BIOCHEMICAL AND BIOTECHNOLOGICAL IMPLICATIONS OF COLD ADAPTED BACTERIA ISOLATED FROM GANGOTRI GLACIER, WESTERN HIMALAYA, INDIA

THESIS

SUBMITTED TO THE

BABASAHEB BHIMRAO AMBEDKAR UNIVERSITY

(A CENTRAL UNIVERSITY)

LUCKNOW

FOR THE AWARD OF DEGREE OF

DOCTOR OF PHILOSOPHY

IN

ENVIRONMENTAL MICROBIOLOGY

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Enrolment No.: 389/11 **Year 2016**

SUMMARY AND CONCLUSION

Gangotri glacier is located in Uttarkashi district of Uttaranchal state, India between $30^{o}44´$-$30^{o}56´$ N latitude and $79^{o}04´$-$79^{o}15´$E longitude. It is about 30 km long flowing in a NW direction and its width varies from 0.5 to 2.5 km. Bacteria that are especially adapted to low-temperature habitats have been described as either psychrotrophic or psychrophilic. Gangotri glacier is highly rich in microbial diversity would be most appropriate to screen microbes for the production of cold active enzymes. The present work was focused on isolation and characterization of cold adapted bacteria capable of producing cold active enzyme protease and lipase.

The present studies revealed the presence of bacteria in samples of Gangotri glacier. Total bacterial count varied from $10x10^{6}$ to $16x10^{6}$ cfu g^{-1} of soil at 37°C. The lowest bacterial counts were found at Gaumukh, Gangotri and Bhatwari while highest bacterial count were found at Haridwar.

One hundred twenty bacterial isolates were screened from the eight sample of Gangotri sample on PSC solid agar medium containing skimmed milk at temperature from 20 ± 2^{0}C. Ten isolates, designated as AAPRT-3, BBPRT-7, BBPRT-13, CCPRT-12, DDPRT-6, EEPRT-5 FFPRT-10, GGPRT-6, GGPRT-8, and HHPRT-8 were selected as potent protease producing strains. These isolates were subjected to the taxonomical characterization. Phylogenetic analysis using 16S rRNA indicated that strain AAPRT-3 and DDPRT-6, shared high similarities with *Bacillus subtilis*. BBPRT-7 shared high similarity with *Bacillus licheniformis*, BBPRT-13 with *Pseudomonas mandelii*, GGPRT-8 With

Pseudomonas fluorescens, FFPRT-10 with *Pseudomonas fluorescens*, HHPRT-8 and CCPRT-12 with *Pseudoalteromonas flavipulchra*, EEPRT-5 with *Staphylococcus aureus* and GGPRT-6 with *Exiguobacterium alkaliphilum*.

Among these ten potent protease producing isolates three isolates were selected as potent protease producer strain designated as DDPRT-6 (*Bacillus subtilis*), BBPRT-7 (*Bacillus licheniformis*) and FFPRT-10 (*Pseudomonas fluorescens*) at alkaline pH different temperature (4-37^{0}C) were taken for further studies.

Extracellular protease from DDPRT-6 (*Bacillus subtilis*), BBPRT-7 (*Bacillus licheniformis*) and FFPRT-10 (*Pseudomonas fluorescens*) was subjected to partial purification. Enzyme from DDPRT-6 (*Bacillus subtilis*) was partially purified to 2.49 purification fold with the increase of specific activity 561.98 U/mg and yield of 82.4 % by ammonium sulphate fraction and dialysis. Enzyme from BBPRT-7 (*Bacillus licheniformis*) was partially purified to 5.39 purification fold with the increase of specific activity 379 U/mg and yield of 66.6 % by ammonium sulphate fraction and dialysis. Enzyme from FFPRT-10 (*Pseudomonas fluorescens*) was partially purified to 3.3 purification fold with the increase of specific activity 1430.55 U/mg and yield of 45.04 % by ammonium sulphate fraction and dialysis.

Effect of pH on enzyme activity was examined in the pH range 7-11.5.The optimum pH for maximum alkaline protease activity was determined by performing protease activity assay in buffers with different pH (pH 7-11.5). Enzyme from DDPRT-6 (*Bacillus subtilis*) shows maximum activity at pH 10 and enzyme was more than 80% stable at pH range 9-10. The BBPRT-7 (*Bacillus*

licheniformis) shows maximum activity at pH 10 and had the widest pH range for activity, with more than 100% stable at pH range 9-10. Enzyme from FFPRT-10 (*Pseudomonas fluorescens*) shows maximum activity at pH 9 and more than 100% stable at pH range 9-10. The protease was active over a broad pH range. Optimal proteolytic activity occurred at neutral or moderate alkaline pH for the three protease. None of the enzymatic activities had a sharp dependence on pH. All of them was inactivated at pH 11.5. Enzymes from BBPRT-7, DDPRT-6 and FFPRT-10 had the widest pH range for activity, with more than 80% of their maximal activity occurring at pH 9.0-11.5. All proteases had maximal activity at neutral pH and showed high residual activity over a broad range of pH.

When incubations at different temperatures with azocasein as substrate were done, the effect of temperature on the purified proteases was observed at Temperature range 10-50^{o}C. Enzyme from DDPRT-6 (*Bacillus subtilis*) shows optimum temperature at 20^{0}C and the enzyme was found stable at a temperature between 10-30^{0}C. The BBPRT-7 (*Bacillus licheniformis*) shows optimum temperature at 20^{0}C and the enzyme was found stable at a temperature between 10-30^{0}C. Enzyme from FFPRT-10 (*Pseudomonas fluorescens*) optimum temperature at 30^{0}C and the enzyme was found stable at a temperature between 10-35^{0}C. BBPRT-7, DDPRT-6 was the most active at 20^{0}C and FFPRT-10 at 30^{0}C. The three proteases showed to be thermolabile, compared with the commercial enzymes, when they were incubated for 1 h at various temperatures. The all tree psychrotolerant proteases reach thermal inactivation at temperatures 10^{0}C lower than the mesophilic metalloprotease. The enzyme was found stable at temperature between 10-30^{0}C.

Purified protease from DDPRT-6 (*Bacillus subtilis*) exposed tremendous stability and compatibility with a wide range of locally available commercial detergents at 20^0C. Purified protease from BBPRT-7 (*Bacillus licheniformis*) shows enhanced activity with Tween 80, wheel, surf and enzyme was sensitive to tide and SDS. The purified enzyme from FFPRT-10 (*Pseudomonas fluorescens*) was stable in presence of Tween 80, surf, SDS and wheel while inhibited by tide. The effect of various metal ions on protease production was evaluated. The purified proteases of DDPRT-6 and BBPRT-7 were inhibited by EDTA while protease activity of FFPRT-10 was increased. Proteolytic activities of all three enzymes were affected in the presence of 5 mM $Zn^{2+,}$ Hg^{2+} and Cu^{2+}. DDPRT-6 was the most sensitive among these protease producing strains.

One hundred bacterial colony were isolated on trybutyrin agar plates from eight samples of Gangotri glaciers. Out of 100 positive isolates eight isolates showed good zone of hydrolysis revealing to be capable of producing lipase. Eight lipase producing strains are designated as AAPRTL-10, BBPRTL-5, CCPRTL-8, DDPRTL-14, EEPRTL-5, FFPRTL-9, GGPRTL-06, GGPRTL-5. These isolates were subjected to the taxonomical characterization. Phylogenetic analysis using 16S rRNA indicated that starin AAPRTL-10 shared high similarity with *Pseudoalteromonas lipolytica*, FFPRTL-9 with *Bacillus sonorensis*, BBPRTL-5 with *Bacillus sphaericus*, DDPRTL-14 with *Arthrobacter psychrolactophilus*, GGPRTL-5 with *Stenotrophomonas maltophilia*, EEPRTL-5 with *Pseudomonas vancouverensis*, CPRTL-8 with *Aeromonas veronii*, GGPRTL-6 with *Bacillus licheniformis*.

Among these isolates GGPRTL-5 is potent lipase producing isolates at temperature 20^0c. The strain GGPRTL-5 was selected as the best producer of lipase and was identified as *Stenotrophomonas multophilia* on the basis of its taxonomical characterization.

Most purification schemes for lipases are based on multi step strategies. Cold active lipase was partially purified by precipitating with ammonium sulfate (40-60%) and using a single step ion-exchange chromatography on a DEAE-cellulose. Partially purified lipase was eluted out as fractions (with 0.1-1 M NaCl gradient) from DEAE-cellulose column with 28.80 fold purification and specific activity of 82.10 U mg^{-1}.

The maximum activity of lipase of GGPRTL-5 at pH 8 at 20^0C and the enzyme was stable between pH 8 and 9 at the indicated pH range when incubated at 20^0C for 24 h, but its activity decreased at pH at 10.

The activity of cold active lipase was determined at a wide range of temperature 10-50^0C. The optimum temperature for lipolytic activity of GGPRTL-5 was determined to be 20^0C. The enzyme activity was almost constant within 25-30^0C and gradually declined at temperature beyond 35^0C. The enzyme was stable up to 30^0C for 1 h and decreased at higher temperature.

The metal ion like Ca^{2+}, Mg^{2+}, Na^{+}, Fe^{2+} and Zn^{2+} at a concentration of 5.0 mM were able to enhance the activity of purified lipase while other ion like Hg^{2+},Cu^{2+},Co^{2+} had shown negative effect on the enzyme activity. Among the different inhibitors EDTA inhibit the cold active lipase. In contrast, lipase is stable in presence of DTT, SDS and Tween 80. The metal ion like Ca^{2+}, Mg^{2+}, Na^{+}, Fe^{2+} and Zn^{2+} were able to enhance the activity of purified lipase. Enhanced activity of

enzyme was due to ion shown that the lipase was metal dependent enzyme. Cold active enzymes produced from isolate GGPRTL-5 (*Stenotrophomonas maltophilia*) showed maximum activity at pH 9.0 and temperature 20°C and thus we can say that the enzyme may have various applications in industry.

The conclusions resulting from these investigations would provide a baseline data about cold adopted bacteria and their industrial significance. The improved understanding of the nature of the bacterial community in glacier ice developed in this study will also help provide a better foundation for more detailed studies of the ecophysiology and ecology of glacier ice bacteria and for the biotechnological applications of cold adapted bacteria in general. We have isolated three cold active protease producing strain DDPRT-6 (*Bacillus subtilis*), BBPRT-7 (*Bacillus licheniformis*), FFPRT-10 (*Pseudomonas fluorescens*). BBPRT-7, DDPRT-6 was the most active at 20^0C and FFPRT-10 at 30^0C. Enzymes from BBPRT-7, DDPRT-6 and FFPRT-10 had the widest pH range for activity, with more than 80% of their maximal activity occurring at pH 9.0-11.5. This pH dependence for activity makes these enzymes interesting for industrial applications, in contrast with other cold proteases with a narrow pH profile. Purified protease from DDPRT-6 (*Bacillus subtilis*) exposed tremendous stability and compatibility with a wide range of locally available commercial detergents at 20^0C.The current consumer demands and increased use of synthetic fibers, which cannot tolerate high temperatures, have changed washing habits towards the use of low washing temperatures. It was found that the lipase enzymes produced by cold adapted bacteria GGPRTL-5 showed maximum activity at pH 9.0 and temperature 20°C may have application in detergent industries.

This study is a one step effort for search of a novel cold active enzyme. Research outcome may give clear picture and base line data about cold active bacteria present in Indian glaciers and search of their biotechnological and industrial implications. In view of the implications of cols adapted bacteria and prospects of their enzymes it is high time for scientist to give their attentions for more focused study. More organized study is needed to explore implications of cold adapted bacteria and their enzymes isolated from Indian glaciers and in this regard attention is needed from government, regularity authorities, research institutes and other agencies concern.