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Enhancement of protease production by *Pseudomonas aeruginosa* isolated from dairy effluent sludge and determination of its fibrinolytic potential

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ABSTRACT

Objective: The present study aimed at isolating proteolytic bacteria from dairy effluent sludge, designing the process parameters for the enhanced production of protease and determination of its fibrinolytic potential. **Methods:** The dairy sludge was processed according to the microbiological criteria for the isolation of proteolytic bacteria. All the isolates were screened for their protease production ability and the isolate showing highest proteolysis was selected for further studies. Effects of various media components and process parameters like carbon and nitrogen supplementation, temperature, pH and incubation period were investigated. Partial purification of the protease was done using ammonium sulphate fractionation, following which its molecular weight and fibrinolytic activity were determined. **Results:** Based on the biochemical studies, the selected isolate was identified as *Pseudomonas aeruginosa*. The highest protease yield was obtained with maltose and yeast extract as supplements. The optimum pH, temperature and incubation period for protease production by the isolate was found to be 7.0, 37℃ and 48 h respectively. The partially purified enzyme preparation showed a single protein band in sodium dodecyl sulphate polyacrylamide gel electrophoresis, revealing the apparent molecular weight of the enzyme to be 35 kDa. The efficient removal of the blood stain emphasized its fibrinolytic potential. **Conclusions:** From the present study it is envisaged that cultural parameters significantly affect the protease production. Based upon the fibrinolytic activity, this protease may find broad applications in detergent and pharmaceutical industries.

1. Introduction

Proteases or proteinases are proteolytic enzymes which catalyze the hydrolysis of proteins. Based upon their structures or properties of the active site, there are several kinds of proteases such as serine, metallo, carboxyl, acidic, neutral and alkaline proteases. Proteases are industrially important enzymes and constitute a quarter of the total global enzyme production[1]. Proteases are industrially important due to their wide applications in leather processing, detergent industry, food industries, pharmaceutical, textile industry etc[2,3].

Proteases are obtained from plants, animal organs and microorganisms, with the majority obtained from microbial sources. Currently, a large proportion of commercially available proteases are derived from bacteria and fungi. Selection of the right organism plays a key role in obtaining high yield of desirable enzymes. On the other hand, it is a well-known fact that extracellular protease production in microorganisms is greatly influenced by media components, especially carbon and nitrogen sources and metal ions[4] along with other growth parameters.

Wastes particularly from dairy, meat and poultry processing industries act as sources for large amount of protein-rich material that can be biologically transformed into recoverable products. Owing to their high protein content, they may serve as excellent sources for isolating proteolytic microorganisms[5]. The present study focuses on the isolation of proteolytic bacteria from dairy sludge, determination of the factors that affect the maximum protease production and its fibrinolytic potential.

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2. Materials and methods

2.1. Sample collection and isolation of proteolytic bacteria

Sludge sample was aseptically collected from the sedimentation tank of a dairy industry, using pre-sterilized zip lock cover and sterile spatula and processed within an hour from the time of collection. Bacteria were isolated from 1 g of the sludge by serial dilution and agar plate method using nutrient agar medium.

Skimmed milk agar medium containing (g/L): skim milk powder, 100; peptone, 5; agar, 20 and distilled water at pH 7±0.2, was used for enzymatic screening wherein, the respective isolates were inoculated on the plates as a single line and incubated at 37℃ for 24 h. Following incubation, the plates were flooded with 10% (w/v) solution of $HgCl_2$ in 20% (v/v) HCl to determine the proteolytic potency of the isolates[6].

Comparison between the proteolytic potential of the isolates was made based on the zone of casein hydrolysis and individual colony diameter. The zone of hydrolysis was measured to the nearest mm. The isolate demonstrating the highest zone of casein hydrolysis was maintained on nutrient agar slants at 4℃ until use.

2.2. Identification of the isolate

The selected isolate was identified based on its morphological and biochemical characteristics. The morphological characterization involved culturing the isolate on nutrient agar plates for studying the appearance of the colonies following which gram's staining and motility test were performed.

The biochemical characterization of the isolate was performed by indole test, methyl red test, Voges Proskauer test, oxidase test, gelatin liquefaction test, lecithinase production test and nitrate reduction test. The isolate was tested for its ability to produce non-fluorescent and diffusible pigment, ability to utilize various sugars and amino acids like maltose, D-ribose, mannitol, L- valine, β-alanine and L- arginine. Bergey's Manual of Determinative Bacteriology (9th Edition) was used as a reference to identify the isolates[7].

2.3. Optimization of cultural conditions

The isolate yielding the highest protease activity was grown in the mineral salt medium (MSM) (g/L: KH_2PO_4, 0.42; K_2HPO_4, 0.375; $(NH_4)_2SO4$, 0.244; NaCl, 0.015; $CaCl_2.2H_2O$, 0.015; $MgSO_4.7H_2O$, 0.05; and $FeCl_3.6H_2O$, 0.054; pH 7±0.1) supplemented with different carbon sources such as 1% (w/v) (glucose, fructose, maltose, sucrose and lactose) and 1% (w/v) nitrogen sources (peptone, yeast extract, beef extract, ammonium chloride, ammonium sulphate and sodium nitrate). Effect of various physical parameters such as initial pH (4, 5, 6, 7, 8 and 9), temperature (25, 32, 37, 40, 45 and 50℃) and incubation period (24, 48, 72, 96 and 120 h) were optimized by conventional methods for maximal enzyme production. All the experiments were conducted in triplicates.

2.4. Protease assay

The protease activity was estimated by the method described by Beg *et al*.[8]. Following incubation, the bacterial broth was centrifuged at 5000 rpm for 20 min at 4℃ to obtain the cell free supernatant (CFS). 1 mL of CFS was added to 1 mL of 1% (w/v) casein solution in glycine-NaOH buffer of pH 10.5 and incubated for 10 min at 60℃. The reaction was stopped by addition of 4 mL of 5% trichloroacetic acid. The reaction mixture was centrifuged at 3000 rpm for 10 min and to 1 mL of the supernatant 5 mL of 0.4 M Na_2CO_3 was added followed by 0.5 mL Folins-Ciocalteu reagent. The amount of tyrosine released was determined using a UV-VIS spectrophotometer (SANYO Gallenkamp, Germany) at 660 nm against the enzyme blank. One unit of protease activity was defined as the amount of enzyme required to release 1 μg of tyrosine per mL per min under standard assay conditions.

2.5. Estimation of total protein content

The soluble protein content of the enzyme sample was determined by Lowry's method[9] using crystalline bovine serum albumin as the standard.

2.6. Molecular weight determination of protease

Protease was purified by 40% ammonium sulphate precipitation method. The precipitate obtained by centrifugation was dissolved in 0.1 M phosphate buffer (pH 7) and dialyzed overnight against 0.01 M phosphate buffer at 4℃. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was employed for the molecular weight determination of the dialyzed sample, using a broad range pre-stained protein marker (NEW ENGLAND Biolabs, UK).

2.7. Determination of fibrinolytic activity

4 pieces of cotton fabric were individually impregnated with 500 μL of blood and the blood stains were allowed to dry. Then the fabrics were soaked in 2% (v/v) formaldehyde for 30 min and rinsed with water to remove excess formaldehyde[10]. Upon drying, the fabric pieces were separately incubated with 1 mL of the partially purified protease, 1 mL of the partially purified protease with detergent, 1 mL of sterile distilled water with detergent and 1 mL of sterile distilled water at 37℃ for 1 h. Following incubation, the fabric pieces were rinsed with water, dried and checked for the extent of

blood removal.

2.8. Statistical analysis

Effect of each parameter was studied in triplicate and the data are graphically presented as the mean ± standard deviation of triplicates (n = 3). ANOVA was performed using Microsoft Excel 2007. P values < 0.05 were considered significant with a confidence limit of 95%.

3. Results

3.1. Isolation and screening of proteolytic bacteria

A total of 26 bacteria were isolated from the dairy sludge examined. When tested for their proteolytic potential, 11 isolates (DS3, DS6, DS7, DS8, DS9, DS11, DS15, DS16, DS17, DS18 and DS20) demonstrated clear zones around the streak on the skimmed milk agar (an indication of protease production). Among these isolates, DS18 demonstrated the highest zone of proteolysis (24 mm) as compared to the other isolates (Figure 1) and therefore it was selected for further studies.

Table 1.
Morphological characterization of the selected bacterial isolate.

Characteristics	Result
Shape	Circular
Color	Green
Opacity	Translucent
Texture	Mucoid
Spreading nature	Non spreading
Elevation	Flat
Margin	Smooth
Gram Staining	Negative rods, scattered
Motility Test	Motile

3.2. Identification of the selected bacterial isolate

Based on the morphological characters the isolate DS18 was found to be gram negative short rod showing motility. Biochemical characterization revealed the identity of the isolate as *Pseudomonas aeruginosa*. The detailed report of morphological and biochemical characterization has been presented in Table 1 and Table 2.

Table 2.
Biochemical characterization of the selected bacterial isolate.

Biochemical Tests	Result
Diffusible, non- fluorescent pigment	+
Growth at 4℃	–
Growth at 41℃	+
Gelatin Liquefaction	+
Lecithinase (egg yolk reaction)	+
Nitrate as nitrogen source	+
Indole	+
Methyl red test	+
Voges Proskauer test	–
Oxidase reaction	+
Utilization of Maltose	–
Utilization of D-Ribose	–
Utilization of Mannitol	–
Utilization of L- Valine	–
Utilization of β - Alanine	+
Utilization of L- Arginine	–

+, positive; –, negative

3.3. Effect of additional carbon source

Our study showed that maltose was the best carbon source showing a protease activity of 293.06 U/mL (Figure 2).

3.4. Effect of nitrogen supplementation

It is well documented in the literature that nitrogen is metabolized to produce primarily amino acid, nucleic acid, protein and cell wall components. These nitrogen sources have regulatory effect on the enzyme synthesis. Production of protease is highly dependent on both carbon and nitrogen sources available in the medium[11]. Testing the effect of various nitrogen sources on protease production, it was found that yeast extract gave the highest enzyme activity (317.89 U/mL). Another notable finding of the study was that the inorganic nitrogen sources proved to be inferior as compared to the organic nitrogen sources in protease

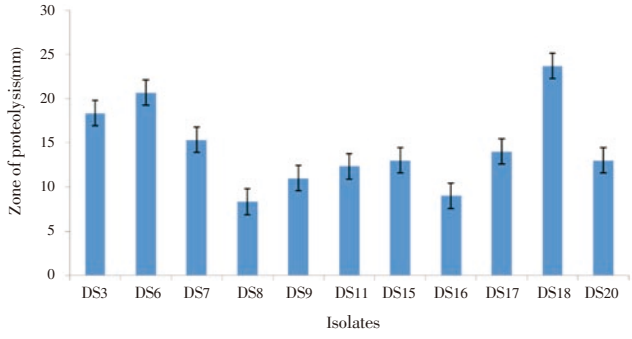

Figure 1. Zone diameter of proteolytic activity by the bacterial isolates. Data represent mean ± S.D. (n=3); P < 0.05

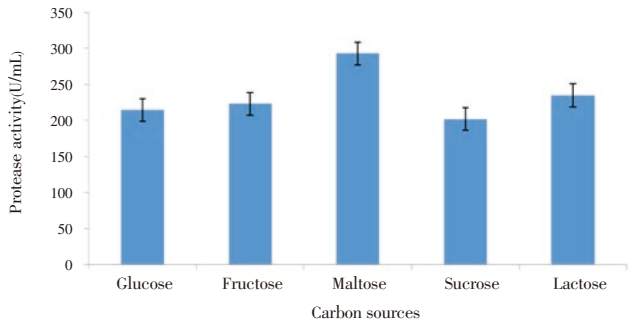

Figure 2. Effect of additional carbon source on protease production. Data represent mean ± S.D. (n=3); P < 0.05

production (Figure 3).

3.5. Effect of initial pH of the medium

Among the physical parameters, pH of the growth medium plays an important role by inducing physiological changes in microbes and their enzyme secretion. The obtained results demonstrated that though protease production was detected over a broad pH range from 4.0 to 9.0, maximum enzyme production (346.84 U/mL) was noted at neutral pH (Figure 4).

3.6. Effect of incubation temperature

The incubation temperature of a fermentation process has a profound role to play on the growth and in turn on

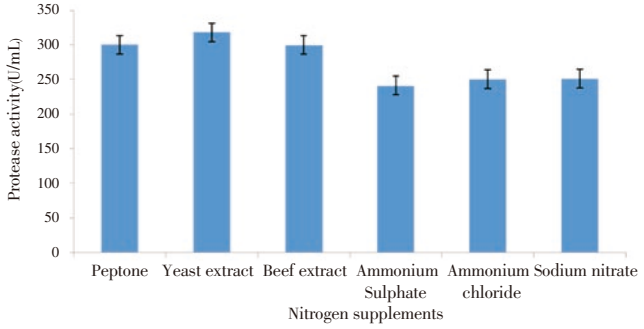

Figure 3. Effect of additional nitrogen source on protease production. Data represent mean ± S.D. (n=3); $P < 0.05$

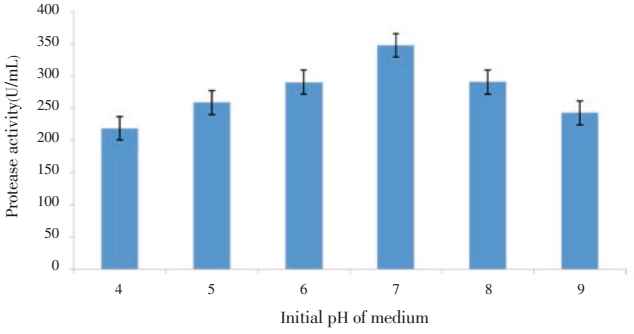

Figure 4. Effect of initial pH of medium on protease production. Data represent mean ± S.D. (n=3); $P < 0.05$

observed after 48 hours of incubation.

3.8. Molecular weight determination

The molecular weight of the partially purified protease, as analyzed by SDS-PAGE, showed a single protein band of approximately 35 kDa.

3.9. Fibrinolytic potential of the protease

The degree of blood removal from the cotton fabric was found in the order of: partially purified protease with detergent > partially purified protease > sterile distilled water with detergent> sterile distilled water.

the metabolic activities of the microbial cells. When the optimum temperature for the production of protease was investigated from 25℃ to 50℃, 37℃ was found to be the optimum temperature for protease production (349.70 U/mL). The incubation at temperatures other than 37℃ was found to decrease the production of protease (Figure 5).

3.7. Effect of incubation time

The incubation time for achieving the maximum enzyme level is governed by the characteristics of the culture and is based on growth rate and enzyme production. The enzyme production varies with incubation time[12]. Figure 6 shows the effect of incubation time on protease production. The maximum amount of enzyme production (349.30 U/mL) was

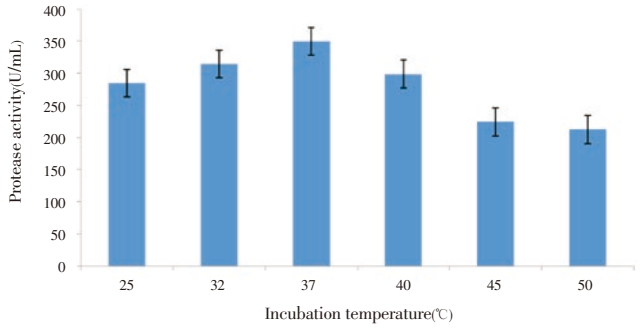

Figure 5. Effect of incubation temperature on protease production. Data represents mean ± S.D. (n=3); $P < 0.05$

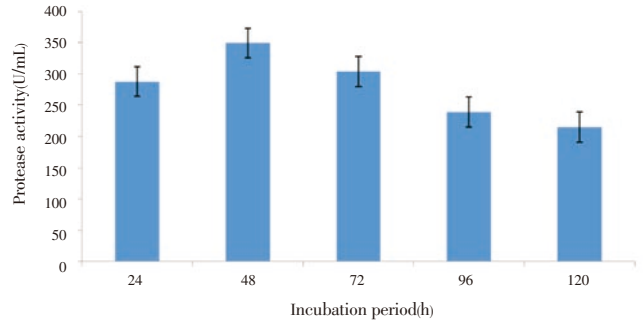

Figure 6. Effect of incubation period on protease production. Data represent mean ± S.D. (n=3); $P < 0.05$

4. Discussion

Proteases (EC 3.4.21-24 and 99; peptidyl-peptide hydrolases) are enzymes that hydrolyze proteins via the addition of water across peptide bonds and catalyze peptide synthesis in organic solvents and in solvents with low water content. In all living organisms, proteolytic enzymes are widely found and are essential for cell growth and differentiation[13].

The isolate could secrete extracellular protease and used maltose as sole carbon source for protease production. Likewise, glucose resulted in the reduction in protease production which could be attributed to catabolite repression by high glucose available in the medium. This observation is

in agreement with that of Afify *et al*[14]. However, increased yields of alkaline proteases were reported by several other workers who used different sugars such as lactose, sucrose and fructose[15,16].

Protease production is believed to be affected by the presence of complex nitrogen sources in the growth medium and each organism vary from the other in terms of its requirement for specific nitrogen source[17]. An earlier study concluded that organic nitrogen sources such as casein-gelatin, peptone, yeast extract and beef extract had significant effects on extracellular protease production by a halophilic *Bacillus* sp, whereas simple inorganic nitrogen sources in the form of ammonium compounds showed reduced growth and protease production[18].

In parallel to our findings, a maximum protease yield of 998 U/mL with specific activity of 256 U/mg protein was obtained from the culture *Streptomyces pseudogrisiolus* NRC-15 using yeast extract[19]. Our result is further supported by a study on protease production by *C. cornatus* wherein the best enzyme yield was obtained using yeast extract, followed by casein while beef extract acted as a poor organic nitrogen source[20].

On the other hand, beef extract among the different organic nitrogen sources and ammonium carbonate among the different inorganic nitrogen sources led to a high proteolytic activity by *Bacillus* sp. at 48 h of incubation[21]. Similarly, among the tested nitrogen compounds, 0.1% (w/w) beef extract followed by yeast extract served as the best co-nitrogen sources for protease production by *Bacillus subtilis*[22].

Microbial cells are significantly affected by the pH of their immediate environment because they apparently have no mechanism for adjusting their internal pH. Different organisms have different pH optima and decrease or increase in pH on either side of the optimum value results in poor microbial growth[12].

Studying the effect of physical factors on the production of organic solvent-tolerant protease by *Pseudomonas aeruginosa* strain K, the highest enzyme yield was observed at pH 7.0. Neutral media increased the protease production as compared to acidic or alkaline media[23]. This is in complete agreement with the present finding. Likewise, when the protease production medium for *B.subtilis* was adjusted at different pH values with different buffers, results indicated that the best buffer was phosphate buffer and the optimum pH for production of protease was recorded at 7.0. A notable decline in the enzyme productivity occurred at both higher and lower pH values[24].

Certain *Bacillus* species produced protease over the entire range of pH investigated (pH 5 - 10). The optimum pH for maximum protease production from *Bacillus* SNR01 was at pH 7.0[25]. Radha *et al*.[26] studied the production and optimization of acid protease by *Aspergillus* sp. from soil. They reported a gradual increase in protease at pH from 3.0 to 5.0, whereas, it declined at neutral and alkaline pH. Fungal acid proteases have an optimal pH range from 4 - 4.5 and they can be stable at pH values from 2.5 - 6.0. Maximum production of enzyme and fungal dry mass were observed at pH of 5. Similarly, Kumar *et al*.[27] reported that the optimum pH was in the acidic range of 5.5- 6.5 for acid protease production from solid tannery waste by *Synergistes* species.

The growth and enzyme activity of microorganisms is greatly influenced by different incubation temperatures. The growth of microorganisms can be inhibited at one temperature but it can be activated at another temperature. Temperature significantly regulates the synthesis and secretion of bacterial extracellular proteinase by changing the physical properties of the cell membrane[28]. Therefore, temperature is a critical parameter that should be controlled in order to obtain an optimum proteinase production.

In concurrence to our result are the previous findings, where the bacterial isolates like *Pseudomonas aeruginosa* MTCC 7926, *Serratia liquefaciens* preferred 37℃ for maximum production of protease[29,30]. The production of alkaline protease by *Bacillus halodurans* was investigated, wherein the maximal cell growth was seen at 50℃ and maximum enzyme production was found at 37℃[31].

It is very essential to detect the optimum incubation time at which an organism exhibits highest enzyme activity since organisms show considerable variation at different incubation periods[32]. Our study is in agreement with previous workers, who reported high proteolytic activity by *Bacillus* sp. using beef extract for an incubation time of 48 h[33]. In another study, *Pseudomonas aeruginosa* showed maximum protease activity at pH 9.5, temperature 37℃ and 48 h of incubation time[34]. Protease production from *Pseudomonas fluorescens* was found to be maximum at 24 h beyond which the enzyme activity gradually decreased from 48 to 168 h, which is in contrast to the present finding[1].

A comparative account of hydrolytic activities of exogenous protease from four strains of Pseudomonas sp. viz., *Pseudomonas* sp. C 61, *Pseudomonas* sp. C 20, *Pseudomonas fragi* ATCC 4973 and *Pseudomonas fluorescens* demonstrated molecular weights of 46.80, 49.20 and 46.10 kDa respectively. Following purification by hydrophobic interaction chromatography, the protease from *Pseudomonas aeruginosa* was homogeneous on SDS-PAGE and its molecular weight was estimated to be around 36 kDa. It also showed a clearing zone in the activity gel containing gelatin as a substrate[35]. This result is in perfect correlation to the result obtained in the present study.

A solvent tolerant, thermostable and alkaline metalloprotease was reported in cell free broth culture of alkalophilic *Pseudomonas aeruginosa* MTCC 7926. The purified protease had a molecular mass of 35 kDa[36].

When applied alone, this protease removed blood stain from the fabric significantly. The washing efficiency of the detergent was also remarkably increased with addition of the enzyme. Hence we advocate the usage of this enzyme as formulations in detergent preparation. Similar result was

obtained earlier. There are different reports for proteases that can act in the presence of detergent and they were used for bio-formulation[37].

The result of the present study elucidated that dairy effluent sludge can be a very good source for isolating proteolytic bacteria. The study gains its importance since literature is scanty in terms of proteolytic enzymes from *Pseudomonas aeruginosa*. Considering the fact that enzyme production by microorganisms is under the influence of various growth conditions, the present investigation determined the optimum environmental and nutritional parameters for maximum production of protease from the isolate. The fibrinolytic nature of the enzyme alone and in synergy with the detergent evokes the idea that this particular organism may be exploited in the pharmaceutical and detergent industries in future.

Conflict of interest statement

We declare that we have no conflict of interest.

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