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ALKALINE PROTEASE PRODUCING BACTERIA ISOLATED FROM THE SOIL OF MANGO ORCHARD AND IDENTIFIED ON THE BASIS OF 16S rDNA SEQUENCING

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ABSTRACT

Five alkaline protease producing bacteria were isolated from the soil of mango orchards. The total protein was estimated and protease activity was measured against casein in 0.05 M buffer (carbonate: bicarbonate) at different temperature and pH. Alkaline proteases from all five isolates were thermostable and found to retain almost full activity after 1h incubation at 40°C. The protease exhibited good stability in presence of some locally available commercial detergents like Surf Excel, Ariel, Wheel, Rin, Sunlight and Tide and found to retain almost 50% activity after 10 min incubation at 35°C. Indicating that these enzymes could be used in detergent industries. One of the five isolates was identified as *Pseudomonas aeruginosa* [MTCC 7807] by 16s rDNA gene sequence analysis. Morphologically they are rod shaped revealed by scanning electron microscopy.

Keywords: Bacteria, Alkaline protease, detergent compatibility, 16s rDNA sequencing.

INTRODUCTION

Proteases are responsible for the hydrolysis of proteins to peptides and corresponding amino acids. From industrial perspective these have great importance and have a demand of nearly 60% of the world enzyme market (Kalisz, 1988; Kumar, 2002) in detergent, food, tannery industry and also in silver recovery and hydrolysis of gelatin from X-ray films (Kumar and Takagi, 1999, Denizci *et al*., 2004). Off all proteases, alkaline proteases are of great importance as these industrial enzymes have a market value of $1 billion world- wide (Madan *et al*., 2002).

The soil of mango orchards of Malda district, W.B., India (longitude 24°40́ 20″ N to 25°32́ 08″ N and latitude 87°45́ 50″ E to 88°28´10″ E) is rich in bacterial flora. Five bacterial strains out of thirty-six diversified bacterial isolates are screened as an alkaline protease producing strains. Two strains out of five strains gave high enzyme activity in crude condition. One strain is identified as *Pseudomonas aeruginosa* [MTCC 7807] and another soil strain referred to as soil isolate 1. The protease activity reached maximum level after 72h culture in an alkaline medium with pH 10.5. The enzyme from five isolates was tested for compatibility with some commercially available detergent powder (Madan *et al*., 2002). One Gram negative rod among the five isolates was identified as *Pseudomonas aeruginosa* [MTCC 7807] by 16s rDNA gene sequence analysis. Since this process has an accuracy of 90% for speciation (Moore *et al*., 2006; Anzai *et al.*, 2000; Lagace *et al.,* 2004). The scanning electron micrograph revealed the morphology and size of the five bacterial isolates. The activity and stability of the alkaline protease from these isolates in a high pH and temperature and its compatibility with detergents suggests its usefulness in industrial applications. The present study reports the isolation of thermostable alkaline proteases from soil bacterial isolates of mango orchards and the bacteria was identified based on 16s rDNA gene sequences.

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MATERIALS AND METHODS

Screening of protease producing isolates

Soil samples from different mango orchards of the Malda district were collected for the isolation of bacterial colonies. Separate bacterial isolates were screened for alkaline protease production in a medium containing 0.5% tryptone, 0.25% yeast extract, 0.1% glucose, 1% sodium caseinate, 0.44% trisodium citrate $2H_2O$, 20mM $CaCl_2$ 6 H_2O and 1.5% agar pH 10.0. After incubation for 3 days at 40^0C the colonies that produce clear zones around its were picked up and cultured in the same liquid medium (Eftekhar *et al*., 2003; Dodia *et al.,* 2006; Jasvir *et al.,* 1999; Kanekar *et al.,* 2002) at 40^oC for 72h with agitation of 250 rev min^{-1} (Kumar, 2002). The 72h old cultures in the above mentioned broth was centrifuged at 10,000g for 30 min. The enzyme in the cell-free supernatant was used as crude enzyme extracts (Madan *et al*., 2002). The total protein content of the five isolates was measured by the method of Lowry (Lowry *et al*., 1951) and bovine serum albumin (BSA) was used as standard.

Protease assay

Protease activity of all five isolates was determined by casein digestion method using 0.05M carbonate: bicarbonate buffer (pH 10.5) at 40^oC. 100 µL of enzyme

Table 1. Crude enzyme activity of alkaline proteases from five bacterial isolates.

Bacterial isolates	Volume of Culture filtrate (mL)	Total protein (mg)	Enzyme activity at 40°C (μM mL^{-1} min^{-1})
Pseudomonas aeruginosa [MTCC 7807]	450.0	1490.00	1.00
Soil isolate 1	450.0	1626.00	1.20
Soil isolate 2	450.0	1537.00	1.00
Soil isolate 3	450.0	1278.00	0.70
Soil isolate 4	450.0	1310.00	0.80

Table 2. Percentage homology based on 16s rDNA nucleotide sequences to determine the close homologs of the strain (§, *Pseudomonas aeruginosa* identified on the basis of 16s rDNA sequence analysis).

S. No.	Isolates	Percentage Homology										
		1	2	3	4	5	6	7	8	9	10	11
1	§ Our strain	*	99	99	99	99	99	99	99	99	99	99
2	AF094713		*	99	99	99	99	99	99	99	99	99
3	AB015516			*	99	99	99	99	99	99	99	99
4	DQ115539				*	100	99	99	99	99	99	99
5	EF064786					*	99	99	99	99	99	99
6	AB015249						*	99	99	99	99	99
7	AB015573							*	99	99	99	99
8	AB126582								*	100	100	100
9	AJ249451									*	100	100
10	DQ464061										*	100
11	AB117953											*

was incubated in 2.9 mL of 0.05M carbonate: bicarbonate buffer with 0.5% casein at 40°C. The reaction was stopped after 9 min with 10% ice-cold trichloroacetic acid. After centrifugation at 10,000 xg for 10 min the protein in the supernatant was estimated by Lowry's method (Lowry *et al*., 1951) considering tyrosine as standard. One unit (U) of protease activity was defined as the amount of enzyme liberating 1μg of tyrosine equivalent to min^{-1} under the assay conditions and expressed as unit mL^{-1}.

Compatibility of the alkaline protease with detergents

Solutions of some locally available commercial detergents at a concentration of 7mg mL^{-1} in double distilled water were boiled for 10 min to destroy any previously present protease and cool down at room temperature then added 100μL of crude enzyme in each detergent solution and incubated at 35°C in different time intervals. The activity was then assayed according to the Madan *et al*. (2002).

Scanning electron microscopy of the strain

All the five strains were subjected for scanning electron microscopy (SEM) to study the morphological details. The specimen was prepared according to the methods of Felgenhauer (Felgenhauer, 1987); fixed in 3% glutaraldehyde in 0.1M-phosphate buffer (pH 7.0) at room temperature for 3h and dehydration was done with ethanol followed by post fixation in 1% osmium tetroxide (OsO_4) in buffer for 2h. The microphotograph was taken with a CambridgeS4-10 scanning electron microscope.

DNA extraction for 16s rDNA gene amplification in PCR reaction

DNA was isolated from one of the five bacterial isolate (MTCC 7807) for the amplification of the 16s rDNA gene for identification purposes. DNA was isolated (Lu *et al*., 2000 and Lopez *et al*., 2003) from the bacteria grown on nutrient agar plates after suspending it in 1mL lysis buffer

(1% Triton X-100, 10mM Tris pH8.0 and 1mM EDTA) and then pelleted by centrifugation at 13,000xg for 5 min. Pellet was boiled for 30 min in 100µL same lysis buffer. Solvent extraction by chloroform was followed by centrifugation at 13,000xg for 5 min. DNA was precipitated after the addition of equal volume of isopropanol and chilling at -20°C.

PCR amplification

For PCR amplification the reaction mixture containing 50ng template DNA, PCR buffer (10mM Tris HCl, pH 8.3; 50mM KCl; 2.5mM $MgCl_2$; 0.001% gelatin), two universal PCR primers each of 0.2µM concentration, dNTPs each in 0.2mM concentration and 1.5 U *Taq* DNA polymerase (Genei, Bangalore) in a total volume of 25µL was prepared. A pair of universal primer was used to amplify the rDNA of one soil isolate. The universal primer was as follows- forward primer 17-mer 5′-TGCCAGCAGCCGCGGTA-3′ and reverse primer 18-mer 5′-GACGGGCGGTGTGTACAA-3′ (Giovannoni *et al*., 1990; Widmer *et al*., 1998). The thermal profile was as follows, first denaturation at 94°C for 1 min, then 40 cycles of 94°C for 1 min, annealing at 60°C for 1 min and extension at 72°C for 1.5 min and lastly at 72°C for 7 min for final extension. 10µL of PCR product was loaded onto 2% agarose gel to determine the size of the product (Lopez *et al*., 2003). The PCR product was cloned into pGEMT easy vector and cloned DNA was bi-directionally sequenced using the forward, reverse and an internal primer. The sequencing data was aligned for finding the closest homologs for the microbe (Sequencing done at Bangalore Genei). The 16s rDNA sequence of 1497bp was submitted to GenBank (Accession no-EF426771).

16s rDNA gene sequencing

One out of five alkaline protease producing bacteria was identified based on 16s rRNA gene sequencing analysis.

RESULTS AND DISCUSSION

Isolation of protease producing microorganisms

Soil dilutions of different mango orchards of the Malda district were found to contain 36 different isolates on the nutrient agar plates. Among these isolates only five strains (*Pseudomonas aeruginosa* MTCC7807, soil isolate 1, soil isolate 4, soil isolate 2 and soil isolate 3) shown the clear haloes around the periphery of colonies when grown on an alkaline protease-screening medium (Eftekhar *et al*., 2003; Chu, 2007; Naidu *et al.,* 2005) (Fig. 2). The gram characters were determined and except *Pseudomonas aeruginosa* MTCC7807 other four were *+ve*, endospore forming, and rods. All strains were found to produce acid from D-mannitol, while only soil isolate 4 and soil isolate 2 were capable of H_2S production in SIM medium. The scanning electron micrograph of the strain *Pseudomonas aeruginosa* MTCC7807 (Fig. 1) confirmed its rod shape and size ranging from 1.54µm to 1.65 µm in length and 0.63µm to 0.68 µm in breadth.

Isolation of crude alkaline protease

The results of crude protease isolation from all five isolates are summarized in Table-1. The enzyme production started after a 12h lag phase and exponentially increased with the increase of cellular growth and reached the maximum level within 60-72h. For casein hydrolysis the temperature and pH profiles of the protease from all five isolates were determined to be within 35-60°C temperatures and 8.0-11.0 pH ranges, respectively (Fig. 3A and 3B). However all isolates showed maximum activity at pH 10.5 and temperature 40°C.

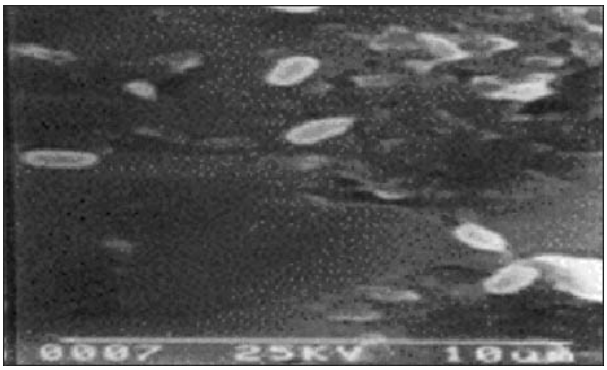

Fig. 1. Scanning electron microphotograph of one of the representative bacterial isolates, *Pseudomonas aeruginosa*.

Compatibility of enzyme with commercial detergents

Protease from five isolates when tested for their activity and stability in presence of different commercial detergents retained 35 to 57% of its original activity after 10min incubation. The enzyme activity in presence of detergents was observed highest in case of the strain soil isolate 1 followed by *Pseudomonas aeruginosa*, isolate 2, isolate 4 and isolate 3. Table 3 summarizes the percentage of retention of enzyme activity by the isolates with different commercial detergents.

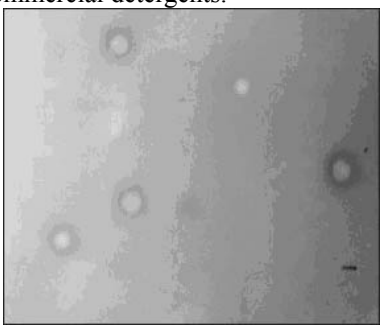

Fig. 2. Formation of clear haloes around the colonies of *Pseudomonas aerugnosa* indicating the positive result of Alkaline protease production at pH 10, and at temperature 37 degree Celcious.

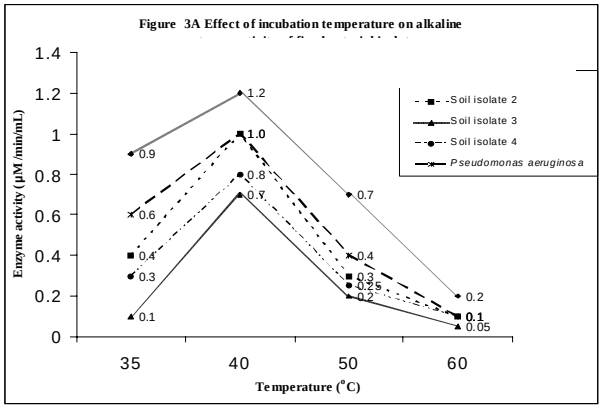

Fig. 3A. Effect of incubation temperature on alkaline protease activity of five bacterial isolates.

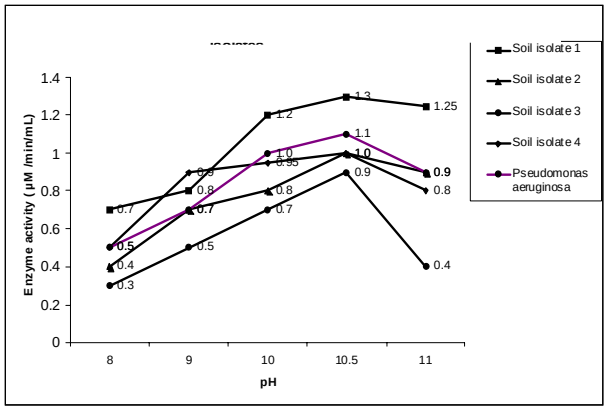

Fig. 3B. Effect of pH on alkaline protease activity of five bacterial isolates.

Table 3. Compatibility of alkaline proteases produced by five bacterial isolates with different commercial detergents.

% of Retention of protease activity after min

Detergents	Soil isolate 1		*Pseudomonas aeruginosa* [MTCC 7807]		Soil isolate 2		Soil isolate 4		Soil isolate 3	
	10min	30min	10min	30min	10min	30min	10min	30min	10min	30min
Control	100	100	100	100	100	100	100	100	100	100
Surf Excel	40	31	39	29	37	28	36.5	23	35	22
Ariel	48	36	47	31	42	29	40	29	39	26
Wheel	57	42	53	42	51	40	51	41	48	39.5
Rin	51	40	50	40	49	34	50	41	49.5	33
Sunlight	55	44	52	42	53	40	49	37	47	39
Tide	52	40	50	39	52	39	50	39.5	48	37

16s rDNA sequence analysis

The sequence of the 1497 bp of 16s rDNA gene of one strain out of five was done by BigDye terminator sequencing method using Applied Biosystem DNA Sequencer of Model ABI 3700 (at Bangalore Genei). The strain was identified and the sequencing data was compared with Ribosomal Database project and GenBank database. It was identified as *Pseudomonas aeruginosa* and its nearest homolg was found to be *Proteobacterium* sp. [ABO15516]. The phylogenetic position of the strain was determined by constructing the phylogenetic tree based on comparison of 16s rDNA sequences of reference *Pseudomonas* and *Proteobacterium* strains (Fig. 4). Information about other close homologs for the microbe can be found from the alignment view in Table 2.

The isolates exhibited good potentiality to produce protease under high alkaline conditions (pH 8-11) and were active against casein in a broad range of pH and temperature. The 16s rDNA sequence analysis was performed as the process has more accuracy over the conventional biochemical processes in determining the species. Nevertheless, this approach of analyzing 1497bp nucleotide sequence allowed the strain *Pseudomonas aeruginosa* to compare with other *Pseudomonas* sp and *Proteobacterium* sp. However, a considerable amount of reports regarding the alkaline protease producing bacteria makes our strains to compare with others. The protease produced by the mutant strain of *Bacillus polymyxa* (Madan *et al*., 2002) showed maximum activity against casein at pH9.25 that are less than our five strains though it showed highest activity at 70^0C. The strain *Bacillus polymyxa* (Emtiazi *et al*., 2005) and a bleach stable protease-producing strain of *Bacillus* sp. (Gupta *et al*., 1999) can produce the enzyme optimally at pH 7.0 and pH 10.0 respectively. Takami *et al*. (1989) reported thermostable alkaline protease from *Bacillus* sp. no.AH101 with stable and optimum temperature about 80°C and pH 12.0-13.0.

The compatibility of the enzyme with commercial detergents was tested with a view to exploit the enzyme in detergent industry. The mutant strain of *Bacillus polymyxa* (Madan *et al*., 2002) reported to retain more than 50% activity after 30 min incubation at 35°C. Protease from *Conidiobolus coronatus* (NCL86-8.20) (Phadatare *et al*., 1993) retained more than 80% activity in presence of commercial detergents. Samal *et al*. (1990) reported the protease BPN' from *Tritirachium album* Limber a highly unstable enzyme in all detergents and retaining only 4% activity after 10 min.

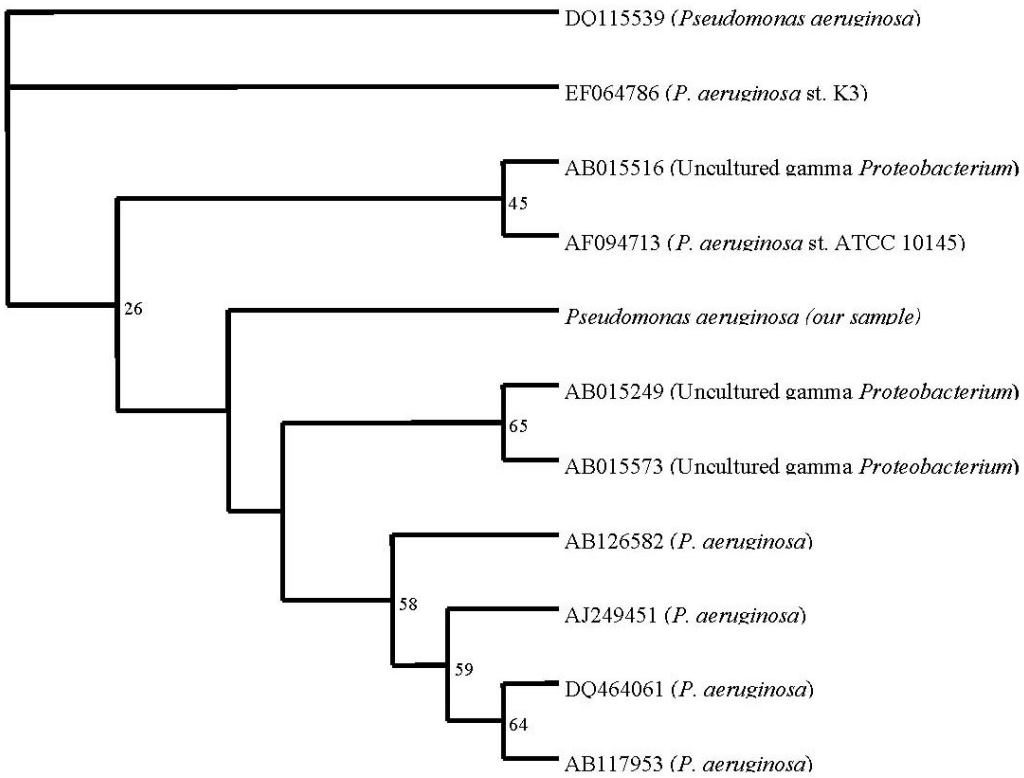

Fig. 4. Phylogenetic dendogram based on 16s rDNA sequence data indicating position of the isolate among the other representatives of the genus *Pseudomonas* and *Proteobacterium*. The sequences used in the analysis were obtained from Ribosomal Database project and GenBank. The scale bar indicates evolutionary distances.

The above discussion indicates that the thermostability with high pH tolerance of the enzymes from the five bacterial isolates supports their potentiality to use in detergent industry. However, more research is suggested before its exploitation commercially.

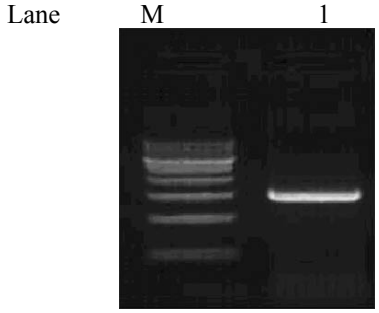

Fig. 5. The PCR product of 16s rDNA gene was fractionated in 2% agarose gel electrophoresis. Lane M, 500bp DNA ladder marker and lane 1, 1497bp amplified product of rDNA gene of *Pseudomonas aeruginosa.*

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