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RESEARCH ARTICLE

ISOLATION, SCREENING AND CHARACTERIZATION OF COLD ACTIVE ALKALINE PROTEASE

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ABSTRACT

Cold active enzymes are produced by microorganisms existing permanently in cold habitats located in polar zones at high altitudes or deep sea. Among cold active enzymes cold active proteases constitute an important group of enzymes which have high catalytic efficiencies at lower temperatures and have important applications in industries like detergents, food, pharmaceutical, silk, leather and environmental bioremediation. The aim of the present study was isolation and screening of Cold active alkaline protease producing bacteria from the soil sample collected from Manasbal Lake, Kashmir, India. With this aim the Soil sample was collected from Manasbal Lake, Kashmir and bacterial microflora can be isolated and 50 numbers of isolates were isolated. These isolates were screened for protease activity and out of 50 isolates screened for protease activity only 3 isolates showed protease activity at 10°C. These isolates were identified as *Bacillus sp.*, *Micrococcus sp.*, and *Staphylococcus sp.*, on the basis of morphological and biochemical characterization and named as MLP-1, MLP-2 and MLP-3. The mlp-1 enzyme was characterized and determination of optimum pH and temperature can be done at which the enzyme showed a maximum activity. The mlp-1 enzyme was cold active and alkaline as it showed a maximum activity at pH 12 and temp 4°C.

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INTRODUCTION

Proteases are enzymes which catalyze the hydrolysis of peptide bonds. Microorganisms are capable of producing these enzymes intracellularly and extracellularly. The isolation of proteases especially the extracellular proteases of microbial origin is easy and economical. Alkaline proteases are referring to proteolytic enzymes which work optimally in alkaline pH (Barett, 1994 and Gupta *et al.*, 2002). The vast diversity of proteases, in contrast to the specificity of their action has attracted worldwide attention in attempts to exploit their physiological and biotechnological applications (Fox *et al.*, 1991; Poldermans, 1990) like food and feed industry, peptide synthesis, leather industry, management of industrial household waste, photographic industry, medical usage, silk gumming and detergents industry etc. The use of alkaline proteases in detergents, food, leather and silk industries has remarkably increased in recent years (Joo *et al.*, 1995). Of all proteases, alkaline proteases produced by *Bacillus species* are of utmost importance due to the stability of their enzymes under different environmental conditions (Kazan D *et al.*, 2005). Moderate halophiles constitute a very interesting group of organisms with great potential for use in biotechnology because of their high activities at high salt concentrations (Ventosa *et al.*, 1995). Recently, there has been an increased emphasis on cold active enzymes produced by microorganisms existing permanently in cold habitats located in polar zones at

high altitudes on deep sea. Cold active enzymes are characterized by high catalytic efficiency at low and moderate temperature at which homologous mesophilic enzymes are not active. Microorganisms that can degrade proteins rapidly at low temperatures are necessary for treatment of protein containing Wastewater because the temperature of such wastewater drainage is relatively low (approx. 5-25°C). The naturally occurring alkaline environments comprise alkaline soils, soda lakes, alkaline springs etc. Isolation and screening of bacteria from these natural environments can be supposed to be useful for obtaining bacterial strains with the potential of yielding alkaline protease. The aim of this study was isolation and screening of Cold active alkaline protease producing bacteria from the soil sample collected from Manasbal Lake, Kashmir, J&K, India. The promising strains in this study may be used in various economic industrial applications.

MATERIALS AND METHODS

Collection of soil sample

The soil sample was collected from Manasbal lake Kashmir, J&K, India. The soil sample was collected in polythene bag of capacity 1 kg. Sample collection was performed by the following standard methods of APHA. The soil sample was carried to the laboratory in a well packed box sealed in ice to avoid the contamination and stored at -4°C.

Isolation of Bacterial microflora from soil sample

Isolation of Bacterial microflora from soil sample from the soil sample by Serial Dilution method. The soil sample was

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suspended in water by vigorous vortexing and serial dilutions were made upto 10⁻⁹ in sterile water. 0.1 ml of appropriate dilution was added to Petri plate on skim milk and Trypticase soy agar containing pH 9 and incubated at 10° C. A clear zone of skim milk hydrolysis around the colonies indicated alkaline protease production by the organism. These colonies were picked and purified by streaking on skim milk agar. The purified proteolytic isolates were stored and maintained in nutrient agar slants (pH 9) by sub culturing at monthly intervals. More than 50 isolates were thus collected.

Screening of isolates for protease activity at 10°C

The isolates were screened for protease activity using a 1% Casein or skimmed milk as a substrate at 10°C.

Morphological and Biochemical characterization of protease active isolates

The morphological characterization of protease active isolates was done by examining the Colony size, Margin Color, Arrangement Texture and Gram's staining of bacterial colonies. The detection of biochemical characteristics which aid in the identification and classification of microorganisms that appears morphologically identical. A series of biochemical tests used to identify the microorganisms are as follow:

IMViC Tests

IMViC reactions are a set of four useful reactions that are commonly employed in the identification of members of family enterobacteriaceae. The four useful reactions are: Indole test, Methyl Red test, VogesProskauer test and Citrate utilization test.

Indole Test

Some bacteria can produce indole from amino acid tryptophan using the enzyme tryptophanase. The tubes of Tryptone/Peptone were inoculated using sterile technique with given bacterial cultures. One tube was served as control. Incubate at 37°C for 24-48 hours. Few drops of Kovac's reagent were added to the culture medium Production of indole is detected by using Ehrlich's reagent or Kovac's reagent. Indole reacts with the aldehyde in the reagent to give a red color. An alcoholic layer concentrates the red color as a ring at the top indicates positive indole test.

Methyl Red test

This test is used to detect the ability of an organism to produce and maintain stable nucleic acid end products from glucose fermentation. The tubes of MR-VP broth were inoculated using sterile technique with given bacterial cultures. One tube was served as control. Incubate at 37°C for 24-48 hours. Test the presence of mixed acids by the addition of methyl red indicator. A red color represents a positive test.

Voges Proskauer Test

While MR test is useful in detecting mixed acid producers, VP test detects but yleneglycol producers. The tubes of MR-VP broth were inoculated using sterile technique with given bacterial cultures. One tube was served as control. Incubate at

37°C for 24-48 hours. Test the presence of acetyl-methyl carbinol by the addition of Barrit's reagent. Shaked well and allowed to stand for 15 minutes. The reagents used for the VP test are Barrit's A (α -naphthol) and Barrit's B (40% KOH). When these reagents were added to the broth in which acetyl-methyl carbinol is present, they turn a pink burgundy color represents a positive VP test. This color may take 20-30 minutes to develop. The presence of rose colouration is a positive result.

Citrate Utilization Test:

This test is used to differentiate among enteric bacteria on the basis of their ability to utilize/ferment citrate as the sole carbon source. Bromomethyl blue is green when acidic (pH 6.8 or below) and blue when alkaline (pH 7.6 or higher). Simmon's citrate agar slants were prepared. Slants were inoculated by means of streak inoculation and incubated 37°C for 24-48 hours. The change in color was examined. Observe the slant culture for the growth and colouration of the medium.

Fermentation of glucose, lactose and sucrose

In this set of tests, it was able to determine if the bacterium can ferment glucose, can hydrolyze lactose into glucose and galactose and then ferment either of the monomers released, usually only the glucose, and can hydrolyze sucrose into glucose and fructose and then ferment either of the monomers released. Fermentation simply uses an organic molecule as an electron acceptor, with the result being the production of organic acids (and a pH change in the medium). We will also be able to determine if the bacterium can produce a gas usually carbon dioxide during the fermentation process. Using an aseptic technique, a small inoculum of each isolate was transferred into each of the three broths (glucose, sucrose, lactose). Incubated the inoculated broth at 37°C for 24-48 hours. Observed each broth for Growth or no growth, Growth with red color (i.e., no change in color compared to the uninoculated control) - indicates the bacterium cannot ferment the sugar in the tube (either cannot ferment the either of the monomers or cannot hydrolyze the dimer to release monomers) or doesn't produce any organic acids if fermentation does takes place.

Triple sugar iron agar test

This test is used to differentiate among members of the Enterobacteriaceae and to distinguish between the gram-negative enteric bacilli from other groups of intestinal bacilli based on carbohydrate fermentation and the production of hydrogen sulfide. The TSI agar slant were prepared and the pure culture into the medium, upto the butt of the TSI tube, and then streak the needle back and forth along the surface of the slant. Incubate at 37°C for 24 to 48 hours. Examine slants to observe alkaline slant (red) and acid butt (yellow) with or without gas production (breaks in the agar butt) or Hydrogen sulfide (H₂S) production.

Catalase test

Catalase is an enzyme that decomposes hydrogen peroxide into oxygen and water. This test is commonly used to differentiate Streptococci (negative) for Staphylococci (positive). During

aerobic respiration in the presence of oxygen, microorganisms produce hydrogen peroxide which is lethal to the cells. With loop cells were transferred from the culture of a well isolate colony to a glass slide. The 1-2 drops of 3% Hydrogen peroxide were added to the bacterial cells. Observe each culture for the presence or absence of gas bubbles.

Urease test

This is a useful diagnostic test for identifying bacteria, especially to distinguish members of the genus proteus from the gram negative pathogens. Prepared urea agar medium by adding filtered urea to the basal medium and the medium to solidify in a slanting position to form a slope. Inoculate slope heavily over the entire surface and stab with loop/nichrome wire. Incubate inoculated slope at 37°C in a water bath or no a hot block or in an incubator. Examine slopes after 4 hours and after overnight incubation. Examine the slants as to their color for the presence of urease (red color) or for no urease (yellow color).

Hydrogen Sulphide production test

The hydrogen sulphide production can be detected by incorporating a heavy metal salt containing lead ion as a H₂S indicator to a nutrient culture medium containing a cysteine and sodium thiosulphate as the sulphur substrates. Hydrogen sulphide a colourless gas when produced reacts with the metal salt forming visible black ferrous sulphide precipitates. Prepared SIM agar slants in test tubes. Aseptically inoculate each experimental organism into its appropriately labeled tube by means of stab inoculation. The last tube will serve as control. Incubated all cultures for 24 to 48 hours at 37°C. Examine the tubes for the presence or absence of black colouration along the line of stab inoculation.

Characterization of mlp-1 enzyme

Determination of optimum pH

Flasks with broth containing the optimum concentration of substrate (0.1 M Sodium Acetate Buffer) and 1% Casein are taken and the pH of the broth is adjusted to 8.0, 10.0, 12.0 and 14.0 in different flasks using 1N HCl and 1N NaOH and sterilized. The cultures are inoculated and incubated at temperature 20°C. At the end of incubation period the cell free culture filtrate is obtained and used as enzyme source and Absorbance is taken at 540 nm.

Determination of optimum temperature

Flasks with broth containing the optimum concentration of substrate (0.1 M Sodium Acetate Buffer) and 1% Casein at pH 12 was inoculated with overnight grown selected bacterial strain. The broth was incubated at different temperatures from -20°C, -10°C, 0°C, 4°C, 10°C, 15°C, 20°C for 24 hr. At the end of incubation period the cell free culture filtrate is obtained and used as enzyme source and Absorbance is taken at 540 nm.

RESULTS AND DISCUSSION

Microbial profile of soil sample

From the soil sample 50 numbers of isolates were isolated.

Screening of protease activity at 10°C

The 50 isolates were screened for protease activity at 10°C temperature out of which only three isolate showed a protease activity at 10°C. They are abbreviated as MLP-1, MLP-2 AND MLP-3.

Morphological Characterization

The 3 protease active isolates were recovered from soil sample were characterized on the basis of colour, shape, texture, margin and arrangement are showed in Table 1. From staining results we come to know about type, shape of bacteria from Gram's staining. Results indicated that all the protease active isolates are Gram positive bacterial strains are showed in Table 2.

Table 1. Morphological characters of protease active isolates

S.NO.	SHAPE	SIZE	COLOUR
MLP-1	Round	Small	White cream
MLP-2	Round	Moderate	Yellow
MLP-3	Round	Large	White cream

Table 2. Morphological characterization of protease active isolates

STRAIN NO	STAINING	CELL ARRANGEMENT
MLP-1	Gram +ve	Rod
MLP-2	Gram +ve	Cocci
MLP-3	Gram +ve	Cocci

Biochemical characterization of protease active isolates

Biochemical characterization was done to characterize the protease active isolates. The results of various biochemical tests performed are shows in Table 3 and the protease active isolates MLP-1, MLP-2 AND MLP-3 are identified as *Bacillus sp.*, *Micrococcus sp.*, and *Staphylococcus sp.*, as showed in Table 3.

Table 3. Biochemical characters of protease active isolates

Sample no.	Indole test	MR	VP	Citrate utilization	urease	Catalase	Lactose fermentation	Glucose fermentation	Sucrose fermentation	TSI/Aar test	H ₂ S test	Identified organism
MLP-1	-	-	-	-	+	+	-	+	-	-	-	<i>Bacillus sp</i>
MLP-2	-	+	-	-	+	+	-	+	-	-	-	<i>Micrococcus sp</i>
MLP-3	-	+	-	-	+	+	-	+	-	-	-	<i>Staphylococcus sp</i>

Table 4. Absorbance (O.D) of mlp-1 enzyme at different pH

pH	Absorbance
8	0.002
10	0.012
12	0.022
14	0.014

Table 5. Absorbance (O.D) of mlp-1 enzyme at different Temperatures

Temperature	Absorbance
-20	0.001
-10	0.002
0	0.004
4	0.008
10	0.007
15	0.004
20	0.002

Characterization of mlp-1 enzyme

Determination of optimum pH

The influence of pH on enzyme activity of mlp-1 was shown in Fig 1. The protease enzyme showed an activity in the range from 8 to 14 with a maximum observed at pH 12.

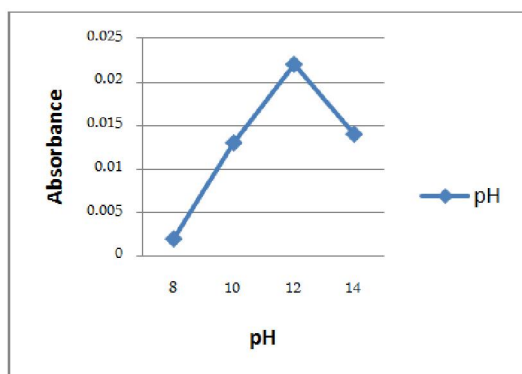


Fig. 1. Influence of pH on enzyme activity

Determination of optimum temperature

The influence of temperature on enzyme activity of mlp-1 was shown in Fig 2. The protease enzyme showed an activity in the range from -20°C. to 20°C with a maximum observed at 4°C.

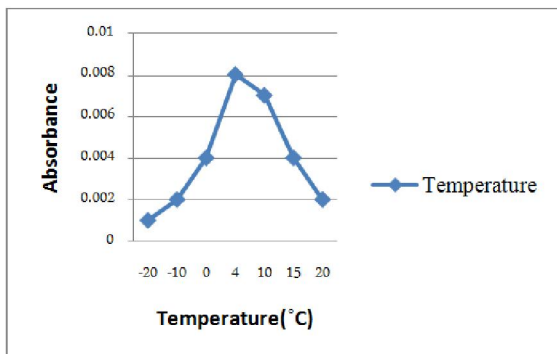


Fig. 2. Influence of Temperature on enzyme activity

DISCUSSION

In the present study the soil sample was collected from Manasbal lake Kashmir (J&K). The isolation of bacterial microflora can be done from the soil sample using a Trypticase Soy Agar media. The 50 isolates were screened for protease activity at 10°C temperature out of which only three isolate showed a protease activity at 10°C. All the protease active isolates were identified upto genus level by morphological and biochemical characterization. They are abbreviated as MLP-1, MLP-2 and MLP-3 and identified as *Bacillus sp.*, *Micrococcus sp.*, and *Staphylococcus sp.* The mlp-1 enzyme from MLP-1 strain was further characterized to determine the optimum pH and temperature at which mlp-1 enzyme showed a maximum activity and found that it showed an maximum activity at pH 12 and temperature 4°C. So we can say that the mlp-1 enzyme from MLP-1 strain i.e. *Bacillus species* was cold active and alkaline could proved it as a promising candidate for industrial use.

Conclusion

From the present study it can be concluded that the mlp-1 enzyme from isolate MLP-1 as *Bacillus sp.*, was cold active alkaline protease as it showed an maximum activity at pH 12 and temperature 4°C. Proteases produced from *Bacillus sp.*, are by far the most important group of enzymes being industrially exploited.

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