Research Article

SCREENING AND MOLECULAR CHARACTERIZATION OF *BACILLUS* SP PR01 FOR THE PRODUCTION OF PROTEASE ENZYME

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ABSTRACT

The current study was attempted to isolate and characterize the protease producing *Bacillus* sp. Among the 3 strains isolated from soil sample collected from kuppam region, Chennai, India. One strain (PR01) exhibited potent proteolytic activity. Based on the morphological, biochemical and molecular characterization, the isolate (PR01) was identified as *Bacillus* sp. The strain was designated as *Bacillus* sp PR01. The maximum growth of *Bacillus* sp PR01 was observed at pH 7. The optimum temperature for the growth of *Bacillus* sp PR01 was found to be 27°C and incubation time for 2 days. The maximum growth was noted when yeast extract was used as a nitrogen source and dextrose was used as the carbon source. The production of enzyme *Bacillus* sp PR01 was carried out in production medium. The total protein concentration was determined and the specific activity was also performed by casein hydrolysis assay.

Keywords: proteolytic activity, *Bacillus* sp, Chennai

INTRODUCTION

Microbial proteases are one of the important groups of industrially and commercially produced enzymes and the initial screening methods for protease detection are of utmost importance². Many microorganisms were widely screened by using a number of substrates including, skimmed milk agar⁶ and casein-agar¹ for protease secretion. Identification and characterization of microbial proteases are prerequisites for understanding their role in the pathogenesis of infectious diseases as well as to improve their application in biotechnology. As well the discovery of new active metabolites must be followed by adequate biological testing⁷ for the rapid and sensitive techniques for the detection and characterization of microbial proteases which are highly desirable³. In the present study, an attempt was made to isolate and determine protease activity which is widespread applications in enzyme screening, especially protease.

MATERIALS AND METHODS

Collection and isolation of protease from soil samples

One soil sample was collected in sterile bag from Chennai location, Tamil Nadu, India. 1 g of soil sample was serially diluted in saline from the range 10^{-1} to 10^{-10} and inoculated by pour plate method and was incubated at 37°C for 24 h. Individual colonies were selected and streaked on nutrient agar plates for further studies. Pure culture of the soil isolates were maintained at 4°C.

Primary Screening

Casein Hydrolysis Method

Skim milk agar was prepared by adjusting pH of the medium to 7.0. A single line streak inoculation of the 3 isolates was made from their respective pure culture plates onto the labelled sterile skim milk agar plates. The plates were incubated for 24 h at 37°C and observed for the lysis.

Secondary Screening

Secondary screening was performed with the culture filtrate

of the potent isolate using well diffusion method. After two days of incubation, the cultures were harvested, centrifuged at 10000 rpm for 15 minutes at 4°C and the supernatant was collected. Culture filtrate of each isolate was placed at 100 µL in each well and incubated at 37°C for 24 h the development of clear zone around the well was observed and noted.

Morphological and Biochemical Characterisation

The potent producer of protease producing isolate was further studied for its morphological including gram staining and biochemical observations. The results were compared using Bergey's manual.

Enzyme production

A total of 50 mL of production medium was prepared and sterilized and was inoculated with a loop full of the bacterial strain PR01 and was incubated at 32°C for 24 h in a shaker at 120 rpm. The entire content of the inoculated production medium was centrifuged at 10,000 rpm for 20 minutes. The supernatant was filtered through a Whatman no. 1 filter paper. The extract was then preserved in the refrigerator at 4°C.

Enzyme assay

The culture supernatant of around 0.5 mL was added to 2 mL of casein solution 2.0 % (w/v) dissolved in Tris – HCl (pH 8.0) and incubated at 37°C for 10 minutes. The reaction was terminated by the addition of 2.5 mL of 0.1M trichloroacetic acid (TCA) and further incubated at 37°C for 30 minutes. It was centrifuged at 10,000 rpm for 10 minutes at 4°C. To 2.5 mL of supernatant, 3 mL of 0.5M Na₂CO₃ and 0.5 mL of 1N Folic Ciocalteu reagent were added, mixed well and incubated for 10 minutes. Absorbance was measured at 660 nm. The standard curve was prepared using different concentration of tyrosine. One unit of enzyme activity was defined as the amount of enzyme required to liberate 1 µmol of tyrosine per min under the defined assay conditions.

Optimization of protease production

The effect of incubation period on protease production was studied by growing potent isolate (PR01) on production medium up to 2 days. The impact of pH and temperature on protease production was investigated by subjecting to various pH (6.0– 8.0) and temperature (4–55°C) settings. The optimum carbon utilization for protease production was

determined by amending different sources such as Lactose, dextrose, xylose, sucrose, maltose and mannitol into the medium at a concentration of 0.5 % and nitrogen sources such as peptone, tryptone, ammonium sulphate, yeast extract and beef extract were supplemented as to study their influence on protease production.

Figure 1: Isolation of protease producing bacteria

Figure 2: Primary screening of PR01 showing zone of clearance

Figure 3: Selection of Potent strain PR01 by secondary screening

Figure 4: Optimization of pH

Figure 5: Optimization of temperature

Figure 6: Optimization of incubation time

Figure 7: Optimization of carbon source

Figure 8: Optimization of Nitrogen sources

Table 1: Morphology of isolated organisms from soil sample

Organism Name	Morphology
PR01	Elevated white colonies
PR02	Irregular, pale yellow
PR03	colonies and circular white colonies

Table 2: Biochemical characterization of *Bacillus*

TEST	PR01
GRAM STAINING	+
INDOLE	-
M.P	+
V.P	+
CITRATE	-
UREASE	-
NITRATE	-

Table 3: Determination of protease activity and yield of crude enzyme from isolate PPR01

Fraction	Volume	Potein content mg/ml	Total protein mg*ml	Tyrosine ug/ml	Activity units	Total activity units*ml	Specific activity (Units/mg)	Yield%	Purification fold
Crude enzyme	100	2.8	280	80.6	33.6	3359.4	12.00	100.00	1

RESULTS AND DISCUSSION

A total of 3 organisms were isolated from 1 soil sample (Figure 1) and studied for its morphological characteristic to identify the genus *Bacillus* (Table 1). The isolated organisms were subjected to primary screening which revealed the potential producers of protease enzyme. Interestingly, the isolate obtained from the soil, designated as PR01 remarkably hydrolyzed the substrate and produced a prominent and maximum clear zone of 15 mm in diameter when compared to other strains isolated (Table 2 Figure 2). Casein or skimmed milk agar plate assays allow principally for qualitative determinations of protease activity. The hydrolysis zone produced on the casein agar could be related to the

amount of protease produced⁴. The culture supernatant of the potent isolate PS01 hydrolyzed casein by forming halo zone (Figure 3), the morphological characterization of the isolate was found to white colored colonies on nutrient agar. The microscopic observation upon gram staining the PR01 strain was found to be gram negative rod shaped bacteria. It also showed the absence of capsule and endospore formation under the microscope. Based on the biochemical tests thegenus of the isolate was found to be *Bacillus* sp. (Table 3). Protease production is associated with growth of the organism in suitable production medium. It has been studied that extreme values of pH leads to the decrease in growth rate of the organism than that would be produced at optimum pH.

The effect of pH on growth is represented in (Figure 4) Based on the results obtained, the optimum pH for the growth of the organism was found to be 7 and the maximum biomass obtained was 1.38 g/L. It has been found that the optimum production for the growth of the isolate PR01 was found to favor the multiplication of the cells. The maximum biomass obtained at 27°C was found to be 1.33 g/L (Figure 5). The maximum growth rate was obtained at 24 h and the maximum biomass was found to be 0.9 g/L. The growth rate had decreased after 24 h (Figure 6). The maximum cell growth and biomass (1.3 g/L) were obtained at an incubation time of 24 h and the growth rate and biomass was slightly decreased at incubation times of 12 and 48 h. The maximum cell growth and biomass (1.7 g/L) were obtained when carbon source was dextrose and the growth rate and biomass was slightly decreased when sucrose and mannitol were used as the carbon source. Very less biomass and cell growth were obtained when carbon sources was xylose, maltose, and lactose. The effect of different carbon sources is represented in (Figure 7). The maximum cell growth and biomass (1.2 g/L) were obtained with nitrogen yeast extract and the growth rate and biomass was slightly decreased when beef extract and tryptone were used (Figure 8). Casein assay showed the specific enzyme activity of the crude supernatant was found to be 12.00 U/mg. (Table 3)

CONCLUSION

In conclusion, though many studies are focused on protease production very few reports have been published to screen proteases with potent activity. The results indicate that extracellular protease produced by *Bacillus* sp PR01 will

serve as an effective microbial protease enzyme. Further studies are needed to study for its complete potential of the enzyme protease.

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