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RESEARCH ARTICLE

ISOLATION AND SCREENING OF KERATINSE PRODUCING BACTERIA FROM CHICKEN FARM BED

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ABSTRACT

Currently, there are about 195 nations in the world, with a population of 7.8 billion. This much higher the population directly influences higher food demand. Due to this interlink massive amount of food waste has been collected from the meat industries; especially poultry plays a major role by discharging feathers into dumps. There are about 8.5 billion tons of feathers that are wasted every year which lead to high global environmental risk and cause different human disease. This high risk is due to typical structure of feather. They have a matrix protein called keratin that makes degradation more tedious under normal condition; they are insoluble in nature with high rigidity due to the disulfide bridges. By extraction of keratin from these waste feathers are used for multipurpose such as cosmetics, biomedical, textile use, bio-fertilizers etc. In addition, as per trend by utilizing nature's gift the microorganisms we can breakdown these matrix proteins easily. By this environmental friendly technique our works gets easier and are also cost effective. By consumption of keratin as substrate some microorganisms produce an extracellular enzyme called keratinase which has the ability to cleave the protein. The produced keratinase is a wealth out of waste which has wide biochemical properties and also used for waste water treatments. On whole the feather considers as waste is completely turned to verity of useful products ubiquitous like biodiesel, biofertilizer, biodegradable plastics, biofilms, skin, hair and other biomedical treatments.

Keywords: Poultry waste, chicken feather bioconversion, keratinase, keratin, microbial enzymes, *Bacillus subtilis* strain KBT.

1. INTRODUCTION

The world is facing the consequences of environmental crisis due to enormous pollutions mainly due to land fill dumping with non-degradable wastes. The products like feathers, hair, horn, skin and nail are trashes that are huge in volume containing over 90% of protein which are tedious to degrade. Predominantly due to poor management of chicken feather which is an organic waste that accumulated in bulk quantities as a by-product in poultry industry has become one of the major pollutants due to its refractory nature. In general, each bird has up to 125 gram of feather that represent 5-7% of the total weight of mature chickens [1]. Meanwhile, more than 400 million chickens being processed every week worldwide [2]. Hence, the accumulation of feather waste reaches five million tons [3]. Most feather waste is land filled or burned that cause global environmental issue such as pollution of both air and underground water resources and protein discharge [4, 1].

Chicken feathers contain more than 85% of crude protein, 70% of amino acids, high-value elements, vitamins and growth factors [5]. Researchers have shown great interest in applying these materials to various products such as feed [6], fertilizer [7] and biofilm [8] etc., chicken feathers have high mechanical stability and are not easily hydrolyzed by common proteolytic enzymes. The refractory nature is due the presence of a protein called keratin which is present in it.

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Keratin is an insoluble protein and is resistant to degradation by common peptidases such as trypsin, pepsin, and papain this resistance is due to the constituent amino acid composition and configuration that provide structural rigidity [9, 10]. The mechanical stability of keratin and its resistance to biochemical degradation depend on the tightly packed protein chains in α-helix (α-keratin) and β-sheet (β-keratin) structures. In addition, these structures are cross-linking by disulfide bridges in cysteine's residues also has hydrogen bonding, and hydrophobic interaction [11, 9, 12]. Chicken feathers are degraded mainly by physical methods (pressurized hydrolysis, and puffing) and chemical methods (acid and alkali) [13, 14, 15]. However, these methods have limitations such as high energy consumption during the production process and substantial of damage to the products [16]. In recent years, biotechnological methods have been used to degrade keratin. Microbial processes are not only environmentally friendly [17] but also maintain the original structure and activity of the products [18]. Currently, studies on biodegradation are focused on the screening and identification of microorganisms that can degrade feathers [19, 20, 21].

Hence, this current study was undertaken for screening, isolation and identification of keratinase producing bacteria from poultry dumped soil for feather degradation and production of value added products using chicken feather waste.

2. MATERIALS AND METHODS

2.1. Feather meal powder preparation

Chicken feathers were collected from chicken stall and processing plant and cleansed with tap water. The main purpose is to clean the feathers from stains, oil and grease before processing it. The feathers were then washed with teepol or soap water and dried under sunlight. The dried feathers are then blended and kept carefully in an autoclave cover and sealed.

2.2. Extraction of keratin crude

2.2.1. Dissolving of Chicken Feathers

Two liters of 0.5 M sodium sulfide solution is prepared in a 2 L conical flask. Fifty grams of blended chicken feathers were weighed and added to the sodium sulfide solution. The pH was maintained about 10-13 and incubated in the shaker at 30 °C for 6 hours. The solution is then filtered and centrifuged at 10,000 rpm for 5 min. The liquid supernatant was carefully collected then filtered using filter paper to make it particle free.

2.2.2. Preparation of Ammonium Sulfate Solution

Seven hundred grams of ammonium sulfate was dissolved in 1 L deionized water. The solution was stirred in cold condition until all the ammonium sulfate particles were dissolved. The solution was then filtered to make it particle free.

2.2.3. Protein Precipitation

The feather filtrate solution collected earlier was placed in a beaker and stirred. Ammonium sulfate solution was added slowly in drop wise. The ratio of feather filtrate solution and ammonium sulfate solution added was 1:1. The solution was then centrifuged at 10,000 rpm for 5 min and the solids particles were carefully collected. The liquid supernatant was collected separately.

2.2.4. Protein Purification

Collected solid particles were added into 100 mL deionized water and stirred (washing). The solution was centrifuged at 10,000 rpm for 5 min and the solids were gathered carefully. The collected solid particles were then dissolved in 100 mL of 2 M sodium hydroxide solution. The solution was then centrifuged again at 10,000 rpm for 5 min and all the liquids were collected carefully and stored while the solids were discarded. The precipitating, washing and dissolving steps were repeated 3 times.

2.2.5. Biuret test

One percentage copper sulphate solution and 1 % potassium hydroxide solution was prepared. Five ml of the sample was mixed with 5ml of potassium hydroxide solution in 1:1 ratio. Two to three drops of copper sulphate solution was added then colour changes in the solution occur from blue colour to violet confirms the presence of protein. The precipitating, washing and dissolving steps are repeated 3 times.

2.2.6. Isolation of keratinase producing bacteria

The soil sample was collected from feather waste dumping site of NGGO colony at Coimbatore in sterilized sampling bags. The samples were brought to the laboratory and processed for analysis on a subsequent day. One grams of soil sample suspended into the 10 ml sterile saline contained in a test tube. The saline sample was serially diluted to 10^{-1} to 10^{-9} fold after the tests were labeled based on dilution number. These suspensions were reinoculated in keratin agar media by spread plate technique. 100 µL of serially diluted samples from 10^{-5}, 10^{-6} and 10^{-8} dilution were taken to make spread plate using keratin agar medium. Inoculated keratin agar plates were labeled as 10^{-5}, 10^{-6} and 10^{-8} respectively then incubated for growth at 37 °C for 48 hours. After incubation colonies which were gowned on the keratin agar plates were sub cultured and screened for keratinolytic activity.

2.2.7. Screening of keratin producing bacteria

The selected isolates were screened for keratinolytic activity, this was done by inoculating the colonies on the screening medium (Keratin solution: 10 ml/L, NaCl: 0.5 g/L, KH_2PO_4: 0.7 g/L, K_2HPO_4: 1.4 g/L, $MgSO_4.7H_2O$: 0.1 g/L, Agar: 15 g/L, pH: 7.5). Screening medium was prepared; isolates were plated and incubated at 37 °C for a week. After incubation clear zone around the colonies was confirmed by addition of 10 % of TCA (Trichloro acetic acid) over the agar plate. Clear zone forming colonies were isolated and pure culture was maintained for further studies.

2.3. Morphological identification of keratinolytic bacteria

2.3.1. Gram staining

Bacterial smears of 16-18 h old were made on clean grease free slides, heat fixed and stained as follows. The slide was flooded with crystal violet for a minute, drained and rinsed with water; followed by grams iodine for one minute, drained and rinsed with water. Decolorized with acetone alcohol for 30 seconds and later counterstained with safranin for one minute and observed under an oil immersion microscope. Violet or purple cells were identified as Gram positive and pink ones as Gram negative.

2.4. Molecular identification of keratinolytic bacteria

2.4.1. Genomic DNA Isolation

Culture isolate of 1.5 mL transferred to microfuge tube and centrifuged at 13000rpm for 2 min. supernatant was discarded and the pellet was suspended with 400µl of STE buffer and vortexd well followed by 50 µL of 10 %SDS was added and mixed gently by inversion and incubated in water bath for 55 ºC for 15min. Further 50 µL of tris saturated phenol: chloroform: isoamyl alcohol was added and vortexd well then centrifuged at 14000 rpm for 10min at 4 ºC. Aqueous phase was carefully transferred to another 1.5 mL centrifuge tube with this 2 volume of ice cold 100 % ethanol was added mixed well and incubated at -20 ºc for 30 min and then centrifuged at 12000 rpm for 15 min. Supernatant was discorded and the dry pellet was dissolved in 50 µL of TE buffer. Isolated genomic DNA was stored at -20 ºC.

2.4.2. PCR studies

Isolated DNA was subjected into 16S rRNA gene amplification using universal bacterial primer such as 8(F) 5′-GGTTACCTTGTTACGACH-3′ and UI492R(R) 5′- AGAGTTTGATCCTGGCTCAG -3′. Amplification reaction was carried out by preparing 20µl reaction mixture consisting of : 10 µL of ready master mix (containing the DNTPs, Taq DNA polymerase, MgCl2 and the reaction buffer); 1 µL template genomic DNA, 1 µL each of forward and reverse primers and 7 µL of nuclease free water. Entire reaction was carried out in Bio- Rad thermal cycler and the reaction parameters comprised of one cycle of initial denaturation at 94 °C for 5 min followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 52°C for 1min and extension at 72 °C for 1.5 min and the final extension was at 72 °C for 10 min. PCR was performed in a thermocycler (Eppendorf, Germany); a Doc Print transilluminator (Vilber Lourmat, France) was used for visualization and documentation. Amplified product was subjected to electrophoresis on a 1.5 % agarose gel and was found to be 1.5 kb in size.

2.4.3. Enzyme production

Bacterial culture of 2.5 mL was inoculated in keratinase enzyme production medium (Media

preparation (g/l): Keratin solution: 10 mL, NH_4Cl: 1 g, NaCl: 1 g, K_2HPO_4: 0.6 g, KH_2PO_4: 0.8 g, $MgCl_2.6H_2O$: 0.48 g, Yeast extract: 0.2 g, pH: 7.5) and incubated at 37 ºC for a week it was kept in the shaker. After a week of incubation culture was centrifuged at 5000 rpm for 10 minutes. Finally supernatant of crude enzyme was collected and stores in cold room.

2.5. Enzyme purification method

2.5.1. Ammonium sulphate precipitation

The crude keratinase enzyme supernatant was partially purified by 70 % saturation with solid ammonium sulfate (47.2g / 100mL). Then the mixture was allowed to stand for 30 min to 1 h. The precipitate was removed by centrifugation at 12000 rpm for 20 min at 4 °C and the pellet obtained was dissolved in 10mM Tris Hcl buffer with pH 7.5. The precipitated enzyme with 70 % saturated ammonium sulfate was assayed for protein and keratinase concentration as before. Protein concentration (mg/mL) of the enzyme was measured as absorbance at 280 nm using UV spectrophotometer. The precipitated enzyme was dissolved in 10 mM Tris-HCL buffer pH 7.5.

2.5.2. Partial purification: Dialyzing the protein sample

The enzyme sample was dialyzed in the membrane by tying one end with a thread and the samples were filled in through the other end. The tubing was checked for leakage. The dialysis bag containing sample was placed in the beaker containing 800 mL of 1mM Tris-HCL buffer pH 7.5 by frequently changing the buffer overnight at 4 °C on a magnetic stirrer. It was then kept on magnetic stirrer in cold room for overnight. It was set in such a way that the dialysis bag slowly floats around the top of the solution. The dialysis buffer was replaced with fresh buffer every 2 hours. After dialysis, the volume was measured and the samples were analyzed for the keratinase and protein concentration as before. Protein and keratinase activity was determined in all these fractions. The proteins were collected in a centrifuge tube and stored at 4 ºC.

3. RESULTS

3.1. Feather meal production

The finely powered feather meal was stored in a sterile autoclave cover and stored for further analysis (Fig 1)

Fig 1. Feather meal from collected chicken feather waste

3.2. Keratin production

The crude keratin was produced from feather meal. Further protein conformation was done by performing Biurets test change in color from blue to violet confirms the presence of keratin protein. Further extracted protein was quantified as 0.65 mg/mL.

3.3. Isolation and Screening of Keratinase producing bacteria

The keratinase producing bacteria were isolated by clear zone formation around the colony which confirms that isolate has keratinolytic activity (Fig 2).

Fig. 2. Screening of keratinolytic bacteria

3.4. Morphological identification of isolate

The Grams staining seems to identify that the isolate is a Gram positive, rod shaped, motile bacteria.

3.5. Molecular Identification of Keratinase producing bacteria

Genomic DNA of the keratinase producing isolate was isolated. The quality and quantity of DNA samples were determined by electrophoresing the extracted DNA on 1% agarose gel (Fig. 3). A single band of high molecular weight were obtained on agarose gel electrophoresis showing that genomic DNA was intact and free from any mechanical or chemical shearing and therefore it was pure enough for subsequent PCR amplification. Isolated genomic DNA was amplified by 16S rRNA Universal primer in PCR and the amplified product was has 1500 bp (Fig 4).

The PCR products after their cleaning-up were sequenced by Sangar sequencing the obtained sequences were subjected to Bioedit. Total of 475bp partial 16S rRNA sequence was retrieved in FASTA format and subjected for BLAST search in GenBank. BLAST result showed that the query sequence was similar to *Bacillus subtilis* with 99.65% similarity and E value 0.0. Hence the isolated bacteria were identified as *Bacillus subtilis* strain KBT.

Fig. 3. Isolated Genomic DNA on agarose gel

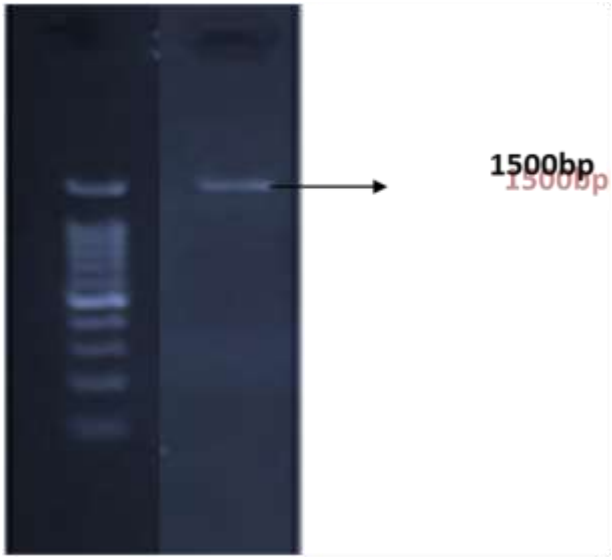

Fig. 4. PCR amplified product

3.6. Feather degradation

Comparing with water and uninoculated control culture treated feathers shown more degradation and also increased percentage of degradation of feather were noted in increased time periods of 7, 14 and 24days of incubation due to the increased production of keratinase enzyme by *Bacillus subtilis* strain KBT (Fig 5). The duration of incubation and feather filtrate weight percentage was given in table 1.

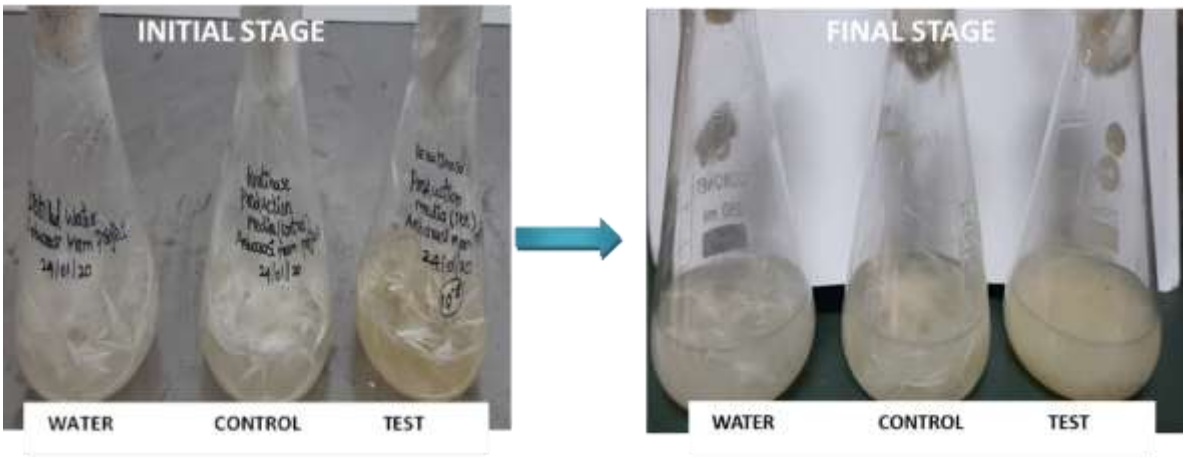

Fig. 5. Chicken feather degradation after inoculation with *Bacillus subtilis* strain KBT

Table 1. Percentage of feather degradation after incubation by the activity of keratinase enzyme

Sample	Initial conc	Final conc	% of Degradation	Keratinase enzyme activity	Keratinase specific activity
Control-1(water)	1.00g	0.99g	1%	-	-
Control-2(medium)	1.00g	0.99g	1%	-	-
7-days	1.00g	0.94g	6%	1.58	0.094
14-days	1.00g	0.86g	14%	2.43	0.144
21-days	1.00g	0.70g	30%	3.60	0.159

3.7. Production and partial purification of keratinase enzyme

Keratinase enzyme was extracted from 48 hrs culture for keratin production medium. The concentration of protein and specific activity of the extracted crude enzyme found to be 2.1mg/ml and 0.13mg/ml, respectively. The After partial purification with 70 % of ammonium sulphate the protein concentration and specific activity noted as 1.9 mg/mL and 0.25 9 mg/mL, respectively. After dialysis Concentration of protein and specific activity quantified as 0.93 mg/mL and 0.27 9 mg/mL, respectively.

3.8. Characterization of extracted keratin solution FTIR

In order to analysis the data, The FT-IR spectrum has been divided into four regions (Fig 6).
Region I (4000 to 3200 cm-1) concerned with water and carboxylic group in chicken feather. In this region the focus is on the revelation of the nature of hydrogen bonding and the carboxylic acids.
Region II (3200 to 1400 cm-1) the bands for functional groups are observed. The functional groups are hydrogen stretching, stretching vibrations lipid acyl group, asymmetric stretching in lipids and proteins were confirmed and the β pleated structures conformation has been obtained.
Region III (1400 – 900 cm-1) has significant importance in the context of biological minerals and

their combinations. The spectra of chicken feather indicates the presence of glucose, deformation of carbohydrates and the characteristics of phosphate ion, carbon ion and also of some functional groups concerned with protein – the Keratin.

Region IV (800 – 600 cm-1) related to cis – double bond (=CH), N-H wagging, CO_2 absorption and SO42- ions and NH_2.

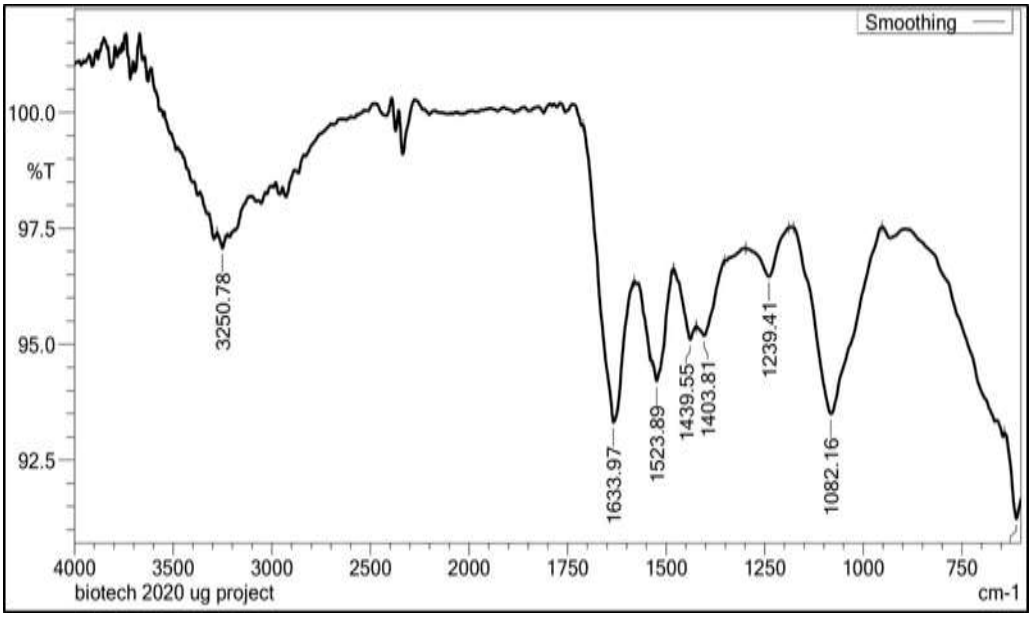

Fig. 6. FT-IR spectroscopy results of extracted keratin

4. CONCLUSION

In this preliminary study, we have isolated and identified chicken feather degrading bacteria *Bacillus subtilis* strain KBT from the soil of a chicken farm bed and screened for their degrading capability of chicken feathers. Result of this study indicates that *Bacillus subtilis* strain KBT is a potential keratinolytic organism and can be used for the biodegradation of keratin in feather industries and can be employed in the production of keratinase. Addition to this, we can turn feather waste into some value added products.

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