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CHARACTERIZATION OF A THERMOSTABLE SERINE KERATINASE FROM NEWLY ISOLATED THERMOPHILIC *BACILLUS LICHENIFORMIS*

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Abstract: *Facultatively thermophilic bacterial strain capable of producing thermostable keratinase was isolated from habitats that are naturally thermophilic. Based upon biochemical characterization the bacterium was identified as Bacillus licheniformis. Bacteria produced keratinase in the medium containing horn meal as sole source of carbon and nitrogen. Effect of different parameters on enzyme activity was studied. Keratinase was active in broad temperature range from 50^{o}C to 80^{o}C. Optimum temperature was found to be 50^{o}C. Incubation at 70^{o}C for one hour retained 53.84 % residual activity indicating its thermostable nature. Enzyme also exhibited stability at broad pH range being active from pH 4 to 9. Optimum pH was found to be 7. EDTA at 1mM concentration inhibited the keratinase activity by 50 % while PMSF inhibited the total activity of the enzyme. Present study offers interesting potential of enzyme use in hydrolysis of keratinolytic waste for generation of value added products for varied biotechnological applications.*

Keywords: *keratinase, thermostable, Bacillus licheniformis, horn meal, PMSF*

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INTRODUCTION

Keratins are insoluble, protective, structural proteins consisting of parallel polypeptide chains which are arranged in α-helical or β-conformation (Nelson and Cox 2000). These proteins form hard but not mineralized structures in many groups of organisms in the form of epidermis, epithelial cells, appendages viz; hairs, nails, horns, feathers, scales and wool, claws of birds, quills and porcupines (Gupta et al., 2006) where they play structural and protective role. Keratins can be classified as one of the extremely hard to degrade animal proteins. Extensive disulphide linkages, tight packaging of polypeptide, stabilization of structures by hydrophobic and Vanderwaal's interaction are responsible for its stability and resistance to degradation (Santos et. al, 1996). Keratins are almost pure proteins i.e. 90 % or more. They are produced as waste in large amount from human sources, meat and leather industries in the form of hairs and other substances (Suzuki et al. 2006). Worldwide, annually more than 10,000 tonnes of keratin is generated as a waste product at poultry processing plants. Dumping of such wastes around processing plants creates environmental pollution owing to recalcitrancy of keratins. Various methods have been adopted to treat keratinous wastes. Disposal through landfilling and combustion without significant advantage harms environment due to high energy demands and carbon dioxide production (Mokrej et al. 2010). Conversion of keratin waste into meal by chemical processes is environmentally unsafe since they require the treatment of keratins with strong acids and bases (Gessesse et al. 2003)

Use of keratinolytic microorganisms and their keratinases presents a alternative approach to treat keratinous waste that is eco-friendly and safe. The thermophilic microbes capable of degrading keratin, through fermentation process can be effectively used to treat keratin waste with simultaneous production of biotechnologically significant value added products.

Thermostable keratinase from different groups of bacteria have been reported by Sangali et al. 2000, Riessen et al. 2001, and Phantange et al. 2010. In present studies, the facultatively thermophilic *Bacillus licheniformis* possessing ability to produce a thermostable keratinase using horn meal as sole source of carbon and nitrogen has been isolated and its enzyme is characterized.

MATERIAL AND METHODS

Material:

All chemicals and reagents were purchased from Sisco Research Laboratory Pvt. Ltd. and Lobachemie Pvt Ltd. Keratin azure was procured from Sigma Aldrich, India.

Enrichment and Isolation:

Samples collected were subjected to enrichment. Appropriately diluted samples were inoculated in the enrichment medium containing (per 100 ml) meat extract, 0.2 g; KH_2PO_4, 0.1 g; K_2HPO_4, 0.3 g; $MgSO_4.7H_20$, 0.01 g; $FeSO_4.7H_2O$, 0.001g; $MnSO_4$, 0.001 g, horn meal, 1 g. (pH =7). The medium was incubated at 55^oC shaker water bath (50 rpm). After incubation for a week, the loopful of enriched broth was streaked on sterile horn meal agar plates and sterile casein agar plates (Composition of both media was kept same with omission of meat extract. Horn meal medium agar and casein medium agar contained horn meal and casein as sole carbon and nitrogen respectively each at 10 gL^{-1}Concentration of agar powder was 4 g per 100 ml). Plates were incubated at 50^oC for 48 – 96 hrs. Plates showing clear zone of hydrolysis around colonies were considered positive for enzyme production and studied further.

Maintenance of cultures:

All isolated cultures were preserved and maintained at 4^oC on Cysteine trypticase agar with periodic transfer after 3 months. Purity was checked by streaking cultures on CTA plates. Stock cultures were maintained at -20^oC as glycerol stocks.

Identification of Isolates:

Microorganisms showing high enzyme activity were considered for further studies and identified by morphological, physiological and biochemical characterization. Bergey's manual of Systematic bacteriology volume 2 and Dworkin et al. 2006 was followed throughout the course of identification.

Enzyme production:

Enzyme production was carried out in 2 litres stirred bioreactor (Schott Duran, Germany). Composition of optimized production medium was, horn meal, 5 %; KH_2PO_4, 0.7%; K_2HPO_4, 0.3%, $MgSO_4$, 0.01 %, Trace element solution, 0.1 ml: Vitamin solution, 0.1 ml. Trace element and vitamin solution was sterilized separately by membrane filtration and added to final medium after cooling. Preparation of same was followed by Atlas RM 2010. 400 ml of

medium was sterilized and inoculum (10^8 cells per ml, O.D= 0.25 at 660 nm) was added at concentration of 2 %. Incubation period was 7 days at 50°C with stirring at 200 rpm.

Enzyme assay:

The enzyme activity was determined spectrophotometrically by the method of Suntornusk et al. 2003 using keratin azure as chromogenic substrate. After 24 hrs of incubation, 1 ml of broth was centrifuged at 7000 rpm (4°C) for 20 minutes and supernatant was used as source of extracellular keratinase. 0.1 g % suspension of fine keratin azure powder was used prepared by suspending fine powder in 0.01M Tris-HCl buffer, pH 7. Reaction mixture for determining enzyme activity consisted of 1ml of enzyme solution and 1 ml of keratin azure suspension. Mixture was incubated at 50°C for one hour with agitation (200 rpm). After incubation the mixture was boiled for 5 min, followed by centrifugation at 4000 rpm for 15 min to remove the substrate. The absorbance was read at 595nm. All assays were carried out in triplicates. Blank (control) was run by boiling the enzyme and substrate before carrying out reaction.

One unit of enzyme activity was defined as the amount of enzyme causing 0.1 increase in absorbance between sample and control at 595nm in one hour under the standard reaction conditions specified.

Effect of various parameters on activity of enzyme:

Effect of temperature:

Aliquots of centrifuged supernatant obtained from fermentation broth were incubated at different temperatures for 30 minutes. The enzyme activity was determined by the method of Suntornusk et al. 2003. Temperatures of incubation were 40°C, 50°C, 60°C, 70°C and 80°C.

Effect of pH:

Aliquots of centrifuged supernatant were incubated in buffers of different pH for the period of 30 minutes at 50°C and the residual enzyme activity was determined by the method of Suntornusk et al. 2003. glycine-HCl, Acetate, phosphate and tris buffer of pH 2, 4, 7 and 9 respectively were used.

Effect of inhibitors:

Aliquots of centrifuged supernatant were incubated with EDTA (Ethylenediamine tetraacetic acid) and PMSF (Phenyl methyl sulphonyl chloride) at concentration of 1 mM, 5 mM and 10

mM for the period of 30 minutes at 50°C and pH 7. The residual enzyme activity was determined by the method of Suntornusk et al. 2003.

RESULTS:

Enrichment and isolation:

Colonies either showing clear zone of hydrolysis on casein medium and horn meal medium agar plates or only growth on horn medium agar plates indicative of keratinolytic activity were isolated and purified. Based on time course of enzyme production, one isolate among total of 5 showing substantial activity (Figure 1a and 1b)) was selected for further study. The isolate, based upon results of biochemical characterization (Table 1) and referring Bergey's manual of determinative bacteriology, 9th edition was identified as *Bacillus licheniformis*

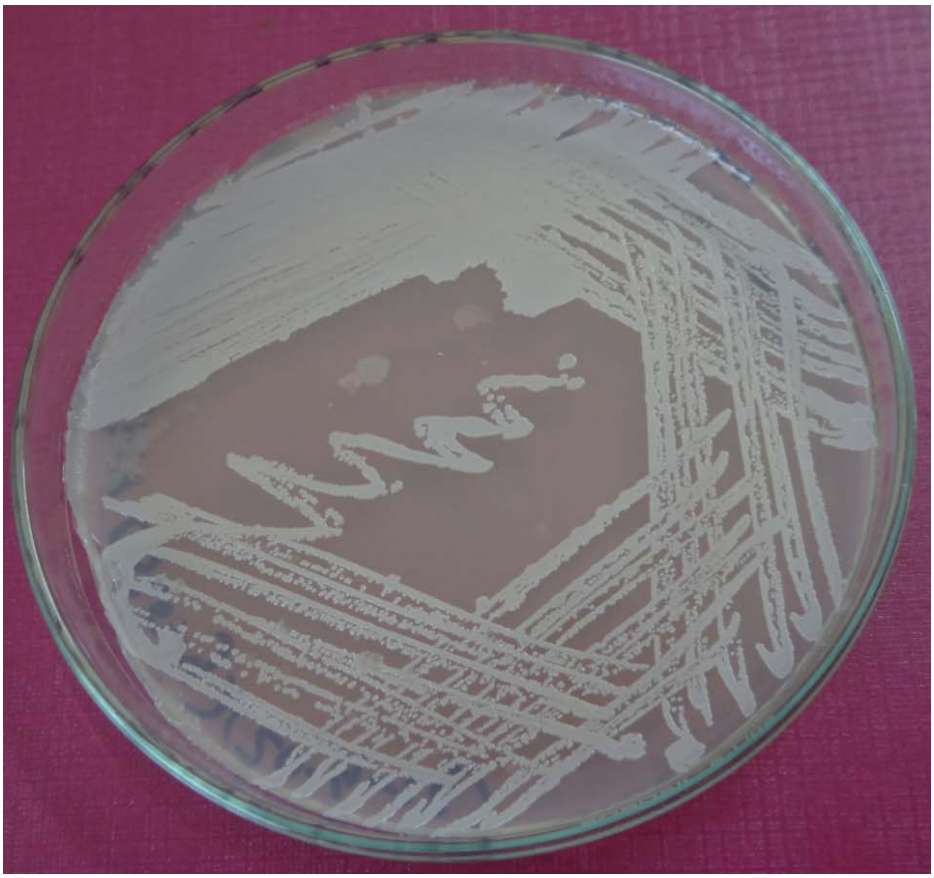

Figure 1a: Colonies of *Bacillus licheniformis* on nutrient agar plates after incubation at 55°C for 24 hrs.

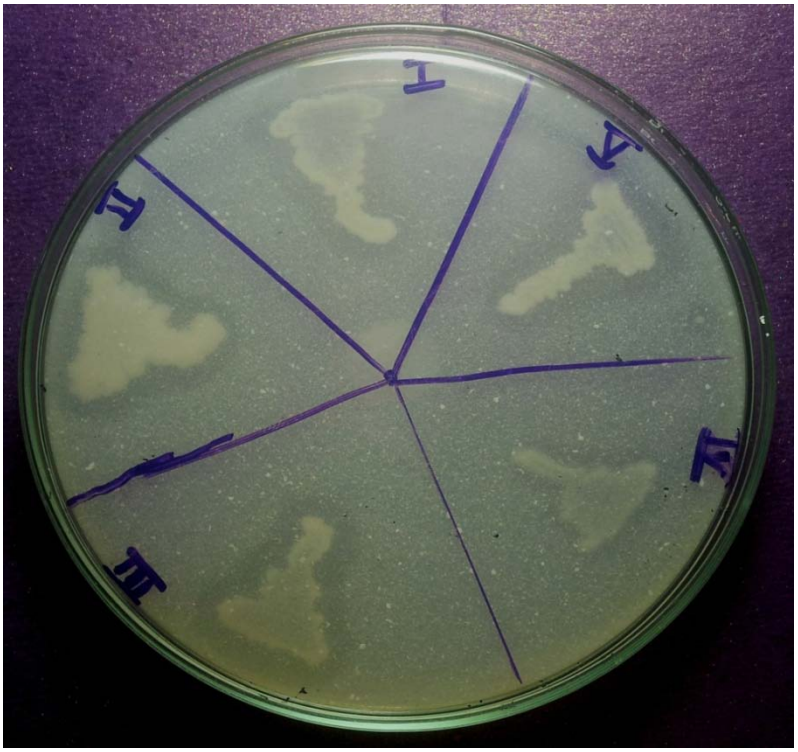

Figure 1b: Isolates showing clear zone of hydrolysis on casein medium agar plates after incubation of 48 hrs at 55^{o}C.

No	Characteristics	Result
01	Colony Characters on Nutrient agar medium	5 mm, irregular, cream, smooth, opaque
02	Gram staining	Gram positive rods
03	Filamentous growth	+
04	Endospore formation	+
05	Cocci in tetrads	-
06	Motility	-
07	Rod shaped in young cultures	+
08	Requirement of oxygen	Facultative
09	Catalase	+
10	Oxidase	-
11	Marked acidity from glucose	+
12	Nitrate reduced to nitrite	+
13	Growth at 3 % to 12 % NaCl concentration	+ till 7 %

Table 1: Result of biochemical characterization of isolate.

Enzyme activity:

Bacillus licheniformis was able to produce extracellular keratinase using horn meal as sole source of carbon and nitrogen. Study on time course showed that maximum enzyme production occurs on 5th day of incubation after which it gradually decreases (Figure 2)

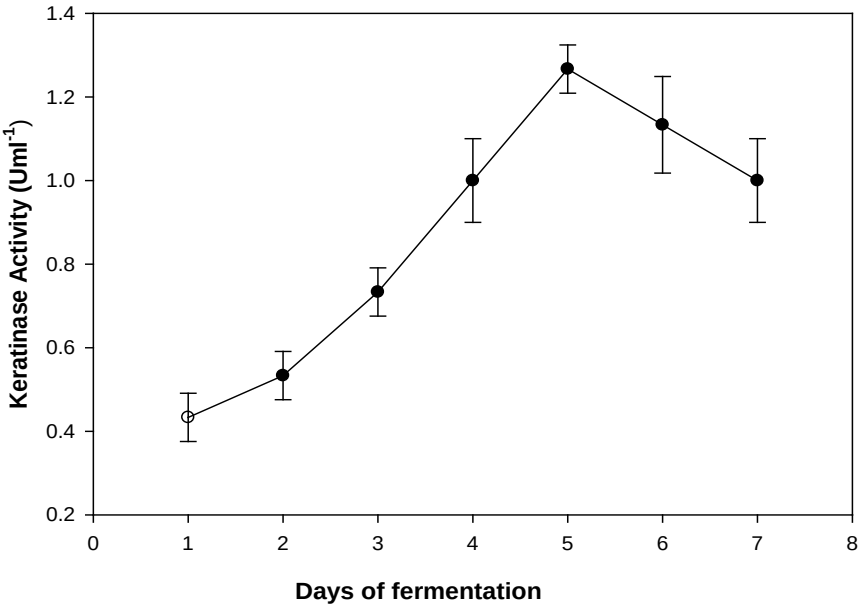

Figure 2: Time course of keratinase activity of *Bacillus licheniformis*

Effect of different parameters on enzyme activity:

Effect of temperature:

Keratinase activity was highest at 50^{o}C (1.42 Uml^{-1}). As the temperature of incubation was increased, the residual activity decreased gradually, being low at 80^{o}C (0.4 Uml^{-1}). (Figure 3)

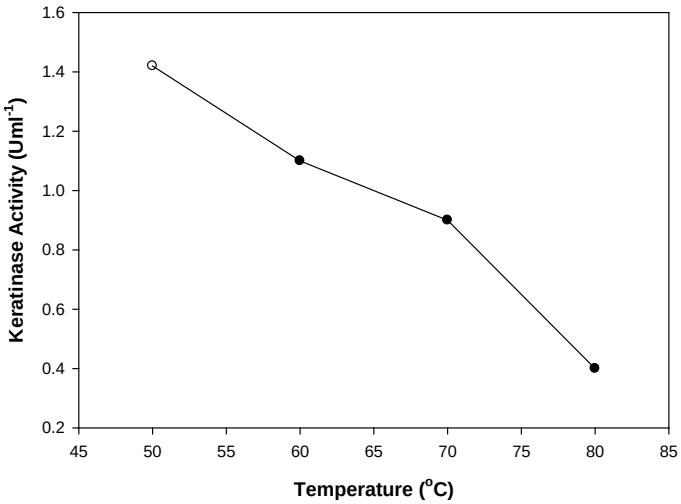

Figure 3: Effect of temperature on Keratinase activity

Effect of pH:

Keratinase was found to be active in broad pH range ranging from 4 to 9. The highest activity (1.45Uml^{-1}) was at pH 7. Residual activity decreased on both sides of pH. Activity at pH 2 was 0.10 Uml^{-1} and pH 9 was 0.9 Uml^{-1} (Figure 4)

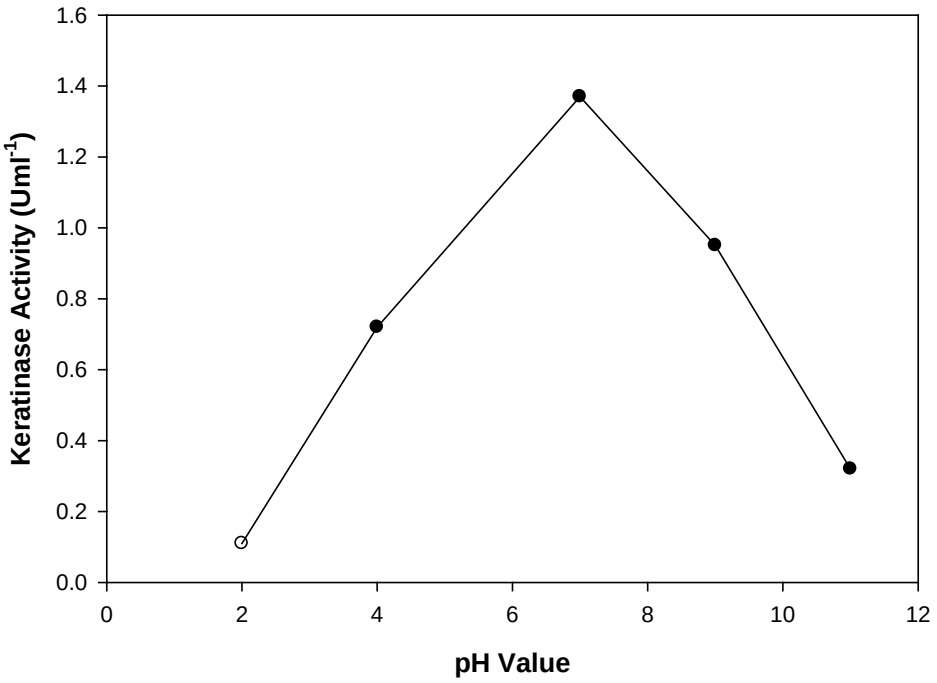

Figure 4: Effect of pH on Enzyme activity

Effect of Inhibitors:

EDTA at increasing concentration decreased the activity of keratinase, while PMSF at all tested concentration inactivated the enzyme, the results of which are summarized in table 3

Inhibitor	Concentration (mM)	Enzyme activity (Uml^{-1})
EDTA	1	0.503
	5	0.340
	10	0.170
PMSF	1	0.0
	5	0.0
	10	0.0

Table 2: Effect of inhibitors on enzyme activity

DISCUSSION:

Thermophilic bacterium was isolated from naturally occurring thermophilic sites capable of producing the extracellular keratinase using horn meal as sole source of carbon and nitrogen. On the basis of biochemical characterization based on Bergey's manual of systematic bacteriology volume 2 and Bergey's manual of determinative bacteriology, 9th edition, the bacterium was identified as *Bacillus licheniformis*. The keratinase that is secreted by bacterium has temperature optima of 50^{o}C and optimum pH of 7. The enzyme is highly thermostable as indicated by the fact that 53.84 % of the activity is retained after 30 minutes at 70^{o}C. Similar kind of thermostable keratinase from *Bacillus licheniformis* with 45^{o}C as optimum temperature has been reported by Phantange et al. 2010 but information regarding the activity at higher temperatures (above 55^{o}C) is not mentioned. Also the *Bacillus licheniformis* reported in same study was grown at moderate temperatures. Since *Bacillus licheniformis* isolated in present studies is thermophilic (able to grow well at temperature of 55^{o}C) could be efficiently used in fermentation process at elevated temperature for production of extracellular keratinase. Use of thermophilic microbes in bioprocesses to produce thermostable enzymes are superior due to their inherent properties (Haki et al. 2003) and allow the performance of industrial processes even at harsh conditions under which conventional proteins are completely denatured.

The optimum pH for enzyme activity was 7. Extreme pH on both sides (pH 2 and 11) tends to inactivate the enzyme. The reason for such observation is already well established fact that very alkaline and acidic pH disrupts non covalent interactions in the three dimensional structure of protein that stabilizes it. However, keratinase retained 69.34 % residual activity at pH 9 after 30 minute incubation, indicating that it is quite active at alkaline pH. Alkaline active extracellular keratinase by *Bacillus pumilus* with pH optima of 8 (pH stability 7.7 to 10) has been reported by Ganesh Kumar et al. 2007.

PMSF at concentration 1 mM and greater fully inhibits the keratinase activity suggesting that it belongs to serine protease family. Lin et al. 1995 from study on DNA nucleotide sequences of keratinase gene, reported that keratinase produced by *Bacillus licheniformis* belongs to serine protease family. Inhibition of partial activity by EDTA may be due to its metal ion chelation activity required for activity of enzyme.

Keratins in the form of horns are readily available and cheap source of substrate that could be utilized in microbial bioprocesses at elevated temperatures to convert them into animal meal and other industrially important value added products with simultaneous treatment of keratinous waste. Also, other keratinous waste could be used as substrate for production of thermostable extracellular keratinase that could find applications in industries that use proteases for process.

CONCLUSION:

The objective of the present study was to isolate thermophilic bacteria capable of producing extracellular keratinase possessing high thermal stability. The bacterium was identified as *Bacillus licheniformis* and produced 1.47 Uml^{-} keratinase after 5 days of incubation with $50^{o}C$ and 7 as optimum temperature and pH respectively. Identification of bacterium by molecular method and purification of enzyme and study of its kinetic parameters is being presently carried out. *Bacillus licheniformis* and its keratinase offers interesting potential biotechnological applications in bioprocesses at elevated temperatures involved in treatment of keratinous waste.

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