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Optimization of Keratinase Enzyme Production from Chicken feathers Using *Bacillus subtilis NCIM 2724*

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Abstract - The Present study deals with the Keratinase enzyme production using Bacillus subtilis NCIM 2724 under submerged fermentation using chicken feather as a substrate. Optimization of fermentation conditions for maximum production of keratinase were carried out by one factor at a time procedure. Various combination of substrates were also tried to achieve maximum keratinase production. The crude enzyme showed maximum keratinase activity of 59.03 IU/ml. The optimum conditions for maximum keratinase activity were found to be pH – 8.0, temperature – 40^{0}C, feather powder – 2.0% as a substrate and an incubation time of 48 hrs respectively, which indicated that the crude Keratinase enzyme produced by the Bacillus species was classified as alkaline proteases. FTIR Fourier Transform Infra-red spectroscopy showed that the change in the functional group was catalysed by unique enzymes of Bacillus species.

Key words: Keratinase, powdered chicken feather, submerged fermentation, FTIR

1. INTRODUCTION

Feathers which are pure Keratin proteins are produced in large amounts as a waste at Poultry processing plant. A total of 5-7% weight of mature chicken Comprises of feathers**.** Feather waste constitutes beta-keratin, an insoluble protein**.** In addition to this, feather waste is produced at the rate of 22 million Kg per year (US) alone). Keratin is a tough protein substance, is the chief constituent of epidermal layer of skin, hair, feather, nail, hoof, wool and certain shell. As much as 90% of feather is made up of keratin, the fibrous and insoluble structural protein. Mechanical stability of keratin and its resistance to biochemical degradation depend on tight packing of protein chain in the (α-helix) or β–sheet (β-keratin) structure and linkage of the structures through disulphide bonds **[1]**. Worldwide 24 billion chickens are killed annually and around 8.5 billion tonnes of poultry feather are produced. According to a recent report in leading newspaper India's contribution alone is 350 million tonnes. The poultry feathers are dumped used for land filling incinerated or buried which involves problems in storage, handling, emission control and ash disposal. Discarded feather also causes various human ailments including chlorosis, mycoplasmosis and fowl cholera **[2]**. Keratinase producing microorganisms have the important Industrial application in fermentation technology. Submerged fermentation of poultry Waste by microorganism producing keratinase helps in the conversion of non-soluble Keratin feather into soluble protein or polypeptide **[3].** These protein by-product may be used as animal and livestock feed and as leather filling agents. Keratinase has also emerging applications in dehairing process in leather industry Instead of sodium sulphides and also used as a detergent to remove Strains on cloth.

Bacterial strains are known which are capable of degrading feathers. These bacterial strains produce enzyme which selectively degrades the beta-keratin found in feathers. This energy makes it possible for the bacteria to obtain carbon, sulphur and enzyme for that growth and maintenance from the degradation of beta keratin **[4].** Several workers have reported that the Genus Bacillus are the prime source of extracellular keratinase enzyme which could degrade keratin substrate and environmentally safe **[5].** Most of the keratinases reported to date have been found to be serine proteases and a few metallo proteases have shown Keratinolytic activity. Almost all keratinases are inducible and different keratin-containing materials such as feathers, hair and wool can be used as substrates for keratinase production**[6]**.In fact, the global enzyme market is expected to reach U.S dollar 7 billion by 2015 **[7]**. Thus keratinase with robust characteristics like oxidation stability, alkaline stability, detergent compatibility and temperature tolerance can fetch a good market among detergent proteases **[8]**. Additionally, keratinases are postulated for utilization in food, feed, detergent, leather and biomedical industries **[9]**.

The present research reported the optimization of process parameters for maximum keratinase production by *Bacillus subtilis NCIM 2724 under* submerged fermentation using the classical method.

2. MATERIALS AND METHODS

2.1. Preparation of substrates and media

White chicken feathers were used in this study. They were first washed extensively under tap water to remove blood and any dust particles. This was followed by washing with0.1% Triton x-100, and then abundantly with distilled water **[10].** The samples were later oven dried at 75^0C for 8 h. The powders were kept at room temperature and used for further studies.

The medium used for keratinase production contained the following constituent: The feather meal media 2 (g/l): NH_4Cl, 0.5; Nacl, 0.5; K_2HPO_4, 0.3; KH_2PO_4, 0.4; $MgCl_2.6H_2O$, 0.1; Yeast extract, 0.1 and feather, 10; pH 7.5 **[11].**

2.2 Microorganism and culture conditions

Bacillus subtilis No- 2724 used in this study was purchased from NCIM Resource National Chemical Laboratory, Pune - Maharashtra. The medium used for the growth and maintenance of the culture comprises of (g /L):Sodium chloride (NaCl) – 0.5, Ammonium Chloride (NH_4Cl) – 0.5, Di-potassium hydrogen orthophosphate (K_2HPO_4) – 0.3, Potassium hydrogen phosphate (K_2HPO_4) – 0.3, Magnesium Sulphate ($MgSO_4$) – 0.1,Yeast extract – 0.1,Distilled water – 1000 ml(pH 7.5 ± 0.2).

Bacterial cells in agar slants were incubated at 37°C for 24 h and the stock culture was stored at 4°C.

2.3. Production of Keratinase enzyme

Keratinase enzyme was carried out by seeding inoculums (2%w/v) in 250ml Erlenmeyer flasks containing 50 mL fermentation medium (feather meal medium 2) and incubated at 37^0C on a shaker operated at 200 rpm for 5 days. After incubation the culture medium was filtered through Whatmann No.1 filter paper to remove the non-degraded residues. Further, the cells were removed by centrifugation at 10,000 rpm for 10 minutes and the culture supernatant was used as crude enzyme extract and examined for enzyme activity **[12].**

2.4. Keratinase enzyme assay

The keratinolytic activity was assayed with 1 ml of crude enzyme properly diluted in phosphate buffer (0.05 M of PH 7.0) was incubated with 1 ml keratin solution at 50^0C in a water bath for 10 min, and this elevated temperature was used for the enzyme incubation to accelerate substrate hydrolysis**[12]**. After incubation at 50^0C for 10 min, Reaction was terminated by the addition of 2 ml of 0.4 M chilled Trichloroacetic acid then the reaction mixture was allowed to stand in ice for 15 min to precipitate the insoluble proteins. The undigested proteins were removed by centrifugation at 10,000 rpm for 5 min and amino acid released was assayed. The supernatant was separated by centrifugation at 10,000 rpm for 10 min; the acid soluble product in the supernatant was neutralized with 5 ml of 0.5 N sodium bicarbonate solution. The colour developed after adding 0.5 ml of 3 fold diluted Folin-Ciocalteau reagent was measured at 660 nm. The amount of tyrosine released was read from the tyrosine standard graph. One unit of keratinase activity was defined as the amount of enzyme required to release 1μ g of tyrosine under the given set of assay conditions [**13**].The quantity was determined from a standard tyrosine solution(50-500μg/ml) using a spectrophotometer. All assays were done in triplicate [**14].**

2.5. Growth curve

Sterile nutrient broth medium was inoculated with 4%(v/v) inoculums of the 24 h old culture of the *Bacillus subtilis NCIM 2724.*The culture was incubated at 37^0C in an orbital shaker at 200 rpm. The culture samples were withdrawn at regular 4 h intervals for a period of 48 h and the growth was determined by reading the optical density of the sample at600 nm in an UV Spectrophotometer against an uninoculated broth which acts as a control **[13].**

2.6. Factors affecting Keratinase production

2.6.1. *Effect of initial pH and temperature on Keratinase production*

The effect of initial pH of the medium on Keratinase production was observed by using different pH like6, 7, 8, 9, 10 and 11 and the effect of initial temperature of the medium on Keratinase production was observed by using different temperatures like 20^0 C, 30^0C, 40 ^{0}C, 50^0 C, 60^0C, 70^0C and 80^0C. Inoculated mediums were incubated at the above mentioned temperatures and the absorbance of the medium was measured using spectrophotometer at 660 nm **[15].**

2.6.2. Effect of Incubation time on Keratinase production

The *Bacillus* species was inoculated into the feather meal medium and incubated at 40^0C. The Keratinase activity was measured at regular intervals for 4 days (12h, 24h, 36h, 48h, 60h, 72h, and 84h). From the

graph the time required to attain the higher enzyme production was determined.

2.6.3. Effect of substrate concentration on Keratinase production

The amount of Keratinase production depends on substrate concentration and cultivation conditions. The effect of substrate concentration was studied by varying the powdered chicken feather concentration from (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, and 4.0) %(w /v). The culture was incubated at 40^{0}C for 48 h. At the end of incubation the supernatant was collected by centrifugation and Keratinase activity was measured for determining the optimum substrate concentration **[16].**

2.6.4. Effect of Inoculum concentration on Keratinase production

The effect of Inoculum concentration was studied by varying the inoculum concentration such as (1, 2, 3, 4, 5, 6, 7) % (w/v) to test its ability to induce keratinase production in the production medium. The culture was incubated at 40^{0}C for 48 h. At the end of incubation the supernatant was collected by centrifugation and Keratinase activity was measured for determining the optimum inoculums concentration **[17].**

2.6.5. FTIR

Fourier transform infrared spectroscopy was used to investigate the changes of functional groups in feather degradation. Dry samples were grinded with KBr and made into transparent pellets at 1MPa pressure. The measurements were carried out in the mid-infrared range from 4000 to 400cm^{-1}.Fourier Transform infrared spectroscopy (FTIR) is a powerful tool for identifying types of chemical bonds in a molecule by producing an infrared absorption spectrum that is like a molecular finger print. Feather sample degraded by *Bacillus subtilis NCIM 2724*was collected after 48 h of incubation. The feather residue was air dried and used for FTIR analysis **[18].**

3. RESULTS AND DISCUSSION

3.1. Effect of Incubation Period

As shown in Fig.1, after 12h incubation, the culture entered stationary phase and the Keratinase production was greatly increased and reached a maximum at 48h. There after the Keratinase production was declined. The results of Keratinase production had a positive impact on the fermentation period. Enzyme production was higher after 36h of incubation, and reaches maximum at 40^{0}C.Similar result was given by **[19]**, on *Bacillus* sp. *JB 99*which took considerably less period of incubation 36 h for maximum keratinase enzyme production .

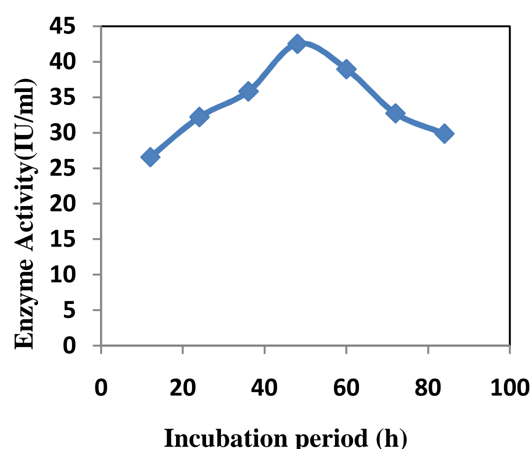

Fig.1. Effect of Fermentation period on Keratinase production

3.2. Effect of pH and temperature on Keratinase production

The pH and temperature are two vital factors that aid in providing a particular characteristic feature to the microorganisms such as thermophilic, mesophilic, psychrophilic, alkaliphilic or acidophilic **[12]**. Alkaline protease production by microbial strains strongly depends on the extracellular pH, because culture pH strongly influences many enzymatic processes and the transport of various components across cell membranes, which in turn supports cell growth and enzyme production **[20]**. The high optimum pH is a feature of alkaline proteases **[21]**. In this study, optimum biosynthesis of Keratinase was observed at pH of 8.0 as shown in Fig.3 and decreased thereafter. The analysis of temperature dependent *Bacillus subtilis NCIM 2724* Keratinase activity revealed that the catalytic behaviour of the enzyme increased with increasing temperature, up to 40^{0}C as shown in Fig.2 and increasing the incubation temperature beyond 40^{0}C drastically reduced enzyme activity. Similar kind of protease properties were studied on *Bacillus subtilis* **[22]**.

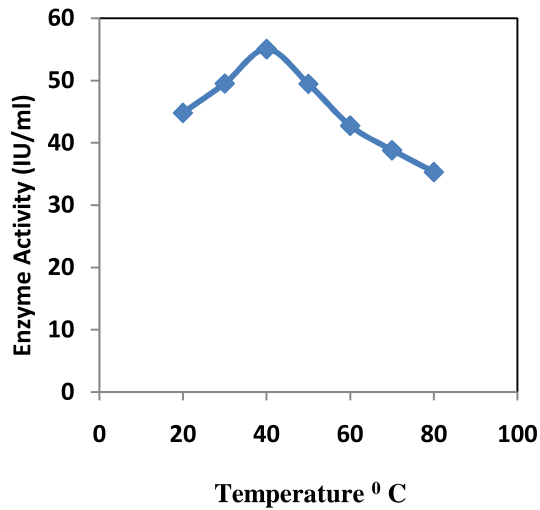

Fig.2. Effect of Temperature on Keratinase production

3.3. Effect of Substrate concentration on Keratinase production

Significant improvement in yield of Keratinase was observed on the medium with 2% powdered chicken feather and is shown in Fig.4. This was because the amount of substrate supplied for growth and enzyme production was sufficient. Similar kind of enzyme production was observed on *Bacillus megaterium (A1)* **[23]**.

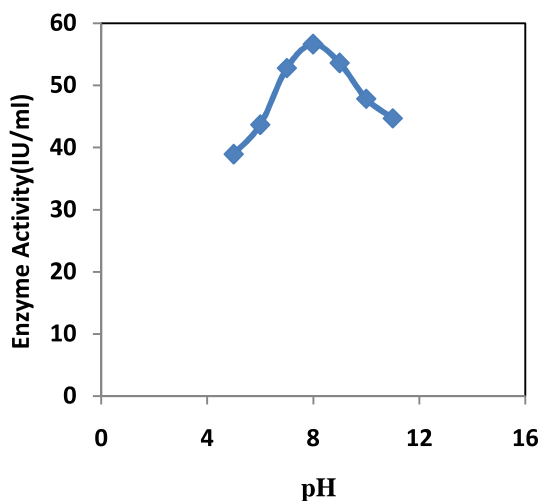

Fig.3. Effect of pH on Keratinase production

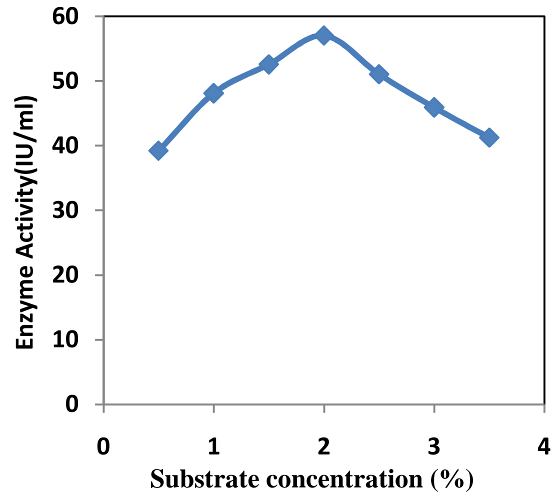

Fig. 4. Effect of Substrate concentration on Keratinase production

3.4. Effect of Inoculum concentration on Keratinase production

Maximum Keratinase enzyme activity of 36.03 IU/ml was recorded at 48h, at 4% of inoculum concentration . Sivakumar et al., reported that 4% of inoculum size gave the maximum keratinase production with chicken feather as substrate **[17]** which is in good agreement with the present work.

3.5. FTIR

The result of FTIR studies indicates a pronounced difference between the control and the experimentally treated feather samples. In control Keratin exhibited a peak value of 3295.63cm^{-1} whereasexperimental one such as feather treated with *Bacillus subtilis NCIM 2724* showed the peak value of 3294.99 cm^{-1}. It indicated that due to degradation of Keratin OH stretches carboxylic acid. Similarly in a control treated the second peak values were found to be 3080.25 cm^{-1} while comparing the peak obtained in experimental treated *Bacillus subtilis* was found to be 3078.22 cm^{-1}. The peak difference was exhibited because of Aliphatic, CH subsequent stretch. Simultaneously the peak obtained in control recorded on 1640.55

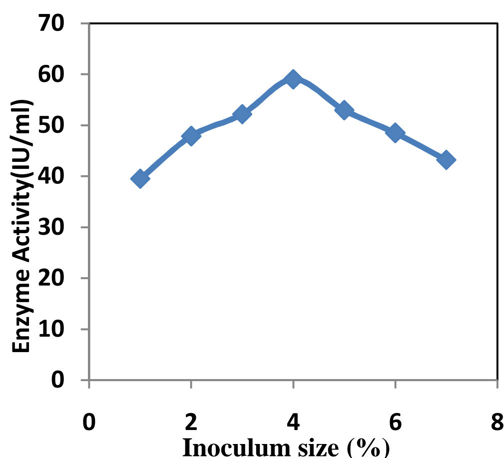

Fig.5. Effect of Inoculum size (%) on Keratinase production

cm^{-1}. Similarly in experimental treated with *Bacillus subtilis NCIM 2724*, it showed a dual peak entity such as C=N or C=O which was a separate entity (carboxyl) which is an indication of carbon and nitrogen binding entities. On analysing the final control sample the peak revealed a value of 1640.55 cm^{-1}. Besides on treatment with *Bacillus subtilis* the peak value was found to be 1159.71& 1084.75cm^{-1} which is an indication of Benzo thio diazole, i.e., a peak corresponding to 5 member rings was recorded **[23]**.

4. DISCUSSION

Several external factors affect the Keratinase enzyme production. This factor includes variations inthe pH, temperature, fermentation period substrate concentration& inoculum size. Changes in pH affect the enzyme production but it may also change the properties of the substrate. Keratinase enzyme production was observed at pH range from 5, 6, 7, 8, 9, 10&11 for *Bacillus subtilis NCIM 2724* respectively. Maximum enzyme production was achieved at medium pH of 8.0 for *Bacillus subtilis.* For pH values less than and greater than 8, the production rate was decreased gradually as shown in Fig.3. **[24]** reported that pH 9.5 gave the maximum keratinase production utilizing *Bacillus sp.001A and also* found that the optimum pH for Keratinase production by *Bacillus sp.001A* waspH9.5.**[25]** also reported similar results of keratinase enzyme production by *Bacillus licheniformis PWD-1 at* pH 8.7.

Maximum Keratinase production of 55.017 was observed at 40^{0}C. Keratinase production was observed to be low at 50^{0}C and 60^{0}C because of an absence of bacterial cell growth at such high temperatures, whereas the highest bacterial growth and keratinase production were found at the incubation temperature of 37^{0}C. Keratinase is mainly produced during the late exponential or stationary phase of microbial growth, whereas keratin degradation takes from 24 h to several days **[26]**.

In the present study, maximum keratinase enzyme of 42.523 IU/ml was produced by *Bacillus subtilis NCIM 2724 at 48* h (Fig.1), whereas further incubation did not result in any significant increase in enzyme production. The amount of Keratinase production depended on substrate concentration and cultivation conditions. The effect of various chicken feather powder concentrations on Keratinolytic enzyme production is shown in Fig.4. In this work 2.0%(w/v) feather powder gave the highest activity for *Bacillus subtilis NCIM 2724*.The time required to attain the maximum keratinase enzyme production as determined, and reaches maximum at 48h of incubation. **[27]** reported the keratinolytic enzyme production increased as the amount of feather increased from (0.5-1.5%, w/v), but when the concentration was raised to 2% (w/v), enzyme production slightly decreased. The highest enzyme production was attained at 1.5%(w/v) feather powder. Because higher substrate concentrations (3% and 5% w/v) may cause substrate inhibition or repression of keratinase production. On the other hand, high feather concentration increased medium viscosity which possibly resulting in oxygen depletion for bacterial growth.

An inoculums concentration higher than the optimum value may produce a high amount of Biomass which rapidly depletes the nutrients necessary for growth and product synthesis. On the other hand, lower inoculums levels may give insufficient biomass and allow the growth of undesirable organisms in the production medium. This increases the necessary time to grow to an optimum number to consume the substrate and synthesis the desired product. In the present study, the highest enzyme activity (59.03 IU/ml) as shown in Fig.5 was obtained at an inoculums level of 4% by *Bacillus subtilis NCIM 2724,* under SmF. Also the most studied Keratinolytic bacteria are *Bacillus sp.*. which have been described to possess feather – degrading activity **[28, 29]**.The growth curve of the *Bacillus subtilis NCIM 2724* has been studied by culturing the bacterium in powdered feather medium at pH 7.5 and temperature of 37^{0}C in an orbital shaker at 120 rpm. The growth curve shown in Fig.6 indicates logarithmic growth till 16thhr and reached an early stationary phase by 20th hour of growth.

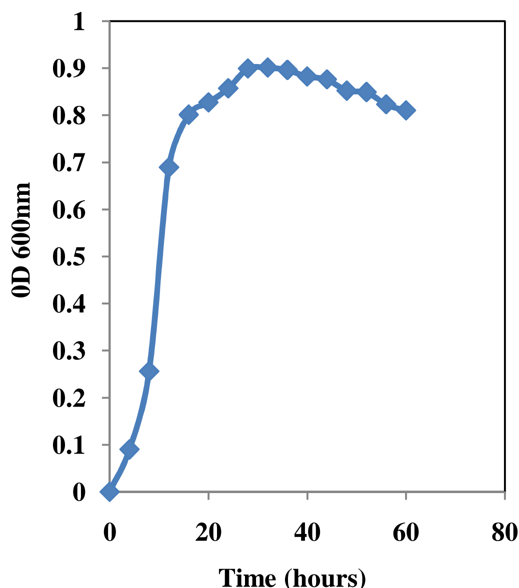

Fig. 6. Growth curve of the Bacillus subtilis NCIM 2724

The functional groups of the feather were directed by FTIR, and the result is given in Fig.7. FTIR spectra of degraded feather displayed that transmittance peaks nearby 669.44, 1236.69, 1542.60, 1651.82, 2960.72 and 3294.99cm^{-1}. The peak located in the range of 2847.27-3078.22 cm^{-1} indicate the presence of CH groups and the broad peak around 3294.99 cm^{-1}is usually caused by the vibration of hydrogen bonded –OH groups. The transmittance peaks for the amide I (1651.82cm^{-1}) and amide II (1542.60cm^{-1}) suggests the presence of an α- helix structure in the sample. The peak near1072.59 was observed, and this fact indicated that C-C groups existed in each of the two samples additionally, as shown in Fig.6 compared with the processed (incubated with *Bacillus subtilis NCIM 2724*) and unprocessed feather powder, the peaks of the disulphide bonds of the processed feather weaker than the native feather powder was observed, it exhibited the disulphide bond structure of the feather was attacked by *Bacillus subtilis* **[30]**. The present result shows good agreement with **[30]**.

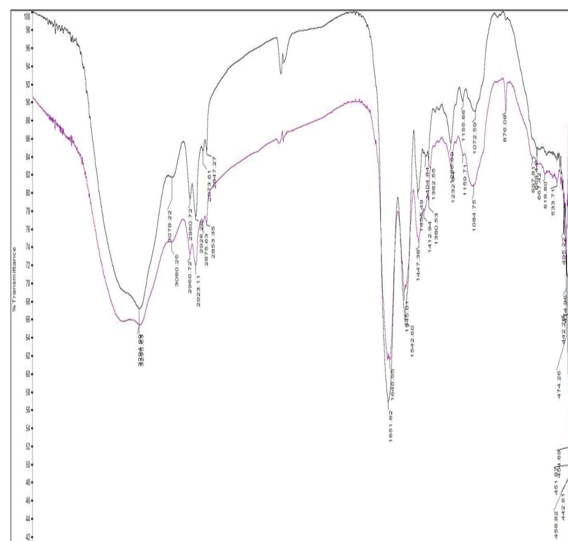

Fig.7 IR spectra of degraded feathers and native feather powder

5. CONCLUSION

The objective of the present investigation was to optimise the process parameters for keratinase enzyme production. The *Bacillus subtilis NCIM 2724*was found to be a profound producer of keratinase enzyme. The maximum extracellular, active alkaline serine keratinase production was achieved after 36 h of incubation time and reaches maximum at 48 h. Optimum pH and temperature for keratinase production was found to be 8.0 and 40^{0}C respectively. Further in the present study, the classical ''one variable at a time approach'' was employed to evaluate the effect of operating conditions, including incubation time, temperature and initial pH of the medium on keratinase production.

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