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Isolation and Characterisation of Keratinase Producing Bacterial Strains

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Abstract : *The aim of the current study was to isolate keratinolytic bacteria from the soil samples collected from different poultry shops in Patna (Shivpuri and Danapur,) The bacterial isolation was performed by the standard serial dilution and spread plate technique. The isolated strains were screened for their keratinolytic activity by inoculating them in basal media enriched with chicken feather waste. Among the eight keratinolytic isolates, two strains designated as S-2 and S-6 showed effective feather degradation in the medium and were selected for further study. The enzyme activities of these two strains were studied by "Agar Well Diffusion" method, and the zones of hydrolysis were found to be 26 mm for S-2 and 31 mm for S-6. The optimum enzyme activity of both the selected strains was observed at temperature 35ºC; and at pH 7 for S-2 and pH 8 for S-6, respectively.*

Key words: *Keratinolytic bacteria, feather waste degradation, keratinase, alternative protein.*

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Introduction :

Keratin is an insoluble protein macromolecule with very high stability and low degradation rate. Keratin is mainly present in hair, feather, nails, wool and horns. High protein content of keratin waste can be used as a good source of protein and amino acids by systemic recycling (Onifade *et al.,* 1998). Feathers are largely produced as a waste by-product at poultry plants (Williams *et al*., 1991). They are insoluble structural proteins cross-linked by disulfide, hydrogen and hydrophobic bonds but could represent a rich protein resource because they contain over 90% (w/w) keratins. Recycling of feather can provide a cheap and alternative protein feed stuff. Further this can be used as animal feed and for many other purposes. However, poor digestibility of keratin is a problem in recycling (Takami *et al.,* 1992*;* Williams *et al*., 1991). Feathers contain over 90% of protein and conversion of feather into feed by keratinolytic bacteria may prove to be an economically viable option. The keratinolytic microorganisms and technologies developed for feather degradation not only remove the waste feather efficiently from the nature but also make the by-product of the process a valuable protein supplement. Further, this feed is relatively superior to other protein supplement like

soya bean meal. The protein rich concentrated feather meal can also be used for organic farming as semi slow release nitrogen fertilizer. Use of keratinase enzyme in leather industry was known long back in dehairing process as an alternative to chemical processing (Hadas & Kautsky, 1994; Choi & Nelson, 1996).

Keratinase is an extracellular enzyme used for the bio degradation of keratin and is produced only in the presence of keratin substrate. It attacks the disulfide bond of keratin to degrade it. The mechanical stability of keratin depends on the tight packaging of proteins in alpha-helix or beta-sheet structures and their high degree of cross-linkages by disulfide and hydrogen bonds. Some microbes have been reported to produce keratinase in the presence of keratin substrate. These microorganisms have ability to degrade chicken feathers, hair, nails, wool etc. (Gradišar *et al.,* 2005; Cai *et al.*, 2008). Keratinases from microorganisms have attracted a great deal of attention in the recent decade, particularly due to their multitude of industrial applications such as in the feed, fertilizer, detergent, leather and pharmaceutical industries.

The purpose of the present study was due to the manifold benefits obtained from them. The keratinolytic bacteria will not only help in the disposal of vast quantity of waste feathers of poultry industry and prevent environmental pollution and protein wastage but will also provide useful end product that can be utilized for various purposes.

Materials and Methods :

The soil samples were collected from different poultry shops of Patna *viz.* Danapur and Shivpuri. Isolation of bacteria was performed by serial dilution and plating method on Nutrient Agar (NA) plates. The bacterial isolates obtained were further sub cultured on NA plates to obtain pure culture (Aneja, 2003). Pure isolates were maintained in NA slants at 4ºC for further studies. The bacterial isolates were inoculated in the basal medium enriched with chicken feather waste. The pH was adjusted to 8.0. The medium was incubated in a rotary shaker at a speed of 150 rpm at 37°C for 24h. After incubation, the cells were removed by centrifugation at 10,000 rpm for 10 minutes and the supernatant was collected and examined for enzyme activity. The Casein agar plates were prepared, wells were made in the agar surface using sterilized gel borer, and 10 ml cell free supernatant was transferred in to the well using a micropipette (Cai *et al*., 2008). The plates were incubated at 37ºC for 24 h. The plates were observed for zone of hydrolysis. The isolates were further characterized by biochemical tests that included Indole test, MRVP test, citrate utilization test, hydrogen sulphide test, catalase test, Litmus milk test and carbohydrate fermentation test (Cappuccino and Sherman, 1996). Effect of pH and temperature on enzyme activity was studied by inoculating the isolates in TSB medium at different pH (3, 4, 5, 6, 7 and 8) and temperatures (4^0C, 30^0C, 35^0C, 40^0C and 45^0C) for 24 h and the absorbance was measured at 590 nm using spectrophotometer (Tamilmani *et al*., 2008).

Results and Discussion :

Physical and Chemical Characteristics of soil samples collected from Shivpuri and Danapur: The physical and chemical characterizations of soil samples are shown in the Table 1.

Table 1. Showing physical parameters of soil samples

Sl. No.	Sample	Temperature (^{0}C)	Moisture (in gm)
1.	Shivpuri	27	0.931
2.	Danapur	21	0.1231

Bacteria isolated from different soil samples: From the different soil samples collected

from the poultry shops of Shivpuri and Danapur in Patna, a total of 8 bacterial strains were obtained. The number of isolates obtained from each of the soil sample is shown in Table 2.

Table 2. Showing no. of isolates obtained from different soil sample.

Sl. No.	Soil Sample	Dilution	No. of colonies
1.	Shivpuri	10^{-5}	1
		10^{-4}	2
2.	Danapur	10^{-5}	2
		10^{-4}	3

Screening of Keratinolytic bacteria : All the 8 bacterial isolates were screened for their keratinolytic activity in the basal medium incorporated with chicken feather waste. Among them two strains designated as S- 2 and S- 6 showed effective feather degradation in the medium (Fig. 1) and were selected for further study.

Fig 1. Showing feather degradation in the basal medium

Assay of enzyme activity: The selected isolates were assayed for their enzyme activities in the Casein Agar plates by Agar Well Diffusion method. The zones of hydrolysis formed by the plates were found to be 26 mm for S-2 and 31 mm for S-6 (Fig. 2).

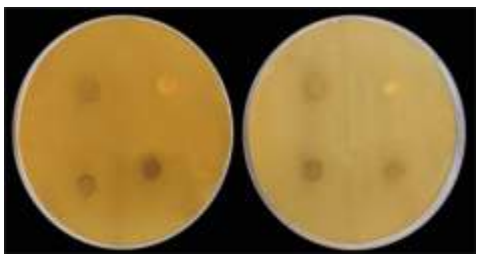

Fig 2. Showing zone of hydrolysis on Casein agar plate

Effect of pH on enzyme activity: The enzyme activities of the selected strains were studied by inoculating the isolates in TSB medium at the specified pH. The absorbance was measured at 590 nm using spectrophotometer after 24 h. The data are presented in Table 3.

Table 3. Showing Absorbance of the selected strains at 590 nm at different pH

pH	S-2	S-6
3	0.126	0.116
4	0.121	0.129
5	1.226	0.679
6	0.117	0.808
7	1.416	0.893
8	0.795	0.939

Effect of temperature on enzyme activity: The enzyme activities of the selected strains were studied by inoculating them in TSB medium and incubating them at the specified temperatures for 24 h. The absorbance was measured at 590 nm using spectrophotometer. The data are presented in Table 4.

Table 4. Showing Absorbance of the selected strains at 590 nm at different temperatures:

Temperature (^{0}C)	S–2	S–6
4	0.269	0.279
30	0.331	0.313
35	0.553	0.447
40	0.407	0.383
45	0.393	0.110

General morphological characters of the isolates: The isolates were Gram stained and examined microscopically. Both of them were Gram positive and rod shaped bacterial strains.

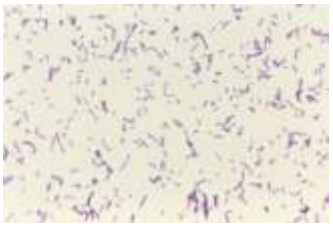

Fig 3. Showing gram positive rod shape bacteria

Biochemical characteristics of the isolates: The specified biochemical tests were performed on the two selected strains. Both the strains showed positive results for Indole, methyl red, citrate utilization, hydrogen sulphide production, Litmus milk and carbohydrate fermentation tests whereas the results were negative for Voges-(Fig. 4-7) Proskauer and catalase tests. The data are represented in Table 5.

Table 5. Showing biochemical characteristic of the isolates S–2 and S– 6

Biochemical Test	S–2	S–6
Indole Production	+	+
Methyl Red	+	+
Voges-Proskauer	–	–
Citrate Utilization	+	+
Hydrogen Sulphide	+	+
Catalase	–	–
Litmus Milk Reaction	+	+
Carbohydrate fermentation	+	+

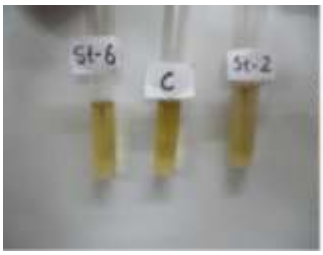

Fig 4. Showing positive H_2S test

Fig5. Showing positive citrate test

Fig 6. Showing positive carbohydrate fermentation test

Fig 7. Showing positive litmus milk test

Keratin is a strong protein found in skin, hair, nails, horns, teeth. Keratin is difficult to dissolve due to the presence of cysteine disulfide that can form disulfide bridges. Microorganisms can degrade the keratin by the production of keratinase (an extracellular enzyme). Some bacteria, actinobacteria and fungi are reported to carry keratinolytic activity. In the current study eight keratinolytic bacterial strains were isolated from the soil samples collected from poultry shops (Shivpuri, Danapur) of Patna. The isolates were designated as S-1, S-2, S-3, S-4, S-5, S-6, S-7 and S-8. Out of these, two strains showed effective feather degradation in the medium and, therefore, were selected for the present study. The micro morphological and biochemical characteristics of the strains S-2 and S-6 suggest that the isolates may be *Bacillus* species; however, it can be confirmed only after 16S rRNA analysis. Previous studies conducted on the isolation of keratinolytic organism from soil and other natural sources; report that *Bacillus* sp. are potential keratinolytic organism and have possible use in field studies for biodegradation of feathers.

Conclusion :

The objective of the present investigation was to isolate and optimize conditions for keratinase producing bacterial strains. Results of this study indicate that the S-2 and S-6 isolates are potential keratinolytic organisms and can be used for the biodegradation of keratin in feathers. However, a lot more parameters need to be taken into consideration before certifying them for commercial use.

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