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Screening of Keratinase Producing Bacteria by Using Feathers of Owl, Crow, Hen, Sparrow, Peacock and Cock.

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ABSTRACT

Enzymes are used in industries for variety of purposes. Microbial keratinase enzyme offer a substantial advantage of degrading keratin waste. In large amount of keratin are present in the soil, nail, hairs, feather and poultry. In many microorganisms are secreted keratinase degrading enzyme used in our study were isolated from poultry waste soil and feathers. The isolates were obtained from culture filtrate was measured in units by keratinolytic activity and their activity was measured used by different incubation periods pH, temperature and different agitation. The highest enzyme activity was observed at 50°C and pH 8 .The enzyme was stable at 40 to 50°C. It was found to be highly stable within the range of 5- 8.5 pH values. Complete feather degradation was achieved at pH 8, inoculum size 2%, agitation rate 200 rpm, and incubation temperature 37°C, incubation period for 48 h. Feather keratin is highly resistant to degradation, but some keratinase produced microorganisms can easily degraded insoluble keratins. These keratinase produced species have an important application in removal of poultry waste and recycled into valuable by product. Maximum feather degrading activity attained at the optimal conditions. In practical applications, keratinase is a useful enzyme for promoting the hydrolysis of feather keratin and improving the digestibility of feather meal. The aim of this study to identify new keratinolytic bacteria and its optimum parameters for higher feather degradation at pH 8 and 500C, with potential application in biotechnological processes.

Keywords: Keratinase, Keratinolytic, Poultry, Bacillus, Feather.

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INTRODUCTION

Enzymes act as biocatalysts which have become an important part of many industries for effective degradation of non-degradable substances. Microbial keratinase is an enzyme capable of degrading the insoluble structural protein found in feather known as keratin. Keratin present in feathers is not degraded by usual proteolytic enzymes such as trypsin, pepsin and papain. These are due to decomposition and molecular conformational of the amino acids found in keratin [1,2]. Several strains of microorganisms produce intracellular and extracellular enzymes during their regular metabolism which have wide commercial applications [3-6]. Animal waste can be used as a powerful nutrient source. But the waste like feathers and hair obtained as a byproduct of meat and dairy processing cannot be degraded easily and hence they form a source of pollution. There are certain strains of microorganisms which produce extracellular keratinase enzyme which can serve as a substantial source for the degradation of keratin waste [7-11]. Accumulation of keratinase waste are produced huge amount in environmental nature [12, 13]. Optimization of enzyme parameters such as pH and temperature. The media composition is important for enzyme activity [14]. Different strains of bacteria isolated if cultured under optimum conditions and the kerazyme activity .In this report we have described the isolation of three different strains of bacteria, their growth characteristics using feather as nutrient medium and the activity of kerazyme secreted by the stains under optimum pH and temperature conditions. Keratinase production is affected by various nitrogen sources and its concentration, such as raw feather, feather meal, powdered chicken nails, bovine hair or wool [15]. In recent years, feather treated with microbial keratinase is attracting wide attention with several applications. Keratinase -treated feather is increased considered as a viable source of dietary protein in food and feed supplements, as the enzyme-treated end product was retain their high nutritive value. Keratinase are projected to generate a potential worldwide market similar to other proteases. Keratin lytic enzymes have several current and potential applications in agro industrial, pharmaceutical, and biomedical fields. Their use in biomass conversion into biofuels may address the increasing concentration on the energy conversation and recycling [16].Keratinase enzymes are useful in processes related with the bio diversion of keratin waste in feed and fertilizers. Other promising applications have been associated with keratin lytic enzymes, including enzymatic de haring for leather and cosmetic industry, detergent uses. The use of keratinase to enhance drug delivery in some tissues and hydrolysis of prion proteins arise as outstanding applications for enzymes [17-19].

MATERIALS AND METHODS

Collection of samples

The soil samples and five different waste feathers required for isolation of bacteria were collected from a poultry industry Vellore district near Anaicut village. The feathers were washed properly 3-4 times with tap water then followed by distilled water, and allowed to dry in the sun for one day. After drying the different feathers were allowed to pretreated with chloroform: Ethanol (3:1) and kept suspended for 2 days. The pretreated feathers were kept for sun drying for 2 days. After feathers were cut into small pieces about 2 inches.

Feather medium preparation

The feather meal broth used for isolation, maintain and, growth of bacteria was made by adding 0.5 g of NH_4cl,0.5 g of Nacl,0.3 g of K_2HPO_4,0.4 g of KH_2P0_4,0.1g of Mgcl.$6H_20$,0.1 g of yeast extract, and 10 g of cut different feathers. The pH was maintained at 7.5 and was prepared 250 ml of volume. Feather agar plates was prepared by adding 7 g of feather agar medium was prepared in the lab and add 4 g of Miller Luria Bertani Agar and 2 g of Agar in 100 ml of distilled water. The broth and agar medium was sterilized at 121°C for 20 minutes.

Isolation of microorganisms

The soil samples were collected from different poultry in Vellore. The glassware's should be clean and sterilized and weigh 1 g of soil then dissolved in 100 ml of sterilized distilled water in a conical flask. The best results were observed when we combined the isolation and screening techniques. After serial dilutions of the soil sample were done 0.1 ml of the diluted sample was introduced onto the LB agar and spread plated following which the sterilized .Feather piece was gently pressed onto the agar. Take a 7 test tubes filled 9 ml of

sterile distilled water in a concentration of about 10^{-1} to 10^{-7} the simultaneously prepared control tube also were serial diluted and 10^{-4} and 10^{-5} concentration of sample was used for plates. These was done using pour plate and spread plate techniques to obtain pure cultures. The colonies were inoculated in fresh medium and subculture to obtain were inoculated in fresh culture medium and subculture to obtain sufficient quantity of individual colonies. The different strains of bacteria were obtained using Gram Staining.

Determination of growth characteristics

The culture inoculums obtained after 5 days of incubation were kept in an orbital shaker at 100 rpm. Periodically O.D values at 280nm were calculated using calorimeter for a duration of 48 hours.

Screening of keratinolytic bacteria

We screened the pure cultures used for certain bacterial species. Instead of hair- sterilized and pretreated; feather was used. After streaking onto LB agar plates, the feather was gently pressed down with sterile forceps on the streaked line and left to incubate at 36^{o}C for 2-5 days. After 2 days those colonies that were growing on the feather were singled out and subculture. These colonies were observed to have feather degrading properties and thus keratinolytic bacteria. The isolate of different strains was cultivated for 24 hours in feather medium. The culture medium was centrifuge at 10,000rpm for 10 minutes, and the supernatant was collected for enzyme preparation. The culture filtrate used for enzyme source where 1 ml of the solution was added to 3.8 ml of 0.1 mole l-1 tris buffer (pH 7.7) containing feather. The solution was incubated for 30 min at 55°C. The reaction was stopped by add 1 ml of TCA and placing the test tubes in ice. The amino acids liberation were measured at 280 nm in a UV spectrophotometer as against a blank.

Identification of bacterial strain

The potential bacterial isolate were identified using staining, biochemical characterization.

Staining

The refractive index of bacteria is nearly same as water, when the bacterial strain observed under microscopic it will be invisible to naked eye. Hence stains were used to observe the bacterial morphology.

Gram staining

In Gram staining three major steps were followed such as smear preparation, staining and decolourization.

Smear Preparation

Smear preparation was followed as described in simple staining.

Staining

In the smear crystal violet was added and it was kept undisturbed for 60 seconds. Then the slide was washed with distilled water. Gram's iodine was added to the smear and allowed to act for 60 seconds then Gram's iodine was washed with distilled water. Decolourizer was added from the top of the slide till the dye colour disappears, then counter stain was added to the smear and incubated for 30 seconds then it was washed with tap water. The slide was dried with blotting paper and observed under microscope by high power oil immersion.

Motility test

The fresh bacterial culture was taken and placed in the center of cover slip were the all the corner of cover slip has Vaseline. Depression microscope slide is placed on the cover slip and gentle press was made.

Then the slide was inverted so the culture was hanging on the cover slip. The slide was observed under microscope.

Biochemical characterization

Identification of genus and species was achieved by various biochemical characterizations.

Catalase test

Using a sterile loop, culture was taken and placed in the center of slide. Few drops of hydrogen peroxide were added to the culture using a fresh dropper. Result was observed for one to two minutes.

Oxidase test

In sterile Petri plate a filter paper is placed, using a swab bacterial culture was placed in the filter paper. To the culture few drops of tetra methyl-p-phenylenediamine is added with the help of sterile dropper. Results were observed within few seconds.

Citrate test

In a sterile test tube Simmons citrate agar was added and kept it in a slant position till the agar gets solidify. Using a cotton swab the pure bacterial culture was streak in the slant. The slant was incubated at 37ºC for 24 hours. Result was observed.

Starch hydrolysis test

Pure bacterial culture was inoculated in starch plate the plate was incubated at 39ºC for 48 hours. After the incubation iodine was flood in the plate. Result was observed in few minutes.

Indole test

In the sterile test tube nitrate broth was added to this sterile broth pure bacterial culture was inoculated and incubated for 48 hours at 37ºC. To the broth few drops of sulfanilic acid and α-naphthylamine was added. Result was observed during the addition of α-naphthylamine.

Lactose test

Phenol red lactose broth was transferred to sterile test tube and inoculated with the bacterial culture. The culture was incubated at 37ºC for 24 hours then the results were observed. The yellow color shows the fermentation of lactose.

Methyl red

Pure culture was inoculated in the MR-VP broth by using a sterile loop. The broth was incubated for 48 hours at 37ºC. To the broth 5 drops of methyl red was added and the result was observed without mixing.

H_2S Formation

Pure bacterial culture was transformed aseptically to TSI agar slant. The culture was incubated for 24 hours at 37ºC. After incubation period the slant was observed for color change.

Optimization of keratinase enzyme

Effect of various incubation times on keratinase production

Feather minimal medium was prepared and incubated at various time intervals (24, 48, 72, and 96 hrs) .0.5% inoculums from 24hrs Luria broth of the culture was inoculated in 100ml of basal feather medium and kept in shaker with 120rpm at 37 °C for 24hrs.

Effect on pH on keratinase production

Feather minimal medium prepared in different PH (2, 4, 6, 8, & 10).0.5% inoculums from 24hrs Luria broth of the culture was inoculated in 100ml of basal feather medium and kept in shaker with 120rpm for 24h.

Effect of temperature on keratinase production

Feather minimal medium was prepared in different temperature(20,30,40,50,&60).0.5% inoculums from 24h nutrient broth of the culture was inoculated in 100 ml of basal feather medium 80°C and kept in shaker with 120rpm for 24 h.

Immobilization of keratinase enzyme

Production of beads formation

4grams of calcium chloride was weighed and dissolved in 100ml of distilled water and kept in 4°cfor 2 hours. To prepare 0.1N NaCl sodium alginate for that weighed 0.685grams of sodium chloride and 3.5 grams sodium alginate was dissolved in 100ml of distilled water. In a beakers, 20 ml of Sodium Alginate is taken. To prepared 3ml of enzyme filtrate is added in beaker containing Sodium Alginate. Hence Sodium Alginate solutions are prepared. Now $CaCl_2$ is taken into a beaker. Then using dropper Sodium Alginate solution of crude enzyme is added drop by drop into beaker containing $CaCl_2$ so that there are of beads formation. When Sodium Alginate solutions is added drop by drop into beaker containing $CaCl_2$ after then beads are formed. Beads are dissolved in phosphate buffer and take O.D at 24 hours interval for 5 days at 280nm.

RESULTS AND DISCUSSION

Samples were collected in Vellore poultry. Feathers are pretreated for screening.

Figure 1: Collection of samples

Figure 2: Pre-treated feathers

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Feather medium preparation

The production medium was prepared for different salts and added feather .From that petri plates colonies were inoculated to production medium.

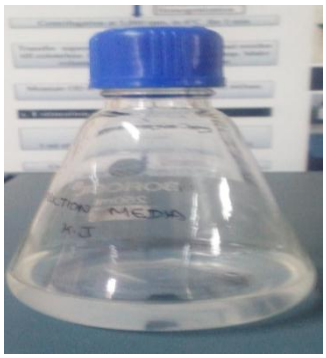

Figure 3: Production medium

Isolation of microorganisms

Keratinase enzyme has been isolated from poultry soil and feather .The keratinase enzyme are isolated from Baiting method.

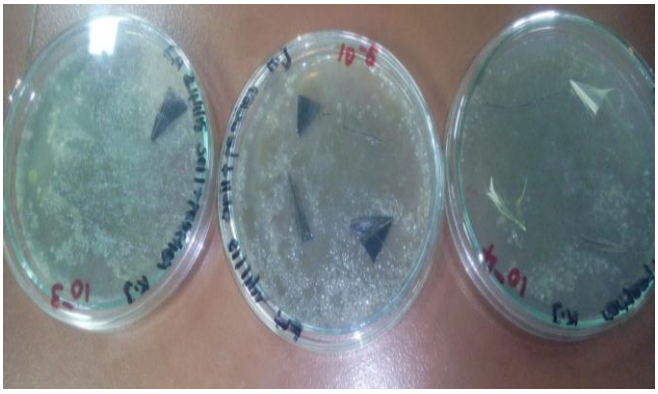

Figure 4: Baiting technique

Streaking method

Streaking plates techniques the colonies were grown after using for further analysis of keratinase enzyme.

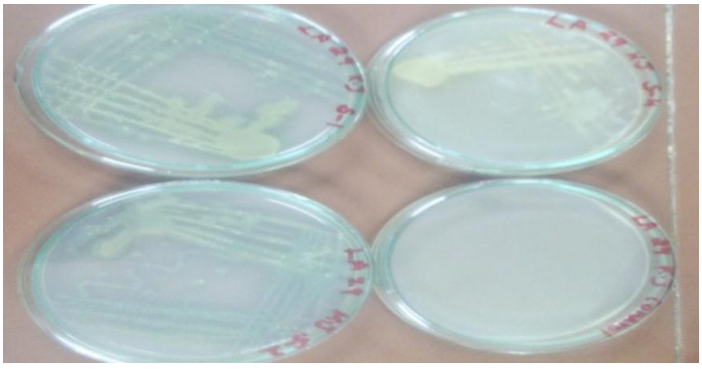

Figure 5: Streaking plates

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Screening of keratinolytic bacteria

Prepared production medium in conical and inoculation sample was taken from streaking plates then they are kept in orbital shaker system for 24hrs.The production medium was inoculated by soil microorganism after degrading the large amount of enzymes are present in that medium.

Figure 6: Shows the feather degradation

Staining: Gram staining, spore staining and motile were done to identifying the keratinase producing bacteria.

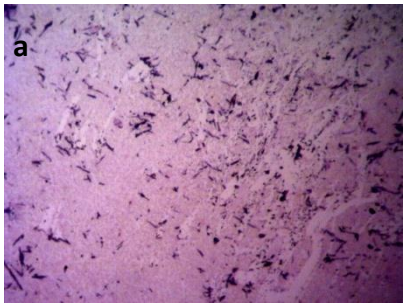

Figure: 7 a. Gram staining

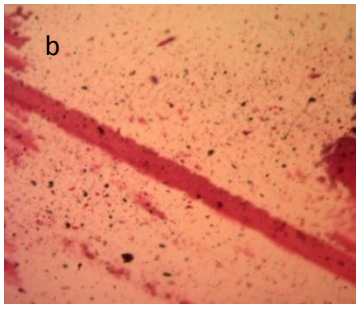

Figure: 8 b. Spore staining

Biochemical characteristics

Bio chemical Test	VIT-JS1	VIT-JS2
1.Catalase test	-ve	-ve
2.Oxidase test	-ve	-ve
3.Citrate utilization test	+ve	+ve
4.Starch hydrolysis test	+ve	+ve
5.Indole test	-ve	-ve
6.Nitrate test	+ve	+ve
7.Lactose test	-ve	-ve
8.Methyl red test	+ve	+ve

Figure 9: Biochemical characteristics of keratinase enzyme: 0ptimization of keratinase enzyme

Effect of pH on keratinase production

Table: 1 Effect of different pH on keratinase enzyme

pH	Enzyme activity
2	0.01
4	0.02
6	0.05
8	0.51
10	0.015

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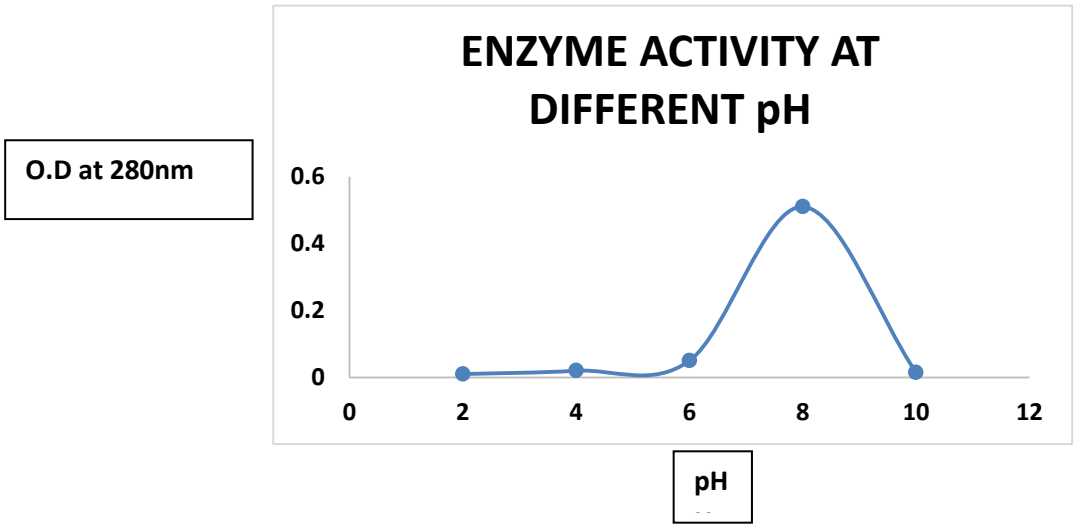

Figure 10: Effect of different temperature on keratinase enzyme

Table: 2 Effect of temperature on keratinase enzyme

Temperature (^{0}C)	Enzyme activity
20	0.04
30	0.07
40	0.09
50	0.12
60	0.06

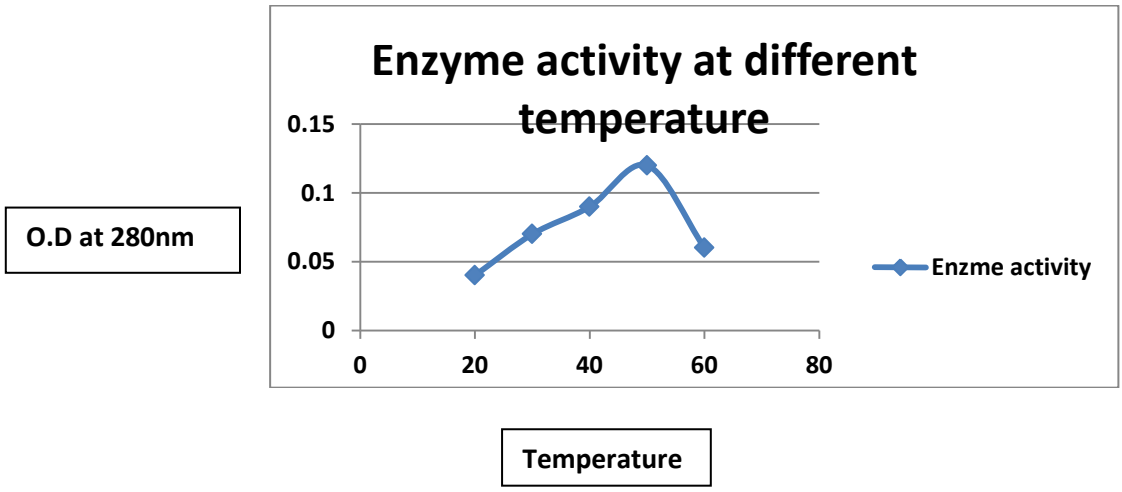

Figure 11: Effect of different temperature on keratinase enzyme

Immobilization of keratinase enzyme

Keratinase beads are formed by using production medium. They are preserved and maintained the enzyme activity.

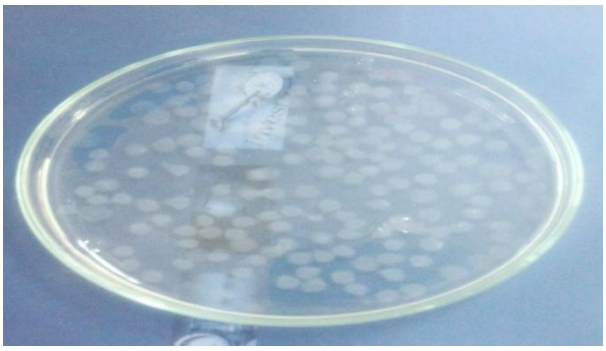

Figure 12: Sodium alginate beads formation of keratinase enzyme

DISCUSSION

The isolated Bacillus sps microorganisms are responsible for the degradation of feather keratin from poultry waste soil. The screening of microorganisms were done by soil samples used by serial dilution followed by baiting method. These microbes degrade keratin to a good extent and can be used as potential keratin degrading organisms. The optimization of keratinase in different pH and temperature shows their enzyme activity for feather keratin degradation yields better results from poultry wastes.

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