Research Paper

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Extracellular Alkaline Protease from a Newly Isolated Bacillus stratosphericus DF: Isolation and Identification

Biochemistry

KEYWORDS : Alkaline protease, Bacillus stratosphericus, Skim milk, 16S rRNA sequence

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ABSTRACT

Bacterial extracellular alkaline proteases are of great importance due to its wide spectrum applications in detergent industries, bioremediation, food industries, and leather processing and bio-film degradation. The aim of the current study was to isolate protease producing bacteria from the soil sample collected at dump yard of dairy form industry, Vishakhapatnam. Total 60 bacterial colonies were isolated from the collected soil sample and all the bacterial strains were purified and tested for proteolytic activity. Out of these, one isolate named DF was selected on the basis of maximum hydrolysis for further study and it was also found to produce the highest yield quantitatively (492 U/ml). The selected strain DF was identified as Bacillus stratosphericus on the basis of morphology, biochemical tests and 16S rRNA sequence analysis. The present study is the first report to examine and describe production of alkaline protease from Bacillus stratosphericus.

Introduction:

The enzymes are considered as "green chemicals" owing to their eco-friendly in nature and possess wide range of applications from industrial sector to house-hold products [1]. Proteases, also known as peptidyl/peptide hydrolases (EC 3.4.21-24 and 99), are industrially useful enzymes, which catalyze the hydrolysis of a peptide bond in a protein molecule [2]. They represent one of the three largest groups of industrial enzymes and account for approximately 60% of the total enzyme sale in the world and they are the leaders of the industrial enzyme market worldwide [1][3].

Proteases are obtained from plant, animal and microbial sources. Microorganisms are the preferred source of these enzymes because of their rapid growth, the limited space required for their cultivation and the ease with which they can be genetically manipulated to generate new enzymes with altered properties that are desirable for their various applications [4][5]. They are capable of producing these enzymes intracellularly and extracellularly. The isolation of proteases especially the extracellular proteases of microbial origin is easy and economical. Seasonal fluctuations in the availability of raw material usually do not affect the enzyme production by microbes. There are possibilities for genetic and environmental manipulations to improve yield and properties of the enzymes. [4]

Microbial proteases are classified as acidic, neutral and alkaline depending on the pH at which they show maximum activity. Amongst these, alkaline proteases are of great interest because of their high proteolytic activity and stability under alkaline conditions. These enzymes find applications in detergents, feather processes, food processing, silk gumming, pharmaceuticals, bioremediation, biosynthesis and biotransformation. The majority of commercial alkaline proteases are produced by bacteria, especially Bacillus spp. [6] [7]. The lack of pathogenicity and the ability to grow in simple culture medium can also be accounted for their application in industry [8]. Several Bacillus species involved in protease production are eg. B.cereus, B.sterothermophilus, B.mojavensis, B.megaterium and B.subtilis [9].

Keeping in view the importance of protease, in this paper we report the isolation and selection of a bacterium (DF), which is a potent producer of extracellular alkaline protease, its identification and enzyme production.

2. Materials and Methods:

2.1 Microorganism:

An extracellular alkaline protease producing bacterial strain was isolated from soil sample collected at dump yard of dairy form industry in Visakhapatnam and was used for the present study. It was maintained by monthly sub culturing at 37°C and stored at 4 °C.

2.2 Isolation of Bacterial Strains:

Soil samples were collected from different regions of Visakhapatnam. About 1g of sample was suspended in sterilized distilled water and was subjected to serial dilution. 0.1ml from an appropriate dilution was spread on a nutrient agar plate and incubated at 37°C for 24 h. Well isolated colonies were picked and further purified by repeated streaking on nutrient agar plates.

2.3 Screening of Strains for Proteolytic Activity:

The isolated pure colonies were screened for proteolytic activities on Skim milk agar medium (skim milk 1%, Peptone 1%, sodium chloride 0.5%, Agar-Agar 2%, pH 10). The pH of the medium was adjusted to 10 with 1N NaOH before sterilization at 121°C for 15 minutes. The inoculated plates were incubated at 37°C for 24-48hrs and a clear zone of hydrolysis gave an indication of protease producing organisms. Depending on the zone of clearance, strain DF was selected for further experimental work.

2.4 Protease Production:

For enzyme production, the selected strain was cultured in 250 ml of Erlenmeyer flasks containing 100 ml culture medium, which consists of 1g tryptone,0.1g of dextrose,0.25 g of yeastextract,0.1g of KH2PO4.The inoculated medium was placed in a thermostatic orbital shaker for 48 hrs at 37°C and 150 rpm. The culture was centrifuged at 10,000 rpm for 15 min to obtain crude enzyme.

2.5 Analytical Methods

2.5.1 Protease Assay:

Alkaline protease activity was determined with a modification of the method[10]. 1ml of suitable diluted enzyme solution was added to 1 ml 1% (w/v) casein solution (dissolved in 0.1 M Glycine-NaOH buffer with pH of 10) and incubated at 37°C for 20 min. The reaction was terminated with 4 ml of 10% (w/v) trichloroacetic acid and the mixture filtrated through a filter paper. The filtrate absorbance was determined using Lowry method and extrapolated against a tyrosine standard curve. One unit of alkaline protease activity is defined as the amount of enzyme required to liberate 1 µg of tyrosine per minute under the experimental conditions.

2.5.2 Protein Assay:

Protein concentration of enzyme samples in each step was determined by the method of Lowry et al. with bovine serum albumin (BSA) as the standard [11].

2.6 Identification and Taxonomic Studies of the Selected Strain:

Identification of the selected isolate was studied based on different morphological, physiological and biochemical characteristics. The data was compared with standard description given in Bergey's Manual of Determinative Bacteriology [12]. Further 16s RNA analysis of the selected isolate was carried out for in depth phylogenetic analysis.

2.7 Nucleotide Sequence Accession Number:
The nucleotide sequence of *Bacillus stratosphericus* DF described in this paper has been deposited in NCBI GenBank Data Library and assigned the accession number **KC866366**.

3. Results and Discussions:
A total of 60 bacterial isolates were obtained from soil sample, collected at dump yard of dairy form industry, Visakhapatnam. All isolates were screened for proteolytic activity by skim milk agar plate method. Among them, 48 isolates showed the proteolytic activity.Out of these, one isolate named DF showed maximum proteolytic zone of 30mm and was selected for further studies.The ability of strain DF for production of proteolytic enzyme was measured in liquid medium upto 96 hrs. Maximum protease activity of 492 U /ml was observed at 48th hr of production. On third day, increase in protease activity is negligible and after third day, decline in protease activity was observed. It was observed that DF exhibited protease production at 37°C, 150 rpm and an initial pH 10. Therefore, these conditions were employed for cultures used in future experimentation.

Table 1 and 2 shows the results of the microbial characteristics of strain DF. The colony appeared to be irregular, smooth, raised, entire, cloudy on nutrient agar. It is a rod shaped, endospore forming gram-positive bacterium. It was able to hydrolyse casein and gelatine but was negative for starch hydrolysis.16s rRNA gene sequence (Accession No-KC866366) analysis of the isolate DF has revealed 99% similarity with Bacillus stratosphericus strain 41KF2a, followed by construction of phylogenetic tree by neighbour joining method revealed that the strain DF is *Bacillus stratosphericus.*

Table 1: Morphological and Cultural Characteristics

TESTS	RESULTS
Colony morphology	
Size	Medium
Colour	Cloudy
Form	Irregular
Surface	Shine and smooth
Texture	Moist
Elevation	Raised
Margin	Entire
Microscopic observation	
Gram's reaction	+ve
Shape	Rods
Size	Medium
Arrangement	2-3 chains
Spore formation	+ve (Ellipsoidal spore in unswollen sporangia)
Motility	-ve
Growth at Temp.	
Room temp	+ve
37°C	+ve
45°C	+ve
Growth at pH	
5	+
5.7	+
6.8	+
8.0	+
9.0	+
10.0	+
11.0	+
Growth at NaCl (%)	
2.5	+
5.0	+
7.0	-
Oxygen requirement O/F	Facultative

Table 2: Biochemical characteristics

TESTS	RESULTS
Catalase test	+
Oxidase test	+
Nitrate test	-
H2S test	-
Urease test	-
Indole test	-
Methyl red test	-
Voges proskauer test	-
Citrate test	-
Starch hydrolysis	-
Gelatin Hydrolysis	+
Casein hydrolysis	+
Hemolysin	-
Aminoacid & derv utilization	
Arginine	-
Lysine	-
Ornithine	-
Carbohydrate utilization	
Starch	-
Raffinose	-
Sucrose	+
Lactose	-
Maltose	-
Trehalose	-
Cellobiose	-
Melibiose	-
Ribose	-
Arabinose	-
Xylose	-
Rhamnose	-
Dextrose	+
Manose	-
Galactose	-
Fructose	-
Aldonitol (ribitol)	-
Manitol	-
Sorbitol	-
Dulcitol(galacitol)	-
Inositol	-
Myo-inositol	-
Salicin	+
Special tests	
Esculin	+
ONPG	+

4. Conclusion:
Here in this paper, we report screening and identification of a new extracellular alkaline protease producing *Bacillus stratosphericus* DF. To date, no report is available on isolation and production of protease by *Bacillus stratosphericus* and this is the first report on *B.stratosphericus*, producing an alkaline protease from dump yard of dairyform industry.

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