Degradation of chicken feather using keratinase enzyme produced by *Bacillus thuringiensis* SJAMB and biofertilizing prospective for agriculture practices

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Abstract

Abundance of keratinase waste is generated from industries and their destruction can cause much environmental deterioration. Chicken feathers are non-degradable because of the presence of insoluble protein keratin and resistance to degradation by common protease enzyme, but several microorganisms are capable of degrading keratin by the enzyme keratinase. This study was planned to evaluate the production of keratinase from Bacillus thuringiensis SJAMB isolated from poultry waste for the capability to disintegrate the chicken feather proficiently. Enzyme activity was analyzed and the degraded feathers were identified by weight loss. SEM analysis revealed the variations of the feather keratin during degradation. The crude keratinase was purified by column chromatography and ammonium salt precipitation and the molecular weight of protein was examined by SDS-PAGE as 56 kDa. Degraded feather used as biofertilizer showed good growth of the green gram plant than urea and chemical fertilizer and this was observed in the day 1 and 13 respectively.

Finally, the chlorophyll content was estimated using UV spectrometer at 663 nm and 345 nm and it was observed that the plant treated with degraded feather showed highest chlorophyll content of about 2.502 followed by plant treated with the enzyme (2.418) and urea (2.372) respectively. This study explored the chicken feather degradation using keratinoltyic bacteria resolving the mechanism of keratin hydrolysis by other processes and replace the currently used hazardous fertilizers.

Keywords: Keratin, *Bacillus thuringiensis*, Keratinase, protease, fermentation, biofertilizer.

Introduction

Keratinase waste produced in the industries in billions of tons and their destruction can cause a number of environmental degeneration[3]. Although, the agricultural waste produced by most of the poultry farms are the chicken feathers. Globally, estimation of feathers generated annually is about several million tons[5,24] and bulk quantities of feathers generated are byproducts in poultry industries that are rich in proteins with β-keratin which constitutes about 91% of feather protein. The feather recalcitrant is due to the presence of keratin, trypsin, pepsin and papain that are considered to be the commonest proteases and this declines the degradation process in nature. Consequently, large number of chicken feathers accumulated cause serious pollution problem[16,20].

Keratinaceous wastes from chicken feather are protein reserve that are important and can be used by bioconversion into high value products using microbial keratinase[1]. Recently, microbial enzyme keratinase used to treat feathers has several applications and vast group of microorganisms reported to produce keratinase are bacteria, fungi and actinomycetes. Among these microorganisms, keratinase from bacteria and fungi was found to exhibit activity under temperatures ranging from 28 – 90 ºC or sometimes higher and pH from 5 – 13[9]. Among the bacteria, many species of *Bacillus* revealed to be capable for the production of keratinase on commercial scale[7,8].

Keratinous waste degraded by kertinase produced by microbes is effective and low-cost method for effective treatment of waste rich in keratin involved in waste management, environmental safety resource generation and non-protein nitrogenous compounds. Microbial degradation of feathers serving as a nitrogen source releases tryptophan that helps in IAA synthesis. During feather degradation, tryptophan released reacts as a precursor for indole acetic acid, the plant hormone which boosts water holding capacity, nitrogen and carbon content of the soil and promotes organic farming and soil activities. The enzyme keratinase has its application as biofertilizer and feather hydrolyzate enhances plant growth employing as nitrogen fertilizer[21,23].

Consequently, chicken feathers are readily accessible which are low priced and are converted into dietary proteins used as feed stock for animals strengthens a bio economical approach[14]. Indeed, the processing of feathers requires several procedures namely chemicals and steam pressure cooking in converting into animal feeds using feathers and the process requires more energy. During treatment process, some amino acids have broken up.

Even though these feathers from the poultry farm are used in various field, still without proper treatment, a huge quantity of feathers are liberated in the environment[15]. A number of mechanisms are proposed and it is known that sulfitolysis and proteolysis takes part in the keratinolytic process. The cleavage of disulfide bonds is sulfitolysis and cleavage of protein is proteolysis[13].

Many researchers have reported that for designing the products of keratinase, the disulfide reductase enzyme is useful. Additionally, breakage of disulfide bonds can be facilitated by adding some chemicals that are useful for the increase in the efficiency of keratinolytic activity. Over a period of time, keratinase that degrades chicken feathers, can be used in several fields such as biofertilizers, pharmaceuticals, clothes, leather industry, decoration, cosmetics and animal feed.

As a result, this process is ecofriendly and can replace the currently used hazardous fertilizers by using the compost from poultry manure with indigenous microorganisms that degrade keratin. The present study exploits the degradation of keratin in chicken feathers by using the enzyme keratinase produced by a bacterial isolate with keratinolytic activity from soil of poultry farm and applied as biofertilizer for agricultural practices.

Material and Methods

Isolation and identification of keratinolytic bacteria: The soil samples were collected from nearby poultry farm, Coimbatore, Tamil Nadu. The pour plate technique was followed for the isolation of keratinolytic bacteria and this method is performed by serially diluting the soil samples and plated in nutrient agar plates and incubated at 37 ºC for 24 hrs. Subsequently the pure cultures isolated following incubation are inoculated in specific medium such as casein agar and starch agar to detect the zone of hydrolysis by the bacteria[18]. The selected bacteria were identified by extracting the genomic DNA and 16S rRNA amplification was done in a reaction using 2 µl of total DNA, 2 µl of primers, 8 µl of PCR master mix and 6µl of distilled water.

The 16S rRNA primers with subsequent sequences are 5'-AGAGTTTGATCCTGGCTCAG-3' (forward primer) and 5'-ACGGCTACCTTGTTACGACTT - 3' (reverse primer). This process was carried out with primary denaturation for 5 mins at 94 ºC and subsequently denaturation by 32 cycles for 1 min at 94 ºC, annealing for 30s at 53 ºC, extension at 72 ºC for 90 s and last extension for 10 mins at 72 ºC. The amplified DNA was analyzed by using agarose gel electrophoresis (1% w/v in 1x TE buffer stained with ethidium bromide) and sequenced using BLAST tool with the existing sequences in the GenBank and the phylogentic tree was constructed for the identified keratinolytic bacteria.

Production of keratinase enzyme using solid state fermentation: The production medium used for the keratinase enzyme was feather meal medium (K_2HPO_4 – 0.3g, KH_2PO_4 – 0.5g, NaCl – 0.5g, Feather – 10g for 1000 ml of distilled water). So, 1ml of culture and the feather meal preparation was fermented and incubated for 7 days at 37 ºC. Two different carbon and nitrogen sources were employed to optimize the production of keratinase in the fermentation medium. The carbon sources used are glucose and sucrose and the nitrogen sources are beef extract, yeast extract and peptone[2]. Each day the biomass is estimated and this is done for alternative days by using 2ml of culture suspension centrifuged for 6 mins at 6000rpm. The supernatant was discarded and the pellet is weighed. At the end of the incubation period (7^{th} day), UV spectrometric readings were taken and enzyme activity was calculated.

Determination of enzymatic activity: To determine the enzymatic activity, different carbon and nitrogen sources are added in the broth culture and incubated for 7 days. Following incubation, centrifugation of the broth was done at 10,000rpm for 5 minutes and 1ml of supernatant is examined for the keratinase activity. The supernatant that contains crude keratinase is then precipitated and purified.

Feather degradation: The feather meal or feather that is still present in the medium was filtered using Whatmann no. 1 filter paper and dried for 48 hrs. The substrate degradation was determined by weight loss.

Precipitation and purification: Cell free supernatant of feather culture was purified by precipitation method. The enzyme keratinase was precipitated by addition of ammonium sulfate and gentle stirring was done until 80% of saturation was reached. The keratinase enzyme was purified using column chromatography and the column was equilibrated with phosphate buffer. Then 2ml of protein sample was loaded on the column and the eluted proteins were collected in fractions.

Analysis of feather degradation by Scanning electron microscope: The change in the structure of feathers during fermentation process was observed by Scanning electron microscope at 15kV.

Protein analysis: SDS - PAGE was done to confirm the keratinase enzyme produced by *Bacillus* sp.

Plant growth using degraded feather as biofertilizer: Green gram plant is selected for the test. Degraded feathers are used as biofertilizer for the plant growth and finally the plant growth is compared with keratinase and urea. Followed by the growth of the plant observed using degraded feather, the root and shoot sizes of each plant are measured and then compared with the plant growth using chemical fertilizer (urea) and the enzyme keratinase.

Chlorophyll estimation: The plant growth after 13 days was checked for its chlorophyll content. The chlorophyll content was estimated by crushing the leaves with mortar and pestle and the extract is filtered and 2ml of acetone is added and incubated at room temperature (dark incubation) for 24 hrs. Spectrometric readings were taken and calculated using this formula:

Chlorophyll a (mg/g) = 12(A663) – 2(A645) × v/1000 ×W
Chlorophyll b (mg/g) = 22(A645) – 4(A663) × v/1000 ×W
Total chlorophyll (mg/g) = 20(A645) – 8(A663) × v/1000 ×W

where A = absorbance at specific wavelength, V = final volume of chlorophyll extract in 50% acetone and W = weight of the fresh tissues extracted.

Results and Discussion

Isolation and identification of keratinolytic bacteria: The keratinolytic bacteria were isolated from the poultry soil sample using serial dilution and the morphological characteristics of the isolate appearing as circular, colorless, smooth colonies (Fig. 1). Following isolation, the bacterial culture streaked on Casein and starch agar plates showed zone of hydrolysis (Fig. 2 a and b) proving that the keratinolytic bacteria have the ability to hydrolyze casein (milk protein) and starch. The zone formed indicates the confirmation of keratinolytic bacteria. The molecular identification of the bacteria was done by Sanger sequencing method. The isolate *Bacillus* sp. was confirmed as *Bacillus thuringiensis* SJAMB showing 90-9% similarity with MK757603 *Bacillus thuringiensis* strain RD. Subsequently, the sequence was submitted in NCBI and using GenBank Acc. No OQ569362, the phylogenetic tree was constructed with other *Bacillus thuringiensis* (Fig. 3).

Superior sources in isolating bacteria for degrading the feather are the poultry industry soil and the isolates are usually identified by 16S rDNA sequencing[11,12]. The bacteria isolated used for degrading the keratin rich materials present in chicken feathers were reported due to the secretion of proteases keratinase by these organisms that is accountable for the cleavage of the keratin proteins. Bacterial species capable to produce the keratin degrading enzymes are *Bacillus*, *Pseudomonas*, *Xanthomonas*, *Brevibacillus* and *Serratia*[17,22].

Production of keratinase enzyme using solid state fermentation and determination of enzymatic activity: The media supplemented with carbon (glucose and sucrose) and nitrogen (yeast extract, beef extract and peptone) sources in the production media for biomass estimation for alternative days showed high biomass yield in carbon and nitrogen sources as glucose and peptone respectively in day 3 (Fig. 4). UV spectrometric readings of the culture media taken at the end of the incubation period (7th day) supplemented with glucose as carbon source and yeast extract as nitrogen source proved high enzymatic activity (Fig. 5). Sharma et al[19] investigated that the keratinase enzyme production by *Bacillus* sp. grown in basal medium showed keratinolytic activity moderately to highest level and the isolates BF11 and BF21 that exhibit highest keratinase in basal medium compared with two liquid medium such as Luria broth and nutrient broth respectively.

Fig. 1: Isolation of keratinolytic bacteria *Bacillus thuringiensis* SJAMB showing growth in nutrient agar

Fig. 2: Keratinolytic activity of *Bacillus thuringiensis* SJAMB (a = Zone of hydrolysis in Caesin agar plate, b – Zone of hydrolysis in Starch agar plate).

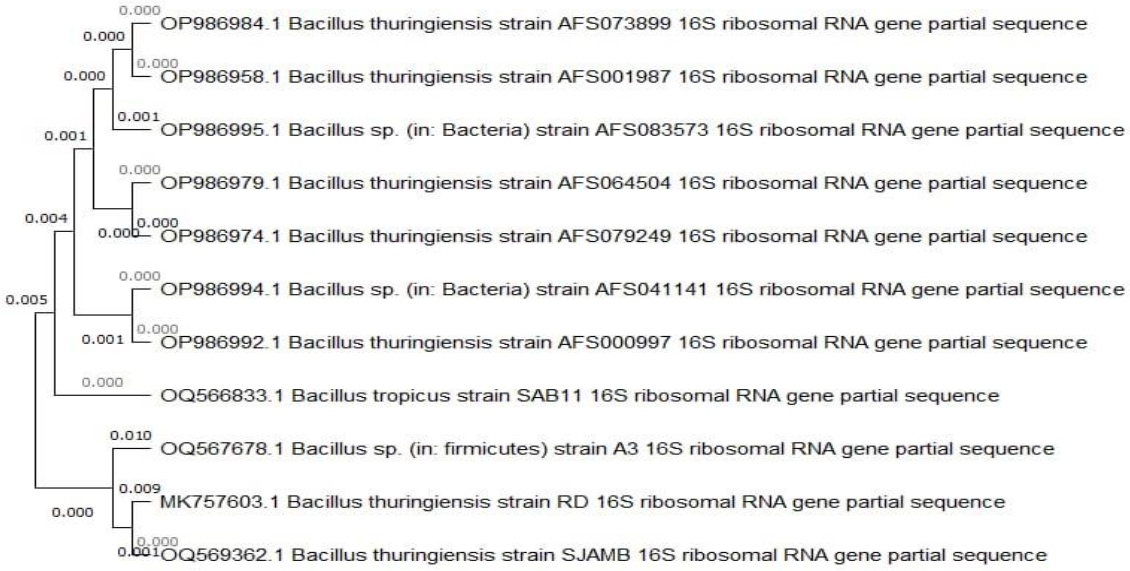

Fig. 3: Phylogenetic tree of *Bacillus thuringiensis* SJAMB

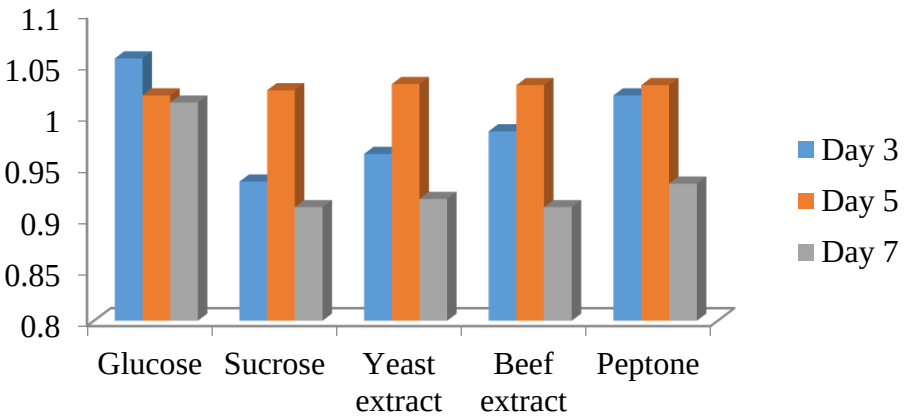

Fig. 4: Biomass estimation using different carbon and nitrogen sources in the broth medium

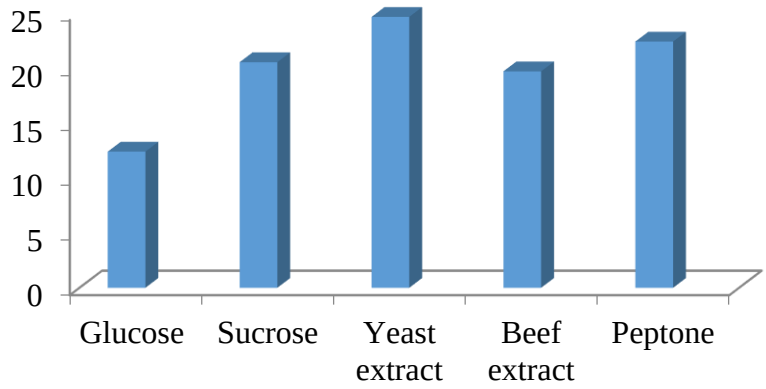

Fig. 5: Enzyme activity using different carbon and nitrogen sources in the broth medium

The isolates showed low production of keratinase in both the liquid medium than basal medium, but variation was not seen in these media for the production of biomass. The optimized condition of the organism to produce keratinse enzyme using different carbon source in the fermentation broth was found that starch and galactose supported highest production of keratinase but glycerol, lactose and galactose were not used by the organism.

Moreover, the best carbon source for keratinase production was found to be glycerol and starch by MBF21. In accordance with the molecular detection, the organism

MBF11 was *Bacillus subtilis* and BF21 as *Bacillus cereus*. In addition, it was investigated that to improve the alkaline protease and keratinase production in *Bacillus* sp., soya bean meal was used as nitrogen source[11,25]. More over yeast extract was found to produce keratinase, but NH_4Cl has no significant effect for the MBF isolates for the production of keratinase.

Feather degradation and analysis by Scanning electron microscope: Filtered and dried feathers from the growth culture were checked for the weight loss. The growth culture supplemented with nitrogen source (peptone) showed maximum of 36% of degradation of feathers (Fig. 6). The degraded feathers when viewed under Scanning electron microscope at 15 kV showed better degradation of the feather by the enzyme keratinase when compared to the control feather (Fig. 7). Feathers containing keratin rich waste are resistant to degradation by certain proteases and microorganisms degrade the keratins that accumulate in nature[26].

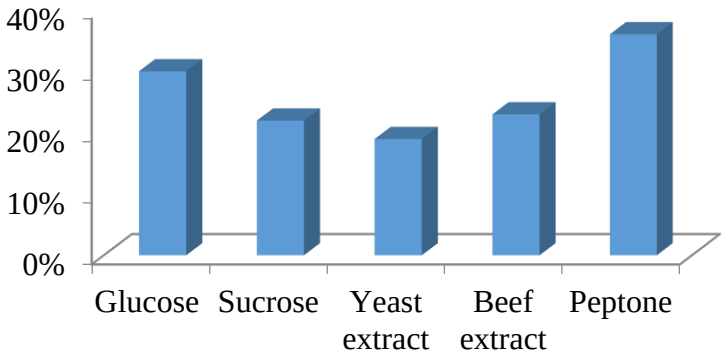

Fig. 6: Feather degradation using carbon and nitrogen sources in the culture medium

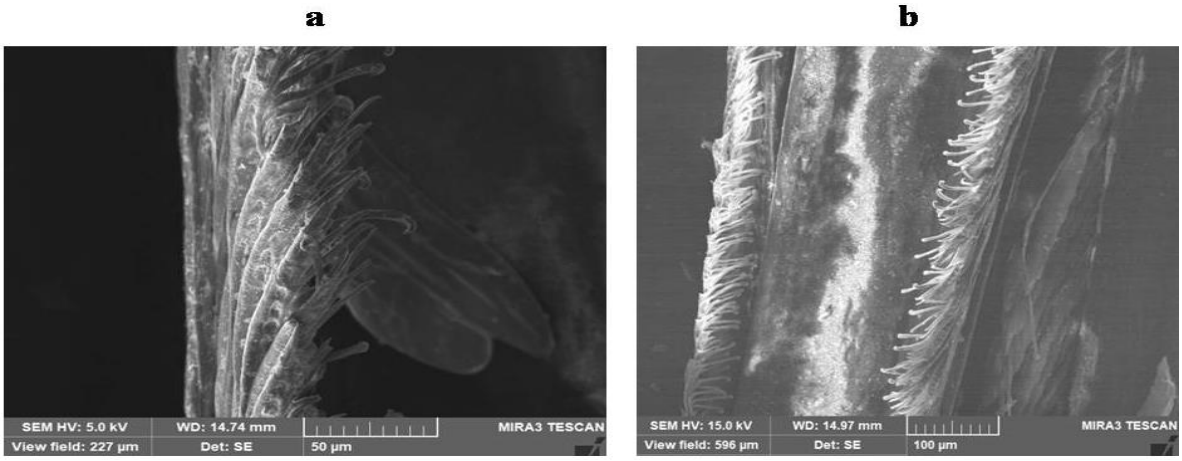

Fig. 7: Scanning electron microscope showing feather degradation (a – Control feather, b – Treated feather)

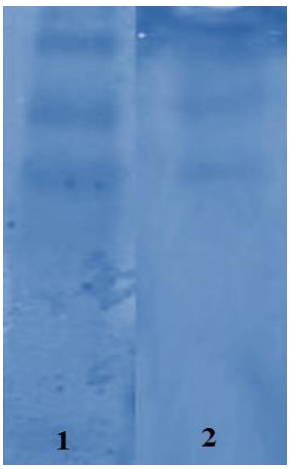

Fig. 8: Protein analysis (Lane 1 – SDS marker, 2 – Enzyme 56 kDa)

The bacteria *B. velezensis* NCIM 5802 produced keratinase during fermentation found to degrade keratin entirely in 84-92 h and it efficiently degrades the chicken feathers in soluble proteins and amino acids. Bacterial adherence, colonization to damage the barbs of the feathers was found as the first step in degradation revealed by Scanning electron microscope and this is followed by fracturing that are extensive and barbs and barbules disintegration. Later the invasion and decomposition of the bacteria and recalcitrant part of the feather are mostly weakened[19].

Precipitation, purification and protein analysis: Ammonium sulfate precipitation is done by adding 8g to 15 ml of supernatant which helps in the quick precipitation of cellular proteins. The crude keratinase was purified by column chromatography technique and the eluted proteins were collected in fractions and spectrometric readings were taken at 260nm. The protein analysis confirms that keratinase has the molecular weight of about 56 kDa (Fig. 8)

Plant growth using degraded feather as biofertilizer: Green gram plant is used for this test. Urea, enzyme and degraded feather as biofertilizer were used for the growth of the green gram plant and the growth of the plant was observed for about 13 days. Fast growth was seen in plant treated with degraded feather whereas it was moderate in enzyme treated plant**.** The plant treated with chemical fertilizer showed less growth when compared with these two in day 4 and day 13 respectively (Fig. 9). The root and shoot size were measured and shown in fig. 10. Finally, keratinolysis results in complete breakdown of the keratinous material that is amorphous porous in nature. Many researchers have reported that degradation of feather keratin using bacteria was found to degrade keratin from 1 to 6 days eventually[6,10,12].

Many researchers have proved that numerous microorganisms can bale to degrade poultry waste by keratinolytic and proteolytic secretion[22]. From different resources, the keratin containing waste is degraded by various microorganisms that are isolated from different environment rich in keratin. Value added products obtained from the microbial conversion of feathers are biofertilizers and animal feed used in poultry industries[4]. Therefore, processing of feathers using microbes is a positive approach to create resourceful products such as biofertilizers.

Chlorophyll estimation: The leaf extract of each plant was checked for its chlorophyll content by UV spectrometer reading at absorbance 663 nm and 645 nm. The growth and chlorophyll content of the normal plant are compared with enzyme (keratinase), degraded feather treated plant. The plant in which degraded feathers were used as a biofertilizer, showed high chlorophyll content in it (Table 1).

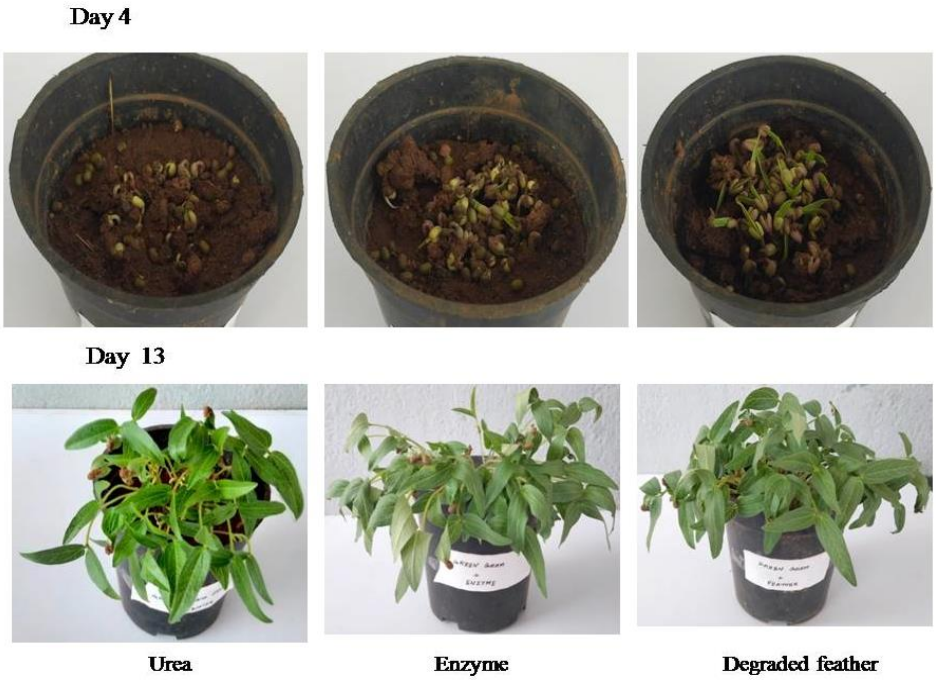

Fig. 9: Growth of green gram plant using urea, enzyme and degraded feather

Table 1
Chlorophyll content of green gram plant leaf extract

Result = + 1.000000 xAbs (663.0nm) + 1.000000 xAbs (645.0nm)				
S.N.	**Name**	**Abs (663.0nm)**	**Abs (645.0nm)**	**Result**
1	Urea	1.298	1.074	2.372
2	Feather	1.252	1.205	2.502
3	Enzyme	1.218	1.201	2.418

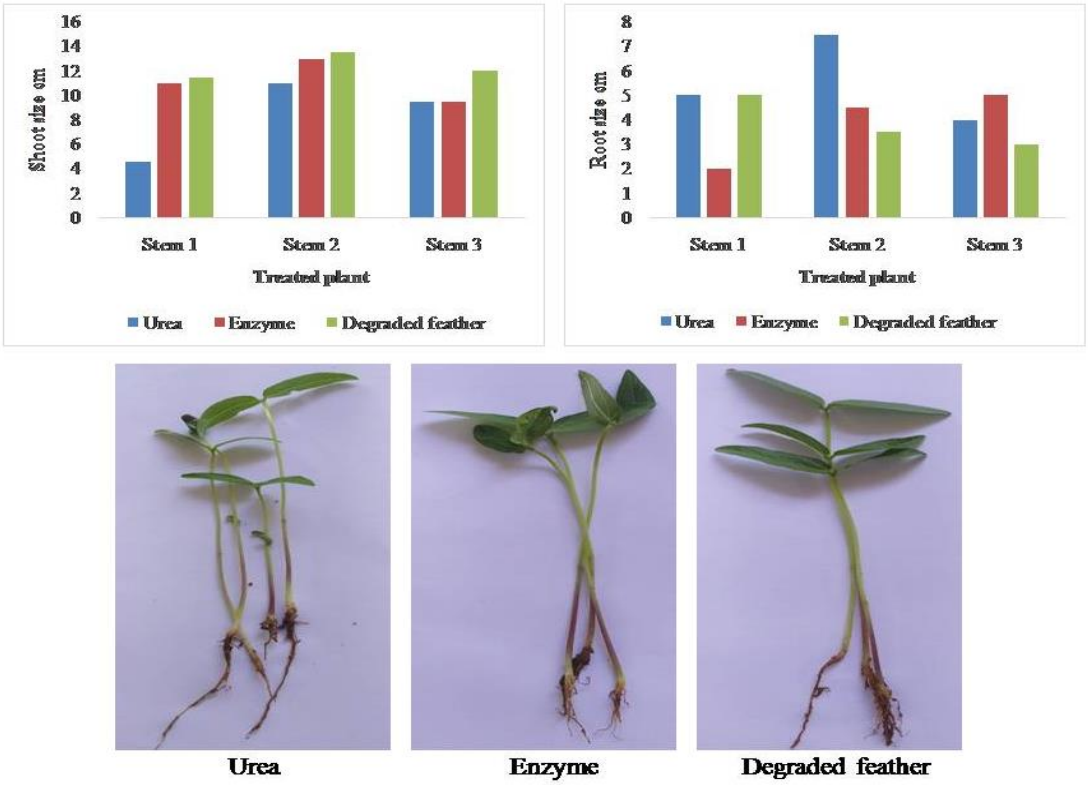

Fig. 10: Shoot and root size of the green gram plant

Hence, the growth of plant using degraded feather (biofertilizer) is compared with urea (chemical fertilizer). Plant treated with feather showed high value followed by enzyme treated plant and urea treated plant.

Conclusion

Bacillus thuringiensis SJAMB isolated from poultry farm with potential keratinolytic activity was evaluated. Different carbon and nitrogen source supplemented in the culture medium showed highest keratinase enzyme production and activity when the bacteria utilize glucose as carbon source and beef extract as nitrogen source. The degradation of feather was observed in the growth culture supplemented with protein source such as peptone at maximum percentage and confirmed using Scanning electron microscope.

The results prove that the keratin degradation of chicken feather and potential of *Bacillus thuringiensis* SJAMB in poultry waste are valuable as biofertilizer production in addition to the management of keratin waste.

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