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Isolation of Protease Producing Bacteria from Soil and Characterization of the Protease

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A B S T R A C T

Microbes are famous source of industrially important enzymes which can easily be modified by biotechnological methods. Proteases are group of commercially important enzymes which can cleave proteins into smaller fragments. This enzymes are widely used at an industrial scale for processing of various foods, cheese production, in leather industry etc. Proteases also serve as signalling molecules. Soil is a rich and biodiverse source of a huge number of microorganisms. The present study is based on the characterization of the protease producing bacteria from the soil present outside and adjacent areas of a sweetshop in Burdwan. Screening of the bacteria was done by employing skim milk agar plating technique at 37°C and observed after 24 hours. Proteolytic activity of the crude protease extract from the bacteria was detected by using azocasein as substrate. Two bacteria was initially screened; one was giving white colony and the other was giving yellow colony. White colony bacteria showed to give higher activity. The optimum pH for the protease activity was found to be 8. 5 for both bacteriabut the optimum temperature was 37°C for yellow and 60°C for white. Bacteria isolated were coccus shaped and Gram-positive in nature. The isolated white bacteria was identified by 16s rDNA sequencing as *Staphylococcus* species.

Introduction

Enzymes are the biological catalysts that function to enhance the metabolic rate of reactions (1). They have wide range of industrial applications for multiple purposes like organic synthesis, chemical synthesis, detergents, food production, pharmaceuticals and fermentation (2,3,4). Proteases are a group of enzyme involved in digesting protein chains into short peptides and finally splitting them further into amino acid residues by breaking the peptide bonds between them (5,6,7). These are ubiquitous in nature and are produced by all living organisms starting from plants, animals and all forms of microbes such as algae, fungi, molds and bacteria mainly *Staphylococcus*, *Bacillus*,

Pseudomonas, *Helomonas*, *Arthrobacter* and many more. Some of the proteases attack the terminal amino acids from the protein chain and therefore are called as exopeptidase (such as amino acid peptidase, Carboxypeptidase A) and others attack internal peptide bonds and thus are called endopeptidase (such as trypsin, chymotrypsin, pepsin, papain and elastin).

Proteases can be used effectively for the degradation of protein containing wastes, to remove clogs from drainage pipes and thus considered as "biocleaner" (8,9,10,16). Proteases have many physiological and pathological roles such as protein catabolism, blood coagulation, tissue rearrangement, cell growth and migration, tumor growth, morphogenesis in development, transport of secretory proteins across the membranes, activation of zymogens (14,15).

Protease containing subtilisin group are used in the treatment of burns and wounds, oral administration produces an anti-inflammatory response in burnt patients and speeds up the process of healing (11,12). Protease enzymes can produce eco-friendly products and so they have wide application in biotechnological industries (1,19). . Proteases are extracted commercially from different plants, animals and microbial sources. The most easily available source is the microbial production of proteases.

They are very easily grown and can be manipulated by the application of genetic engineering. 60% of the total enzyme market is been shared by the enzyme produced by the bacteria. Protease production by microorganisms is greatly influenced by media components like nitrogen and carbon sources and by physical factors such as pH, temperature, agitation, inoculum density and incubation time (2,20). Microbial enzyme production on an industrial scale was 1st started by Takamine, in 1980, he started the production of Takadiastase enzyme which was actually α-amylase that contained substantial amount of proteases (2,21). Since then many kind of protease production took place in the market.

Among the variety of different microorganisms Bacillus sp. was known to produce maximum amount of protease commercially (2,22). Our study was aimed at finding new strains of microorganisms producing proteases in the soil. We have screened two bacteria for protease property and identified one bacterium by 16s rDNA sequencing method as Staphylococcus species.

Materials and Methods

Soil sample

Soil sample was collected from near and adjacent areas of a sweet shop in Burdwan District, West Bengal, in a sterilized bag and carried to the laboratory for further analysis.

Isolation and screening of bacteria

1 gm of the soil sample was weighed and serially diluted (10^-1 to 10^-6). 0. 1ml of the aliquots were spread plated on Skimmed Milk Agar and incubated 24 hours at 37°C. Caseinolytic activity of the bacteria was noted by the hydrolytic zones formed on the plates. Colonies showing higher zones were streak plated on Nutrient Agar which were stored at 4°C for further studies.

Cell lysate preparation

Bacteria was grown in Nutrient broth which was centrifuged at 6000rpm for 10 mins. The pellet was dissolved in 200mM lysis buffer (containing TRIS-Cl and EDTA; pH=8), lysozyme (20mg/ml) and protease inhibitor cocktail and incubated for 1 hour at 37°C.

Partial purification of crude enzyme

Partial purification of the enzyme by Zambare method in which the cell lysate was precipitated with ammonium sulfate(at 40% saturation) and at 5000rpm for 20 minutes at 4°C. After the centrifugation was done, the protease precipitate was loaded in dialysis bag for dialysis which was dipped in 100ml of 200mM phosphate buffer(pH=8) for 6 hours with a change of buffer after every 2 hours. After this the partially purified protease was collected and protease activity and also its molecular weight was determined.

Estimation of protein concentration by Lowry's method

Unknown samples were incubated with TRIS-Cl buffer, Reagent A(2% of sodium carbonate mixed with 0. 1N sodium hydroxide), Reagent B (1. 56% Copper sulfate with mixed 2. 37% sodium potassium tartrate) and 2 ml Folin-Ciocalteau reagent in dark for 30 minutes and the amount of the tyrosine released was measured calorimetrically at 660nm.

Estimation of protease activity by using azocasein as substrate

Enzyme activity was determined by using azocasein as substrate. Protease activity of the cell lysate or extract after ammonium sulphate precipitation was measured by azocasein assay (degradation of azo group from azocasein in per unit time can be detected by monitoring absorbance at 405 nm). 0. 1ml of the crude enzyme was incubated with 0. 25 ml of azocasein (2% azocasein in 0. 1M Tris HClbuffer;pH=7) for 60mins at 37°C and the reaction was stopped by the addition of 0. 2ml of TCA(5%).

Effect of pH on protease activity

To study the effect of pH the azocasein assay was carried out by changing the pH of the reaction (pH=4,5,6,7,7. 5,8,8. 5,10) and incubating at 37°C. The absorbance value was converted to enzyme activity in terms of degradation of azo dye per unit time per unit protein with the help of extinction coefficient of azocasein37 litres g-1 and cm-1.

Effect of temperature on protease activity

To study the effect of temperature the azocasein assay was carried out at different temperature(4°C,25°C,37°C,55°C,80°C) at a constant pH 7. 0. Enzyme activity was calculated as earlier.

SDS PAGE of the protein extract from bacterial lysate

Molecular weight of the protease was determined by SDS-PAGE in which 15% gel (Resolving and Stacking gel) is prepared as per the Lammeli method,1970 (4,30). After running the electrophoresis the gel was stained with 0. 1% Coomassie Brilliant Blue, protein band was visualized after de-staining with methanol, acetic acid and water in the ratio of 4:1:5. The molecular weight of the partially purified protease was estimated by using standard protein marker of known molecular weight.

Genomic DNA isolation and PCR amplification of 16s rDNA

Genomic DNA was isolated by phenol-chloroform extraction method(30) and then agarose gel electrophoresis was carried out, the DNA bands were then visualized by Gel-Documentation System. They were then amplified by universal primers 27F and 1492R.

Sequencing of 16s rDNA and BLAST analysis

16S rDNA sequencing was performed in

order to identify the unknown bacterial isolate. 16S rDNA sequencing was outsourced from eurofins technologies for species identification. NCBI website is used to perform BLASTn against 16s rDNA database for prokaryotes.

Molecular phylogenetic analysis

The top 10 sequences from the BLASTn result was obtained and used for phylogenetic tree formation. Phylogenetic tree was obtained by using phylogeny. fr website following the neighborhood joining method.

Results and Discussion

Isolation and screening of bacteria

Two strains of bacteria were screened among others which showed clear zones on the Skimmed Milk Agar plate indicating caseinolytic property of proteases (Figure 1).

These two strains were isolated and allowed to grow on nutrient agar media. Pure colonies were obtained with distinct morphological features.

Characterization of bacteria

After sub-culturing on the Nutrient agar plates from the milk agar plates by streak plate method, the following characteristics of the colonies were observed in Figure 2 and table 1.

Protein concentration of bacterial extract

White and Yellow both bacterial cell lysates showed high protein concentration as determined by Lowry's method using BSA standard curve.

Figure 3 showed that white bacterial lysate has 1. 49 mg/ml and yellow bacterial colony has 1. 28 mg/ml protein.

Determination of protease activity by using azocasein as substrate

Estimation of the protease activity was estimated quantitatively by using azocasein substrate. Degradation of azo dye was measured by absorbance at 440nm.

From the equation, enzyme activity was determined:-

Enzyme activity =(Δ Absorbance/min.) / Є
(Є is the molar extinction coefficient and Є1% of azocasein is 37 litres g-1 and cm-1

Effect of pH on protease activity

A standard protease assay using azocasein was carried out by varying pH(4-10) for 1 hour at 37°C. The optimum pH of the protease enzyme was found to be 8. 5. Protease from white bacteria showed higher activity (Figure 4).

Effect of temperature on protease activity

A standard protein assay using azocasein was carried out at constant pH 7 for 1 hour by varying the temperatures(4-80°C). The optimum temperature of the protease from yellow bacteria was found to be 37°C. Protease from white bacteria showed optimum temperature at 60^{o}C (Figure 5).

SDS PAGE of bacterial cell lysate

SDS PAGE image showed (Figure 6) protein bands in both the samples and the major bands were found to be around 63kDa and below 48kDa.

Genomic DNA of the two identified bacterial strains

Genomic DNA was clearly observed in 1% agarose gel (Figure 7).

Table. 1 Colony morphology

Color	Shape and Size
White	Large and round
Yellow	Small and round

Table.2 BLAST result for 16s rDNA sequence of the white bacteria

Microbial Strain	Accession no	Query Value	E Value	Per. Ident
Staphylococcus pasteuri **strain ATCC 51129**	NR_114435. 1	96%	0	99. 07%
Staphylococcus warneri **AW 25**	NR_025922. 1	96%	0	98. 79%
Staphylococcus lugdunesis **strain ATCC 43809**	NR_024668. 1	96%	0	98. 34%
Staphylococcus kloosii **strain ATCC 43959**	NR_024667. 1	96%	0	98. 19%
Staphylococcus cohnii **subsp. Urealyticus strain CK27**	NR_037046. 1	96%	0	98. 19%
Staphylococcus gallinarum **strain VIII1**	NR_036903. 1	96%	0	98. 19%
Staphylococcus petrasii**strain CCM 8418**	NR_118450. 1	96%	0	98. 19%
Staphylococcus saprophyticus **subsp. saprophyticus**	NR_074999. 2	96%	0	98. 04%
Staphylococcus edaphicus **strain CCM 8730**	NR_156818. 1	96%	0	98. 04%
Staphylococcus saprophyticus **strain NBRC 102446**	NR_114090. 1	96%	0	98. 04%

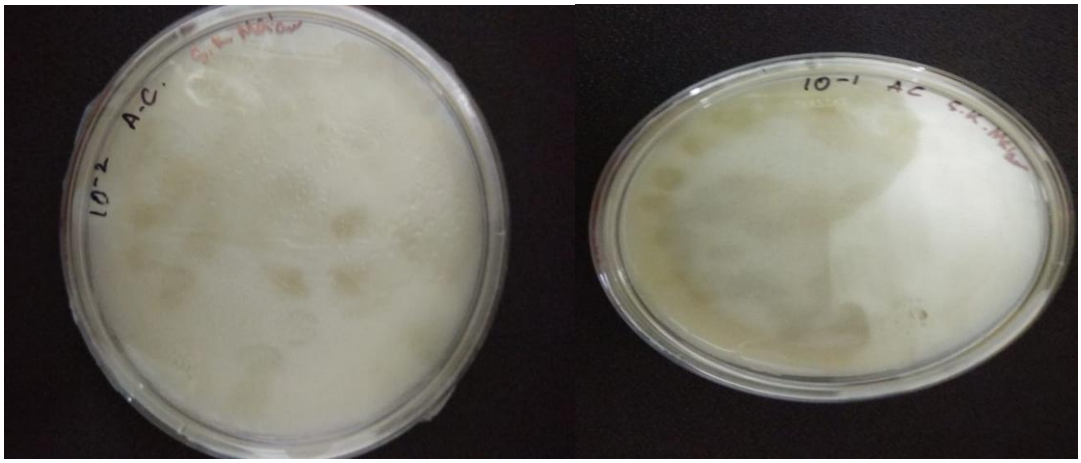

Figure.1 Screening of soil bacteria on skim milk agar plates showing casinolytic property

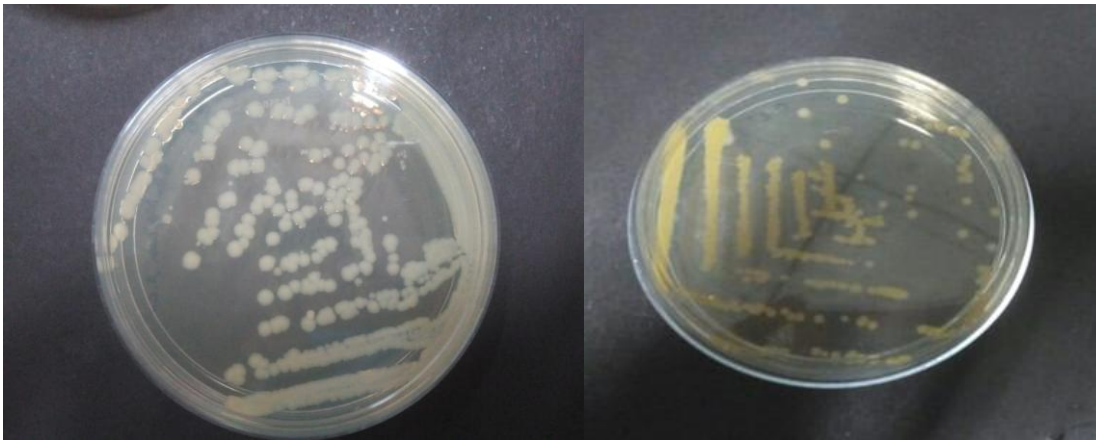

Figure.2 Single colony isolation on nutrient agar plates. A White colonies and B. Yellow colonies

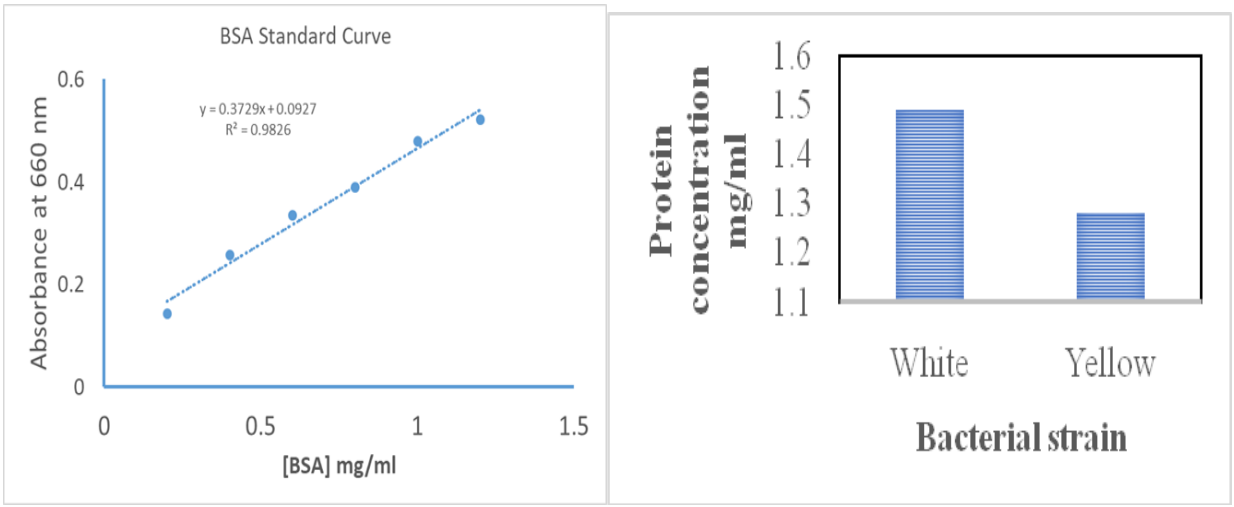

Figure.3 A. Standard curve of protein concentration using BSA as standard. B. Protein concentration in different crude extract

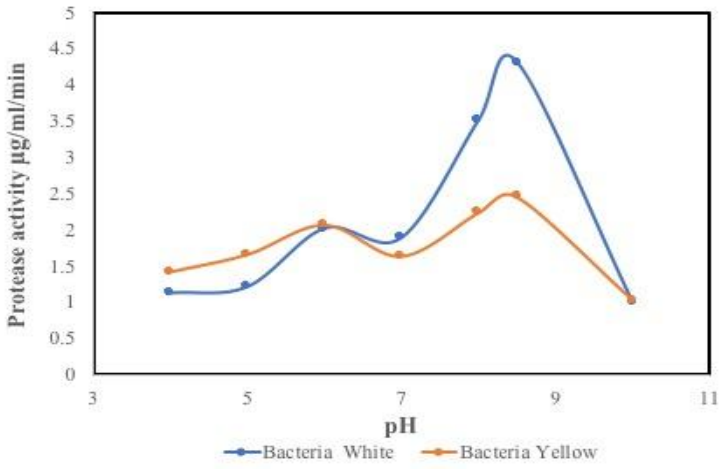

Figure.4 Characterization of protease activity with respect to pH

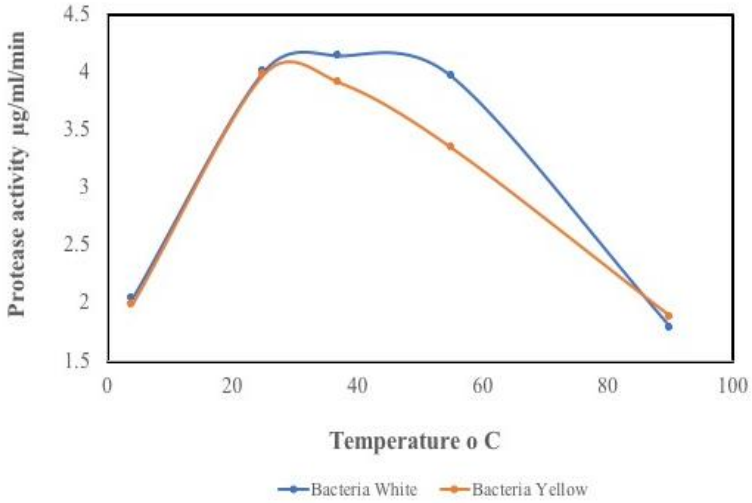

Figure.5 Characterization of protease activity with respect to temperature

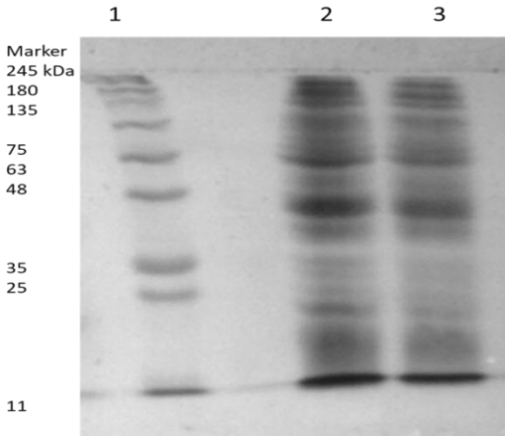

Figure.6 SDS PAGE showing protein bands in the crude cell lysate
15 % resolving gel showing lane 1 marker, lane 2 cell lysate from white bacteria and lane 3 cell lysate from yellow bacteria

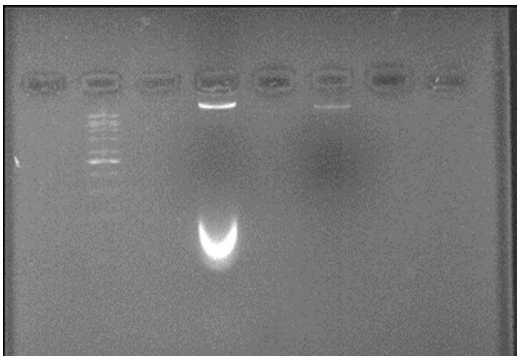

Figure.7 Genomic DNA observed in 1% agarose gel

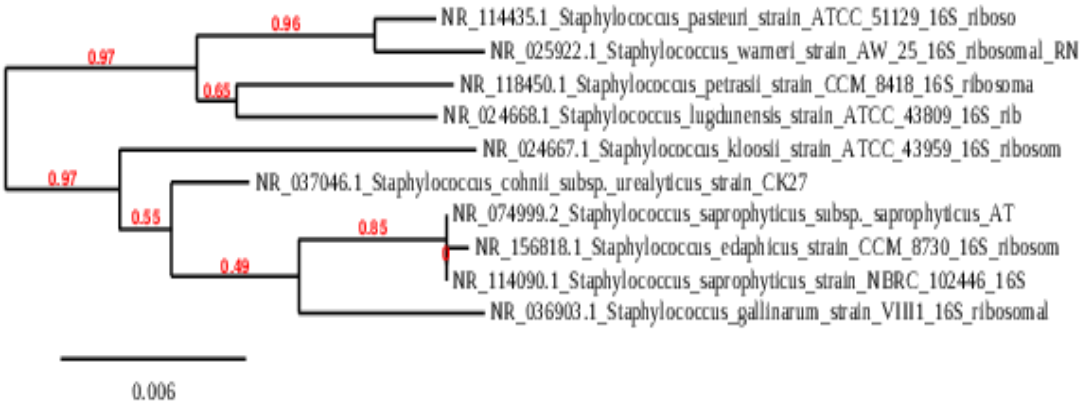

Figure.8 Phylogenetic tree showing the matches with high similarity in BLASTn result

Sequencing of 16s rDNA and BLAST

16S rDNA sequencing was outsourced from eurofins technologies for species identification. The obtained nucleotide sequence was used as query fordoing BLAST (Basic local alignment search tool) in NCBI website against 16s rDNA nucleotide database for prokaryotes. It was identified that the present study was with bacteria *Staphylococcus* and the highest degree of homology was identified and listed (Table 2).

The analysis suggested that the protease producing bacteria shared 99% sequence similarity with 16SrDNA sequence of *Staphylococcus pasteuri* strain ATCC 51129 (NR_114435. 1). The strain shared 98. 79% sequence similarity with *Staphylococcus warneri* AW 25 (NR_025922. 1); 98. 34% sequence similarity with 16S rDNA sequence of *Staphylococcus lugdunesis* strain ATCC 43809(NR_024668. 1); 98. 19% sequence similarity with 16SrDNA sequence of *Staphylococcus kloosii* strain ATCC 43959(NR_024667. 1), *Staphyloccocus cohnii* subsp. urealyticus strain CK27(NR_037046. 1), *Staphylococcus gallinarum* strain VIII1 (NR_036903. 1) and *Staphylococcus petrasii* strain CCM 8418(NR_118450. 1);98. 04% similarity with *Staphylococcus saprophyticus* subsp. saprophyticus ATCC 15305(NR_074999. 2)

Molecular phylogenetic analysis

Top 10 sequences are downloaded in fasta format. These sequences were used to do multiple sequence alignment and phylogenetic tree construction with the help of phylogeny. fr website. The phylogenetic tree was shown in Figure 8.

Two bacteria strains from the soil of adjacent

areas of a sweet shop are identified as protease producing bacteria. One of them is identified as Staphylococcus sp. The two proteases are highly active against azocasein at alkaline pH and at elevated temperature. They can be further explored.

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