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Isolation And Characterization Of Environmental Protease-Producing Bacteria For Enhanced Bread-Making Applications

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ABSTRACT

This study was aimed at isolating and characterizing acid protease producing bacteria from different environmental sources for its possible application in bread making industry. Now, soil samples were collected from different ecological niches such as the kitchen waste, slaughterhouse, vegetable market, and meat shop. Fifteen bacterial isolates with strong proteolytic activity were identified from a casein agar medium (pH 6.0)] Through morphological, cultural, and biochemical tests, these isolates were characterized. Based on the zone of hydrolysis and enzyme index measurement, the Kitchen Waste Soil Sample isolates (KWSS1 and KWSS2) showed the highest proteolytic activity. Based on the bacterial resources, the isolated proteases were most notably tested for enzyme activities in bread-making applications, which demonstrated differences in bread texture and consistency. Enzymatic activities of isolates from slaughterhouse (SHSS2) and meat shop (MSSS1) led to softer bread texture indicating their possible application in industrial bread making. By isolating and utilising environmental acidic protease-producing bacteria in the production of bread, this study contributes significantly to the advancement of bread quality and texture.

Keywords*: Acidic protease[1], Bacterial isolation[2], Bread-making[3], Enzyme activity[4], Food biotechnology[5]*

1. INTRODUCTION

Proteases are enzymes that catalyse the hydrolysis of peptide bonds, leading to the generation of oligopeptides and amino acids. Production of these enzymes is one of the largest groups of industrial enzymes, representing around 60% of total worldwide enzyme sales (Anwar & Saleemuddin, 1998; BCC Report, 2018). Applications of proteases are found in detergent, food, pharmaceutical, leather, textile and waste management industries (Banerjee & Ray, 2017; Basher & Umesh, 2018). Of different protease sources, microbial proteases, particularly bacterial proteases gained goose over biochemical diversity, fast growth, less space requirement in fermentation and amenability for genetic manipulation (Bhunia et al., 2012; Aguillar et al., 2008) Bacterial proteases can be categorized according to their optimum pH as acidic, neutral or alkaline proteases. Apart from alkaline proteases, their application in detergent formulations has been studied significantly (Banik & Parkash, 2004; Baweja et al., 2016), while acidic proteases are likely to find application in food industries, viz., bread-making (Anwar & Saleemuddin, 1998).

Proteases are important in the baking industry, as they alter gluten proteins, thereby affecting dough rheology and also bread texture and quality (Ahmed et al., 2008). In particular, acidic proteases are able to hydrolyze gluten proteins under the slightly acidic conditions present in bread dough, which could enhance dough handling properties, bread volume, and texture (Cheng et al., 1995). In particular, compared to alkaline proteases, acidic

proteases derived from bacterial sources have been less thoroughly investigated, especially for use in bread-making applications. Isolation of protease-producing bacteria from various environmental sources is a strategic way of finding novel enzymes with unique properties that are suitable for specific industrial use (Alnandi, 2012; Asha & Palaniswamy, 2018). Soil samples taken from diverse ecological niches possess a variety of microbial communities adaptable to different environmental conditions, therefore they are a potential source of novel bacteria producing protease (Agarwal et al., 2016).

In this study, we aim to isolate and characterize different acidic protease-producing bacteria from the environment and test their application in bread-making. The specific objectives are as follows:

- Isolation of bacteria producing acidic proteases from different ecological niches
- Isolation and screening of effective acidic protease producing bacterial isolates
- Morphological, cultural and biochemical characterization of the isolates selected
- Assessment of the enzymatic activity of purified proteases for bakery applications

2. LITERATURE REVIEW

Proteases form one of the largest industrial enzyme groups, with the worldwide enzyme sales indicating an approximate 60% share for protease (BCC Report, 2018). These enzymes have wide industrial applications for detergent, food, pharmaceutical, leather and textile industries (Banerjee & Ray, 2017). Bacterial sources of microbial proteases have specific advantages over plant and animal proteases, such as biochemical diversity, susceptibility to genetic manipulation, rapid growth and limited space needed for microorganisms' cultivation and production (Bhunia et al., 2012). Based on pH optima, bacterial proteases are divided into three groups (Anwar & Saleemuddin, 1998): acidic, neutral, and alkaline proteases.

Alkaline proteases have been thoroughly investigated as potential detergent components (Banik & Parkash, 2004; Baweja et al., 2016). These enzymes work exceptionally well in removing protein stains from fabrics in alkaline conditions. A soil isolate Bacillus koreensis BK-P21A producing a solvent stable extracellular protease with potential application in detergent formulations was characterized by Anbu (2013). Acidic proteases are, on the other hand, applicable in food industries including bread-making (Anwar & Saleemuddin, 1998). Proteases are one of the most important within the baking industry where the modification of gluten proteins affects the rheology of dough, texture and quality of the bread (Ahmed et al., 2008). In particular, acidic proteases can hydrolyze gluten proteins at the slightly acid-dominating environment, which can contribute to better dough handling properties, bread volume, and texture (Cheng et al., 1995).

Isolation of protease producing bacteria from variety of environmental sources is a strategy to explore new enzymes with desired characteristics for industrial application (Alnandi, 2012; Asha & Palaniswamy, 2018). Microbial communities that survive in various ecological niches are known to be adapted to different environmental conditions, and they remain potential source of novel protease producing bacteria (Agarwal et al., 2016). Almalika et al. (2017) isolated Enterobacter aerogenes strains producing halotolerant alkaline protease from marine environments, characterized their halotolerant ability and predicted application prospects in bioprocesses and foods. The isolation of protease-producing bacteria has been previously identified in a number of origins including among others soil (Agarwal et al., 2016), marine environment (Almalika et al., 2017) and food waste (Asha & Palaniswamy, 2018). Most of the studies have been published on alkaline proteases, with

few studies on acidic proteases, especially for bread-processing applications. The present study attempts to fill this gap by isolating and characterizing acid protease producing bacteria from various environmental sources and testing their potential application in bread-making.

3. MATERIALS AND METHODS

3.1 Media

3.1.1 Casein Agar Medium (g/L)

- Casein: 0.9g
- Nutrient Agar: 6.5g
- Sodium Chloride (NaCl): 5g
- Distilled Water: 300ml
- pH: 6.0

3.1.2 Casein Broth (g/L)

- Casein: 0.25g
- Nutrient Broth: 0.65g
- Sodium Chloride (NaCl): 5g
- Distilled Water: 300ml
- pH: 6.0

3.2 Chemicals and Reagents

Analytical grade chemicals and reagents obtained from Hi-Media such as Casein, Nutrient agar, Sodium chloride (NaCl) and from Sigma such as Hydrochloric acid (HCl) were used for most of the investigations.

3.2.1 1N HCl Solution (100ml)

- Concentrated HCl: 8.3ml
- Distilled Water: 91.7ml

3.3 Microbiological Methods

3.3.1 Collection of Samples

Soil samples for the isolation of protease-producing bacteria were collected with a sterile spatula from 5-6 cm depth of surface soil and transported in sterile polybags. This experimental study conducted a process of sample collection on the following, Field soil, Sludge soil, Slaughterhouse soil, Kitchen waste soil, Meat shop soil, Fish shop soil, Vegetable market soil. Sample collecting bags were stamped with dates.

3.3.2 Isolation of Acidic Protease-Producing Bacteria

A gram of soil sample was weighed and added to the casein broth (Enrichment media) and incubated in a rotary shaker incubator at 120 rpm at 40°C for 24 h. The casein broth inoculum was taken as 1ml and inoculated to the sterilized 9ml of water blanks. Each soil sample was serially diluted by sterile distilled water blanks and the dilutions were made up to dilution 10^{-6}. 0.1ml of suitable dilution were spread on casein agar plate of pH 6.0 and incubated at 37°C for 24–48h (Anbu et al., 2013). The colonies showing clear zone of proteolysis were selected after incubation and pure cultured by inoculating to fresh casein agar plates using spotting method and incubation for pure cultures for 24−48 hours to confirm protease production based on clear zone development.

3.3.3 Screening of Acidic Protease-Producing Bacteria

HCl solution on each of the casein agar plates for 10-15 minutes. The generation of defined zones surrounding the colonies indicated acidic protease secretion. Those colonies having a well-defined zone surrounding the growth were defined as protease positive isolates (Olajuyigbe & Ajele, 2005).

3.3.4 Qualitative Estimation of Protease Production

The protease-producing ability of bacterial isolates was assessed by spotting the isolates on casein agar medium plates at pH 6.0. Plates were subjected to 37°C incubation for 72 hours and were examined daily for the formation of halozone around colonies, indicating casein hydrolysis during the incubation. Isolates producing protease were selected according to the zone of clearance. The zone of inhibited growth was measured as an index of protease activity.

Enzyme index (EI) = (Diameter of colony + Halozone) – Diameter of colony

Where, A = Diameter (Colony + Halozone), B = Diameter of colony.

3.3.5 Characterization and Identification

Morphological Characterization

The isolated bacteria were identified based on colony morphology including size, shape, color, margin, and elevation, growth condition, Gram's reaction, motility test, etc.

Simple Staining (Aneja, 2009)

- The process of simple staining was performed as given below:
- A clean glass slide was obtained with a droplet of water placed on it.
- One slide was made for each bacterial culture by smearing the bacteria on the slide.
- The heats were fixed by passing each moran over a flame.
- The stain was poured out, and the smear washed gently with running water.
- Slides were dried using a blotting paper.
- The slides were looked at under a microscope.

Negative staining (Aneja, 2009)

- Negative staining was done as follows:
- A clean slide was taken and one drop of nigrosin was added on the end of the slide.
- A loop of culture was taken from each bacterial culture, and an inoculation loop was used to smear the loop into the CLED agar.
- A clean slide was dragged with a smear at an angle of 30° over the entire surface of the bottom slide.
- The smear was left to dry on air.
- Preparation was inspected with an oil immersion objective under a microscope.

Gram's Staining (Aneja, 2009)

- Gram's staining procedure was performed as below:
- Water droplet added to a clean slide.
- Loopful of sample was used to make a smear of suspension on slide.
- The slide was heat-fixed by passing it over a flame.
- The crystal violet stain was flooded onto the mixed smear and let sit for 1 minute.
- The slide was washed under running tap water.

- The slide was immersed in Gram's iodine for 1 min.
- Gram's iodine solution was washed away with running tap water.
- The slide was washed with decolorizer (acetone or alcohol) for around 20-40 seconds.
- The slide was rinsed with tap water and counterstained with safranin for approximately 1 minute, washed with water.
- The slide was air-dried, a cover slip was mounted, and the slide was microscopically examined.

Biochemical Test (Aneja, 2009)

Catalase Test

- The catalase test was performed according to the following procedure:
- Inside a petri dish was a clean slide.
- A colony that was well-isolated was taken from a sterilized inoculating loop and placed on the slide.
- A few drops of 3% hydrogen per- oxide were placed on the colony, using a dropper.
- This colony was not with the hydrogen peroxide.
- Bubbles were rapidly elaborated.

3.4 Enzymatic Activity of Acidic Protease in Bread

The isolated bacteria were injected with the enzyme to check for the consistency of the flour for baking bread and the texture of the bread. The enzyme activity was measured by the DNSA method (3,5-Dinitrosalicylic acid). The optical densities were measured at a wavelength of 540nm with the 10^{-1}, 10^{-2}, 10^{-3} dilutions of buffer and substrate (B+S), buffer and enzyme (B+E), and buffer, enzyme, and substrate (B+E+S). The bread was baked at 191 °C for 30 min, and the consistency and texture were assessed.

4. RESULTS

4.1 Collection of Samples

Soil samples were collected from various sites including Kitchen waste soil, Slaughterhouse soil, Meat shop soil, Sludge soil, Fish shop soil, Vegetable market soil, and Field soil, which were collected in sterile polybags.

4.2 Isolation of Acidic Protease-Producing Bacteria from Various Ecological Niches

Samples were enriched under protease-producing microorganism-favorable screen with casein broth medium adjusted to pH 6.0. A total of 15 morphologically different bacteria were isolated from the soil samples collected. Among them, protease-producing ability was determined by the formation of clear zones of hydrolysis around the colonies on the casein agar plates, which were at pH 6.0 and incubated for 48 h, for a total of 7 isolates.

The distribution of the isolated bacteria from different soil sample sources are presented in Table 1. Two isolates were recovered from Kitchen waste soil (KWSS1, KWSS2), Slaughterhouse soil (SHSS1, SHSS2), and Meat shop soil (MSSS1, MSSS2), whereas one isolate was recovered from Vegetable market soil (VMSS1).

Table 1: Isolation of bacteria from different sources and their numbers

S. No.	Types of sample	Isolates	Total number of isolates	Number of Positive isolates

1	Kitchen waste soil sample	KWSS1, KWSS2	5	2
2	Slaughter house soil sample	SHSS1, SHSS2	4	2
3	Vegetable market soil sample	VMSS1	3	1
4	Meat shop soil sample	MSSS1, MSSS2	2	2

4.3 Screening of Acidic Protease-Producing Bacterial Isolates

Screening was performed by flooding the casein agar plates with 1N HCl solution. Clear, transparent zones of hydrolysis were observed around the colonies of all the positive isolates, confirming their ability to produce acidic proteases.

4.4 Quantitative Estimation of Acidic Protease Activity

7 positive isolates had a halozone size varied from 1.4 to 3.4 cm. The highest protease production based on zone size and enzyme index was in isolate KWSS1 from Kitchen waste soil (zone size: 3.4 cm; E.I: 3.0), followed by KWSS2 also from kitchen waste soil (zone size: 3.0 cm; E.I: 2.6). In contrast, VMSS1 from Vegetable market soil showed the lowest protease production (zone size: 1.4 cm; E.I: 1.1).

Table 2: Quantitative estimation based on zone hydrolysis produced by different bacterial isolates

S. No.	Sample	Bacterial isolates	Zone of hydrolysis (cm)	Enzyme index
1	Kitchen waste soil sample	KWSS1	3.4	3
2	Kitchen waste soil sample	KWSS2	3	2.6
3	Slaughter house soil sample	SHSS1	2	1.7
4	Slaughter house soil sample	SHSS2	1.5	1.2
5	Vegetable market soil sample	VMSS1	1.4	1.1
6	Meat shop soil sample	MSSS1	1.8	1.5
7	Meat shop soil sample	MSSS2	2.2	1.9

4.5 Characterization and Identification of Potential Isolates

Morphological and cultural characteristics of the isolated bacteria were studied based on colony morphology, microscopic examination, and biochemical tests. Table 3 summarizes the results of these characterizations.

Table 3: Morphological and cultural characteristics of acidic protease-producing bacteria

S. No.	Sample	Bacterial isolates	Colony morphology	Simple staining	Negative staining	Gram's staining	Catalase test

1	Kitchen waste soil sample	KWSS1	Irregular, Convex, Lobate, Yellow color	Rod shaped	Capsulated	Gram negative (Bacillus)	Positive
2	Kitchen waste soil sample	KWSS2	Irregular, Raised, Undulate, Pale yellow color	Rod shaped	Capsulated	Gram positive (Bacillus)	Positive
3	Slaughter house soil sample	SHSS1	Circular, Flat, Entire, Creamish white color	Rod shaped	Non-capsulated	Gram positive (Bacillus)	Positive
4	Slaughter house soil sample	SHSS2	Irregular, Umbonate, Undulate, White color	Rod shaped	Capsulated	Gram negative (Bacillus)	Positive
5	Vegetable market soil sample	VMSS1	Circular, Convex, Entire, Orange color	Rod shaped	Non-capsulated	Gram negative (Bacillus)	Positive
6	Meat shop soil sample	MSSS1	Irregular, Raised, Undulate, Light orange color	Rod shaped	Capsulated	Gram positive (Bacillus)	Positive
7	Meat shop soil sample	MSSS2	Filamentous, Umbonate, Lobate, Cream color	Rod shaped	Non-capsulated	Gram negative (Bacillus)	Positive

All bacterial isolates were rod-shaped (Bacillus) and catalase-positive, indicating their aerobic nature. Among the 7 isolates, 3 were Gram-positive and 4 were Gram-negative. Isolates were also capsulated and non-capsulated isolates were also obtained. The colony morphology of the isolates varied according to shapes (irregular, filamentous, circular), elevation (raised, convex, flat, umbonate) and margin (entire, undulate, lobate.

4.6 Enzymatic Activity of Acidic Protease in Bread

The enzymes produced by the isolated bacteria were evaluated for their effect on bread texture and consistency. Table 4 summarizes the results of this evaluation, including the optical density measurements at different dilutions and the observed bread characteristics.

Table 4: The enzymatic activity of acidic protease with different dilutions and at a particular wavelength in baked bread

S. No.	Types of sample	Isolates	Optical Density with dilution 10^{-1}, 10^{-2}, 10^{-3} (λ-540nm)	Consistency	Texture
1	Kitchen waste soil sample	KWSS 1	B+S: 0.159, 0.147, 0.130 B+E: 0.148, 0.134, 0.126 B+E+S: 0.155, 0.142, 0.131	Thick	Hard
2	Kitchen waste soil sample	KWSS 2	B+S: 0.112, 0.150, 0.142 B+E: 0.154, 0.138, 0.135 B+E+S: 0.154, 0.133, 0.127	Thick	Hard
3	Slaughter house soil sample	SHSS1	B+S: 0.124, 0.140, 0.160 B+E: 0.101, 0.100, 0.099 B+E+S: 0.143, 0.124, 0.121	Moderate	Moderate
4	Slaughter house soil sample	SHSS2	B+S: 0.144, 0.134, 0.130 B+E: 0.141, 0.131, 0.122 B+E+S: 0.133, 0.120, 0.119	Less thick	Soft

5	Vegetable market soil sample	VMSS 1	B+S: 0.155, 0.145, 0.147 B+E: 0.134, 0.123, 0.121 B+E+S: 0.152, 0.141, 0.136	Thick	Hard
6	Meat shop soil sample	MSSS1	B+S: 0.149, 0.140, 0.132 B+E: 0.156, 0.135, 0.146 B+E+S: 0.138, 0.136, 0.134	Less thick	Soft
7	Meat shop soil sample	MSSS2	B+S: 0.132, 0.142, 0.114 B+E: 0.148, 0.133, 0.142 B+E+S: 0.153, 0.144, 0.140	Thick	Hard

The bread texture and consistency varied depending on the bacterial isolate used. The isolates KWSS1, KWSS2, VMSS1, and MSSS2 produced enzymes that resulted in thick and hard bread, while SHSS2 and MSSS1 produced enzymes that yielded less thick and soft bread. SHSS1 produced bread with moderate thickness and texture.

5. DISCUSSION

The isolation and characterization of acidic protease-producing bacteria from different environmental sources were the objectives of this study, as well as testing the application potential in bread-making. Seven acid protease producing bacteria had been isolated and characterized from different soil samples. The casein agar medium (pH 6.0) used in the screening of protease-producing bacteria is appropriate for acidic protease producers. Flooding of the colony forming isolates with 1N HCl solution resulted in formation of clear zone of hydrolysis around their colonies, showing its ability to produce acidic proteases. The quantitative determination of protease activity in the isolation study based on the zone diameter and the enzyme index showed two isolated from the kitchen waste soil (KWSS1 and KWSS2) with the highest proteolytic activity, while, in the isolate from the meat shop soil (MSSS2) was found below that. The enzyme index was highest (3.0) for KWSS1, similar to the one reported by Asha & Palaniswamy (2018) for a Bacillus cereus strain isolated from soil (2.8).

Morphological and cultural characterization of the isolates showed that all of the isolates confirmed the presence of rod-shaped bacteria (Bacillus) and this is similar to previous findings which indicated that Bacillus species are among the most prominent protease producers in soil samples (Alnandi, 2012; Bhunia et al., 2012). All isolates were positive for catalase, suggesting their aerobic nature. Of these isolates, 3 were Gram-positive while 4 were Gram-negative, indicative of the variety of protease-producing bacteria found in the soil samples. The assessment of the baking properties of the purification proteases showed that they led to interesting bread textural and rheological properties. This study shows that the enzymes of SHSS2 and MSSS1 showed better results in the qualities of bread which is thickness and soft texture respectively as these characteristics are highly appreciated in the bread-making. The isolates also suggest potential applications for the baking industry. On the contrary, the enzymes from KWSS1, KWSS2, VMSS1, and MSSS2 made thick and hard bread, which might be not expected for some kinds of bread products.

These variations between the quality of bread can be related to the proteolytic activity and specificity of different bacterial isolates. SHSS2 and MSSS1 protease may hydrolyze gluten proteins more efficiently and adjust the bread structure, in the other hand other isolates it may affect different specificities which effects negatively to the characteristics of bread. These results agree with previous studies indicating that proteases can affect bread quality through the modification of gluten proteins (Ahmed et al., 2008; Cheng et al., 1995). But this is the first study to show how acidic proteases from different bacteria source impact bread texture and consistency.

6. CONCLUSION

The current study aimed to isolate and characterize some acid protease-producing bacteria from natural sources or environments and explore their possible application in the bakery industry. The main findings and conclusion of this study are:

- Nine strains of acidic protease producer bacteria were isolated, with the Kitchen waste soil isolates (KWSS1 and KWSS2) showing the highest proteolytic activity.
- All isolates were identified as rod-shaped (Bacillus) and catalase-positive, comprised of Gram-positive and Gram-negative characteristics, revealing a diversity of protease-producing bacteria in the soil samples.
- Based on qualitative analysis of the isolated proteases in the preparation of the bread, it was observed that the bread samples were thinner and softer when using the isolated proteases from Slaughterhouse soil isolate SHSS2 and meat shop soil isolate MSSS1, indicating their beneficial effect in baking industry.
- Bread-quality differences highlight the use of targeted protease sources relative to specific applications in bread production.

The results of the work add to the existing knowledge about acidic proteases produced by bacteria and provide instruction to the baking sector. Further investigation may involve the purification and characterization of the identified proteases (SHSS2 and MSSS1), optimizing their production conditions, and assessment of industrial performance across different bread formulations.

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