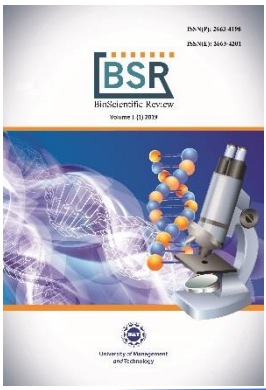

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Production of Extracellular Protease from Bacterial Co-cultures using Solid State Fermentation

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Abstract

Proteases (also known as peptidases or proteinases) are hydrolytic enzymes that cleave proteins into amino acids. They comprise 60% of the total industrial usage of enzymes worldwide and can be obtained from many sources. The current study aims to isolate and screen protease-producing bacterial strains from the soil and to produce protease from the bacterial co-cultures using solid-state fermentation (SSF). Primary screening of the protease-producing bacterial strains was carried out on skim milk agar and they were sub-cultured and preserved on the nutrient agar for further testing. Thirty-two compatibility tests of twenty-seven bacterial isolates were performed and SSF was carried out. Afterward, absorbance was taken at 660 nm against tyrosine as standard. According to the results, the bacterial co-culture 19 showed the highest absorbance with an enzyme activity of 10.2 U/ml. The bacterial strains of the co-culture 19 were identified through morphological and biochemical tests. Bacterial strain 1 was observed as cocci and irregular, while bacterial strain 2 was bacillus and rod-shaped. Both strains were positive for gram staining, catalase test, casein hydrolysis test and methyl red test. As for endospore staining, bacterial strain 1 was spore forming while bacterial strain 2 was a non-spore former. It was concluded that the bacterial co-culture 19 can act as a potent co-culture for protease production. Compatibility test was carried out to enhance the production of protease by utilizing cheap and readily available agro-waste products, which benefit the industry by being cost effective and the environment by being eco-friendly.

Keywords: bacterial co-culture, enzyme assays, hydrolytic enzymes, proteases, solid-state fermentation

1. Introduction

Proteases are enzymes that hydrolyze proteins into amino acids by cleaving the peptide bond. They are also known as peptidases or proteinases [1]. Hydrolytic enzymes comprise 60% of all commercially produced enzymes and proteases account for 20% of the hydrolytic enzymes [2].

Extracellular proteases are important in protein hydrolysis carried out in various industries such as in the detergent industry [3]. Various sources can be utilized to obtain proteases such as animal, bacterial, fungal, and plant sources. Microbial proteases are preferred because of the presence of all the desired characteristics for industrial applications.

Proteases can be divided into two main groups, endopeptidases, and exopeptidases. Endopeptidases cleave the peptide bonds present in the proteins to form amino acids, while exopeptidases cleave the peptide bonds present on the terminals of the protein structures. Endopeptidases can be further classified based on the catalytic triad and pH. Based on the catalytic

triad they can be further divided into serine protease, cysteine protease, aspartic protease, and metalloprotease. Based on pH, they are further divided into acidic, neutral, and alkaline proteases. Acidic proteases are mainly obtained from fungal sources, while neutral and alkaline proteases can be produced from plants and bacteria, respectively. As for as, exopeptidases are concerned, they are grouped into aminopeptidases and carboxypeptidases which can further be divided into various sub-groups [4].

Proteases obtained from animal sources such as chymotrypsin, trypsin, and pepsin are used for digestion purposes. Those obtained from plants such as bromelain, papain, and ficin are utilized for meat tenderization and fruit ripening. As for microbial proteases, they can be either be obtained from bacteria or fungi. For example, subtilisin and bacillopeptidases are obtained from bacteria, and aspergillopepsin is obtained from fungi [5].

The proteases available in the market are of microbial origin which makes them suitable for various biotechnological applications due to their high yield in a small amount of time, minimum space requirement, and cost-effectiveness. They can grow on waste materials that are cheap to find and also benefit the environment through the consumption of waste materials such as substrates [6, 7].

Bacterial proteases are used in various industries such as the baking industry – for the breakdown of proteins in various bakery items; the detergent industry – for removing protein-based stains; the leather industry – for the processing of leather and the de-hairing of the animal skin; the cosmetic industry – used in masks and creams for the regeneration of the skin; the pharmaceutical industry – as a food supplement and in various ointments and creams and other medicines mainly used for the treatment of stomach related diseases; the meat industry – for the tenderization of meat; the dairy industry – for the production of cheese and the infant formula milk powder; the food and feed industry – in animal and baby food; and finally, they can be used also for research in synthetic organic chemistry [5].

Industrially important protease-producing bacteria belong to the *Bacillus* genus. Some *Bacillus* species are *Bacillus cereus* strain AT, *Bacillus megaterium* RR2, *Bacillus cereus* FJ10, *Bacillus licheniformis* N-2, *Bacillus amyloliquefaciens, Bacillus licheniformis* KBDL, *Bacillus subtilis* and *Bacillus cereus.* They are used in detergent, leather, silver recovery, food, feed industries, and also for medical purposes. Besides *Bacillus* species, *Pseudomonas aeruginosa* is also an important bacterial strain used to obtain protease in the detergent industry [4].

The objective of this study is to isolate and screen high potential protease-producing bacterial strains from the soil samples and to produce protease from the bacterial co-culture using SSF.

2. Materials and Methods

2.1. Sample Collection

Soil samples were collected from various locations in Lahore in zip-lock bags. The date, time, and location of each sample were noted properly and the samples were transported to the lab aseptically. Soil samples were collected mostly from the sewerage, dirty drainage, farms, slaughterhouses, and junkyards. The reason to choose these locations was that they reportedly have

maximum protease-producing strains.

2.2. Isolation and Screening of Bacterial Isolates

Serial dilution and spread plate methods were used for the isolation of bacterial strains from these samples [8]. Sterile skim milk agar (1%) was poured into Petri plates and incubated for 24 hrs at 37°C for the contamination check. Afterward, 1 g of soil sample was dissolved in 100 ml distilled water and diluted from 10^{-1} to 10^{-10}. Moreover, 1 ml of each aliquot was spread on skim milk agar plates and incubated for 48 hrs at 37°C. Zones of hydrolysis were observed and the strains were then sub-cultured on the sterile nutrient agar plates and incubated for 24 hrs at 37°C. Isolated colonies were preserved on the sterilized nutrient agar slants at 4°C for further testing.

2.3. Compatibility Test

Two different bacterial strains were streaked in the shape of a plus (+) sign on the nutrient agar plates to check their compatibility. The plates were labeled and incubated at 37°C for 48 hrs. After incubation, the strains that fully crossed each other showed that they favored each other's growth. On the contrary, if the growth of the strains stopped in the center of the plate, it was determined that they inhibited each other. The strains that favored mutual growth were selected for further testing.

2.4. Vegetative Inoculum Preparation and Glycerol Stock Preparation

Luria-Bertani broth was added into cotton-plugged test tubes and autoclaved. After sterilization, the colony was added into it and the test tubes were placed on the rotatory shaker at 37°C overnight. For glycerol stock preparation, 800 µl of 30% glycerol was added into a 1.5 ml tube (Eppendorf) along with 300 µl of vegetative inoculum. The tubes were then frozen to preserve the strains for the long term.

2.5. Solid-state Fermentation (SSF)

Tests were carried out in 500 ml Erlenmeyer flasks containing 5.0 g of wheat bran as a solid substrate along with 20 ml of production media containing KH_2PO_4 (0.1%), NaCl (1%), $MgSO_4$ (0.01%), and NH_4NO_3 (0.5%) with pH 7.2. The flasks were cotton plugged and sterilized and afterward, they were allowed to cool at room temperature. Later on, 2 ml inoculum of each co-culture was added into the flasks which were then incubated at 35°C for 48 hours. After the incubation, the fermented matter was mixed with 50 ml distilled water and placed on the incubator shaker for 1 hour at 37°C. After shaking, the fermented medium was separated using the muslin cloth. The filtrate was centrifuged at 10,000 rpm for 15 mins. The cell-free supernatant was separated and used for further study [9].

2.6. Enzyme Assay

Filtrate supernatant (0.25 ml) along with 1.25 ml of 0.1M phosphate buffer and 1% freshly prepared casein solution was added into the test tube and incubated for 30 mins at 30°C. After incubation, 3 ml chilled trichloroacetic acid (TCA) was added to stop the reaction and placed in the fridge for 10 mins at 4°C. The media was then centrifuged at 5000 rpm for 15 mins. Afterward, 0.5 ml supernatant was taken in a clean test tube to which 2.5 ml of 0.5M sodium carbonate was added and incubated for 20 minutes at 37°C. Finally, 0.5 ml Folin–Ciocalteu reagent was added and absorbance was taken at 660 nm using spectrophotometer against tyrosine as standard [9].

2.7. Tyrosine Standard Curve

For the tyrosine standard curve, the dilutions of tyrosine standard solution were formed by adding 1 ml, 2 ml, 3 ml, 4 ml, and 5 ml of tyrosine standard solution with 9 ml, 8 ml, 7 ml, 6 ml, and 5 ml of distilled water in each test tube respectively to obtain the final volume of 10 ml. A blank dilution was also formed by adding only 10 ml of distilled water with no amount of tyrosine standard solution. Moreover, 1 ml of each aliquot was taken in new test tubes along with 5 ml of 2.47 M sodium carbonate and 1 ml of Folin–Ciocalteu reagent and incubated in the water bath for 20 mins at 40°C [9]. After incubation, absorbance was taken at 660 nm and the tyrosine standard curve was drawn on MS Excel. A previously reported method was used to measure the tyrosine released [9] which states that "protease activity was expressed in microgram of tyrosine released by 1 ml of an enzyme in 30 minutes at 30°C on tyrosine equivalent."

2.8. Identification of Bacterial Strains

The morphological determination of bacterial strains was carried out based on the shape of the colony, elevation, and margins. Endospore staining, gram staining, and biochemical tests including casein hydrolysis test, catalase test, and methyl red test were performed and the bacterial strains were identified according to Bergey's manual of determinative bacteriology.

3. Results

3.1. Isolation and Screening of Protease Producing Bacterial Isolates

Twenty-seven bacterial isolates were obtained from five soil samples. The plates were incubated and the zones of hydrolysis were observed (Figure. 1A). The colonies showing the zones of hydrolysis were streaked on the nutrient agar plates to obtain the isolated colonies which were then preserved on the nutrient agar slants (Figure. 1B, Figure. 1C).

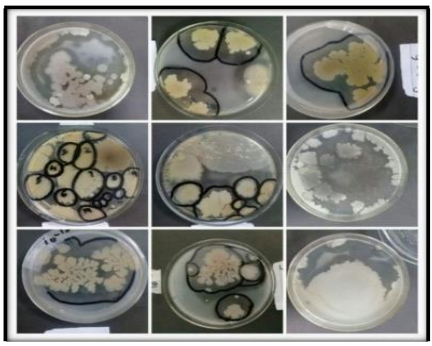


Figure 1A. Protease producing strains showing the highest zones of hydrolysis on skim milk agar plates

Figure 1B. Streaking of bacterial isolates on a nutrient agar plate

Figure 1C. Preservation of bacterial isolates on the nutrient agar slants at 4°c

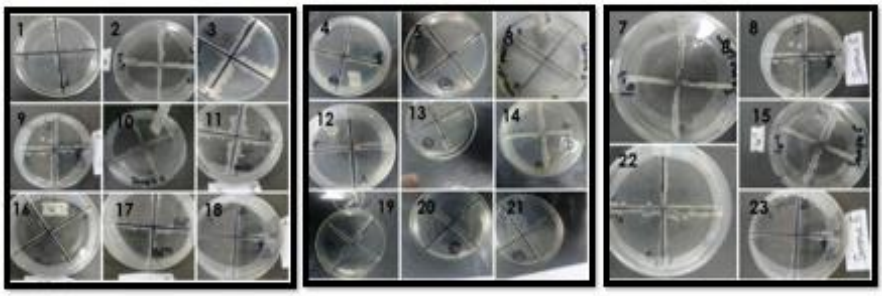


Figure 2B. Positive bacterial co-cultures

3.2. Compatibility Test

The isolated strains were then tested for compatibility using the co-culturing technique. A total of 32 co-culture tests were performed out of which 23 came positive while the remaining 9 came negative (Figure. 2A, 2B, 2C, and 2D).

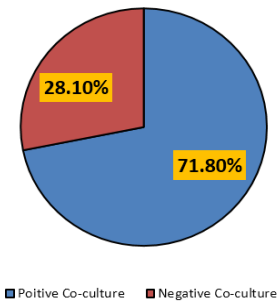


Figure 2A. Percentage of compatibility test

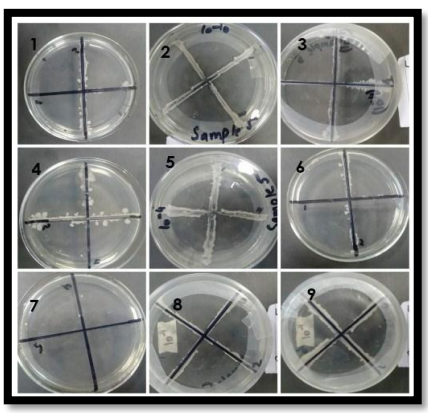


Figure 2C. Negative bacterial co-cultures

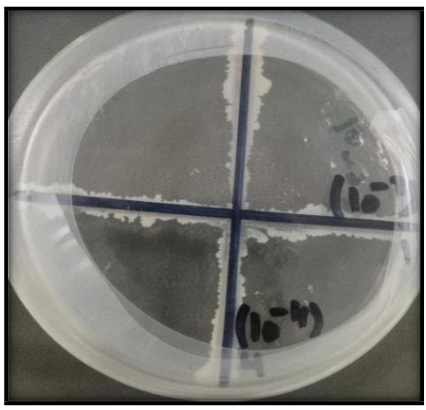


Figure 2D. Positive bacterial co-culture no. 19

3.3. Solid-state Fermentation (SSF) and Enzyme Assay

The co-culture strains that showed the highest zone of hydrolysis were used for SSF (Figure. 3A, Figure. 3B). Moreover, enzyme assay was performed and absorbance was taken at 660 nm. The curve value was calculated as y = 0.0115x + 0.0183. Out of the total 23 positive co-cultures, 7 co-cultures (CC-8, CC-9, CC-10, CC-16, CC-17, CC-18, CC-19) with the highest zones on skim milk agar were selected for enzyme assay. Of these positive co-cultures, the co-culture no. 19 yielded the maximum protease production with an enzyme activity of 10.2 U/ml. This co-culture was selected for further studies (Figure. 4A, 4B, 4C) (Table 1A, Table 1B).

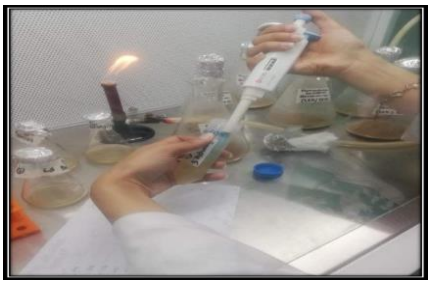


Figure 3A. Inoculum preparation for SSF

Figure 3B. Solid-state fermentation media

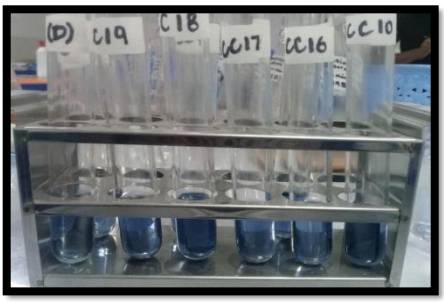


Figure 4A. Dilutions for enzyme assay

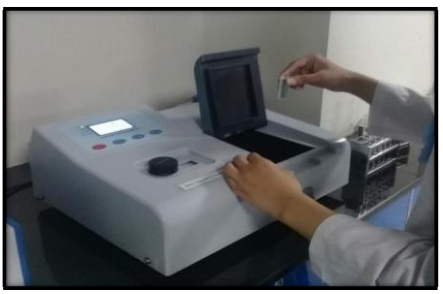


Figure 4B. Enzyme assay

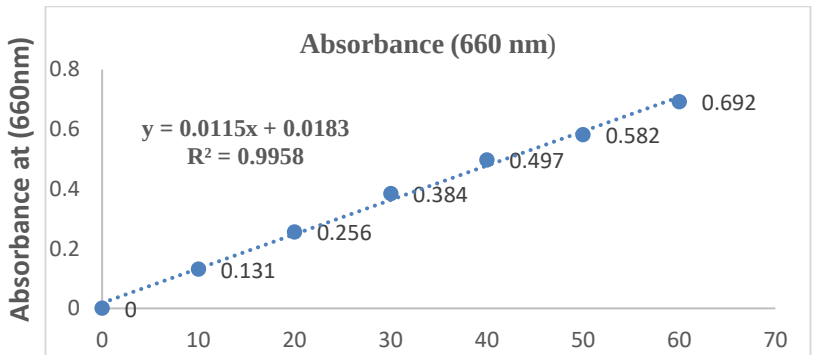


Figure 4C. Tyrosine standard curve

Table 1A. Different Dilutions of Tyrosine Standard Solution

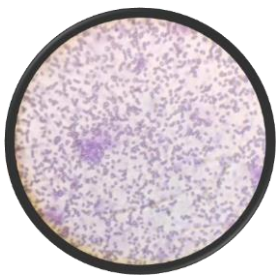
Standard Solution No.	Volume of Tyrosine Solution (ml)	Volume of Distilled Water (ml)	Tyrosine Concentration (µg/ml)	Absorbance at 660nm
1	0	10	Blank	0
2	1	9	10	0.131
3	2	8	20	0.256
4	3	7	30	0.384
5	4	6	40	0.497
6	5	5	50	0.582
7	6	4	60	0.692

Table 1B. Determination of the Protease Activity of the Selected High Potential Bacterial Co-cultures

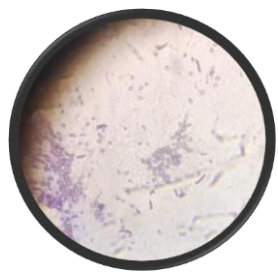
Sr. No.	Fermentation Co-culture No.	Absorbance at 660nm	Concentration of Tyrosine Released (ug/ml)	Enzyme Activity (U/ml)
1	Control	0.267	21.6	4.8
2	CC 8	0.490	41.0	9.1
3	CC 9	0.444	37.0	8.2
4	CC 10	0.517	43.3	9.6
5	CC 16	0.476	39.8	8.8
6	CC19	0.544	45.7	10.2
7	CC 17	0.480	40.1	8.9
8	CC 18	0.393	32.5	7.2

3.4. Identification of Bacterial Strains

Bacterial isolates of the co-culture 19 were further identified according to Bergey's manual of systematic bacteriology. Bacterial strain 1 of the co-culture 19 was observed as cocci and irregular, while bacterial strain 2 was bacillus and rod-shaped. Both strains were positive for gram staining, catalase test, casein hydrolysis test, and methyl red test. As for endospore staining, bacterial strain 1 was spore-forming while bacterial strain 2 was non-spore former (Figure. 5A, Figure. 5B) (Table 2).

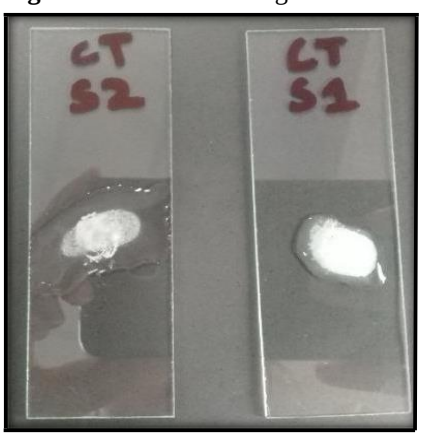


(a) Gram-positive Cocci

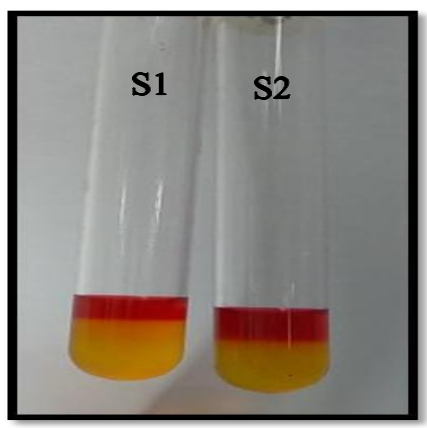


(b) Gram-positive Rod-shaped

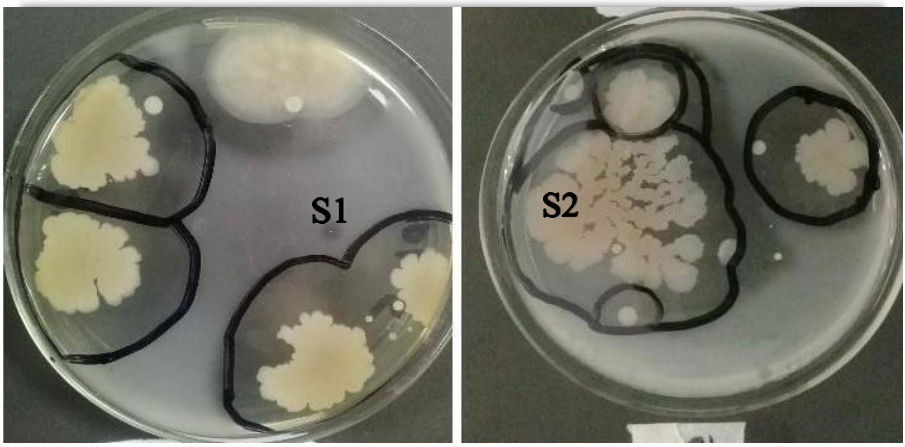
Figure 5A. Gram Staining of the Bacterial Co-culture 19 Strains



(a) Catalase test



(b) Methyl red test



(c) Casein hydrolysis test

Figure 5B. Biochemical Tests of the Bacterial Co-culture 19 Strains

Table 2. Morphological and Biochemical Testing of the Co-culture 19

Co-culture No. 19	Shape and Colony Morphology	Gram Staining	Endospore Staining	Catalase Test	Casein Hydrolysis Test	Methyl Red Test
Bacterial Strain 1	*Cocci* Irregular	Positive	Positive	Positive	Positive	Positive
Bacterial Strain 2	*Bacillus* Branched Rod-shaped	Positive	Negative	Positive	Positive	Strongly Positive

Discussion

The selection of a productive bacterial culture is vital for the prosperity of the economic biosynthesis of protease. In the current study, the production of microbial protease (especially from bacteria) was preferred over other sources such as animal, plant, and fungal sources. This is because bacterial proteases are suitable for various biotechnological applications. Hence, they benefit the economy due to their high yield in a small amount of time, the minimum space requirement for growth, and cost-effectiveness since they can grow on waste materials [1].

In this study, twenty-seven different bacterial isolates were obtained from soil samples using the serial dilution method [8]. Soil samples were collected from various locations in Lahore such as dirty drainage, sewerage, junkyards, farms, and slaughterhouses. These locations reportedly yield maximum protease-producing bacterial strains [10].

The strains showing the highest zones of hydrolysis and a positive co-culture were screened by protease production using SSF.

Gram staining was performed for the characterization of bacterial isolates based on their cell wall composition. All the bacterial strains were identified as gram-positive since they showed the

purple color when observed under 100X using a microscope [11, 12].

The compatible strains forming the highest zones of hydrolysis on isolation media were further screened using SSF [9]. Solid-state fermentation was selected over submerged fermentation because it is cost-effective, easy to perform at a large scale, and uses much less water as compared to the submerged fermentation. Moreover, the solid substrates needed in SSF are mainly agro-waste products that are cheap and easily available in the market. Also, it benefits the environment by consuming waste materials as substrates [7].

In the current study, wheat bran was selected as the solid substrate [13]. The reason to select wheat bran as substrate was that it is cost-effective and easily available in the market. Besides, there are 16 grams of protein per 100 grams of wheat bran. Protein is an important component consumed by the protease-producing strains during fermentation to produce protease enzymes. After SSF, enzyme assay was performed and absorbance was taken at 660 nm against tyrosine as standard. Finally, the amount of tyrosine released was calculated by the known tyrosine standard curve [14].

High-quality protease production is crucial in the manufacturing of industrial goods. Hence, the search for a bacterial strain capable of producing industrial-grade protease remains in progress [15]. Recent years have witnessed widespread use of microorganisms to produce various enzymes on an industrial scale as it offers many economic and technical advantages [16].

Conclusion

The biosynthesis of protease by the bacterial co-culture no. 19 using SSF was analyzed. The results obtained in the current study suggest that the chosen co-culture may act as a dynamic co-culture for enzyme production through SSF by utilizing wheat bran (WB) as a solid substrate. It seems that wheat bran is a readily available agro-waste substance that can act as a satisfactory economic medium for fermentation to obtain the maximum yield of protease. It can replace the more expensive substrates currently used for proteinase production. The maximum activity of protease was obtained as 10.2 U/ml by the strains of the bacterial co-culture 19 at 37 °C after 48 hours of incubation.

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