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SCREENING AND PRODUCTION OF EXTRA CELLULAR FEATHER DEGRADING ENZYME FROM BACTERIAL ISOLATES

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ABSTRACT

Keratins are most abundant protein in epithelial cells of vertebrates and represent the major constituents of skin and its appendages such as nail, hair, feather and wool. Keratin comprises long polypeptide chains, which are resistant to the non-substrate specific proteases. The durability of keratins is a direct consequence of their complex architecture. Keratin molecules form parallel-intertwined heterodimers consisting of one each of acidic Type I Keratins and basic or neutral Type II keratins. Antiparallel couplets of heterodimers comprise protofilaments, which pair to form protofibrils. Each filament of keratin, in turn, consists of four bundled protofibrils. The protein chains are packed tightly either in alpha chain (-keratin) or beta sheet (-keratin) structure. Keratins belong to the super family of intermediate filament protein. The hydrophobic interactions make keratinous material water insoluble and extremely resistant to degradation by proteolytic enzymes such as trypsin, pepsin and papain. That's why the term Keratinase refers to the group of proteolytic enzymes, which are able to hydrolyze insoluble keratin more efficiently than other proteases are called Keratinases. The Present study is much concerned with the screening of Microbes which have potent keratinolytic activity and to determine the impact of physical parameters (Temperature, pH) on keratinase. The isolate was found to produce 68% of crude extract of enzyme per hundred ml of broth and has 50 U/ml activity at 35^{0}C where as 22 U/ml of activity at pH 8.

KEYWORDS: Keratins, Water insoluble proteins, Keratinase enzyme

The day by day increase in consumption of meat received from chicken is causing harsh effect to environment as the waste from the chicken birds more particularly the feather are not properly treated, while in the nature the deteriorization of feather is slow originating sulphureous composites, causing an environmental problem. To combat with the problem, the existing methods (physical, chemical or dumping in soil/incineration) are not sufficient to deal expediently. So it needs modern well-organized and well-equipped process to save the environment and surrounding from the hazardous substances, which are the outcome of untreated waste. And also to take the benefit from the metabolite produced by the organism for degradation of feather, for various industrially important processes. In contraposition to physical & chemical process, The Biotechnological approaches show significant importance in such waste management, which includes use of Keratinase enzyme. A group of proteolytic enzymes which are able to hydrolyze insoluble keratins more efficiently than other proteases are called keratinases.

Feather represents 5-7% of the total weight of mature chicken, World-wide poultry processing plants produce millions of tons of feathers as a waste product annually, which consists of approximately 90% keratin; the keratin is largely responsible for their high degree of recalcitrance if remain untreated.

The best studied keratinase from the dermatophytic genera microsporum, and Trichophyton as well as bacteria of the genera Bacillus and Streptomyces. Most keratinases have some common characteristics despite of their different origin. They belong mainly to the extracellular serine protease, with the exception of keratinase from yeast, which belong to aspartic proteases.

The molecular masses of the enzymes range from 20 kDa to 60 kDa. They are mostly active in alkaline environments, with optimal activity at temperatures up to 50°C. Thermostable keratinases with optimal temperatures of around 85°C and a higher molecular mass have been reported.

The stability of Keratinases is influenced by physical factors, (temperature, pH) chemical substances (Organic solvents: methanol, ethanol and isopropanol).

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These factors apparently induce the activity of enzyme resulting in modified catalytic activity. Certain substances lead to inhibition of the enzyme, example EDTA. It was verified that EDTA decreases the catalytic activity by 30% when incubated with 5 to 10mM concentration.

However much current studies are concerned on potential use of keratinases of bacterial origin for the industrial treatment of keratin containing compound.

The present study is concerned on the identification and screening of bacterial isolates with keratinolytic activity and its production. As keratinases has following Industrial applications.

Useful in hydrolysis of poultry feather.

Used for de-hairing in leather industry, detergent industries.

In textiles for waste bioconversion.

In medicine for drug delivery through nails.

In cosmetics for degradation of keratinized skin.

MATERIALS AND METHODS

The chemical used during project work is of pure grade, produced from Hi-Media Pvt. Ltds. Mumbai. The glassware used were of Borosil, all the glassware used for practical purpose was first clean with acid and rinsed with double distilled water.

Preparation of Feather Basal Broth

Feather were washed, dried and cut into smaller pieces, and hammer milled prior to use being added to the medium. The medium was sterilized by autoclaving at 121°C (15 lbs) for 15 min.

Isolation of Feather Degrading Microorganism

One gm soil was obtained from Local poultry farm and added to 9 ml of saline water. This initial dilution was activated by heat shock at 70°C for 15 min. This heat treated sample then serially diluted to reduce initial number of microorganism (dilution up to 10^{-9} in saline water prepared in tubes) and were plated on nutrient agar medium and incubated at 35°C for 24 hrs. The appeared colonies were checked for spore formers and streaked on Nutrient Agar slants for further study (Savitha et al., 2007).

Screening on Casein Agar Plates

Casein agar plates were prepared, and the colonies from nutrient agar plates was taken by making suspension and spreaded on casein agar for testing caseinolytic activity of the organism. Bacteria were inoculated on plates and incubated at 37°C for 24 hrs. Colonies showing zone of clearance on the plates were selected. (Zerdani et al., 2004).

Identification of Isolated Feather Degrading Bacteria

The bacterial isolates were studied for cultural, morphological and Biochemical characters. Gram staining, Spore staining, motility test, catalase test, IMViC test and Starch hydrolysis etc. (Williams et al., 1990).

Production and Extraction of Enzyme

Inoculum Development

The selected bacterial colony after its identification and characterization inoculated into the feather basal broth medium. Prepared as described above. And incubated on orbital shaker incubator at 35°C for 7 days.

Production of Enzyme

After seven days of incubation, 10 ml of culture medium was transferred to 1.0 liter medium. The medium was prepared similarly as previously described. All incubations were done at 35°C with shaking at 150 rpm in a controlled environment shaker.

Extraction of Enzyme

A. Filtration

The culture medium was filtered through Whatmann No.1. Filter paper to remove undegraded residues. (Xiang et al .,1992).

B. Centrifugation

The filtrate was then subjected to centrifugation at 10,000 rpm for 10 min to remove bacterial residue. After centrifugation ammonium sulphate was added to the supernatant to achieve 30% saturation, which gives the precipitation of enzyme in suspension, now this Crude enzyme then used for enzyme assay and characterization.

C) Assay for Keratinase Activity

Keratinolytic activity of culture filtrates was measured spectro-photometrically; the test described below was developed in order to simplify analytical work on Keratinase. Azo-keratin hydrolysis provides a colorimetric assay for enzymatic activity on keratin (Shih et al .,1993).

Synthesis and Enzymatic Hydrolysis of Azo-Keratin

Azo-keratin was prepared by a similar method similar to a known procedure for azoalbumin. The use of azoalbumin as a substrate in the colorimetric determination of peptic and tryptic activity. Ball-milled feather powder was prepared. (Shih et al., 1993). 1 g portion of the feather powder (the keratin source) was placed in a 100-ml round-bottomed reaction flask with 20 mL of deionized water. The suspension was mixed with a magnetic stirrer. Two ml of 10% NaHCO (w/v) were mixed into the feather suspension (Lin et al., 1992). In a separate 10-ml tube, 174 mg of sulfanilic acid were dissolved in 5 mL of 0.2 N NaOH. Sixty-nine mg of NaNO were then added to the tube and dissolved. The solution was acidified with 0.4 mL of 5 N HCl, mixed for 2 min and neutralized by adding in 0.4 mL of 5 N NaOH This solution was added to the feather keratin suspension and mixed for 10 min. The reaction mixture was filtered and the insoluble azo-keratin was rinsed thoroughly with deionized water. The azo-keratin was suspended in water and shaken at 50°C. for 2 hr and filtered again. This wash cycle was repeated until the pH of the filtrate reached 6.0-7.0 and the spectrophotometric absorbance of the washing at 450 nm was less than 0.01 (Burtt and Ichida, 1999). Finally, the wash cycles were repeated at least twice using 50 mM potassium phosphate buffer, pH 7.5. The azo-keratin was washed once again with water and dried in vacuum overnight at 50°C. The resulting product is a chromogenic substrate that can be incubated with enzyme solution to produce and release soluble peptide derivatives that cause an increase in light absorbance of the solution (Burtt and Ichida, 1999).

Enzymatic Hydrolysis of Azo-keratin

This procedure tested the keratinolytic activity of keratinase on azo keratin to begin the process, 5 mg of azo-keratin was added to a 1.5-ml centrifuge tube along with 0.8 mL of 50 mM potassium phosphate buffer, pH 7.5. This mixture was agitated until the azo-keratin was completely suspended. A 0.2-ml aliquot of supernatant of crude enzyme was added to the azo-keratin, mixed and incubated for 15 min at 50°C with shaking. The reaction was terminated by adding 0.2 mL of 10% trichloroacetic acid (TCA). The reaction mixture was filtered and analyzed for activity (Burtt and Ichida, 1999).

The absorbance of the filtrate was measured at 450 nm with a UV-160 spectrophotometer (LaboMed.Inc). A control sample was prepared by adding the TCA to a reaction mixture before the addition of enzyme solution A unit of keratinase activity was defined as a 0.01 unit increase in the absorbance at 450 nm as compared to the control after 15 min of reaction (Burtt and Ichida, 1999).

Enzyme Characterization

Enzyme characterization was done by Allpress et al. (2002) method.

Temperature

The effect of temperature on keratinase activity was determined by the addition of 20 µl keratinase (3 mg/ml) to 1.5 ml phosphate buffer (100 mmol 1, pH 7.5) containing 15 mg azo keratin and incubating at a range of temperatures (25, 30, 35, 40, 45 and 50C) for 10 h. Peptide release was determined spectro-photometrically (280 nm; 1 unit of activity (U) the amount of enzyme causing an increase of 1.0 A280 unit within 1 minute).

pH

The effect of temperature on keratinase activity was determined by the addition of 20 µl keratinase (3 mg/ml) to 1.5 ml phosphate buffer (100 mmol) with different pH (5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0) containing 15 mg azo keratin, and incubating at 35°C for 10 h. Peptide release was determined spectrophotometrically (280 nm; 1 unit of activity (U) the amount of enzyme causing an increase of 1.0 A280 unit Within 1 minute).

Feather Degradation by Crude Enzyme Preparation

A 0.1 g of autoclaved native whole feather was inoculated in the test tube containing 10ml of crude enzyme preparations. After incubations for 12 h at 40°C, the test tube was inspected for visible degradation of feather against feather containing distilled water as a blank.

RESULTS AND DISCUSSION

Isolation and Identification of Feather-Degrading Microorganism

It was found that a previously enriched, feather-degrading microbial culture exhibits Keratinolytic activity contained and shows clearing zone when streaked onto the

casein agar plates Cells of the isolate were grown on basal feather agar and transferred at frequent intervals to the basal medium, containing finely chopped feathers. Cells were also grown on keratin containing media and sub cultured at regular intervals. Eventually, after several weeks of repeated selection and sub culturing a pure culture of the bacterium was obtained.

Identification and Characterization of Feather Degrading Isolate

Microscopic observation of the isolate showed a straight rod with endospores. The bacterium grew aerobically, strongly catalase positive, Gram negative and was highly motile. Additional morphological, physiological and biochemical Tests were conducted (Table-1)

Table 1: Morphological and Biochemical Characteristics of Keratinolytic Isolates

Characteristics	Bacterial Isolates
Colony Property	On nutrient agar, Colonies are large circular, smooth round, waxy, slightly yellow to white, mucoid without pigment.
Gram's nature	Negative Rods.
Spores Stain	Ellipsoidal and cylindrical, central sub terminal, swelling the sporangium.
Motility	Motile
Catalase	Positive
Indole Test	Positive
Methyl Red Test	Negative
Voges-Proskauer Test	Positive
Carbohydrate Fermentation	
Lactose	Acid With Gas
Dextrose	Acid With Gas
Sucrose	Acid With Gas
Starch Hydrolysis	Negative
Gelatin Hydrolysis	Positive
Nitrate Reduction	Positive

Collectively, these characteristics indicate that the isolate was of the Genus *Bacillus*

Degradation of Feathers by Isolate

It was observed that aerobic growth by the isolate on feathers, with the Feathers as its primary source of carbon, nitrogen, energy and sulfur, resulted in nearly complete. Degradation of the keratin after 7 to 10 days of incubation at 37°C. Biodegradation was measured as the increase in the absorbance at 450 nm by the Azokeratin hydrolysis enzyme assay.

Characterization of Keratinase

The organism grew well and completely degraded poultry feathers in the medium. This intense feather degrading activity was achieved in room temperature, 35°C and with initial pH adjusted from 7.0 to 8.0. Similar growth curves were observed with in this range of temperature and pH. Maximum enzyme activity and enzyme yield were observed at 35°C in alkaline pH 8.0. In all cases, the pH values and temperatures increased from 6.0 to 9.0 and 25 to 50°C respectively. The results for effect of various temperature and pH in table 1&2 as well as graph 1&2.

Table 2 : Effect of Temperature on Enzyme Activity

S.N.	Temperature	Enzyme Activity U/ml
1	20	18
2	25	30
3	30	40
4	35	50
5	40	35
6	45	30
7	50	20
8	55	18

Graph 1: Effect of Temperature on Enzyme Activity

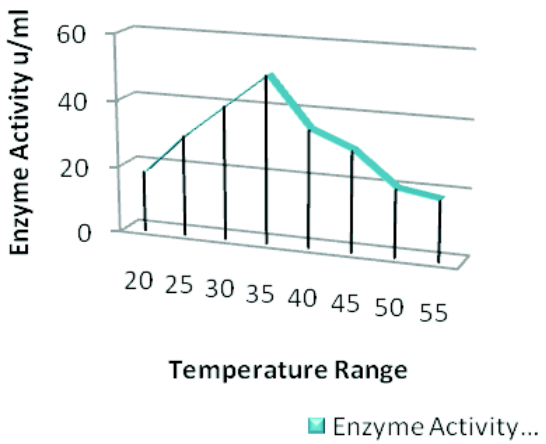
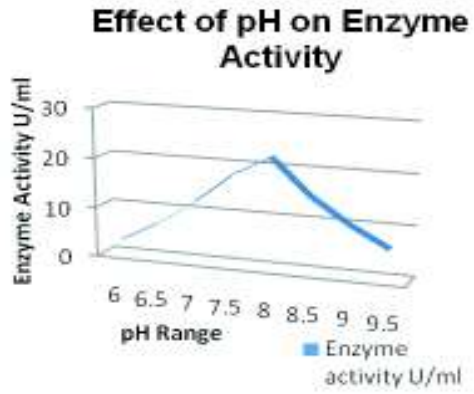


Table 3: Effect of pH on Enzyme Activity

S.N.	pH	Enzyme Activity U/ml
1	6	3
2	6.5	7
3	7	12
4	7.5	18
5	8	22
6	8.5	15
7	9	10
8	9.5	6

Graph 2: Effect of pH on Enzyme Activity



DISCUSSION

Bacteria were isolated from a poultry processing plant that owned keratinolytic activity and ability to degrade keratin wastes. These bacteria present different characteristics, such as a broad temperature range of growth. The optimal proteolytic activities were detected between 30 and 37°C, whereas previously described keratinolytic bacteria mostly have feather-degrading activity at elevated temperatures. An optimum keratin-degrading activity at mesophilic temperatures should be a desirable characteristic because these microorganisms may achieve hydrolysis with reduced energy input. The potential use of keratinases is in different applications where keratins should be hydrolyzed, such as the leather and detergent industries, textiles, waste bioconversion, medicine, and cosmetics for drug delivery through nails and degradation of keratinized skin.

CONCLUSION

A feather-degrading bacterium was isolated from poultry waste. This bacterium was grown in basal media with feathers as its primary source of carbon, nitrogen, sulfur and energy. The organism is rod Shaped, highly motile, endospore forming catalase positive and gram negative. Phenotypic characterization carried out in our laboratory showed that this novel gram-negative bacterium

belongs to Bacillus genus. This novel keratinolytic isolate could be a potential candidate for degradation and utilization of feather keratin. In this study, the optimum conditions for Keratinase, synthesized by bacteria were determined which was primary and essential step for the production of adequate amount such industrially important enzyme.

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