# Studies on Microbial Communities from Satopanth Glacier Western Himalaya and Evaluation of their Biotechnological Applications

THESIS

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# 5. SUMMARY AND CONCLUSION

Satopanth Glacier, a major peak of Garhwal division is situated in Western Himalaya, Uttarakhand between 30°42'55"- 30°50'32" N latitude and 79°13'55"- 79°29'40" E longitude and altitude is 4,600 masl. Satopanth glacier is situated at the head of Alaknanda River and draining in north-westerly direction. It is approximately 15 km long and with an average width of 750 m and covering an area of 21.17 sq. km. (Nainwal et al., 2008). Glacier is one of the richest bio resource of unique microflora particularly bacteria, fungi and actinomycetes which have immense bio potential (Trivedi et al., 2012). Therefore monitoring and biological assessment of the Satopanth glaciers is important to assess the overall reservoir health. Cold adapted microorganisms are abundant and play an important role in the cold environment. Cold adapted microorganisms have distinct features due to their unique physiological and metabolic characteristics; henceforth, they are easily adapted to the extreme environments such as low temperature, acidic or alkali environment, and low nutritional content. These unique features have attracted many scientists to explore in depth. Cold adapted microorganisms were already proven as a potential agent for the production of many metabolites including industrial enzymes. These organisms are active not only in permanently cold areas but also in habitats which experience seasonal variation in temperature during late fall and spring. Therefore, it would be of much interest to study the nature of extracellular enzymes. The one main objective of the present study is to evaluate the biotechnological importance of cold adapted bacteria isolated from the soil samples of Satopanth Glacier, Western Himalaya. Present research is an also attempt to assess the bacteriological contamination in glacial water runoff from Satopanth and Alaknanda river system. With this in view, seven soil samples were collected from uppers stretch of

Satopanth and twelve water samples from glacier runoff for further study of biotechnological implication of cold adapted bacteria and for assessment of pollution load respectively.

The present studies revealed the presence of bacteria in samples of Satopanth glacier. Result indicate that total viable count in soil from Satopanth glacier varied from $2.7\times10^4$ to $12.7\times10^4$ cfu $g^{-1}$ soil at 20±2°C. The lowest bacterial counts were found at SG-4 while highest bacterial count was found at SG-7. Of the numerous CFUs appearing on the culture media plates, fifty seven distinct representative isolates were picked both based on unique morphotypes and also randomly from each plate for further studies. Due to their biotechnological importance twenty one isolates from upper stretch of glacier were selected and studied in detail to establish their taxonomic identity and their phylogenetic position. The results indicated that the twenty isolates may belong to five different genera on the basis of morphological and biochemical characteristics. These were primarily identified as the genera, *Bacillus, Pseudomonas, Micrococcus*, *Stenotrophomonas*, *Arthrobacter*. These glaciers are supposed to be highly diverse in bacterial population surviving at low temperature. The results suggested that the soil of Satopanth glacier could be an affluent source of technologically significant microbial pool.

To assessment of bacteriological contamination of study area further research has been done. The Total viable count from glacial runoff and Alaknanda river system value showed a regular trend. The highest TVC was noted in Badrinath ghat of Alaknanda River and Mana village, where the values were as high as 86.3 $\times10^4$ and 68.5 $\times10^4$, respectively. The lowest Value 8.4 $\times10^4$ and 12.3 $\times10^4$ recorded in Satopanth top and Narayan Parvat, respectively. Results for FC and FS counts have also shown a similar trend to TVC and TC. The MPN of faecal coliform organisms fluctuated from 2 to 75

MPN/100 ml. i.e., highest FC count was observed in Alaknanda at Badrinath (SR-11) 75 MPN/100 ml and lowest count was at Satopanth top (SR-2) 2 MPN/100 ml.

The present study revealed resistance against common antibiotics among the bacterial population in glacier and glacial runoff regions with low anthropogenic activities is very unusual, most of the *E. coli* isolates from Alaknanda river system exhibited multiple antibiotic resistance (MAR). Maximum resistance (for four out of seven antibiotics) was observed for Nalidixic followed by Kanamycin, Tetracycline, and Gentamycin in the isolates from Badrinath Dham. While minimum resistance (for one antibiotic) was found in isolates from three stations namely Chakratirath, Alkapuri and Narayan parvat. While isolates from three stations viz; Satopanth tal, Satopanth top and Shahsradhara showing susceptibility for all seven antibiotics.

In order to establish their phylogeny and their ability to grow at low temperature, and to generate cold adapted enzymes, we screened protease, lipase and amylase producing bacteria and further purification and characterization of all these three enzymes. In best of our knowledge till the date this part of Himalayas, remained unexplored for microbial diversity and their biotechnological potential. Therefore keeping in mind the potential applications of cold adapted microbes and their extracellular enzymes, we screened bacteria resembling protease, lipase and amylase activity from study area.

Twenty one isolates from seven soil samples of glacier were examined for protease production on PSC agar medium containing skimmed milk at temperature 20±2$^{o}$C. Eleven isolates screened for protease production, among the isolates, five isolates were found to be capable of producing protease at alkaline pH and at two different temperature 4$^{o}$C and 20$^{o}$C. Among them strain SGPR10 yielded highest proteolytic activity with a ratio of solubilisation zone front to colony diameter i.e. one potent protease

producing bacterial isolates, SGPR10 has been taken for further purification and characterization of enzyme.

This isolate SGPR10 was subjected to the morphological and biochemical characterisation according to Bergey's Manual of Systematic Bacteriology (Holt et al., 1994) and phylogenetic analysis (Tamura et al., 2013). Based on the morphological, physiological, and biochemical characteristics and phylogenetic analysis, the isolate SGPR10 was identified as *Bacillus aryabhattai.*

Extracellular protease from *Bacillus aryabhattai*, SGPR10 culture was subjected to partial purification. Enzyme was partially purified to 3.1-purification fold with the increase of specific activity 1328.32 U/mg and yield of 31.5% by ammonium sulphate fraction and dialysis.

The optimum temperature for purified enzyme activity was determined by assaying the caseinolytic activity at different incubation temperatures 4, 10, 20, 30, 40, and 50$^{o}$C. Enzyme from *Bacillus aryabhattai*, SGPR10 shows optimum activity at temperature 10$^{o}$C and found stable between 10-30$^{o}$C. The proteases showed to be thermolabile, compared with the commercial enzymes, when they were incubated for 1 h at various temperatures.

The optimum pH for maximum alkaline protease activity was determined by performing protease activity assay in buffers with different pH (5.0-11.0). Enzyme from *Bacillus aryabhattai*, SGPR10 shows optimum pH 10.0 and the enzyme was found stable between 8.0-10.0. These results indicated that the extracellular protease from *Bacillus aryabhattai*, SGPR10 is an alkaline protease. In broad-spectrum, all currently used detergent compatible proteases are alkaline in nature therefore they are suitable for laundry detergents, which is generally in the range of 8.0 to 12.0 (Rao et al., 1998).

Partially purified enzyme from SGPR4 was incubated with different detergent viz. SDS, Tween 80 and different commercial detergents like Wheel Tide (1%, w/v or v/v) for 1 h at 20ºC and then enzyme assay were performed. The enzyme exhibited unusual stability in presence of 1% SDS with 60.61% residual activity after 1 h at 20ºC. The enzyme also exhibited enhanced activity in detergent Tween 80, commercial detergent Wheel and Tide. In conclusion, results obtained in our study suggest that among the twenty one strains, SGPR10 proved to be potential alkaline protease producer; hence it was subjected to further characterization & identified as *Bacillus aryabhattai* strain SGPR10 by 16S rDNA identification and used for further study. The protease produced by strain SGPR10, was an alkaline protease and was active in alkaline conditions as well as stable at low temperature, indicating its potential use in detergent formulations & other industrial applications.

Isolation of lipase producing strains was carried out using tributyrin agar medium. Lipolytic bacteria are widely distributed in nature, with around 20% of several thousand microbes isolated from soil are found to be lipase producers as tested on solid media for lipase production (Jaeger and Eggert, 2002). Total twenty-one bacterial colonies were isolated on nutrient agar media from seven soil samples from Satopanth Glacier, Western Himalaya. Out of twenty-one, on the basis of zone formation, seven isolates were showing positive results while among them, four isolates were found to be capable of producing lipase at two different temperature 4ºC and 20ºC both. Among them, on the basis of zone diameter, one potent lipase producing isolate SGPR4, has been selected for further identification, partial purification and characterization.

On the basis of a clear zone around the colony on tributyrin agar a potential isolate, designated as SGPR4 had been selected for morphological and biochemical characterization (Holt et al., 1994). On the basis of colony morphology, microscopic

observations, biochemical and phylogenetic analysis the isolate SGPR4, was identified as *Pseudomonas* sp. maximum similarity with *Pseudomonas fluorescens.*

Most purification schemes for cold active lipases are based on multi step strategies. Cold active lipase was partially purified by precipitating with ammonium sulfate (60%) and using a single step ion-exchange chromatography on a DEAE-cellulose. Partially purified lipase was eluted out as fractions (with 0.1–1M NaCl gradient) from DEAE-cellulose column with 6.44-fold purification and specific activity of 120.47 U/mg.

There is a significant effect of temperature on cold active lipase production. Beyond the optimum temperature a sharp fall in the lipase production was observed. The activity of cold active lipase was determined at a wide range of temperature 10ºC-60ºC. Enzyme from *Pseudomonas* sp., SGPR4 shows optimum activity at temperature 20ºC and the enzyme was found stable between 20ºC-30ºC.

The pH of medium strongly affects many enzymatic processes and transport of compounds across the cell membrane (Kuddus and Ramteke, 2008). For determination of activity at different pH, buffers were used. In the present study cold active lipase from *Pseudomonas fluorescens,* SGPR4 shows optimum activity at pH, 8.0 and the enzyme was found stable between pH 7.0- 9.0. However, it was not stable at acidic pH. Lipases performing high stability and activity over a wide range of pH and activity under non-traditional conditions are of great interest. The relatively high thermolability of cold-adapted enzymes may therefore be advantageous as in order to avoid changes to food ingredients caused by undesirable side-reaction that would otherwise occur at higher temperatures.

Twenty-one isolates were examined for extracellular amylase production on starch agar media at 20±2ºC. Out of twenty-one, on the basis of hydrolysis zone, five

isolates (SGPR1, SGPR6, SGPR11, SGPR20 and SGPR21) were screened for production of cold-adapted extracellular amylase. Among these, three isolates were found to be capable of producing amylase at pH 8.0 and at temperature 4ºC and 20ºC. On the basis of solubilisation zone formation, i.e. on the basis of diameter of hydrolysis zone among them one potent amylase producing bacterial isolate, SGPR6 has been taken for further purification and characterization of enzyme.

On the basis of a clear zone around the colony on starch agar a potential isolate, designated as SGPR6 was selected for morphological and biochemical characterization (Holt et al., 1994). Based on the morphological, physiological, and biochemical characteristics and phylogenetic analysis, the isolate SGPR6 was identified as *Bacillus cereus.*

Amylase from *Bacillus cereus*, SGPR6 was subjected to partial purification. Amylase from *Bacillus cereus*, SGPR6 was subjected to partial purification. Most purification schemes for amylase are based on multi step strategies. Cold active amylase was partially purified by precipitating with ammonium sulfate (40-60%) and using a single step ion-exchange chromatography on a DEAE-cellulose. The amylase exhibited a specific activity of 116.23 U/mg, corresponding to a purification factor of 1.62-fold and a total yield of 12.29%.

Optimum temperature for alpha amylase produced by *Bacillus cereus,* SGPR6 was tested by incubating the enzyme at various temperatures such as 10°C, 20°C, 30°C, 40°C, 50°C and 60°C. *α*-amylase from *Bacillus cereus,* SGPR6 shows optimum temperature at 20ºC and the enzyme was found stable at a temperature between 20-40ºC. Therefore research suggests considerable different approach of *Bacillus cereus,* SGPR6 towards temperature among all other previous studied psychrotrophic amylolytic bacteria. Results from the present study provide baselines of evidence that *Bacillus*

*cereus,* SGPR6 could be a good candidate for the efficient for biotechnological application.

The effect of pH on enzyme activity was examined at different pH values (pH 5.0 to 11.0) at 20°C for determination of relative activity at pH, different buffers were used. The optimum pH for $\alpha$-amylase activity from *Bacillus cereus,* SGPR6 ranged from pH 5.0 to 11.0 with an optimum activity at pH 9.0. and the enzyme was found stable between pH 8.0-10.0. In the present work, the most desirable properties of amylase from *Bacillus cereus*, SGPR6 was its high activity at low temperature and stability at slightly alkaline pH, which also permitted its biotechnological application in various industries. For example, it could be applied as a detergent additive, as a desizing agent in textile processing and leather processing and in the food industry.

Researchers around the world are putting a lot of plunge towards understanding the pathways involved in production of novel secondary metabolites and to understand their species richness, functional and phylogenetic diversity and response under changing abiotic and biotic factors as they are answerable for functioning of the ecosystem. This study improved our understanding for the bacterial community in glacier ice and this study will also help to provide a better foundation for more understanding about ecophysiology and ecology of glacier microflora in general and cold adapted bacteria in particular. However, in our best knowledge not as much as study available for bacteriological contamination from runoff of Satopanth and Alaknanda river system, which have great relevance to geological exploration and tourism, in addition to environment and public health safety. It is also necessary to understand the pathogenic bacteria genera in the Himalayas river system and to develop measures that can serve as indicators of water pollution. Therefore this study may be relevant and useful for conservation of glacial as well as river system for the safety of aquatic life environment

and human health. This is a one attempt also for search of novel cold active enzymes from glacier colonizing cold adapted bacteria. Research findings 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 cold adapted bacteria and prospects of their enzymes it is high time for scientist to give their attentions for more focused study. Conclusive statement is that the cold active enzymes might also have tremendous applications in industrial processes, which require low temperatures. Therefore it could be cost effective and environment friendly. More organized study is required to explore biotechnological 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.