International Journal of Advanced Biotechnology and Research(IJBR)
ISSN 0976-2612, Online ISSN 2278–599X,
Vol5, Issue4, 2014, p576-581
http://www.bipublication.com

Isolation, Screening and Characterization of Cold Active Alkaline Protease from wular Lake of Kashmir Region

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[Received 10/08/2014, Accepted-07/10/2014]

ABSTRACT

Bacterial alkaline proteases are of great importance due to its wide spectrum applications in detergent industries, bioremediation, food industries, and leather processing, bio-film degradradation, 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 for industrial purposes. In present study total of 24 isolates were isolated from the soil sample of Wular lake 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 8-12 and at low temperature of 0-10ºC. Out of these, WLP1 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 12 and 10ºC respectively which showed that the protease from WLP1 is cold active in nature and has great application in biotech industries.

Key words: Lake, alkaline protease, cold active, Bacillus, WLP1.

INTRODUCTION

Proteases are the most important industrial enzymes. Proteolytic enzymes are degradative enzymes which catalyze the cleavage of peptide bonds in other proteins [1]. They execute a wide variety of functions and the amount of enzyme produced greatly depends on various important biotechnological applications [2]. Proteases are currently classified into 6 groups: Serine proteases, Threonine proteases, Cysteine proteases, Aspartic acid proteases, Mettaloproteases and Glutamic acid proteases [3].

With increasing emphasis on environmental protection, the use of microbial enzymes particularly from extremophiles has gained considerable attention during the last several years in many industries, including manufacturing of chemicals, textiles, pharmaceuticals, paper, food and agriculture chemicals [4]. Also alkaliphiles are reported to be a rich source of alkaline active enzymes, for example, amylase, protease, cellulose, xylanase and other enzymes that have numerous applications in many industrial

processes due to an interest in their physiological adaptation to high pH [5].

Microbial proteases, especially from *Bacillus* species have traditionally held the predominant share of the industrial enzyme market of the worldwide enzymes sales with major application in detergents formulations [6].

Microorganisms are considered potentially to be the most suitable sources of alkaline protease for industrial application. Among the various bacteria Bacillus Sp. are found to be the major group producing protease [7].

Alkaline proteases, which are referring to Proteolytic enzymes which work optimally in alkaline pH are the main enzymes among proteases and constitute 60-65% of the global industrial enzyme market [1] and has been estimated to reach US $ 4.9 billion by 2013 [8-9].

Though several microorganisms such as bacteria, fungi, yeast, plant and mammalian tissues are known to produce alkaline proteases [10-11]. Using of cost effective growth medium for the production of alkaline proteases from an alkaliphilic Bacillus Species is especially important [12]. Among bacteria, Bacillus species are specific producers of extracellular alkaline proteases [13].

Microorganisms growing at low temperature regions are important for their metabolic contribution in the ecosphere as well as for their enzymes that provide a wide biotechnological potential, offering numerous economic and ecological advantages over the use of organisms and their enzymes which operate at higher temperatures [14-19].

Cold-active proteases have high catalytic efficiency at low temperature due to their high flexibility and higher turnover number (kcat) at the expense of Km or by optimizing both parameters [20]at which homologous mesophilic enzymes are not active, and are thermolabile.

These properties of cold-active enzyme are concern for both basic research and industrial application. The application of such enzymes enables lowering of the temperature and shortening of processing times without a loss of efficiency, which leads to save energy consumption. Proteases are one of the most important industrial enzymes, and they are a convenient tool whenever protein removal is needed [21].

Cold-active enzymes have relatively low optimal temperatures hence they are potentially useful in some industrial applications such as food processing, detergent additives and biotransformation of chemicals [17]. Thus, it is desirable to search for new source of cold-active proteases with novel properties from as many different sources as possible. There are several reports on cold-active proteases produced by microorganisms from different habitat [22]. The aim of this study to isolate cold active alkaline protease producing bacteria from the soil of Wular Lake of Kashmir and optimization of temperature and pH conditions for production of enzyme.

MATERIAL AND METHODS

Collection of sample

The soil sample used for the isolation of alkaline protease was collected from Wular lake (34°20′N 74°36′E), which is one of the largest fresh water lake in Asia, located in Bandipora district of the Indian state of Jammu and Kashmir. The sample from the above habitat was collected randomly from the upper layer of soil not exceeding 5-6 cm depth using pre-sterilized spatula and was transferred into sterilized polythene bags. The sample was then brought and stored under cold conditions until processed.

Isolation of bacterial flora from soil.

The soil sample was taken in 1gm quantity and was suspended in water by vigorous vortexing and serial dilutions were made upto 10^{-8} in distilled water. 200µl of appropriate dilution was added to petri plate on casein agar medium ranging pH from 8-12 and incubated at 10°C for 72hrs.

Screening of cold active alkaline protease

A clear zone of casein agar hydrolysis around the colonies indicated alkaline protease production by

the organism (Fig 1). These colonies were marked, picked and purified by streaking on casein agar medium and skimmed milk agar medium. The purified proteolytic isolates were stored and maintained in glycerol stocks. 24 isolates were thus collected.

Fig 1- Showing clear zone of casein agar hydrolysis.

Morphological Characterization of WLP1 enzyme

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**.**

Identification of isolate WLP 1 showing protease activity.

The Indole test, Methyl Red test, Voges Proskauer test and Citrate utilization test, Urease test, Catalase test, Lactose-Glucose-Sucrose fermentation test, TSI Agar test and H_2S were carried out for phylogenetic identification of positive isolate. With the help of these biochemical tests we were able to identify the micro-organism upto the genus level.

Optimization of pH and Temperature conditions.

The optimum pH for maximum alkaline protease activity was determined by performing protease activity assay in buffers with different pH (pH 8-12). Following buffers were used for the production of enzyme activity for the different pH ranges: pH 8.0 to 10.0, 50 mM sodium citrate; pH 11.0 to 12.0, 50 mM sodium phosphate; pH 13.0 to 14.4, 50 mM Tris-HCl at 4°C. For that purpose, 1% casein (substrate) used in the assay was dissolved in the buffers.

The optimum temperature for enzyme activity of WLP1was measured by determining its hydrolytic activity at different temperatures (-20 to 20°C) for 30 min at pH 12.0.

RESULTS

In the present study, 24 bacterial isolates were isolated from the soil sample of Wular Lake which showed proteolytic activity at alkaline pH ranging from 8-12 and low temperature ranging from 0-10°C.Out of these 24 isolates, the WLP1 showed maximum protease activity and the isolate was further characterized biochemically in order to establish its phylogeny. The WLP1 isolate was further characterized on the basis of colour, shape, texture, margin and arrangement.

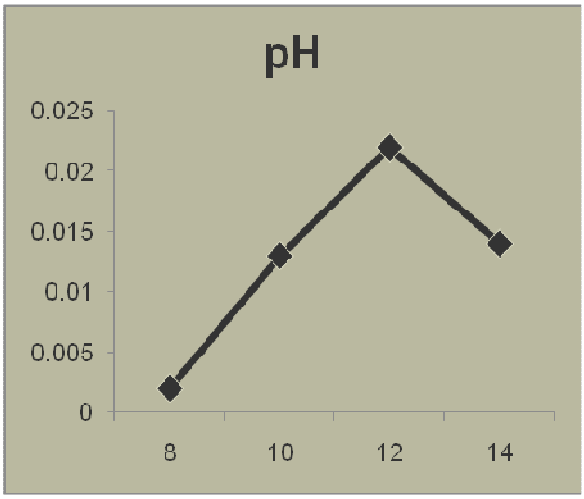
Gram staining results allow us to figure out the type and shape of bacteria. Results indicate that the protease active isolate(WLP1) is Gram positive bacterial strain and belongs to Bacillus species as shown in table 1.

Indole Test	**-ve**
MR Test	**-ve**
VP Test	**-ve**
Citrate Utilization Test	**-ve**
Urease Test	**+ve**
Catalase Test	**+ve**
Lactose Test	**-ve**
Glucose Test	**+ve**
Sucrose Test	**-ve**
TSI Agar Test	**-ve**
H_2S test	**-ve**
Identified Organism	**Bacillus Sp.**

Table 1. Biochemical characters of protease active isolate- WLP1

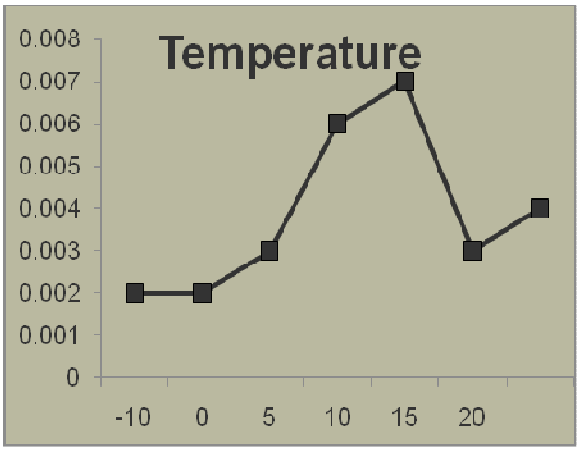
After identification of the isolate, the optimum pH for maximum alkaline protease activity was determined by performing protease activity assay in buffers with different pH (8-12) and results

indicate that the isolate WLP1 shows maximum activity at pH 12 as shown in graph 1.



Graph 1. Influence of pH on enzyme activity

The optimum temperature for enzyme activity of WLP1 was measured by determining its hydrolytic activity at different temperatures (-20 to 20°C) for 30 min at pH 12.0 and it showed the maximum activity at 10°C (Graph 2).



Graph 2. Influence of Temperature on enzyme activity

DISCUSSION

The main objective of this study was to isolate cold active protease producing extracellular enzyme from soil sampleofWular lake of Kashmir region. Isolates was characterized biochemically, in order to establish the phylogeny and the ability to grow at low temperature (0-35°C) and alkaline pH ranging from 8-14 with high levels of activity. Previously cold active proteases have been reported from microorganisms from cold habitats such as arctic regions, polar regions, deep sea and glacier soils, glacier ice, permafrost, cold desert soil, sub-Antarctic sediments, sub-glacial water, alpine regions and other cold regions on earth [23].

24 Isolates that grow and showed activity between 0-10°C and at pH around 8-12 were isolated and picked from water sample collected at Wular Lake, Kashmir. The positive isolate was identified by the clear zone around the colonies.

Out of these 24 isolates, the one which showed maximum activity was abbreviated as WLP1. The WLP1showed maximum activity at pH 12 and at temperature 10^0C, proving that the isolate is cold active and can be very useful tool in biotechnology industries. WLP1 was further characterized biochemically to identify up to the genus level and after performing all the biochemical tests, the microorganismwas identified as *Bacillus* strain.

The protease reported by Mohsinet. al, 2011 [24] showed optimal activity at 11.5 pH and 50^0C temperature and the protease reported by Pradeep et.al, 2012 [25] showed optimum activity at pH 10 and 37^0C temperature proving that the protease is alkaline in nature but do not show maximum activity at cold temperature. In the present study, the WLP1 showed maximum activity at 12 pH and 10^0C which shows that the protease is cold active in nature that concern for both basic research and industrial application. Hence, WLP1 can be used in wide range of applications such as pharmaceuticals, silk recovery, feather degradation, household process and few other areas of concern.Further research needs to be carried out for the search of new source of cold-active proteases with novel properties from as many different sources as possible.

CONCLUSION

It was found that the enzymes showed maximum activity at pH 12.0 and temperature 10°C and thus we can say that the enzymes produced from isolate WLP1 is cold active and alkaline in nature and has

great importance in biotechnology industry as well as other industries.

AKNOWLEDGEMENT

Authors are thankful to hon'ble V.C and management, Arni University for providing the necessary research facility.

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