SCREENING AND CHARACTERIZATION OF KERATINASE FROM *Bacillus licheniformis* ISOLATED FROM NAMAKKAL POULTRY FARM

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Abstract: Keratin are insoluble fibrous proteins found in hair, wool, feather, nail, horns and other epithelial covering which is rich in beta helical coil linked through cysteine bridges. Keratinase (EC 3.4.4.25) belongs to the class hydrolase which are able to hydrolyse insoluble keratins more efficient than other proteases. The bacteria *Bacillus licheniformis* showing higher keratinase activity was screened out of the ten different bacterial strains isolated. The ability of *Bacillus licheniformis* to utilize chicken feather powder as a substrate was tested. It was found that maximum enzyme activity was 10.76U/ml. Similarly optimum temperature and pH for the enzyme activity was found to be 60°C and 7.0 respectively. The k_m and V_{max} values were 0.22 mg/ml and 0.01 U/ml respectively. The enzyme is stable (30-40°C) and active around wide pH range (6-8). Among the various metal ions tested zinc, magnesium were found to enhance the enzyme activity where as mercury, copper, cadmium, 1, 10 phenanthroline and EDTA completely inhibit the enzyme activity. It was found from this study, organism such as *Bacillus licheniformis* isolated from poultry soil can be used as a potential candidate for degradation of feather and for dehairing process in leather industry. [Researcher 2010;2(4):89-96]. (ISSN: 1553-9865).

Key words: Feather, Keratin, *Bacillus licheniformis*, degradation, dehairing

1. Introduction

Keratinase producing bacteria are *Bacillus licheniformis.* (Zerdani *et al*., 2004; Ramnani *et al*., 2005; Korkmaz *et al*., 2004; Manczinger *et al*., 2003; Williams *et al*., 1990; P.Tamilmani *et al*., 2008). *Burkholderia*, *Chryseobacterium*, *Pseudomonas*, *Microbacterium sp* (Brandell and Riffel, 2006) were isolated and was studied with respect to different parameter. This enzyme has been produced by fungi, including the species of *Aspergillus, Onygena, Absidia* and *Rhizomucor* (Friedrich *et al*., 1999), some species of dermatophytes, including *Trichophyton mentagrophytes, T. rubrum, T.gallinae, Microsporum canis* and *M. gypseum* (Wawrzkiewicz *et al.*, 1991). The enzyme is a potential enzyme for removing hair and feather in the poultry industry (Takami *et al*., 1992), for nutritional upgrading of feather meal and conversion of feathers into a feed protein in feed industry (Williams *et al*., 1991), and for clearing obstructions in the sewage system during waste water treatment and eco-friendly de-hairing process in leather industry. However, much current research is centered on the potential use of keratinase of bacterial origin for the industrial treatment of keratin-containing compounds. Keratinolytic enzymes have been studied from a variety of fungi, but to a lesser extent in bacteria. With this background, this study was designed to identify feather degrading bacteria from Namakkal poultry farm soil which produce high amount of keratinase.

2. Materials and Methods:

2.1 Isolation of microorganism:

Samples (soil and feather) were taken from the poultry farm in the town of Namakkal (Tamilnadu). Serial dilution for each sample was prepared by adding 1 g of the soil sample to 9 ml of sterile saline. Then serial dilution up to 10^{-9} was done using sterile saline. All the dilutions were plated on Nutrient Agar medium and incubated at 37°C for 24 hours. The colonies appeared was checked for the presence of spore and streaked onto agar slants for further characterization.

2.2 Characterization:

All the collected strains were grown on Nutrient Agar medium for fresh cultures. Spore production and localization were examined by microscopic observations. The identification was done according to the method described by Larpent and Larpent-Gourgaud (1985).

2.3 Identification of Isolated feather degrading bacteria:

The organism was identified and confirmed by carrying out tests like Gram Staining, Spore staining, Motility test, Catalase Test, Starch hydrolysis test and casein hydrolysis test

2.4 Screening for Keratinolytic bacteria:

Among the different bacterial colonies obtained on the spread plated agar plate. Ten different morphologically different bacterial colonies were identified and each inoculated onto a sterile feather meal agar plate. The inoculated plate was then incubated at 37°C for 48 hours. The strain that shows high zone of clearance was selected and it was sub cultured. The strain was further grown on nutrient broth containing feather meal and kept for incubation at 40°C for 7days with shaking at 150 rpm. Then the culture supernatant was assayed for kerotinolytic activity. The strain, which degraded keratin effectively and it was further identified by 16s rRNA amplification and nucleotide sequencing done at the Institute of Microbial Technology (IMTECH), Chandigarh, India.

2.5 Preparation of keratin solution:

Keratinolytic activity was measured with soluble keratin (0.5%, w/v) as substrate. Soluble keratin was prepared from white chicken feathers by the method of (Wawrzkiewicz et *al.,* 1987). Native chicken feathers (10 g) in 500 ml of dimethyl sulfoxide were heated in a hot air oven at 100 °C for 2 h. Soluble keratin was then precipitated by addition of cold acetone (1 L) at −70 °C for 2 h, followed by centrifugation at 10, 000×g for 10 min. The resulting precipitate was washed twice with distilled water and dried at 40 °C in a vacuum dryer. One gram of quantified precipitate was dissolved in 20 ml of 0.05M NaOH. The pH was adjusted to 7.0 with 0.1M Hydrochloric acid and the solution was diluted to 200 ml with 0.05 mol/L Phosphate buffer (pH 7.0).

2.6 Keratinase activity assay:

The keratinolytic activity was assayed 1.0 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 °C in a water bath for 10 min, and the reaction was stopped by adding 2.0 ml 0.4M Trichloroacetic acid (TCA). After centrifugation at 1450×g for 30 min, the absorbance of the supernatant was determined at 280 nm against a control. The control was prepared by incubating the enzyme solution with 2.0 ml TCA without the addition of keratin solution.

One unit (U/ml) of keratinolytic activity was defined as an increase of corrected absorbance of 280 nm (A_{280}) (Gradisar *et al*., 2005) with the control for 0.01 per minute under the conditions described above.

2.7 Characterization of crude enzyme:

Effect of pH:

The optimum pH of the crude enzyme was found by dissolving the keratin at various buffers using 0.05 mol/L acetate buffer (pH 4-5.5), 0.05 mol/L phosphate buffer (pH 6-7.5), 0.05 mol/L Tris-HCl (pH 8-9), Based on the above mentioned pH the keratin solution is prepared for various buffers. The experiment on the effect of pH on enzyme stability was carried out by incubating the enzyme solution at pH ranges of 4-9. Then the enzyme activity was determined by the standard enzyme assay.

Effect of temperature:

With the optimum pH of the crude enzyme as constant the optimum temperature was found by incubating the enzyme with the substrate (keratin solution prepared according to optimum pH) at varying temperature range from 30 to 80°C. The experiment on the effect of temperature on enzyme stability was carried out by incubating the enzyme solution at temperature ranges of 30-80°C Then the enzyme activity was determined by the standard enzyme assay.

Substrate concentration:

The k_m and Vmax value for the crude keratinase is determined by using different concentrations of keratin. For this stock solution of keratin is prepared by dissolving 0.5 g in 50 ml phosphate buffer of pH 7. The stock solution is diluted for different

concentrations (0.1-0.7 g) in a series of test tubes using phosphate buffer.

Effect of Metal ions on enzyme activity:

To study the influence of enzyme inhibitor, crude keratinase (1ml) was incubated with varying concentration (1-3mM) of 1.10 Phenanthroline and EDTA in 0.05mol/l phosphate buffer (pH 7) at 37°C for 30min. The effect of divalent cation on keratinolytic activity was determined by incubating the crude enzyme in the presence of Zinc, magnesium, copper, mercury, cadmium (Zn^{2+}, Mg^{2+}, Cu^{2+}, Hg^{2+}, Cd^{2+}) at (1-3mM) concentration for 1h at 37°C. Then the enzyme activity was determined using lysis of feather powder as described above

3. Results Analysis

Bacterial selection and identification:

A total of ten keratinase producing bacteria were screened for feather degrading properties. It was found that the NS-3 strain was only the feather degrading strain (shown in table-1) capable of growing and degrading feather at 40°C within 7 days. The NS-3 strain (NS- Namakkal soil) which appeared singly or in chain and was straight rods, Gram positive and endospore-forming organism. It was aerobic, motile, and strong oxidase and catalase positive. Additional morphological, physiological and biochemical test were conducted as shown in (Table 2). The feature agreed with description of Bergey's Manual of systematic Bacteriology. It was identified to be *Bacillus licheniformis.* On the basis morphological characteristics and 16s rRNA studies, the strain was found to be *Bacillus* sp., .having 99% similarity with *Bacillus licheniformis.* From this result, the NS-3 strain was identified as *Bacillus licheniformis.*

Degradation of feather by *Bacillus licheniformis*:

Bacillus licheniformis was able to grow and produced keratinase in nutrient medium in which feather meal served as a additional carbon and nitrogen source and acted as enzyme inducer, resulted in nearly complete degradation of the feather after 7 days incubation at 40°C at 150 rpm. Keratinase activity was associated with growth at the maximum level of 10.76 U/ml. Kerotinolytic activity was measured in the absorbance at 280nm by the standard enzyme assay method.

Characterization of keratinase:

The strain *Bacillus licheniformis* grew well and completely degraded poultry feather in the nutrient medium. The intense feather degrading was achieved in 40°C and initial pH adjusted to 7and 8. Similar growth curve were observed with in this range of temperature and pH. The keratinase was active in neutral and alkaline condition with an optimum activity at pH 7 as shown in (Figure 1.) depending on the buffer used. The enzyme was stable over a wide range of pH values, with the highest stability at pH 6-8 for 30 minutes as shown in (Figure 2.) The enzyme had an optimum activity at the temperature of 60°C and was rapidly inactivated at higher temperature. Above 80°C, the keratinase was no longer active. The enzyme was unstable at high temperatures but was stable at moderate temperatures as shown in (Figure 3.) The effect of substrate concentration on keratinase production was investigated. The affinity of the keratinase for keratin were determined at 60°C and pH 7.0 by a Line weaver Burk plot. The k_m and V_{max} values for keratin were 0.22 mg/ml and 0.01 U/ml respectively. Keratinolytic activity of keratinase was completely inhibited by EDTA, 1, 10 Phenanthroline by 83 and 94% respectively. This observation indicates that the crude keratinase belongs to the group of metalloproteases. Among the metal ions the Zn^{2+} and Mg^{2+} enhance the keratinolytic activity by 1.34 and 1.71 folds respectively and Cu^{2+}, Hg^{2+} and Cd^{2+} inhibits the keratinolytic activity by 92,94 and 93% respectively. The optimum activity was observed at 1mM as shown in (Table 3) (data not shown for 2 and 3mM concentration).

TABLE-1: Keratinase production by bacterial isolates from soils in Namakkal poultry farm.

Isolate	Keratinase (U/ml
NS-1	0.21
NS-2	2.36
NS-3	10.76
NS-4	0.17
NS-5	4.94
NS-6	0.98
NS-7	2.00
NS-8	2.38
NS-9	3.35
NS-10	1.56

Abbreviations: NS- (Namakkal soil strain)

TABLE-2: Results Of Morphological and Biochemical Tests for *Bacillus licheniformis*

EXPERIMENTAL DETAILS	OBSERVATIONS
Gram stain	Positive
Shape and arrangement	Rods in Single and in Chains
Endospore stain	Positive
Litmus milk reactions	Peptonization
Carbohydrate fermentations with lactose	Acid with Gas
Sucrose	Acid with Gas
Dextrose	Acid with Gas
Nitrate reduction	Positive
Motility test	Positive
Indole production	Positive
Methyl red test	Negative
Citrate utilization	Positive
Catalase activity	Positive
Starch hydrolysis	Positive
Caesin hydrolysis	Positive
Urease activity	Positive

TABLE-3 Effect of Inhibitors and Metal ions:

Metal Ions	Concentration (mM)	Relative Activity (%)	Inhibition (%)	Enhancement
Crude	-	100	-	-
Zn^{2+}	1	134.02	-	1.34 folds
Mg^{2+}	1	171	-	1.71 folds
Hg^{2+}	1	5.86	94	-
Cd^{2+}	1	6.83	93	-
Cu^{2+}	1	7.18	92	-
1,10 Phenanthroline	1	5.48	94	-
EDTA	1	16.44	83	-

Results represent the average of three experiments

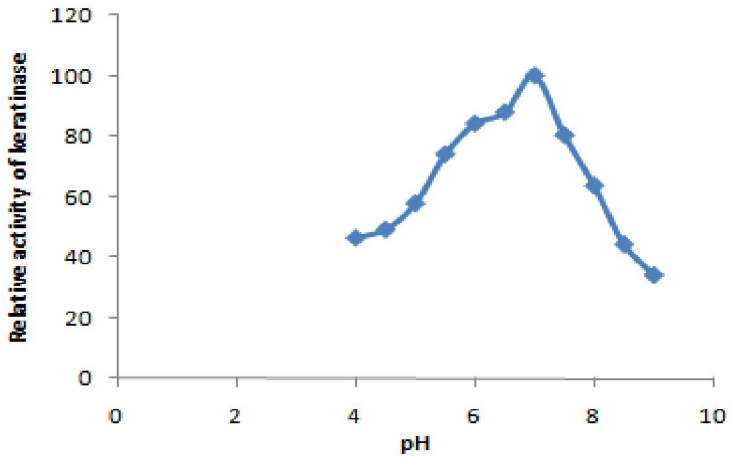

Figure 1. Effect of pH on keratinolytic activity

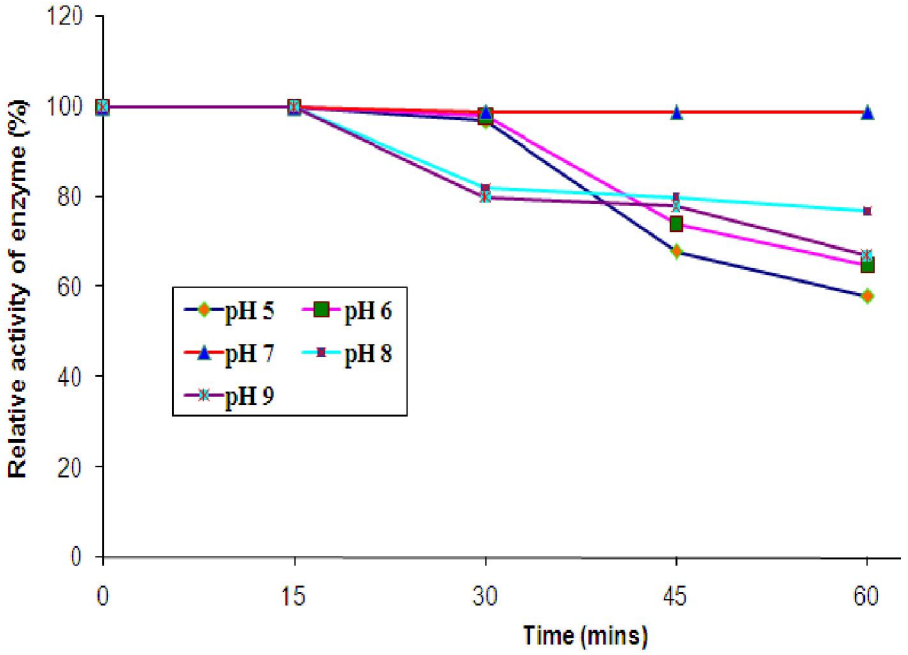

Figure 2. pH stability on keratinase from *Bacillus licheniformis*

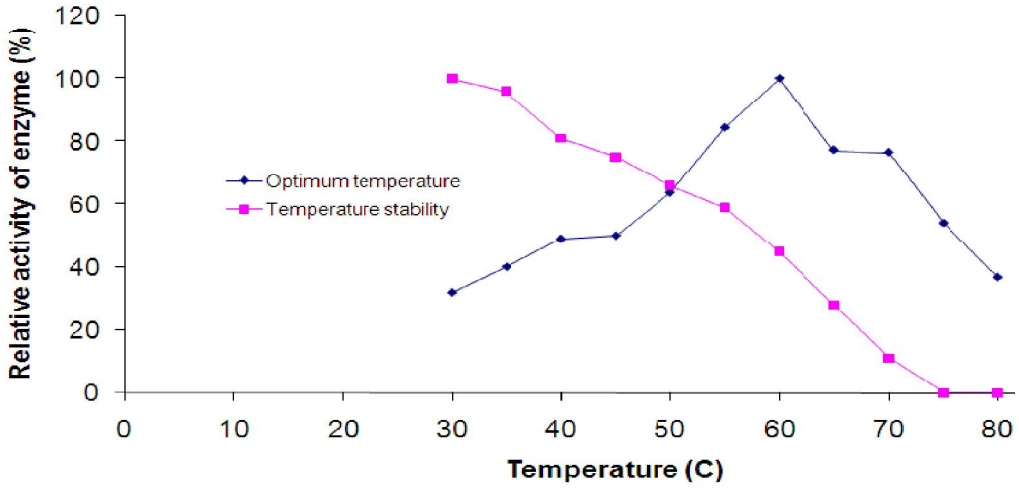

Figure 3. Optimum temperature and temperature stability of keratinase from *Bacillus licheniformis*

4. Discussion:

A bacterium isolated from poultry waste has been shown to degrade feather keratin. The bacterium isolated from anaerobic habitat however, showed maximum growth under aerobic condition, as would be expected of a member of the family bacillaceae (William *et al*., 1990). These bacterium present different characteristics, such as broad temperature range of growth. The optimal kerotinolytic activity was detected at 50°C, whereas previously described kerotinolytic bacteria mostly have feather-degrading activity in the temperature ranges from 40-60°C. *Bacillus* species have been reported to produce kerotinolytic protease (Lin *et al*.1992; Kim *et al*. 2001; Lee *et al*.2002; Savitha G. Joshi *et al*. 2007). However, this strain isolated from namakkal soil grew and

degraded feather well at elevated temperatures. The keratinase from *Bacillus licheniformis* was induced by feather meal. However the induction of keratinolytic enzyme produced by the species of *Bacillus* with feather powder, guinea pig hair human hair and nails, and cow horn and hooves was reported (Cheng *et al*., 1995; Lal *et al.*,1999 Tamilmani *et al.*, 2008). The presence of this species in a poultry waste may be that the bacterium is indigenous to the chicken gut. However, more likely that, it was indigenous to the environment in which poultry excreta are collected. The environmental also contain feathers and the isolate may have adapted to utilize this substrate. The most studied kerotinolytic bacteria are *Bacillus licheniformis* which have been described to possess feather degrading activity. Micro organism growing on nutrient medium containing feather meal also act as carbon and nitrogen source presented variable activity on keratin, suggesting that this enzyme may be inductive. Substrate level in the medium may regulate enzyme secretion. *Bacillus licheniformis,* showed to be more adapted to keratinase production using keratin as substrate since maximum kerotinolytic activity of the isolate was observed during early growth, and the strain displayed a higher total activity during incubation. The enzyme activity was studied over a broad range of temperature (30-80°C). The optimum temperature of keratinase from *Bacillus licheniformis* 60°C was slightly higher than that of other bacillus kerotinolytic protease (50-55°C) (Lin *et al.*1992; Cheng *et al*.1995; Balaji *et al*.2008). The enzyme was stable at high temperatures partly because its catalytic site was well protected by a substrate. Further increase in the temperature to 80°C reduced the activity. The active range of pH 5.0 and 7.0 whereas the optimum pH for the keratinase was found to be at pH 7.0, The enzyme was stable at the range of 6-8(Cheng *et al.*1995). the activity decreased at pH 3.0 and 8.0. Keratinolytic bacteria often exhibit optimal growth and activity at higher temperature (Lin *et al.*1999) and some mesophilic bacteria exhibit the optimal enzyme production and activity ranging from 20 to 30°C (Allpress *et al.* 2002). Here the keratinase secreted by the isolate was active at a broad range of temperature and maximum activity continued beyond ambient temperature. The Keratinase from *Bacillus licheniformis* is very likely to be a serine protease since it was mostly inactivated by 1, 10 phenanthroline and EDTA and its pH optimum was in a basic range. Several reports have shown serine protease to be slightly affected by metalloprotease inhibitor (Manachini *et al.*1998). Divalent metal ion activated the keratinase activity of *Bacillus licheniformis* A metal ion probably act as salt or ion bridge to maintain the structure conformation of the enzyme of the enzyme or to stabilize the binding of the substrate and enzyme complex. Therefore, this enzyme could be applied to digest keratin substrate under various metal ion surroundings. The enzyme showed partial on chicken feather and hair since these substrates contained disulphide linkages which are a crucial structural feature of their molecules. This result indicates that this enzyme could not completely cleave only peptide bonds in the substrate. Pre-treatment of these substrate such as chicken feather, nail, and human hair by physical method or reducing agents, or detergents, or activation of the enzyme by adding metal salts are required for improvement of their degradation. Keratinase produced Baci*llus licheniformis* plays a part feather degradation. The bacterium and its enzyme could be used to improve nutritional values of animal feed containing feather, or keratin, or poultry processing waste in namakkal region.

5.Conclusion:

Bioconversion of feather with *Bacillus licheniformis,* has great potential to protect our environment. This novel keratinolytic isolate could be a potential candidate for the degradation of feather keratin and also in de-hairing process in leather industry and can be used as additives in poultry field.

Acknowledgement:

The authors are grateful to the Management of Kumaraguru College of Technology, Coimbatore, Tamil Nadu, India, for their valuable support to carry out this research project.

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