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Molecular Characterization, Statistical Analysis of Protease and its Application

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ABSTRACT
Proteolytic enzymes are currently attracting enormous attention because of their biotechnological potential. Currently, bacterial proteases are in great demand because of potential industrial applications that are found in soil or mud. This research was conducted to find the proteolytic bacteria isolation from PKC (Palm Kernel Cake) sample collected from Oil Palm India Limited, Kollam, Kerala and it was screened for a novel protease producing bacteria in Skim milk agar medium. Among the isolates, the S1 isolate exhibited high proteolytic activity and was selected as a novel protease-producing bacteria, which was identified by morphological and biochemical characteristics as Pseudomonas aeruginosa. The optimization study was carried out for pH, carbon, substrate, surfactants, nitrogen, metal ions, and inoculum size for determining the maximum production of protease. The results are stated thereby.

Key words: Protease, *Pseudomonas aeruginosa*, Statistical analysis

INTRODUCTION

Microbes are preferred to many as they are cheap sources, their enzyme contents are predictable and growth substrates are obtained as standard raw materials. The probability of the enzyme industry shows a bright development with the raised market position for the existing and new enzymes. Protease enzymes are a mixture of different enzymes, which include proteinases, peptidases, and amidases and are incorporated in a sub-class of the enzymes hydrolases Intact protein molecules hydrolyze to proteases, peptones, and some amino acids by the enzyme proteinases. (Munawar et al. 2014). A large array of proteases are present in microorganisms, which are intracellular and/or extracellular. Proteases are vital for various cellular and metabolic processes, such as sporulation and differentiation, maturation and utilization of enzymes and hormones, and maintenance of the cellular protein pool. For the hydrolysis of proteins, extracellular protease is very important in cell-free environments and this helps the cell to utilize and absorb hydrolytic products (Kalisz 1988). Extracellular proteases have also been commercially exploited to assist protein degradation in various industrial processes (Kumar and Takagi 1999, Outtrup and Boyce 1990). The present work describes the protease enzyme used as an alternative method for dehairing property where protease enzymes were extracted and purified from bacteria, further optimization technique was performed to design the medium to attain maximum quantity through statistical analysis, and its application study for dehairing property was analyzed.

METHODOLOGY

Study area

Soil samples were collected from the Palm oil industry, Kollam. The samples were collected in a sterile polythene bag by a sterile spatula and transferred to the lab and stored at 4°C. 1g of soil sample was added to 100 mL of mineral salt media and incubated at 35°C in a 250 mL flask in a rotary shaking incubator at 120 rpm for 5 days. 10mL of this media was transferred aseptically to sterile mineral media and primary and secondary screening was performed.

Assay of protease

In the present work the isolate used was isolated and showed potential protease production on a starch casein agar plate with a zone of hydrolysis. The clear zone of hydrolysis around the bacterial colony is due to hydrolysis of casein by protease enzyme by the isolate S1. The best isolate that showed marked degradation activity was selected for optimization studies S1 was inoculated into the mineral salt

medium at pH 7 and incubated at 30°C. Influence of various conditions - pH, temperature, duration of incubation, different carbon and nitrogen sources on enzyme production were studied. Samples were drawn periodically at 24 h intervals till 144 h and analyzed for enzyme activity.

Direct dehairing activity of the protease

The hair removing activity of the culture filtrated was tested on 8½×8½ leather for a different time interval. Culture supernatant containing enzymes had a direct effect to remove hair from leather. Hair removing efficiency increases with incubation time and amount of enzyme. In the conventional methods of hair removing, the use of microbial enzymes as an alternate technology helps to minimize the pollution loads from leather Industry.

RESULTS AND DISCUSSION

Identification of the potent isolates

Palm Kernel Cake (PKC) samples were collected from the Palm oil industry, Kollam (Fig. 1) in sterile plastic bag and sealed and it was used for isolation of protease producing bacteria. The potential isolates obtained through primary and secondary screening (Fig. 2) were characterized based on the gram-negative characteristics and biochemical properties. The results showed that the isolate is a gram-negative rod, the biochemical characterizations were explained (Table 1). Genomic DNA was isolated using NucleoSpin® Tissue Kit (Macherey-Nagel). Sequencing of 16S rDNA region using universal primers 5'CAGGCCTACCACATGCAAGTC3', 5'GGGCGGWGAGTACAAGGC3'. The sequence was then submitted to GenBank and is available as accession no. (KY432822) and named the organism *Pseudomonas aeruginosa* S1 and the sequence is available in the GenBank database for extracellular protease production employing submerged fermentation.

Optimization analysis

Confirmation of the predicted results was done by manual optimization. Among the various conditions of influence - pH, temperature, duration of incubation, different carbon and nitrogen sources (organic and inorganic) showed better results (Fig.

Table 1. Biochemical characterization of isolate S1

Test	Response of the organism
Gram staining	-
Indole	-
Methyl red	-
Voges proskauer	-
Simmon citrate agar	+
Oxidase	+
Catalase	+
Glucose	+
Triple sugar iron agar	+
Nitrate reduction	+

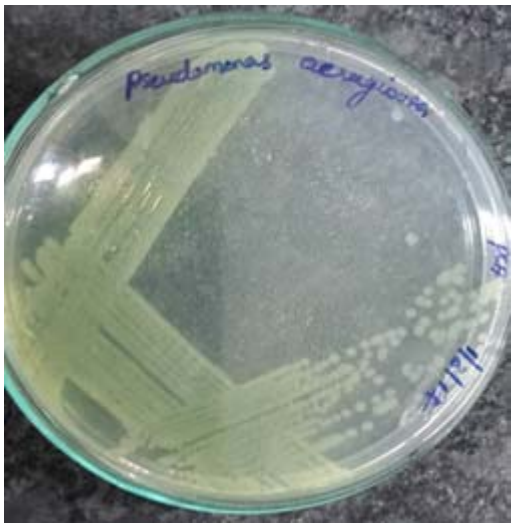

Figure 1. Colony morphology of S1 on nutrient agar plate

Figure 2: Protease activity of S1 on skim milk agar plate

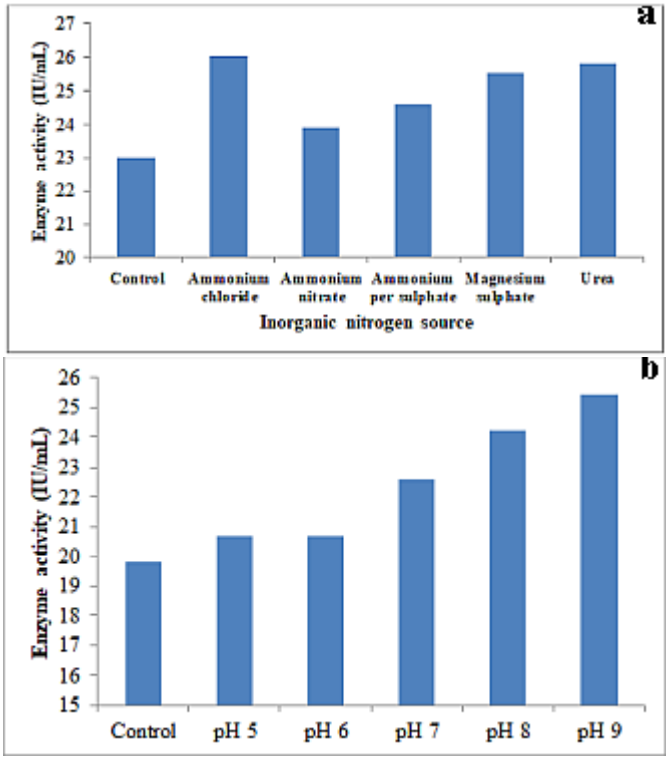

Figure 3. Effect of inorganic nitrogen source (a) and pH (b) on protease activity

3). Based on optimized parameters the designed medium for protease production was obtained (Table 2). Protease activity of 77.08 IU/mL having 70 h of incubation, 7 pH, 42.83°C temperature, 2mg/mL of carbon source glucose, 1 mg/mL of nitrogen source yeast extract, 0.3mg/mL of metal ion magnesium sulfate. For purification of protease from potential bacteria, the culture filtrate of PS1 were subjected to purification strategy i.e. acetone precipitation showing 2.87 purification fold, 50.5% yield and specific activity of 2.46 U/mg.

Direct dehairing activity of protease

The hair removing activity of the culture filtrate was tested on 8×8½ leather sample for different time interval. Culture supernatant containing enzymes had direct effect to remove hair from leather. Hair removing efficiency increased with incubation time and amount of enzyme. So the use of microbial enzymes as an alternate technology to the conventional methods, and highlights the importance of these enzymes in minimizing the pollution loads. The protease isolated from the strain S1 dehaired the sample with in 10 hr indicating that the novel

Table 2. Optimized conditions for protease

Parameters	Conditions
Incubation time	60 h
pH	7
Temperature	42
Carbon source	Glucose
Nitrogen source	Yeast extract
Metal ions	$MgSO_4$

protease from the isolate has advantage over the already reported proteases and a wide application in leather industry (Fig 4).

The partial 16S rDNA gene sequence of the isolate showed 98% similarity to *Pseudomonas aeruginosa*. It is a known fact that *Pseudomonas* is a highly versatile organism that represents the most abundant genus of bacteria playing a major role in dehairing treatment (Rossello-Mora et al. 1994). Majority of the proteases used for dehairing of goat skins were from the strains belonging to the genus *Bacillus* viz.

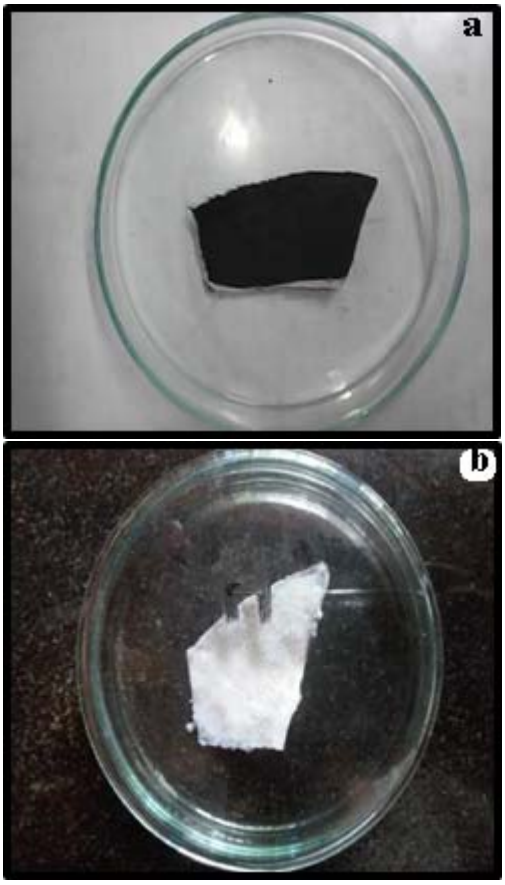

Figure 4. Direct dehairing activity of the enzyme - a - Control, b - 100% dehaired

B. subtilis, *B. megaterium*, *B. licheniformis*, *B. halodurans*, *B. cereus* and *Bacillus* sp. Dehairing of goat skins by protease from *Elizabethkingia meningoseptica* resulted in complete removal only after 18 hr (Nagal et al. 2010).

In most of the earlier studies dehairing assays used very small pieces of skins ranging from 2x2 cm to 5x5 cm by dipping the skin pieces in enzyme solutions with or without lime and sulfide held in alkaline pH range and temperatures ranging from 30-40°C which are optimum for the enzyme action (Pillai and Archana 2008, Nadeem et al. 2010, Prakash et al. 2010, Haddar et al. 2011, Saleem et al. 2012). Haddar et al. (2011) reported dehairing of goat skin pieces with *B. licheniformis* protease (7000 U/ml) and found no hair removal at 25°C while incomplete dehairing at 30°C and complete hair removal only at 37°C after incubating for 24 hr under shaking conditions. Excessive damage to the skin can occur when dehairing has to be carried out at elevated temperature which is difficult to maintain at commercial scale (Edmonds 2008). Though one can argue that temperatures vary in different regions of the country and may reach around 40°C in summers in south, skins are normally piled up and left overnight when temperatures are lower than the day temperatures. Therefore, proteases active over a broad temperature range and suitable for application throughout the year and all over the country are advantageous.

CONCLUSION

The protease isolated from the strain S1 dehaired the sample within 10 hr which indicates that the novel protease from the isolate has advantage over the already reported protease and a wide application in leather industry.

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Conflict of interest: Authors declare no conflict of interest

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