Optimization of Fermentative Production of Keratinase by *Bacillus subtilis* Strain S1 in Submerged State Fermentation Using Feather Waste

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**Abstract**

Keratinases are well-recognized enzymes with the singular power of taking down the recalcitrant structural proteins such as keratin. Their prospective in bio-waste management of feather waste has been well recognized since long. In present scenario, they have acquired importance in various other industrial applications. The present study mainly focused on the isolation of keratin degrading bacteria and its use in optimization of culture conditions to maximize the keratinase production. The isolated strain was identified as *Bacillus subtilis* strain S1 according to morphological and biochemical characteristics followed by 16S rRNA sequencing (accession no: LC054177). The influence of cultivation temperature and initial pH of the medium on keratinase production revealed the optimal values of the temperature and pH as 40 °C and 7, respectively. Maximum keratinolytic activity was observed at 72 h after incubation. Optimized value for inoculum size and substrate concentration was found to be 5% and 1% respectively, 150 rpm found to be the optimum agitation level. The best additional nitrogen source was Beef extract and all the additional carbon sources showed a negative effect on keratinase production. These results indicate that this bacterial strain shows a high potential for keratinase production in submerged-state fermentation, and use of feather waste as the substrate can be implemented for keratinous solid waste management.

**Keywords**

*Bacillus subtilis*, Keratinase production, Submerged-state fermentation, Feather waste.

**Introduction**

Keratinases are well-acknowledged enzymes with the extraordinary power of taking down the unmanageable structural proteins such as keratin. Their prospective in bio-waste management of feather waste has been well established since long. In present scenario, they have acquired significance in various other industrial applications. A massive amount of fibrous insoluble protein in the form of feathers, hair, nails, horn, and other are available as byproducts of agroindustrial processing plants (Onifade *et al.*, 1998). These keratinous wastes are very tough to degrade because of the densely packed polypeptide and several hydrogen bonds and hydrophobic interactions, in addition to several disulfide bonds. Keratin is the insoluble structural protein of feathers and wool and is known for its high stability (Bradbury 1973). In spite of their prominent resistance, keratins do not mount up in nature and can be hydrolyzed by a number of
microorganisms. Keratinolytic enzymes are produced by fungi, actinomycetes, and bacteria and have been repeatedly isolated from soils where keratinous materials are deposited (Kaul and Sumbali, 1997; Riffel and Brandelli, 2006). Among bacteria, keratinolytic activity has been extensively acknowledged for strains from the genera Bacillus and Streptomyces (Lin et al., 1999; Kim et al., 2001; Bressolier et al., 1999). Keratinolytic enzymes from bacteria may have significant uses in biotechnological processes concerning keratin-containing wastes from poultry and leather industries through the expansion of nonpolluting processes.

Keratinase producing microorganisms have the significant industrial application in fermentation technology. Submerged fermentation of poultry waste by microorganism producing keratinase helps in the translation of non-soluble keratin (feather) into soluble protein or polypeptide (Suntornsuk and Suntornsuk, 2003). Insoluble feather keratins can be transformed after enzymatic hydrolysis to feedstuffs, fertilizers, glues, and films or used for the production of the infrequent amino acids serine, cysteine, and proline (Papadopoulos et al., 1986; Onifade et al., 1998; Gupta and Ramnani 2006). Keratinase has also promising application in dehairing process in leather industry as a replacement for sodium sulphides (Alexandre et al., 2005) and also used as a detergent to get rid of strains on cloth (Gessesse et al., 2003).

Valorization of keratin containing wastes like feathers from poultry farms and hair from leather industries may have the prospective in expansion of non-polluting processes. The mammoth augment of the poultry industry has generated bulky amounts of feathers as byproduct. The consumption of agroindustrial residues may correspond to an added significance to the industry. The whole story has encouraged the exploration for alternatives to convert unmanageable keratinous waste into precious products. In this regard the present study was conducted to optimize the keratinase production from feather waste.

Materials and Methods

Isolation and screening of the feather degrading microorganisms

The soil samples were collected from poultry waste dump sites in Allahabad, India. For each sample, 1 g of soil was suspended in 50 mL sterile distilled water. The supernatant was diluted and then laid on skimmed milk agar plate. After incubation at 37°C for 48 h, clearing zones around the colony were observed to signify the protease production. A single colony with a clearing zone was picked up and inoculated on feather meal agar plate containing the following (g/L): feather meal (10.0), NaCl (0.5), K₂HPO₄ (0.3), and KH₂PO₄ (0.4). The isolated strain, which showed growth on the feather meal agar plate, was selected for further studies.

Taxonomic studies and 16S rRNA sequencing

Bacterial identification was conducted based on morphological and biochemical tests. The 16S rRNA gene of the isolated strain was sequenced after genomic DNA extraction and PCR amplification. Two bacterial 16S rRNA primers, forward primer - (CCGAA TTCGTCGACACACAGGTTGATCCTGG CTCAG) and reverse primer - (CCCGG GATCCAAGCTTACGCTTATTTACGGTACCTGTTAC GACTT), were used for gene amplification and sequencing. PCR was run for 35 cycles under the following steps: 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min. A Bio Edit version 7.2.5 DNA Analyzer (Applied
Biosystems) was used for sequencing. The 1478-bp sequence was submitted to the Data Bank of Japan (DBJ). The nucleotide sequence of the strain was compared to any similar database sequence in the GenBank using the program BLAST version 3.2.2 via the NCBI site. The 16S rRNA sequences were aligned using clustal W program, and the phylogenetic tree was prepared using MEGA4 Software.

Optimization of culture conditions for enzyme production

The influence of different culture conditions on enzyme production was examined by using a medium containing feather meal as a sole source of carbon and nitrogen. The medium contained the following (g/L): chicken feather meal (10.0), MgSO4.7H2O (0.2), K2HPO4 (1.0), CaCl2 (0.1), and KH2PO4 (0.4). The pH of the medium was adjusted to 7.0. The influence of incubation period on keratinase production was examined at 24, 48, 72, 96, 120 and 144 h. The influence of temperature on keratinase production was examined at 25, 30, 35, 40, 45, 50, 55 and 60°C. The keratinase production was also investigated in media with various initial pHs ranging 3.0-11.0. Different inoculum sizes ranging 1-3% were also investigated. Different agitation levels ranging 50-300 rpm were also checked. Effect of substrate concentration ranging 1-3% was examined. The effect of additional carbohydrate and nitrogen sources on the keratinase production was also examined. Data were taken in triplicate. Extraction was done by taking samples from each flask and centrifuged to remove the cells and residual feathers, and keratinase activity of the supernatant was determined.

Keratinase assay

Keratinase activity was determined by taking 20 ml of 0.1 mol⁻¹ Tris buffer (pH 8) containing 0.1% feather and 40 μl of enzyme solution and was incubated for 30 minutes at 55°C. The reaction was stopped with 500 μl of 0.1 mol⁻¹ trichloroacetic acid (TCA) in 0.1 mol⁻¹ Tris buffer, pH 8.

The amino acid liberated were measured as the absorbance at 590 nm against a reagent blank and the quantity was determined from a standard tyrosine solution (50-500 μg ml⁻¹) using a spectrophotometer(Alessandro and Adriano 2006).

Results and Discussion

Isolation and screening of keratinophilic isolate

The feather-degrading microorganism isolated from poultry waste dump site showed clear zone on skimmed milk agar plate and demonstrated pronounced growth in feather meal medium which conforms it a keratinolytic organism.

The identification of this bacterial isolate was based on cell and colony morphology, growth characteristics, several biochemical tests, and 16S rRNA sequence analysis.

On the basis of morphology and different biochemical tests the isolate was identified as Bacillus subtilis.

It has been further characterized on the basis of 16s rRNA studies, and was found to be Bacillus subtilis (LC054177) having 99% similarity with Bacillus subtilis strain PWK36 Accession Number KJ620422.

The phylogenetic tree constructed from the sequence data by using clustal W and MEGA version 4 software program (Fig. 1) which showed the detailed relationships between the isolated strain and other closely related species of the genus Bacillus.
Optimization of Keratinase production by *Bacillus subtilis*

**Effect of incubation period**

The effect of incubation period for keratinase production from *Bacillus subtilis* was studied for the incubation period from 0 to 144 h as shown in (Fig. 2). It was observed that the maximum enzyme production was attained after 72 h of incubation period. Incubation beyond the optimum time showed a rapid decline in the enzyme yield, as compared to maximum (13.74 U/ml) at 72hrs. An increase in the enzyme production from 0 h towards 72 h was observed. After 72 h of incubation a decrease in the trend of enzyme activity towards 144 h was observed with minimum (1.19 U/ml) at 144 h of incubation period. The optimum incubation period in this study was found similar to the results of (Lin and Yin 2010) who observed maximum keratinase production after 72 h.

**Effect of incubation temperature**

The effect of incubation temperature for keratinase production from *Bacillus subtilis* was studied for the incubation temperature from 25°C to 60°C as shown in (Fig. 3). It was observed that the maximum enzyme production was attained at 40°C of incubation temperature. Incubation beyond the optimum temperature showed a rapid decline in the enzyme yield, as compared to maximum (13.25 U/ml) at 40°C. An increase in the enzyme production from 25°C towards 40°C was observed. After 40°C of incubation temperature a decrease in the trend of enzyme activity towards 60°C was observed. Minimum (1.90 U/ml) enzyme activity was observed at 25°C and in this case no activity was detected at 55 and 60°C of incubation temperature. The optimum cultured temperature in this study was found similar to those in the previous reports. *Bacillus* sp. FK46 (Suntornsuk et al., 2003), *Lysobacter* sp. (Allpress et al., 2002), and *Stenotrophomonas* sp. D-1 (Williams et al., 1990), showed optimum temperature for growth and keratinolytic enzyme production ranging from 20 °C to 40 °C. (Lin et al., 1999) indicated that the optimal range of temperature for keratinase production by feather-degrading *B. licheniformis* was between 40°C and 45°C. Sahoo et al., (2012) carried out fermentation reaction at temperature range of 25–45°C for optimizing keratinolytic enzyme production by *B. weihenstephaensis PKD5*, and found optimum temperature of 40°C for maximal enzyme production. Suh et al., (2001) recorded maximum temperature for keratinase production of 40°C with *Bacillus subtilis* and *Bacillus pumilis*.

**Effect of Inoculum Size**

The effect of inoculum size on the production of keratinase by *Bacillus subtilis* was studied for inoculum sizes of 1 to 6 % (v/v) as presented in (Fig. 4). From the results it was observed that the maximum production (14.04 U/ml) was obtained at 5% of inoculum size. *Bacillus subtilis* showed higher production of keratinase as the inoculum size was increased above 2%. Minimum enzyme activity of (4.69 U/ml) was observed at 1% of inoculum size. Results of the present study was found similar to the previous studies as many workers have described that higher keratinase production is obtained at higher percentage of inoculum sizes, for instance (Lateef et al., 2010) observed maximum keratinase production at 5% concentration of inoculum size with *B. cereus LAU08* strain.
**Fig. 1** Phylogenetic position of *Bacillus subtilis* S1 (LC054177) based on 16S rRNA sequence within the genus *Bacillus*. The sequences were aligned using the Clustal W program and MEGA4 software.

**Fig. 2** Effect of Incubation period on keratinase production by *Bacillus subtilis* S1.

**Fig. 3** Effect of Incubation temperature on keratinase production by *Bacillus subtilis* S1.
Fig. 4 Effect of inoculum size on keratinase production by *Bacillus subtilis* S1

![Inoculum Size vs Keratinase Production Graph](image1)

Fig. 5 Effect of pH on keratinase production by *Bacillus subtilis* S1

![pH vs Keratinase Production Graph](image2)

Fig. 6 Effect of agitation level on keratinase production by *Bacillus subtilis* S1

![Agitation Level vs Keratinase Production Graph](image3)
Fig. 7 Effect of substrate concentration on keratinase production by *Bacillus subtilis* S1

![Graph showing the effect of substrate concentration on keratinase production by Bacillus subtilis S1.]

Fig. 8 Effect of additional carbon source on keratinase production by *Bacillus subtilis* S1

![Graph showing the effect of additional carbon source on keratinase production by Bacillus subtilis S1.]

Fig. 9 Effect of additional nitrogen source on keratinase production by *Bacillus subtilis* S1

![Graph showing the effect of additional nitrogen source on keratinase production by Bacillus subtilis S1.]

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Similarly, Suntornsuk and Suntornsuk (2003) found 5% of initial inoculum as optimal for keratinase production by Bacillus sp. FK 46. Sivakumar et al., (2013) observed 4% inoculum size to be optimum for keratinase production by B. cereus TSI.

**Effect of pH**

The effect of pH on keratinase production from Bacillus subtilis was studied for the range of pH 3 to pH 10 as shown in (Fig. 5). According to the results an increase in the enzyme production from pH 3 towards pH 7 was observed and maximum enzyme production from the bacterium was attained at pH 7. pH beyond the optimum showed a gradual decrease in the enzyme yield, as compared to maximum (13.18 U/ml) at pH 7. After pH 7 a decrease in the trend of enzyme activity towards pH 10 was observed. The present results are in accordance with some of the previously reported keratinases, indicating that the keratinase produced by Bacillus species might be most active in neutral or basic conditions. Jeong et al., (2010) found 7.0 as an optimum pH for keratinase production by Stenotrophomonas maltophilia R 13. According to Suntornsuk and Suntornsuk (2003) during production of keratinase, keratin utilization occurs more rapidly and to a great extent at pH 7.5. Kim et al., (2001) reported the optimum pH for keratinase production by B. cereus at 7.0.

**Effect of agitation level**

In the present study effect of different agitation level i.e. 100 rpm, 150 rpm, 200 rpm, 250 rpm and 300 rpm was studied for keratinase production by Bacillus subtilis as shown in (Fig. 6). According to results obtained in the study the shaking speed at 150 rpm yielded maximum keratinase production. But the production of keratinase start decreasing after 150 rpm and found lowest production at 300 rpm. Generally, increased shaking speed provided high oxygen transfer rate supporting cell growth. But high shaking speed (200-300 rpm) may give good bacterial growth but it lowers rate of keratinase production possibly because of too high dissolved oxygen and too much shear stress which may have repressed keratinase synthesis and excretion. While at low shaking speed (100 rpm), low keratinase production was observed, this could be due to at low shaking speed bacterial cells and substrate were not well mixed with heterogeneous distribution and lower oxygen dissolved at this speed results in low keratinase production. Similar results were reported in some of the previous studies such that (Pissuwann and Suntornsuk, 2001) reported shaking speed of 150 rpm yielded maximum keratinase production by Bacillus sp. FK 28. (Hossain et al., 2007) reported 120 rpm of agitation speed for maximum keratinase production from B. licheniformis MZK-3. But on the other hand present study was found contradictory to the results of previous studies for example Cai et al., (2011), Jeong et al., (2010), Park and Son (2007), Refai et al., (2005) reported 200 rpm of agitation speed for maximum keratinase production by B. pumilus FH-9, B. megaterium F7-1, Stenotrophomonas maltophilia R-13 respectively.

**Effect of substrate concentration**

In the present study the effect of substrate concentration on the production of keratinase by Bacillus subtilis was studied for a substrate concentration range of 1% to 3% which is shown in (Fig. 7). According to the results the highest keratinase production was obtained at 1% feather powder concentration. Keratinase produced at a level of 0.5% feather powder was less than that produced at 1% feather powder. This may be because the amount of substrate supplied for growth and enzyme
production was insufficient, while substrate concentration above 1% i.e. from 1.5%-3% showed a decreasing trend in keratinase production. This decreasing trend in keratinase production above 1% feather powder concentration is due to substrate repression on keratinase production. High feather powder concentration may also have increased the medium viscosity which possibly results in oxygen limitation for bacterial growth. The results in the present study were found in accordance with the previous studies in the keratinase production. Cheng et al., (1995) reported that 1% feather powder gave the highest keratinase activity for B. licheniformis PWD-1. Brandelli and Riffle (2005) also indicated that the production of keratinase by Chryseobacterium sp. Kr6 was repressed by higher percent of keratin substrate in the production medium.

**Effect of additional carbon source**

The effect of different carbon sources such as Lactose, maltose, fructose, glucose, sucrose, dextrose and mannitol on keratinolytic enzyme production from Bacillus subtilis is shown in (Fig. 8). Bacillus subtilis produced appreciable level of keratinolytic enzyme when cultivated in a medium containing feather as the sole carbon source. Additionally, extra carbon sources adversely affected the production of keratinase enzyme and lower enzyme production was achieved as compared to control in which only feather was used as carbon source. A decrease in the keratinase production due to the addition of conventional carbon sources is reported in the present study were in agreement with the earlier research, for example, Anbu et al., (2007), Ignatova et al., (1999), Yamamura et al., (2002) described that the decrease in keratinase production by adding conventional carbon sources may be due to the catabolic repression of keratinase. Kainoor and Naik (2010) illustrated that keratinase production from Bacillus sp. JB 99 was most inhibited in the presence of dextrose, followed by citric acid and glucose. Ramnani and Gupta (2004) in case of Bacillus sp. MIR-99 also found that addition of glucose, glycerol and sucrose to the production medium suppressed the enzyme secretion.

**Effect of additional nitrogen source**

The effect of different nitrogen sources such as beef extract, peptone, yeast extract urea, ammonium nitrate, ammonium sulfate and ammonium chloride on keratinolytic enzyme production from Bacillus subtilis was studied as shown in (Fig. 9). Bacillus subtilis produced appreciable level of keratinolytic enzyme when cultivated in a medium containing feather as the sole nitrogen source. Additionally, extra nitrogen sources such as beef extract, peptone, and yeast extract had a positive influence on enzyme production, resulting in considerable increases in enzyme production over the control. On the other hand, when other nitrogen sources such as urea, ammonium nitrate, ammonium sulfate and ammonium chloride were used as extra nitrogen sources in production medium, reduced enzyme production considerably. Maximum enzyme (13.71 U/ml) production was obtained in presence of Beef extract as additional nitrogen source and minimum (2.62U/ml) in presence of urea. These results were in accordance with those in some previous investigations. For instance, (Park and Son 2009) found that beef extract, casein, gelatin, skim milk, tryptone, and yeast extract had a positive influence on enzyme production by Bacillus megaterium F7-1. (Malviya et al., 1992) also found similar results for Chrysosporium queenslandicum. However, results of this study were also found in contrast with some studies of the previous literature for example (Refai et al.,
2005) showed that extra NH4Cl as nitrogen source have a favorable effect on keratinase production by B. pumilus FH9. (Nilegaonkar et al., 2007) reported that increased level of keratinase production by B. cereus MCM B-326 was observed to be up due to the addition of ammonium chloride and sodium nitrite compared with other inorganic nitrogen sources.

Keratinases are the special class of proteolytic enzymes which have gained importance in various industries such as in pharmaceutical, cosmetic, leather and feed processing industry, as well as in keratin waste treatment. Various production parameters were optimized to produce keratinase by Bacillus subtilis S1. This strain displayed good capability of keratinase production after 72 h of incubation at 40°C and pH 7.0, with agitation level of 150 rpm in a 1% feather concentration. The best additional nitrogen source was Beaf extract and all the additional carbon sources showed a negative effect on keratinase production. These results indicate that this bacterial strain shows a high potential for keratinase production in submerged state fermentation, and use of feather waste as the substrate can be implemented for keratinous solid waste management.

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