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ORIGINAL ARTICLE

Isolation, Screening and Identification of Protease producing bacteria from soil and fruit peel waste

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ABSTARCT
Microbial enzymes are used in various industries, including baking, brewing, paper manufacturing, pulp processing, and detergents. The study aimed to isolate and identify the protease-producing bacterial strains from different soil samples and fruit peel waste. A total of 20 bacteria were isolated, and their protease activity was determined on skimmed milk agar. The protease activity was observed by the occurrence of a clear zone around the isolates. Among 20 isolates, 5 bacterial isolates were showed zone formation around colonies on skimmed milk agar plates. These five bacterial isolates were designated as 1C1, 1C2, 1C3 ,2C1 and 3C2.The protease producing isolates were identified by cultural characterization, microscopic observation and biochemical identification. The isolates were determined to be Micrococcus spp. (1C1 and 1C2), Lactobacillus spp. (1C3) and Bacillus spp. (2C1 and 3C2). The proteolytic bacterial isolates were further evaluated for protease production using fermentation broth. The highest enzyme activity 0.10784 U/mL/min. was recorded by the bacteria isolate 1C1 (Micrococcus spp.) at 72 hours of incubation. These bacterial isolates can be used as microbiological tool for various industries.
Key words: *Skimmed milk agar, Micrococcus spp., Protease, Fruit peel waste*

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INTRODUCTION

Enzymes or biocatalysts are specialized proteins produced by living organisms and used to enhance the metabolic rate of reaction. These Protein catalysts are extremely effective and safe for the environment. They are distinguished by their specificity, strong catalytic activity, and capacity to function at both low and high temperatures [1]. The enzyme has wide applications in various industries such as food, pharmaceutical, textile, etc. for synthesizing chemicals, food production, and fermentation processes.

Enzymes are extensively investigated from an industrial standpoint aside from the fundamental approach. Due to the advantageous fusion of enzyme technology with scientific progress, rapid translation technology, and commercial-scale manufacturing processes, the last few decades of the twentieth century have seen astounding advancements and improved living standards. Protease is the most important industrial enzyme from an economic point of view because it accounts for about 60% of the industrial market worldwide [2] Protease (also known as proteinases, or proteolytic enzymes) is an enzyme that can hydrolyze the peptide linkage in proteins. Proteolysis of proteins can be used to produce hydrolyzed products with different functional properties, which can be used in various industries.

Plants and animals produce a wide amount of protease, but protease is produced exclusively from microorganisms [3]. Proteases are found in all organisms such as bacteria, fungi and algae where this enzyme plays a vital role in metabolic and physiological processes [4]. Several bacteria such as *Pseudomonas sp.*, *Bacillus sp*., *Staphylococcus sp*., and *Aeromonas sp.* are protease-producing bacteria. Microbial proteases are commercially important enzymes for having a huge range of applications in various industrial, Biotechnological, medicinal and basic research fields [5]. Microbial protease account for approximately 40% of the total worldwide enzyme sales [6]. Protease has enormous applications in both physiological and

commercial fields [7]. Various industrial applications have protease catalyse or hydrolyze protein portraying a vital role. Due to their numerous uses in the agricultural, pharmaceutical, photographic, leather, detergent, and food sectors, proteases play a crucial role. Processes for bioremediation are one of the protease's significant biotechnological applications [1]. The range of protease applications has broadened with the emergence of new areas in biotechnology, including clinical, pharmaceutical, and analytical chemistry [7].
The main purpose of this study is the isolation, screening, and identification by morphological and biochemical characterization of potent protease-producing bacteria from soil and fruit peel waste.

MATERIAL AND METHODS

Chemicals

The chemicals used throughout the study were of analytical reagent grade and obtained from Sigma, Aldrich and Merck.

Sample Collection

Different soil samples were collected from near and adjacent areas of Zundal village of Gandhinagar and Ahmedabad. Some fruit peel waste samples were collected from local vendors. All the samples were collected in a sterilized bag and carried to the laboratory for further analysis.

Isolation and Screening of Protease-Producing Bacteria

Protease-producing bacteria were isolated by using the serial dilution method. One gram of different soil samples and fruit peel waste samples were added to 9 mL of sterile distilled water and performed serial dilutions up to 10^{-3} dilutions under aseptic conditions. From each dilution 0.1 mL sample was spread on nutrient agar medium plates. All the nutrient agar plates were incubated at 37^0 C for 24 hours. Single bacterial colonies were selected and subcultured on nutrient agar slants and maintained at 4°C.

Screening of protease-producing bacteria

For Primary screening or qualitative protease assay, the isolated bacterial colonies were grown on Skim milk agar (SMA) that contained (g/L): 1 g glucose, 2.5 g yeast extract, 28 g skim milk powder, 5 g casein and 15 g agar powder. All SMA plates were incubated at 37°c for 24 hours. The selection of protease-producing bacteria is based on the formation of a clear zone around the colonies [6]. Secondary screening or quantitative assay was carried out based on the study of a zone of proteolytic activity of the different isolates. The colony showing the highest zone was selected for further studies.

Cultural, Morphological and Biochemical Characteristics of Isolates

The isolate was subcultured on a nutrient agar medium. Colony characteristics such as size, shape, texture, consistency and transparency were noted down. Gram staining was carried out for the selected isolates. Biochemical tests like Indole test, Methyl red test, Voges Proskauer, Triple sugar iron (TSI) agar test and Carbohydrate utilization test were performed for the selected isolates.

Crude enzyme preparation/ protease production

The determination of Protease enzyme activity was carried out by using a modified method of Cupp-Enyard [8]. For crude enzyme, the selected bacterial isolates were grown in 50 mL of fermentation broth (glucose -5 g/L, peptone-7.5 g/L, casein-1 g/L, $MgSO_4 \cdot 7H_2O$–5g/L, KH_2PO_4-5 g/L, $FeSO_4.7H_2O$-0.1 g/L and D/W-1000 mL) and incubated at 37°C for 24 hours. After incubation, the broth was centrifuged at 7000 rpm for 10 minutes. The supernatant was used as a crude enzyme source for the determination of enzyme activity.

Protease enzyme assay

Protease activity was determined by using Casein as a substrate. For enzyme assay, the reaction mixture was prepared by taking one mL of crude enzyme and one mL of 1 % Casein (dissolved in 50 mM sodium phosphate buffer, pH 7.5) in a tube. The reaction mixture was incubated at 37 °C for 10 minutes. After 10 minutes, the reaction was stopped by adding 3 mL of 10% trichloroacetic acid (TCA) in a tube. After that entire mixture was centrifuged at 5000 rpm for 15 minutes. After centrifugation, one mL of filtered solution,2.5 mL of 500 mM sodium carbonate solution and 1 mL of Folin & Ciocalteu's phenol reagent (1N) were added to the tube. The tube was kept in the dark for 30 min at room temperature. The absorbance was recorded at 660 nm by using a spectrophotometer and the standard curve was plotted [9].The concentration of tyrosine in the filtrate was calculated from the standard graph. One unit of protease activity was defined as the amount of enzyme that liberated one micromole of tyrosine per mL per minute under standard reaction conditions. All the experiments were performed in triplicates and mean value was presented. The potential isolate with higher protease activity was chosen for carrying out further characterization and identification steps.

RESULTS AND DISCUSSION

Isolation of protease-producing bacteria

A total of 20 bacteria were isolated from different samples such as fruit peel waste and soil samples collected from near and adjacent areas of Zundal, Ahmedabad. All isolated bacterial colonies were spot inoculated on the skim milk agar plate for primary screening of potential protease-producing bacteria. Out of 20 colonies, five bacterial colonies showed a zone of casein hydrolysis on skim milk agar plate considered positive protease producers. Among five protease-producing bacterial isolates, 1C1,1C2 and 1C3 were obtained from fruit peel waste, 2C1 and 3C2 were isolated from Zundal soil and Ahmedabad soil respectively.

In the present investigation, among five bacterial isolates, 1C1 (2 mm), 1C2 (3 mm),1C3 (8 mm), and 2C1 (6 mm) were found to produce a smaller casein hydrolysis zone in comparison with 3C2 (Table 1 and Figure 1). Bacterial isolate 3C2 showed a maximum zone (10 mm) of casein hydrolysis.

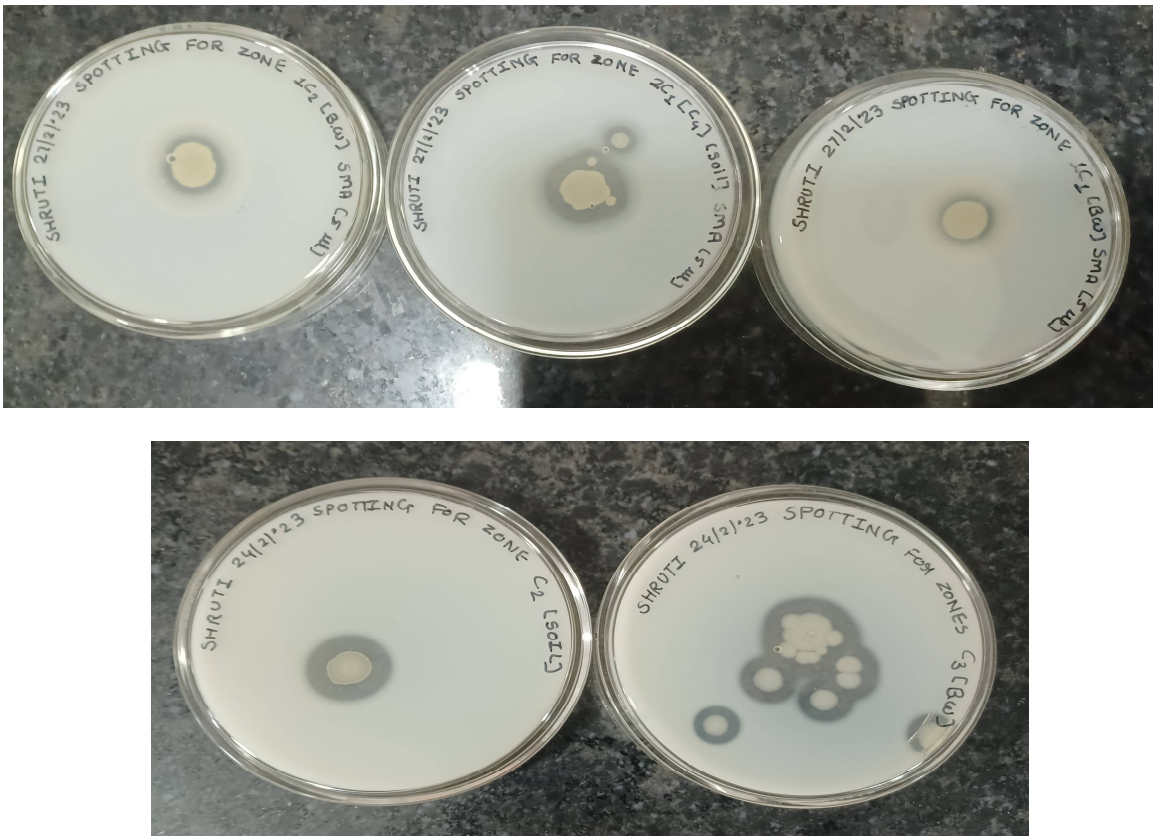

Figure 1: Zone of casein hydrolysis by protease-producing bacterial isolates on milk agar plates

Table 1: Zone (mm) of casein hydrolysis on skim milk agar plate by selected bacterial isolates

Bacterial isolates	Diameter of colony (d) mm	Diameter of zone+colony (D+d) mm	Zone of casein hydrolysis (D-d) mm
1C1	15	17	2
1C2	15	18	3
1C3	07	15	8
2C1	05	11	6
3C2	10	20	10

The screening of 6 bacterial isolates on skim milk agar plate was reported by Alnahdi [10]. Proteolytic activity was expressed as the diameter of the clear zone of hydrolysis around bacterial colonies. A similar screening method using skim milk agar medium and gelatin agar medium has been used earlier by many researchers. [2,11,12, 13 and 14].

Cultural, Morphological and Biochemical Characteristics of Selected Isolates:

The selected five bacterial isolates namely 1C1, 1C2, 1C3, 2C1 and 3C2 were grown on a nutrient agar medium to study their morphological characteristics. Among the five bacterial isolates, the colonies of

1C1 and 1C2 were entire, convex, circular, smooth and opaque. The colony of 1C3 was characterized by small, smooth, convex and translucent whereas 2C1 and 3C2 showed gray-white, round, opaque, flat and dry colonies on nutrient agar. The isolate 1C3 was grown on MRS (deMan Rogosa Sharpe) Agar plate, whereas 2C1 and 3C2 were grown on Brain Heart Infusion Agar plates. The potent bacterial isolates were identified based on morphological characterizations. The morphological characteristics are shown in Figure 2. The results revealed that among all five isolates, 1C1,1C2 were gram-positive cocci and isolates 1C3, 2C1, 3C2 were gram-positive rod-shaped bacteria. The biochemical characterization of all five bacterial isolates was carried out and is tabulated in Table 2.

Table 2: Biochemical characterization of selected bacterial isolates

Biochemical tests	Selected Bacterial Isolates				
	1C1	1C2	1C3	2C1	3C2
Carbohydrate Fermentation Test	+	+	+	+	¬
Catalase Test	+	+	¬	+	+
Indole Test	¬	¬	¬	¬	¬
Citrate Test	¬	¬	¬	+	+
Urease Test	¬	¬	¬	¬	¬
Gelatine Test	¬	¬	+	+	¬
Methyl Red Test	+	+	+	¬	¬
Voges-Proskauer Test	¬	¬	¬	¬	¬

Here, '+' Sign indicates Positive result; '-'sign indicates Negative result

The results of the biochemical test revealed that the isolates 1C1, 1C2, 1C3 and 2C1 showed Carbohydrate Fermentation Test positive. IMViC test shows that 2C1 and 3C2 were positive for Methyl Red Test, 2C1 and 3C2 were positive for Citrate Test. Among all five isolates, 1C3 and 2C1 were able to liquefied the gelatin whereas 2C1 and 3C2 were positive for the catalase test. From the results of biochemical tests and colony morphology, the bacterial isolates 1C1 and 1C2 were identified as *Micrococcus spp,* 1C3 as *Lactobacillus spp.* The bacterial isolates 2C1 and 3C2 were identified as *Bacillus spp*. The efficacy of *Bacillus spp*. in protease production among the isolates aligns with previous studies. [10,15]

The protease assay of five isolates was carried out by growing the organisms in liquid medium. All bacterial isolates produced protease enzyme at varied levels (Figure 2). The highest enzyme activity was observed at 72 hours of incubation. Among five bacterial isolates, the maximum protease activity 0.10784 U/mL/min. was found by *Micrococcus spp*. (1C1) at 72 hours. The protease activity 0.038 EU/mL and 0.024 EU/ mL was found at 48 hours of incubation by *Micrococcus luteus*-4 and *Micrococcus varians*, respectively [16]. *Bacillus pseudomycoides* showed higher protease activity 0.082 U/mL at room temperature [17]. The maximum protease production by *Bacillus sp.* 0.031 U/mL was observed at 24 hours of incubation [18].

Other three isolates 1C2, 1C3 and 3C2 showed 0.04592 U/mL/min.,0.10576 U/mL/min. and 0.057280 U/mL/min. protease activity respectively. The lowest enzyme activity (0.02864U/mL/min.) was observed by isolates 2C1.

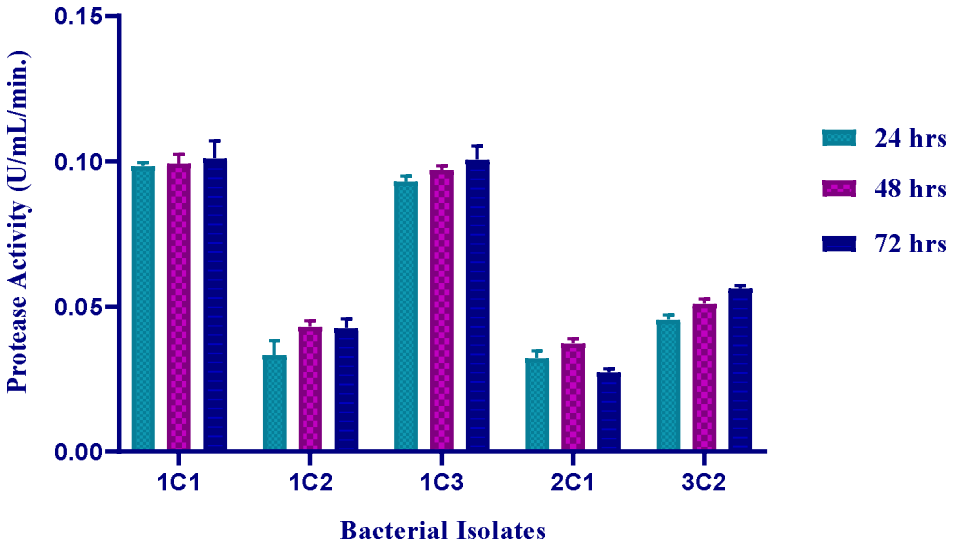

Figure 2: Protease activity of different bacterial isolates in production medium

CONCLUSION

The present study has exposed that a total of 20 bacteria were isolated from different samples such as fruit peel waste samples and soil samples. Five bacterial strains showed potent proteolytic activity on the skim milk agar plate assay method. The proteolytic bacterial isolates were further screened for protease production using fermentation broth. The bacteria isolate 1C1 has higher protease activity (0.10784 U/mL/min.). Based on morphological and biochemical characteristics, the 1C1 Strain was identified as *Micrococcus* species. This strain might be used for many industrial applications.

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