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Research Article

Production, Optimization, and Partial Characterization of Alkali-stable Alpha-Amylases from *Alkalihalobacillus* Isolates

Sunita R Borkar[1,2]* and Saroj N. Bhosle[1]

[1]*Department of Microbiology, Goa University, Taleigao Plateau, Goa, India*

[2]*UG and PG Department of Microbiology, P.E.S's R.S.N College of Arts and Science, Farmagudi, Ponda, Goa, India*

***Corresponding Author:** Sunita R Borkar, Associate Professor in Microbiology, Department of Microbiology, Goa University, Taleigao Plateau, Goa, India.

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Abstract

Alkali-stable amylases offer greater advantages over other amylases in biotechnological applications. In the present study, we aimed to produce, optimize and characterize amylase from alkaliphilic strains SB-D and SB-W isolated from sediment samples of an agrochemical factory. Both the strains were identified as *Alkalihalobacillus* sp. by 16S rRNA sequencing. The strain SB-D efficiently hydrolyzed 1% starch with optimized conditions of 2% inoculum, pH 10.3, 25°C, at 200 rpm after 24 h, while for SB-W it was 2% inoculum, pH 10.3, 55°C, at 250 rpm and after 16 h. Further, crude amylases from strain SB-D and SB-W were partially purified to 2.9 and 1.8 fold, respectively by ammonium sulfate precipitation method and showed multiple protein bands on SDS-PAGE. Interestingly, amylase from SB-W retained 80% activity in the presence of butanol, isopropanol and ether and showed activity at a broad pH range of 7.3-11.3 and temperature of 25˚C -75˚C. In comparison with commercial α-amylase; amylase from both strains showed a similar pattern of reducing sugar and iodine blue value. Furthermore, amylases required metal ions for their activity and were stable in the presence of detergent additives. Conclusively, SB-D and SB-W are potential producers of thermo-alkali tolerant and detergent-stable amylases of biotechnological significance.

Keywords: Agrochemical Factory; *Alkalihalobacillus* sp. Strain SB-D; *Alkalihalobacillus* sp. Strain SB-W; Alkali Stable

Introduction

Amylases are widely distributed in living organisms and are produced by members of all domains of life, thereby playing a crucial role in the field of biotechnology. Among extremophiles**,** alkaliphilic microorganisms producing alkaline amylase have enormous potential which can be exploited for industrial uses since alkaline conditions reduce bacterial contamination, increase substrate solubility and enhance enzymatic reactions [1]. Amylolytic enzymes from the genus *Bacillus* have several applications in the paper, textile, food, starch, adhesive and sugar industries [2-4].

Alkaliphilic *Bacillus* sp. is one of the dominant genera among the Gram-positive isolates retrieved from soda lakes [5] and their sediment [6]. The first amylase-producing alkaliphilic *Bacillus* strain was reported by Horikoshi in 19717. Industrial applications of these microorganisms and their alkaline amylases have been investigated extensively [7-11].

Amylases are categorized as *α*-amylase, *β*-amylase and glucoamylase based on the difference in their catalytic mechanisms, structures and amino acid sequences in 3-D form [12,13]. Alpha-amylase (E.C. 3.2.1.1); also known as 1, 4-α-D-glucanglucanohydrolase(GH) hydrolyzes α-1, 4-glycosidic linkages in starch and related carbohydrates made up of glucose (http://www.cazy.org/Glycoside-Hydrolases.html) [14,15]. α-amylases hold the maximum market share of enzyme sales with major applications in the starch industry, baking, analytical chemistry, automatic dishwashing detergents, textile desizing, medicine, pulp and paper industry [8,16]. The demand for alpha-amylase; specifically amylases for use in detergents has also been growing for several years due to high stability at alkaline pH, temperature and resistance against bleaching components [17].

Although amylases are produced by different organisms, their production and activity are optimum under different physicochemical factors and nutritional conditions. Carbon, nitrogen, phase of growth, the extent of aeration and agitation, temperature and pH have been found to influence the production as well as activity of amylase [18-21].

The aim of the present study was to i) optimize the physicochemical parameters for maximum production of amylases by strain SB-D and SB-W, ii) identify strain SB-W by 16S rRNA sequencing and iii) partially purify and characterize amylases from SB-D and SB-W.To our knowledge, this is the first report on the production and purification of amylase from alkaliphilic strains belonging to the genus *Alkalihalobacillus* isolated from spent water of an agrochemical factory, in Goa India.

Materials and Methods

Screening and Selection of potential amylase producers

Eighty-four alkaliphilic isolates growing at pH 10.3 were isolated from the spent waters of an agrochemical factory and screened for amylase production on starch agar [22] (Borkar., *et al*. 2003). Briefly, a loopful of each 18 h old culture was spot inoculated on Horikoshi II medium [23] (Borkar and Bhosle 2021) at alkaline and neutral pH with 1% soluble starch (w/v) (Hi media). The plates were incubated at 25ºC After 48 h, plates were flooded with 1% iodine in 2% potassium iodide solution and the zone of hydrolysis of starch was measured in mm. Two potential isolates, *Alkalihalobacillus* sp. strain SB-D (KJ372395) [23] and SB-W were selected for further studies.

Genotypic characterization of the isolate SB-W

DNA extraction from the isolate SB-W was done as per Prabhu., *et al*. [24]. The DNA amplification, purification of PCR product, and DNA sequencing followed by the building up of a phylogenetic tree were carried out as described for the SB-D strain by Borkar and Bhosle [23]. Further, the sequence was deposited in NCBI with accession number KJ372396.

Amylase production by strain SB-D and SB-W

Inoculum for each strain was prepared by transferring a loopful of culture from a slant into a 100 ml Erlenmeyer flask containing 20 ml of liquid Horikoshi II medium (pH 10.3). The medium consist of g/l: 10 starch, 5 polypeptone, 5 yeast extract, 1 KH_2PO_4, 0.2 $MgSO_4$, 20 agar, pH 10.3 adjusted using 10% Na_2CO_3. The flasks were incubated at 25°C and 55ºC at 200 rpm. After 24 h, 1% inoculum of each culture was inoculated in 50 ml of sterile Horikoshi II medium (pH 10.3) in 250 ml Erlenmeyer flasks. The flask containing culture SB-D was kept at 200 rpm at 25°C since no growth was observed at 55ºC, while SB-W was incubated at 25°C and 55°C. The aliquots of each culture were removed every 4 h to monitor growth at 600 nm and residual starch was measured using the iodine blue value. The maximum utilization of starch and iodine blue value was observed after 24 h and 16 h for strain SB-D and SB-W, respectively.

Preparation of cell-free supernatant (CFS) and amylase assay

The culture broth of each strain SB-D and SB-W was centrifuged at 10,000 x g for 20 minutes at 4°C (Remi RC 24 centrifuge) and CFS was used as a crude enzyme source. 50µl of CFS obtained at different time intervals of incubation of SB-D and SB-W as described above were placed in wells (6 mm) which were bored on starch agar plates (pH 10.3). The plates were incubated at 25ºC for12 h and hydrolysis of starch was observed by flooding the plate with 1% iodine in 2% potassium iodide solution.

Quantitative amylase assay

The reaction mixture consisted of 1.2 ml of 1% starch solution (w/v) prepared in 0.2 M carbonate bicarbonate buffer (pH 10.3), 0.6 ml of 0.2M carbonate bicarbonate buffer (pH10.3) and 0.2 ml of the crude enzyme. The reaction mixture was incubated for 30 minutes and used to determine residual starch by iodine value [25] and amylase activity by DNSA [26] by minor modifications in the assays.

Residual starch by iodine blue value

The reaction mixture (0.1 ml) was treated with 2.4 ml of 0.3 % iodine in 3% KI solution which was further diluted to 4%. Readings were taken at 620 nm against iodine solution as blank. The iodine blue value was calculated as given in the equation.

Amylase activity by DNSA method

The reaction mixture (1ml) containing CFS and starch was treated with 1 ml Di-Nitro-Salicylic Acid (DNSA) reagent. The reaction mixture was kept in a boiling water bath for 10 min, cooled and added with 2 ml of distilled water. The absorbance of the reaction at 540 nm was then used to estimate units of enzyme activity from the maltose standard. One unit of enzyme activity was defined as the amount of enzyme that produced 1 µg of reducing sugar at 25°C for strain SB-D and 55°C for strain SB-W for 30 minutes. Separately, the protein was determined by Folin Lowry's method with bovine serum albumin as the standard [27].

Optimization of culture conditions for maximum growth and optimum production of amylase

Based on growth curves, each culture was grown separately in Horikoshi II medium by substituting soluble starch with different carbon sources (1%) and yeast extract with organic and inorganic nitrogen sources (0.5%). Furthermore, cultures were also grown at different pH (4.3 13.3), temperature (5°C-75°C), agitation rates (100, 150, 200, 250 and 300 rpm), inoculum (1-8%) and different concentrations of yeast extract (0%-1.1%) and starch (0%-7%) for 24 h in case of SB-D and 16 h for SB-W. Each culture was incubated at previously optimized conditions. Growth was monitored by measuring the turbidity at 600 nm, residual starch by the iodine blue value method and amylase activity by the DNSA method.

Partial purification of amylase from strain SB-D and SB-W

Strain SB-D and SB-W were grown separately in 500 ml of Horikoshi II liquid medium under optimized production conditions. After 24 h, each culture broth was centrifuged at 10,000 x g for 20 minutes at 4°C. CFS obtained was treated separately with ammonium sulfate with constant stirring to achieve 80% saturation. The saturated supernatants were kept overnight at 4°C and precipitates obtained were collected by centrifugation. The precipitates were dissolved in small volume of carbonate bicarbonate buffer (pH 10.3) and the solutions were dialyzed (Dialyzed bag, cellulose tubing, molecular cutoff, 12 kDa, Sigma) twice over the course of 10 h against the same buffer. Protein concentration by Lowry's and amylase activity by DNSA were determined before and after the concentration step.

Molecular characteristics and zymogram of amylase from strain SB-D and SB-W

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) of amylase was performed. After the run, the gel was immersed in a staining solution (Coomassie brilliant blue R-250) to stain the protein bands. The molecular weight of the protein separated was determined by the mass with known proteins present in the molecular protein marker run separately. Additionally, in gel amylase activity, staining was carried out by incorporating 0.5% starch in the resolving gel consisting of 10% SDS. After electrophoresis, the gel was washed with buffer to remove bound SDS from the protein and a gel and flooded with lugols iodine to observe for starch hydrolysis as clear zone against the blue background.

Physicochemical characterization of the amylase

The amount of reducing sugar released was measured by the DNSA method after 60 minutes. The activity was expressed as % relative activity concerning maximum activity.

Type of amylase

The enzyme assay for the partially purified amylase from strain SB-D and strain SB-W was carried out as described earlier. The relation of residual starch concentration to sugar formed was plotted to determine the type of amylase using commercial alpha-amylase (Sigma) as the reference standard.

Effect of pH and Temperature on amylase activity and Stability

Partially purified amylase was assayed under pH ranging from 3.3 to 12.3, and temperature of 5°C to75°C. The pH and temperature stability profile of the enzyme(s) were determined by pre-incubating the enzyme at different pH (4.3-12.3) and at various temperatures (25°C-80°C).

Stability of amylase in the presence of metal ions, organic solvents and detergent additives

Amylase from each strain was incubated in the presence of metal ion solutions ($CaCl_2$, $MgC1_2$, $CuSO_4$ and KC1) at 0.1mM/l. The residual activity was assayed after 30 min. A reaction without metal salts was used as a negative control. Furthermore, the stability of each amylase in the presence of hydrophilic and hydrophobic organic solvents (Methanol, butanol, ethanol, isopropanol, chloroform and di ethyl ether at a concentration of 50%(v/v) and in the presence of detergent additives (TritonX-100, Tween80, SDS and EDTA) at 1% concentration was carried out. After 30 minutes, enzyme activity was determined in terms of relative activity.

Results

Detection of amylolytic activity on a starch agar plate

Screening results of alkaliphiles for amylase activity revealed that cultures SB-D and SB-W produced maximum amylase activity in comparison with other isolates. Amylolytic potential indicated the maximum hydrolysis of starch for SB-D and SB-W at pH 10.3 and 25ºC (Supplementary Figure S1).

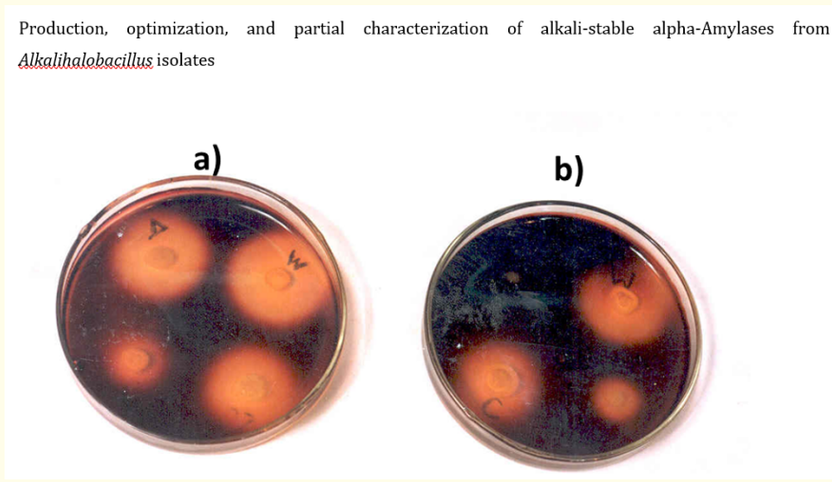

Figure S1: Agar plate test used for detection of amylase activity by SB-D and SB -W at a) pH 7.4 and b) pH 10.3. The strains were cultured in Horikoshi II agar plate for 48 h at 25˚C at neutral and alkaline pH.

Molecular characterization of isolate SB-W

The 16S rRNA gene sequence of SB-W showed 98.25 % similarity with *Alkalihalobacillus halodurans* AB021187 (Figure 1) followed by similarity to other *Alkalihalobacillus* sp. Based on molecular characterization, the strain SB-W was affiliated with the genus *Alkalihalobacillus* and designated as *Alkalihalobacillus* sp. strain SB-W. The present study reported for the first time the isolation of amylase producer *Bacillus halodurans (*reclassified now as *Alkalihalobacillus halodurans*) from highly alkaline spent waters of agrochemical factory.

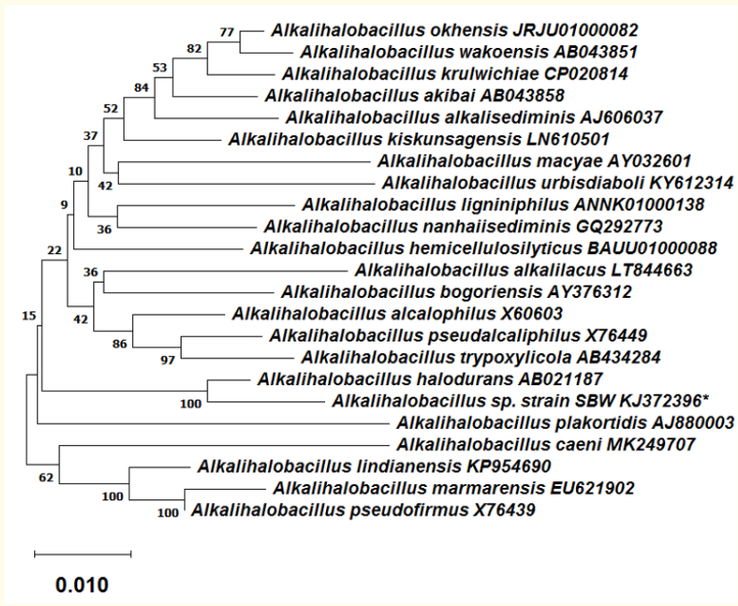

Figure 1

Amylase production and optimization from strain SB-D and SB-W

During the monitoring of the growth at 600 nm for SB-D and SB-W in various formulations of Horikoshi II medium, the isolates could grow in the absence of starch utilizing peptone and yeast extract as carbon and nitrogen sources (Table 1). However, starch as a source of carbon was found to exhibit good growth and initiate amylase production. Amongst the nitrogen sources, polypeptone, yeast extract, and meat extracts supported maximum growth; while inorganic nitrogen sources such as diammonium hydrogen orthophosphate and ammonium dihydrogen phosphate supported maximum growth (Table 1). Additionally, on scoring the organisms as CFU/ml, viable count of the organism producing amylase ranged from 10^2 to 10^9 CFU/ml at alkaline pH and an exponential increase in the counts with increase in the incubation was noted (Results not presented). Isolate *Alkalihalobacillus* sp. strain SB-D showed a continuous growth profile without a lag phase in Horikoshi II medium. However, utilization of starch, and hence enzyme production was found to be initiated only after 8 h of incubation (Supplementary figure S2), while, the growth rate of *Alkalihalobacillus* sp. strain SB-W was found to be faster at 55°C, with a sharp reduction in residual starch within 8 h (Supplementary figure S3).

Further, culture SB-D showed optimum growth and the least residual starch in the supernatant of the culture broth grown at 25°C. However, SB-W showed growth over a wide range of temperatures between 25°C-65°C with the least residual starch in the supernatant of the culture grown at 55°C (Figure 2a).

Carbon sources	O.D. 600 nm		Nitrogen source	O.D. 600 nm	
	SB-D	SB-W		SB-D	SB-W
Glucose	2.64	2.34	Control	1.35	1.25
Fructose	2.34	2.13	Organic Nitrogen Source		
Rhamnose	0.35	0.32	Peptone	2.28	2.16
Xylose	2.23	2.13	Yeast extract	2.50	2.23
Lactose	0.64	0.34	Meat extract	2.49	2.32
Sucrose	2.00	2.06	Soyabean meal	1.54	1.25
Maltose	0.34	0.45	Casein	1.45	2.21
Glycerol	2.80	2.60	Chitosan	0.75	0.34
Sorbitol	2.77	2.45	Urea	1.08	1.23
Xylitol	1.33	1.24	Peptone + Yeast extract	2.80	2.60
Mannitol	2.81	2.34	Inorganic Nitrogen Source		
Chitin	0.43	0.32	Ammonium nitrate	0.19	0.05
Starch	2.79	2.63	Diammoniumsulphate	0.27	0.15
Chitosan	0.74	0.34	Potassium nitrate	1.57	0.34
Tributyrin	1.18	2.30	Sodium nitrate	1.12	1.13
Tween80	1.90	2.42	Sodium nitrite	0.89	0.45
Coconut oil	2.24	2.73	Diammonium hydrogen orthophosphate	1.75	1.62
Longifolene	2.66	2.72	Ammonium dihydrogen phosphate	1.34	1.23

Table 1: Monitoring of growth of SB-D and SB-W in different carbon and nitrogen sources.

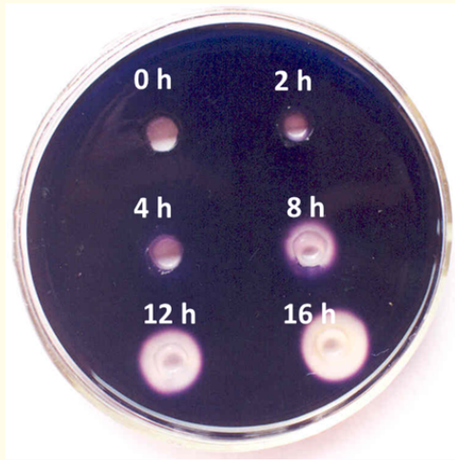

Figure S2: Amylase activity of culture supernatant of Alkalihalobacillus sp. strain SB-D on starch agar during growth.

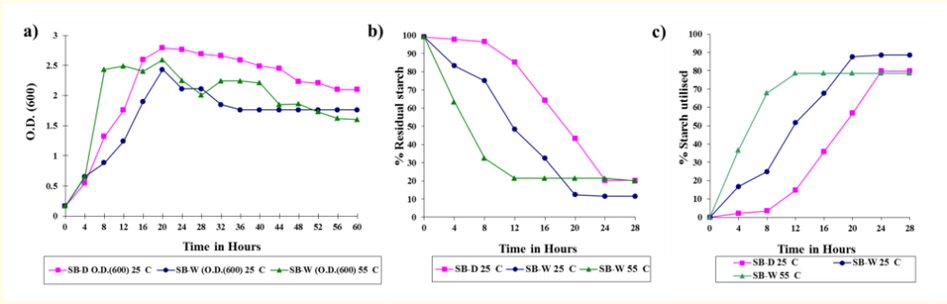

Figure S3: Growth Profile (a), Residual starch during growth (b) and starch utilized during growth (c) of SB-D and SB-W in Horikoshi II medium.

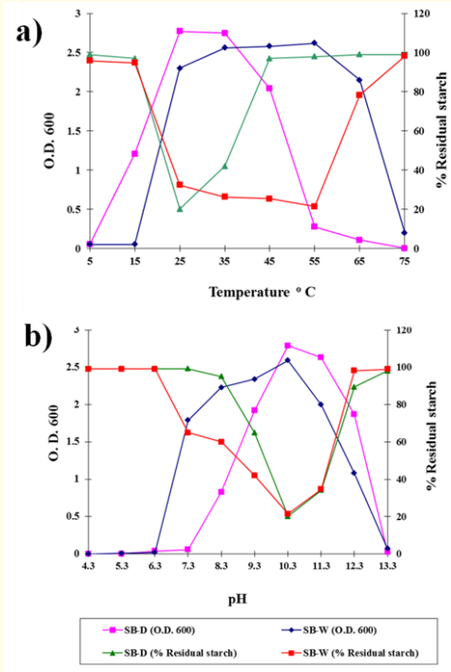

Figure 2

Significantly, growth and starch utilization showed distinct relationships at different pH. *Alkalihalobacillus* sp. strain SB-W showed maximum growth and starch utilization at pH 10.3 (Figure 2b); while growth of SB-D was initiated at pH 8.3 with zero utilization of starch. Above pH 8.3, the growth and starch utilization were linearly related with the optimum being at pH 10.3. Interestingly, an agitation rate of 200 rpm and 250 rpm promoted the growth of SB-D and SB-W, respectively (Figure 3a); while an inoculum size of 2% was required for maintaining the least residual starch in the culture supernatant by both the isolates (Figure 3b) although 3% inoculum size was optimum for growth of the cultures. As seen in Figure 3c, 0.5% yeast extract supported maximum growth and utilization of starch by *Alkalihalobacillus* sp. strain SB-D. With, *Alkalihalobacillus* sp. strain SB-W optimum growth was observed at 0.5% yeast extract that declined with increasing concentration. However, the conversion of starch continued up to 0.7% yeast extract and with SB-D there was no reduction at 1% starch concentration (Figure 3d).

Partial purification of crude amylase from strain SB-D and SB-W

Crude amylase from both the strains SB-D and SB-W were partially purified using ammonium sulfate precipitation followed by

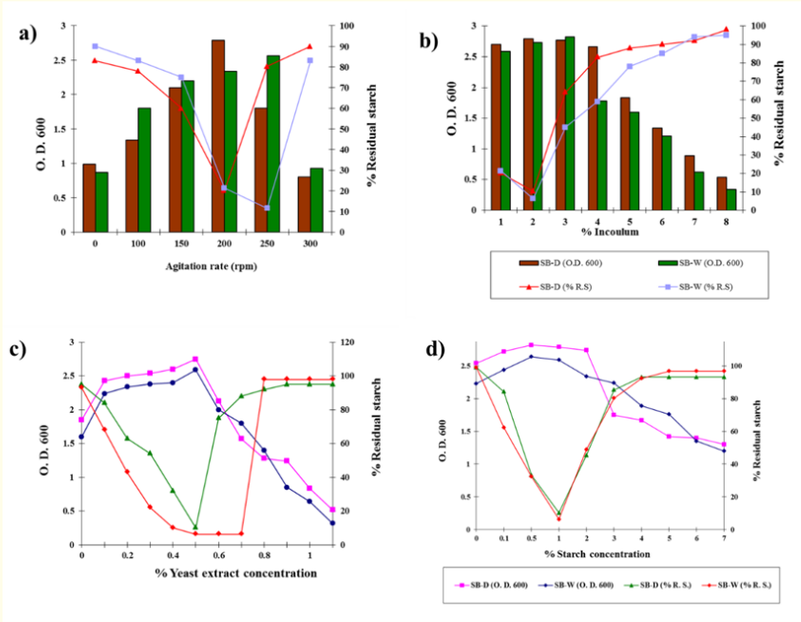

Figure 3

dialysis through a molecular cutoff of 12 kDa. The activity was found to increase with purification by 2.91 and 1.84 fold with SB-D and SB-W, respectively as compared to crude enzyme (Table 2). However, further purification needs to be carried out to determine the major unit of amylase produced by both strains.

SDS-PAGE and Zymography

SDS-PAGE profile of crude amylase showed multiple protein bands (Figure 4a); while partially purified enzyme showed the presence of 4 brightly stained protein bands of molecular mass 142 kDa, 67 kDa, 32 kDa and 12.4 kDa from *Alkalihalobacillus* sp. strain

Purification steps	Strain SB-D					Strain SB-W				
	Protein (mg/ml)	RS (mg/ml)	Enzyme Units	SA (U/mg)	FP	Protein (mg/ml)	RS (mg/ml)	Enzyme Units	SA (U/mg)	FP
CFS	0.506	0.24	24	47.43	1	0.597	0.35	35	58.62	l
$(NH_4)_2SO_4$ precipitation	0.67	0.58	58	86.56	1.82	0.742	0.65	65	87.6	1.49
Dialysis	0.47	0.65	65	138.2	2.91	0.724	0.78	78	108.33	1.84

Table 2: Partial purification of crude amylase from strain SB-D and SB-W.
CFS, cell free supernatant; RS, residual sugar; SA, specific activity; FP, fold purification.

SB-D, whereas eight bands were observed for SB-W amylase (Figure 4b), indicating multimeric nature of both the amylase. Further, activity staining of partially purified amylases from SB-D and SB-W showed diffused clearance of starch on in-gel starch zymography confirming the active nature of the enzyme (Figure 4c).

Determination of the type of amylase, optimal pH and temperature for amylase from strain SB-D and SB-W

Determination of the type of amylase showed a relationship between iodine blue value and reducing sugar similar to commercial α-amylase (Figure 5). A pH of the purified amylase from SB-W revealed 2 pH optima of 7 and 10.3 and pH optimum of 10.3 for amylase from SB-D (Figure 6a). Amylase of *Alkalihalobacillus* sp. strain SB-D showed activity at an optimum temperature of 25°C while for the enzyme from SB-W, the activity was maximum at 55°C irrespective of the pH being either 7 or 10.3 (Figure 6b). The stability of the amylase at different pH and temperature for 60 minutes was determined under optimum assay conditions. Interestingly, the amylase from *Alkalihalobacillus* sp. strains SB-W.

was found to be stable between pH 7.3 and 10.3 and temperature up to 65°C (Supplementary figure S4a and S4b); while SB-D had more thermolabile properties being stable only up to 35°C and pH between 9.3-10.3 (Supplementary figure S4c and S4d).

Activity and stability of amylase in the presence of metal ions, organic solvents and detergent additives

Amylase from SB-D showed maximum relative activity (97%) in the presence of K^+ followed by Ca^{2+} (95%) and Cu^{2+} (94%); while

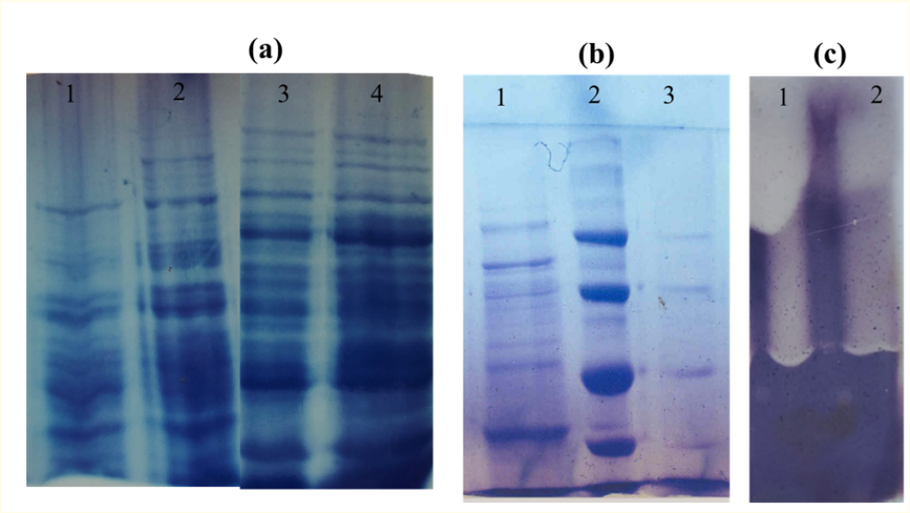

Figure 4

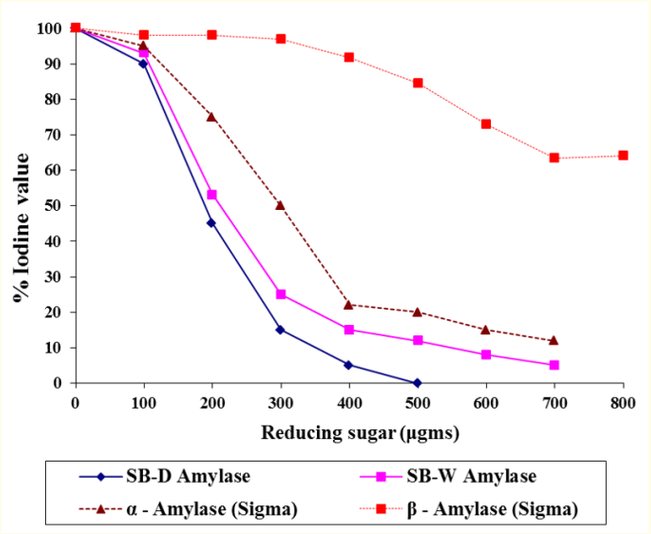

Figure 5

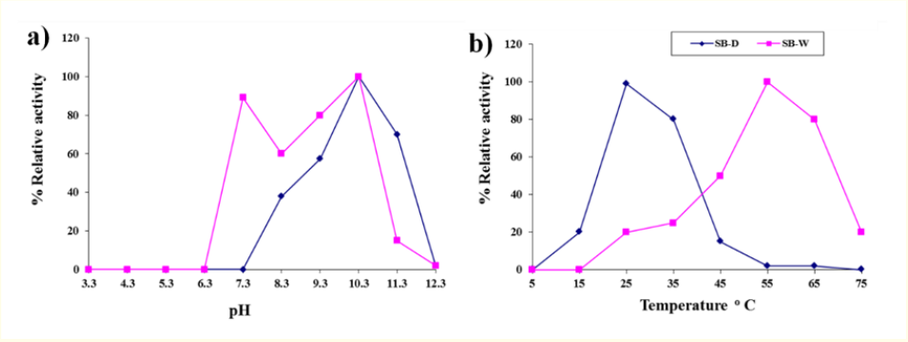

Figure 6

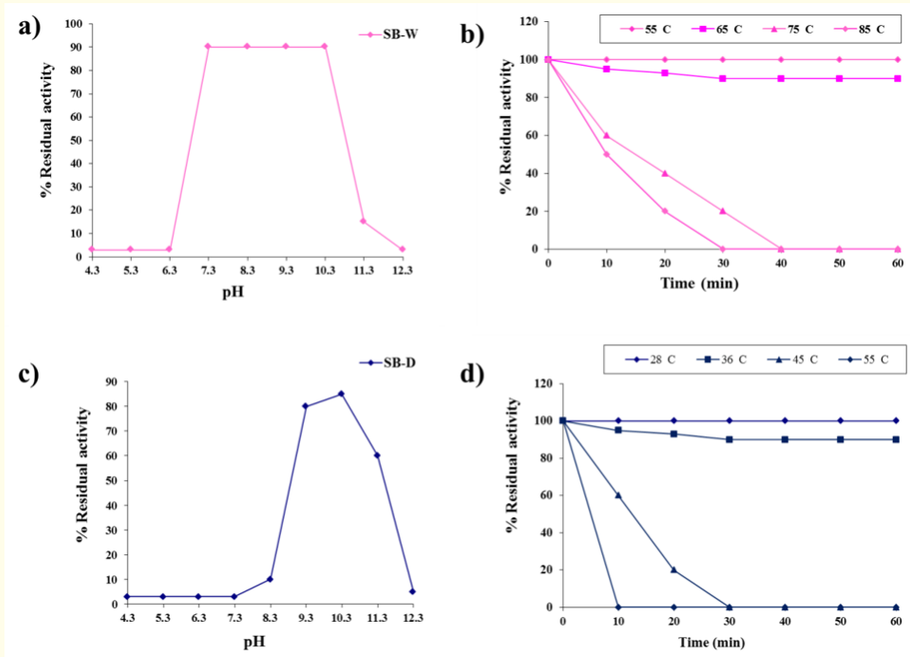

Figure S4: pH stability of SB-W amylase after 1 h at 55˚C (a) and SB-D after 1 h at 25˚C (c); Thermostability of amylase from SB-W (b) and SB-D (d).

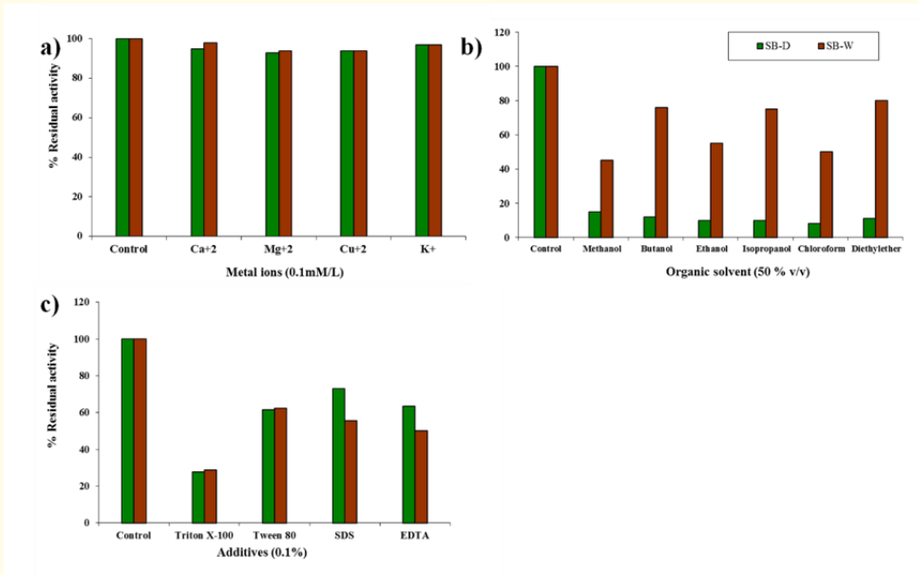

Figure 7

SB-W amylase was strongly stimulated by Ca^{2+} (98%) followed by Mg^{2+} (94%) and Cu^{2+}(94%) (Figure 7a). Activity of *Alkalihalobacillus* sp. strain SB-W amylase in presence of organic solvents monitored at 50% concentration revealed the retention of 80% activity in butanol, isopropanol and diethyl ether (Figure 7b). However, SB-D amylase showed least activity in presence of organic solvents. Further, the amylase from SB-D was found to be stable in the presence of SDS followed by EDTA> Tween 80>Triton X-100; while SB-W amylase showed enhanced stability in the presence of Tween 80 (Figure 7c).

Discussion

Agrochemical factory is located at Zuarinagar, Goa, India and is involved in the production of urea and nitrogen, phosphorus and potassium fertilizer. The spent waste waters of this industry are highly alkaline and could be favorable for the proliferation of alkaliphilic/alkali tolerant organisms [23]. Alkaliphiles have been isolated from various sources such as soda lakes and soda deserts found in Egypt, Rift Valley of Africa and western US and alkaline salterns including acidic soils. This reflects the interesting property of alkaliphiles of change the pH of the environment to suit their

growth are phenomenon very interesting from ecological point of view. The greatest enzymatic diversity on earth resides in microorganisms. Amongst the bacteria, organisms belonging to the genus *Bacillus* have been widely used for the commercial production of multiple enzymes [2,9,17].

Alkalihalobacillus sp. SB-D was found to grow and produce amylase at pH 10.3; while *Alkalihalobacillus* sp. SB-W could grow and produce amylase at neutral and alkaline pH indicating them to be obligate and facultative alkaliphile, respectively. Recently, *Bacillus* has been reclassified into novel genera such as *Alkalihalobacillus, Metabacillus, Mesobacillus, Neobacillus, Cytobacillus and Peribacillus* using phylogenomic approach [28]. Among these, *Alkalihalobacillus* is Gram stain variable, rod-shaped, endospore forming consisting of 39 species. All the members of the genus *Alkalihalobacillus* have been retrieved from diverse environments such as saltpan, soda lake, hypersaline lake, compost, sea water, sediment, sea urchin, rhizosphere soil, non-saline forest soil, goldmine, mangrove sediment, mural paintings [29]. Several species from this genus are known to produce cellulase, xylanase, protease and cyclodextin glucanotransferase [29]. The present study reported for the first time the isolation of *Bacillus lehensis* (reclassified now as *Alkalihalobacillus lehensis*) and *Bacillus halodurans (*reclassified now as *Alkalihalobacillus halodurans*) from highly alkaline spent waters of an agrochemical factory. Strain SB-D grew at pH 10.3, fermented glucose, arabinose and produced exoploymer and amylase [23]; while strain SB-W produces amylase maximally active at pH 10.3 and 55°C suggesting it to be alkali and thermo-stable enzyme. Although, strain SB-W showed maximum similarity to genus *Alkalihalobacillus*, the former was the only strain retrieved from spent waste water of agro-chemical factory producing amylase [23].

The results of optimization of culture conditions for maximum growth and amylase production revealed that SB-D and SB-W utilize other available carbon sources than starch to initiate and maximize growth followed by amylase production. Further, the culture SB-D and SB-W utilized starch at 25°C and 55°C, respectively. Such utilization of starch at 55°C in *Bacillus halodurans* has also been previously reported [18-20,30]. Most members of the genus *Alkalihalobacillus* are mesophilic and grow in the temperature range of 4°C to 45°C with optimum between 25-37°C. Nevertheless, the growth and production of amylase by strain SB-W at 55°C cannot be ruled out and is even likely since, commercially used amylases from *B. subtilis, B. licheniformis* or *B. amyloliquefaciens* showed excellent thermostability; although these bacteria were retrieved from moderate temperature conditions of gastrointestinal tract of ruminants, plant roots and soil [31].

For utilization of nitrogen sources, an increase in yeast extract amount showed a sharp inhibition of utilization of starch which is perhaps due to competitive utilization of the carbon source. This is also reflected in the extent of growth of the culture SB-D and SB-W on yeast extract alone. An increase in concentration of starch to 1% led to optimum growth; although 0.5% concentration showed maximum amylase secretion. Such an effect of the carbon source on the extent of growth of Gram-positive cultures is related to the stress due to excess nutrients and induction of sporulation [32]. Interestingly, it was observed that an increase in inoculum concentration above 1% for *Alkalihalobacillus* sp. strain SB-D and SB-W inhibited amylase production; although exhibiting optimum growth. Such a positive noticeable effect of inoculum levels with high inoculum densities inhibiting amylase production has also been reported [18]. Generally, it has been reported that cultural conditions have a profound influence on alkaline amylase production [18,33-36].

Crude amylase from SB-D and SB-W was partially purified to 2.91 and 1.84 fold, respectively using ammonium sulfate precipitation. The individual partially purified amylase revealed multimeric proteins which showed positive activity on starch in-gel zymography. Similar multiple amylolytic proteins are also observed in *Bacillus* and *Lactobacillus* [37]. Further, partially purified *Alkalihalobacillus* sp. strain SB-D and SB-W amylase was categorized as α-amylases as reported by Swamy., *et al*. [38]. Furthermore, amylase from both cultures is relatively thermo-stable and active either at pH 7 or 10.3. Alkaline α-amylase of the genus *Bacillus* has been reported [28,39-44]. In the present study, the activity and stability of amylase from SB-D and SB-W in the presence of metal ions, organic solvents and detergent additives was evaluated under standard assay conditions. Alpha amylases of *Alkalihalobacillus* sp. strain SB-D and *Alkalihalobacillus* sp. Strain SB-W showed maximum activity in the presence of K^+ and Ca^{2+}, respectively. Most alkaline amylases reported from the genus *Bacillus* are stimulated and inhibited by Ca^{2+} and Hg^{2+}, respectively [1,45,46]. To produce α-amylase of industrial grade, many researchers have also assessed the role of specific metal ions, salts, chelating agent, moistening agents, organic solvents and detergents on the very versatile α-amylase so that it can resist and tolerate various non-favorable environmental conditions [47,48]. The activity and stability of amylase from both the strains in the presence of metal ions, organic solvents and detergent additives was evaluated for biotechnological applications in textile, detergent and food industries. Amylases are extensively used in the formulation of enzymatic detergent with 90% of liquid detergents containing amylase [49]. Interestingly, SB-D and SB-W amylases were relatively stable in the presence of hydrophilic and hydrophobic solvents and detergent additives. Amylases from alkaliphilic *Bacillus* sp. which are stable in the presence of organic solvents and detergent additives have been reported [50-53]. Such stability of amylases from *Alkalihalobacillus* sp. strains SB-D and *Alkalihalobacillus* sp. Strain SB-W in the presence of physicochemical parameters and chemical additives is essential in the industrial processes carried out at extreme conditions [54-56].

To summarize, we infer that, the *Alkalihalobacillus* sp. strains SB-D and SB-W showed maximum production of amylase at 25°C and 55°C, respectively at pH 10.3. *Alkalihalobacillus* sp. SB-D amy-

lase functioned between pH 9-11 and temperature below 35°C. *Alkalihalobacillus* sp. SB-W amylase was the highly thermo-stable and organic solvent-tolerant type of alpha-amylase. Hence, these bio-prospecting strains *Alkalihalobacillus* SB-D and SB-W are excellent candidates for the production of thermo-alkali stable amylases that can be employed in biotechnological applications.

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Conflict of Interest

None.

Availability of Data Material

None.

Code Availability

None.

Author Contribution

Author S. Borkar designed, performed the experiments, analyzed the data, and drafted the manuscript. S. Bhosle helped in the data analysis and corrected the manuscript.

Ethical Approval

None.

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