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Research Article
Production and Partial Purification of Hyperthermostable Alkaline Amylase in a Newly Isolated *Bacillus cereus* (sm-sr14) from Hot-spring Water

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Abstract

Background and Objective: Thermostable amylases from bacteria are of great importance for industrial purposes and for today's biotechnological applications. This research aimed to study industrially important and physico-chemical stress-resistant bacterial amylase and its applications. **Methodology:** Amylase producing bacteria was collected from Bakreshwar hot-spring water, India by serial-dilution plating method on starch agar media. The strain, sm-sr14 was selected due to its strong hydrolysis efficiency. The strain was identified by polyphasic approach using biochemical, physiological properties and 16S rDNA homolgy. The optimization for amylase production, enzyme characterization and production using solid-state natural substrate were carried out with special emphasis on its heat stability by DNS method. The SPSS 10 was used for statistical analysis. **Results:** A hyperthermostable (60% residual activity up to 250°C) and highly alkaline (8-13; optimum 10) amylase producing *Bacillus cereus* sm-sr14 (Accession No. KM251578.1) was obtained from hot spring water. The amylase activity was found to be optimum at 50°C for 30 min incubation. The divalent cations Mn^{2+} and Ca^{2+} played critical role by 56% increase of enzyme activity. The EDTA (3 mM) moderately inhibits enzyme activity whereas, it was increased by the same concentration of β-mercaptoethanol. Enzyme was partially purified by cold acetone precipitation. The molecular mass was found to be 81 kDa by SDS PAGE. This amylase was found to be highly catalytic towards natural solid substrates like potato, rice, wheat and corn which was almost similar to laboratory-grade purified soluble and insoluble starch. **Conclusion:** The present findings suggested isolation and characterization of a hyperthermostable alkaline amylase with some important biotechnological potential ranging from food, fermentation, textile, detergent to paper industries.

Key words: Alkaline amylase, hyperthermostable, *Bacillus cereus*, solid rice/potato utilization

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Thermostable enzymes are stable and active at temperatures which are even higher than the optimum temperatures for the growth of the microorganisms[1]. Thermostable enzymes are gaining wide industrial and biotechnological interest due to the fact that their enzymes are better suited for harsh industrial processes[2]. One extremely valuable advantage of conducting biotechnological processes at elevated temperatures is reducing the risk of contamination by common mesophiles. Allowing a higher operational temperature also has an appreciable influence on the solubility and bioavailability of organic compounds and thereby provides efficient bioremediation. Other advantages of carrying out industrial processes at elevated temperatures include higher reaction rates due to a decrease in viscosity and an increase in diffusion coefficient of substrates and favourable equilibrium displacement in endothermic reactions[3,4]. Such enzymes could also be used as models for the understanding of thermostability and thermoactivity, which is also useful for protein engineering[5].

Starch, the plant materials and the substrate of amylase is one of the most abundant and accessible energy sources in the world. It consists of amylose and amylopectin. Rice (*Oryza sativa*), Corn (*Zea mays*) and wheat (*Triticum* sp.) are the most used crop for domestic consumption and in starch processing industries. The potato (*Solanum tuberosum*) and tapioca (*Manihot esculenta*) are also important consumable crops while sorghum (*Sorghum bicolor*), sweet potato (*Ipomoea batatas*), arrowroot (*Maranta arundinacea*), sago (*Cycas revoluta*) and mung bean (*Vigna radiata*) are used to a moderate extent[6]. Amylases are enzymes which hydrolyze starch molecules act on α-1,4-glycosidic bonds to give diverse products including dextrin and progressively smaller polymers composed of glucose units[7]. The α-amylase (1,4-α-D-glucan-glucanhydrolase, EC. 3.2.1.1) is a widely distributed secretary enzyme. The α-amylase of different origin has been extensively studied[7].

Processed starch is mainly used for glucose, maltose and oligosaccharide production, but a number of products can also be produced via cyclodextrin. The glucose can be further converted to high-fructose syrups, crystalline dextrose and dextrose syrups, which are used in food applications. Glucose can also be fermented to produce ethanol, amino acids or organic acids which are of great practical importance[8]. Amylases have several different industrial applications including those for animal feed, foods, pharmaceuticals, detergents and biofuels[9].

Each application requires unique properties with respect to specificity, stability and temperature and pH dependence. Therefore, screening of the microorganism with novel amylase activities could facilitate different useful purposes[10]. A cold active α-amylase from *Bacillus cereus* suggests that a networks in structurally critical regions of few amino acids serves for its cold adaptations[11]. This present studies focus on the high temperature adaptation of the experimental α-amylase. Taken together, this enzyme has broad range of temperature withstanding ability.

In the present investigation, the amylase producing thermpohilic strain have been isolated/identified and eventually designated as sm-sr14. The outstanding properties of the enzyme were extensively characterized and the bacterial strain was explored at physiological and metabolic level with a molecular (16S rDNA) identification. This enzyme is highly temperature resistant in dry and moist environment with wide pH variations. The enzyme is noticed to be detergent tolerant. For the basic science study on the structural-functional stability of this enzyme protein is ideal. This amylase was found to be highly catalytic towards natural solid substrates like potato, rice, wheat and corn and that activity was almost similar to laboratory-grade purified soluble and insoluble starch. The long term goal of this work was to elicited the molecular mechanism of the hyper-thermostability of the isolated amylase and its possible use in harassed industrial environment like high temperature, pH zone. Finally, this enzyme can be utilized at application level for commercial purpose.

MATERIALS AND METHODS

Isolation of amylase producing thermophilic bacteria: Working on high amylase producing bacteria from different habitat a substantially higher amylase producing strain was isolated from hot spring water of Bakreshwar, India (23.88°N, 87.37°E) and eventually investigated further for characterization and it was designated as sm-sr14. Water samples from this hot spring (>80°C, pH 7.8) were collected in sterile plastic container. For an enrichment of thermophilic bacteria, 1 mL water samples was inoculated in triplicate in Luria Bertani[12] broth (pH >7) (50 mL) and incubated in a rotary shaker incubator (200 rpm) at 50°C for the isolation of thermophilic bacteria and subsequently sub-cultured on the same solid medium. All the isolates were cultured in pure form after several successive passages and preserved at -20°C with glycerol (30 % v/v).

Primary selection of starch hydrolysing ability among the isolates was made by allowing them to grow individually on starch agar media. Starch hydrolysis was observed surrounding the colonies after adding lugol's solution[13]. Strain sm-sr14 was selected for further study due to its higher thermophilic nature and starch hydrolysis activity. That was grown in starch broth media and amylase activity was determined on cell free supernatant by standard 3,5-dinitrosalicylic acid (DNS) method[14].

Characterization of the strain sm-sr14: The strain sm-sr14 was characterized after an extensive study with a polyphasic approach. The standard microbiological techniques were followed to determine colony morphology, arrangement and endospore property. Various biochemical properties like sugar utilization, extra-cellular enzymatic profile and antibiotic susceptibility profile were determined after growing it on various specific media. Growth characteristics of the strain (i.e., optimized media pH, incubation temperature, optimum incubation time and salt tolerance) were also determined[15].

Scanning Electron Microscopy (SEM) study of sm-sr14: A physical measurement and morphological details were revealed after scanning electron microscopy study of the strain (Vega© Tescan, Chezc Republic). Sample preparation for SEM observation was followed by graded ethanol dehydration and then was taken up to critical dry point[16].

16S rDNA amplification and phylogeny construction for sm-sr14: Cells were grown in LB media for 24 h and genomic DNA was isolated from the cell pellet from the function of post lysis with lysozyme (20 mg mL^{-1}) and SDS (10%) treatment in tris buffer pH 8[17]. Phenol chloroform extraction was performed twice followed by the precipitation of DNA with isopropanol. The 16S rRNA gene was amplified using a primer 27F 5'-AGTTTGATCCTGGCTCAG-3' and 1492R 5'-GGCTTACCTTGTTACGACTT-3'[18]. The PCR conditions were programmed as follows: Initial denaturing temperature of 94°C for 5 min and then 30 cycles were followed each cycle of 1 min at 95°C, primer annealing for 1 min at 55°C and primer elongation 5 min at 72°C. A final extension was done for 10 min at 72°C. Amplified products were purified with PCR purification kit (Himedia, Mumbai, India). Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with same primers using BDT v3.1 cycle sequencing kit on ABI 3730xl Genetic Analyzer.

Consensus sequence of 1464 bp was generated and used to carry out BLAST with the nr database of NCBI GenBank. Based on maximum identity sequences were carried for multiple alignments by Clustal W. A distance matrix was generated using Ribosomal Database Project (RDP) and the phylogenetic tree was constructed using Molecular Evolutionary Genetics Analysis (MEGA6)[19]. The evolutionary history was inferred using the Neighbour-Joining method[20]. The evolutionary distances were computed using the Kimura 2-parameter method[21].

Optimization of various physico-chemical parameters for amylase production: For optimum production of amylase from strain sm-sr14, some external parameters were regulated by One Variable At A Time (OVAT) fashion during amylase production. Most desired media for amylase production was checked by growing the strain in different media formulation (Table 1). Amylase production at maximum level was determined by growing the strain in optimum media at different initial media pH (2-12) and incubation temperature (20-80°C) separately. Culture was inoculated with optimum amylase production conditions as determined in earlier steps and optimum incubation period for best amylase activity was evaluated from culture broth drawn at every 6 h interval[22]. The enzyme production was determined in terms of maximum enzyme activity obtained by DNS assay method[14].

Table 1: Different type of formulated media

Media	Composition (%)
SP	Starch 2, peptone 0.5, beef 0.3, NaCl 0.5
SB	Starch 2, beef 0.3, NaCl 0.5
SG	Starch 2, gelatin 0.2, NaCl 0.5
SGB	Starch 2, gelatin 0.2, beef 0.3, NaCl 0.5
MS1	Starch 2, sucrose 3, $NaNO_3$ 0.3, KCl 0.05, $FeSO_4$,$7H_2O$ 0.001, $MgSO_4$ 0.05, K_2HPO_4 0.1
MS2	Starch 2, $(NH_4)_2SO_4$ 0.2, $MgSO_4$, $7H_2O$ 0.05
MS3	Starch 2, yeast extract 0.05, $MgSO_4$ 0.03, K_2HPO_4 1, $(NH_4)_2SO_4$ 1.05, $CaCl_2$ 0.05, $FeSO_4$ $7H_2O$ 0.001
MS4	Starch 2, $MgSO_4$ 0.05, K_2HPO_4 0.1, $NaNO_3$ 0.2, KCl 0.05, $FeSO_4$ $7H_2O$ 0.001
MS5	Starch 2, $MgSO_4$ 0.05, K_2HPO_4 0.1, $NaNO_3$ 0.2, KCl 0.05, $FeSO_4$ $7H_2O$ 0.001, peptone 0.1, yeast extract 0.1

MS: Mineral salt

Characterization of enzyme activity: Cell free culture broth (100 µL) of sm-sr14 was taken as enzyme source and various physico-chemical parameters were optimized for the enzyme activity. Enzyme substrate reactions were carried out for 30 min at different pH (2-14) and temperature conditions (5-70°C). Effect of glucose, metal-ions, metal chelating agents, reducing agents, oxidizing agents, detergents and various solvents on enzyme activity was also determined by standard methods[23]. Enzyme stability at different critical conditions was checked. The crude cell free enzyme was incubated in a wide range of temperature (0-250°C) for 2 h. Samples were then brought at room temperature and residual was dissolved in 500 µL tris buffer and the activity was checked against 1% soluble starch. Similarly, pH stability was evaluated by incubating at a pH range (4-14) and NaCl stability was determined by incubating it in graded concentration of NaCl (0-5 M)[24].

Substrate specificity of the enzyme: The substrate specificity of the enzyme was studied by using different concentration (%) of substrates (soluble starch, insoluble starch, rice, potato, wheat and corn). The reaction mixture was composed of different (%) of the above substrate, tris-HCl buffer (0.1 M, pH 10) and 100 µL enzyme with control. The mixture was incubated at 50°C for 30 min. The reaction was terminated by addition of DNS solution.

SDS-polyacrylamide Gel electrophoresis (PAGE) and in-gel localization of active protein: The cell free culture supernatant was mixed with acetone (1:2) and kept overnight at 4°C. Post centrifugal precipitation (10,000 rpm for 15 min) was collected and dissolved in 5 mL tris buffer (0.1 M, pH 10) added with Mn^{2+} (1 mM). It was then concentrated by freeze drying to a final volume of 500 µL. This partially purified sample was analysed through denaturing (10%) PAGE. Protein bands were visualized after silver staining of the gel[25]. The molecular weight was calculated from a semi-logarithmic plot of the molecular masses of standard protein versus relative mobility of each band observed. For zymogram (in-gel enzyme assay) analysis SDS-PAGE was performed in a 10% resolving gel containing 1% soluble starch[26]. Protein sample was prepared with non-reducing sample loading buffer without heat exposure and electrophoresed at 50 mA. After electrophoresis, the gel was washed for 1 h with 25% isopropanol to remove SDS followed by a washing with distilled water immersion in tris buffer (pH-10) for 1 h at 50°C. Subsequently, the in-gel amylase band was visualized after treating the gel with lugol staining solution. A transparent zone on dark blue background indicated amylase position that was comparable with silver stained gel bands. The similar electrophoresis procedure (silver staining for protein band and in-gel assay for amylase activity) was employed to verify the purified amylase activity after it was treated with different temperature (100-250°C).

Statistical analysis: The SPSS 10, Microsoft Excel 7 and sigma plot was used to draw different type of bar and line diagram[22]. Further statistical analysis was done in this study.

RESULTS

Physiological and biochemical characterization of sm-sr14: Among 11 isolated strain from hot spring water, the strain sm-sr14 was selected due to its comparatively higher thermophilic nature and greater amylase activity. The strain appeared to be rod shaped with occasional chain formation with 2-5 bacteria. Electron microscopic study despicable the strain was about 1.5-2 µm long with 0.5-1 µm diameter (Fig. 1). Morphological characterization shows that it is a Gram positive, endospore forming, non-capsulated bacteria that produce creamy white, large smooth colony (3-4 mm diameter) of irregular margin. The strain was found positive for indole, methyl red assay (Table 2). It was able to produce H_2S on sim agar media and successfully able to utilize rhamnose, sucrose, galactose and raffinose as sole carbon source among the sugars employed for determining its sugar utilization pattern (Table 2). Extracellular enzyme production profile by sm-sr14 indicates its ability for the production of amylase, cellulase, caseinase, gelatinase and lipase. The strain showed resistance to antibiotics like methicillin, streptomycin, co-trimoxazole etc (Table 2).

16S rDNA analysis and molecular characterization of sm-sr14: It was found from the BLAST analysis that the strain sm-sr14 possess 16S rRNA gene sequences which was similar (99%) to many strains of *Bacillus cereus*. However, in phylogenetic tree it form a separate cluster with *B. cereus* SBTBC-008 and hence from polyphasic characterization it can be proposed that the strain is a new strain of *Bacillus cereus* sm-sr14 (Fig. 2) with GenBank accession KM251578.1.

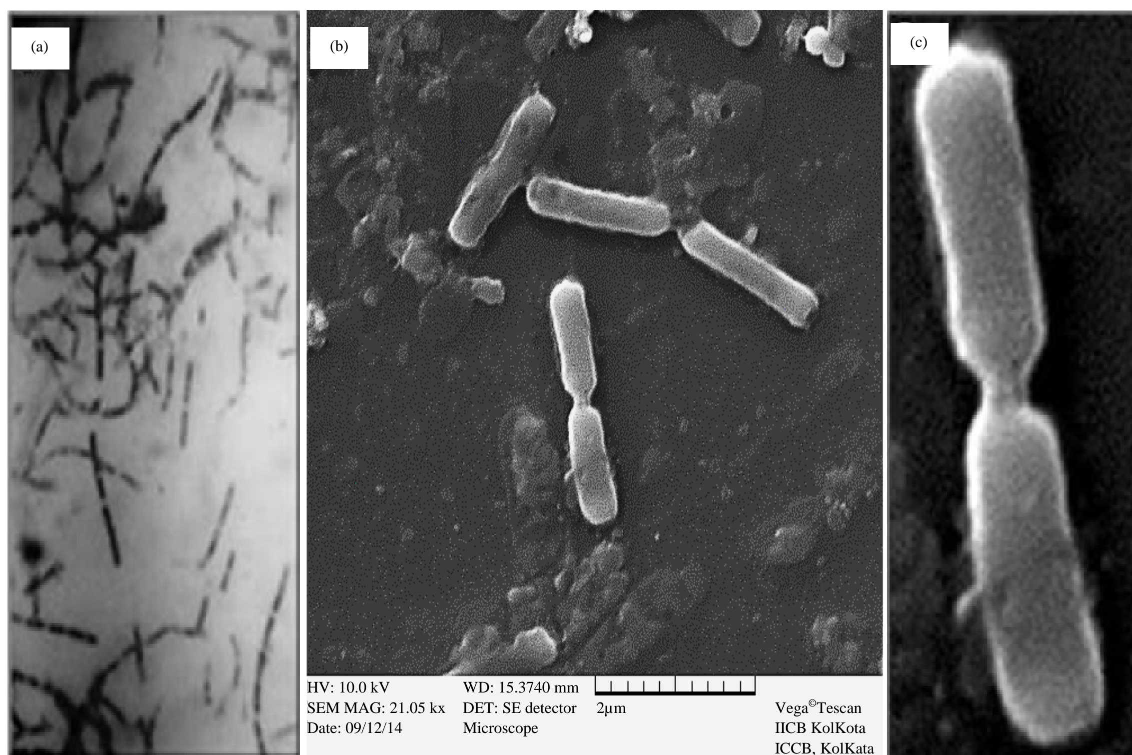

Fig. 1(a-c): Microscopic view of strain sm-sr14 (a) Compound microscopy after Gram staining and (b-c) SEM view

Table 2: Characterization of sm-sr14 from various aspects

Parameters		
Morphological	Colony	Large colony (3-4 mm diameter), irregular margin, milky white
	Cellular	Long slender rod shaped bacteria exist mostly in chain. Length 1.5-2 µm and 0.2 µm diameter. Gram positive, endo-spore forming
Biochemical	Indole, methyl red, H_2S production,	+
	Voges prouskuer, citrate utilization	-
Sugar utilization	Rhamnose, sucrose, galactose, raffinose,	Huge growth
	Mannose, lactose	Moderate growth
	Fructose, dextrose, maltose, xylose	
NaCl tolerance	0, 1, 3 M	+, ++, +, respectively
Extracellular enzyme	Catalase, gelatinase, amylase, caseinase,	+
	Lipase, cellulase, nitrate reductase	
	Urease , phenylalanine deaminase	-
Antibiotic susceptibility	Penicillin, amplicillin, methicillin, streptomycin, Co-trimoxazole	Resistant
	Tetracyclline, kanamycin, neomycin, Norfloxacin, chloramphenicol, nalidixic acid, rifampcin	Sensitive

+: Positive and -: Negative

Optimization of growth and culture media: The strain was found to grow best at pH 8 though significant growth was observed in a wide range (pH 6-10). The temperature optima for its growth was 40°C while it is able to grow well in between 30-60°C with maximum thermo tolerance up to 80°C (Fig. 3). The growth pattern as well as amylase production by sm-sr14 were determined in different formulated media. It was found that among the selected media MS5 (mineral salt media) was most suitable for its growth as well as amylase production. The growth pattern and enzyme-production was verified in MS5 media at different type of starch substances as sole carbon source while keeping constant all other ingredients. It was noticed that bacterial growth was considerable in all type of starches. Amongst the natural sources of starch, potato and rice yielded considerable amylase in this study (Fig. 4).

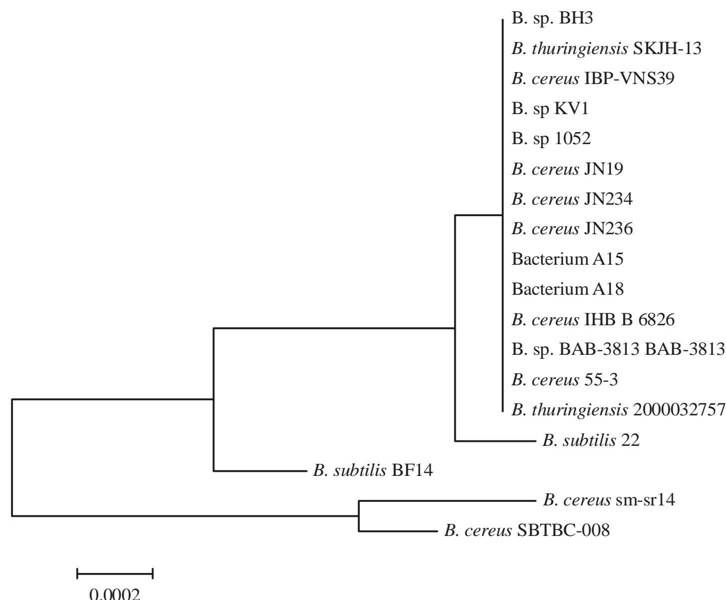

Fig. 2: Position of strain sm-sr14 in phylogenetic tree

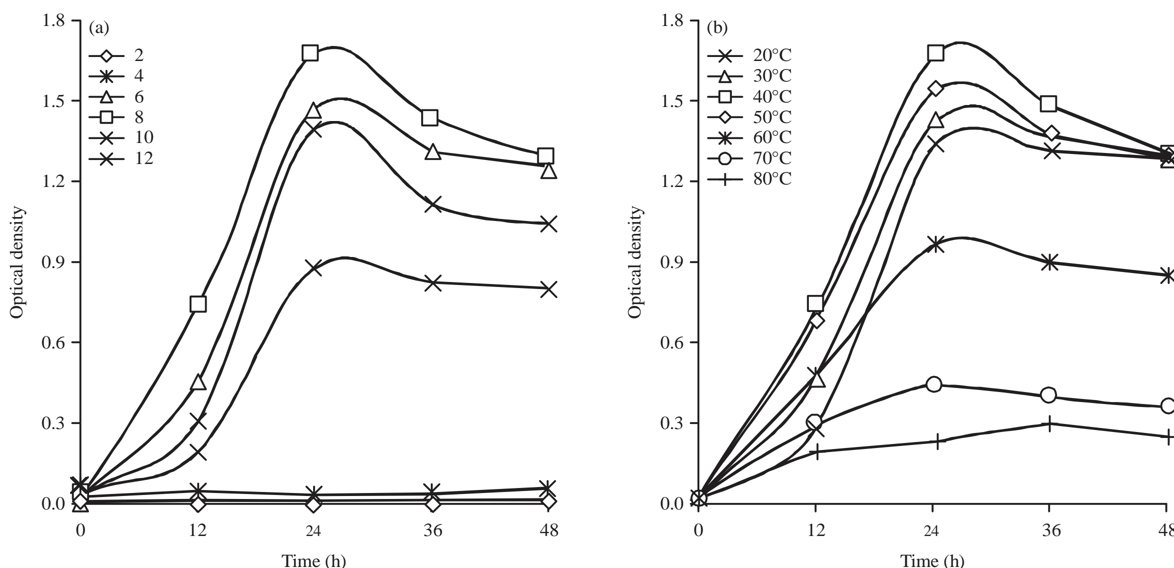

Fig. 3(a-b): Growth optimization of strain sm-sr14 at different physical parameters, (a) Various pH and (b) Various temperature

Optimization of enzyme activity: Amylase production in MS5 media was highly correlated with optimum growth conditions (40°C, pH 8) (Fig. 5). The amylase produced by this strain was also characterized from various aspects. This enzyme showed maximum activity within 30 min incubation with soluble starch at 50°C. However, pH 10-12 was found as optimum pH for this enzyme activity (Fig. 6).

Enzyme stability on pH, temperature and salt strength: The enzyme was found to retain its activity after incubating it for 2 h at up to 120°C (moist heat). It was also quite stable at 250°C (dry heat) temperature as retain significant activity (~60%) at that condition. It was also noted that its stability at even incubating at 5 M NaCl for 24 h. The enzyme was found to remain functionally active after incubation for 2 h at wide range of pH (6-12) conditions. However, enzyme activity was totally lost when incubation was done at pH 4 and 14 for 24 h (Fig. 7).

Effect of various effectors on enzyme activity: Table 3 shows that various influencing chemicals may enhance or depress the enzyme activity. The Mn^{+2} played a critical role

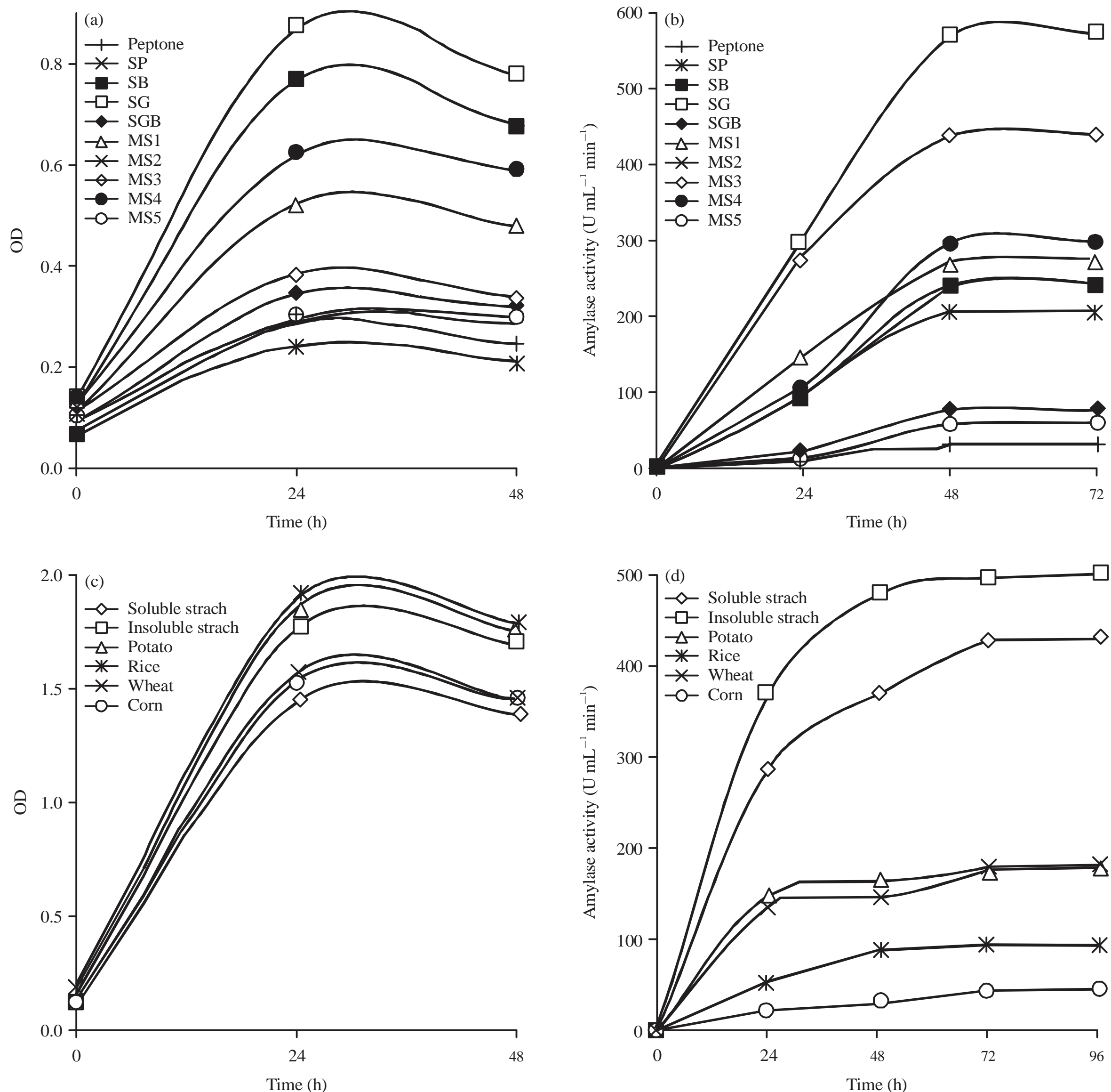

Fig. 4(a-d): Optimization for nutritional status of bacterial growth and amylase production (a, c) Bacterial growth in different media and different starches, respectively and (b, d) Amylase production in different media and different starches, respectively

as metal ion in enzyme activity. Enzyme activity was increased about 56% after incubating at 1 mM $MnSO_4$. However, Ca^{+2} and Ba^{+2} also showed significantly enhanced enzyme activity whereas Hg^{+2} (5 mM) depressed the enzyme activity by 45%. Glucose, lactose and glycerol lowered enzyme activity among which lactose almost repressed 94% activity. The detergents like SDS and tween 80 also were able to enhance enzyme activity at 1 mM final concentration.

Substrate specificity of enzyme: Studied on different substrate of sm-sr14 amylase revealed that insoluble starch was the most suitable and specific substrate among the others (Table 4). That enzyme was also showed activity in different solid substrates. From this study, the enzyme was found highly suitable to work on different types of substrates.

Gel zymogram study of α-amylase and its molecular weight determination by SDS-PAGE: Molecular mass of the enzyme was analysed by determining its position with respect to marker proteins on polyacrylamide gel (Fig. 8). The enzyme was located after comparing its in-gel substrate hydrolyzing ability and the mass was estimated about 81 kDa. The enzyme

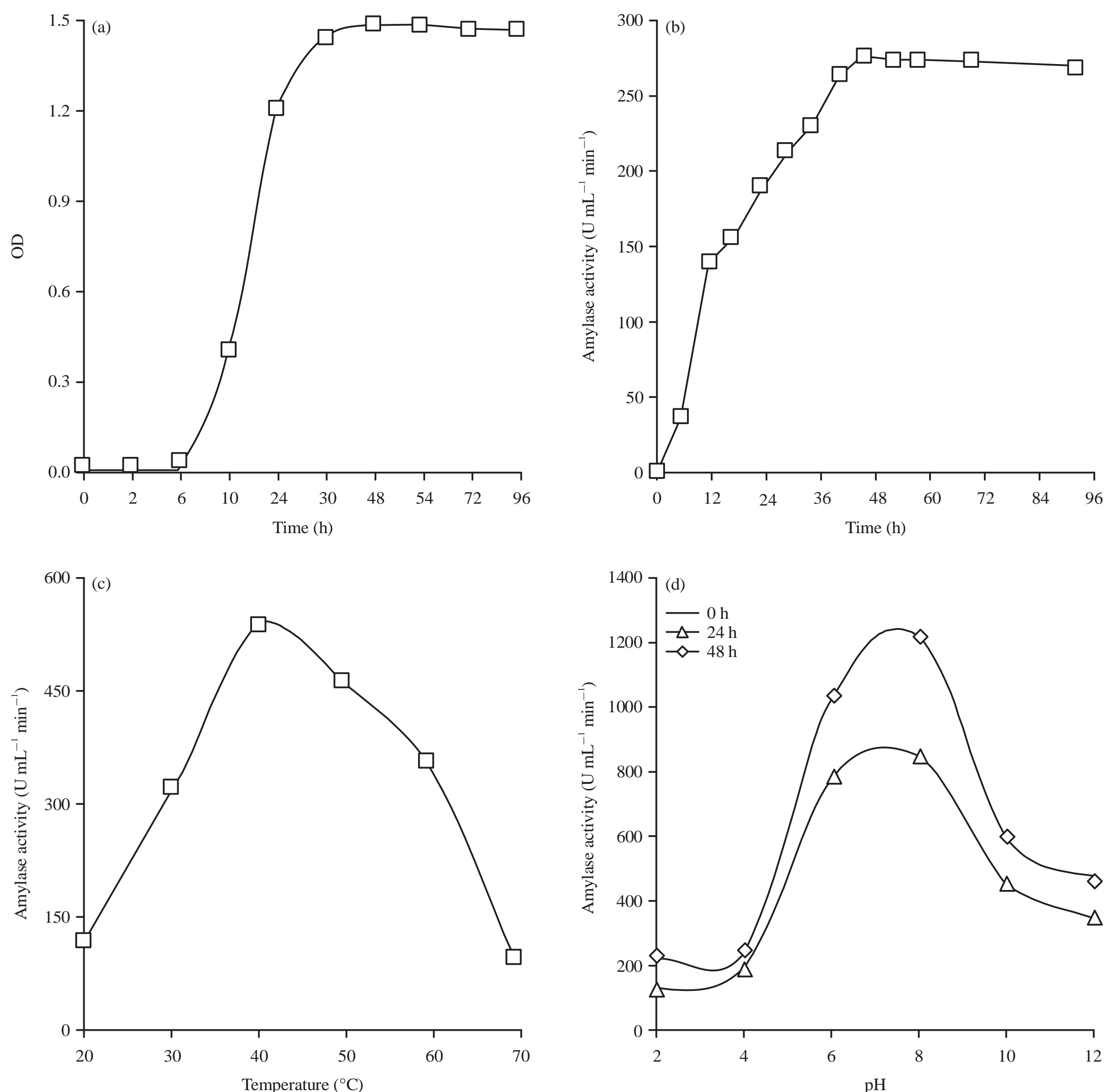

Fig. 5(a-d): Study of bacterial growth and characterization of amylase production, (a) Bacterial growth curve, (b) Amylase production at different time point of growth and (c-d) Optimum temperature and pH for amylase production

was found to retain its activity after incubating it for 2 h at up to 120°C (moist heat). It was also quite stable at 250°C (dry heat) temperature as retain significant activity (~60%) (Fig. 9). In left panel the enzyme activity center on gel band is noticed close to the region of ~80 kDa. The bands in lane 2, 3, 4 represented the amylase protein after treated with 100, 120 and 250°C for 2 h and lane 5, 6, 7 are the corresponding enzyme activities. Middle panel demonstrates the silver-stained enzyme protein band in lane 1, 2 (after 250°C treatment for 2 h). Lane 3, 4, 5 (triplicate) showed corresponding amylase activities in a starch gel with same amount of protein after similar treatment. Right panel picture demonstrate the enzyme activity assay from 250°C (2 h) treated BSA (lane 1), similarly treated purified commercial amylase (2), blank (3) and lane 4, 5, 6 and 7 are the present purified-amylase (5, 10, 20, 30 μL, respectively) after similar treatment. Taken together, Fig. 9 demonstrates a significant stability in the current purified-amylase up to 250°C temperature.

DISCUSSION

The presently studied sm-sr14 strain was fitted in the genus *Bacillus* after studying with different characterization

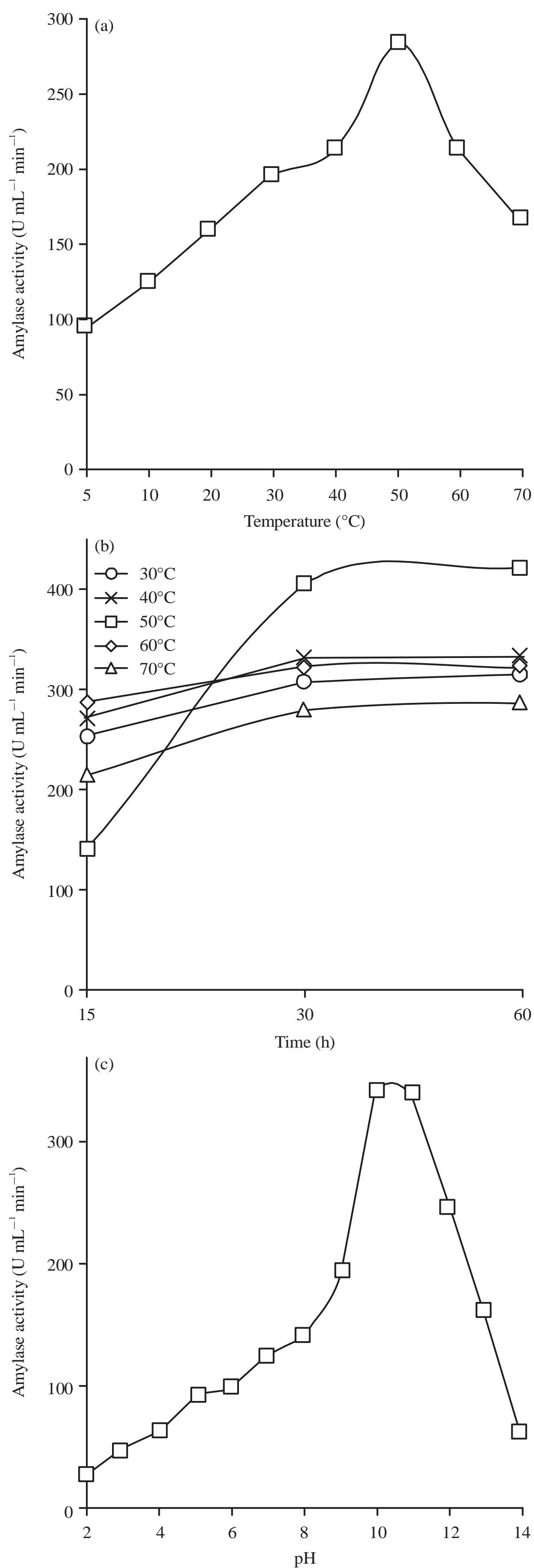

Fig. 6(a-c): Characterization of amylase activity, (a) Different temperature activity, (b) Optimum reaction time determination and (c) Different pH activity

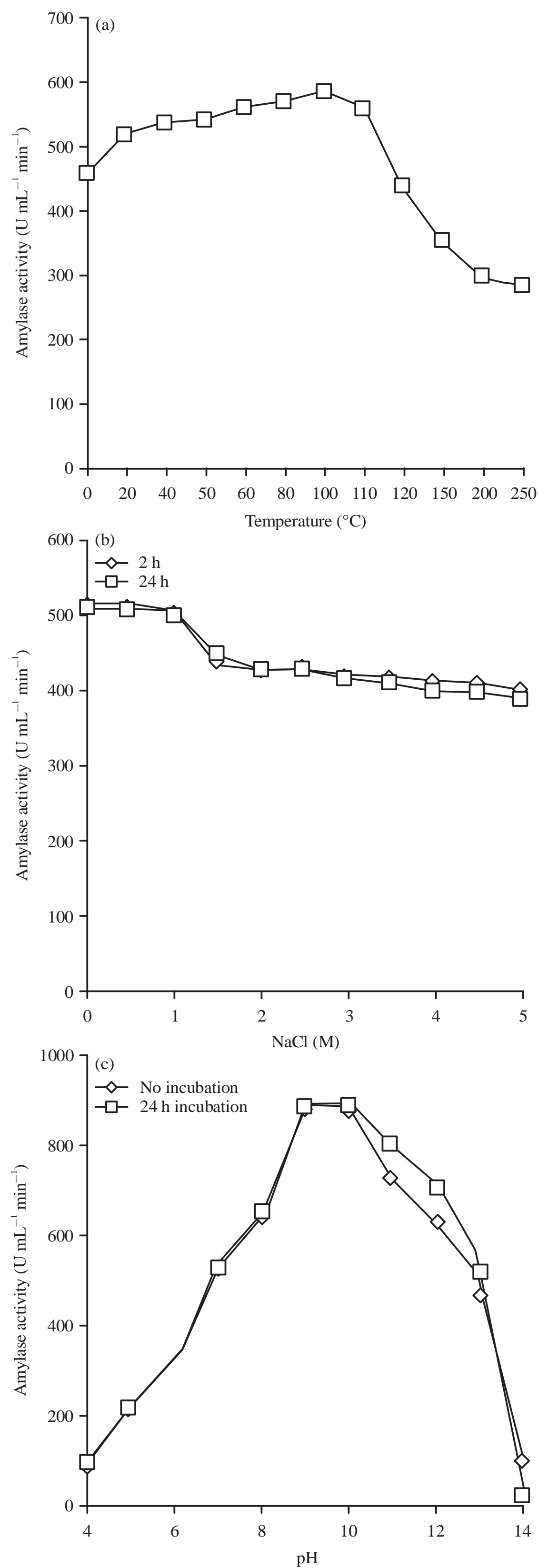

Fig. 7(a-c): Determination of enzyme stability, (a) Temperature, (b) Salt and (c) pH

Table 3: Various effectors on enzyme activity

Metal ions	Effectors	Concentration (mM)	Residual activity (%)
Crude (Untreated)	-	-	100
	$KMnO_4$	1	95
		3	94
		5	86
	$AgNO_3$	1	116
	KI	1	106
	$NaASO_2$	1	113
	$SnCl_2$	1	95
	$HgCl_2$	1	99
		3	71
		5	55
	$CuCl_2$	1	115
	Lead acetate	1	102
	$CdCl_2$	1	102
	$(NH_4)_2SO_4$	1	101
	$BaCl_2$	1	131
	MnO_2	1	139
	$MnSO_4$	1	156
	$CaCO_3$	1	144
	NaN_3	1	119
	$K_2Cr_2O_7$	1	99
		3	80
		5	71
Sugars	Glucose	1	24
	Lactose	1	6
Alcohol	Glycerol	1	56
	Methanol	1	123
Organic compound	Glycine	1	122
	Urea	6000	99
		8000	77
		10000	64
Organo sulfur	DMSO	1	136
Chelating agent	EDTA	1	100
		3	95
		5	88
Denaturing agent	β-mercapto	1	157
	Ethanol	3	117
		5	93
	SDS	1	128
Surfactant	Triton-X	1	101
	Tween-80	1	156

Table 4: Enzyme activity on different substrate

Substrate (5%)	Amylase activity (U mL^{-1} min^{-1})
Soluble starch	480
Insoluble starch	519
Potato	478
Rice	472
Wheat	465
Corn	398

approaches. Further, it was assigned as *Bacillus cereus* (GenBank accession-KM251578.1) in phylogenetic tree. Besides thermophilicity, the strain could be able to grown at alkaline pH (8-12) (Fig. 3) and moderate saline environment (0-3 M) which indicate its high biotechnological potential. Strain sm-sr14 was best grown in the presence of 1 M NaCl,

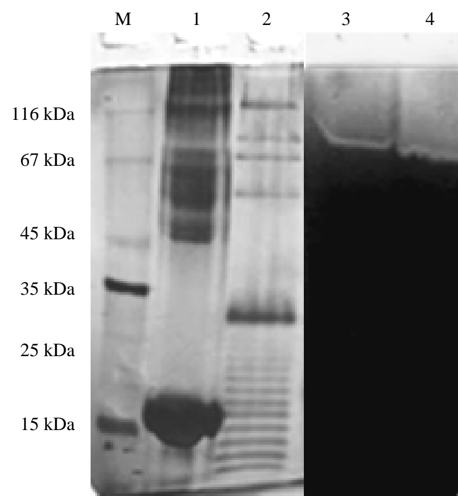

Fig. 8: PAGE analysis of bacterial extra cellular proteins and in gel localization of amylase within it

Lane M: Marker, lane 1: Bacterial cell lysate, Lane 2: Acetone precipitated lyophilized protein, Lane 3 and 4: Substrate hydrolysis in-gel

while growth was arrested in presence of 3 M NaCl in the media. The strain had the capability to utilize various sugars efficiently, so it could be cultivated using various cheap carbon sources. Differential antibiotic resistance property of this strain was also comparable to *Bacillus* sp. The TDSAS2-2CS-2010, *Bacillus cereus* ATCC 13061, *Bacillus cereus* 10876 and *Bacillus cereus* ATCC 53522[27-29]. But, this strain showed even a wider range of antibiotic resistance. Several *Bacillus* sp., were known to produce variety of extracellular enzymes and they had a varied range of industrial applications. Of these enzymes, amylases were of particular importance to the industry. The strain sm-sr14 was found to produce significant amylase in MS5 media (Table 1) with peptone and yeast extract. The optimum culture conditions for amylase production by sm-sr14 was growth associated; reaching to a maximum level around 48 h while production started at 12 h duration of incubation. The enzyme activity of sm-sr14 was found to be comparatively higher than that of obtained by Asoodeh *et al.*[30], Bezbaruah *et al.*[31], Kubrak *et al.*[32] and Asgher *et al.*[33]. Bacterial enzymes were of particular interest for their lower harvesting period. The mesophilic *B. licheniformis* and *B. amyloliquefaciens*, which were the main sources of commercial amylase at present, were reported to require a batch time of 48-72 h incubation for the optimum production of the enzyme protein[34,35]. The optimum temperature for production of amylase was found to be 40°C in the present study. Temperature may influence at gene expression level for amylase synthesis at the level of translation, thus might increases the stability, hence, the

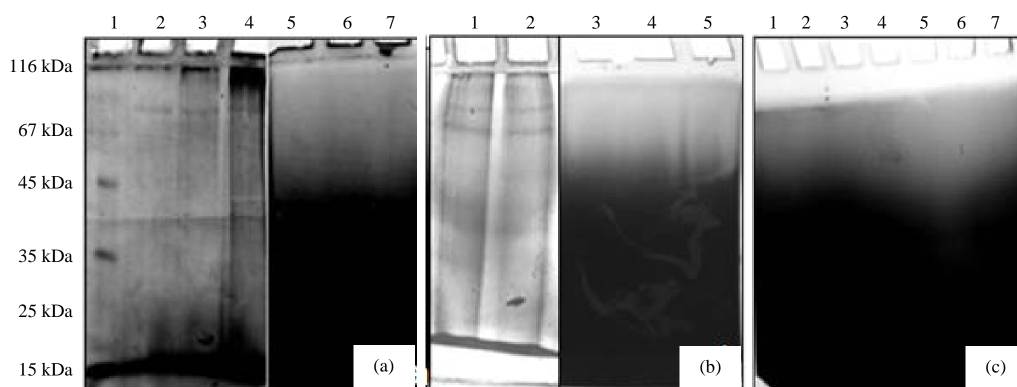

Fig. 9(a-c): In-gel thermostability determination of extra-cellular amylase produced by *Bacillus cereus* sm-sr14, (a) Lane 1: Marker Lane 2: 100°C treated enzyme, Lane 3: 120°C treated enzyme, Lane 4: 250 °C treated enzyme, Lane 5: 100°C treated enzyme, Lane 6: 120°C treated enzyme, Lane 7: 250°C treated enzyme, Lane 1-4: Silver stain, Lane 5-7: Zymogram, (b) Lane 1 and 2: 250°C treated enzyme, Lane 3-5: 250°C treated enzyme 30 µL, Lane 1-2: Silver stain, Lane 3-5: Zymogram and (c) Lane 1: 250°C treated BSA, Lane 2: 250°C purified commercial α-amylase, Lane 3: Blank, Lane 4: 250°C treated enzyme 5 µL, Lane 5: 250°C treated enzyme 10 µL, Lane 6: 250°C treated enzyme 20 µL, Lane 7: 250°C treated enzyme 30 µL, Lane 1-7: Zymogram analysis

amount of the protein. However, the elevated temperature may also increased the secretion of an extracellular protein; possibly by changing the physical integrity of the plasma membrane[12]. One report suggest that *Bacillus cereus* str. The α-amylase may be maximally active at 65°C, pH 8.0 and 89% of its activity was sustained even at pH 11[10]. The production rate of sm-sr14 amylase was also found to be most suitable in alkaline environment. The media pH was an important determinant in nutrient absorption and stimulation of enzyme production via signaling pathways with released of extracellular enzymes based on amylolytic mechanism of signal peptidases[36]. Soluble starch serveed as the best carbon source for maximum growth and enzyme production, a phenomenon that had been described for a number of amylase producing *Bacillus*[37-39]. Peptone (0.1%) was found to provide critical nitrogen level in the enzyme production-media in the current study. The released NH_4^+ ions from peptone stimulate the growth as well as increase the enzyme yield. Insoluble starches were preferable for commercial production of amylase due to its low cost and high availability. The enzyme was optimally active and stable at pH 10, though it remains active at a wider range of basic pH (8-13). The stability of the enzyme at alkaline pH indicate its amino acid composition such, that contain possibly more acidic amino acid that were exposed to the external medium and probability of Na^+ cycle that facilitates the solute uptake. A greater surface-occurrence of acidic amino acids was reported relating to protein stability and integrity[40]. Enzyme was thermostable and retained activity at higher temperature range (up to 120°C moist heat and 250°C dry heat) while very few strains of thermophilic *Bacillus* had been reported so far that produce highly thermostable amylases[41]. The present findings were clearly evident from the Fig. 9. Several repetition data suggested that this enzyme was very much stable against a very high moist as well as dry heat. The presence of higher number of nonpolar amino acids and hydrophobic core with interplaying week forces had been shown to confer protein thermostability in report[40]. The thermal stability of the enzyme and complete retention of activity over 110°C (even a 50% retention from an incubation at 200-250°C) provides its better industrial application potency like foods, pharmaceuticals, detergents and biofuels etc. Such thermo-tolerance can be explained by the presence of polyamines in the protein structure. However, increased number of hydrogen bonds, salt bridges and higher proportion of thermophilic amino acids may also contribute towards the thermal stability of the enzyme[40]. A previous *in-silico* study revealed that, apart from thermal stress, hydrophobicity and compact coiling-structure had been implicated as a protein stability determinant against other type of stress like fungal infection-associated damages[42]. The current data suggested that the present strain could be exploited in different harsh practical situation (higher temperature, higher pH and higher salt concentration) and application requirements become more demanding for industrial interests. Earlier studies had shown that amylases of different sources remain active at pH range of 5-10 and

temperature 30–60°C[38,43,44]. The combination of very high alkali and thermo-stability with some saline-resistance properties had equipped this enzyme more suited in critical reaction environment. Sugars were inhibiting to this enzyme activity probably by blocking the substrate binding site to form un-dissociated enzyme-product complex. The report on metals like Hg^{2+}, Pb^{2+} inhibition of microbial amylase and these findings support the present results. Here Hg^{2+} moderately inhibited the amylase activity[45,46]. Inhibitory activity found with Hg^{2+} on amylase activity might be due to the competition between the exogenous cations and the protein-associated cations, resulting in decreased metallo-enzyme activity[38]. The Mn^{2+} and Ca^{2+} ions augmented the enzyme activity possibly playing the co-factors of this enzyme. Most of the amylases were known to be metal ion-dependent (Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Fe^{2+}, etc.) enzymes[47,48]. The stabilizing effect of Ca^{2+} on thermo stability of the enzyme could be explained due to the salting out of hydrophobic residues in the protein by Ca^{2+}, thus ensuing the adoption of a compact structure[49]. The result demonstrated that Mn^{2+} and Ca^{2+} (1 mM) enhanced (50%) the enzyme activity. Such divalent ions stimulate the enzyme activity by forming a link bridge between enzyme and substrate combining with both and so holding the substrate and the active site of the enzyme at their proper location[50]. The α-amylase from sm-sr14 was active in the presence of EDTA at 1 mM concentration. But with higher concentration (3-5 mM), the enzyme was slightly (5-12%) inactivated suggesting its resistance towards the metal chelating agent. These iso-enzymes referred to metallo-amylases in which the activity of enzyme was dependent on the some kinds of ions. Thus, addition of chelating agents (3-5 mM) to the reaction mixture results in the formation of complexes with the ions in the active site, which cause certain inhibition of enzyme activity in the present study. It was found that mercaptoethanol (3 mM) that act as a reducing agent, enhanced enzyme activity. This result may suggest the absence of thiol groups at catalytic site of enzyme. In addition, enhanced enzyme activity may be attributed to the reduction in aggregate size by destroying the intermolecular disulfide linkages and protection of thiol groups that stabilize the three dimensional structure of enzyme[51]. This enzyme was found to retain its activity at even 6M urea where other α-amylases were found to be inactive[48]. In addition, urea was found to passively unfold proteins by decreasing the refolding rate of local parts of the protein[52]. This enzyme was found to remain active in the presence of detergents like SDS, tween 80 and triton X which enhanced utilization of potentiality of this enzyme at various commercial purposes like detergent and textiles industries. The reason may be that surface active agents might have increased the turnover number of amylase by increasing the contact frequency between the active site of the enzyme and the substrate by lowering the surface tension of the aqueous medium and with the maintenance of open form of amylase which may enhanced the activity. The observed stability of enzyme in polar or less polar system suggests the exposed active site at any environmental condition and thus maintains the flexible conformation over nonpolar to polar and non-aqueous to aqueous. As water was responsible to maintain the structural flexibility and mobility of the protein molecule, the organic solvent may cause deamidation of Asn and Gln residues and hydrolysis of the peptide bond, which ultimately lead to unfolding of enzyme molecules and loss of enzymatic activity[53]. The polyacrylamide gel electrophoresis showing the amylase activity band (in-gel assay or zymography) corresponds to the silver stained band and the protein marker[54-57]. This enzyme was calculated to have molecular weight 81 kDa that was similar to the result obtained by *B. subtilis*[44]. Lin *et al.*[38] reported an amylase with 42.8 kDa molecular mass from *Bacillus* sp. and opined that *Bacillus* sp., could produce five forms of amylases but in the present study, one protein band was detected having starch hydrolyzing ability. Beside high amylolytic activity, this strain also manifest moderate cellulolytic, lipolytic, gelatinase and nitrate reductase activities which may be exploited for different commercial purpose and environmental issues. The extra-cellular amylase of the current strain sm-sr14 showed unique properties of hyperthermostability, resistant to strong alkali, urea and detergent which might have great industrial application. Further studies are necessary for the elucidation of structural basis of this enzyme for its thermostability. That may help for the designing of engineered protein for adverse condition. Proper scaling up of this enzyme for industrial level may be the objective for its utilization.

CONCLUSION AND FUTURE RECOMMENDATIONS

Thermostable enzymes are very significant for various industrial applications. It reduces the time span of reaction and saves energy thus reduces production cost. The amylase produced by strain sm-sr14 showed high thermal stability that shows great biotechnological importance. In present study *Bacillus cereus* sm-sr14 produces 81 kDa amylase with thermophilic, alkaliphilic and thermostable property. However, further investigation is required to ascertain the molecular characteristics for recreated protein structure, kinetic properties and substrate specificity to access the true potential of this microbial amylase. The present enzyme can be directly used for agricultural purpose, alcohol production, feed processing and for environmental management.

SIGNIFICANCE STATEMENTS

In this study hyperthermostable (>150°C) and highly alkaline (pH 10) amylase-producing *Bacillus cereus* was isolated which is highly catalytic towards natural solid substrates. This amylase is important for basic research to study the molecular basis of hyper-thermostability in protein structure that can be recreated for commercial use in extreme environment. The present enzyme can be directly used for agricultural purpose, alcohol production, feed processing and for environmental management.

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