Characterization of Extracellular Thermophilic Amylase from Geobacillus sp. Isolated from Tattapani Hot Spring of Himachal Pradesh, India

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Abstract: Geobacillus kaustophilus PW11, Geobacillus thermoleovorans PW13 and Geobacillus toebii PS4 were isolated form Tattapani hotspring of Himachal Pradesh, India and characterized for extracellular amylase activity. All the three Geobacillus spp. exhibited thermophilic amylase activity, which was predominantly extracellular. The activity was optimum at 90°C and pH 7.0. Interestingly, no amylase activity was observed at temperature less than 40°C, indicating its thermophilic nature. Moreover, pre incubation of enzyme at 100°C enhanced the amylase activity. The specific activity of amylase of PW11, PW13 and PS4 was 3898, 3597 and 3289 U mg⁻¹, respectively at 90°C and pH 7.0. Starch, amylopectin and dextrin were observed to be the best substrates for amylase activity. Further, amylase activity of PW13 and PS4 remained unaffected in the presence of Triton X 100 (0.5%), but decreased by 40% in case of PW11 isolate. Among the metal ions tested, Mn²⁺, Co²⁺ and Fe²⁺ enhanced the amylase activity of PW11, PW13 and PS4 by 2-5 folds. While Hg²⁺ (1mM) strongly inhibited enzyme activity of PW13 and PS4, Cd²⁺ completely inhibited amylase activity of PW11. Amylase activity of PW13 and PS4 was enhanced by 2-2.5 folds in the presence of EDTA (5 mM). The thermophilic amylase activities of PW11, PW13 and PS4 are highly advantageous for downstream industrial applications.

Keywords: Thermophilic, amylase, extracellular, geobacillus, tattapani, thermophiles, hotspring.

1. INTRODUCTION

Thermophiles are extremophiles that require high temperature for growth. The hydrolytic enzymes (lipases, amylases, proteases, chitinases, xylanases and pullulanases) produced by thermophiles are known as thermostoys, and mostly resistant to harsh conditions. The advantages of using thermostable enzymes in industrial processes include the decreased risk of contamination, reduced cost of external cooling, increased solubility of many polymeric substrates, and decrease in viscosity, improved transfer rates and consequently increased reaction rates.

The amylases (EC 3.2.1.1, 1.4-α-D-glucanohydrolase) hydrolyze long-chain carbohydrates by acting at random locations along the starch chain, ultimately yielding maltotriose and maltose from amyloose, or maltose, glucose and "limit dextrin" from amylpectin. Since the industrial processes of starch liquefaction and saccharification require high temperature, intensive research has focused on the isolation of thermophilic microorganisms that produce thermally stable enzymes [1]. Moreover, extracellular enzymes are cost effective and easy to purify as compared to intracellular enzymes. The stability and specificity of α-amylases to temperature, pH, substrates, and other harsh conditions remain a crucial factor in exploiting this enzyme in different fields such as food, feed, detergents, textile, paper, pulp, chemical, pharmaceutical industries and bioethanol production. Therefore, the search for novel and specific starch degrading enzymes is imminent. Thermococcale α-amylases are generally active in a broad temperature range, between 40-140°C [2], whereas amylase produced by Pyrococcus strain DSM3638 showed maximum activity at 100°C [3]. Extracellular α-amylase from Pyrococcus furiosus [4] and P. woesei [5] has also been reported. A hyperthermostable α-amylase was reported in Geobacillus sp. [6] and Bacillus thermoleovorans [7-8]. The thermostable amylases from B. licheniformis, Thermococcus litoralis and Sulfolobus solfataricus have been reported [9-11]. Many hot springs of India such as Tarabalo (Odisha), Bakreshwar (West Bengal), Soldhar (Uttar Pradesh) Suryakund (Jharkhand), and Manikaran (Himachal Pradesh), have been explored for the isolation of thermophilic microorganisms and their enzymes [12-16]. Tattapani hot spring is one of the volcanic regions of Himachal Pradesh, India, situated in the snowy mountains of Himalayas. However, the thermophilic microbes of Tattapani hot spring and their enzymes have not been reported till date. Considering the wealth of amazing biodiversity of thermophiles, the present study was undertaken to determine amylolytic characteristics of thermophilic bacteria isolated from water and soil sediment of Tattapani hot spring. In the present study, thermophilic Geobacillus species of Tattapani hot spring were screened and characterized for extracellular and thermophilic amylase activity.

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2. MATERIALS AND METHODS

2.1. Thermophilic Bacterial Strains

The thermophilic bacteria used in this study were isolated from water and soil sediments of Tattapani hot spring, Himachal Pradesh, India, and reported elsewhere (Sharma et al., under review). The optimum temperature and pH for growth of these organisms was 90°C and pH 7.0. The bacterial isolates were identified by 16S rDNA analysis using 27 F (5′-AGAGTTTGTATCTGGCTC-3′) and 1492 R (5′-ACCTGGTTACGACTT-3′) primers. 16S rDNA showed that bacterial isolates belong to genus *Geobacillus*, and the sequences have been deposited in NCBI GenBank with accession numbers KF751758 (*Geobacillus kaustrophilus* strain PW11), KF751757 (*Geobacillus thermoleovorans* strain PW13) and KF751759 (*Geobacillus toebii* strain PS4).

2.2. Characterization of α-Amylase from Thermophilic Bacterial Isolates

The thermophilic bacterial isolates PW11, PW13 and PS4 were cultured in LB liquid medium. Thermophilic bacterial isolates were grown at 90°C and as a control, *E. coli* strain DH5α (laboratory strain) at 37°C for 24 h with shaking at 200 rpm. Equal number of cells (3 μl cultures normalized to A<sub>600</sub> = 0.1) were spotted on LB agar medium supplemented with 1% starch to qualitatively screen amylase activity. As LB agar medium is not stable at 90°C, the plates were incubated at 65°C for 24 h. The starch agar plates were flooded with 1% Gram’s iodine solution. The appearance of clear zone around the bacterial growth indicated the utilization of starch, thus indicative of amylase activity. Quantitatively, amylase activity was measured by DNS method [17]. Cells were separated by centrifugation at 5000 rpm for 5 min at 4°C. Cell free spent medium was used as extracellular source, whereas whole cell extract (prepared by ultra-sonication, followed by centrifugation) was used as intracellular source of enzyme. Assay reaction contained 10 μl of 1% starch prepared in 0.1M potassium phosphate buffer, and 10 μg of total protein (enzyme). Total proteins present in the cell free spent medium and whole cell extracts were quantified by Bradford Method [18]. One unit of amylase activity is defined as the amount of enzyme required to release one micromole of glucose or maltose in one minute under defined assay conditions. Specific activity is defined as units of enzyme activity per milligram of proteins.

**Determination of optimal pH, temperature, thermal stability and preference of substrates for amylase activity**: Quantitatively, the optimal pH and temperature for amylase activity was determined by incubating the assay reaction at different temperatures ranging from 40-100°C and by adjusting the pH 5 and 6 by using citrate-phosphate buffer, pH 7 using phosphate buffer and pH 8 and 9 using Tris-HCl.

Thermal stability of amylase was studied by pre-incubating the cell free spent medium at 100 °C in 0.1M potassium phosphate buffer, pH 7. The amylase activity was determined at regular interval of 1 h, for 6 h. To evaluate the preference of substrate for amylase activity, 10 μl of 1% dextran, dextrin, maltose, amylopectin, glycogen, amyhum, starch or amylase were used as substrate in amylase assay.

**Effect of chaotropic agents, metal ions and solvents on amylase activity**: In order to test the effect of chaotropic agents, metal ions and solvents on amylase activity, enzyme preparation was pre-incubated with different concentrations (1, 5 and 10 mM) of inhibitors like phenyl methyl sulfonyl fluoride (PMSF) and ethylene diamine tetra acetic acid (EDTA); salts of metal ions (Ni<sup>2+</sup>, Ca<sup>2+</sup>, Mn<sup>2+</sup>, Mg<sup>2+</sup>, Zn<sup>2+</sup>, Co<sup>2+</sup>, Cu<sup>2+</sup>, Hg<sup>2+</sup>, Fe<sup>2+</sup> and Cd<sup>2+</sup>) and detergents (0.5 and 1%) as such as sodium dodecyl sulfate (SDS) and triton X-100. Various solvents such as ethanol, phenol, n-butanol, cyclohexane, hydrogen peroxide, pyridine and toluene (0.5 and 1% v/v) were assessed for the effect on amylase activity. The relative activity was calculated as compared to the amylase assay without adding inhibitors, metal ions and detergents. The amylase enzyme activity was calculated as units of amylase activity per milligram of proteins.

3. RESULTS

3.1. Thermophilic *Geobacillus* species PW11, PW13 and PS4 Produce Extracellular Amylase

To examine the thermophilic bacterial isolates for amylase production, the starch agar plate spotted with equal number of bacterial cells were flooded with Gram’s iodine. A clear zone of diameter of 24, 22 and 21 mm was observed for PW11, PW13 and PS4 isolates respectively, thus indicating the production of amylase by the three thermophilic isolates (Fig 1A). Quantitative amylase assays revealed that the amylase activity of all the three isolates was predominantly extracellular (data not shown).

To study the effect of temperature and pH on the amylase activity, enzyme assays were performed at temperatures ranging from 40-100°C (Fig. 1B). In an independent experiment, effect of different pH ranging from 5-10 was studied. Thermophilic bacterial isolates PW11, PW13 and PS4 showed amylase activity in a pH range of 5.8 (Fig. 1C) and temperature between 50-100°C (Fig. 1B). Interestingly, the amylase activity of all the thermophilic bacterial isolates increased with every 10°C rise in temperature (Fig. 1B). There was only a marginal (13%) decrease in the amylase activity at 100°C, and no activity was observed at temperature less than 40°C (Fig. 1B), indicating that the amylase activity of PW11, PW13 and PS4 is thermophilic in nature.

3.2. Amylase Activity is Enhanced by Pre-Incubation at 100°C

Thermophilic enzymes are activated by elevated temperatures [1]. To study the thermostability of amylase, enzyme preparation was pre-incubated at 100°C, and amylase activity was determined at regular intervals of 1 h, for 6 h using starch as a substrate. The amylolytic activity was increased by 182, 178 and 128 U mg<sup>-1</sup> for PW13, PW11, and PS4 isolates respectively, with increase in incubation time within the first 2 h (Fig. 1D). The amylase activity was stable up to 4 h at 100°C for all the three isolates. After that, there was a 20-30% decrease in amylase activity (Fig. 1D).
Fig. (1). Comparative characterization of α-amylase activity of thermophilic Geobacillus species. Qualitatively, α- amylase activity was tested by spotting equal number of cells of thermophilic bacterial isolates and E. coli strain (DH5α) on LB agar medium supplemented with 1% starch. The plates were incubated at 65°C for 24 h and flooded with Gram’s iodine and observed for the zone of clearance (A). Quantitatively, effect of temperature (B), and pH (C) on amylase activity was studied by incubating the assay reactions at temperature ranging from 40-100°C and pH 5-10. Thermostability of amylase was performed by pre incubating the cell free spent medium at 100°C for different time intervals and measuring the amylase activity at 100°C (D). Amylase assays were performed in the presence of different substrates (starch, dextran, dextrin, maltose, amylopectin, glycogen, amylum) (E). Specific amylase activity (U mg⁻¹) of three independent experiments was plotted with standard deviation as indicated.
Interestingly, amylase activity was stable even at 120°C for 3 h, following decrease in enzyme activity for PW11, PW13, and PS4 respectively (data not shown). Thus, amylase activity of PW11, PW13 and PS4 is activated by high temperatures.

3.3. Amylase Activity of was Enhanced in the Presence of Mn²⁺, Co²⁺ and Fe²⁺

Metal ions exhibit varied effect on enzyme activity depending on the mechanism of enzyme catalysis. Therefore, the effect of various metal ions on amylase activity of PW11, PW13 and PS4 was studied. Amylase activity of PW13 and PS4 was increased by 2-5 folds in the presence of Ni²⁺, Mn²⁺, Co²⁺ and Fe²⁺ (Fig. 2A-C). The amylase activity of PW11, PW13 and PS4 was enhanced by 2.8, 3.1 and 1.3 folds, respectively in the presence of Mn²⁺ (10 mM) (Fig. 2A-C). Amylase activity of PW11, PW13 and PS4 was increased by 1.8, 3.4, 3.2 folds in the presence of 10 mM Co²⁺ and 2.4, 2.8, 3.9 folds in the presence of 10 mM Fe²⁺ respectively. No significant effect of other metal ions (Mg²⁺, Cu²⁺ and Ca²⁺) was observed on amylase activity. Mercury

![Fig. (2). Effect of metal ions on the amylase activity of thermophilic microbial isolates.](image-url)

Reaction mixture was supplemented with salts of metal ions (Ni²⁺, Ca²⁺, Mn²⁺, Mg²⁺, Zn²⁺, Co²⁺, Cu²⁺, Hg²⁺, Fe²⁺, Cd²⁺) and enzyme assay was carried out at 90°C, pH 7 for 30 min. The relative amylase activity was plotted against the various metal ions and their concentration as indicated for PW11 (A), PW13 (B) and PS4 (C). Reaction without supplementation of metal ions was served as control and considered as 100% amylase activity. Data of three independent experiments was plotted with standard deviation.
chloride (1mM) inhibited the amylase activity by 90 and 55% for PW13 and PS4 isolates respectively. Amylase activity of PW11 was inhibited by 92% in the presence of Cd²⁺. The extracellular amylase activity of all the bacterial isolates was independent of Ca²⁺ ions.

3.4. Amylase Activity of PW11, PW13 and PS4 was Stable in the Presence of Organic Solvents and Chaotropic Agents

The amylase activity of PW11 isolate was not inhibited in the presence of toluene (0.5%), whereas it was marginally reduced to 92.8 ± 4.9 in the presence of ethanol (0.5%). In the presence of butanol (0.5%) or cyclohexane (0.5%), amylase activity was reduced to 75 and 71% respectively (Fig. 3A). With increase in concentration of solvents (0-1%), the relative amylase activity of PW11 was decreased by 30-70% in the presence of cyclohexane, n-butanol and ethanol. The amylase activity of PW13 and PS4 was unaffected in the presence of cyclohexane and n-butanol (0.5%). The amylase activity of PW13 was enhanced by 125% ± 2 in the presence of 0.5% ethanol and 110% ± 4.6 in the presence of 0.5% cyclohexane (Fig. 3B). Amylase activity of PS4 was increased in the presence of 0.5% n-butanol (118% ± 2) or 0.5% toluene (111% ± 3.2) (Fig. 3C). Presence of phenol and H₂O₂ significantly inhibited amylase activity of all three thermophilic bacterial isolates (Fig. 3A-C).

Among the detergents tested, SDS (0.1%) had inhibitory effect on amylase activity of PW11 and 44 ± 3.3% amylase activity was retained. The amylase activity of PW11 was decreased from 40-70% with increase in concentration of Triton X-100 (0.1-1%) (Fig. 4A). In contrast, amylase activity of PW13 and PS4 remained unaffected in the presence of SDS or Triton X-100 (0.1%) (Fig. 4B, C).

Amylase activity of PW11, PW13 and PS4 was completely inhibited even in the presence of low concentration of PMSF (1mM). Amylase activity of PW11 was decreased by 10-30% with increase in concentration of EDTA (1-10 mM). There was 2-2.5 folds increase in amylase activity of PW13 and PS4 in the presence of EDTA (5mM) (Fig. 4D-F).

4. DISCUSSION

Thermophilic bacterial isolates PW11, PW13 and PS4 showed maximum amylase activity at pH 7 and 90°C. α-amylose of PW11, PW13 and PS4 was stable at 100°C for 4 h and 10% decrease in activity was observed after 4 h of incubation. Similarly, α-amylose activity of thermophilic Bacillus sp strain SMIA-2 (isolated from a soil sample of Campos dos Goytacazes City, Rio de Janeiro, Brazil) showed optimum activity at pH 7.5 and 70°C. The amylase activity was stable for 2 h at 50°C and there was 4, 13 and 38% decrease in amylase activity at 60, 70 and 90°C respectively [19].

Geobacillus sp. NMS 2 isolated from soil samples of a hot water spring of Sri Lanka showed optimum extracellular amylase activity at 50°C and pH 6.9 [20]. The amylase activity of Geobacillus species isolated in the current study was enhanced by 2-5 folds in the presence of Mn²⁺, Co²⁺, Fe²⁺ and Ni³⁺. The amylase activity of PW13 and PS4 isolate was inhibited (90 and 55%) respectively by Hg²⁺ (1mM), whereas no loss of amylase activity was observed in the presence of Hg²⁺ (5mM) for PW11 isolate. Moreover, the amylase activity of PS4 was marginally inhibited (<10%) in the presence of Cd²⁺. It was reported that the amylase activity of Geobacillus sp. LHB was increased by 0.2-1.0 folds in the presence of Mn²⁺, Ca²⁺, Cr³⁺ and Al³⁺, while it was reduced by 0.2-0.6 folds in the presence of Mg²⁺, Ba²⁺, Ni²⁺, Zn²⁺, Fe³⁺, Cu²⁺ and EDTA [21].

The amylase activity of Geobacillus stearothermophilus was activated by Ca²⁺, Mn²⁺, and Triton X-100, but strongly inhibited by Cu²⁺, Zn²⁺, Fe²⁺, and Hg²⁺ [22]. α-amyloses from B. licheniformis and B. lichenoliquefaciens were used in the liquefaction of starch, which require Ca²⁺ for their stability and/or activity [4-5]. α-amylose from PW11, PW13 and PS4 was independent to Ca²⁺ requirement and more suitable for saccharification and liquefaction of starch.

The amylase activity of PW11, PW13 and PS4 was stable in the presence of Triton X 100 (0.1%), EDTA (1 mM), and organic solvents such as ethanol, cyclohexane, toluene and n-butanol (0.5%). Amylase activity was reduced by 20-70% in the presence of phenol (0.5%). It was reported that the extracellular amylase activity of Bacillus tequilensis RG-01 was increased to 232, 160, 168.8, 120, 170.2, 133, 144, and 120% in the presence of n-dodecane, isooctane, n-decane, xylene, toluene, n-hexane, n-butanol, and cyclohexane respectively [23]. In the presence of benzene, methanol and ethanol, the amylase activity was inhibited by 72, 93.3, and 78.3%, respectively, as compared to un-supplemented control. Amylase activity of Bacillus tequilensis RG-01 was inhibited by 5% in the presence of Triton-X-100. On the other hand, addition of SDS, Tween-40, Tween-60, and Tween-80 (1%) was found to enhance the amylase activity by 20, 10, 15, and 9% respectively. A stimulatory effect of SDS (6%) on amylase activity of Bacillus sp. strain TS-23 was also reported [24]. The extracellular amylase activity of Anoxybacillus sp. IB-A (isolated from hot spring of Bakreswar, India) was enhanced in the presence of EDTA (112.81%), SDS (110.39%), and Triton X100 (121.6%) [25].

On the other hand, amylase activity of Amy-K38 was not affected in the presence of EDTA (1 mM) [26], but α-amylose from Thermus filiformis Ork A2 was inhibited by 12% in the presence of 10 mM EDTA [27]. α-amylose of A3-15 isolate was inhibited by 5% in the presence of 5 mM EDTA or 3 mM PMSF.

CONCLUSION

In conclusion, extracellular α-amylose produced by Geobacillus species was stable and functional at high temperature (70-100°C) in the presence of organic solvents and detergents. The special properties of the isolated Geobacillus species have potential applications in food, textiles, paper, starch saccharification, bakery, pharmaceuticals and detergent industries.
Fig. (3). Effect of different solvents on the amylase activity of thermophilic bacterial isolates: Enzyme assays were carried out in the presence of different solvents at concentration of 0.5% and 1% and the relative amylase activity of PW11 (A), PW13 (B) and PS4 (C) was plotted against the indicated concentration of solvents. Reaction without supplementation of solvents was served as control and considered as 100% activity. Data of three independent experiments was plotted with standard deviation.
CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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Fig. (4). Effect of chaotrope agents on amylase activity. The relative amylase activity of PW11 (A and D), PW13 (B and E) and PS4 (C and F) was tested in the presence of indicated concentration of ionic (SDS) and non ionic (Triton X-100), EDTA and PMSF. Reaction without supplementation served as control and set as 100% activity. Data of three independent experiments was plotted with standard deviation.
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