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PRODUCTION OF KERATINASE ENZYME FROM THE CHICKEN FEATHER WASTE OBTAINED FROM THANJAVUR DISTRICT TAMIL NADU

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ABSTRACT: Chicken feathers, consists of 90% keratin (a recalcitrant protein) which cannot be easily hydrolyzed by common proteolytic enzymes. These feathers constitute a sizable waste disposable problem. Hence an eco-friendly alternative to this problem is mandatory. In the present investigation, the feather degrading microorganisms were isolated from feather dumped soil in Thanjavur District. The results were compared in accordance with the Bergey's manual of Determinative Bacteriology. In the present investigation, the degradation of chicken feathers was performed in feather minimal medium (pH 7.5) in which chicken feather acts as the sole carbon and nitrogen sources. *Bacillus subtilis* effectively degraded the chicken feathers within 17 days. The result for FTIR study indicated a pronounced with control Bacillus treated feather samples. The feather keratin inoculated with *Bacillus subtilis* analyzed by biodegradation carried for 20 days. Different spectral wavelength of 3405.47cm^{-1} and the other peaks include 3270.62 and 1452.46 respectively. Within the mid infrared wavelength (400-4000cm^{-1}) indicated bending, vibration of functional group and molecular bond in protein sugar and polysaccharides. The highest activity of keratinase was observed after 48 hours of incubation after cultivation in feather meal for *Bacillus subtilis* (176 U/ml). It was found out that an enhanced keratinase production was observed at pH 7.0, temperature 40oC, carbon sources (Dextrose and fructose) and nitrogen sources (yeast extract). The above results indicated that *Bacillus subtilis* produced appreciable levels of keratinase enzyme using feather as substrate and this could open new opportunities to reduce these recalcitrant feather waste and thereby reducing the pollution.

KEYWORDS: Keratinase enzyme, Chicken feather wastes, Thanjavur and *Bacillus subtilis*

INTRODUCTION:

Every year more than 20,000 tons of feathers are produced as waste poultry farming. Insoluble and hard-to-degrade animal proteins are ubiquitously present throughout animal bodies e.g., nails, horns, hair, wool and feather. Feather wastes are generated in large quantities as a byproduct of commercial poultry processing **[1]**. The feather of poultry was recognized as an important bioresource with an annual production up to several million tons by the chicken industry in the world **[2].** Feather consists of almost 80 - 90 % pure β-keratin. Because animals do not secrete enzymes that can break down disulfide bonds or cross-linkage of keratin, they are unable to digest feather protein **[3].** A total of 5-7% weight of mature chicken comprises of feather. Feathers function as insulation, protection, waterproofing, coloration and flight. Typically, as each bird has up to 125 grams of feather, the weekly worldwide production of feather waste is about 3000 tons **[4].** Discarded feather also causes various human ailment including chlorosis, mycoplasmosis and fowl cholera **[5].**

The feathers which are hydrolyzed by mechanical or chemical treatment can be converted to feed-stuffs, fertilizers, glues and foils. This process in addition to being energy intensive, it also results in the loss of some essential amino acids **[6].** Chicken feather contains approximately 90% keratin, 8% water and 1% lipids. Keratin is rich in cysteine bonds and its hydrophobic side chains make it water proof by mass **[7].** Keratin is a protein with 95% amino acids and has a molecular weight of 10.168 kilo Dalton. Keratins were the most abundant proteins in epithelial cells of vertebrates and represent the major constituents of skin and its appendages such as nail, hair, feather and wool. The protein chains were packed tightly either in α – helix (α-Keratins) or in β –sheet (β-keratins) structures which fold into a final three-dimensional form. The pungent smells of burning hair and rubber are due to the sulfur compounds formed. Extensive disulfide bonding contributes to the insolubility of keratins, accepts in dissociating or reducing agents **[8].**

The advantages of enzymatic dehairing include hair- saving dehairing process, a reduction of sulfide content in the effluent, recovery of hair which is of good quality and elimination of the bate in the de- liming **[9,10,11].** A number of feather degrading species of bacteria, actinomycetes and fungi have been used for the production of keratinase enzyme in submerged as well as in solid state fermentation. Most of keratinase enzyme from microbial sources is extracellular and inducible by keratin waste. These enzymes make it possible for the bacteria to obtain carbon, sulfur and energy for their growth and maintenance from the

degradation of beta keratin **[12].** Therefore, the present work continues in the way of effective degradation of feather keratin using low cost and expense and also to prove the physiological parameters influence the effective degradation of keratin which in turn useful for the environmental application to clean up the world causing pollution. Further the application of keratinase will be carried to study the effectiveness of its application in industry.

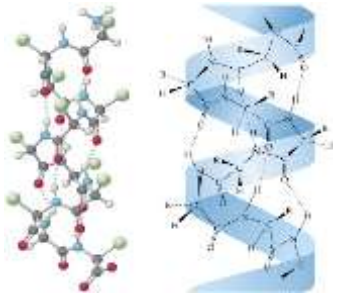

Fig.1: The Structure of keratin

MATERIALS AND METHODS:

Collection of soil sample

The feather dumped soil samples were taken from the dumping sites of Thanjavur District. The soil samples were collected from the superficial layer, at a depth of 3-5 cm. These samples were placed in a sterile plastic bag.

Enrichment for the isolation of keratinolytic bacteria

About 100 g of the collected feather dumped soils were kept in a plastic container along with 2 g of chopped chicken feather (about 3 cm size), homogenously mixed and incubated at room temperature. The samples were kept moistened with water at 2 days interval for 2 weeks. From this sample, about 1 g of partially degraded feather-soil mix was inoculated in a 500 ml Erlenmeyer flask containing 100 ml of peptone-yeast extract medium (g/l): peptone – 10 g, yeast extract – 5 g, NaCl – 5 g, pH – 7) for 24 hours at 37^{o}C in an orbital shaker at 100 rpm.

Isolation of microorganisms from enriched culture

About 1 g of the enriched sample was serially diluted in sterile saline up to 10^{-8} dilutions.

A suitable dilution of this sample was spread plated on the nutrient agar plates. The plates were incubated at 37^{o}C and the number of the colonies was counted after 24 hours of incubation. The appeared colonies were checked for spore formers and streaked on Nutrient agar slants for further study. For long term preservation of the pure cultures, maintained the culture in 20% glycerol stock.

Screening on keratin agar

The isolates were screened for its keratinolytic activity in Feather meal agar at 37^{o}C for 72 hours. The strain which exhibited largest clearing zones were selected, identified and grown in cultivation medium for enzyme production **[5].**

Identification and characterization of isolated organisms

The Morphological characteristics such as abundance of growth, pigmentation, optical characteristics, size and shape were studied on Nutrient agar plates.

Gram's staining:

The isolate was smeared on the glass slide and heat fixed. The crystal violet dye was added, kept for 1 min and washed in running tap water. Then the gram's iodine was added, kept for 1 min and washed in running tap water. It was decolorized with ethanol and then counter stained with safranine and washed in running tap water. It was observed in compound microscope. The bacteria that retained the crystal violet strain (appear violet) was designated as gram positive and those cells that stained with pink colour are called gram negative.

Spore staining (schaeffer- fulton method):

The isolate was smeared in the slide and heat fixed. Then the slide was flooded with malachite green and steam heat the slide for 2-3 min. Cool the slide and washed in running tap water. It was then added with counter stain safranin and kept for 30 sec. Wash the slide with running tap water. Air dried the slide and examined under oil immersion, in microscope. The spores appeared green in colour while the vegetative cells appeared red in colour.

Hanging drop method

One loop full of inoculum was kept at the center of the cover slip. The cavity of slide was placed over the coverslip and turned over to prepare a hanging drop. It was viewed under microscope. The motility was determined from the swarming movement of the microorganisms.

Bio chemical characterization indole production test

Indole production test is used to test whether the organism can have the ability to produce indole. Peptone broth was prepared, sterilized and cooled. Inoculate the test organism to the sterile peptone broth and incubate the tubes at 37^{0}C for 24 hrs. The culture producing the

cherry red colour ring following the addition of kovac's reagent indicated as positive. The absent of red colouration indicate a negative result.

Methyl red test

Methyl red test is employed to detect the ability of microorganisms to oxidize glucose with the production of high concentration of acid end products. The isolated 24 organisms were inoculated into test tubes containing sterile MR-VP broth and incubate the tubes for 24 to 48 hrs at 37^0C. After incubation, add 7-8 drops of methyl red indicator and appearance of red color indicated the positive result.

Voges-Proskauer test

This test is also known as the acetoin production test. This test is used to differentiate the capacity of organisms to produce some nonacidic (or) neutral end product such as acetyl methyl carbinol (or) 2, 3, -butanediol. The isolated organisms were inoculated into sterile MR-VP broth tubes and incubate for 24 hrs at 37^oC. Development of deep rose color following the addition of Barritt's reagent A and B indicated the positive result. The absence of deep rose color is a negative result.

Citrate utilization test

Some of the organisms were capable of utilizing citrate as the sole carbon source and mono ammonium phosphate at the sole source of nitrogen. As a result, the pH of the medium change, this was indicated by changes in the indicator present in the medium. Simmon's citrate medium was prepared, sterilized, and kept in a slanting position and allowed the tubes to solidify. The test organism was streaked on the slant and incubated at 37^0C for 24hrs. The change of color from green to Prussian blue coloured slant indicated the positive result.

Triple sugar iron test

TSI test is used to differentiate the isolate according to the ability to ferment lactose, sucrose and glucose and production of hydrogen sulfide. Triple sugar iron agar medium was prepared and sterilized. Kept the tubes as slant and butt and allow it to solidify. Streak a loop full of test organisms on the surface of the slant and incubate at 37^0C for 24 hrs. Acidification of the medium caused by the isolates attacking one of the sugars causes the phenol red indicator to change to yellow colour. Gas production is indicated by bubble formation in the butt. Hydrogen sulphide production causes the formation of a black precipitate at the junction between the slope and the butt**.**

Nitrate reduction test

This test is used to detect whether the organism reduced the nitrates to nitrites or not. Nitrate

broth was prepared and sterilized. Inoculate one loop full of test culture and incubate at 37^0C for 98hrs. Following incubation, add 0.1 ml of test reagent (sulphanilic acid and α-napthalamine) to the test culture. A red colour developing within a few minutes indicated the presence of nitrites and hence the ability of the organisms to reduce nitrates.

Gelatin hydrolysis

Gelatin is an incomplete protein lacking the essential amino acids tryptophan and it acts as nutrient sources for many microorganisms. When gelatin is enzymatically hydrolyzed into amino acids, it loses its stability to become gel even at low temperature. Prepare nutrient gelatin tubes and sterilized. Inoculate the test organisms on to the tubes and incubate at 37^0C for 24 – 48 hrs. Following incubation, the tubes are placed in a refrigerator at 4^0C for 30 min. Cultures those remain liquefied after 30 min showed positive result and that remained solid showed negative result.

Starch hydrolysis

This test is to detect the ability of microorganisms to hydrolyze the starch by means of the extracellular enzyme like amylase. Starch agar medium was sterilized and make a single line of streak of the organisms across the center of the starch agar plate. Incubate the plates at 37^0 C for 24 hrs for sufficient growth. After incubation, flood the plate with iodine solution. Hydrolysis is indicated by clear zones around the growth which indicated the positive result**.**

Biodegradation studies (in synthetic medium)

Feather degradation by the selected Bacillus strains was carried out in a 250 ml Erlenmeyer flask containing 100 ml of basal feather medium with 1 g of chicken feathers. Bacterial culture (5% inoculum) grown on nutrient broth at 37^oC, 150 rpm for 24 hours was used as inoculum. After inoculation, the flasks were then kept at 37^oC at 150 rpm for 20 days. The degraded feather residue was air dried and the degraded end products were analyzed by Fourier transform infrared spectroscopy (FTIR).

Fourier transform infrared spectroscopy

(FTIR) Fourier Transform Infrared Spectroscopy (FTIR) is a powerful tool for identifying types of chemical bonds in molecules by producing an infrared absorption spectrum that is like a molecular "fingerprint". FTIR can be used to identify chemicals from spills, paints, polymers, coatings, drugs and contaminants. FTIR is perhaps the most powerful tool for identifying types of chemical bonds (functional groups). The wavelength of light absorbed is characteristic of the chemical bond as can be seen in the annotated spectrum. FTIR can be used for some quantitative analysis. Usually these are rather simple types of tests in the

concentration range of a few ppm upto the percent level.

Sample preparation for FTIR

Chicken feather sample degraded by *Bacillus sp* was collected after 20 days of incubation. The chicken feather residue was air dried and used for FTIR analysis. Sample for FTIR can be prepared in a number of ways. For liquid samples, the easiest is to place one drop of sample between two plates of sodium chloride (salt). Salt is transparent to infrared light. The drop forms a thin film between the plates. Solid chicken feather samples were milled with potassium bromide (KBr) to form a very fine powder. This powder is then compressed into a thin pellet which can be analyzed. KBr is also transparent in the IR. Alternatively, solid samples can be dissolved in a solvent such as methylene chloride and the solution placed onto a single salt plate. The solvent was then evaporated off, leaving a thin film of the chicken feather residue on the plate. Solutions can also be analyzed in a liquid cell. This is a small container made from NaCl (or other IR transparent material) which can be filled with liquid, such as the extract for EPA 418.1 analysis. These create a longer path length for the sample, which leads to increased sensitivity. Sampling methods include making a mull of a powder with hydrocarbon oil or pyrolyzing insoluble polymers and using the distilled pyrolyzatee to cast a film. Materials can be placed in a attenuated total reflectance (ATR) cell and gases in cells.

Assay for keratinase activity

Azo-keratin hydrolysis provides a calorimetric assay for enzymatic activity on keratin. The inoculum was incubated in 0.9% NaCl at 37^0C for 24 hours before inoculating into the basal feather broth for the crude enzyme assay **[13].** A 5%inoculum was inoculated in 100 ml of basal feather medium at 37^oC and 120 rpm.

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. **[14].** About 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% NaHCO3 (w/v) were mixed into the feather suspension **[16].** 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 NaNO2were 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 0.4mLof 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 hours and filtered again. This 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 **[16].** Finally, the wash cycles were repeated at least twice using 50mM 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 **[16].**

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. A0.2-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 **[16].** The absorbance of the filtrate was measured at 450 nm with a UV-vis spectrophotometer. 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 **[16].** The enzyme activity was calculated by the following equation: $U = 4 \times \eta \times A280 / (0.01 \times T)$ Where, η – dilution rate, 4 – final reaction volume (ml), T – Time of incubation in minutes.

Medium optimization for keratinase production

Effect of various carbon sources on keratinase production feather minimal medium was prepared with different carbon sources of 1% strength (dextrose, fructose, sucrose, lactose, starch and mannitol). 0.5% inoculations from 24h nutrient broth culture was inoculated in 100ml of basal feather medium and kept in shaker with 120rpm at 37ºC for 24hrs. Effect of various nitrogen sources on keratinase production Feather minimal medium was prepared with different carbon sources of 1% strength (sodium nitrate, yeast extract, gelatin, peptone and casein). 0.5% inoculums from 24 hrs culture of the organisms was inoculated in 100ml of basal feather medium and kept in shaker with 120rpm at 37ºC for 24hrs. Effect of various pH on keratinase production feather minimal medium was prepared in different pH (5, 6, 7, 8, 9, 10). 0.5% inoculations from 24h nutrient broth culture was inoculated in 100ml of basal

feather medium and kept in shaker with 120rpm at 37ºC for 24hrs. Effect of various temperature on keratinase production feather minimal medium was prepared and 0.5% inoculums from 24hrs nutrient broth culture was inoculated in 100ml of basal feather medium subjected to 29 various temperature (30, 40, 50, 60, 70, 80ºC) and kept in shaker with 120rpm for 24hrs.

Quantitative estimation of protein

The quantitative estimation of protein was determined by the method of **[17]**, using bovine serum albumin as the standard.

Procedure for standard (BSA) preparation

Different concentrations of Standard (BSA) are prepared by mixing stock BSA solution (1mg/ml) by adding water in the test tube. The final volume in each of the test tubes is 5 ml. the BSA range is 0.05 to 1 mg/ml. From these different dilutions, pipette out 0.2 ml of protein solution to different test tubes and add 2 ml of Alkaline Copper Reagent. Mix the solutions well. The test tubes were incubated at room temperature for 10 min. Then add 0.2 ml of Folinciocalteau reagent to each tube. The test tubes were again incubated at room temperature (in dark) for 30 minutes. The absorbance was taken at 660 nm immediately. Plot the absorbance against protein concentration to get a standard calibration curve. Protocol for protein estimation of whole cell extract About 1ml sample of whole cell extract was taken and 0.2 ml of alkaline copper reagent was added. It was incubated at room temperature for 10 minutes. To that, 500µl of diluted Folin's reagent (1:1 with distilled water) was added. The test 30 tubes were incubated at room temperature for 30 minutes. Sample was read at the absorbance of 660 nm (immediately).

$$\text{Protein (mg / ml)} = \frac{\text{O.D of the sample X Concentration of Standard X O.D of Standard}}{\text{ml of Sample used}}$$

Goat hides dehairing assays

Freshly salted goat hide was cut into pieces of approximately 2.5×2.5 cm and washed with distilled water repeatedly (400%v/w of salted hide) with 0.3% calcium carbonate. The soaked hide pieces were incubated with enzyme (1%w/w of hide) in tap water having pH 7.0 at room temperature(37ºC) along with control i.e., Hide pieces were incubated without enzyme, under the same conditions. After 12h, pieces were dehaired by mechanical means and visually analyzed for color **[18,19].**

RESULTS:

Isolation of feather degrading bacteria

The feathers which were incubated with feather decomposed soil effluents in the laboratory for two weeks were found to be partially degraded. About 10 grams of the samples was subsequently enriched with 2 g of chopped chicken feather in the peptone yeast extract medium for the isolation of bacteria. The diluted culture of this enriched sample was transferred on to nutrient agar plate for the isolation of single colonies. The obtained colonies with different morphological characteristics were re-streaked onto the nutrient agar plate to obtain pure culture of these isolates. Two dominant colonies were selected and the obtained isolates were designated as K1 and K2.

Screening on feather minimal medium

Feather minimal medium with keratin act as a sole carbon source was used for screening the keratinolytic activity of the isolate. Due to the production of keratinase enzyme, the isolates produced a remarkable zone of clearance (22 mm and 8 mm) in the feather minimal medium (Table.1).

Morphological and Biochemical characterization

The strain K1 was a gram positive, short rod-shaped spore forming organism. Further biochemical tests were conducted to ascertain the genus of the bacteria. The results were depicted in (Table.2). Indole was not produced by the organism. Glucose was not oxidized to produce acid end products in methyl red test. Acetoin and butane diol were not produced in vogesproskauer test. Citrate was utilized as its sole carbon source. Nitrates were reduced to nitrites. The organism utilized starch and gelatin. The strain fermented sugars like dextrose, Mannitol, fructose and sucrose. From the obtained results, K1 was confirmed as *Bacillus subtilis* and Colony appeared as, on nutrient agar plate the isolate K1 produced whitish to brown, irregular, dull with rough surface, undulate colonies.

Degradation of chicken feather by *Bacillus subtilis* in synthetic medium

Bacillus subtilis was able to grow and produce keratinase in feather minimal medium in which chicken feathers served as an additional carbon and nitrogen source and acted as an enzyme inducer, resulted in complete degradation within a short period of 18 days.

FTIR

The result of the FTIR studies indicated a pronounced difference between control and the experimental treated feather samples. In control keratin exhibited the peak value of 1240.23cm^{-1} followed by similar peak of 416.23, 663.51cm^{-1}, 451.34cm^{-1} respectively. The above IR absorbance is

a finger print region that is it hails. Within cm^{-1} the mid IR (400- 4000cm^{-1}) which is due to stretching, bending, vibration of functional group molecular bonds in protein, sugar, lipopolysacharides. Another prominant peak of 1240.23cm^{-1} indicates a mixture region on fatty acids, bending, vibration, keratin protein and phosphate carrying compound. Fourier transform infrared spectroscopy analysis conserved with biodegradation of keratin from feather waste using *Bacillus* subtilis was studied. The result for FTIR study indicated a pronounced with control Bacillus treated feather samples. The feather keratin inoculated with *Bacillus subtilis* analyzed by biodegradation carried for 20 days. Different spectral wavelength of 3405.47cm^{-1} and the other peaks include 3270.62 and 1452.46 respectively. Within the mid infrared wavelength (400-4000cm^{-1}) indicated bending, vibration of functional group and molecular bond in protein sugar and polysaccharides**.**

Keratinase enzyme assay

The enzyme assay was performed by using azokeratin as substrate. Azokeratin was synthesized by the method of **[16].** The keratinolytic activity of selected bacteria *Bacillus subtilis* was monitored during growth in feather minimal medium after 24, 48 and 72 hours. The strain grown well in basal medium with 1% chicken feathers as a sole source of carbon, nitrogen and sulphur at 37^{o}C with pH 7.5. *Bacillus subtilis* species showed good growth and protein synthesis with a varying level of keratinolytic activity (Table.3) on feather meal. The high keratinase activity was demonstrated after 48 hours of incubation. After 72 hours, the keratinolytic activity was strongly decreased. The protein content decreased with decrease in keratinase activity**.**

Effect of carbon sources on keratinase activity

The supplementation of additional carbon sources to the fermentation medium may influence the production of enzyme. (Table. 4) revealed that the effect of carbon sources on keratinase production after 48 hours of incubation period at 37°C. Among the different carbon sources tested, the maximum Keratinase production was recorded in dextrose(140U/ml) supplemented medium and minimum Keratinase production was recorded with Starch as carbon source (88U/ml) for *Bacillus subtilis***.**

Effect of nitrogen sources on keratinase activity

The effect of different kinds of organic nitrogen sources on Keratinase production was studied after 48 hours of incubation period at 37°C. The maximum amount of Keratinase production was observed in yeast extract (114 U/ml) supplemented medium and minimum enzyme activity was observed in casein (100U/ml). Yeast extract was found to be the desirable nitrogen source for enhancing the production of keratinase enzyme. It is noteworthy

to observe that, with the exception of dextrose and yeast extract, the extra additional carbon and nitrogen sources to the fermentation medium led to the remarkable reduction in feather solubilization. The results were presented in Table.5

Effect of pH on keratinase activity

The growth and keratinolytic enzyme synthesis were greatly influenced by initial medium pH. In the present investigation, the enzyme stability was studied at 34 various pH ranging from 5-10 after 48 hours of incubation period at 37°C was depicted in Table. 7. The results exhibited a gradual increase in the activity with increasing pH upto the optimum followed by the gradual fall in the activity. The activity was decreasing under acidic conditions and stable at basic pH. The maximum Keratinase production was observed at pH 7.0 (120 U/ml) (Table.6)

Effect of temperature on keratinase activity

Temperature is an important environmental factor affecting the growth and production of metabolites by microorganisms. The effect of temperature on keratinase activity was recorded from 30-80oC. The maximum Keratinase production was obtained at 40°C (122U/ml). The influence of temperature on the enzyme activity and stability was presented in Table.7. With the increase in temperature, the enzyme was inactivated considerably.

Application of keratinase enzyme in dehairing process

Temperature plays an important role in the keratinase production and feather solubilization. In the present study, the keratinolytic activity was detectable between 30 and 80^{o}C. The maximum activity was obtained at temperature 40^{o}C for *Bacillus subtilis* with 122U/ml. [**19**] studied the keratinolytic abilities of *Bacillus polymyxa* and *Bacillus cereus* in feather minimal medium. They found out that 50^{o}C and 45^{o}C were the optimum temperature for *B. polymyxa* and *B. cereus.*

DISCUSSION:

The *Bacillus subtilis* effectively remove the goat skin hair with in 24hrs. This strain is selectively degrading the goat hair only without damaging other layers of the goat skin. It was similar to that the enzymatic dehairing process has been gaining importance as an alternative chemical methodology in present day scenario as this process is significant in the reduction of toxicity in addition to improvement of leather quality **[20].** However, most of the proteases reported so far were unsuitable for dehairing purpose because of the associated collagen-degrading activity **[21]**. They selectively degrade the soft keratin tissue in the follicle, thereby pulling out intact hairs without affecting tensile strength of the leather [**18**]. Keratinase similarly from *B. cereus* exhibited a remarkable dehairing capabilities was reported by **[21].**

The ability of *Bacillus subtilis* to grow and produce appreciable levels of keratinase using feather as substrate could open new opportunities for the achievement of efficient biodegradation of keratin containing wastes and thereby help to reduce the environmental impact of such bio waste.

Table 1: Screening for keratinolytic activity of K1 and K2

S. No	Isolates	Keratinolytic Activity (in mm)
1	K1	22
2	K2	08

Table 2: Morphological, physiological and biochemical characteristics of K1

	Characterization of Bacteria	Observation
Cultural characteristics	**Colony Morphology**	Creamy white irregular undulate colonies
Microscopic characters	**Gram staining**	Positive rods
	Spore staining	Present
	Motility	Motile
	Indole Production	Negative
	Methyl red	Negative
	VogesProskauer	Negative
	Citrate utilization	Negative

Biochemical Characteristics	**Triple sugar iron agar**	Alkaline slant
	Nitrate Reduction test	Positive
	Gelatin Hydrolysis	Positive
	Starch hydrolysis	Positive

Table 3: Keratinase production by different bacterial species on feather minimal medium

Bacteria	**Growth time (In hours)**					
	25		**48**		**72**	
	Protein content (mg/ml)	**Keratinolytic activity (U/ml)**	**Protein content (mg/ml)**	**Keratinolytic activity (U/ml)**	**Protein content (mg/ml)**	**Keratinolytic activity (U/ml)**
Bacillus subtilis	**0.72**	**88**	**0.96**	**176**	**0.79**	**76**

Table 4: Effect of various carbon sources on keratinase Production

Carbon sources	**Keratinase activity (U/ml)**
Fructose	140
Lactose	102
Sucrose	98

Mannitol	112
Starch	88
Dextrose	**140**

Table 5: Effect of various Nitrogen sources on keratinase Production

Nitrogen sources	**Keratinase activity (U/ml)**
Casein	100
Yeast extract	**114**
Peptone	108
Gelatin	104
Sodium nitrate	106

Table 6: Effect of Various pH on Keratinase Production

pH	**Keratinase activity (U/ml)**
5	116
6	114

7	**120**
8	118
9	112
10	96

Table 7: Effect of Various Temperature on Keratinase Production

Temperature(^{0}C)	**Keratinase activity (U/ml)**
30	120
40	122
50	120
60	116
70	114
80	112

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