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Isolation and Characterization of Psychrotrophic Protease Producing Bacteria from Gangotri Glacier, India

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Abstract: A Psychrotrophic bacterium producing cold active protease upon growth at low temperature was isolated from soil of Gangotri glacier identified as Bacillus sp. Out of the ten selected protease producing isolates the best one BBPRT-7 was further characterized on the basis of their morphological and biochemical characterization as given in Bergey's Manual of Systematic Bacteriology. The bacterial strain BBPRT-7 showed maximum activity at ph 10.0 and temperature 20°C. These Cold-active proteases are generally characterized by high-catalytic efficiencies at lower temperature and lower thermal stability. These cold active proteases have enormous implications in industry. Bacterial alkaline proteases are of great importance due to its wide spectrum applications in detergent industries, bioremediation, food industries, and leather processing, bio-film degradation, pharmaceuticals industry, meat tenderizers, protein hydrolyzates. As per our study isolate BBPRT-7 may have applications in detergent industry.

Keywords: Psychrotrophic, Protease, Gangotri, Bacteria.

I. INTRODUCTION

Cold environments represent a large proportion of Earth's area, including the Arctic, the Antarctic, oceans, and mountain areas (Cowan et al, 2007). The world's oceans occupy 71% of the earth surface and 90% of their volume is below 5°C, the polar regions represent 14% of the earth surface and if one includes alpine soils and lakes, snow and ice fields, fresh waters and caves, more than 80% of the earth biosphere is below 5°C. Despites of unfavorable condition at low temperature various microorganisms survive at these harsh environments. Cold adapted bacteria do not merely survive or endure such extremely inhospitable conditions but are irreversibly adapted to these environments, as most of them are unable to grow at mild (or mesophilic) temperatures. Cold adapted microorganisms are of particular importance in global ecology since the microorganisms capable of coping with low temperatures are widespread in these natural environments where they often represent the dominant flora and they should therefore be regarded as the most successful colonizers of our planet (Russell, 1990). Cold active enzymes are produced by microorganisms existing in permanently cold habitats located such as glaciers and polar zones. The cold active enzymes of microorganism adapted to permanently low temperature have attracted much less attention than the enzymes of thermophiles. two properties of cold-active enzymes that have the most obvious biotechnological application are their high catalytic activity at low temperatures and low thermostability at elevated temperatures. Among these, cold microbial proteases are of great commercial value, representing a significant fraction of the world market of cold active enzymes (Kuddus and Ramteke 2012).

Proteases are important industrial enzymes accounting for 60% of total global enzyme sales (Chun et al 2007, Nunes and Martins 2001, Singh et al 2001, Maugh T1984, Outturp and Boyce 1999, Ward 1985, Chu W-H 2000). Bacterial alkaline proteases are of great importance due to its wide spectrum applications in detergent industries, bioremediation, food industries, and leather processing, bio-film degradation, pharmaceuticals industry, meat tenderizers, protein hydrolyzates, food products and even in the waste processing (Furhan, and Sharma 2014). Economic benefits can be achieved by using cold-active proteases as they allow working at low temperatures even in an industrial scale (S. Joshi and T. Satyanarayana 2013).

The advance in enzyme technology and Industrial biotechnology offer a way for industrial application of protease. The aim of this study to isolate cold active protease producing bacteria from the soil of Gangotri glacier, Western Himalaya, India and optimization of temperature and pH conditions for production of enzyme.

II. MATERIAL AND METHODS

A. Collection of Sample

The sample from the above habitat was collected randomly from different sites of Gangotri glacier and was transferred into sterilized polythene bags. Temperature of Gangotri glacier ranges from 5 to 20°c. The sample was then brought and stored under cold conditions until processed. Extreme care was taken at all times during the whole sampling processes to ensure the minimal contamination.

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B. Isolation of Bacterial Flora from Soil -
Bacterial counts were determined in 1gm soil. Soil was suspended in 10 ml sterile phosphate buffer saline in 100 ml sterile flasks. A decimal dilution series of the supernatant to 10^{-5} was prepared in sterile saline. Appropriate dilution of sample was spreaded on PSC agar media containing skimmed milk. Bacterial cultures growing in a temperature range between 4 and 20^0C were picked and further checked by growing them on skimmed milk agar plates containing peptone (0.1%), NaCl (0.5%), Agar (2%), Skimmed milk (10%) and incubated for 4–5 days.

C. Screening of Cold Active Alkaline Protease–
A clear zone of skim milk hydrolysis appeared after 48 h of incubation. One hundred twenty bacterial colonies were isolated on skimmed agar medium from eight soil samples of Gangotri glaciers. The proteolytic activity was assayed using skimmed milk agar and expressed as diameter of clear zone around bacterial colony at temperature 20^oc, one isolated was selected as potent protease producer strain, designated as BBPRT-7.

D. Morphological Characterization of Isolates BBPRT-7
The morphological characterization of protease producing isolates active isolates was done by examining the Configuration, margin, elevation size, Margin Color, Arrangement Texture and Gram's staining of bacterial colonies**.**

E. Identification of Isolate BBPRT-7 Showing Protease
Biochemical tests like MR-VP, citrate utilization, Indole production, H2S production, Catalase test, Oxidase test were done. Microorganism was identified up to genus level with the help of these biochemical tests.

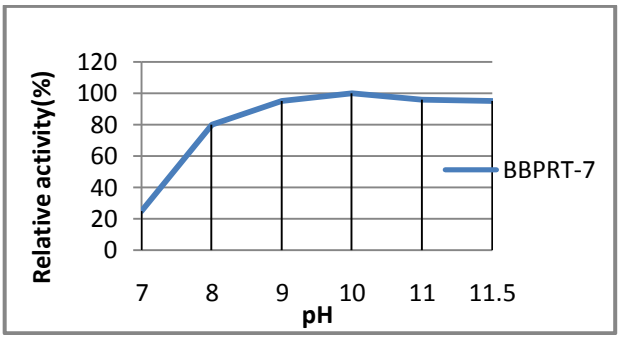
F. Production of enzyme and partial purification
The microorganism was grown at 20^0c in ten 500ml Erlenmeyer flask containing 100ml PSC medium and 10% skimmed milk for 24 hour at shaker. Cell free supernatant was subjected to Ammonium sulphate fractionation. Fraction was dialyzed against glycine NaOH buffer. The purified enzyme served as a enzyme source for further characterization.

G. Effect of ph on Protease Activity-
To study effect of pH on proteolytic activity of partially purified enzyme and stability 0.2 ml of cold active protease of strain BBPRT-7 was added to the substrate mixture containing 1.5 ml of 1% (w/v) casein, and 0.1 mM $MgCl_2$ in phosphate buffer having(pH 7-8), glycine buffer pH(9-11) and incubated at 20 °C for 60 min. After incubation the proteolytic activity was determined by the protease assay (1%v/v).

H. Effect of Temperature on Protease Activity
For determination of the protease activity at different temperature, the reaction was carried out at various temperatures between 10 and 50^oC. A 0.2 ml of cold active protease of strain BBPRT-7 was added to the substrate mixture containing 1.5 ml of 1.0% (w/v) casein in 100 mM Tris-HCl in 1 mM $MgCl_2$ at pH 10.0 and incubated at 10, 20, 40, 60, 80, 100, 120°C for 1 h. After the incubation, the proteolytic activity was determined by the protease assay. For the evaluation of the effect of temperature on enzyme stability, the proteases were incubated for 1 h at pH 10.0 (enzyme assay buffer) at different temperatures ranging from 10 to 50^OC. After that, enzyme solutions were cooled and the residual activity was measured using the standard protease assay.

III. RESULTS

In the present study, One hundred twenty bacterial isolates were isolated from various soil samples that have been collected from the soil from the Gangotri glacier located in western Himalaya of India, however, ten isolates were identified as protease producing species. Out of the ten selected protease producing isolates the best one BBPRT-7 was further characterized on the basis of their morphological and biochemical characterization. For the identification of strain of interest cultural characteristics, morphological characteristics, and biochemical tests (KR Aneja 2007) were conducted and identified on the basis of characters as given in Bergey's Manual of Systematic Bacteriology (Holt et al 1984). Results indicate that the protease active isolate (BBPRT-7) is Gram positive bacterial strain and belongs to Bacillus species as shown in (table 1)



Graph-1 Effect of pH on enzyme activity

Table 1: Biochemical characterization of BBPRT

Characterization test	Result
Gram's reaction	Positive / Rod
Catalase	Positive
Starch hydrolysis	Positive
Nitrate reduction	Positive
Gelatin hydrolysis	Positive
Indole Test	Negative
MR Test	Positive
VP Test	Positive
Citrate Utilization Test	Positive
TSI Agar test	Negative
H_2S Test	Negative
Sucrose test	Negative
Possible identification	Bacillus

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Protease produced by BBPRT-7 was taken for further study. In our study total activity of protease enzyme was 70.2 µg/min/ ml and after ammonium sulphate fractionation (40-60) it was 379 µg/min/ ml (Table 2 and 3)

Table.2-Enzyme activity of BBPRT-7

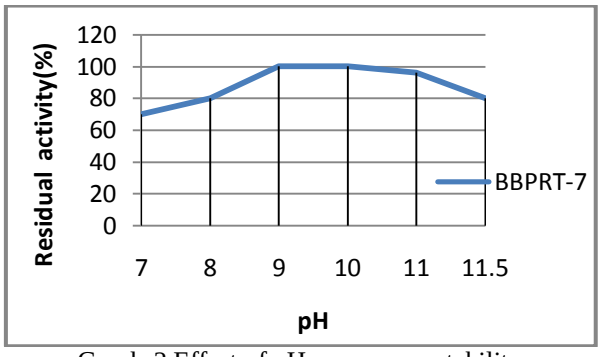
S.No.	Isolate	Activity(µg/min/ ml)
1.	BBPRT-7	70.2

Table.3-Enzyme activity after partial purification

S. No.	Isolate	Fractionation	Activity (µg/min/ ml)
1.	BBPRT-7	40-60	379

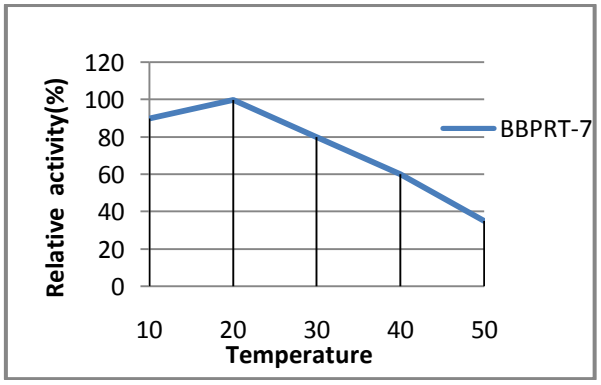
After the identification of isolate BBPRT-7, the optimum pH for maximum alkaline protease activity was determined by protease assay in buffer with different pH (7-11) and results were shown in graph 1.

The highest enzyme activity was obtained at pH 10. However, increased alkalinity was favorable up to pH 11. Enzymes from BBPRT-7 had the widest pH range for activity, with more than 80% of their maximal activity occurring at pH 8.0–11.5 (Graph 2)

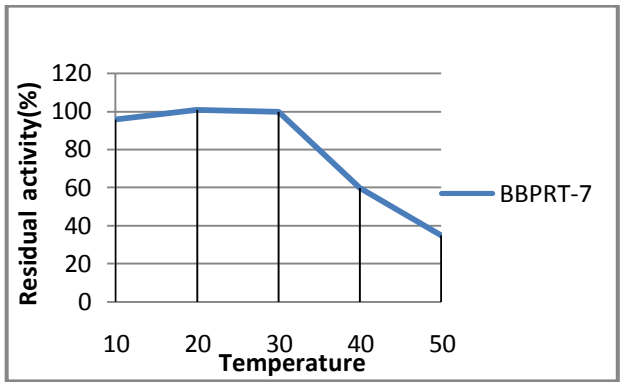


Graph-2 Effect of pH on enzyme stability

The enzyme displayed significant activity within a temperature range of 10–50°C with maximum activity at 20°C and at pH 10.0 (Graph.3). The enzyme found to be stable at temperature between 10-30°c (Graph 4)



Graph- 3 Effect of temperature on enzyme activity



Graph – 4 Effect of temperature on enzyme stability

IV. DISCUSSION

Owing to the characteristics of the cold adapted enzymes, much attention is being focused on isolation and characterization of extracellular enzymes produced by psychrophiles and Psychrotolerant microorganisms (Brenchley, 1996).In the present study we isolate the cold adapted bacteria from the Gangotri glacier, western Himalaya India. The total 120 strains were isolated from the soil and the potential cold active proteolytic strain BBPRT-7 was found gram positive rod shaped bacterium. The biochemical tests showed indole negative, methyl red negative, vogues proskauer positive and citrate positive, bacteria does not produce hydrogen sulphide gas. Temperature is one of the most important factors affecting the enzyme production. The isolated bacillus BBPRT-7 was found to grow well between 10 and 30°C showing an optimal growth at 20°C. The isolated strain thus can be classified as a psychrotroph according to the definition of Morita et al.'s (1997) which describes them as psychrotrophs (now psychrotolerants), as they are able to grow at 0°C but have optimum growth temperatures 15–25°C. lower in comparison with proteases from mesophilic isolates. The extracellular protease secreted by sample of Gangotri glacier, was partially characterized, and most of its properties were found to be distinct from those of other proteases from Bacillus strains that are mesophilic. Ammonium sulphate fractionation of 40-60 of the enzyme shows enzyme activity 379 µg/min/ ml and it may have some industrial application. The enzyme produced by BBPRT-7 is similar in optimum growth temperature of bacteria isolated from Gangotri glacier by Baghel et al (2005).Maximum enzymatic activity was found at 20°C by most isolates. It was indicated that isolates were belonged to psychrotrophic family not to obligate Psychrophiles (Feller and Gerday. 2003).

The BBPRT -7 protease is nearly a neutral protease, with an optimal pH of 10, most known Bacillus species produce commercial proteases that are highly active at pH 7.0 and 11.0, with an optimum around pH 8.0-10.0 (Davail et al., 1994; Hutadilok-Towatana et al., 1999; Jaouadi et al., 2008 Moreover, the BBPRT-7 protease maintains its highest activity at 20°C, which is one of the typical characteristics found in cold-active enzymes (Wang et al., 2005; Zhang and Zeng, 2008).

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The stability curves are shown in graph-2 was described above, showed high RA over a broad range of pH. This Ph dependence for activity makes these enzymes interesting for industrial applications, in contrast with other cold proteases with a narrow pH profile (Fernandez et al., 1996; Secades et al., 2001; Irwin et al., 2001. In the present study, the BBPRT-7 showed maximum activity at 10.0 pH and 20^{O}C like to cold adapted Bacillus strain islolated from soil of Wular Lake of Kashmir. (Furhan J and Sharma S 2014) Microbial alkaline proteases dominate commercial applications with a significant share of the market captured by alkaline proteases from Bacillus sp. for laundry detergent applications (Kumar and Takagi 1999).The field of cold-active protease research is still wide open and expected to achieve spectacular success in the nearest future.

V. CONCLUSION

It was found that the enzymes showed maximum activity at pH 10.0 and temperature 20°C and thus we can say that the enzymes produced from isolate BBPRT-7 is cold active and alkaline in nature and has great importance in biotechnology industry as well as other industries.

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