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STUDIES ON KERATINOLYTIC ACTIVITY OF ALKALINE PROTEASES FROM HALOPHILIC BACTERIA

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ABSTRACT

Alkaline proteases producing halophilic bacteria isolated from Sambar Lake, Rajasthan were investigated for keratinolytic activity. The aim of this study was to optimize culture conditions for maximum enzyme production as well as characterization of proteases with keratinolytic action. Casein was used as sole carbon and nitrogen source for alkaline protease production at 37°C and 150 rpm. Higher keratinolytic activity was obtained when the production medium was supplemented with chicken feathers at a concentration of 1g/L. Optimum pH and temperature for maximum activity was found to be 9.0 and 50°C respectively. The enzyme is stable at alkaline pH (8-9). It has high thermostability at 50°C and lost 25% of its keratinolytic activity after 8 h of incubation at 50°C.

KEYWORDS:Alkaline proteases, Feather degradation, Halophiles, Keratinase, Thermostable

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INTRODUCTION

Alkaline proteases account for a major share of the enzyme market all over the world because of their applications in various industrial sectors such as detergents, leather, food & feed industry and waste management etc.[1] A major source of bacterial alkaline proteases is *Bacillus* species, which secrete commercially applicable alkaline proteases with good stability at higher pH and temperature values.[2] Keratinolytic proteases (Keratinases) are of special interest as they can target the hydrolysis of insoluble keratin substrates which are recalcitrant to the commonly known proteolytic enzymes.[3] Feathers are composed of over 90% protein (keratin) and produced in large amounts as a waste by poultry processing applications worldwide. Accumulation of feathers will lead to environmental pollution and feather protein wastage. Chemical and mechanical hydrolysis of keratin wastes is successful but they have several disadvantages of being energy intensive, polluting and leading to loss of essential amino acids. Hence, keratinolytic enzymes may have important uses in the biotechnological conversion of keratin-containing wastes from poultry to the development of non-polluting processes. Hydrolysed feather keratins can be used in feedstuffs, fertilizers, and films etc.[4,5] Halophiles are of interest due to their fascinating property of adaptation in extreme environments and differentiated according to their salt requirements: slight halophiles grow optimally at (2–5%) NaCl, moderate halophiles grow optimally at (5–150%) NaCl and extreme halophiles grow optimally above (15–30%) NaCl. Halophilic enzymes find rapidly increasing use in biotechnological applications owing to their halo-tolerance, often thermostability for long incubation periods and capability to retain activity in presence of high levels of organic solvents.[6,7] Halophilic enzymes are distinguished from their non halophilic counterparts by maintaining soluble and active conformations in high concentrations of salt upto 5 M NaCl and exhibiting unusually high stability.[8] Recently, halophilic B*acillus* sp. has been reported towards the production of extracellular alkaline proteases.[9-12] Although alkaline proteases with keratinolytic activity from non halophilic sources have been well documented,[13-16] keratinolytic alkaline proteases from halophiles are least reported. Given the potentials of halophilic enzymes, the aim of this study was to optimize cultural conditions for production of alkaline proteases from halophilic bacteria and evaluation of their keratinolytic activity.

MATERIALS AND METHODS

The chemicals used were of analytical grades and purchased from Merck and Himedia respectively. The chicken feathers were procured from the local market of Ranchi, Jharkhand. It was washed properly with distilled water to remove the blood stains and dust attached on the surface and dried at room temperature.

Bacterial Strains and Culture Conditions

The halophilic bacteria used in this study were isolated from sludge samples of Sambar Lake, Rajasthan. Isolated cultures showing clear zones of casein hydrolysis on 12% Modified Growth Media (MGM) plates were purified and used for the study. The medium contains $MgSO_4$ 14g/L, $MgCl_2$ 12g/L, NaCl 120 g/L, KCl 2.8 g/L, $CaCl_2$ 0.55 g/L. Yeast extract 1 g/L and casein 5 g/L were sterilized separately and mixed properly before inoculating with the purified strain. The pH of the medium was adjusted to 8.0 before sterilization. The production medium was kept at 37°C for 72 h at 150rpm.

Alkaline Protease Production in Shake Flasks

The medium used for the alkaline protease production was 12% MGM supplemented with and without chicken feathers (1g/L). 250 ml Erlenmeyer flask containing 50 ml of the sterile production medium were inoculated with purified isolate and incubated at 37°C in an incubator shaker at 150 rpm. In one set, chicken feather was added in the production medium, while in other set, only casein was used for the enzyme production. The inoculum

size used for the production of enzyme was 5% (v/v) from an over night grown seed inoculum (12h age). The flasks without feathers were removed after 3 days of incubation while the flasks with feathers were removed after 7 days of incubation period. The production broth was harvested by centrifugation at 10,000 rpm for 10 minutes at 4^0C to collect the supernatant (crude enzyme) and used for enzyme assay.

Enzyme Assay

1. ***Alkaline protease activity***

   Alkaline protease activity was determined by a method described by Makhija *et al*..[17] According to this procedure 1 ml of 1% casein solution in 0.1 M tris-HCl (pH 9.0) was incubated with 1 ml of suitably diluted enzyme supernatant at 50^OC for 30 minutes and the reaction was stopped by adding 0.5 mL of 10% trichloroacetic acid (TCA). The precipitated protein was removed by centrifugation at 10,000 rpm for 10 minutes. 1 ml of the supernatant was used to measure released tyrosine at 660 nm. One unit of alkaline protease activity (U) was defined as µg of tyrosine released/ml/min under the defined assay conditions.

2. ***Keratinase activity***

   Keratinolytic activity was assayed with keratin powder as substrate using modified method of Riffel *et al*..[18] 2 ml of 2% keratin solution in 0.1 M Tris-HCl (pH 9.0) was incubated with 1 ml of enzyme supernatant at 50ºC for 60 min and the reaction was the stopped by the addition of 1 ml of 10% trichloroacetic acid. After centrifugation at 10,000 g for 5 min, the absorbance of the supernatant fluid was determined at 440 nm. One unit of enzyme activity (U) is defined as a change of absorbance of 0.01 against the control at 440 nm for 60 min at 50ºC. The control was prepared by addition of TCA before incubation immediately after enzyme addition.

3. ***Protein content***

The total protein contents of the samples were determined usi ng the Lowry method.[19]

Optimization of culture conditions

1. ***Effect of different concentrations of NaCl***

   The effect of different concentrations of sodium chloride on production of enzyme was performed by varying the concentration of sodium chloride at 0, 6, 12 and 18% (w/v) respectively keeping other parameters constant and the activity was evaluated at 50 ^{0}C and pH of 9.0.

2. ***Effect of different concentrations of casein***

   The effect of different concentrations of casein on production of alkaline proteases was studied at 0, 2.5, 5, 7.5, and 10 (g/L) respectively and activity was monitored at optimal conditions.

3. ***Effect of different concentrations of feather***

   The effect of varying feather concentrations on production of enzyme alkaline proteases was investigated at 0.5, 1.0, and 2 (g/L) respectively and activity was measured at each parameter.

4. ***Effect of incubation period***

   The effect of optimal incubation period for maximum enzyme production and subsequently activity was determined by incubating production medium for different incubation periods viz. 24, 48, 72, 96 h at 37 º C.

Characterization of enzyme

1. ***Effect of temperature on enzyme activity and stability***

   The effect of temperature on keratinolytic activity was determined by incubating the reaction mixture at different temperatures ranging from 37 to 70°C. To determine the enzyme stability with respect to temperature, the crude enzyme was incubated at different temperatures (50, 60°C) and relative activities were assayed at different time intervals.

2. ***Effect of pH on enzyme activity and stability***

   The effect of optimum pH for maximum enzyme activity was determined by assaying the activity at 50°C with different

pH values ranging from 4.0 to 11.0 using different buffers of 0.1 M strength. The following buffers were selected for this study: Sodium acetate (pH 4.0–5.0), Potassium phosphate (pH 6.0-7.0), Tris–HCl (pH 8.0-9.0), Glycine– NaOH (pH 10.0-11.0). Similarly for pH stability, the enzyme was diluted in different buffers (pH 4.0–11) and incubated at room temperature for 1 h and the relative activity was measured as per assay protocol.

3. ***Effect of varying substrate concentrations on enzyme activity***
The effect of different concentrations of keratin on keratinase action was studied at optimal conditions and line- weaver burk plot was constructed to calculate Vmax and Km values for keratinolytic activity. Different concentrations of keratin (5-50 mg) were used for observing the maximum rate of enzymatic reaction. All the experiments were performed in triplicate and mean values are reported.

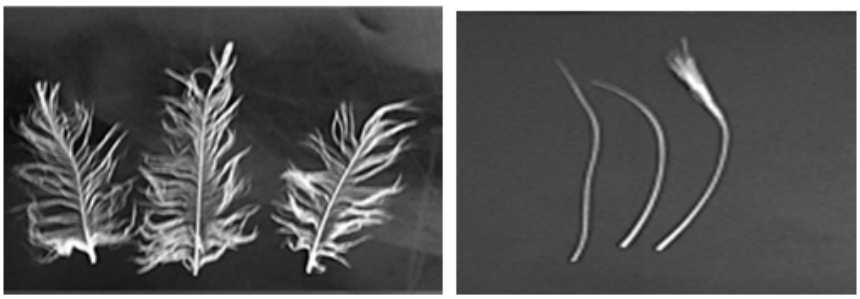
RESULTS

Zone of clearance and keratinolytic activity
During preliminary investigations, three halophilic alkaline protease producing bacterial isolates were selected for enzyme production on the basis of clearance on casein agar plates as shown in Table1. One of the isolates NPBT002 was identified as *Bacillus* sp. by Institute of Microbial Technology, Chandigarh, India and used for further analysis. When observed for the keratinolytic activity by the selected isolates in liquid culturing conditions, it was found that 90% (w/v) of chicken feather was degraded in the production medium supplemented with chicken feather after 7 days of incubation at optimal conditions confirming the keratinolytic activity of the alkaline proteases (Figure 1)

Table 1
Diameter of zone of clearance on 12%MGM- casein agar plates

S. No.	Name of isolates	Zone of clearance (mm)
1	NPBT001	8
2	NPBT002 (*Bacillus* sp.)	10
3	NPBT003	12

It was also observed that in the presence of both casein and feather in production medium, keratinolytic activity was 1.4 times greater than in presence of casein alone. This may be due to the initial utilization of casein by isolates for production of alkaline proteases which subsequently degrade the feather and hence higher activity was obtained. The isolates when evaluated for alkaline protease activity, no significant variation was observed.



(a) **(b)**

Figure 1
Feather degradation by different halophilic bacterial isolates.
(a) Control – Non inoculated production media; (b) Feather degradation after 7 days of incubation

Optimization of different culture conditions
The studies of varying sodium chloride concentration on the maximum production of enzyme were represented in Figure 2. It was observed from the studies that increasing the concentration of

sodium chloride increases the enzyme activity up to 12% of sodium chloride concentration and then started decreasing. The finding of the experiment indicates halophilic nature of *Bacillus* sp. as the bacterium requires sodium chloride as main growth constituents. Very low activity was found when the production medium was devoid of the sodium chloride.

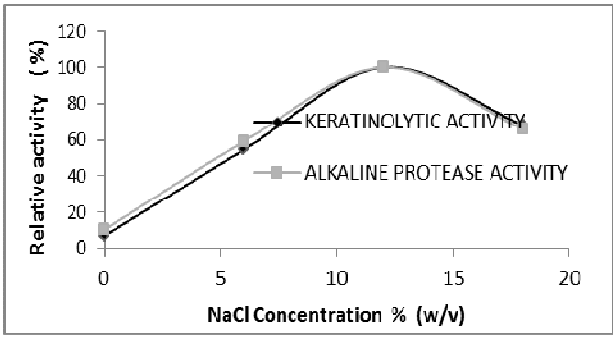


Figure 2
Effect of concentration of sodium chloride on production of enzyme

Optimizing the concentration of casein for maximum production of alkaline proteases as well as keratinolytic action, it was found that a concentration of 5 g/L is optimum for maximum production of enzymes (Figure 3). Figure 4 shows the results for activities of enzymes produced in the presence of different concentrations of feather. The highest levels of activities were obtained when the production medium is inoculated in presence of 1g/L of chicken feathers.

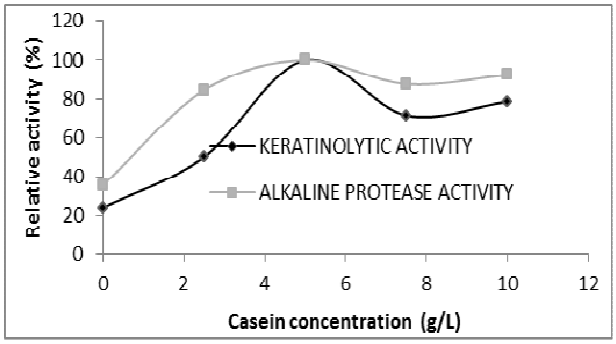


Figure 3
Effect of concentration of casein on production of enzyme

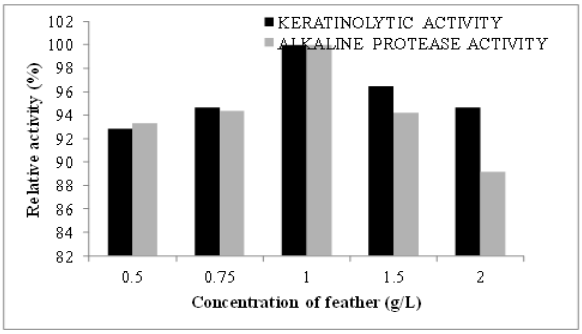


Figure 4
Effect of concentration of feather on production of enzyme

The studies on optimization of incubation periods on the maximum production of enzyme revealed that the activity increased with increasing incubation periods and reached to a maximum at 72 h of incubation (Figure 5a) and then decreased. It is also reported from other halophilic alkaline protease producers that a broad incubation period ranging from 24-96 h is required for maximum activity of alkaline proteases.[9,11,20,21] It was also observed that as time increases the total protein content kept increasing till 96 hours irrespective of decrease in enzymatic activity after 72 h of incubation (Figure 5b). The protein contents of supernatants with and without feathers were 3.94 and 2.8 mg/ml.

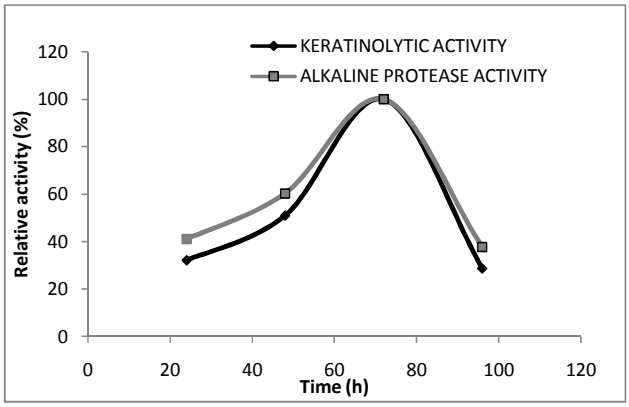


Figure 5a
Effect of incubation time on enzyme production

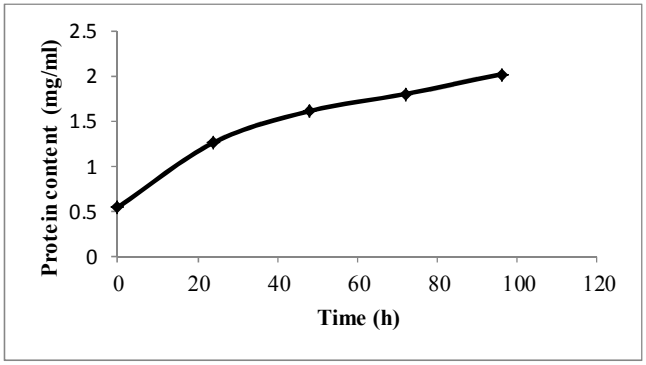


Figure 5b
Effect of incubation time on protein content

Enzyme characterization

Effect of temperature on keratinolytic activity and stability

The effect of temperatures on keratinase activity was studied in a temperature range of 37-70°C as shown in Figure 6a. An increase in activity was observed with increasing temperature till 50°C (optimum temperature) above which the keratinolytic activity declined sharply. The decreased in enzyme activity above optimum temperature might be due to the denaturation of enzyme protein. An Optimum temperature of 50°C for protease activity of halophilic bacterium NB2-1 and *Halobacillus* sp. SR5-3 has also been reported.[22,23] The enzyme produced from this *Bacillus* sp. is thermostable in nature as it retains 75% of the original keratinolytic activity at 50°C after 8 h of incubation. Further studies showed that the half-life of enzyme is about 3 h at 60°C (Figure 6b).

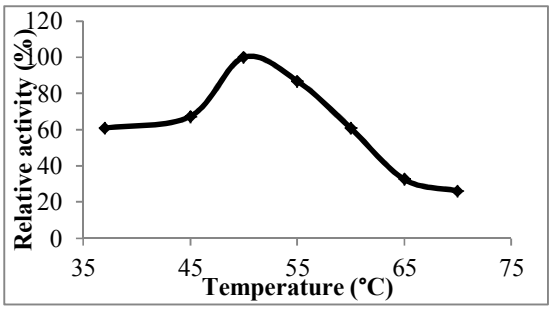


Figure 6a
Effect of temperature on keratinolytic activity

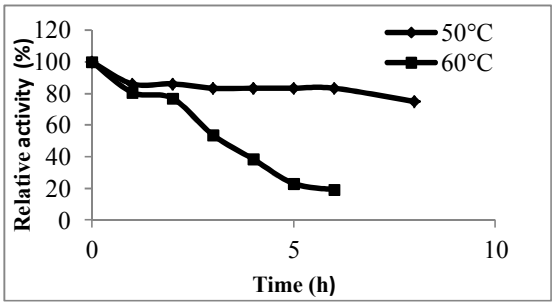


Figure 6b
Stability of keratinolytic activity of enzyme at different temperatures

Effect of different pH on keratinolytic activity and stability

The effect of different pH on keratinase action revealed that by increasing pH of buffer from 4 to 11, there is sharp increase in activity of enzyme till pH 9 (Optimum pH). Further increase in pH of reaction mixture above 9.0, the activity of enzyme decreased with a 22% of relative activity at pH 11.0 (Figure 7a). The optimum pH of 9.0 for alkaline protease activity has also been reported by certain halophilic bacteria.[23-25] The enzyme was found to be stable at alkaline pH (8 and 9) and more than 60% activity was retained at room temperature for 1 h at pH 10.0 and 11.0 as shown in Figure 7b.

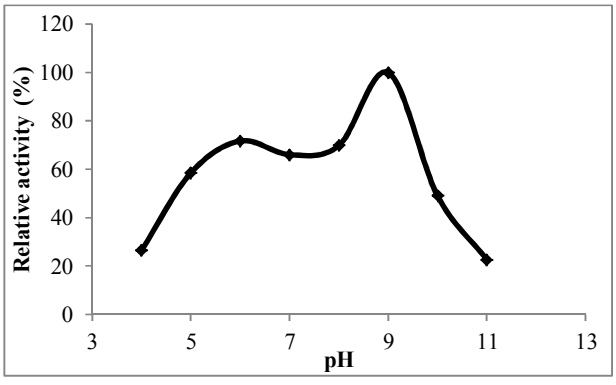


Figure 7a
Effect of pH on keratinolytic activity

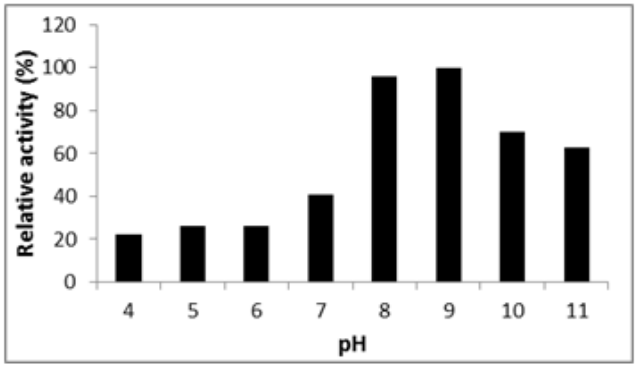


Figure 7b
Stability of keratinolytic activity of enzyme at different pH

Effect of keratin concentrations on keratinolytic activity

The effect of different concentrations of keratin on keratinase activity was studied in the range of 5-50 mg and a double reciprocal plot between 1/S and 1/V (Figure 8) was constructed to find out the maximum rate of reaction (Vmax) and Michaelis Menten constant (Km).

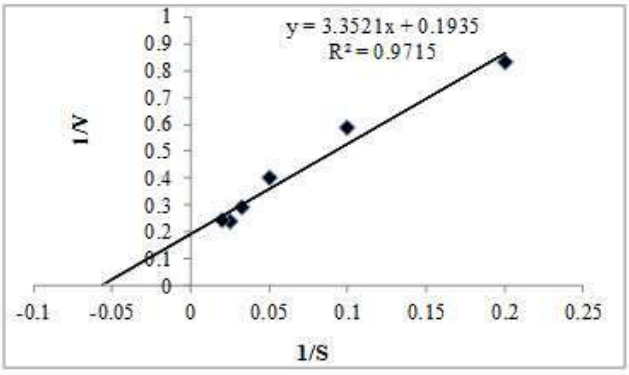


Figure 8
Double reciprocal plot of keratinase

The calculated values for Vmax and Km were 5.0 U and 16.67 mg respectively for keratinolytic activity at optimal conditions.

DISCUSSION

In recent years, the number of biotechnological uses of halophilic microorganisms has increased due to the halotolerance and high stability of enzymes derived from these microorganisms. During the last decade, various studies have been carried out on production and characterization of alkaline proteases from halophilic bacteria[1-4]. One of the rare studies on halophilic proteases exhibiting keratin degradation exists from actinomycetes but no true alkaline protease producing halophilic bacteria with keratinolytic activity have been reported. In this study, halophilic *Bacillus* species producing alkaline proteases was shown to degrade chicken feathers. Various recent studies showed the feather degradation by non halophilic *Bacillus* sp. In one of the study where chicken feather was used for the production of enzyme by *B. licheniformis* RPK showed an optimum pH of 9.0 and a temperature of 60 ^{0}C for the maximum activity.[26] Similarly, *B. subtilis* AMR produces keratinase with an optimum pH of

9.0 and 50 ^{0}C temperature for maximum activity using human hair a sole source of carbon.[27] As far as stability of keratinases are concerned no detailed studies has been carried out. *B. subtilis* SLC has also been evaluated for the production of keratinase that showed an optimum pH of 10.0 and a temperature of 60 ^{0}C for the maximum activity in a medium supplemented with feather (1%).[28] Alkaline protease and keratinolytic activities by the isolate *Bacillus* sp. were investigated in modified mineral medium and observed that the maximum activities were achieved at 12% NaCl concentration in the production medium though the activities have also been noticed at 18% of NaCl (65% relative activity) indicating the halophilic nature of the isolate. It was also found that the activities increased with increasing incubation periods and reached to a maximum value at 72 h of incubation. The incubation periods for maximum production of alkaline proteases from other halophilic sources also varied from 48-96 h of production time.[11,20,21] Enzyme activities also responded to changes in pH and temperature. In our studies optimum temperature and pH for both alkaline protease activity and keratinolytic activity were found to be 50°C and 9.0 respectively. The enzyme secreted by this bacterial isolate showed a broad range of pH stability. Similar to these results, halophilic bacterium NB2-1 and *Halobacillus* sp. SR5-3 showed the optimum temperature of 50°C for maximum enzyme activity.[22,23] The optimum pH for maximum keratinase activity ranged from 8.5 to 10.0 as observed from various halophilc sources. [28, 24-25] The keratinase produced from halophilic *Bacillus* sp.has both alkali and heat stable properties. Its ability to retain activities at 50°C up to 8 h is quite high and thus suitable for industrial biotechnology applications. Owing to the demonstrated feather degrading properties of the enzyme, it could be used in poultry processing wastes to improve nutritional values of animal feeds containing feather, or keratin.

CONCLUSIONS

The studies on production and characterization of alkaline proteases exhibiting keartinolytic activity from halophilic bacteria, *Bacillus* sp. showed that the 90% degradation of the chicken feathers was observed at 7 days of incubation at 37°C and 150 rpm. The microorganism requires 12% of sodium chloride for maximum production of enzymes. The enzyme retained comparatively more activity at 50°C than at 60°C. High thermostability of enzyme in alkaline pH also suggests its potential application in detergent industry besides its major application in feather degradation (poultry, animal feed) and keratin hydrolysis.

ACKNOWLEDGEMENTS

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REFERENCES

1. Gupta RG, Beg QB and Lorenz PL, Bacterial alkaline proteases: molecular approaches and industrial applications. *Appl Microbiol Biotechnol*, 59: 15-32, (2002).
2. Kazan D, Denizci A, Öner M and Erarslan A, Purification and characterization of a serine alkaline protease from *Bacillus clausii* GMBAE 42. *J Ind Microbiol Biotechnol*, 32: 335-344, (2005).
3. Brandelli A, Daroit D and Riffel A, Biochemical features of microbial keratinases and their production and applications. *Appl Microbiol Biotechnol*, 85: 1735-1750, (2010).
4. Onifade AA, Al-Sane NA, Al-Musallam AA and Al-Zarban S, Potentials for biotechnological applications of keratin-degrading microorganisms and their enzymes for nutritional improvement of feathers and other keratins as livestock

feed resources. *Biores Technol*, 66: 1–11, (1998).
5. Gupta R and Ramnani P, Microbial keratinases and their prospective applications: an overview. *Appl Microbiol Biotechnol*, 70: 21–33, (2006).
6. Jerry E, Biotechnological uses of archaeal extremozymes. *Biotechnol Adv,* 19: 261-278, (2001).
7. Madern D, Ebel C and Zaccai G, Halophilic adaptation of enzymes. *Extremophiles,* 4: 91-98, (2000).
8. Hough DW and Danson MJ, Extremozymes. *Curr Opin Chem Biol*, 3:39-46, (1999).
9. Sehar S and Hameed A, Extracellular alkaline protease by a newly isolated halophilic *Bacillus* sp. *Glo J Biotechnol Biochem,* 6: 142-148, (2011).
10. Ghasemia Y, Rasoul-Aminia S, Ebrahiminezhadb A, Kazemib A, Shahbazib M and Talebniab N, Screening and isolation of extracellular protease producing bacteria from the maharloo salt lake. *Ira J Pharm Sci*, 7: 175-180, (2011).
11. Kuberan T, Sangaralingam S and Thirumalaiarasu V, Isolation and optimization of protease producing bacteria from halophilic soil. *Res J Biol Sci*, 1: 163-174, (2010).
12. Ibrahim ASS, EI-Shayeb NMA and Mabrouk SS, Isolation and identification of alkaline protease producing alkaliphilic bacteria from an egyptian soda lake. *J Appl Sci Res*, 3: 1363-1368, (2007).
13. Jaouadi B, Abdelmalek B, Jaouadib NZ and Bejar S, The bioengineering and industrial applications of bacterial alkaline proteases: the case of SAPB and KERAB. Progress in Molecular and Environmental Bioengineering - From Analysis and Modeling to Technology Applications, Angelo Carpi (Ed.) ISBN: 978-953-307-268-5 InTech (2011).
14. Hoq MM, Siddiquee KAZ, Kawasaki H and Seki T, Keratinolytic activity of some newly isolated *Bacillus* species. *J Biol Sci*, 5: 193-200, (2005).
15. Suh HJ and Lee HK, Characterization of a keratinolytic serine protease from *Bacillus subtilis* KS-1. *J Prot Chem,* 20: 165-169, (2001).
16. Paul D, Rahman A, Ilias M and Hoq MM, Production and characterization of keratinolytic protease of *Bacillus Licheniformis* MZK-03 grown on feather mill. *Ban J Microbiol,* 24 : 57-61, (2007).
17. Makhija P, Nigam VK, Mohan MK, Ghosh P and Sasamal D, Characterization of extracellular alkaline proteases produced by halophilic bacteria. *Proceedings of the Indian National Science Academy*, 76 : 362-367, (2006) .
18. Riffle A and Brandelli A, Keratinolytic bacteria isolated from feather waste. *Braz J Microbiol,* 37 :395-399, (2006).
19. Lowry OH, Rosembrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* , 193 :267-275, (1951).
20. Giménez MI, Studdert CA, Sánchez JJ and De Castro RE, Extracellular protease of *Natrialba magadii*: purification and biochemical characterization. *Extremophiles,* 4 :181-188, (2000).
21. Vijayanand S, Hemapriya J and Selvin J, Production and optimization of haloalkaliphilic protease by an extremophile- *Halobacterium* sp. Js1, isolated from thalassohaline environment. *Glo* J *Biotechnol Biochem* ,5: 44-49, (2010).
22. Chamroensaksri N, Akaracharanya A, Visessanguan W and Tanasupawat S, Characterization of halophilic bacterium Nb2-1 from Pla-Ra and its protease production. *J Food Biochem* ,3 : 536–555, (2008).
23. Namwong S, Hiraga K, Takada K, Tsunemi M, Tanasupawat S and Oda K, A halophilic serine proteinase from *Halobacillus* sp. SR5-3 isolated from fish sauce: Purification and characterization. *Biosci Biotechnol Biochem* ,70 : 1395–1401, (2006).
24. Dodia MS, Rawal C, Bhimani H, Joshi R, Khare S and Singh S, Purification and stability characteristics of an alkaline serine protease from a newly isolated Haloalkaliphilic bacterium sp. AH-6. *J Ind Microbiol Biotechnol*, 35 :121-131, (2008).
25. Joshi GK, Kumar S and Sharma V, Production of moderately halotolerant, SDS stable alkaline protease from

Bacillus cereus MTCC 6840 isolated from lake Nainital, Uttaranchal state, India. *Braz J Microbiol,* 38 :773-777, (2007).

26. Nahed K, Safia K, Laila M and Moncef N, Production and biochemical and molecular characterization of a keratinolytic serine protease from chicken feather degrading *Bacillus lichaeniformis* RPk. *Can J Microbiol,* 55 :427-436, (2009).
27. Mazotto AM, Cedrola SM, Lins U, Rosado AS, Silva KT, Chaves JQ, Rabinovitch L, Zingati RB and Vermelho AB, Keratinolytic activity of *Bacillus subtilis* AMR using human hair. *Lett Appl Microbiol*, 50 :89-96, (2010).
28. Cedrola SM, de Melo AC, Mazotto AM, Lins U, Zingali RB, Rosado AS, Peixoto RS and Vermelho AB, Keratinases and sulfide from *Bacillus subtilis* SLC to recycle feather waste. *World J Microbiol Biotechnol*, 28 :1259-1269, (2012).