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Estimation of Keratinase from *Bacillus* sp, *Pseudomonas* sp., and *Vibrio* sp., isolated from Poultry Soil Biodegradation of Chicken Feather

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Abstract
The *Bacillus* sp, *Vibrio* sp, and *Pseudomonas* sp, were isolated from poultry waste. The chicken feathers were collected from the poultry industry at Kadalaiyur. These organisms were identified by Gram's staining, spore staining, simple staining and various microscopic observation and biochemical tests. For degradation of feathers by using microorganisms. The keratinase enzyme was estimated by using the laboratory procedure. Degradation of feather by these organisms was carried out at temperature of 37°C for 14 days and observed visually. Degradation of *Bacillus* sp, *Vibrio* sp, and *Pseudomonas* sp was determined of various pH and temperature. The maximum keratinase activity was obtained at pH 7&8 and temperature at of 37° C. When optimized with different carbon, and nitrogen sources are produced the medium. Degradation of *Bacillus sp, Vibrio sp*, and *Pseudomonas* sp was determined of various pH and temperature bacteria obtained from feather collection, screened for extracellular keratinolytic activity. The selected cultures such as the *Bacillus* sp, *Vibrio* sp, and *Pseudomonas* sp suggested strongly of bacteria that produces keratinase in the cell free culture supernatants. The best biodegradation of feather was obtained using *Bacillus* sp and *Pseudomonas* sp.

Keywords
Feather degradation, Keratiase, Selected Microbes, Optimization, UV Spectrophotometer.

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INTRODUCTION

FEATHER

Feathers are produced as waste of poultry processing plants in large quantities, millions of tons per year worldwide. Poultry feather constitutes the most abundant keratinous material in nature. The main component of feather is keratin, a mechanically durable and chemically un reactive protein, which renters it difficult to digest by most proteolytic enzymes. Recycling of feather is a subject of interest because it is potentially cheap and alternative protein supplement to be used in animal feed (Muthusamy Govarthanan *et al*.., 2011). Worldwide around 25 billion chickens are killed annually. The feather represents 5 to 7 % of the total weight of mature chickens.

FEATHER DEGRADATION

Biodegradation of feather keratin by microorganisms producing keratinases represents an alternative method to improve the nutritional value of feather waste and to prevent environmental contamination. There is growing interest in microbial process of commercial importance in many areas as environmental sciences, biomedicine and biotechnology. Feathers are generated in large amounts as a waste by product as commercial poultry –processing plants reaching millions of tons per year worldwide (T. Jayalakshmi *et al*., 2012)

KEARATIN

Keratin is the key structural material of outer layer of human skin. It is also the key component of hair, nails, horn and wool. It belongs to family of fibrous structural protein in which monomer assembled to form intermediate filament. Keratin has very high stability and degradation rate due to higher degree of disulfide bridges, hydrogen bond and hydrophobic interaction. (Nidhi sahni *et al.,*2015). The protein chains are packed tightly either in α-helix or β-sheet structures, which fold into final 3- dimensional form. Keratins are grouped into hard keratins (feather, hair, wool, and nail) and soft keratins (skin and callus) according to sulphur content. This report describes the protein, amino acid, keratinolytic activity and degradation of feather by *Pseudomonas, Microphilus* and *Leuconostoc sp*., (Tamilkani et al..,2013).

CHARECTERISTICS OF KERATINASE

Keratinases are proteolytic enzyme in nature. It was classified as proteinase; keratinases are only in the presence of keratin containing substrate. It mainly attacks on the disulfide (-s-s-) bond of the keratin substrate. Keratinolytic enzyms are widespread in nature and are produced by a group of microorganisms largely isolated from poultry wastes. (Nidhi sahni *et al.* 2015). The bioprocesses using keratinolytic microorganisms address four important needs:1) Ecological, because the degradation of feather environmental leads to production of sulphur, 2) economical and 3) Nutritional, resulting in a product with better and nutritional value digestibility. Biodegradation of poultry waste by keratinase is an economically friendly process that can play an important role in biotechnological applications such as enzymatic improvement of feather meal, production of rare amino acids (serine, cysteine and proline), peptides used in the leather industry as well as medicine and cosmetic production.(Somayeh mousavi *et al..,* 2013).The aim of the present study is degradation of feather and production enzymes by soil microbes *Bacillus* sp*, Vibrio* sp and *Pseudomonas* sp.

MATERIALS AND METHODS

COLLECTION OF SAMPLES

Chicken feather were collected from the waste of slaughter house in the local market of Kadalaiyur.

ISOLATION OF FEATHER DEGRADING BACTERIA

The collected soil samples were serially diluted up to 10^{-9} and 0.1 ml from 10^{-6} dilution was spread plated on to nutrient agar medium. The petridishes were incubated at or 24 hours. From the growth of colonies, these dilutions were then plated on nutrient agar medium at 37°C for 24 hours. After that the specifically picked microbes were plated on selective media such as *Bacillus* isolation agar medium, TCBS and *Pseudomonas* isolation agar medium. Skim milk agar plates were prepared and colonies from nutrient agar plates were taken by streaking on skim milk agar plates for purification of the organism and incubated at 37°C for 24 hours.

PREPARATION OF INOCULAM

A 100ml nutrient broth solution was prepared and sterilized at 121°C for 20 mins. The medium was inoculated under aseptic conditions with bacteria. The broth culture was incubated for 14 hrs on a rotary shaker (150 rpm) at 30°C and was used for inoculating the production medium.

FEATHER MEAL POWDER PREPARATION

Poultry was washed extensively, boiled for 2-3 hrs. Dried in hot air oven for 4 hrs at 50°C. The fried feather was pulverized, and the powder was used as feather meal.

KERATINASE PRODUCTION

The production was carried out in 500 ml Erlenmeyer flask containing 100 ml of the culture medium (composition: -10g raw feather, NH_4CL 0.5gm, NaCl 0.5gm K_2HPO_4 4gm, KH_2PO_4 0.3gm) for 3 days at optimized temperature at 150 rpm. After 3 days of incubation, the culture medium was filled through Whattsman No.4 filer papaer and filtrate was collected. The filtrate was centrifuged at 10,000 rpm for 10 minutes at 37°C and culture supernatant were used as crud enzymes to examine keratinase activity and protein content. (Srinivasan balakumar et al.., 2013)

DETERMINATION OF KERATINASE ACTIVITY

The residual feather was washed, dried and scaled to calculate DD by using following equation DD (%) = (TF-R) x 100/ TF where, TF is the total feather and RF is the residual feather.

ESTIMATION OF KERATINASE ACTIVITY

The keratinolytic activity was assayed according method of Vigneshwaran *et al.,*1.0 ml of crud enzyme was diluted in 0.05 M Tris-HCL buffer (Ph-8.0) and was incubated with 1ml of 1% soluble keratin

solution at 50°C in a water bath for 10 minutes. The reaction was stopped by adding 2.0 ml of 0.4 M trichloroacetic acid (TCA). The resulted precipitate was removed by centrifugation at 10000 rpm for 10 min. 0.2 ml of the supernatant was taken and diluted with to 0.1 ml with distilled water. To this, 5.0 ml of folin ciocalteau (FC) reagent was added and incubated in the dark for 30 min to allow blue colour development. The control was prepared by incubating enzyme solution with 2ml of TCA without the addition of keratin solution. The absorbance was measured at 660 nm using a UV – spectrophotometer (SL159, ELICO). One unit of alkaline keratinase was defined as the amount of enzyme required to liberate 1µg of tyrosine per min per ml under the standard assay conditions.

PURIFICATION OF KERATINASE

The cell free extract from fermentation broth was partially purified by acetone precipitation method. Keratinase was precipitated by pre chilled acetone (30-80%) fractionation. The acetone was added to the cell free extract in 3:1 ratio and incubated for 60 mins at 20° C. The contents were subjected to centrifugation at 10000 rpm for 10min. The supernatant was carefully discarded carefully, and pellet was dissolved in Trish-acetate buffer (P^H 7).

ANTIMICROBIAL ACTIVITY

Susceptibility to antibiotics was tested as follows: discs (diameter, 6.5 mm) impregnated with antibiotics (Himedia) were laid on mullar-hindan agar plates which had been surface inoculated with the test sample of *Bacillus* sp, *Vibrio* sp and *Pseudomonas* sp, into spread plate method. The following 10 antibiotics discs (mg/disc) were used; amoxicillin, ampicillin, chloramphenicol, kanamycin, methicillin, ciprofloxacin, streptomycin, tetracycline and vanomycin. After 24-48 hours inhibition zones were recorded.

DETERMINATION OF AMINO ACID

The amino acid present in keratinase production to calculate using ninhydrinMethod (somayeh mouysavi et al.,) and separate amino acids where identified and then calculated RF value using this formula.

RF = Distance moved by the amino acids / Distance moved by the developing solvent

PROTEIN ESTIMATION

The amount of protein present in keratinase assayed by the Lowry methods using bovine serum albumin (BSA) as standard. The colour developed was read at 660 nm. (Venkata saibabu et al., 2012). The specific activity (U/mg) of the enzyme was calculated by the following formula.

Specific activity of enzyme = Enzyme activity /Total protein content.

FACTORS AFFECTING KERATINASE ACTIVITY:

Effect of pH (6,7,8), Temperature(35-45°C), sources: carbon, nitrogen)

STATISTICAL ANALYSIS

The result obtained were expressed as standard deviation (SD) for determination. Data were analysed and plot the graph.

RESULT AND DISCUSSION

ISOLATION AND IDENTIFICATION OF FEATHER DEGRADING MICROORGANSMS

The feather dumped soil sample were collected which was serially diluted and inoculated with nutrient agar medium at 37°C for 24 hrs. After the incubation period the colonies were specifically taken and undergo gram's staining, spore staining biochemical tests. The identification of keratinolytic bacteria was based on cell morphology, colony morphology and several other methods. Then isolated cultures were plated on *Bacillus* isolation agar, *Pseudomonas* isolation agar, TCBS. These results suggested that the isolates were idendified as *Bacillus* sp, *Pseudomonas* sp, and *Vibrio* sp.

SCREENING OF FEATHERB DEGRADING MICROBES

The isolated were tested for their capacity to degrade feather wastes. Three isolated culture were screened for keratinolytic activity on the skim milk agar plates. Keratinolytic activity was showed the zone formation produced by the isolates on agar surface with 24 hours incubation at 37^{0}C.

FEATHER DEGRADING IN LIQUID MEDIA

The degradation of feather in basal salt medium, fresh feather meal broth medium by using *Bacillus* sp, *Vibrio* sp and *Pseudomonas* sp under shaking condition for 7 days at 37^{0}C.

KERATINASE ASSAY

Then the filtration and centrifugation process were done to get crude proteins. The crude enzymes of keratinase were obtained which was made to react with prepared TCA. The absorbance values were measured individually at 595 nm by using spectophotometer.

PROTEIN ESTIMTION

The protein content of the feather degradation was measured spectrophotometrically by Lowry's method and compared standard BSA.

DETERMINATION OF AMINO ACID

The amino acid components were separated and observed blue colour spots (except proline) were identified the amino acid value and compared the stranded value and then calculated RF value. (serine, cysteine, proline).

ANTIMICROBIAL ACTIVITY

Antibiotic activity mullar-hinten agar plates were inoculated with the test sample of *Bacillus* sp, *Vibrio* sp, and *Pseudomonas* sp, into spread plate method. The following 10 antibiotic discs (mg/disc) were used: amoxicillin (2.3), ampicillin (3.2), chloramphenicol (2.8), kanamycin (3.4), methicillin (2.6), oxaloacin (3.8), ciprofloxacin (2.4), streptomycin (3.2), tetracycline (3.9), and vancomycin (3.9).

FACTORS AFFECTING KERATINASE ACTIVITY:(PH, Temperature, sources: carbon, nitrogen)

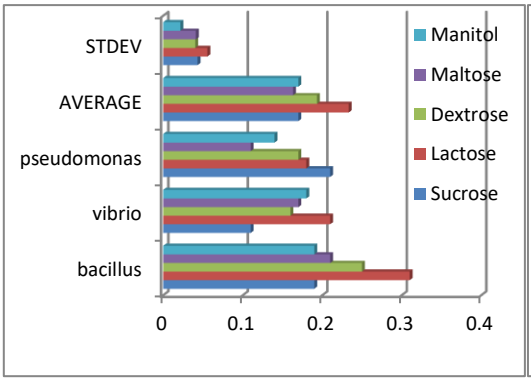
Effect of PH on the enzyme activity was measured at various PH ranges (7.8). The PH was adjusted using the following buffers –phosphate, Tris –HCL and the culture were inoculated in the broth. The tubes were incubated at room temperature 7-14 day's incubation.

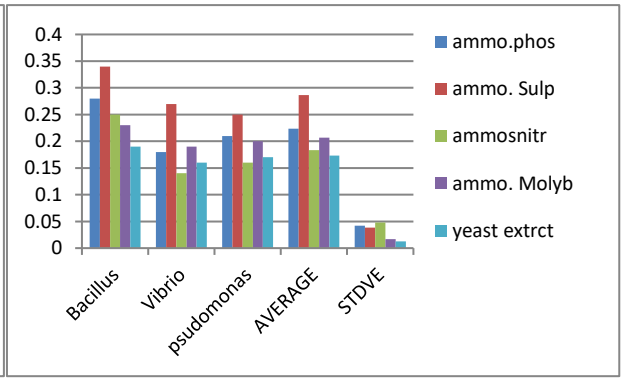
The activity of the enzyme was determined by incubating the reaction mixture at different temperature ranging from 35, 40 and 45°C were incubated.

Sucrose, maltose, lactose, mannitol, dextrose and feather meal at 1% w/v were tested to determine the optimized carbon sources. The tubes were incubated at room temperature 7, 14 days incubation. After incubation growth was recorded and noted. The keratinase production was also optimized by supplementing different nitrogen sources individually at 1% concentration. Ammonium phosphate, ammonium nitrate, ammonium sulphate, ammonium molybdate, and yeast extract. The tubes were incubated at room temperature 7-14 days incubation.

ANALYSIS OF THE SAMPLE

Degraded feathers were analysed by spectrophotometer and it showed the degradation of range of three isolated and also it revealed the crude keratin compounds by UV spectrophotometer.





DETERMINATION OF CRUDE KERATINASE ACTIVITY ON SUBSTRATE LEVEL OF *Bacillus* sp USING SPECTROPHOTOMETER

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