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Production of protease enzyme by isolated fungi and bacteria under solid state fermentation using low cost medium wheat bran

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Abstract : *The present study aimed to isolate microorganisms from soil samples which have the potential to produce protease enzyme on wheat bran media. Screening of the isolates were done on skimmed milk agar plates. A total of twelve strains were isolated. Upon initial identification, out of seven bacterial strains tested, only five showed proteolytic activity and out of five fungal strains tested, only three showed good proteolytic activity. Further extraction and optimization of protease enzyme at different temperature and after different time interval was done. On the fifth day of incubation maximum enzyme activity was observed by bacterial strains. On incubation at different temperatures, at 37°C strain II and IV and at 25°C strain I and III showed maximum enzyme activity. Among fungal strains, strain II showed maximum enzyme activity.*

Key Words: *Skimmed milk agar media, fungi, bacteria, wheat bran.*

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Introduction:

Enzyme is a biocatalyst which accelerates the rate of biological reaction. Proteases occur in all organisms. These enzymes are involved in a multiple of physiological reactions from simple digestion of food proteins to highly regulated cascades (Cai et al. 2008) Microorganisms are the most important sources for enzyme production. Selection of the right microorganism plays a key role in high yield of desirable enzymes. Among these, *Bacillus* genes gained importance at industrial scale. The first detergent containing bacterial enzymes was introduced in the market in 1956 under the trade name bio-40 and today alkaline protease. The *Aspergillus* species produces a large variety of extracellular enzymes, of which proteases are of significant industrial importance (Pandey et al. 2000). At present the overall cost of enzymes production is very high (due to high cost of substrate and medium used) and therefore development of novel processes to increase the yield of proteases with increasing the production cost is highly appreciable from the commercial point of view. To achieve these goals during the recent years, effects have been directed to expose the means to reduce the protease

production costs through improving the yield, and the use of either cost free or low cost feed stocks or agricultural by-products as substrate for protease production. The most widely used application of protease is to remove protein stains such as grease, egg and human sweat and blood by proteolytic degradation to polypeptides that are more soluble and amino acids. Among the vast pool of enzymes, proteolytic enzymes from microorganisms are the most desirable enzyme in the detergent industries worldwide investigating the microbial diversity ; there is always a chance of finding microorganisms producing alkaline enzymes which are suitable for the manufacture of "bio cleaners" (Vishalakshi et.al. 2009)

Materials and Methods:

Soil sample collection: The soil samples were collected from the rhizospheric region of medicinal plants *Aloe vera* and Tulsi as well as dairy waste soil.

Isolation of microorganisms: Collected soil samples were serially diluted up to 10^{-7} dilution. The diluted soil sample were inoculated on Nutrient Agar (NA) and Potato Dextrose Agar (PDA) medium by using spread plate technique under aseptic conditions and incubated at 37°C and 26°C temperature for optimum growth. Colony characteristics were analyzed and gram staining and cotton blue mount was done for their characterization as bacteria and fungi. The different strains were identified after gram staining method and observed through light microscope (magnification 40X)

Screening of isolates: The screening of isolated microbial strains was done on skim milk agar plates (skimmed milk agar media: skim milk powder-100g agar-15g distilled water-1000ml) (Aneja 2013) .

Preparation of solid state fermentation media: The agro waste wheat bran was collected from the market, grounded and washed for further use. Screened microorganisms were grown on enriched agro waste media for protease production. Initially coarse agro waste (100gm) was taken in a flask containing casein-0.5g, sodium chloride-0.5g, distilled water-250ml. The pH 7.2 for bacteria and 5.6 for fungi was adjusted, mixed thoroughly and autoclaved at 121°C and 15lbs pressure for 15 min (Kuberan et al. 2010). The content of the flask were inoculated with 1ml of inoculums after autoclaving and incubated in different incubation temperatures of 25°C, 37°C, 50°C.

Crude protease enzyme extraction from fermented matter: For the extraction of protease production under solid state fermentation a known quality of fermented matter was mixed with NaOH glycine buffer (pH 11). Then the flask was placed on shaker at 120 rpm for 30 min at 37°C. The slurry was then squeezed through muslin cloth filter by Whatman filter paper 1 and then centrifused. The supernatant was used as the crude enzyme extract for the assay (Srinivas et al. 2010).

Protease enzyme assay: The protease activity was assayed by the modified Anson Hagihara method (Hagihara et al. 1958). 1ml of enzyme was added to 6ml of casein (0.6 w/v 0.2 M glycine NaOH buffer pH 10.4) and the reaction mixture was incubated at 37°C for 10 minutes and then 6 ml of tricarboxylic acid (TCA) was added and incubated for 30 minutes at room temperature. The precipitates formed were removed by filtration through Whatman filter paper 1 and to 1 ml of filtrate 2 ml of Folin Ciocalteu's (FC) reagent was added and after 30 minutes optical density was taken at 660 nm. One unit of alkaline protease activity was defined as the amount of enzyme liberated in 1 gram of tyrosine for a minute under assay conditions.

Estimation of protein: The protein content of the enzyme sample was estimated by the Lowry's method (Lowry et al. 1951). Working standard solution volume of 0.2, 0.4, 0.6, 0.8 and 1.0 ml was pipetted into a series of test tubes and the final volume was made up to 1 ml adjusted with distilled water in all the tubes. A tube with 1 ml of distilled water served as blank and 1 ml of the sample was taken for testing to which; 5 ml of reagent C was added and shaken well. The reaction mixture was incubated for 10 minutes. After 10 minutes 0.5 ml of Folin's Phenol reagent was added and kept in dark for 30 minutes. The absorbance of the solution (developed blue colour) was measured at 660 nm. A set of standards were prepared using Bovine Serum Albumin. A standard graph was plotted taking concentration of protein on X-axis and optical Y-axis.

Results and Discussion:

Isolation of microorganism from soil: Seven bacterial strain and five fungal strain were isolated from the rhizospheric region of medicinal plants *Aloe vera* and Tulsi as well as dairy waste soil. Colony characteristics were analyzed and gram staining and cotton blue mount was done for their characterization as bacteria (Table1) and fungi (Table 2).

Table 1. Total number of isolated bacterial pure strain

Strain No.	Colony morphology	Gram Staining
I	White colnies which are convex with entire margin.	Gram +ve
II	White shiny mucoid colonies which have entire margin.	Gram-ve
III	Irregular colonies which are dry, flat and yellow in colour	Gram+ve
IV	Golden round yellow colonies which are convex with entire margin.	Gram-ve
V	White mucoid colonies	Gram-ve
VI	White mucoid colonies which have entire margin	Gram-ve
VII	White colonies which are dry, flat and irregular margin	Gram+ve

Table 2. Total number of isolated fungal strain

Strain No.	Colony morphology	Microscopic characterstics	Identification on microscopic view
I	Grassy green with white margin	Conidial hed are typically radiate, biseriate,Conidia are globase to subglobase.	Aspergillus flavus
II	Light green colony	Sporangiophore is simple branched with column shaped columella.	Mucor
III	White colony with dense cottony growth	Sphorangiophore are smooth walled, non septate, simple or brnched, sporangia greyish black, podery in appearance.	Rhizopus
IV	Black colony	Conidiophores are smooth walled hyaline, conidial head are biseriate.	Aspergillus niger
V	Grey colony	Columnar, uniseriate conidial heas, short smooth walled conidiophores.	Aspergillus fumigatus

Screening of microorganism on skimmed milk agar plates: Isolated microbial pure strains were screened on skimmed milk agar plates. Those microorganisms which formed a clear zone on skimmed milk agar plates were selected for protease enzyme production.

Bacteria: Out of seven, four bacterial strains gave positive results on skimmed milk agar plates. They form a clear area surrounding the bacterial growth due to the presence of extracellular protease enzyme (Table 3).

Table 3. Showing result on skimmed milk agar media (for bacteria)

Strain no.	Result after 48 hours
I	+ve
II	-ve
III	-ve
IV	+ve
V	-ve
VI	+ve
VII	+ve

+ shows "positive", - shows "negative"

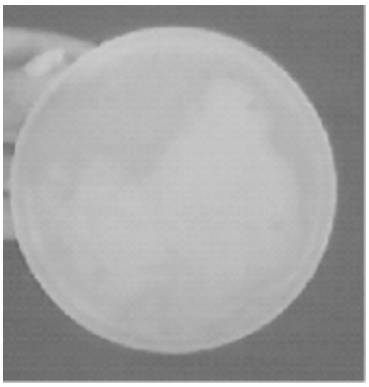

Fig 1. Bacteria showing positive result on skim milk agar plate

Fungi: Out of five, three fungal strain gave positive result on skimmed milk agar plates. They form a clear zone around fungal growth (Table 4).

Table 4. Showing result on skim milk agar media (for fungi)

Strain no.	Result after 48 hours
I	+ve
II	-ve
III	-ve
IV	+ve
V	+ve

+ shows "positive", – shows "negative"

Microbial characterization: On the basis of the various biochemical tests performed on the four bacterial strains isolated the identification was done (Table 5).

Table 5. Different biochemical tests performed on selected bacterial strains

Strain	Methyl red test	Voges-proskauer test	Fermentation test		Nitrate reduction test	Motility test	Amylase production test	Catalaste production test	Indole production test	Citrate utilisation test
			Dextrose	Lactose						
I	-ve	+ve	+ve	-ve	-ve	+ve	-ve	-ve	-ve	-ve
II	+ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve	+ve	-ve
III	+ve	+ve	+ve	-ve	-ve	+ve	-ve	-ve	-ve	-ve
IV	-ve	+ve	+ve	-ve	+ve	+ve	+ve	-ve	-ve	-ve

+ ve = positive result ; –ve = negative result

Enzyme production by bacterial strains :

Optimization of protease production at different time interval:

Extraction of extracellular crude protease enzyme: for the production of enzyme selected microbial strains were inoculated in wheat bran medium and incubated for different time period at different temperature (Table 6).

For bacteria: selected four bacterial strains were inoculated in wheat bran medium and incubated for **three, five** and **seven** days at 37°C (Table 7).

Table 6. Showing Optical Density (O.D) of protease enzyme at different time interval

Strain No.	Optical density at 660 nm		
	3 day	5 day	7 day
I	0.321	1.808	0.891
II	0.406	1.296	0.938
III	0.389	1.324	0.806
IV	0.334	1.034	0.883

Table 7. Showing the concentration of enzyme on different days

Strain No.	Concentration of enzyme (U/ml/min)		
	3 day	5 day	7 day
I	0.00053	0.00267	0.00143
II	0.00067	0.0021	0.00150
III	0.00060	0.00213	0.00127
IV	0.00057	0.0016	0.0014

Maximum production of enzyme was observed on the 5th day by all the four bacterial strain and strain I showed maximum production of enzyme and strain IV showed minimum production of enzyme. The subsequent decrease in the enzyme units could probably be due to the inactivation of

the enzyme by other constituent proteases, the reduced availability of nutrients and production of toxic metabolites (Ikram-ul-haq et. al. 2006).

Estimation of protein: at different temperature:

Table 8. Showing the O.D of protein at different temperature

Strain No.	O.D. at 750 nm		
	25°C	37°C	50°C
I	1.902	0.742	0.030
II	0.889	1.868	0.110
III	0.862	0.722	0.185
IV	1.824	1.568	0.092

At 25°C strain I, III are showed maximum protein content and at 37°C strain II and IV showed maximum protein content but at 50°C all the four strain showed very less protein content (Table 8).

The reduction in enzyme activity may be due to enzyme denaturation by losing its catalytic properties at high temperature due to stretching, breaking of weak hydrogen bonds within the enzyme structure (Ikram-ul-haq et. al. 2006). Previous report estimated the maximum enzyme production was obtained from Wheat bran medium of pH 9 and temperature 45°C (Vishalakshi, et. al. 2009).

Enzyme production by fungal strains :

Selected fungal strain was inoculated in wheat bran medium and incubated it for five days at 27°C.

Table 9. Showing the O.D of protease by fungal strain

Strain No.	O.D at 660 nm
I	0.830
II	1.311
III	1.194

Table 10. Showing the concentration of enzyme by fungal strain

Strain No.	Concentration of enzyme(U/ml/min)
I	0.00133
II	0.0021
III	0.00197

Strain II showed maximum production of protease and strain I showed minimum production of protease (Table 9 and 10). In earlier reports, Pushpa and Madhava Naidu (2010) reported the maximum production of protease from coffee byproducts by *Aspergillus oryzae* at temperature 30°C (Pushpa and Madhava Naidu 2010).

Estimation of total protein

Table 11. Showing the O.D of protein at 750nm

Strain No.	O.D at 750 nm
I	1.697
II	1.964
III	1.884

Strain II showed maximum protein content and strain I is showed minimum protein content (Table 11). The gradual decrease in the enzyme activity was observed with increasing incubation time clearly suggesting the enzyme being produced in the log phase of the growth of the fungus for the utilization of nutrients present in the solid substrate (Alagarsamy et. al. 2006).

Conclusion:

It was observed that microorganisms which were isolated from dairy waste soil showed good proteolytic activity. On incubation on different days, the fifth day showed good enzyme activity by bacterial strain. On incubation at different temperatures, at 37°C strain II and IV showed good enzyme activity and at 25°C strain I and III showed

good enzyme activity. Besides this among fungal strains, strains II and III showed good enzyme activity. The major objective of our research was cost effective production of enzyme and to isolate those microorganisms which have ability to produce protease enzyme. The protease enzyme that we extracted in our research was crude enzyme therefore enzyme activity was less because it was not purified. Finally, from the above result, the role of agro-waste i.e. wheat bran in protease enzyme production was identified and production parameters were determined. These significant results give new hope in the enzyme based detergent industries.

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