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Isolation and Screening of Cold Active Alkaline Protease for Bioremediation

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Abstract—Cold active alkaline proteases from microbial sources are of great importance due to their wide spectrum applications in baking and detergent industries, bioremediation, leather processing, bio-film degradation, pharmaceuticals industry, meat tenderizers, protein hydrolyzates, food products and even in the waste processing. Bacillus sp. - the most widely exploited alkaline proteases producer, are often commercially used in bioremediation mixes. In present study 28 isolates were isolated from the soil sample of Kashmir region and screened for protease activity on skim milk agar and casein agar medium. All the isolates showed protease activity at alkaline pH ranging from 9-12 and at low temperature of 0-30ºC. Out of these, DLCP1 isolate was further characterized biochemically in order to establish their phylogeny and belongs to Bacillus species. Optimization of pH and temperature conditions for the production of enzyme were determined and found to be 9 and 25ºC respectively. The ability to work at low temperature and high alkophilic conditions of this enzyme could make it valuable ingredient for bioremediation processes which require low temperature.

Keywords*: Alkaline protease, cold active, Bacillus, Bioremediation, DLCP1.*

1. INTRODUCTION

Proteases are the enzymes that are plentifully present in nature and its ambiance. Proteases are found on inner and outer surfaces of living organisms that includes plants, animals and microbes [1]. These enzymes facilitate in breakdown of proteins into simpler form that exist between two amino acids of a polypeptide chain by the process of hydrolysis [2].

Alkaline proteases are those enzymes that flourish and stay active in alkaline conditions. Due to their stability in odd conditions, they are considered as the most imperative group of enzymes used for commercial purposes [3].

Alkaline proteases obtained from microorganisms are fundamental group of enzymes for a variety of physiological and commercial purposes. With time, the function of alkaline proteases has extended upto a range of industries starting from detergent industries and gaining value in other sectors such as tanning, photographic industries, pharmaceutical, leather processing, silk degumming, food and dairy, baking, pharmaceutical industries, silver recovery from x-ray films, waste management and others [4].

Enzymes that are shaped from cold-adapted microorganisms, show levels of utmost activity and stability in cold conditions and are recognized as cold-active enzymes [5]. These types of enzymes carry soaring catalytic efficiency at low and moderate temperatures that concerns for both basic research and industrial application [6]. These properties of cold active enzymes differentiate them from mesophiles that don't remain active at low temperatures and makes them useful for various biotechnological purposes.

Extracellular enzymes have been more often than not used in industrial sectors but through recent times these enzymes have they been studied as a means for enhancing bioremediation [7]. Fermentation process is carried out to produce extracellular enzymes and such enzymes have the ability to break down bonds within organic compounds. In other words they transform the complex structures that can be harmful to the nature and its surroundings into simpler or less toxic and more biodegradable forms. Enzymes unlike many microbes show activity and stability in wide range of pH and temperature. They remain effective particularly when they are immobilized on a carrier, and can degrade a wide variety of compounds. Alkaline proteases have shown the capability of replacing chemicals with enzymes that can help to decrease the pathogen counts that can be harmful, reduce the solids content, and increase de-flocculation in sludge [8]. Currently, the widespread use of extracellular enzymes for remediation is inhibited due to high production costs but with continuous research researchers are trying to make the production of enzymes cost effective. Also bench studies and field studies have shown enzymatic treatment to be feasible options for bioremediation.

2. MATERIALS AND METHODS

2.1 Collection of sample producing alkaline proteases from various ecological niches.

The soil sediment samples used for the isolation of alkaline proteases were collected from three different lakes of Kashmir region namely Wular lake, Manasbal Lake and Dal lake. The

samples from the above habitats were collected at random from the upper layer of soil not exceeding 5-6 cm depth under sterile conditions and were transferred into sterilized polythene bags. The samples were then brought and stored under cold conditions until processed.

2.2 Isolation and screening of cold active alkaline proteases

Soil from each lake sediment sample was weighed and taken in 1gm quantity and transferred into test tubes using sterile conditions. Equivalent amounts of distilled water were added into the test tubes and soil sample was mixed with distilled water by vigorous vortexing and serial dilutions upto 10^{-8} were made. 200**µ**l of appropriate dilution was added to petri plates on casein agar medium with pH ranging from 6-12. After that the petri plates were kept under incubation at 10°C for 72hrs. A clear zone around the colonies demonstrated the hydrolysis of casein agar as shown in the figure below (Fig.1) and indicated the alkaline protease production by the organism.

Fig. 1: Clear zone around the colonies indicates alkaline protease production.

These colonies were firstly marked, picked and further purified by streaking on casein agar medium and skimmed milk agar medium. The purified proteolytic isolates that showed the best results were picked, stored and preserved in glycerol stocks for further use. A total of 28 isolates were thus collected.

2.3 Identification of isolate DLCP1 showing protease activity

Various biochemical tests were carried out for phylogenetic identification of positive isolates. The tests showed that the isolate DLCP1 belongs to genus *Bacillus.*

2.4 Optimization of pH and Temperature conditions

Protease activity assay was performed to determine the optimum pH for maximum alkaline protease activity. The activity was carried out in buffers with different pH ranges (pH 6-12). Following buffers were used for the production of enzyme activity for the different pH ranges: pH 6.0 to 8.0, 50 mM sodium citrate; pH 9.0 to 10.0, 50 mM sodium phosphate; pH 11.0 to 12.0, 50 mM Tris-HCl at 4°C. 1% casein was taken as substrate in the assay and dissolved in the buffers.

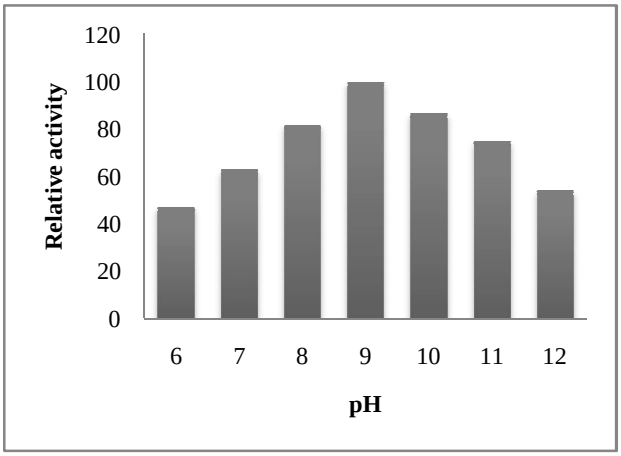
Hydrolytic activity of DLCP1 was measured at different temperatures (0 to 30°C) for 30 min at pH 9.0 to determine the temperature optima for enzyme activity.

3. RESULTS

Twenty eight bacterial isolates were isolated from three different samples of Kashmir region which showed proteolytic activity at alkaline pH ranging from 6-12 and low temperature ranging from 0-10°C.

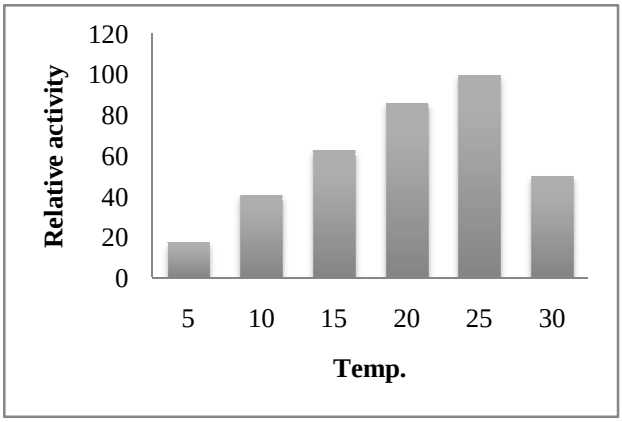
Out of these 28 isolates, the DLCP1 isolate was further characterized biochemically in order to establish its phylogeny and belongs to *Bacillus* species.

After identification of the isolate, pH and temperature optimization was determined by performing protease activity assay. Different buffers with different pH (6-12) were used and results indicate that the isolate DLCP1 shows maximum activity at pH 9 as shown in the graph below (Graph 1).



Graph 1 DLCP1 showing maximum activity at pH 9

Hydrolytic activity of DLCP1 was measured at different temperatures (5 to 30°C) for 30 min at pH 9.0 to determine the temperature optima for enzyme activity and the isolate showed the maximum activity at 25°C as shown in the graph 2 below.



Graph 2 DLCP1 showing maximum activity at 25°C

4. DISCUSSION

The main objective of this study was to isolate cold active protease producing extracellular enzyme from soil samples of Kashmir region. To characterize them biochemically, in order to establish their phylogeny and their ability to grow at low temperature (≤ 10°C) and alkaline pH ranging from 6-12 with high levels of activity, the positive isolates were identified by the clean zone around the colonies. Cold active proteases have been reported from microorganisms that thrive in regions with extreme low temperature. These microorganisms from cold habitats are able to withstand the nature's extreme harsh cold conditions and can be found in arctic and Polar Regions, under the deep sea, glacial soil, glacier ice, permafrost, cold desert soil, sub-Antarctic sediments, sub-glacial water, alpine regions and other cold regions on earth [9].

Twenty eight isolates that grew and showed activity between 0-10°C and at pH around 6-12 were isolated from water samples collected at Wular, Manasbal and Dal Lake, Kashmir. All positive isolates produced active extracellular proteases on skim milk agar medium and casein agar medium when grown at 0-10°C temperature.

Out of these 28 isolates, DLCP1 was further characterized biochemically to identify the isolate up to the genus level and after performing all the biochemical tests, the microorganism was identified as *Bacillus* strain. Among all the bacterial species, *Bacillus* sp. is most commonly used for the production of alkaline proteases that can be further used commercially in bioremediation mixes.

5. CONCLUSION

It was found that the enzyme showed maximum activity at pH 9.0 and temperature 25°C and thus we can say that the enzyme produced from isolate DLCP1 was cold active and alkaline in nature and has great importance in Biotechnology industry as well as in bioremediation and waste management.

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