Partial Characterization of Cold Active Alkhophilic Protease from Kashmir

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***ABSTRACT*- Cold active alkaline proteases from microbial sources are of great importance due to their activity and stability in cold conditions and at alkaline pH. They can be used in diverse industrial applications as in detergents, textiles, baking, brewing, starch, animal food, leather and the pulp industries, including agricultural, chemical, and pharmaceutical applications. In present study 28 bacterial isolates were isolated from the soil sample of Kashmir region and screened for protease activity on skim milk agar and casein agar medium under cold (0-10ºC) and alkaline (pH 9-14) conditions. Among all the isolates showing protease activity, MLP1 isolate was further characterized. The enzyme produced by MLP1 was stable in the alkaline pH range of 9.0-14.0 with the pH 8.0 and optimum temperature at 8ºC. The protease showed highest thermal stability at 6 ºC. Future studies involve the cloning of gene encoding alkaline protease that can be used as a substitute to toxic chemicals used in leather industry and mostly in detergents. The ability to work at low temperature and high alkophilic conditions of this enzyme could make it valuable candidate in the field of enzyme biotechnology.**

Keywords: Alkaline Protease, MLP1, Cold active, Enzyme.

I. INTRODUCTION

Cold-adapted microorganisms that comprise psychrophiles and psychrotrophs are known to produce enzymes with high levels of activity at low temperatures; these enzymes are called cold-active enzymes [1-4]. Mesophilic or thermophilic microorganisms are also known to produce cold active enzymes but are less active at low temperatures [5].

Bacillus sp. is widely used in biotechnology for the large-scale production of enzymes which are useful for various purposes such as proteases. Bacterial microorganisms are considered as a major source to obtain industrially important protease till date. In fact, most of the alkaline proteases are especially derived and extensively studied from high yielding strains of *Bacillus* species [6]. The best known proteases acquire quality to withstand high alkaline and temperature conditions and can be useful in various industries such as baking and detergents [7].

Among the alkalophiles, cold active enzyme producing bacteria from microbial samples find important applications in biotechnological applications such as food industry and bioremediation. Cold active enzymes have many advantages over mesophiles as they reduce the risk of contamination and also save energy [8].

In present time, researchers are now trying to exploit cold active enzymes that display highly specific activity and catalytic efficiency at low temperatures have the capability to adapt to cold habitats [9]. These enzymes play a vital role in many industrial manufacturing processes.

Due to the limited source of information available on cold-active enzymes and their industrial application, it is vital to isolate new cold-adapted bacteria from different ecological niches and to identify production of cold adapted proteases.

In present study, we partially characterized a cold active alkaline protease; examine proteolytic activity and also the production of protease in cold conditions.

II. MATERIAL AND METHODS

A. Collection of soil sample.

The soil sample was taken from Manasbal Lake (34°15′N 74°40′E) of Kashmir region. Using aseptic techniques, soil sample was collected randomly from the upper layer of soil not exceeding 4-5 cm depth using pre-sterilized spatula and were transferred into sterilized polythene bag. The sample was then brought and stored under cold conditions until processed.

B. Isolation and screening of cold active alkaline protease.

Soil sample was taken in 1gm quantity and transferred into test tubes using sterile conditions. Equal 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. 100 l of appropriate dilution was added to petri plates containing nutrient agar medium with 1% casein and incubated at 10°C for 72hrs. A clear zone around the colonies demonstrated the hydrolysis of casein and indicated the alkaline protease production by the organism. Positive colonies were marked, picked and purified by streaking on casein agar medium as shown in the figure below. The purified proteolytic isolate was stored and maintained in glycerol stock.

C. Identification of isolate MLP 1 showing protease activity.

Various biochemical tests were carried out for the phylogenetic identification of isolates showing protease activity.

D. Optimization of pH, temperature and thermal stability.

The influence of pH on the enzyme activity was determined by measuring the enzyme activity at varying pH values ranging from 8 to 12 at 10ºC using different buffers, 50 mM sodium acetate (pH 4.0 and 6.0), 50 mM sodium phosphate (pH 8.0), 50 mM glycine-NaOH (pH 10.0) and 50mM KCl/NaOH buffer (pH 12.0) respectively.

The optimum temperature for protease activity of MLP1 was measured by determining its hydrolytic activity at different temperatures (0°C to 10°C) for 30 min at pH 9.0.

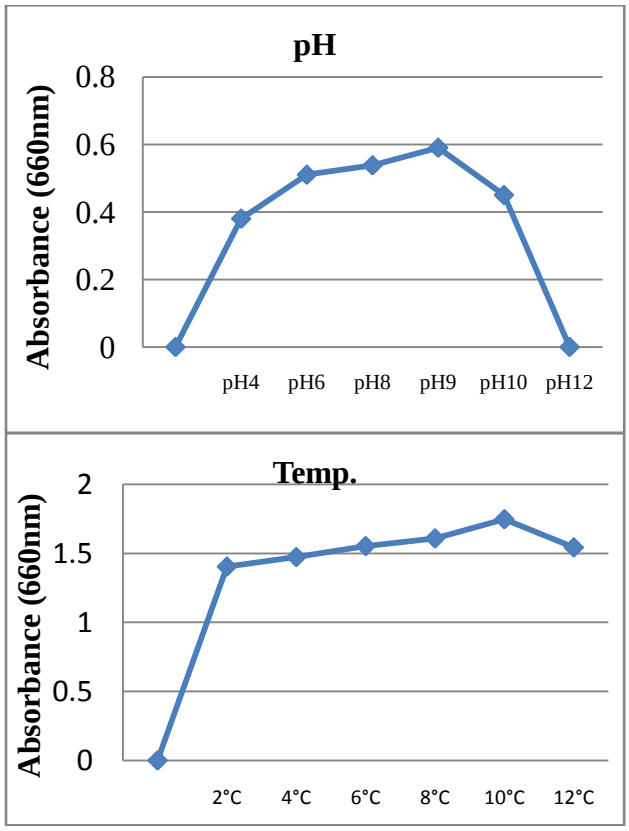
The thermostability of enzyme was determined by preincubating the enzyme for up to 1 hour at temperature ranging between 0°C and 10°C, followed by residual activity determination with added substrate (50µl autoclaved casein) at 10°C.

III. RESULTS

In present study, soil sample from Manasbal Lake of Kashmir was collected. Positive isolates were isolated from the soil sample showing proteolytic activity on casein agar and skimmed milk agar in alkaline pH conditions (8-12) and low temperature ranging from 0-10°C.

MLP1 isolate was further characterized biochemically in order to establish its phylogeny and belongs to Bacillus species.

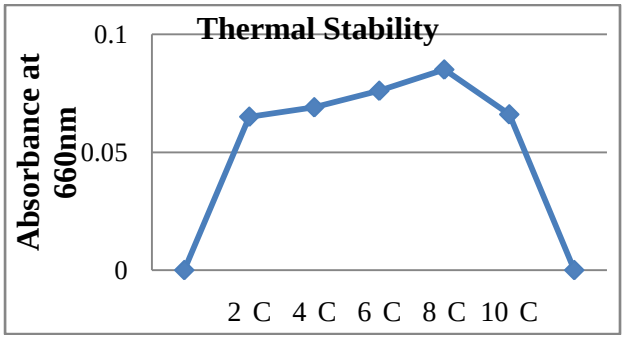
After identification, the optimum pH was determined performing protease activity assay with 1% casein (as substrate) dissolved in a mixed buffer containing sodium acetate, sodium phosphate, glycine-NaOH and KCl/NaOH (50 mM each) and incubated the enzyme in above mixed buffers of different pH values in the range of 8.0-12.0 at room temperature. The results indicate that the isolate MLP1 shows maximum activity at pH 9.



The optimum temperature for enzyme activity of MLP1 was measured by incubating the reaction mixture at different temperatures (-20 to 20°C) for 30 min at pH 12.0.

The hydrolytic activity was determined and it showed the maximum activity at 10°C.

Thermal stability of MLP1 was determined by incubating the culture broth for 1 hour at different temperatures (2°C, 4°C, 6°C, 8°C and 10°C). After incubation, 50µl of autoclaved casein (as substrate) was transferred into each culture tube and again incubated for 30 mins. The isolate showed maximum thermal stability at 8°C.



IV. DISCUSSION

The main aim of this study was to isolate novel enzyme-producing alkaline bacteria from bacterial samples from Kashmir, to characterize biochemically, in order to establish their phylogeny and their ability to grow at low temperature, and to generate cold adapted proteases showing proteolytic activity against casein agar and skimmed milk agar medium. The positive isolates were identified by the clean zone around the colonies. The 28 isolates were screened for protease activity in cold conditions.

Out of these isolates, MLP1 showed best protease activity and was further characterized biochemically to identify up to the genus level and after performing all the biochemical tests, the microorganism were identified as *Bacillus* strain. Previous studies have strongly showed that *Bacillus* Sp. are the most widely exploited alkaline proteases producer, are often commercially used in various biotechnological applications.

MLP1 was competent to grow in cold conditions and to biosynthesize proteases cold-adaptive at low temperature (10ºC). The protease showing proteolytic activity generated by this cold adapted *Bacillus* strain could be valuable candidates in various biotechnological applications when the metabolic ability of the mesophilic protease producing bacteria is reduced due to low temperatures. These enzymes might also have valuable applications in other industrial processes, which require low temperatures.

V. CONCLUSION

It was found that the enzyme showed maximum activity at pH 9.0 and temperature 10°C. The enzyme showed maximum thermal stability at 8°C, proving the enzyme produced from MLP1 to be cold active in nature that retains its activity in alkaline conditions. Thus MLP1 has the potential to be of great importance in Biotechnology industry.

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