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Screening and Characterisation of Keratinase Enzyme Obtained From Keratin Degrading Microorganism Isolated From Sanjan Poultry Waste Dumping Soil

BHARTI AGRAWAL
Department of Microbiology
N.B. Mehta Science College, Bordi, Dist., Palghar (MS)
India
MITTAL DALAL
Dept. of Pharmacology
Rofel Shri G. M. Bilakhia College of Pharmacy, Vapi, Dist., Valsad (GJ)
India

Abstract

A large amount of feather waste is generated from the poultry industry. This waste containing a considerable amount of keratin can be utilized by microorganisms thus leading to its environmentally safe disposal. The objective of the study was to isolate and identify a feather degrading bacterium from poultry farm soil, optimize the cultural conditions and partial purification of keratinase enzyme using ammonium sulphate precipitation, followed by exploring the applications. The study was carried out by collecting soil samples, enrichment using an appropriate enrichment medium, followed by screening, identification and then optimizing cultural conditions. The isolated keratinolytic bacterium carried out complete degradation of the feather waste within 4 weeks and was found to be of Bacillus sp. by biochemical identification. The bacterium presented optimum growth at pH 6.8 and a temperature of 37 ^{o}C. The keratinase enzyme activity was found to be 8.80U/ml/min for isolate P and 13.60U/ml/min for isolate W. The study shows the potential of the feather degrading isolate to carry out environmentally safe disposal of poultry waste and also to produce amino acids from a cheap raw material.

Keywords- Feather- degrading bacterium, Poultry waste, *Bacillus*, Keratinase, ammonium sulphate precipitation.

Introduction

The market demand for contemptible meat, such as poultry and broiler meat and its derivates has been increasing steadily. Feathers are the main waste byproduct of poultry processing industry and gradually increase day by day. Typically as each bird has up to 125g of feather, the weekly worldwide production of feather waste is about 3,000 tons. Growth of the poultry

industry resulted in the generation of an increased quantity of organic solid wastes, including poultry feathers. The United States and the European countries mainly the United Kingdom generate about respectively 4 billion pounds and 150,000 tons of feather waste per annum respectively. The poultry waste generated from processing industries in India is 350 million tons per year [1-3]. These poultry feathers are dumped, used for land filling, incinerated or buried, which involves problems in storage, handling, emissions control and ash disposal. Discarded feather also causes various human ailments including chlorosis, mycoplasmosis and fowl cholera etc[2]. Also the feather flour obtained after baking and milling of feathers is the carrier of the unconventional infectious agent, the prion protein, the causative agent of a group of diseases called transmissible spongiform encephalopathies (TSE) that include mad cow disease, scrapie, kuru and Creutzfeld- Jakob disease[4] . Feather is composed of over 90% (w/w) protein, the main component being ß- keratin, a fibrous and insoluble protein highly cross- linked with disulfide bonds.Considering its high protein content, this waste could have a great potential as a source of protein and amino acids for animal feed and for many other applications. Despite the recalcitrance (hard to deal with), keratin wastes can be efficiently degraded by specific proteases such as keratinase. The production of keratinases has been a domain of saprophytic and dermatophytic fungi, actinomycetes and some *Bacillus* species. Many of the current studies are focussing on potential use of keratinases of bacterial origin for the industrial treatment of keratin containing materials.

Materials and Methods

Sample collection: Soil samples were collected from regular feather dumping site of Sanjan poultry processing plant, Gujarat, India in sterilized sampling bottles. The samples were taken from 30 cm depth from the surface of the soil. The samples were brought into the laboratory and processed for analysis on the same day. The sample was then stored at 4^{o}C for further uses.

Isolation of feather (keratin) degrading micro- organism

Enrichment: 1gm of poultry waste was serially diluted in order to reduce the initial number of micro organisms. This dilution was then inoculated into 250 ml Erlenmeyer flask containing sterile 100ml of soyabean casein digest broth medium. The flask was incubated at room temperature for 2 days. After incubation, one loop full enriched sample from above flask was streaked on the sterile soyabean casein digest agar medium plates. The streaked plates were then incubated at 37 ^{o}C for 24hrs in incubator[5].

Screening on the skim milk agar plates: Sterile Skim milk agar (Himedia) was prepared and the above isolated colonies were inoculated on the milk agar

plates. The plates were then incubated at 37°C for 24 hrs. The strains that produced clearing zones in this medium were selected[6].

Screening on modified feather basal medium: Modified basal liquid medium supplemented with raw feather used for the secondary screening of selected keratin degrading bacterial isolates.Modified basal liquid medium contained the following constituents (g/L): NaCl (0.5), $MgCl_2.6H_2O$ (0.1), NH_4Cl (0.5), KH_2PO_4 (0.3), K_2HPO_4 (0.4), Feathers (10.00); pH 7.5Raw feathers were collected from the poultry farm, washed properly using tap water to remove dust particles from it followed by washing with distilled water. Washed clean white chicken feathers were dried at room temperature. The above medium was taken in 250 ml flask - each flask containing 100ml modified basal medium and 1 gm of clean and dried medium size chicken feather, which was then sterilized by autoclaving. The medium was allowed to cool and then inoculated with selected bacterial isolates. Inoculated flask was then incubated at room temperature for 4 weeks and observed weekly for feather degradation [2,3,7-9].

Identification & Characterization of isolated feather degrading bacteria: Gram staining, motility test and morphological tests[2,5,6,10-12]. IMVIC test, Urease test, Catalase test, Carbohydrate fermentation test. The results of all the above mentioned tests are compared with Bergey's Manual of Determinative Bacteriology, 8th edition[13].

Keratinase enzyme production: The culture of different isolates were first inoculated in the flask containing 100ml of minimal basal medium (g/L) which was prepared as follows: NaCl (0.5), $MgCl_2.6H_2O$ (0.1), NH_4Cl (0.5), KH_2PO_4 (0.3), K_2HPO_4 (0.4), Feathers (10.00); pH 7.5.The above flasks were then incubated at 37°C for 24 hrs. All the above incubated media were then centrifuged at 5000 rpm for 15 minutes. The supernatant were then used for crude enzyme preparation [14].

Keratinase enzyme purification:

By Ammonium sulphate fractionation: Protein in the supernatant was precipitated by adding solid ammonium sulphate (20-100%). The precipitate collected by centrifugation at 1000 rpm for 40 minutes was dissolved in minimal volume of 20mM Tris-HCl buffer, pH 8.0.This is crude enzyme solution. The enzyme solution was desalted by dialysis with a cellulose dialysis bag (35 KDa pore-size) in 20mM Tris-HCl buffer at pH 8.0[14,15]

Determination of Keratinase activity:

Preparation of DMSO-solubilized Keratin: Washed chicken feathers were finely chopped with scissors into powder form. 10 grams of this fine feather powder was reacted with 500 ml of DMSO. This mixture was heated in an oven at 100°C for 120 minutes, instead of refluxing in a reflux condenser. 1000 ml of cold acetone was then added to precipitate the soluble keratin. Precipitation was further carried out in a deep freezer for 2 hours. The precipitate was obtained by centrifugation at 10,000 rpm for 20 minutes. It was washed thoroughly, thrice, with distilled water and dried in a vacuum dryer at 40°C. 1 gram of quantified precipitate was dissolved in 20 ml of 0.05N NaOH solution. The pH of this solution was adjusted to 8 using 0.1 mol/L Tris HCl.

Keratinase Assay: The keratinase activity was assayed by reacting 1 ml of crude enzyme with 1 ml of the keratin solution in a water bath at 50°C with shaking for 10 minutes. The reaction was then stopped by addition of an equal volume of 20% trichloroacetic acid. The precipitate was removed by centrifugation at 10,000 rpm for 15 minutes and the optical density of the supernatant was measured at 280 nm. One unit of keratinase activity was defined as an increase in the corrected absorbance (A_{280}) by 0.01units with respect to a control, per ml per minute under the conditions described above.

It was calculated as: $\boldsymbol{U=4\times n\times A_{280}/(0.01\times 10)}$
Where n is the dilution rate; 4 is the final reaction volume (ml); 10 is the incubation time (min).

During the present study, the enzyme production by the bacterial cells in broth has been quantified as Units of Enzyme Activity per Milliliter of cell free broth per minute.

Optimization of culture conditions:

Effect of Temperature: Tubes of soyabean casein digestive broth were inoculated with one loopful of 24 hr old culture suspension of bacteria. 4 sets were prepared each containing one tube inoculated with different isolates. Set of the tubes were incubated at different temperature such as 8°c, 25°c, 37°c and 50°c for 24 hrs. After incubation the extent of growth in each tubes were visually observed [16].

Effect of pH: Tubes containing soyabean casein digest broth alongwith the prepared buffer of different pH as mentioned in above table was prepared. All the tubes were sterilized by autoclaving. Each tube was then inoculated with

one loopful of different isolates.All the tubes were incubated at 37 ^{o}C for 24 hrs[16].

Results

Isolation and identification:

Soil samples from Sanjan poultry farms in Gujarat were enriched and screened for the feather degrading bacteria. On the basis of screening, two isolates (designated as isolate P & isolate W) were selected for further identification. As per the cultural, microscopic and biochemical tests, these bacteria belong to the genus *Bacillus sp*. (Table 1 & 2).

Optimization of cultural condition: The both bacterium presented optimum growth at pH 6.8 and a temperature of 37 ^{o}C (Table3&4).

Purification of keratinase and degradation of feathers: Both the isolates (i.e isolate P and W) shows complete degradation of the feather within 4 weeks. The keratinase enzyme was purified from both bacterial isolates using ammonium sulphate fractionation and dialysis.The keratinase enzyme activity was found to be 8.80U/ml/min for isolate P and 13.60U/ml/min for isolate W (Table5).

Table1: Morphological Characteristics

Characters	**Isolate-P**	**Isolate-W**
Size	1 mm	1 mm
Shape	Circular	Circular
Colour	Off white	Whitish
Margin	Entire	Entire
Elevation	Concave	Concave
Opacity	Opaque	Opaque
Consistency	Dry	Sticky
Gram Nature	Gram positive Rods	Gram positive Rods
Motility	Non motile	Non motile

Table: 2 Biochemical Tests after 24 hrs incubation at 37^{0}C.

Test	**Isolate-P**	**Isolate-W**
Catalase	Positive	Positive
Urease	Negative	Negative
Indole production	Positive	Positive
Methyl red	Positive	Positive
Voges Proskauer (VP)	Negative	Negative
Citrate Utilization	Positive	Positive
Glucose	+	-
Lactose	-	-
Mannitol	+	+
Sucrose	-	+

Keynotes: - Negative & + Acid

Table 3: Effect of Temperature

Temperature	Isolates	
	P	W
Refrigerator (8^0C)	-	-
Room Temp. (25^0C)	+++	+++
Incubator (37^0C)	+++	+++
Hot Air Oven (50^0C)	-	-

Keynotes: - No Growth, + Scanty Growth, ++ Moderate Growth & +++ Abundant Growth

Table 4- Effect of pH

SCD Broth (ml)	Phosphate/ citric acid buffer (ml)	pH	Growth of isolate P	Growth of isolate W
8.0	2.0	2.8	-	-
8.0	2.0	3.6	-	-
8.0	2.0	4.4	-	-
8.0	2.0	5.2	-	-
8.0	2.0	6.0	++	++
8.0	2.0	6.8	+++	+++
SCD Broth (ml)	**Tris/HCl buffer (ml)**	**pH**	**Growth of isolate P**	**Growth of isolate W**
8.0	2.0	7.6	+	+
8.0	2.0	8.4	+	+
SCD Broth (ml)	**Borax/ NaOH (ml)**	**pH**	**Growth of isolate P**	**Growth of isolate W**
8.0	2.0	9.2	-	-
8.0	2.0	10.0	-	-

Keynotes: - No Growth, +Scanty Growth, ++ Moderate Growth & +++ Abundant Growth

Table 5-Keratinase Activity

Isolates	O.D. at 280nm
P	0.11
W	0.17

FORMULA:

$$U=4\times n\times A_{280}/(0.01\times 10)$$

CALCULATION:

Keratinase activity of Isolate P = $4\times2\times0.11/(0.01\times10)$
= **8.80U/ml/min**

Keratinase activity of Isolate W = $4\times2\times0.17/(0.01\times10)$
= **13.60U/ml/min**

Discussions

Poultry waste dumping soil was collected from Sanjan poultry form, Sanjan. 5 different samples were collected from the dumping sites (predominantly

where feathers were dumped). These were pooled together and enriched and then screened to obtain 17 isolates. Of these, isolates P and W were found to have significant positive effect in the degradation of protein and thus indicating good potential for production of protease enzyme. The bacteria with protease enzymes were expected to utilize the keratin in the chicken feathers as metabolites for its growth and reproduction. Chicken feathers are poorly disposed in landfills. Combustion forms the major way of disposal of these feathers and this has had an adverse effect on the environment. Enzymatic hydrolysis of feather wastes could be a safe method of recycling this organic matter into a form that can be utilized by animals as protein feed supplements. Worapot *et al.,* 2005 and Xiang *et al.,* 1992 studied the feather degrading ability of *Bacillus licheniformis* and concluded that its ability to grow well in thermophilic temperature is very important in efficient degradation of feathers. Our study involves bacillus species operating at 25 to 40^{o}C, which is the normal temp range found in these areas. The bacteria isolated were found to grow optimally at a temperature of 37°C and therefore would require less energy in a controlled process for efficient and faster degeneration of chicken feathers.

Feather degradation in this study was determined visually. Increase in the turbidity of the feather broth was an indication of bacteria growth and subsequent degradation. Feather degradation by isolated *Bacillus sp.* is confirmation that these bacteria possess a protease capable of reducing disulphide bonds of keratin. *Bacillus* is more adaptable to the environment they inhabit than the other bacteria isolated in this study as shown by the dominance of *Bacillus sp* isolated in the whole study. Thus, *Bacillus sp.* has the property of being stable and makes it the best candidate in the biotechnological industry. Further optimisation of the process needs to be done to make it suitable for commercial use. This includes optimisation of feather concentration with the bacteria directly as well as the isolated enzyme. The isolated enzyme free of the live bacterium would be advantageous for use of the degraded products as animal feed supplements.
Also, specific customised functions for different keratin containing waste products can also be explored. However, the cost effectiveness of the use of enzyme also needs to be studied.

Conclusion

In the present study, two feather degrading bacteria were isolated from poultry waste dumping soil. Characterization carried out in the laboratory showed that this novel bacteria belongs to *Bacillus* genus and were found to have proteolytic (especially Keratinolytic) activity & hence may be used for disposal of chicken feathers. The data generated from this research work indicates that Sanjan poultry waste dumping soil could be a rich source of

feather keratin degrading bacteria producing enzymes that could be put into industrial use as exhibited by the two isolates characterized in this study.

Future prospective

There is need to carry out further studies on these isolates to facilitate industrial uses. Further studies and experiment are recommended to establish degradation of the chicken feathers and other keratin containing wastes for production of animal feed & nitrogen supplements and further optimisation of the parameters like temperature, pH media, feather concentration, etc. can be done to enhance the activity of the isolated species. Investigation of the purification and molecular properties of each enzyme obtained from the isolated species is also important. Further work may be done on *Bacillus sp.* especially in the evaluation of their enzymes as catalysts in biotechnological applications involving bioremediation and hydrolytic reactions.

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