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Characterization of Protease Production Efficiency of Bacteria Present in the Poultry Farm Soil

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Abstract: ***Proteases are widely used in food processing industries for the partial digestion of protein present in food stuff. These proteases could potentially be used in the food processing industries for the partial digestion of proteinaceous food stuffs like meat tenderization and partial digestion of baby food. Present research work has been focused on the characterization of protease production efficiency of bacteria present in the soil of poultry farm of Bilaspur city. A total thirty bacterial strains were isolated from domestic poultry farm, Bilaspur (22.09°N 82.15°E). Among thirty bacterial strains, the four bacterial strains were selected by qualitative screening on the basis of clear zone. Further all four bacterial strains were screened for their specific protease activity. The two significant protease producing bacterial strains viz. PDB-2 and PDB-24 were identified as Bacillus sp. PDB-2 and Bacillus sp. PDB-24 and they represented 91% similarity to Bacillus licheniformis and 94% similarity to Bacillus cereus respectively on the basis of morphological, physiological and biochemical characteristics. Specific Protease activity of Bacillus sp. PDB-2 and Bacillus sp. PDB-24 were observed 32.47 ±0.60 and 28.92±0.58 (µmole min^{-1} mg^{-1} ±SD) respectively under an un-optimized condition. Bacillus sp. PDB-2 showed maximum specific protease activity and after optimization, the specific protease activity was found to be increased by 1.27-fold (from 32.47 ±0.60 to 41.52 ±1.56 µmol $min^{-1}mg^{-1}$ ±SD) at 45^{0}C (pH-8.5) for 96 h. Bacterial-derived proteases having significant activity could potentially be used in the food processing industries for the partial digestion of protein complexes present in the edible food-stuffs to increase its digestibility inside the human body.***

Keywords: Food processing industries, Meat tenderization, Poultry farm, Specific protease activity, *Bacillus sp.*, Bacterial-derived proteases

1. Introduction

Proteases (EC 3.4) belong to hydrolases class of enzyme that performs proteolytic action to hydrolyze the peptide bonds of a polypeptide chain [1] and [2]. Proteases are classified into four group's viz., Serine proteases (EC 3.4.21), Cysteine proteases (EC 3.4.22), Aspartic proteases (EC 3.4.23) and Metallo-peptidases (EC 3.4.24) [3]. Proteases are produced by animals, plants, fungi, bacteria and viruses and have a potential application in food processing, pharmaceuticals and detergent industries [4]. Activities of the poultry animals and shedding of their high protein content feathers and nails make the soil rich in protein degrading microbial flora. In case of, large scale production of plants- and animals-derived proteases, a huge land requirement and more time are needed as compared to microbial-derived protease production. So, as an alternative now days, the microorganisms are getting attention for faster rate of enzyme production at lower cost. Moreover, the genetic engineering techniques can also be easily applied in microbial cells to improve the efficiency of enzyme production [5]. Thus, the present research work has been focused on the characterization of protease production efficiency of bacteria present in the soil of poultry farm for its application in food processing industries.

2. Materials And Methods

Sampling and Isolation of Bacteria

Soil samples were aseptically collected in sterile poly bags from the domestic poultry farm, Bilaspur (22.09°N 82.15°E) and brought to the laboratory. Bacterial strains were isolated in the Luria-Bertani (LB) agar media containing (w/v; g l^{-1}) 10.0-peptone, 5.0-yeast extract and 5.0-NaCl in double distilled water (pH- 7.0). The pure cultures of bacterial strains were prepared and stored at 4^{0}C until used.

Qualitative Screening of Protease Producing Bacterial Strains

Skim-milk Agar Media (SAM) consisting of (w/v; g l^{-1}) 5.0-yeast extract, 0.50-$MgSO_4$, 0.25-KH_2PO_4 and 20.0-Skim milk powder in double distilled water (pH- 7.0) was used for the qualitative screening of protease activity of each bacterial strain. The SAM plates were inoculated with a loop full pure culture of each test bacterial strain and incubated at 37^{0}C for 48 h and thereafter, observed for clear zones around the bacterial colonies. The clear zones indicated the positive result for protease production by test bacterial strains.

Protease Production

Bacterial strains, those showed the positive protease activities in qualitative analysis were selected for the further assessment of protease production. Protease was produced by submerged fermentation process as per suggested by Anstrup [6] with some modification. 5.0 ml of overnight bacterial culture (5.7×10^6 CFU ml^{-1}) was added to the 100 ml of production media which consisted of (w/v; g l^{-1}) 5.0-Peptone, 5.0-yeast extract, 0.50-$MgSO_4$, 0.25-NH_4SO_4, 0.25-KH_2PO_4 and 0.2-$CaCl_2$ in double distilled water (at pH -7.0). Production media was supplemented with 2 % skim milk as substrate and incubated in a rotary shaker-incubator at 37°C (120 rpm) for 72 h. After incubation the fermented broth was centrifuged at 5000 rpm for 20 min to remove bacterial cells from the fermented broth. After centrifugation, the clear supernatant was used as the source of crude protease.

Partial Purification of Crude Protease

Partial purification of crude protease was done by the method described by Zambare [7]. Crude protease was precipitated with ammonium sulphate (at 70% saturation) and centrifuged at 5000 rpm for 20 min at 4^{0}C. After centrifugation, the crude protease precipitate was collected and dissolved in a 0.05 mol l^{-1} Tris- HCl buffer (pH 8.0).

This protease precipitate was loaded in a dialysis bag for dialysis and then dipped in beaker containing 100 ml of 0.025M phosphate buffer and for 24 h on a magnetic stirrer for salting out. After dialysis the partially purified protease was collected and its activity was determined.

Protein Estimation

Protein estimation was done by the standard protocol of Lowery's method [8] using Bovine serum albumin as standard.

Protease Assay

Protease assay was done as suggested by Manivannan and Kathiresan [9], with some modification. 0.5 ml of crude enzyme was incubated with 1.0 ml casein solution (2.0% casein in 0.1 M Tris HCl buffer, pH 7.0) for 10 min at 37˚C. The reaction was stopped by the addition of 5.0 ml of 5% Trichloroacetic acid (TCA) and incubated for 30 min. The mixture was filtered and added 4.0 ml of 0.1N NaOH and 0.5 ml Folin-Ciocalteau reagent and incubated for 30 min. The amount of tyrosine released was measured colorimetrically at 670 nm.

Effect of pH, Temperature and Time

The effect of pH (6.0 to 9.0), temperature (30 to 60^0C) and time of incubation (24 to 120 h) on enzyme activity were analyzed.

Identification of Bacterial Strain

The morphological, physiological and biochemical characteristics of significant protease producing bacterial strains were determined by Bergey's Manual of Determinative Bacteriology (BMDB) [10].

3. Result And Discussion

A total thirty bacterial strains were isolated from domestic poultry farm. Qualitative and quantitative screenings were performed for the assessment of protease production efficiency of bacterial strains. Among thirty bacterial strains, the four bacterial strains (PDB-2, PDB-7, PDB-24 and PDB-29), on the basis of clear zone (Fig. 4) were selected for qualitative screening. PDB-2, PDB-7, PDB-24 and PDB-29 showed 15.2, 11.65, 18.47 and 6.79 (mm) clear zone around bacterial colony respectively. Further all four bacterial strains were screened for their specific protease activity. PDB-2, PDB-7, PDB-24 and PDB-29 showed 32.47 ±0.60, 18.65 ±1.02, 28.92 ±1.56 and 9.24 ±0.89 (specific protease activity µmole min^{-1} mg^{-1} ±SD) respectively under an un-optimized condition. The crude protease was partially purified by ammonium sulphate precipitation method before the determination of its enzyme activity.

Two bacterial strains PDB-2 and PDB-24 were morphologically, physiologically and biochemically characterized by the method described in BMDB [10]. Results showed that both bacterial strains, PDB-2 and PDB-24 belong to *Bacillus sps.* and identified as *Bacillus sp.* PDB-2 and *Bacillus sp.* PDB-24 and represented 91% similarity to *Bacillus licheniformis* and 94% similarity to *Bacillus cereus* respectively as per their morphological, physiological and biochemical characteristics as described in BMDB. *B. cereus* CA15 [11], *B. licheniformis* [12] and [13], *B. licheniformis* UV-9 Mutant [14], *Bacillus sp.* NKS-21[15], *Bacillus sp.* Y [16], *Bacillus sp.* CW-1121 [17] and *Bacillus sp.* KSM-K16 [18] have been reported earlier for significant protease production.

During screening of potential bacterial strains, it was noted that PDB- 24 showed maximum protease activity in terms of clear zone during qualitative analysis (Fig. 4) but later in quantitative analysis PDB- 2 exhibited maximum specific protease activity. This may be due to the different conditions used in each technique; means, in quantitative screening experimental flasks were incubated inside shaker at 120 rpm for 72 h, whereas in qualitative screening the plates were incubated inside incubator at 37^0C for 48 h. Also it may possible that for PDB-2, the incubation period and shaking were significant parameters for protease production. Due to maximum specific protease activity yield (32.47 ±0.60 µmol $min^{-1}mg^{-1}$ ±SD) obtained with PDB-2 under an un-optimized system, so this strain was further selected for optimization to enhance its specific protease activity. During the optimization, the pH range, from 6.0 to 9.0 was analyzed and the maximum specific protease activity (38.60 µmol $min^{-1}mg^{-1}$) was obtained at pH-8.5 (at constant temperature-37°C and time-72 h). After increasing the pH from 8.5 to 9.0, the reduction in specific protease activity (from 38.60 to 36.24 µmol $min^{-1}mg^{-1}$) was observed (Table 1 and Fig. 1). This finding indicates that the protease produced was slightly alkaline in nature. Seenivasagham and Rose reported the maximum protease activity at pH 8.0 (37^0C) [19]. Zambare worked on protease production by *Pseudomonas aeruginosa* MCM B-327 and found the optimum pH of 8.0 [7].

The temperature range was (from 30 to 50^0C) and the maximum specific protease activity was recorded at 45^0C (39.35 µmol $min^{-1}mg^{-1}$) under constant pH-8.5 and time-72 h (Table 1 and Fig. 2) and after increasing the temperature from 45 to 50^0C, the specific protease activity was getting down to 37.21 µmol $min^{-1}mg^{-1}$. This may be due to the inhibition of enzyme by temperature. Further the time at a range of 24 to 120 h was assessed. Results showed that, the maximum specific protease activity was found at 96 h (41.52 µmol $min^{-1}mg^{-1}$) and further acceleration in temperature from 96 to 120 h, the specific activity was dropped from 41.52 to 38.57 µmol $min^{-1}mg^{-1}$ (Table 1 and Fig. 2). After optimization, the specific protease activity of *Bacillus sp.* PDB- 2 was found to be increased by 1.27-fold (from 32.47 ±0.60 to 41.52 ±1.56 µmol $min^{-1}mg^{-1}$ ±SD) at 45^0C (pH-8.5) for 96 h (Table 1).

4. Conclusion

The present research work was done to reveal the protease production efficiency of bacterial strains present in the domestic poultry farm. The bacterial strains, *Bacillus sp.* PDB-2 and *Bacillus sp.* PDB-24 were identified for significant protease production. Afterward, *Bacillus sp.* PDB-2 was optimized and specific protease activity was increased by 1.27-fold under optimized condition. This protease enzyme could potentially be used in the food processing industries for the partial digestion of proteinaceous food stuffs. The partial digestion of proteinaceous food makes it easily digestible inside the

human stomach. Further, the study of protease production by *Bacillus sp.* PDB-2 using cheaper substrate is on way to make it cost effective.

Table 1: Specific Protease Activity of Bacillus sp. PDB-2 at Different Parameters

Un-optimized System	*Optimized System*					
Specific protease activity (µmole min^{-1} mg^{-1} ±SD)	*pH*	*Specific protease activity (µmole min^{-1} mg^{-1} ±SD)*[1]	*Temperature*	*Specific protease activity (µmole min^{-1} mg^{-1} ±SD)*[2]	*Time*	*Specific protease activity (µmole min^{-1} mg^{-1} ±SD)*[3]
32.47 ±0.60	6.0	18.31 ±0.86	30	22.47 ±0.68	24	13.98 ±1.46
	6.5	23.49 ±1.14	35	28.64 ±1.27	48	21.67 ±0.91
	7.0	26.78 ±0.95	40	35.41 ±1.34	72	39.33 ±0.82
	7.5	31.47 ±0.84	**45**	**39.35 ±1.29**	**96**	**41.52 ±1.56**
	8.0	34.50 ±1.04	50	37.21 ±1.15	120	38.57 ±1.43
	8.5	**38.60 ±0.97**				
	9.0	36.24 ±0.71				

Un-optimized System - pH - 7.0, Temperature -37 °C and Time – 72 h were constant
Optimized System - [1] pH varied while Temperature (37°C) and Time (72 h) were constant
[2] Temperature varied while pH (8.5) and Time (72 h) were constant
[3] Time varied while pH (8.5) and Temperature (45 °C) were constant

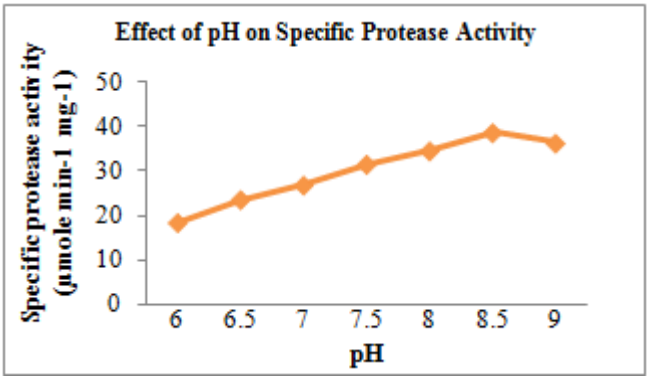

Figure 1: Effect of pH on Specific Protease Activity

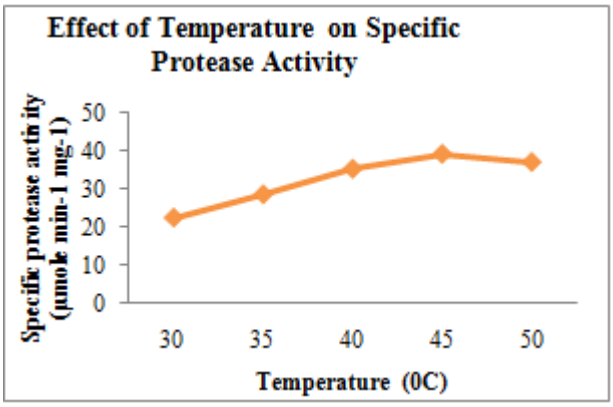

Figure 2: Effect of Temperature on Specific Protease Activity

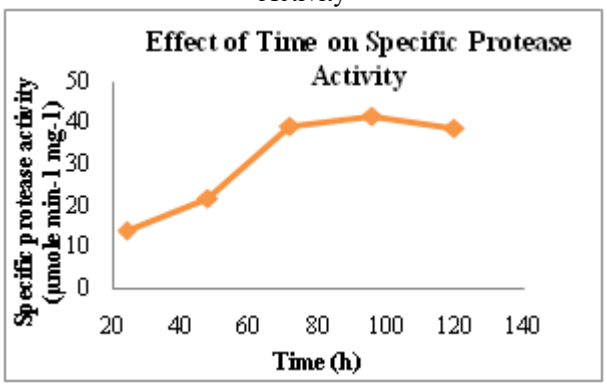

Figure 3: Effect of Time on Specific Protease Activity

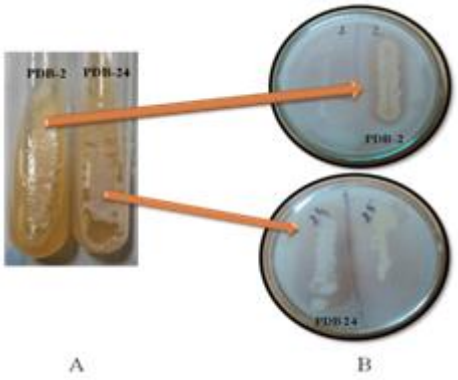

Figure 4: Quantitatively screened bacterial strains (PDB-2 and PDB-24) (A) Agar slants (B) Clear zones in SAM plates

5. Acknowledgement

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