Isolation, Optimization and Molecular Identification of Keratinase producing Bacteria *Bacillus velezensis* Strain VITSJ01 from Chicken Farms

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Abstract

Keratin is a protein that acts as the key structural material in making up hair, nails, feathers, scales etc. It protects epithelial cells from damage or stress. Degradation of these keratin rich materials in nature is done by specific bacteria and other microorganisms that produce an enzyme called Keratinase. The present study aimed at the isolation, optimization and PCR-assisted identification of Keratinase producing bacteria associated with chicken feathers. Keratinase producing bacteria were isolated from soil samples collected from chicken farms. The isolated bacteria were screened primarily on skimmed milk media and the colonies indicating keratinolytic behaviour were classified morphologically, microscopically and biochemically. The potential strains were then subjected to enzyme production in order to measure the enzyme activity. The effects of temperature, pH and period of incubation on the enzyme activity were also studied to identify the optimal range to produce the enzyme. The maximum enzyme activity was observed for the isolate VITSJ01 at an optimum pH of 5 and 37° C temperature.

Further the potential strain was identified as Bacillus velezensis by 16S rDNA sequencing. The isolate was then successfully purified, sequenced and analysed by NCBI-BLAST for similar search. Thus, the purification of microbial keratinase from soil samples could be effectively utilized for degrading feather keratin and feathers can be converted to feedstuffs, fertilizers, glues and films.

Keywords: Keratinase, Keratinolytic activity, Bacteria, Poultry waste.

Introduction

The disposal of poultry waste generated globally, particularly in countries like India, has become a significant cause of concern due to its impact on the environment. Worldwide, around 5 million piles of feathers are generated from poultry plants as waste product annually[5]. Chicken feathers are one of the most substantial waste materials generated by the poultry industry[2]. They are a rich protein source due to their high keratin content, making them an attractive option for valorization[12]. However, the typical ubiquity of chicken farms does not mean that optimal and efficient methods of disposing of the waste generated are being computed.

Historically, chicken feathers have mainly been burnt, buried, or discarded as waste. Unfortunately, these methods are not practical due to their extensive operating costs, utilization of energy, loss of natural resources and significant environmental pollution[8,9]. Therefore, it is crucial to explore new, efficient and eco-friendly alternatives for handling chicken feather waste materials. Thus, degradation of feathers by microorganism appears to be an alternative approach when compared to conventional methods. Certain bacteria isolated from soil have the ability to degrade keratinous materials such as chicken feathers, nails, hair and horns[13]. These bacteria synthesize specific protease known as keratinases which break down the protein matrix of feathers into smaller, digestible peptides.

Basically, 90 percent of the chicken feathers are rich in beta keratin[8]. They are mechanically stable and chemically non-reactive protein. This is mainly due to their molecular arrangement of amino acids, cross linking of disulphide bridges, hydrogen and hydrophobic interactions. All these features make keratin highly insoluble in water, inert and non-degradable by majority of proteolytic enzymes like trypsin, papain, pepsin etc.[4,8] The mechanical firmness of keratin and its resistance to biochemical degradation is due to the tight packing of their production chains. The secondary structure of keratin consists of α- helix and β-sheets linked by disulfide bonds. The enzyme attacks this disulfide bond of keratin to degrade it[10].

Majority of the keratinase enzymes are produced by bacteria, fungi, actinobacteria as well as dermatophytes[13]. They are widely present in various environments and are isolated from soils, sewage sludges and animal wastes[10]. Keratinase from microorganisms is valued due to its activity on degradation of specific insoluble keratin substrates as well as on several protein materials[5]. Therefore, this enzyme is considered as an important biocatalyst having applications in leather, cosmetics, feed, textile and detergent industries[1,3].

Apart from this, the enzyme also has potential applications on drug delivery systems, bio-processing of used X- ray integument as well as in agro industrial waste degradation[7, 13]. Therefore, this study aims to isolate, optimize and identify bacteria that produce keratinase from soil collected from chicken farms using 16S rDNA sequencing.

Material and Methods

Sample collection: The soil sample was collected from the chicken farms in Angamali, Cochin, Kerala. The sample was transferred aseptically in a sterile plastic container and brought to the Marine Biotechnology Laboratory and stored at 4^{o}C till use.

Isolation of keratinolytic bacteria: Isolation was done by serial dilution method. One gram of soil was mixed in 10 ml of sterilized distilled water and was serially diluted up to 10^{-6} dilution. 0.1 ml of 10^{-3} to 10^{-6} dilutions were spread plated onto keratin agar media and incubated at 37^{o} C for 24-48 hours.

Screening for keratinase producing bacteria: The isolated bacteria were screened for the production of extra cellular keratinase enzyme using skim milk agar. The isolates were streaked onto the skim milk agar media and incubated at 37^{o} C for 24 hours. After incubation, the plates were observed for the formation of a clear zone around the colonies.

Identification of Keratinase producing bacteria: The isolates were identified based on their cultural characteristics with respect to color, shape, size and nature of the colony[6]. The morphology of the isolated bacteria was observed by Gram staining and observed under a light microscope. Finally, the isolates were characterized biochemically by Indole test, Methyl Red test, Voges Proskauer test, Citrate Utilization test, Catalase and Oxidase test.

Enzyme production: The potent bacterial isolate was inoculated into the production broth (Keratin azure-50 g, glucose- 2 g, peptone- 5 g, yeast extract- 5 g, K_2HPO_4- 1 g, KH_2PO_4- 3 g, $CaCl_2$- 1 g, $MgSO_4$- 1 g) and incubated at 37^{o} C for 24 hours in a shaking incubator at 120 rpm. After fermentation, the broth was centrifuged at 14000 rpm for 10 minutes and the supernatant was collected to study the enzyme activity.

Enzyme assay: Keratinase activity was measured by adding 1 ml of culture supernatant and 1 ml of keratin azure suspension. The control experiment consisted of keratin azure suspension only. The mixture was kept in a water bath at 50^{o} C for 30 minutes for the reaction to happen. After incubation, the tubes were transferred to a boiling water bath for 5 minutes. The tubes were then cooled down to room temperature. Further, the reaction was stopped by adding 2 ml of trichloroacetic acid (TCA). The reaction mixture was then centrifuged at 3000 rpm for 20 minutes and the supernatant was collected. The absorbance of the supernatant was measured at 595 nm using a spectrophotometer. One unit (U/ml) of keratinase activity was defined as the amount of enzyme causing 0.01 absorbance increase between the sample and control at 595 nm under the specified conditions for all the three samples[11].

Optimization of keratinase production: The effect of temperature, pH and period of incubation on growth and enzyme activity was studied for the sample that showed the highest absorbance.

Effect of temperature: The potential strain was inoculated in nutrient broth and incubated at different temperature (20^{o} C, 30^{o} C, 37^{o} C and 50^{o} C) for 24 hours. The enzyme assay was carried out in the presence of keratin substrate and the absorbance was measured at 595 nm.

Effect of pH: Effect of pH on enzyme production was noticed at different pH values ranging from 5, 7, 8 and 10. The pH of the sample was altered to attain different pH levels and an enzyme assay was carried out using standard assay protocol.

Effect of period of incubation: The optimal incubation time for maximum keratinase production was determined at 24 h, 48 h, 72 h and 96 h. Enzyme production was evaluated by performing enzyme assay using standard assay protocol.

Molecular characterization and identification of the potential isolate: The identification of the potential strain was confirmed by carrying out 16S rDNA sequencing[14]. Genomic DNA was isolated according to the standard protocol[15]. The isolated DNA was then observed by agarose gel electrophoresis. The DNA fragment was amplified and the PCR product was sequenced bi-directionally using universal primers [forward primer (5'-GAGTTTGATCCTGGCTCAG-3') and reverse primer (5'-GAATTACCGCGGCGGCTG-3')]. The amplified PCR product was then analyzed by agarose gel electrophoresis and further sent for sequencing. The sequences were compared with the reference strains for similarity index using the NCBI Blast tool. The phylogenetic tree was constructed for the obtained sequence and its homology was prepared using MEGA 10 software.

Results and Discussion

Isolation and screening of keratinolytic bacteria: A total of five bacteria were isolated from the soil sample collected from the chicken farms in Angamali, Cochin, Kerala. The isolates were named as SJ01, SJ02, SJ03, SJ04 and SJ05.On the basis of a clear zone of hydrolysis around the colonies in skim milk agar, 3 bacterial isolates (SJ01, SJ02, SJ03) were selected and sub-cultured on nutrient agar medium.

Identification of Keratinase producing bacteria: White, translucent, smooth surface colonies with irregular margins and raised elevation were observed**.** The morphology of the three selected isolates was observed by Gram staining. All the isolates were found to be Gram positive rods under 100 X magnification.

From the biochemical test, isolates SJ01, SJ02 and SJ03 showed positive result for VP test which indicates that the isolates have the ability to produce butylene glycol. Indole, methyl red and citrate test are negative for all the isolates (Table 1).

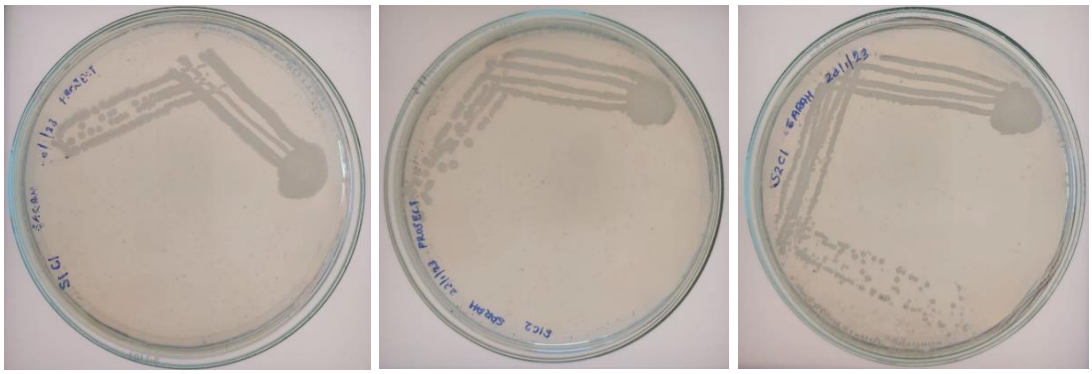
Enzyme assay and Optimization of keratinase production: Among the three isolates, maximum keratinase activity was observed for the isolate SJ01 followed by SJ03 and SJ02 (Figure 2). The isolate SJ01 which exhibited the highest keratinase activity was further optimized for maximum enzyme production at various temperature, pH and period of incubation. The maximum enzyme production was observed at temperature 30º C, 37º C and 50º C by checking its growth pattern. This was further confirmed by enzyme assay which showed that the maximum enzyme activity was observed at 37º C followed by 30º C and 50º C (Figure 3). Similarly, the maximum enzyme activity was observed at pH 5(acidic) followed by pH 7 (neutral).

pH 8 and pH 10, the two alkaline levels showed lower activity (Figure 4). Lastly, the enzyme assay was carried out at different incubation periods. It was concluded that enzyme activity was highest after 24 hours of incubation. The downward trend of the absorbance levels after each day indicates that the enzyme activity peaks are at an optimum period of incubation at 24 hours after which the activity levels decrease with additional time (Figure 5).

Molecular characterization and identification of the potential isolate: The molecular characterization by 16S rDNA of the potential isolate revealed that the bacteria was closely related to *Bacillus* species and therefore named as *Bacillus velezensis* strain VITSJ01 (Figure 6). The sequence of the organism was submitted at NCBI GenBank with an accession number OR858890.1.

Conclusion

Keratins are the broadest and most complex family of cytoskeletal intermediate filament proteins of animal cells. Keratinases are a group of serine hydrolases that are capable of degrading keratin. Due to the molecular configuration of the constituent amino acids, high degree of cross-linkages of disulphide bridges, hydrogen bonds and hydrophobic interactions, keratin is a mechanically durable and chemically unreactive protein. In the present study, keratinase-producing bacteria were isolated from soil samples of chicken farms for the production of one of the industrially important extracellular enzyme such as keratinase.



(a) **(b)** **(c)**

Figure 1: Pure cultures of the isolates (a) SJ01, (b) SJ02, (c) SJ03 on nutrient agar medium

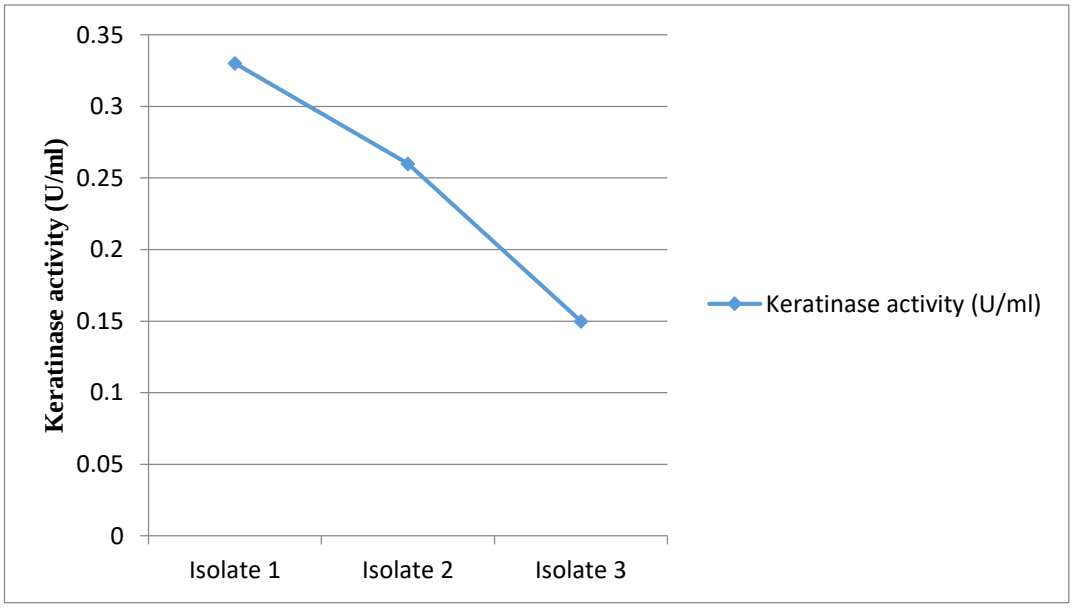


Figure 2: Keratinase activity shown by the potential isolates

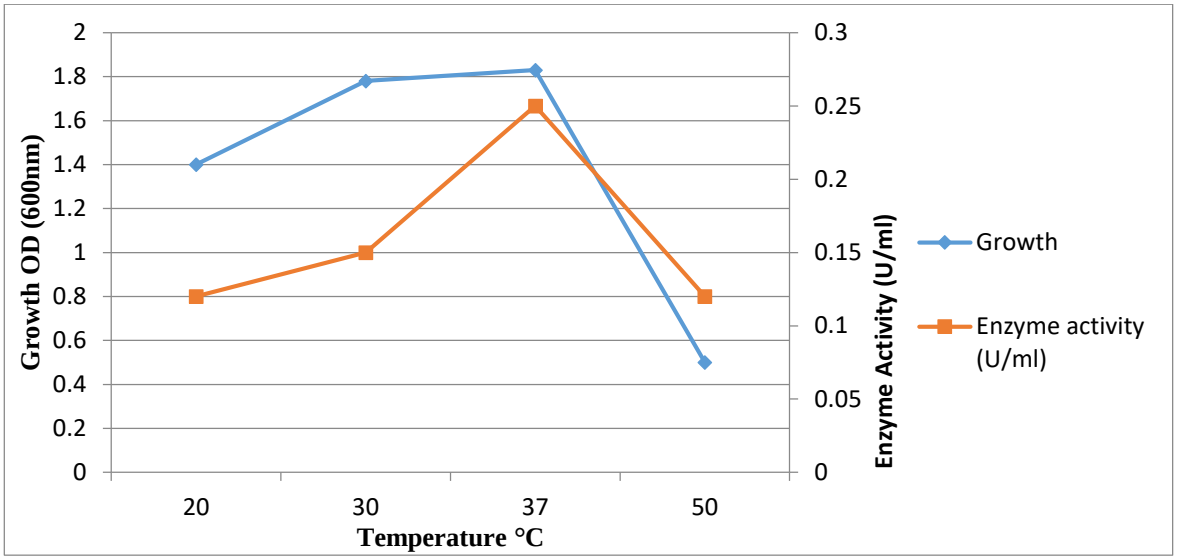


Figure 3: Effect of temperature on growth and keratinase by the isolate SJ01

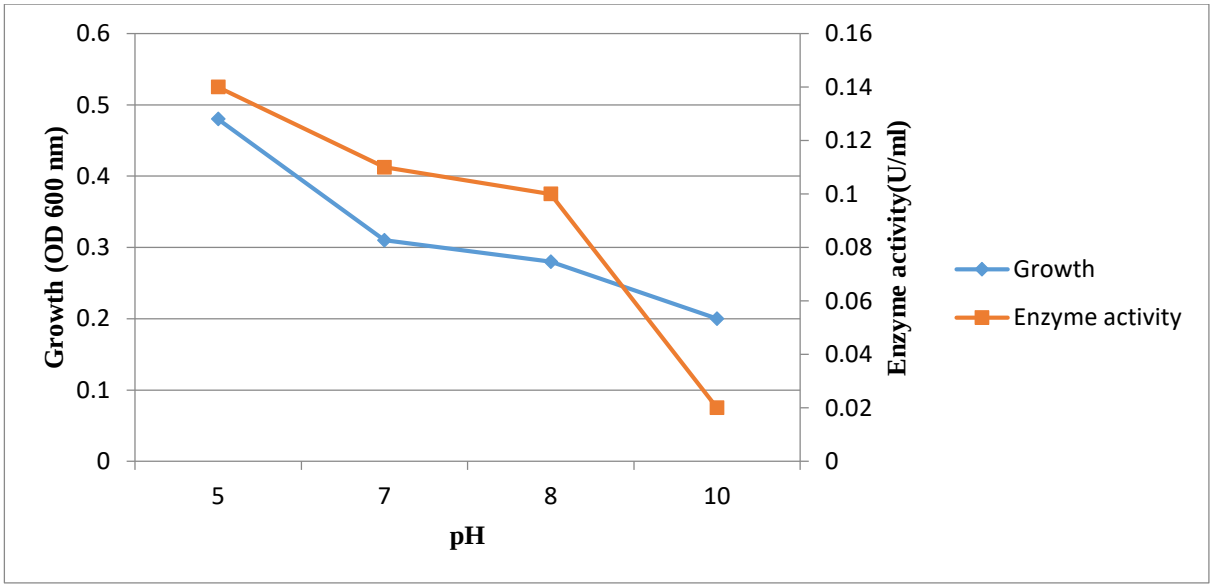


Figure 4: Effect of pH on growth and keratinase by the isolate SJ01

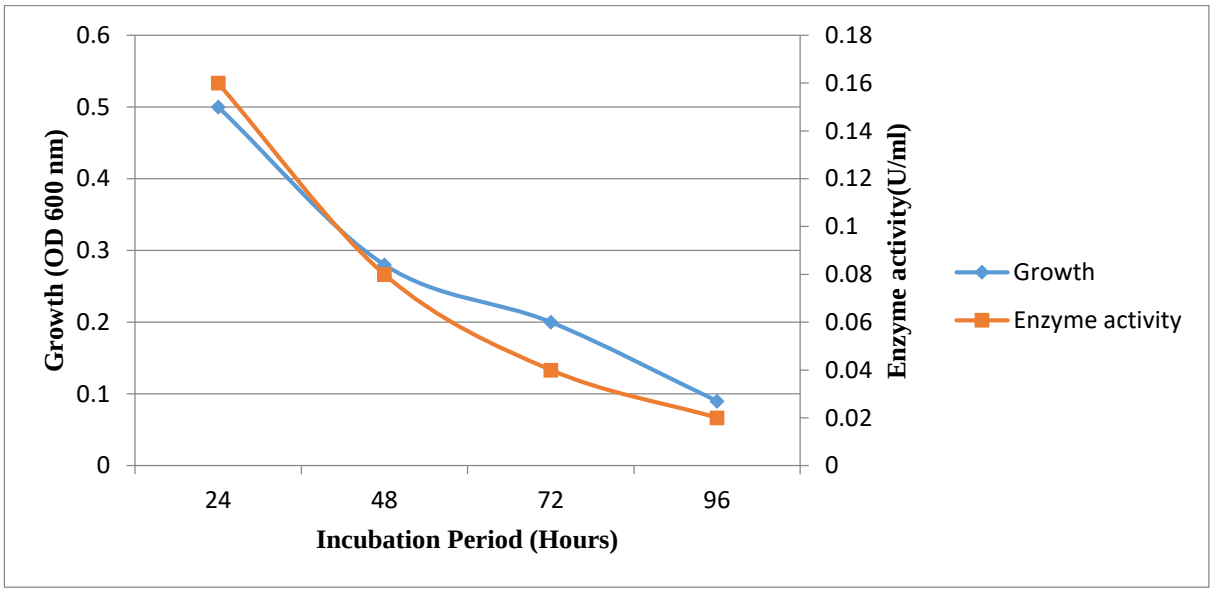


Figure 5: Effect of incubation period on growth and keratinase activity by the isolate SJ01

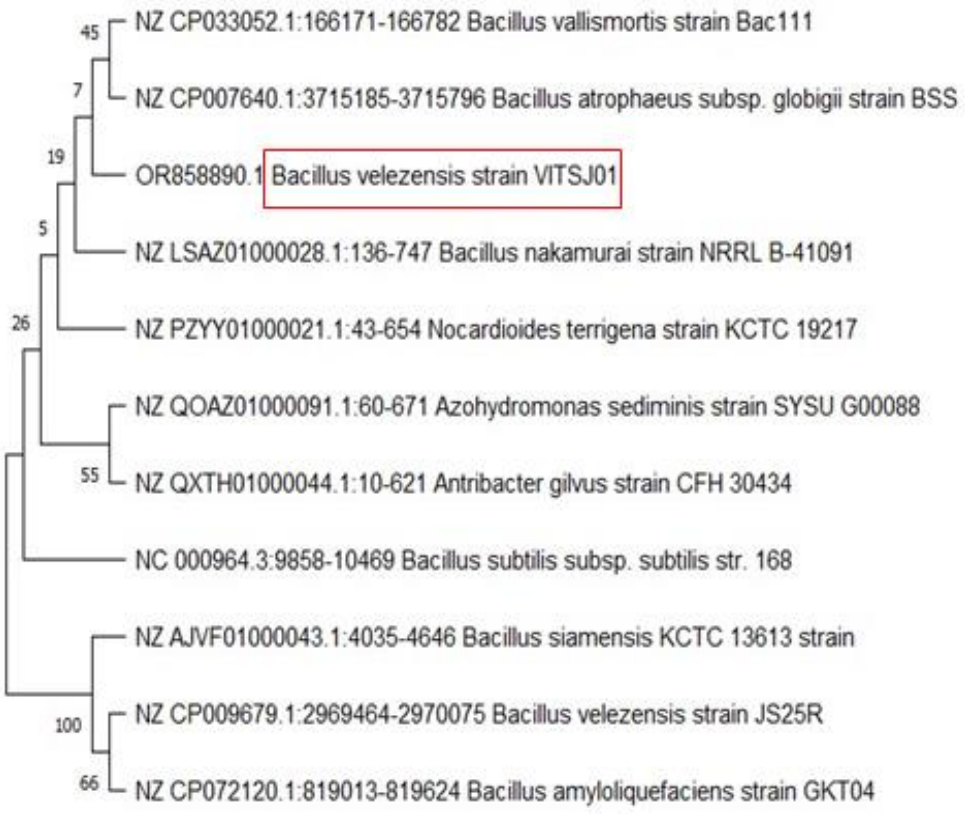


Figure 6: *Bacillus velezensis* strain VITSJ01 phylogenetic tree constructed using neighbor joining method.

Among the isolated bacterial strains, *Bacillus velezensis* strain VITSJ01 showed good keratinolytic activity at optimized conditions. In future, this strain can be employed to digest the recalcitrant feathers into a nutrient-rich feather meal which can be used as a feed supplement. Apart from this, they can be also used to produce low-cost organic manure for the restoration of soil fertility.

Acknowledgement

The authors thank Vellore Institute of Technology and Unibiosys Biotech Research Labs for providing all the facilities for carrying out this work.

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(Received 04th April 2024, accepted 03rd June 2024)