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Studies on Protease Producing Thermophilic Bacteria Isolated from Hot Springs

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Abstract

Microorganisms inhabit every probable area of the earth including unfavorable encampments, too. The bacteria that can survive in extreme conditions are commonly known as extremophilic bacteria. Amongst the extremophilic bacteria, thermophiles have attracted investigators in recent decades for their properties to survive at a very elevated temperature. Proteolytic enzymes of thermophilic bacteria are one of the major classes of enzymes with commercial importance that are utilized for industrial purposes especially in biotechnological industries. In the present study, protease enzyme producing thermophilic bacteria were isolated from two hot springs in the vicinity of Satpuda Ranges, Maharashtra, India. Thus, the study included isolation of thermophilic bacteria and screening of isolates for protease activity. The selected isolates were subjected to proteases production under various pH. From the studies it was seen that 14 isolates produced protease enzyme but the isolate S_25 was exhibiting high activity compared to other isolates. The production of protease enzyme by isolate highlighted the promising information about the hot springs as a potential source of thermophilic bacteria for industrial and biotechnological applications with the special context of extracellular protease enzyme.

Keywords: Hot springs, Satpura Hills, Protease, Thermophilic bacteria

1. Introduction

Microorganisms inhabit at every possible nooks and corners of this planet ranging from favorable environments for their growth to very extreme unfavorable conditions.

Those microorganisms growing at extreme conditions are called as extremophiles [1]. Extremophiles with their various categories, thermophiles (high temperature), halophiles (high salinity), alkaliphiles (high pH), psychrophiles and (low temperature) acidophiles (low pH), have attracted researchers due to understand their survival and stability properties at extreme conditions and their possible applications [2]. Extremophiles such as thermophilic microorganisms (optimum growth temperature around 50°C and above) have received attention as they are sources of thermostable enzymes (examples: proteases, amylases, cellulases, lipase, chitinases, etc); these enzymes possesses special properties that help them sustain at high temperatures and may have biotechnological uses [3]. Based on the range of pH in which they function, proteases can be classified as acidic, alkaline, or neutral, and can be divided into cysteine, serine, and aspartic proteases, and metalloproteases, based on the functional groups present around their active sites [4,5, 6].

Proteases are used at commercial level in detergent industries and important in dish washing in institutional sector as well [7]. Alkaline proteases, a sub class of proteases exhibit high stability and found to be suitable for in detergents. Applications in industries such as washing powders, food industry, leather processing and pharmaceuticals and for studies in biology are some important features of proteases [8].

Thermophiles can be classified into hyperthermophiles (growth optimum, 80–110°C), extreme thermophiles (growth optimum, 60–80°C) and moderate thermophiles (growth optimum, 50–60°C) [9].

There are seven geothermal appearances located in between Satpura hills and Tapi River [10] The hot springs there extended in the belt of area about 55 Km in Dhule, Jalgaon and Nandurbar Districts of Maharashtra. The surface temperature range of these hot springs estimated to be in the range of 38 to 60 C.

Although interest in studying thermophilic bacteria from other hot springs has been demonstrated by previous studies, there is need of research to be carried out on thermophiles from theses hot springs. The aim of this study was isolation of thermophilic bacteria, and to screen and characterize them for protease activity that may possess possible environmental and industrial applications.

2. Materials and Method

Sampling and isolation of thermophilic bacteria

Water and soil (mud) samples were collected from hot springs near Satpuda (Satpura) hills/ranges, Maharashtra, India (at Unapdeo: Shahada- district Nandurbar: sample 1 and, Chopada- district Jalgaon: sample 2) in sterile containers, pH noted with strip method and transported to laboratory and maintained at 4°C. These samples were used to isolate thermophilic bacteria as per Narayan *et al.* [11] with slight changes. The samples were diluted and streaked on nutrient agar plates and plates were incubated at 50°C for 24-48 h. Isolates were labeled accordingly and maintained after checking colony characteristics and used for screening for enzyme protease producers.

Sample analysis and screening of protease producing isolates

Samples were subjected to water analysis for dissolved oxygen (D.O.) and coli form counts (MPN) by standard methods [12, 1]. Bacterial isolates obtained were screened for extracellular protease production on agar media as per Denizci *et al.* [13], Shaheen *et al.* [14], Song and Hu [15] with slight modifications. In brief, using three different combinations (i) nutrient agar, 1% glucose and skim milk, (ii) casein agar and (iii) nutrient agar and skim milk were inoculated and incubated at 50°C for 24-48 h. Zone of hydrolysis/ clearance around the colony was observed and noted. Isolates showing maximum zone of hydrolysis, were further selected for production of protease by submerged fermentation process.

Biochemical characterization

The biochemical characterization for enzyme production ability was performed for the selected bacterial isolates. Characterization with the help of testing of enzymes catalse, oxidase and Extracellular hydrolytic

enzymes; gelatinase, lipase, amylase biochemical was done as per described by Cruickshan [12] and Narayan *et al.* [11].

Production of protease; shake flask experiment:

After the screening, fourteen culture isolates (S_1A, S_1B, $S_1$1, $S_2$2, $S_2$2A, $S_2$3, $S_2$4A, $S_2$5, SMPN, 6, G1, G2, G3, G4) were selected for further study of protease production. The enzyme production and separation were done as per Huang *et al.* [16], Yossan *et al.* [17] by some modifications. The production media (pH 7.5) comprising glucose 1%, peptone 1%, yeast extract 0.02% (w/v), skimmed milk (2%) were inoculated using fresh growth of bacterial isolates in Erlenmeyer flask and incubated at 50°C for 48 hrs in a shaking incubator (Remi, Thane, Maharashtra, India) maintained at 150 rpm. Culture supernatant of grown culture broth was obtained by centrifugation at 8000 rpm for 20 min. The supernatant was assayed for protease activity and subjected for further study.

Protease assay

Protease activity was determined by following the standard assay method using casein as a substrate as per Haung *et al.* [16] and Haddar *et al.* [18] with slight modifications. In short, the protease activity was assayed by incubating 1 ml of the enzyme with 1 ml of 2% (w/v) casein (prepared in 100 mM phosphate buffer; pH 7.0 and 100 mM Tris-HCl buffer; pH 9.0) at 55°C for 10 minutes. Reaction was terminated by adding 10 ml tricarboxylic acid (TCA). TCA soluble fraction containing soluble peptides was estimated using the by referring the standard curve of tyrosine and the enzyme activity was determined accordingly.

Effect of pH on protease production:

The effect of variable pH on the production of protease was analyzed by growing the selected bacterial isolate in the production medium by varying the pH (6.0, 6.5,7,7.5 and 8.0). Isolates were grown in the production media for 48 h incubation period incubating at 50°C.The caseinolytic activity of enzyme was measured after 48 h using the standard assay procedure mentioned as above.

3. Results and Discussion

Isolates of thermophilic bacteria

From the water/ mud samples collected from two different hot springs, about twenty thermophilic bacterial isolates were obtained on nutrient agar. Samples were subjected for determination of dissolved oxygen and MPN (Table 1). The pH of water samples was found to be near neutrality 7.0 and 7.2, respectively. The D.O. was found to be 3.2 nd 3.1 mg/L which seems to be little diminished. As reported by Kumar and Sharma, [1] concentration of dissolved oxygen decreases as temperature increases and it was frond to be in the range of 3.25 to 3.55 at hot springs in Iran. The coliform bacterial count checked by MPN which is broadly used method to study quality of water; with our samples it was found to be zero that indicates absence of coliform bacteria [19].

Table 1: Analysis of water samples collected

Sr. No.	Parameter	Sample 1	Sample 2
1	pH	7.2	7.0
2	Dissolved oxygen (DO)	3.2mg/lit	3.1mg/lit
3	MPN	Nil	Nil

Sample analysis and screening of protease producing producer

Morphologically distinct bacterial colonies isolated from samples were subjected to screening for the protease through repetitive streaking on casein agar and modified nutrient agar (skimmed milk/glucose) plates. Fourteen bacterial isolates showing zone of hydrolysis around growth revealed and considered positive for production of extracellular protease. The zones obtained on nutrient agar (modified) plates were much prominent than on the casein agar plates (Fig 1 a and b). These 14 isolates were then subjected for further study.

Biochemical characterization

All the isolates were found to be positive for oxidase, catalase, amylase and gelatinase while not producing urease and lipase (Table 2).

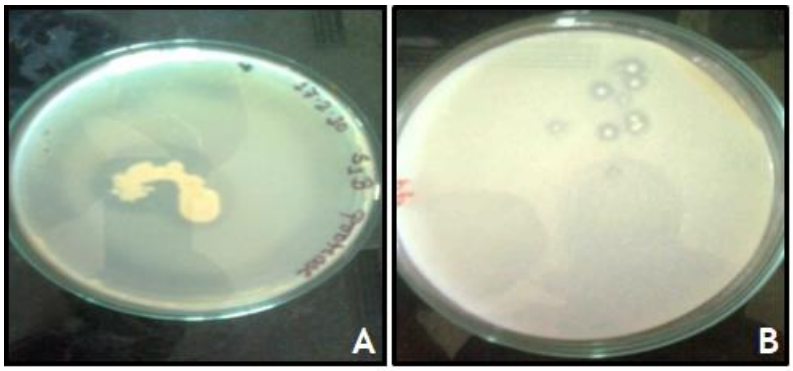

Fig 1: Protease activity of isolate on a) skimmed milk agar and b) casein agar

Table 2: Various enzymes produced by the isolates:

Isolates	Oxidase	Catalase	Gelatinase	Lipase	Urease	Amylase
S_1A	+	+	+	_	_	+
S_1B	+	+	+	_	_	+
S_11	+	+	+	_	_	+
S_22	+	+	+	_	_	+
S_22A	+	+	+	_	_	+
S_23	+	+	+	_	_	+
S_24A	+	+	+	_	_	+
S_25	+	+	+	_	_	+
SMPN	+	+	+	_	_	+
6	+	+	+	_	_	+
G1	+	+	+	_	_	+
G2	+	+	+	_	_	+
G3	+	+	+	_	_	+
G4	+	+	+	_	_	+

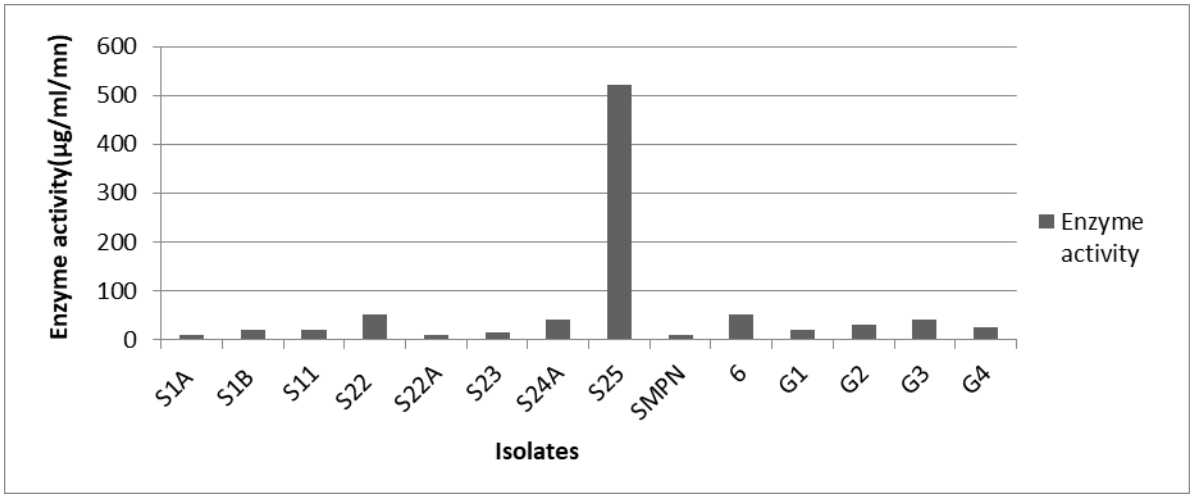

Fig 2: Protease produced by isolates

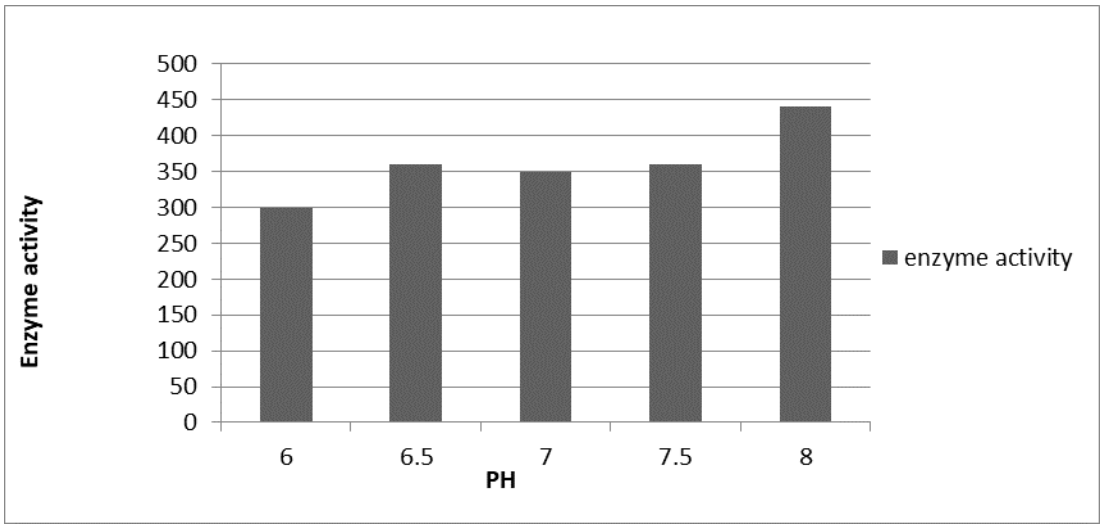

Fig 3: Effect of pH on enzyme production by isolate S25

Production of protease; shake flask experiments

The production of protease is studied in production medium in flask. The enzyme activity of all 14 isolates was determined in that isolate S25 found to be maximum producer amongst tested (figure 2). Hence this isolate was selected for study of effect of pH of growth medium for production of protease enzyme.

The production of protease may be affected by fermentation/growth parameters such as pH, substrate concentration, media composition, temperature, etc. Maximization production of protease from selected isolates is essential to reveal their industrial applications. Further work was carried out for optimization protease production using media with varying pH but other cultural conditions also needed to study.

Influence of variable pH (6.0 to 8.0) on the production of protease by isolate S25 was investigated. The production level of the enzyme was observed to increase with corresponding increase in the pH of production media from 6.0 to 8.0 (Figure 3). Thus it can be said that the trend is increasing as pH of growth medium is considered. The maximal activity of the enzyme (440 U/ml) was obtained at pH 8.0 for an incubation period of 48 h. Kamran et al. [20] have studied effect of pH on enzyme activity of thermophilic bacterial protease and found maximum activity at pH around 8.0. Haung et al also reported similar results of protease activity at pH 7.5 with thermophilic bacillus strains. This goes parallel with our study but we have grown cultures at varied pH rather than carrying activity at varied pH; and isolate shown maximum enzyme activity when grown at pH 8.0.

4. Conclusion

Fourteen thermophilic bacterial isolates were obtained from soil and water samples collected from hot springs from the vicinity of Satpuda ranges (Nandurbar and Jalgaon districts), Maharashtra. Isolates exhibited extracellular protease activity as observed from zone of clearance on skim milk/casein agar. The ability of these isolates to produce protease at pH 8.0 and at a temperature of 50°C was confirmed. Isolate S_25 was found to be promising one that exhibited higher level of protease enzyme production. From the above studies it can be concluded that the studied hot springs are the potential sources for bacteria producing industrially important enzymes. Further studies are required to characterize and identify the isolates and to find out the potential applications of the protease obtained from the isolates.

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Conflict of interest
No conflict of interest influenced in this research

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