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Isolation and characterization of thermophilic *Bacillus sp.* with extracellular enzymatic activities from hot spring of Ganeshpuri, Maharashtra, India

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Abstract
Thermophilic bacillus sp are well studied important group of microorganisms due to their ability to produce enzymes of industrial importance. Thermostable enzymes have great biotechnological potential. In this study, thermophilic bacteria belonging to *Bacillus* genus were isolated from hot springs of Ganeshpuri, Maharashtra, India. Isolates were identified using biochemical tests. All bacteria growing at 55 °C were screened and characterized for extracellular enzymatic activities namely amylase, protease, cellulase and lipase. The study confirmed that the isolated *Bacillus* sp. to be a true thermophile and could be a source of thermostable extracellular enzymes which can be exploited for biotechnological and industrials applications.

Keywords: Thermostable enzymes, Bacillus sp., hot spring, extracellular enzymatic activities

1. Introduction
Enzymes are complex protein molecules produced by living organisms to catalyze the biochemical reactions required for life. Microorganisms requiring extreme environments for growth are called extremophiles and the enzymes they produce are called extremozymes (Burg B, 2003) [1]. Of all extremozymes, thermophilic enzymes have attracted most attention during the past four decades (Rakshit *et al*, 2007) [2]. Such enzymes are of great industrial and biotechnological interest due to the fact that the enzymes are better suited for harsh industrial processes. Thermophiles thus, represent an obvious source of thermostable enzymes, being reasonable to assume that such character will confer their proteins a high thermal stability. Thermostable enzymes are stable and active at temperatures which are even higher than the optimum temperatures for the growth of the microorganisms. (Bora *et al*, 2007) [3]
A hot spring is a spring that is produced by the emergence of geothermal-heated groundwater from the earth's crust and remains one of the natural habitats of the thermophilic bacteria. Hot springs located at Ganeshpuri village and surrounding area is one of the hot springs in Maharashtra state in India which has been not yet fully explored in details microbiologically. However recent review (Patil *et al*, 2015) [4] suggests there are attempts of isolating microbes and identifying bioactive molecules from the same site.
Bacillus sp. is one of the dominant genuses among the thermophilic microorganisms studied. The thermophilic strains of genus Bacillus produces a large range of extra cellular thermostable enzymes, of which amylases, proteases, cellulases and lipases are of significant industrial importance. (Bora *et al,* 2007, Amnin *et al,* 2008) [6, 7].
The aim of this study was to isolate and identify thermophilic *Bacillus sp*. Next is to characterize them for thermostable extracellular enzymatic activities.

2. Materials and Methods
2.1 Sample collection: study site was Ganeshpuri hot springs located in Thane district of Maharashtra, India. Temperature of the water and soil sediment was measured by calibrated thermometer at the site and it was found to be 57 °C. Samples (40 – 50 grams of sediment soil) were collected from different sites of the hot spring in sterile poly bags and immediately brought into the laboratory.

2.2 Isolation and identification of bacteria: Bacteria were isolated in Nutrient Agar (NA) medium following serial dilution technique. Inoculated NA plates incubated at 55 °C for 24 hours.

Isolated bacteria maintained on nutrient agar slants and were subjected to several biochemical tests for identification using Bergey's Manual of Systematic Bacteriology [5]

2.3 Screening for extracellular enzymatic activities: All isolates were screened for production of extracellular enzymes namely amylase, protease, cellulase and lipase at 55 °C. Nutrient agar medium supplemented with 1% starch, 1% gelatin and 1% glycerol tributyrate was used for amylase, protease and lipase activity respectively. 1% Carboxymethyl cellulose agar medium was used for cellulose activity. Extracellular enzymatic activity of isolates was studied using substrate hydrolysis method. Disappearance of substrate around the colony of isolate was indication of production of extracellular enzyme by the isolate (Bora *et al*, 2007) [6].

2.4 Crude enzyme preparation: Crude enzyme production was carried out using nutrient broth supplemented with 1% of respective substrates for amylase, protease and lipase except cellulase for which 1% CMC broth medium was used. Sterile 50 ml broth mediums were inoculated with 5 ml, 24 h old culture of isolates and incubated at 55 °C for 48 h in rotary shaker. Later the broth is centrifuged at 10000 rpm for 10 min in cold centrifuge. Clear, bacteria free supernatant was crude enzyme preparation and used for determining its activity.

2.5 Effect of temperature on enzyme activity and its thermostability: Effect of increasing temperatures on enzyme activities was studies. Enzymes were allowed to react with specific substrates at 25 °C, 35 °C, 45 °C, 55 °C, and 65 °C. Enzyme activities were determined in terms of U/ml (Ajayi *et al*, 2007, Akel *et al*, 2009, Ibrahim *et al*, 2007, Nawani *et al*, 2006) [9-12]. For Thermostability study, enzyme activity was determined after treatment of crude enzyme preparation at 90 °C for 30 minutes.

3. Results and Discussion

All the bacterial isolates were catalase positive, endospore forming, Gram positive rods and hence belonged to genus Bacillus. Endospore staining showed presence of terminal and sub terminal spores. The optimum temperature for growth was found to be in range of 45-55 ºC. Different biochemical tests revealed that the isolates B and H was identified (Table 1) as *Bacillus macerans*, isolate C, D and F were identified as *Bacillus coagulans*, *Bacillus circulans*, *Bacillus stearothermophilus* respectively, isolate E and G was showing similar biochemical results to *Bacillus polymyxa* but with exception of growing at 55 ºC. Isolate M is identified up to genus level as *Bacillus sp.* Earlier studies also reported presence of diversified strains of Bacillus group of microorganisms in hot spring environment with extracellular enzymatic activities in India as well as overseas (Bora *et al,* 2007, Amnin *et al,* 2008, Khalil 2002, Ajayi *et al*, 2007, Akel *et al*, 2009, Ibrahim *et al*, 2007, Nawani *et al*, 2006) [6-12].

All isolates possessed Extracellular enzymatic activity (Table 2) of amylase, 3 isolates gave protease activity and one isolate showed cellulase activity at 55 °C. Only one isolate produced lipase at 55 °C.

The optimum temperatures for crude amylase activities were observed in range of 45-55 °C. In each case enzyme activity increased from 25 °C to 55 °C but reduced at 65 °C. Thermostability studies (Graph I) at 90 °C for 30 minutes revealed that crude amylases from isolates B, C, D, E, F, G, H, and M retained 50%, 40%, 52%, 37%, 46%, 52%, 42%, 27% activities respectively. Amylases from isolates D and G had higher thermostability. There are studies which reported high optimum temperatures and thermostabilities for thermostable amylases. Studies (Rekhadwad 2015 and Jeevan *et al,* 2009) [13, 14] on *Bacillus sp.* reported amylases to have optimum temperatures of 68 °C and 85 °C respectively. Stabilities of crude amylases under study were found to be moderate when compared to same work [13, 14] where amylase retained 85% and 79% activities respectively.

Crude proteases preparation from isolates C and G showed optimal activity at 55 °C while protease from E showed optimum activity at 45 °C (Graph II). Crude protease from isolates C, E, G retained 51%, 41%, 48% activities, indicating that protease from C had high thermostability. Obtained results were well comparable with previous study which showed protease isolated from *Bacillus pumilus* D-6, gave optimum activity at 50 °C and stability of 54-55% when incubated at 70-100 °C (Bajaj *et al,* 2013) [15]. But another study showed higher stabilities and optimum temperatures 50 °C – 75 °C of protease enzyme from different *Bacillus sp.* (Chu *et al,* 2007) [16].

Crude cellulase preparation from Isolate G gave best activity at 45 °C (Graph III). it retained 40% activity at 90 °C. Considerable cellulolytic activity at higher temperature of 65 °C is been reported (Bajaj *et al*, 2009) [17] but thermostability of crude cellulase is significant and comparable as the same study which showed 50% reduction in activity at 90 °C.

Crude lipase preparation of isolate M possessed optimum activity at 55 °C (Graph IV). it retained 60% activity at 90 °C which is highest thermostability among all. Crude lipase enzyme showed better stability than that of the study reported (Bora *et al*, 2012) [18] from a Bacillus strain having optimum temperature of 60 °C and keeping 90% of activity at 60 °C and almost 70% 0f the activity retained at 70 °C.

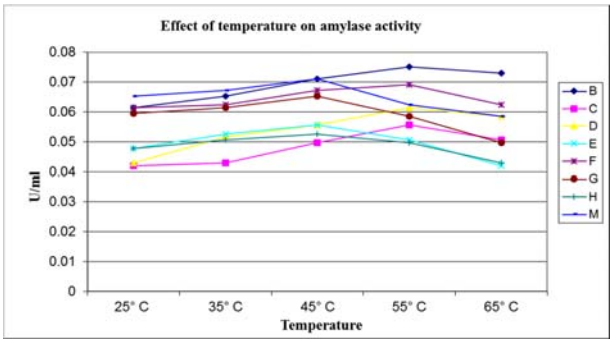
Enzyme activities reported in this study are moderate because of the crude nature of the enzyme Activities will be getting better when enzymes are purified as number of factors influence enzyme activity. However activities and stabilities of crude enzymes were found good enough to be considered for industrial applications. Sometimes there might be requirement of highly thermotolerant enzymes which can be obtained from thermophilic bacteria from natural sites or by improving the enzyme activity by protein engineering so that it could be applied to harsh processes.

Table 1: Biochemical activities of isolates

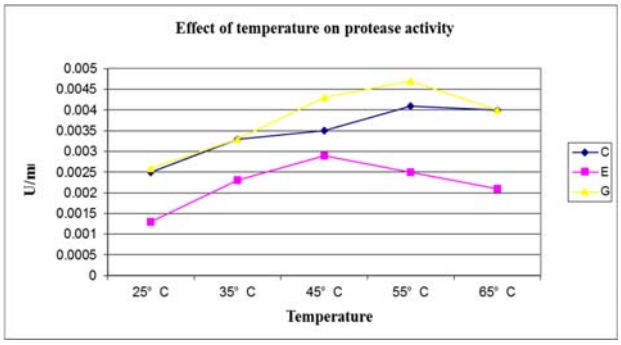
Isolate	V-P reaction	Growth in 7% NaCl	Acid and gas from glucose	Nitrate reduction	Starch hydrolysis	Growth at 65 ºC	Catalase	Acid from glucose	Casein hydrolysis	Bacteria identified as
B	-	-	-	-	+	-	+	+	-	*Bacillus macerans*
C	+	-	-	-	+	-	+	+	+	*Bacillus coagulans*
D	-	-	-	+	+	-	+	+	-	*Bacillus circulans*
E	+	-	-	+	+	-	+	+	+	*Bacillus polymyxa*
F	-	-	-	-	+	+	+	+	-	*Bacillus stearothermophilus*
G	+	-	-	+	+	-	+	+	+	*Bacillus polymyxa*
H	-	-	-	+	+	-	+	+	-	*Bacillus macerans*
M	+	-	-	+	+	-	+	+	-	*Bacillus sp.*

Table 2: Extracellular enzymatic profile of isolates

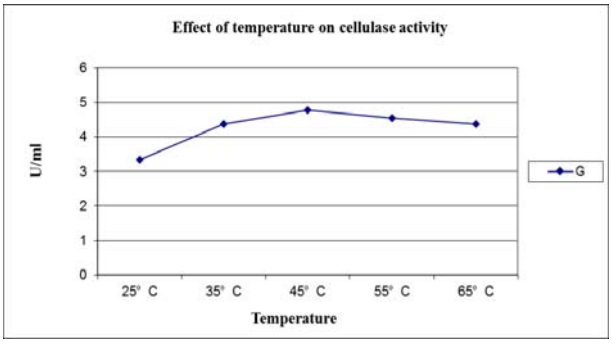
Isolate	Amylase	Protease	Cellulase	Lipase
B	+	-	-	-
C	+	+	-	-
D	+	-	-	-
E	+	+	-	-
F	+	-	-	-
G	+	+	+	-
H	+	-	-	-
M	+	-	-	+



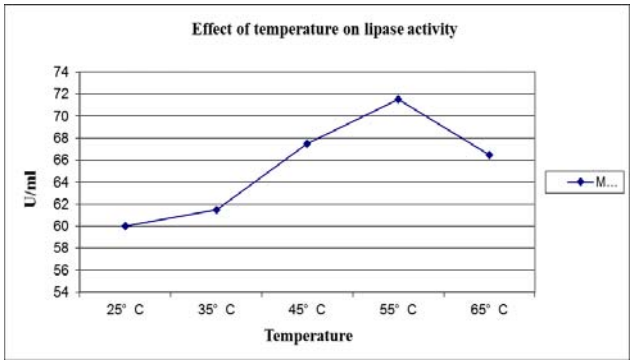
Graph I: Effect of temperature on amylase activity



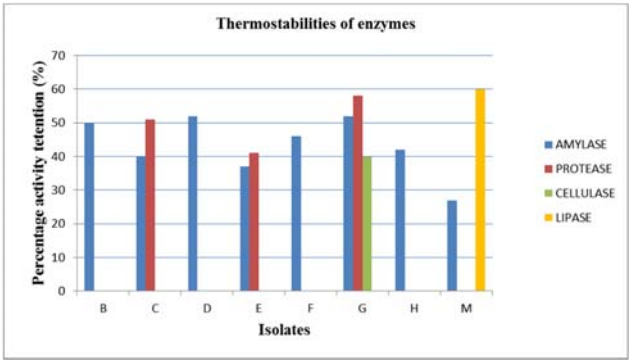
Graph II: Effect of temperature on protease activity



Graph III: Effect of temperature on cellulase activity



Graph IV: Effect of temperature on lipase activity



Graph V: Thermostabilities of all enzymes

4. Conclusion

The result is encouraging as bacteria with enzyme production potential are found in hot spring sediment soil. The present study was a preliminary screening report of diversity of *Bacillus sp.* and their enzyme production potential from hot spring sediment. The study revealed a high taxonomic diversity among the Bacilli isolated. Study clearly revealed new and interesting perspectives showing that bacillus strains isolated from Ganeshpuri hot spring, represents a source of bacterial enzymes that can be exploited potentially for biotechnological purpose. These enzymes can be further studied extensively for different industrial applications such as detergent, textile industry, drugs, toxic wastes removal, bioremediation process, bio-refineries etc.

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