant effect on the enzyme activity at 5 mM concentration. The inhibition by Hg^{2+} may specify the significance of indole amino acid residues in enzyme function and has been reported for microbial α-amylases [28]. Amoozegar et al., has reported that amylase from *Nesterenkonia* sp. was stimulated by Ca^{2+} and strongly inhibited by Fe^{3+}, Cu^{2+}, Zn^{2+} and Al^{3+} [30]. The α-amylase from *Bacillus firmus* was also strongly inhibited by heavy metals such as Ni^{2+}, Cd^{2+}, Zn^{2+} and Hg^{2+} [29]. The α-amylase from *Bacillus firmus* was also strongly inhibited by heavy metals such as Ni^{2+}, Cd^{2+}, Zn^{2+} and Hg^{2+} [30].

Washing performance test. In order to assess the compatibility of thermotolerant alkaline amylase as a laundry detergent additive, the crude enzyme was preincubated with the local commercially available washing detergents in the market for one at 35 and 45°C. Laundry detergents, which comprise the most important part of the detergent market and it was formulated mostly function at a high alkaline pH. The thermotolerant amylase from *Bacillus vallismortis* TD6 showed (Table 3) a significant compatibility towards most of the commercial laundry detergents at both tested temperatures. The crude amylase from TD6 was found to be more compatible with laundry detergent Rin® retaining more than 102 and 112% followed by Ponvandu® retaining 98 and 100% at 35 and 45°C of its initial activity. But considerable loss of amylase activity has been observed in the presence of Ujala®, Wheel®, Arasan®, Mr. White® and Henko®. Some of the detergents such as Surf Excel® and Ariel® showed partial loss of amylase activity which may be recognized as inhibitory effects of components such as anionic surfactants, bleaching agents and water softening builders etc. High stain removal was observed (Fig. 5) when crude enzyme mixed with laundry detergent was added on the stained cotton fabric for washing purpose. Therefore, the thermotolerant amylase from *Bacillus vallismortis* TD6 is suitable laundry detergent additive for washing performance.

In this present investigation, a halotolerant bacterium was isolated from saltern sediments from Tuticorin and identified as *B. vallismortis* TD6. This study was the first report for amylase production from *B. vallismortis*. Culture conditions were optimized with "One at a time" for amylase production. After optimization one fold of amylase production was increased. The strain TD6 was capable of amylase production in alkaline condition and it was active at high temperature and the crude amylase enzyme is compatible with commercial laundry detergents and it can be used for improving the wash performance. Based on the results

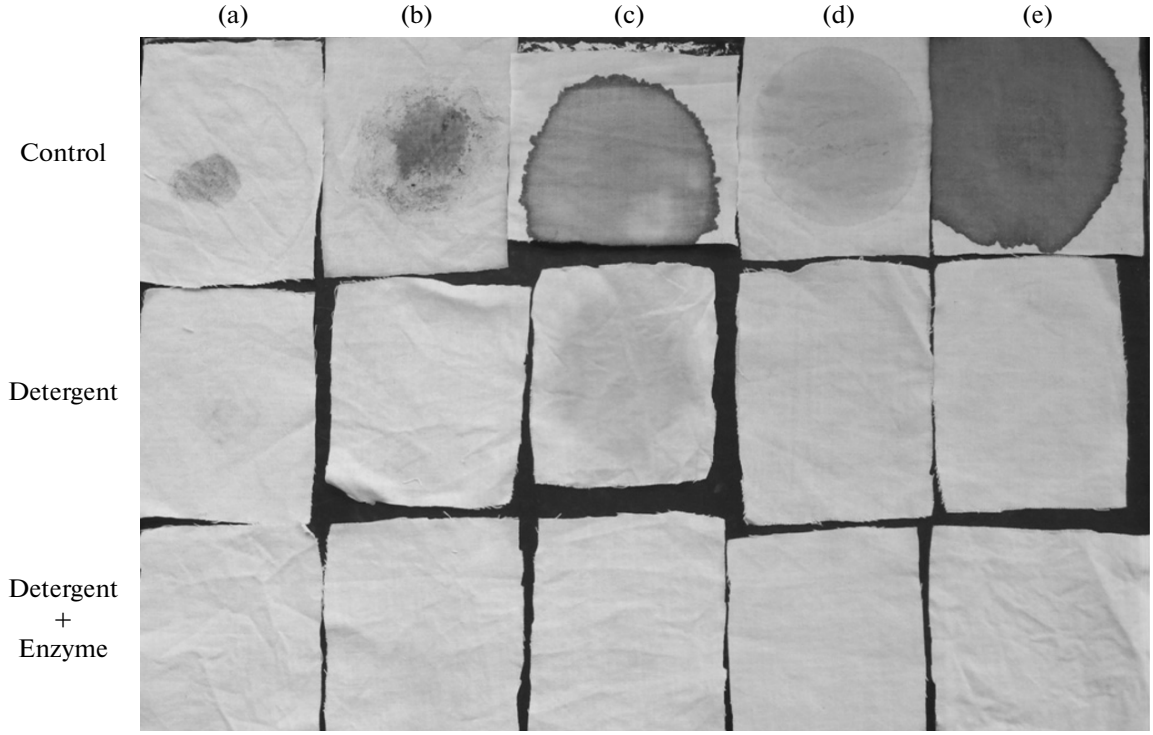

Fig. 5. Wash performance of the crude amylase from *Bacillus vallismortis* TD6 in the presence of the commercial detergent Rin. (1a) Ketchup (2a) Chocolate (3a) Blood (4a) Coffee (5a) Tea (1b) Ketchup with detergent (7 mg/mL) (2b) Chocolate with detergent (7 mg/mL) (3b) Blood with detergent (7 mg/mL) (4b) Coffee with detergent (7 mg/mL) (5b) Tea with detergent (7 mg/mL) (1c) Ketchup with detergent (7 mg/mL) and 50 U/mL of crude amylase enzyme (2c) Chocolate with detergent (7 mg/mL) and 50 U/mL of crude amylase enzyme (3c) Blood with detergent (7 mg/mL) and 50 U/mL of crude amylase enzyme (4c) Coffee with detergent (7 mg/mL) and 50 U/mL of crude amylase enzyme (5c) Tea with detergent (7 mg/mL) and 50 U/mL of crude amylase enzyme.

of present investigation amylase produced by strain, TD6 could be a potential biological ingredient in laundry detergent formulation.

ACKNOWLEDGMENTS

The authors thank VIT University for providing the necessary facility to carry out this work.

REFERENCES

1. Rajagopalan, G. and Krishnan, C., Alpha amylase production from catabolite derepressed *Bacillus subtilis* KCC103 utilizing sugarcane bagasse hydrolysate, *Biores. Technol.*, 2008, vol. 99, pp. 3044–3050.
2. Kondepudi, K.K. and Chandra, T.S., Production of surfactant and detergent-stable, halophilic and alkali-tolerant α-amylase by a moderately halophilic *Bacillus* sp. strain TSCVKK, *Appl. Microbiol. Biotechnol.*, 2008, vol. 77, pp 1023–1031.
3. Mageswari, A., Parthiban, S., Suganthi, C., Karthikeyan, S., Babu, S., and Gothandam, K.M., Optimization and immobilization of amylase obtained from halotolerant bacteria isolated from solar salterns, *J. Genet. Eng. Biotechnol.*, 2012, vol. 10, pp. 201–208.
4. Hough, D.W. and Danson, M.J., Extremozymes, *Curr. Opin. Chem. Biol.*, 1999, vol. 1, pp. 39–46.
5. Patel, R., Dodia, M., and Singh, S.P., Extracellular alkaline protease from a haloalkaliphilic *Bacillus* sp: production and optimization, *Proc. Biochem.*, 2005, vol. 40, pp. 3569–3575.
6. Ollivier, B., Caumette, P., Garcia, J.L., and Mah, R.A., Anaerobic bacteria from hypersaline environments, *Microbiol. Rev.*, 1994, vol. 58, pp. 27–38.
7. Ventosa, A. and Nieto, J.J., Biotechnological applications and potentialities of halophilic microorganisms, *World J. Microbiol. Biotechnol.*, 1995, vol. 11, pp. 85–94.
8. Amoozegara, M.A., Malekzadeha, F., Khursheed, A., and Malik, Production of amylase by newly isolated moderate halophile, *Halobacillus* sp. strain MA-2, *J. Microbiol. Methods*, 2003, vol. 52, pp. 353–359.
9. Prakash, B., Vidyasagar, M., Madhukumar, M.S., Muralikrishna, G., and Sreeramulu, K., Production, purification, and characterization of two extremely halotolerant, thermostable and alkali-stable alpha amylases from *Chromohalobacter* sp. TVSP 101, *Proc. Biochem.*, 2009, vol. 44, pp. 210–215.
10. Jaya Prakash Goud, M., Suryam, A., Lakshmipathi, V., and Singara Charya, M.A., Extracellular hydrolytic enzyme profiles of certain South Indian basidiomycetes, *Afr. J. Biotechnol.*, 2009, vol. 8, pp. 354–360.
11. Babu, K.R. and Satyanarayana, T., Parametric optimization of extracellular α-amylase production by ther-

mophilic *Bacillus coagulans, Folia Microbiol.*, 1993, vol. 38, pp. 77–80.

12. Miller, G.L., Use of dinitrosalicyclic acid reagent for determination of reducing sugar, *J. Anal. Chem.*, 1959, vol. 31, pp. 426–428.
13. Sambrook, J., Fritsch, E.F., and Maniatis, T., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor: Cold Spring Harbor Laboratory Press, vol. 3.
14. Michael, S., Roberts., Nakumura, L.K., and Choa, F.M., *Bacillus vallismortis* sp. nov., close relative of *Bacillus subtilis*, isolated from soil in Death Valley, California, *Int. J. Syst. Bacteriol.*, 1996, vol. 46, pp. 470–475.
15. Khire, J.M. and Pant, A., Thermostable salt-tolerant amylase from *Bacillus* sp., *World J. Microbiol. Biotechnol.*, 1992, vol. 8, pp. 167–170.
16. Vijayabaskar, P., Jayalakshmi, D., and Shankar, T., Amylase production by moderately halophilic *Bacillus cereus* in solid state fermentation, *Afr. J. Microbiol. Res.*, 2012, vol. 6, pp. 4918–4926.
17. Jetendra, K., Roy., Sudhir, K., Rai., Ashis, K., and Mukherjee, Characterization and application of a detergent-stable alkaline α-amylase from *Bacillus subtilis* strain AS-S01a, *J. Biol. Macromolec.*, 2012, vol. 50, pp. 219–229.
18. Burhan, A., Nisa, U., Gokhan, C., Omer, C., Ashabil, A., and Osman, G., Enzymatic properties of a novel thermophilic, alkaline and chelator resistant amylase from an alkalophilic *Bacillus* sp. isolate ANT-6, *Proc. Biochem.*, 2003, vol. 38, pp. 1397–1403.
19. Jin, B., Van-Leeuwen, J.H., and Patel, B., Mycelial morphology and fungal protein production from starch processing wastewater in submerged cultures of *Aspergillus oryzae, Proc. Biochem.,* 1999, vol. 34, pp. 335–340.
20. Asgher, M., Javaid Asad, M., Rahman, S.U., and Legge, R.L., A thermostable α-amylase from a moderately thermophilic *Bacillus subtilis* strain for starch processing, *J. Food Eng.*, 2007, vol. 79, pp. 950–955.
21. Sivakumar, T., Shankar, T., Vijayabaskar, P., Muthukumar, J., and Nagendrakannan, E., Amylase production using *Bacillus cereus* isolated from a vermicompost site, *Intl. J. Microbiol. Res.*, 2012, vol. 3, pp. 117–123.
22. Haseltine, C., Rolfsmeier, M., and Blum, P., The glucose effect and regulation of α-amylase synthesis in the hyperthermophilic archeon *Sulfolobus solfataricus, J. Bacteriol.*, 1996, vol. 178, pp. 945–950.
23. Mukesh kumar, D.J., Andal Priyadharshini, D., Suresh, K., Saranya, G.M., Rajendran, K., and Kalaichelvan, Production, purification and characterization of α-amylase and alkaline protease by *Bacillus* sp. HPE 10 in a concomitant production medium, *Asian J. Plant Sci. Res.*, 2012, vol. 2, pp. 376–382.
24. Thippeswamy, S., Girigowda, K., and Mulimani, V.H., Isolation and identification of α-amylase producing *Bacillus* sp. from dhal industry waste, *Indian J. Biochem. Biophys.*, 2006, vol. 43, pp. 295–298.
25. Rasooli, I., Astaneh, S.D.A., Borna, H., and Barchini, K.A., Thermostable α-amylase producing natural variant of *Bacillus* spp. isolated from soil in Iran, *Am. J. Agri., Bio. Sci.*, 2008, vol. 3, pp. 591–596.
26. Hamilton, L.M., Kelly, C.T., and Fogarty, W.M., Purification and properties of the raw starch degrading α-amylase of *Bacillus* sp. IMD 434, *Biotech. Lett.*, 1999, vol. 21, pp. 111–115.
27. Goyal, N., Gupta, J.K., and Soni, S.K., A novel raw starch digesting thermostable α-amylase from *Bacillus* sp. I-3 and its use in the direct hydrolysis of raw potato starch, *Enzyme Microb.*, vol. 37, pp. 723–734.
28. Gupta, R., Gigras, P., Mohapatra, H., Goswami, V.K., and Chauhan, B., Microbial α-amylase: a biotechnological perspective, *Proc. Biochem.*, 2003, vol. 38, pp. 1599–1616.
29. Shafiei, M., Ziaee A.A., and Amoozegar M.A., Purification and biochemical characterization of a novel SDS and surfactant stable, raw starch digesting, and halophilic α-amylase from a moderately halophilic bacterium, *Nesterenkonia* sp. strain F, *Proc. Biochem.*, 2010, vol. 45, pp. 694–699.
30. Cordeiro, C.A.M., Martins, M.L.L., and Luciano, A.B., Production and properties of α-amylase from thermophilic *Bacillus* sp., *Braz. J. Microbiol.*, 2002, vol. 33, pp. 57–61.