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Isolation and Characterization of Keratinolytic Bacteria from Poultry farm soils

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Abstract

Keratinolytic microorganisms have a great importance in poultry waste degradation and its bioconversion to compost or animal feed. The present study aimed at selection of keratin degrading bacteria from poultry soil. The poultry farm soil samples were added in basal medium with feathers as a source of carbon and nitrogen. The enriched samples were streaked on nutrient agar and hichromebacillus agar for isolation of keratinolytic bacteria.15 isolates were obtained. All the isolates were screened for their keratinolytic potential on milk agar. Out of fifteen isolates five were keratinolytic.Five isolates were further studied for feather degradationin shake flasks.Of the five, four showed complete degradation of feather barbules within 120 h and one isolate showed degradation within 72 h. The isolate was identified morphologically and by 16 s rRNA sequencing as Breuvendimonosterrae. It produced maximum keratinase at 37 °C temperature and at 130 rpm speed within 72 h. Breuvendimonosterrae showed 76U/ml enzyme activity.The bacterium can be used for biotechnological purpose.

Keywords: Keratin, keratinase, Breuvendimonos terrae.

Introduction

Keratin is insoluble and hard to degrade due to extensive disulphide bond and cross linkages. Resistance to proteolysis is due to cross linking of protein chains by cystein bridges. Keratins are found in animal hair, nails, hoofs and feathers. The disulphide bonds of keratin can be reduced by enzyme disulphide reductase[1]. Feathers are generated in large quantities as a byproduct of poultry industry. Billions of chickens are killed annually and near about 8 billion tones of poultry feathers are produced. Nowadays feather waste is utilized as a dietary supplement for animal feed stuffs. Physical and chemical treatment for conversion of feathers into food supplement can destroy amino acids and decrease protein quality. Use of microbial Keratinase for keratin degradation is the innovative solution for recycling feather waste and reducing pollution Conversion of feathers into feather meal, dietary protinfor animal feed by using physical and chemical treatment is significant. These methods can destroy certain amino acids and decrease protein quality and digestibility. Physical and chemical methods can lead to destruction of amino acids as well as decrease the protein content and digestibility. The utilization of agro-industrial residues may increase energy conservation and recycling. To overcome the loss of aminoacids due to keratin hydrolysis microbial keratinases are used. However, the mechanism of keratin biodegradation by microorganisms is not yet completely understood. Comprehensive reviews about keratinases have been published this article presents recent advances on keratinolytic proteases from bacterial origin with emphasis on their biochemical properties and discussion on their current and potential applications in soils and poultry wastes[2].

For conversion of feathers into feed protein ,Keratinase can be used for hair removal[3].

Keratinase belong to class hydrolase. These are metalloproteins and efficient proteolytic enzymes. The enzyme keratinase is a potential enzyme for removing hair and feathers in poultry industry[4]. A number of keratinolytic microorganisms have been reported including species of fungi such as *Microsporum*[5] *Aspergillus*[6], *Bacillus*[7,8], *Streptomyces*[7,9] and other Actinomycetes[7]. In this study we report the isolation of bacterial strain that produces keratinase which degrade feathers within 72 h. of incubation.

Material and Methods

Chemicals: All Chemicals required for experimental work were of analytical grade, pure and purchased from Himedia laboratory. The chemicals include: Yeast extract, Hichrome bacillus agar, K_2HPO_4, KH_2PO, Keratin, Yeast, Trichloroacetic acid, Agar powder, etc.

Collection of soil samples: Soil samples with decaying feathers were collected from poultry farms at Vita region in Sangli District in sterile polythene bags. Soil samples numbered from N1 to N7 were collected at different poultry sites. All the soil samples were processed on the same day. Soil samples were inoculated in basal salt medium along with feathers and kept for enrichment for 3 days at 37°C temperature. Basal salt medium of pH 7.5 containing gl^{-1}; NH_4Cl-1, NaCl-1, K_2HPO_4-6, KH_2PO_4-8, $MgCl_2$-2, and yeast extract-1.

Isolation of keratinolytic microorganisms: Nutrient agar and Hichrome bacillus agar were used for isolation of keratinolytic bacteria. The same media were used for growth and maintainance of bacteria. For rapid identification of *Bacillus* species Hichrome bacillus agar was used[10].

Screening for keratinolytic bacteria: On Hichrome agar, fifteen morphologically different colonies were identified and further inoculated on sterile feather meal agar plates[10] and incubated at 37°C for 48 h. The strains showing zone of clearance were selected as keratinolytic. Furthermore, the bacterial strains were inoculated in modified broth medium containing feathers and all the flasks were incubated at 130 rpm for 5 days. Feather degradation was confirmed visually.

The supernatant was used for keratinase assay and protein determination. Protein was determined by Lowry'smethod[11,12]. The efficient bacterial strains showing degradation after 72 h. were identified by morphological, cultural, biochemical and confirmed by 16 s rRNA sequencing.

Determination of keratinase activity: The keratinase activity of the crude culture broth was assayed after 5 days of incubation.1.0 ml of crude enzyme was diluted in phosphate buffer (0.05 M of pH 7.0).1 ml of keratin solution was added and kept at 50°C for 10 minutes. Reaction was stopped by adding 2.0 ml of 0.4 M trichloroacetic acid. The mixture was centrifuged at 1450x for 30 minutes and absorbancy was measured at 280 nm[13]. The control was prepared by adding enzyme solution with 2.0 ml of trichloroacetic acid without addition of keratin[14].
Enzyme Units per ml were calculated by using following formula:
Enzyme Units per ml= Optical Density X 4 X dilution rate /0.01X T
Where, T=Incubation time, 4 =Total volume used

Protein concentration was determined by Lowry's method with Bovine serum albumin as a standard.

Results and Discussion

After enrichment of poultry soil sample, it was found that five isolates among 15 exhibited feather degradation activity within 120 h. One isolate N7(2) degraded feathers within 3 days. This isolate was identified as *Breuvendimonos terrae* by 16s r RNA sequencing and have gene accession number KC588949. From selected soil sample out of 15 isolates four isolates allowed to grow on milk agar and on feather meal agar. Feather meal powder serves as source of nitrogen and carbon. The strains selected were number N1, N2, N3, N7(1), N7(2), as they showed clear zone of hydrolysis around colony on milk agar on incubation at 37°C for 24 hrs and on feather meal agar on incubation at 37°C for 72 h. These observations suggests that these strains have keratinolytic activity. For isolation of potent strain of keratinolytic microbe, all strains were inoculated in modified basal salt media along with feathers and incubated at 140 rpm for 3-5 days in shaker incubator. Visual detection of feather degradation was carried out every after 24 hrs. It was found that N7 (2) strain degrade feathers within 72 hrs, and having keratinolytic potential. All these strains were also grown on Hichrome bacillus agar for rapid identification of the isolates. N7 (2) showed pale colour colonies on Hichrome while other strains were of purple and pink colour.

Colonies of N7 (2) on Nutrient agar were small, smooth, shiny, and viscous. Colonies of N1 N2 N3 and N7 (1) were large pink and violet. The isolated strain of N7 (2) was Gram negative short rods and motile. Strains N1, N2 N7 (1) were Gram positive rods.

Table-1
Morphological and cultural characters of isolates on Nutrient media

Isolate number	Size	Shape	Colour	Margin	Opacity	Elevation	Consistency	Gram Character
N1	1mm	Circular	White	Even	Opaque	Flat	Moist	Gram positive sporulated rods
N2	2mm	Circular	White	Regular	Opaque	Low convex	Moist	Gram positive rods
N3	1mm	Circular	Creamy	Regular	Semitransparent	Flat	Moist	Gram positive cocci
N7(1)	2mm	Circular	White	Irregular	Opaque	Low convex	Moist	Gram positive short rods
N7(2)	1mm	Circular	Creamy shiny	Regular	Opaque	Low convex	Moist	Gram negative short rods

N7 (1) and N7 (2) are the number of colonies in the same samples.

Table-2
Biochemical results of isolate number N7(2)

Details of experiment	Result
Shape and arrangement	Short rods in single.
Carbohydrates fermentation with	
Glucose	Positive-acid and gas production
Lactose	Negative
Mannitol	Negative
Indole test	Negative
Methyl red test	Negative
VogesPrausker test	Negative
Citrate utilization test	Negative
Oxidase test	Negative
Indole production	Negative
Casein hydrolysis test	Positive

Table–3
Keratinase activity produced by feather degrading bacterial strain

Days	Relative activity %
1	36
2	48
3	76
4	65
5	63
6	56

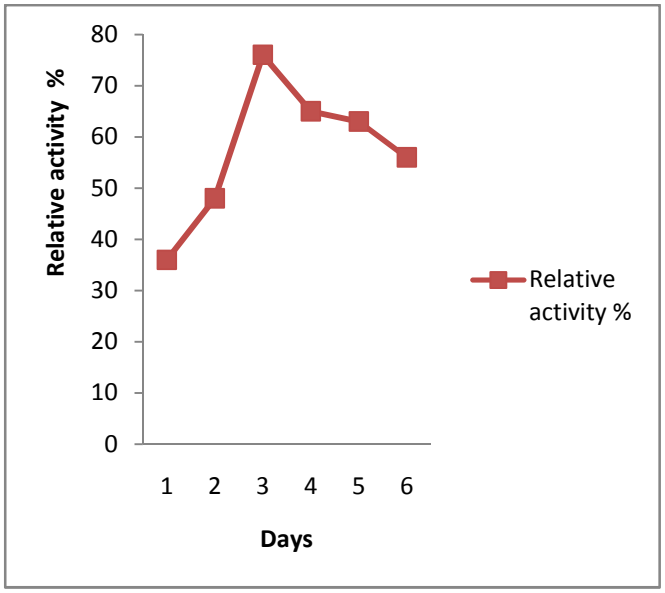

Figure-1
Relative activity of keratinase producing organism

Figure-2
Degradation of chicken feathers by bacterial strain isolated from poultry soil. Feather control without the bacterial strain and feather after 72 hrs of incubation with isolated bacterial strain which show degradation

For the production of keratinase and degradation of feathers, 5% innoculum was used with modified basal salt medium[15,16] and maintained in a shaker at the rotation speed of 130 rpm /min. The growth and feather degradation by Breuvendimonos was optimum at 37^0C temp.

Discussion: The use of microbial keratinase is being explored in applied microbiology where there is a need of active feather keratin degraders. Keratin degradation is mostly performed by Gram positive bacterium, although there are few reports on feather degrading Gram negative bacteria[15]. In this study, the keratinase enzyme producing Gram negative bacterium was isolated and identified as *Breuvendimonos terrae*.

Keratinase produced by this isolate can actively degrade the feather at PH 8.2 when incubated for three days. The isolate was studied for its morphological, and biochemical characters and the results were compared with Bergey's Manual of Determinative Bacteriology. The isolate was examined as Gram negative, motile, nonsporeforming rods and showed negative results for carbohydrate fermentation with lactose, mannitol, indole production, methyl red test, Voges Prasuker test , citrate utilization test and oxidase test and positive for glucose utilization, catalase and casein hydrolysis test.

The identification was confirmed by 16s r RNA sequence analysis indicating that the isolate is *Breuvendimonos terrae.*

The isolated bacterium showed effective degradation after three days. Depending upon the keratinolytic activity, keratinase

enzyme producing bacterium was cultured in basal salt medium. The crude filtrate showed a specific activity of 76 U /ml and 2.5 mg /ml protein content. Similar findings were reported for Bacillus spp[7].

Priliminary identification of bacteria was done by using Hichrome Bacillus agar[10]. It is also reported that use of feather meal in medium enhance keratinase enzyme production. The strain, *Breuvendimonos terrae* produced higher yields in feather meal and raw feathers which have been used as good substrates for production of other keratinolytic enzymes[17]. It is stated that high protein content reached at 72 h. of incubation, but decrease thereafter[18]. Activity was estimated at regular intervals and reached maximum after 72 h. A unique structure of keratin makes it very resistant to proteolytic digestion[6]. Keratinase enzyme secreted by many microorganisms that hydrolyses keratin into smaller molecules[15]. Production of feather hydrolysate by mechanical and chemical treatment leads to destruction of essential amino acids and decreasing nutritive values. The use of microbial keratinase is an obvious choice in biodegradation of keratinous waste. The isolated strain is able to produce keratinase and could be applied for feather degradation into feather hydrolysate.

It is also known that most keratinases are inducible and very specific towards their substrates like hair, wool, nails, feathers, etc[6]. Keratinase enzyme is found to be stable at 30^0C temp and active. Some isolate show partial feather degradation since substrate contain disulphide linkages. Pretreatment of the substrate by physical or by using metal ions may improve degradation[19].

However it is elucidated that for biotechnological application of the reported strain; more detailed understanding of the mechanism of feather degradation and the factors affecting native feather degradation is required. Therefore, additional researches regarding purification and characterization of keratinase enzyme and kinetics have to be done.

The presence of such type of species in poultry waste is due to adaptation to utilize substrates like feathers. The most studied keratinolytic bacterium is *Bacillus licheniformis* which possess high keratinolytic activity. The bacterium and keratinase enzyme could be used for improving nutritive quality of animal feed containing feathers of poultry waste[12].

Conclusion-Keratinolytic microorganism isolated in this work presents high keratinolytic activity. Recycling of poultry waste for environmental protection and for bioconversion of feathers into animal feed protein, the isolate with high keratinolytic activity could be used for biotechnological application in keratin hydrolysis process. Thekeratinolytic activity of keratin degrading isolate will have biotechnological application in various industrial processes involving keratin hydrolysis. Use of microbial kerainase id beneficial and economical approach for keratinous waste disposal and to prevent environmental pollution.

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