Characterization of Amylase from Industrially Important Thermophilic Microorganism: *Geobacillus thermoleovorans* Strain Rekadwadsis

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***Abstract*—The production of extracellular thermostable amylase by *Geobacillus* was detected on nutrient agar plates containing 2.0% soluble starch at 65 ^{o}C. Bacterium was produced 8, 578 U/mL amylase under SmF. Thermostable amylase (size 42 kDa) showed optimum temperature 68 ^{o}C at pH optima 7.5. *Geobacillus* amylase was stable at 90 ^{o}C temperature and retained its 85% initial activity. The thermostable amylase was retained stability and activity in the presence of various denaturing agents such as SDS, Triton X-100, Tween-60, Tween-80. Ca^{+2}, Cu^{+2} and Co^{+2} ions increased activity, while Na_2EDTA, Hg^{+2}, Zn^{+2}, Sn^{+2} showed inhibitory effects. The thermostable amylase was stable, compatible and works at 10 mg/mL concentration of laundry detergents *(Ariel, Ghari, Surf Excel, Wheel, Tide, Nirma, Rin and Henko)*. The K_m and V_{max} values were 2.702 mg/mL and 7692.3 mmol respectively.**

***Index Terms*—extremophiles, *Geobacillus*, amylase, ionic concentration, biocatalysts, thermostability**

I. Introduction

Thermophiles are the extraordinary microorganisms include number of phyla with increasing potential in biotechnology. These are stable to such high temperature only because they are structurally adapted and have thermostable biomolecules. They have several modifications in their structural components and biomolecules [1]. The biomolecules such as proteins, lipids, enzymes, ribosome, RNA and DNA have higher intrinsic stability [2]. Thermophiles and their products such as enzymes, proteins and bioactive compounds have several applications in industries [3] and biotechnological processes due to their thermostability at high temperature [4]. Various kinds of thermostable enzymes plays vital role in industrial processes. The biocatalytic, detergents, food, feed, starch, textile, leather, pulp and paper, and pharmaceutical industries are the major users of thermostable enzymes [4-5]. Thermostable amylolytic enzymes are used for the hydrolysis and modification of starch to produce glucose and fructose syrup and other products [6]. Amylolytic enzymes are also used in agricultural, textiles, paper, and baking industries [4], [7]. Among thermophilic prokaryotes, *Geobacillus* are widely used as producers of thermostable amylases due to increasing industrial attention [8]. In present study, thermostable extracellular amylolytic enzyme was produced by *Geobacillus* at laboratory scale. The thermostable enzyme was stable at high temperature and may have potential industrial applications. The present study reports isolation and identification of thermophilic *Geobacillus*, production, purification and characterization of thermostable amylase.

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II. Materials And Methods

A. Isolation, Identification of Bacterial Strain & Production of Thermostable Amylase

Thermophilic bacteria strain was isolated from Unkeshwar hot spring sediment, Nanded (India). Qualitative starch agar test was performed for preliminary screening for amylase production at 65 ^{o}C. Those isolate showed maximum production of thermostable amylase as selected for further study. The isolated species was tentatively identified on the basis of morphological, biochemical characteristics using Bergey's Manual of systematic bacteriology [9]. The identification of bacterium was confirmed by using 16S rRNA gene sequence analysis. For 16S rRNA analysis, extraction of DNA performed from stable enrichment cultures in nutrient medium and the isolation was done by the phenol chloroform method. The method was modified as follows: cell pellet of 2 mL from each enrichment culture of isolate was suspended in extraction buffer (100 mM Tris-HCl (pH 8.0), 100 mM Na_2EDTA (pH 8.0) Proteinase K (Nitrogen, USA) was added at the final concentration of 100 mg/mL and set was incubated at 55 ℃ for 2 h with continuous shaking. Then 0.5 M NaCl was added and set was incubated at 72 ℃ for 30 min. Subsequently, DNA was extracted by phenol: chloroform: isoamyl alcohol. DNA was washed twice with 70% ethanol and dissolved in Tris-EDTA buffer (pH 8.0). Set was analyzed by electrophoresis on a 0.8% agarose gel and visualized by ethidium bromide staining using UV trans-illuminator. The 16S rDNA of the enriched strains were amplified with a pair eubacteria specific of primers (forward primer 530 F: 5' GTGCCAGCAGCCGCGG 3' & reverse primer 1392 R: 5'ACGGGCGGTGTGTAC 3'. The PCR conditions used were an initial denaturation at

94 ℃ for two minutes, followed by 35 cycles of denaturation at 95 ℃ for one minute, annealing at 55 ℃ for one minute and extension at 72 ℃ for one minute then a final extension was given at 72 ℃ for 10 min. The amplified PCR product was a mixture of 16S rRNA genes from all the strains used for the amplification. The identity of the isolates was determined through a BLAST search [10]. The starch-yeast extract-tryptone broth (SYT broth) [Composition (g/L): soluble starch, 10; yeast extract, 3; tryptone, 3, K_2HPO_4, 1.2; KH_2PO_4, 0.2; $MgSO_4$, 0.02 and $CaCl_2$ 0.01; pH 6.0] was used for the production of thermostable amylase at 65 ^oC for 24 h in an orbital shaking incubator (at 120 rpm). After incubation broth was 15,000 rpm for 20 min at 4 ^oC. The clear supernatant used for further assay and purification [10].

B. Amylase Assay

The amylase activity was determined by using Bernfeld method [11]. Amounts of 1 mL amylase solution and 2 mL 0.5% starch solution in 0.1 M Tris–HCl buffer (pH 6.0) and 1 mL buffer solution were incubated for 30 min at 60 ℃. At the end of this duration, the reaction was stopped by addition of 2 mL DNS (3, 5-dinitro salicylic acid) and kept in boiling water for 5 min. Then, it was diluted by 10 mL distilled water, and spectrophotometric measurements were conducted at 489 nm. All the experiments were performed in triplicate and the standard error was expressed [12-13]. One unit of enzymatic activity was defined as the amount of enzyme that produced 1 μmol of reducing sugar as maltose per minute under the assay conditions.

C. Purification of Thermostable Amylase

The centrifuged clear supernatant was mixed with 70% ammonium sulphate to precipitate thermostable amylase. The precipitated amylase was collected by re-centrifugation at 15,000 rpm for 30 min 4 ^oC. The obtained precipitated enzyme was dissolved in 3 mL of phosphate buffer (0.1 M, pH 6.0). The mixture was added in pre-equilibrated Sephadex gel exclusion column (Sephadex G 80-120, 1.9 X 30 cm). The flow rate for enzyme elution was maintained at 0.5 mL/min using phosphate buffer. The collected active fractions were mixed and loaded on pre-equilibrated (with 10 mM Tris-HCl pH 7.8) anion exchanger. Protein was eluted using 50, 100, 150, 200, 250, and 300 mM NaCl concentrations and collected. The collected active fractions were combined and used in further experiments as the purified enzyme [14].

D. Denaturing SDS-PAGE

SDS-PAGE experiment was performed for the purified thermostable enzyme as per standard protocol given by Laemmli [15].

E. Effect of Temperature and pH on Amylase Stability and Optimization of Parameters

The effect of temperature ranges from 30, 40, 50, 55, 60, 65, 70, 75, 80, 90 and 100 ^oC and pH of 4, 4.5, 5 (0.02 M acetate buffer), 6, 6.5, 7, 7.5, 8, 8.5 (0.02 M phosphate buffer), and 9, 9.5 (0.02M glycine-HCl buffer) on amylase activity was determined [14]. The thermostability of amylase was determined by incubating at 60-90 ^oC for one hour in 0.02 M potassium phosphate buffer (pH 7.5). Then, this incubated enzyme was used for measuring its activity [16].

F. Effect of SDS, denaturing agents, chelating agents and metal ions on activity of amylase

The effect of SDS (Sodium dedeocyl sulphate) at 0.5% and 1%; Triton X-100, Tween-60, Tween-80 at 0.2%, 0.4%, 0.6%; disodium EDTA at 1mM, 2mM, 5mM and 10mM; and heavy metal ions Ca^{+2}, Cu^{+2},Co^{+2}, Hg^{+2}, Zn^{+2}, Sn^{+2} at 1 mM concentrations on activity of amylase activity was determined by pre-incubating with enzyme for one hour [10].

G. Effect of Commercial Laundry Detergents on Activity of Amylase

The compatibility of *Bacillus* sp. strain EF_TYK1-5 amylase with local laundry detergents was studied. Detergents used were *Ariel* (Procter and Gamble, India), *Ghadi* (Rohit Surfactants Pvt. Ltd., India), *Surf Excel* (Hindustan Lever Ltd., India), *Wheel* (Hindustan Lever Ltd., India), *Tide* (Procter and Gamble, India), *Nirma* (Nirma Ltd., India), *Rin*, *Henko* (Hindustan Lever Ltd., India) and *Henko*. Detergents were dissolved in distilled water (1 % w/v) and boiled for 1 minute to denature native enzymes present in the solution. Then it was filtered. The thermostable amylase was pre-incubated with detergents solution (1:1) for 30 minute at 60 oC, and the effect on enzyme activity was determined. The residual activity of amylase was compared with control sample incubated under same conditions (without any detergent). The amylase activity of control sample was taken as 100% [10, 17].

H. Enzyme Kinetics

Substrate concentration is one of the most fundamental factors affecting enzyme activity. The K_m and V_{max} values were determined by varying the substrate concentration from 0.2 to 2.0% [17]. The effect of substrate concentration on activity is expressed in K_m and V_{max} values using Lineweaver-Burk plot [18].

III. Results And Discussion

A. Isolation, Identification of Amylase Producer, Production, Purification of Thermostable Amylase

Thermophilic bacteria capable of amylase production were screened on starch agar qualitatively by incubating at 65 ^oC and isolated by replica plate method. Total six isolates were isolated. Those isolate showing maximum production of amylase at 65 ^oC was selected for further study. In this study, the selected thermophilic species was identified tentatively as *Geobacillus thermoleovorens* and was used for thermostable amylase production. Identification of isolate further confirmed by using 16S rRNA gene sequence analysis and deposited in NCBI repository under the accession number KP053645.

Geobacillus have produced extracellular thermostable amylase (8, 578 U/mL) under SmF after 24 h incubation at 65 °C temperature. Similar study was performed by Rekadwad [17].

B. SDS-PAGE

The fractions collected were analyzed by SDSPAGE. The thermostable *Geobacillus* amylase appeared as a single band with an apparent molecular mass of 42 kDa. Annamalai *et. al.*, recorded similar type of results [19].

C. Effect of Temperature and pH on Enzyme Activity and Stability

The thermostable amylase showed activity and stability at varied ranges (40 and 90 °C) and of temperatures and pH (5.5 to 9.0) with an optimum activity at 68 °C and at pH 7.5 (Fig. 1 and 2). The thermostable enzyme was stable at 90 °C temperature and retained its 85% initial activity. *Bacillus* sp. amylase isolated from Unkeshwar hot spring showed optimum activity at 60 °C temperature and pH 7.0 [10].

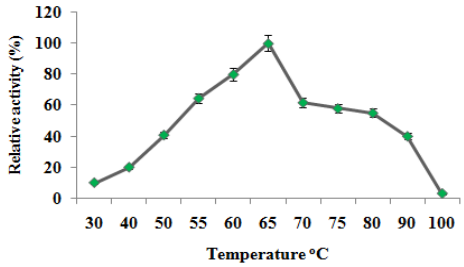

Figure 1. Effect of temperature on thermostable amylase activity and stability.

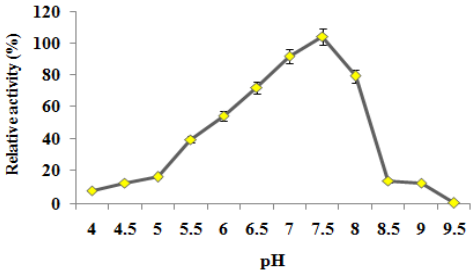

Figure 2. Effect of pH on amylase thermostable activity and stability.

D. Effect of SDS, Denaturing Agents, Chelating Agents and Heavy Metal Ions

The thermostable amylase reported showed stability and activity in the presence of SDS, denaturing agents, chelating agents and heavy metal ions. The Geobacillus amylase retained 94, 90, 83%, 80%, 75, 60 and 60% activity in the presence of 1 mM Disodium EDTA, 2 mM Disodium EDTA, 0.2% Triton X-100, 0.5% SDS, 5 mM Disodium EDTA, 10 mM Disodium EDTA, 0.4% Triton X-100, 1% SDS and 0.6% Triton X-100 respectively (Fig. 3). The activity of thermostable amylase was increased by Ca^{+2}, Cu^{+2} and Co^{+2} ions, while Na_2EDTA, Hg^{+2}, Zn^{+2}, Sn^{+2} showed inhibitory effects on amylase activity (Fig. 3 and 4). Various research groups worldwide reported similar type of results [16], [20-21].

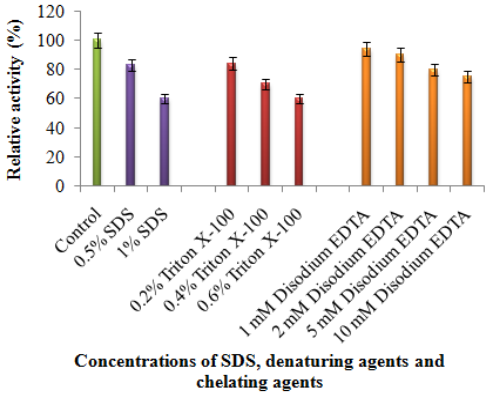

Figure 3. Effect of SDS, denaturing agents and chelating agent on thermostable amylase activity and stability.

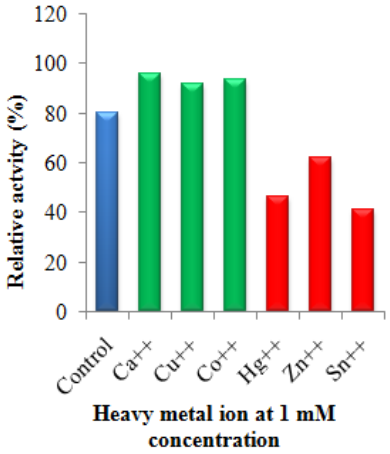

Figure 4. Effect of heavy metal ions (at 1 mM concentration) on thermostable amylase activity and stability.

E. Compatibility and Stability of Thermostable Amylase in the Presence of Commercial Laundry Detergents

The thermostable amylase showed extreme stability in the presence of commercial local laundry detergents such as *Ariel (85%), Ghari (79%), Surf Excel (82%), Wheel (75%), Tide (77%), Nirma (70%), Rin (76%) and Henko (78%)* at high (65 °C) temperature (Fig. 5). Such enzyme gives excellent performance at ordinary washing conditions in combination with detergents. Similar results were recorded by other research groups [10], [14].

F. Enzyme kinetics

The effect of concentration of substrate ranges from 0.2 to 2.0% was studied. The enzyme activity increased with an increase in starch concentration from 200 to 800 µg/mL, but further increase in concentration of starch have produced no significant increase of enzyme activity. The observed K_m and V_{max} values for *Geobacillus* amylase were 2.702 mg/mL and 7692.3 mmol respectively (Fig. 6). Similar studies were performed using *Bacillus* sp. [10], [22].

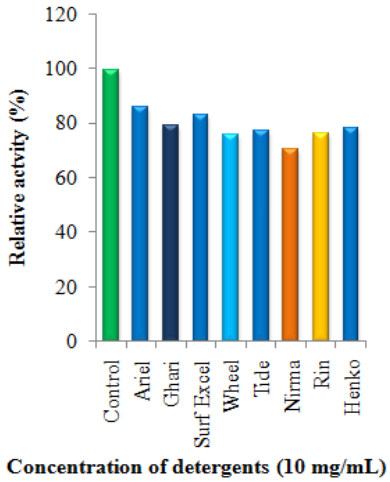

Figure 5. Effect of commercial laundry detergents on thermostable amylase activity and stability.

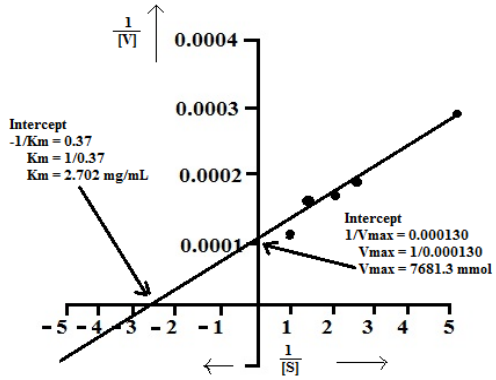

Figure 6. Effect of substrate concentration on thermostable amylase activity and determination of K_m and V_{max} from Lineweaver-Burk plot.

IV. Conclusions

From the results of this study, it can be concluded that isolated thermophile is producing high quantity of thermostable amylase having industrial importance showed high activity and thermostability in presence of various harmful chemicals and commercial local laundry detergents. *Geobacillus thermoleovorans* and *Geobacillus* like thermophilic microorganisms may be commercialized after optimizing conditions for enzyme production.

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