



Research Journal of **Microbiology**

ISSN 1816-4935



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Optimization of Keratin Degrading Enzyme from Thermophillic Strain of *Streptomyces sclerotialis*

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ABSTRACT

Feathers are major byproduct waste of poultry industries and produced in large amount. Keratin can be biodegraded by keratinolytic microorganisms and in this study, 45 keratinase producing actinomycetes and their enzyme production was isolated from hot springs, Rajgir, India. After Screening of keratinase enzyme production, the strain showed highest activity was selected and identified as *Streptomyces sclerotialis* by using polyphasic taxonomy including morphological and biochemical characteristics followed by 16S rDNA and DNA directed RNA polymerase beta subunit (*rpo β*) gene sequencing (accession no: HQ827802 and HQ827815). The effect of various substrates, temperature, pH and protease inhibitors, reducing agents and metals ions supplements in the production medium on enzyme production was studied and found that starch (3.5%) at 120 h, gives maximum activity followed by, protease peptone (1.5%) at 144 h recorded. The optimum temperature and pH recorded 55°C and 9.0, respectively. The enzyme was stable at 55°C for 1 h. Metals like MgSO₄, CaCl₂ were found strong inhibitory effect; whereas, Na₂SO₃ found to be stimulatory for enzyme production. Gel electrophoresis analysis revealed that the crude enzyme was monomeric with only a single protein band of 46 KDa were observed after ammonium persulfate precipitation.

Key words: Thermophillic, keratinase, optimization, serine protease, *rpo β*

INTRODUCTION

Keratins are insoluble proteins and treated as major pollutant from poultry and leather industries (Onifade *et al.*, 1998; Sangali and Brandelli, 2000). These are persistent in nature because of their molecular structures i.e., α -helix and β -sheet structures attached by di-sulfide bonds (Gradisar *et al.*, 2005; Yamamura *et al.*, 2002). Despite the rigid structure, it can be degraded by means of mechanical, chemical and biological methodologies. The major demerit of mechanical and chemical degradation over biological meaning, they might destroy certain amino acids and decrease protein quality and digestibility (Moritz and Latshaw, 2001; Anbu *et al.*, 2005).

Microbial enzymes had attracted attention from researchers all over the world because of their broad biochemical diversity, industrial as well as agricultural importance and feasibility of mass culture and ease of genetic manipulation. There are many microbes reported to degrade keratin such as fungi (Essien *et al.*, 2009; El-Naghy *et al.*, 1998; Gradisar *et al.*, 2000), *Bacteria* (Gessesse *et al.*, 2003; Cai and Zheng, 2009; Joo *et al.*, 2002; Pillai and Archana, 2008; Suh and

Lee, 2001), including actinomycetes (Bressollier *et al.*, 1999; Syed *et al.*, 2009; Tatineni *et al.*, 2008). Keratinase enzyme had several important uses in biotechnological processes such as in the leather industry (Kim *et al.*, 2001), agricultural farming (nitrogen source for plants), cosmetics, wool degradation (Soomro, 2000) and biodegradable films (Kim *et al.*, 2004). Feathers can also be used as biological indicators for heavy metal contamination in environment (Almansour, 2004). Some researchers tried to optimized medium for the production of Keratinase enzymes (Syed *et al.*, 2009; Xie *et al.*, 2010).

Approximately, 8.5 billion tonnes of poultry feather are produced in world annually, while in India alone contributing 350 million tonnes (Agrahari and Wadhwa, 2010). Keratin is largely responsible for their high degree of recalcitrance (Hoq *et al.*, 2005). Feather waste makes a serious problem as environmental pollutant and in outbreaks of H5N1 virus (Saber *et al.*, 2010). There is always a requirement of isolation of enzymes from new sources to meet the industrial and environmental demand. Since feathers are almost pure protein (90% keratin), they are potentially a less expensive alternative source of protein for animal feed. Traditional ways to degrade feathers such as alkali hydrolysis and steam pressure cooking may not only destroy the amino acids but also consume large amounts of energy (Belewu *et al.*, 2008). Biodegradation of feathers by keratinase from microorganisms may provide a viable alternative, i.e., feather hydrolysates of *Bacillus licheniformis* PWD-1 (Tamilmani *et al.*, 2008), *Bacillus subtilis* S14 exhibits remarkable dehairing capabilities (Raju *et al.*, 2007), as well as in newer fields like prion degradation for treatment of dreaded mad cow disease (Tork *et al.*, 2010).

Among actinomycetes, many species of *streptomyces* were reported known to degrade kertain by the secretion of extracellular enzymes such as multiple peptidase (Sinha *et al.*, 1991); serine peptidases (Johnson and Smillie, 1974), respectively. In the present study, we isolated a new keratin-degrading *streptomyces* strain R3 isolated from thermal spring of India. Keratinase enzyme was purified and optimized using solid state fermentation method from thermophilic *Streptomyces sclerotialus* R3.

MATERIALS AND METHODS

Isolation and strain characterization: Water and sediment samples were collected from the Rajgir hot spring, India (Coordinates: 25.03°N 85.42°E), during summer (June-July, 2009 to 2010) at regular time intervals, with the recorded temperatures and pH ranging from 52 to 55°C and 8.5, respectively. Samples were brought to laboratory for further analysis.

A thermophilic actinomycetes *Streptomyces sclerotialus* R3 was isolated by enrichment culture technique. The sediment samples (1 g) was added to Starch- Malt extract media containing K_2HPO_4 -2 g L⁻¹, $FeSO_4$ -0.01 gL⁻¹, NaCl-2 g L⁻¹, KNO_3 -2 g L⁻¹, $MgSO_4 \cdot 7H_2O$ -0.05 g L⁻¹, Soluble Starch-10 g L⁻¹, Malt extract-0.03 gL⁻¹ and Agar-18 gL⁻¹. The pH was adjusted to 7.4 and incubated on incubator at 37°C for 48 h. The isolates were further characterized for its growth parameter like temperature and pH and found that its growth limit is restricted at 60°C upto 12.0 pH. The isolate was identified as *Streptomyces* by its morphological (scanning electron microscopy Fig. 1) and biochemical characteristics as per Bergey's manual of determinative bacteriology (Holt *et al.*, 1994) (Table 1). The potential strain was further identified by 16S rRNA and DNA directed RNA polymerase beta subunit (rpo β) gene amplification and sequencing (accession No. HQ827802 and HQ827815).

Inoculum preparation and enzyme production: The inoculum was prepared by adding a loopful of pure culture into 100 mL of sterile NB broth medium and incubated at 37°C on rotatory

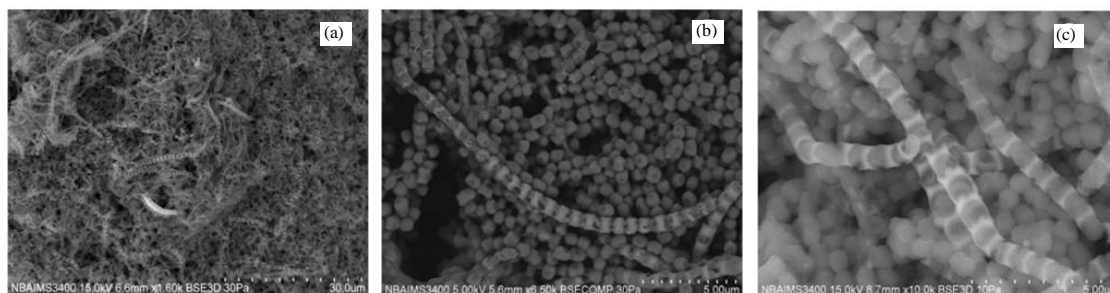


Fig. 1 (a-c): SEM photograph of *Streptomyces sclerotialis* R3 from Rajgir thermal spring, showing straight chain morphology

Table 1: Physiological and biochemical characterization of the *Streptomyces sclerotialis* R3 strain

Parameters	<i>Streptomyces sclerotialis</i> Strain R3
Cell morphology	
Aerial mycelia colour	White
Substrate mycelia colour	Yellow white
Biochemical characteristics	
Gram reaction	+
Pigment production	+
Starch hydrolysis	+
Catalase test	+
Casein hydrolysis	+
Tyrosine degradation	+
Xanthine degradation	+
Nitrate reduction	+
Indole production	+
Gelatin hydrolysis	-
H ₂ S production	+

flask for 48 h. A 10% of inoculum from the culture (at A₆₆₀) was added to basal media containing NH₄Cl-0.5%, CaCl₂-0.2%, MgSO₄- 7H₂O- 0.2%, K₂HPO₄-0.4%, feather powder 0.4%; pH 7.4, for the production of enzyme, feather waste were utilized as a substrate. After incubation at 37°C for 48 h under shaking condition (250 rpm) for seven days, cells were harvested and growth was measured at A₆₆₀. The culture was centrifuged at 10,000 rpm for 10 min at 4°C. Bacteria were removed by filtration and the filtrate was used for crude enzyme preparation (Bressollier *et al.*, 1999).

Solid State Fermentation (SSF): *Streptomyces* spore suspension was obtained by growing the culture in Nutrient broth under room temperature and harvested after 72 h of cultivation. The spore suspension was passed through a 0.5 mm sieve to eliminate mycelia and the spore concentration was estimated by direct microscopic counting using hemocytometer. The spore suspension at 10⁷ spore mL⁻¹ and volume of 20% (v/w) was mixed with the prior autoclaved growth medium. This medium was used as inoculum for SSF process.

Enzyme assay: Keratinolytic activity was measured using a modified protocol of Anson (1938) using feather powder as a substrate. The reaction mixture contains 0.2 mL of culture supernatant

and 0.5 mL of 0.4% (w/v) feather powder and it was incubated at 45°C for 30 min. The reaction was stopped by adding 2.5 mL of Trichloroacetic (10%). The solution was allowed to stand at 37°C for 60 min and then centrifuged at 15,000 g for 15 min and supernatant was measured at 595 nm for amino acid liberation. The quantity was determined from standard tyrosine solution (50-500 µg mL⁻¹) using UV spectrophotometer (Letourneau *et al.*, 1998). One unit of keratinolytic activity was defined as the amount of enzyme required to liberate 1 µmol tyrosine under standard conditions.

ENZYME ACTIVITY: OPTIMIZATION AND STABILITY

Effect of pH and temperature: The pH optimization was performed using different buffers (pH 7 to 11). Enzyme broth was incubated at different pH values for a fixed time and activity was measured at regular time interval at the fixed temperature of 55°C. For temperature optimization, enzyme broth was incubated for fixed period at different temperatures (30-60°C; pH 9.0) and activity was measured to determine the optimum temperature. For enzyme stability, enzyme broth was incubated at the different time interval upto 60 min (i.e., 0, 15, 30, 45 and 60 min) and activity was determined at regular time interval at fixed temperatures (55°C) and pH (9.0), respectively.

Optimization of incubation period: The kinetics of growth and enzyme production was followed at different time intervals (1 to 7 days) and different substrate concentrations (i.e., 0.5 to 5%). The culture was inoculated in basal medium with following respective substrates i.e., starch, sucrose, malt extract, protease peptone, tryptone, soyacake and gelatin, were incubated at 55°C under shaking condition (100 rpm). Culture sample were withdrawn aseptically every 24 h and cell density along with enzymatic activity was measured as described above.

Effect of protease inhibitor, reducing agent and metal ions: The effect of various protease inhibitor compounds (1 mM; PMSF, pCMB, EDTA), reducing agents Na₂SO₃ (1 mM) as well as metal ions (Mg, Ca, Zn) at 10 mM final concentration was studied following pre-incubation with enzyme with for 24 h. After 24 h incubation, enzyme activity was determined.

Ammonium sulfate precipitation: The crude enzyme was first saturated up to 30% with solid (NH₄)₂SO₄ and then centrifuged at 5000 g for 15 min. The supernatant obtained was further saturated up to 70% with solid (NH₄)₂SO₄ and again centrifuged. The pellets obtained were dissolved in minimum volume of 0.1 M Tris-HCl buffer, pH 8.0.

Gel-electrophoresis: For determination of homogeneity and molecular weight we use the methods as described by Lundy *et al.* (1995), enzyme preparation and known molecular weights (Genei, India), were subjected to 12.5% agarose gel electrophoresis.

Statistical analysis: Experiments were performed in triplicate and the results were statistically analyzed using analysis of variance using SPSS software (version 9). Standard error was also calculated. Univariate analysis of variance was employed on the data with initial substrate concentration and keratinase activity, pH and keratinase activity, temperature and keratinase activity, incubation period and keratinase activity and substrate supplementation and keratinase activity were tested for significance. In case of stability of keratinase, time and activity and time and pH were grouping variables.

RESULTS

Strain characterization: Morphological characterization including spore arrangement showed that isolate R3 possesses long straight chain spore arrangement as evident by light and scanning electron microscopy (Fig. 1), was preliminarily identified as belongs to *streptomyces* genera after comparing with Bergey's manual. Physiological and biochemical characteristics along with areal and substrate mycelial colors were also tabulated (Table 1), which showed confirmatory identification of the isolates belonging to the genus *streptomyces* as Gram staining positive as well as ability to degrade casein, tyrosine and xanthine respectively.

Optimization of incubation period: The effect of incubation period on enzyme production was recorded with the maximum release of enzyme on 120 h (7.4 U mL⁻¹), after that it decreases and complete degradation of feather meal occurs at 144 h, respectively.

Optimization of substrate concentration: As the enzyme yield was very low with sucrose, malt extract, tryptone, soycake and gelatin in comparison with starch and protease peptone, therefore these substrates were omitted for substrate concentration optimization (data not shown). Enzyme production was analyzed in terms of activity with variable concentrations (0.5-5%) of starch and protease peptone, after every 24 h. Starch was found to be an excellent medium for the production of enzyme by *S. sclerotialus*. A common trend in production profile was in the range of 3.9-10.3 U mL⁻¹ (Fig. 2) for all the concentrations after 24 h. Starch (3.5%) yield maxima (10.3 U mL⁻¹) at 120 h, then decreased on next day.

Total Extracellular Protein production was recorded to be maximum with 3.5% starch showed maximum protein production (10.3 mg mL⁻¹) after 120 h, while at 1.5 and 4.5% starch concentration it showed 9.0 and 8.6 mg mL⁻¹, respectively. However protease peptone showed maximum protein (4.95) at 1.5% concentration after 144 h. The parallel increase in protein concentration and enzyme release was recorded for most of the substrate concentrations.

Similar trend was observed for total extra cellular protein (Fig. 3). Enzyme yield was slightly lower with Protease peptone as compared to starch. The enzyme activity ranged between 3.2-5.4 U mL⁻¹ after 24 h at various concentrations of peptone. Maximum amount of enzyme was released with 1.5% of protease peptone (5.2 U mL⁻¹) after 144 h. Total protein concentration decreased after 24 h, which may be attributed to simultaneous increase in protease activity. The parallel increase in protein concentration and enzyme release was recorded for most of the substrate concentrations.

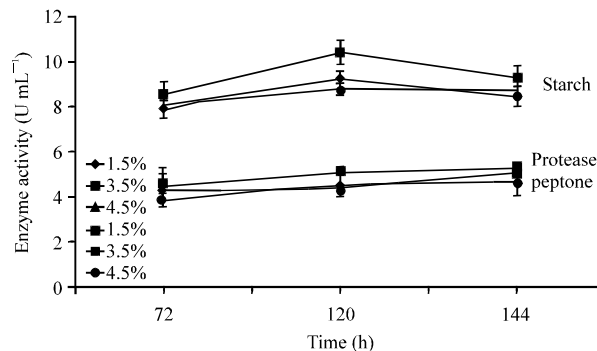


Fig. 2: Effect of different substrates on keratinase production of *Streptomyces sclerotialus* R3. Symbols represents: Starch. Data are represented as Mean of five replications±SD

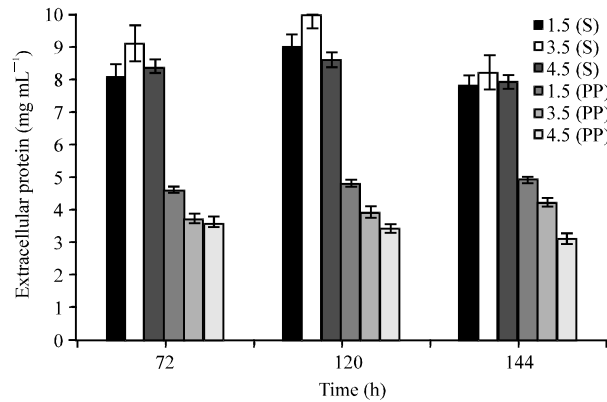


Fig. 3: Effect of Starch and Protease Peptone on extracellular protein production of *Streptomyces sclerotialus* R3. Bars represent concentration of a substrate for extracellular protein production, S: Starch, PP: Protease Peptone. Data are represented as Mean of five replications \pm SD

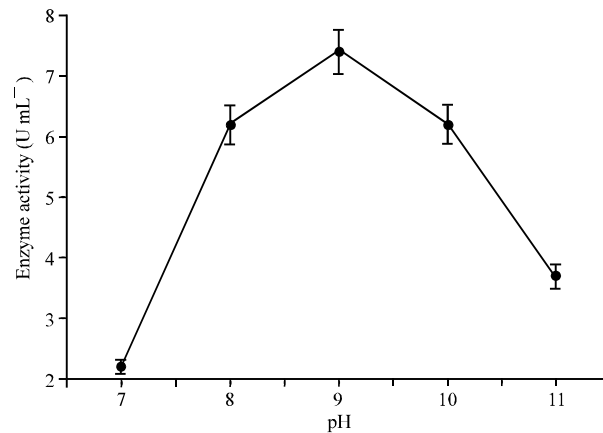


Fig. 4: Effect of pH optimization on protease enzyme of *Streptomyces sclerotialus* R3. Symbol represents pH optimization. Data are represented as Mean of five replications \pm SD

Optimization of pH and Temperature and its stability studies: The crude enzyme used for pH and temperature optimization was obtained after 5th days of fermentation. The time coincides with maximum enzyme activity in the fermentation broth. Enzyme was found to have an excellent activity over a wide range of pH values studied. Maximum activity was recorded at pH 9 (7.4 U mL⁻¹), however, at pH 8 and 10, enzyme retained about 85% of maximum activity (6.2 U mL⁻¹) while at pH 7, 30% activity (2.24 U mL⁻¹) and at pH 11, 50% activity (3.7 U mL⁻¹) was recorded. It is evident that the enzyme was efficacious at alkaline pH values with optimum activity at pH 9 (Fig. 4).

The temperature optimum of protease activity was recorded; the crude enzyme was incubated at five different temperatures for fixed time intervals at pH 9. Maximum activity was (7.4 U mL⁻¹) recorded at 55°C. Approximately 70% of the maximum activity (2.3 U mL⁻¹) was lost at the temperature less than 30 and more than 60°C (Fig. 5). A continuous rise in enzyme activity was recorded from 30 to 55°C.

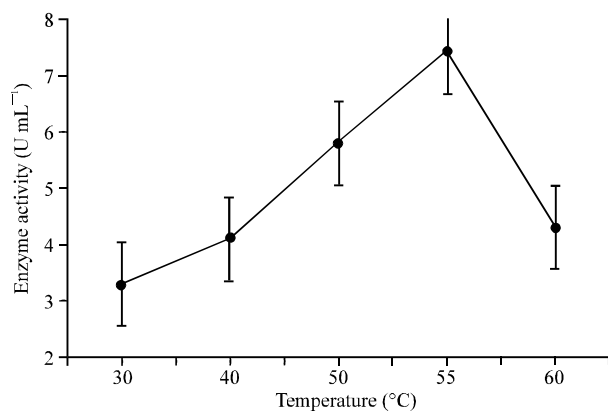


Fig. 5: Effect of temperature optimization on protease enzyme of *sclerotialus* R3. Symbols represent. Data are represented as Mean of five replications±SD

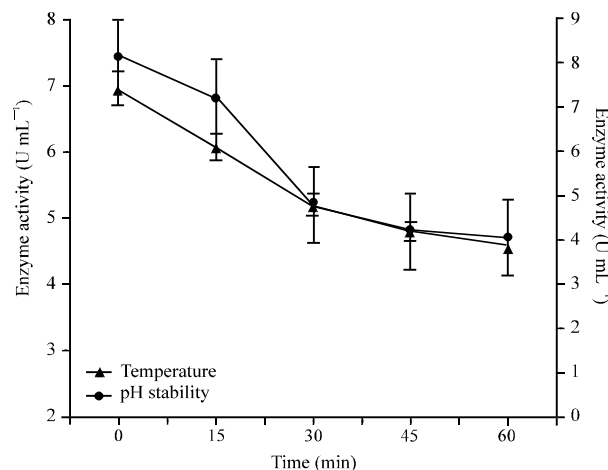


Fig. 6: Effect of enzyme stability on keratinase production of *Streptomyces sclerotialus* R3. Symbols represent of enzyme. Data are represented as Mean of five replications±SD

Enzyme stability studies: Stability of enzyme was checked on its optimum temperature (55°C) and optimum pH 9 up to 1 h. The activity at 0 min (i.e., 7.4 U mL⁻¹) was considered as 100% activity. After 15 min enzyme activity showed 92% activity, 30 min, for 70.2%, 45 min, for 65% and 60 min, for 64%, respectively, with a total loss of 36% activity upto 60, thereafter enzyme was found to stabilize as almost constant activity was recorded thereafter (Fig. 6). The results suggest that enzyme was stable at 9.0 and 55°C pH and temperature with recorded activity of 7.4 U mL⁻¹ upto 60 min, respectively (Fig. 6). On further incubation, a very slow decline in enzyme activity was observed up to 120 min.

Effect of protease inhibitors, reducing agents and metal ions: PMSF and EDTA were strong inhibitory but pCMB were not affect the enzyme activity, while sodium sulfate enhances 102.5% enzyme activity. Metal ions namely Mg²⁺, Ca²⁺ as well as Zn²⁺ reduces its activity by 92, 84 and 33%, respectively (Table 2).

Table 2: Effects of the different compounds on the keratinolytic activity of *Streptomyces sclerotialis* R13 strain

Compounds	Concentration (mM)	Residual activity (%)
Control	0	100
PMSF	1	0
EDTA	1	2
pCMB	1	100
Na ₂ SO ₃	1	102.5
MgCl ₂	10	92
CaCl ₂	10	84
ZnCl ₂	10	33

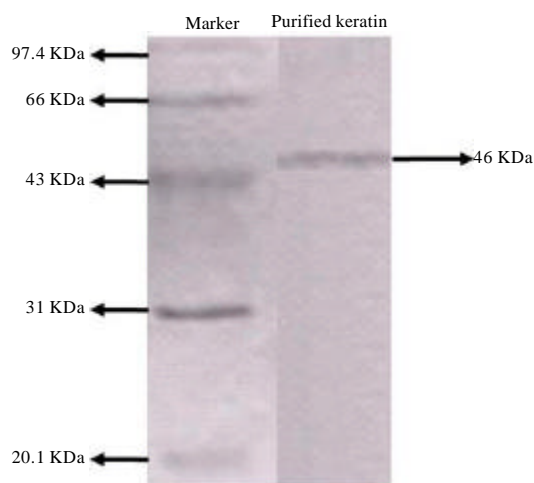


Fig. 7: SDS-PAGE of crude keratinase obtained from cell free broth of feather utilizing *Streptomyces sclerotialis* R3. Lane 1: Molecular weight marker (20.1–97.4 KDa). Lane 2: Purified Keratin

Gel-electrophoresis: SDS-PAGE analysis revealed that the crude enzyme preparations were quite homogeneous and there was only a single protein band which had proteolytic activity. The molecular weight of band was found to be about 46 KDa (Fig. 7). The usefulness of this preparation in its crude form for industrial applications could be exploited.

DISCUSSION

Researcher had paid attention towards extremophilic microorganisms for the production of industrial enzymes. Recently, a novel keratinolytic enzyme was reported to yield maximum enzyme production at the temperature 45°C and pH 9.0 after 120 h (Syed *et al.*, 2009; Nam *et al.*, 2002). Proteolytic enzymes have dominated the detergent enzyme market. Nonetheless, there is always a need for new enzymes with novel properties that can further widen the scope of enzyme-based detergents. Keratinases could help in the removal of keratin that are often encountered in the laundry and on which most normal peptidases fail to act.

Keratinases of high molecular weight may consist of four domains, which contribute to their substrate specificities (Kim *et al.*, 2004). Solid state fermentation process was used in this study, now a days many researchers tried to optimized the enzyme production by using SSF techniques as it reduces optimization time as well as had many advantages over submerged process

(Sivaramakrishnan *et al.*, 2006). Enzyme yield was slightly lower with protease peptone as compared to starch. Rise and fall in the enzyme activity may be because of catabolite repression system. Initially low level of enzyme activity is responsible for breakdown of protein at low pace which are taken up by the cells. The amino acids and short peptides induce the bacterial cells for further production and release of proteases, which ultimately released more amount of product, which might be responsible for maximum enzyme release and release of more amount of product then decline because, enzyme production is repressed by the accumulation of reaction products in the intercellular environment. The results exhibits that, enzyme production was higher on 5th day, which is in agreement with Xie *et al.* (2010). Microbial keratinase enzyme was known to be inducible (Cheng *et al.*, 1995). Stimulation of keratinase activity in *Streptomyces sclerotialus* was in presence of increasing amounts of starch. Since starch is a main component in the media for isolation of actinomycetes. Many *Streptomyces* species have been reported to produce keratinolytic protease (Lal *et al.*, 1999; Wang and Shih, 1999). The molecular weight of keratinase from *S. sclerotialus* (46 KDa) was approved by the others workers also (Bressollier *et al.*, 1999; Gradisar *et al.*, 2000).

Enzymes were significantly inhibited by serine protease inhibitor (PMSF and EDTA) but not affected by cysteine inhibitor (pCMB), moreover it significantly increases by adding sodium sulfite this indicates that the enzymes belong to the monomeric serine protease family and are metal ion related enzymes. Several *Streptomyces* as well as other microbial genera showed keratinolytic activities had been reported belongs to serine proteases family (Hossain *et al.*, 2007; Bressollier *et al.*, 1999; Bockle and Muller, 1997). The divalent metal ion Ca^{2+} and Mg^{2+} showed an inhibitory effect on enzyme production, respectively. The inhibitory effect of metal ions and their possible causes is well documented in the literatures (Valle and Ulmer, 1972; Kumar *et al.*, 1999).

Various keratinous materials such as chicken feather, feather meal, wool and bovine hair have been used as inducers of keratinases (De Toni *et al.*, 2002; Ignatova *et al.*, 1999; Nam *et al.*, 2002; Kumar *et al.*, 2008). It is evident that the enzyme was efficacious at alkaline pH values with optimum activity at pH 9. Similar finding were also reported by Bressollier *et al.* (1999), Korkmaz *et al.* (2003), Letourneau *et al.* (1998), Mohamedin (1999) and Rozs *et al.* (2001).

We also found temperature and pH stability of enzyme activity was 55°C and 9.0 upto 1 h. Our thermal stability findings were also supported by Brouta *et al.* (2001), Letourneau *et al.* (1998), Rozs *et al.* (2001) and Syed *et al.* (2009), while pH stability was observed were also showed good agreement with other known enzymes of keratinolytic *streptomyces* (Bressollier *et al.*, 1999; Korkmaz *et al.*, 2003; Mohamedin, 1999; Syed *et al.*, 2009), respectively.

In conclusion these findings suggest the enzyme is highly stable with only 36% loss in activity (92.4 U mL^{-1}) after 1 h at temperature and pH of 55°C and 9.0, respectively. These results were supported by many workers also Syed *et al.* (2009), Xie *et al.* (2010), Bressollier *et al.* (1999), Gradisar *et al.* (2000), Bockle and Muller (1997) and Kumar *et al.* (1999) but we had purified enzyme with molecular weight corresponds to 46 kDa, showed higher than findings of Xie *et al.* (2010). Hence, the latest trend in enzyme-based detergents is use of rDNA and protein engineering technologies to produce bioengineered enzymes with better stability. These characteristics are important because the pH of laundry detergents is generally in alkaline range and there is variable thermostable at laundry temperatures (50 or 60°C) (Takami *et al.*, 1989; Manchini *et al.*, 1998). Mutations have led to newer protease preparations with improved catalytic efficiency and better stability towards temperature, oxidizing agents and changing wash conditions (Rao *et al.*, 1998).

Thus, the reported enzyme might contribute to detergent formulations. Further work on this protease towards cloning and expression of gene responsible for oxidation-stability is currently underway.

CONCLUSION

The results obtained from this study reveals that, the best strain which showed the highest activity was selected and identified as *S. sclerotialis* after morphological and biochemical properties investigation and further confirmed by 16S rDNA and DNA directed RNA polymerase beta subunit (rpo β) gene sequencing. Medium for the maximum activity of enzyme was optimized and found that starch (3.5%) at 120 h, gives maximum activity followed by, protease peptone (1.5%) at 144 h recorded. The optimum temperature and pH recorded 55°C and 9.0, respectively. The enzyme was stable at 55°C for 1 h. Metals like MgSO₄, CaCl₂ were found strong inhibitory effect; whereas, Na₂SO₃ found to be stimulatory for enzyme production. The present work showed that *S. sclerotialis* can also be used in protease industries (laundries, leather industries etc). This study can also serve as a one forward step for further optimization and purification of this process and other corresponding investigations.

ACKNOWLEDGMENTS

The study was supported by the grant from ICAR Network Project, Government of India, New Delhi, India. Authors are gratefully acknowledged.

REFERENCES

- Agrahari, S. and N. Wadhwa, 2010. Degradation of chicken feather a poultry waste product by keratinolytic bacteria isolated from dumping site at ghazipur poultry processing plant. Int. J. Poult. Sci., 9: 482-489.
- Almansour, M.I., 2004. Using feathers as a biological indicator of lead environmental pollution. Pak. J. Biol. Sci., 7: 1884-1887.
- Anbu, P., S.C.B. Gopinath, A. Hilda, T. Lakshmi priya and G. Annadurai, 2005. Purification of keratinase from poultry farm isolate *Scopulariopsis brevicaulis* and statistical optimization of enzyme activity. Enzyme Microb. Technol., 36: 639-647.
- Anson, M.L., 1938. Plant proteolytic enzymes. J. Gen. Physiol, 22: 79-84.
- Belewu, M.A., A.R. Asafa and F.O. Ogunleke, 2008. Processing of feather meal by solid state fermentation. Biotechnology, 7: 589-591.
- Bockle, B. and R. Muller, 1997. Reduction of disulfide bonds by *Streptomyces pactum* during growth on chicken feathers. Applied Environ. Microbiol., 63: 790-792.
- Bressollier, P., F. Letourneau, M. Urdaci and B. Verneuil, 1999. Purification and characterization of a keratinolytic serine proteinase from *Streptomyces albidoflavus*. Applied Environ. Microbiol., 65: 2570-2576.
- Brouta, F., F. Descamps, T. Fett, B. Losson, C. Gerday and B. Mignon, 2001. Purification and characterization a 43.5 kDa keratinolytic metalloprotease from *Microsporium canis*. Med. Mycol., 39: 269-275.
- Cai, C. and X. Zheng, 2009. Medium optimization for keratinase production in hair substrate by a new *Bacillus subtilis* KD-N2 using response surface methodology. J. Ind. Microbiol. Biotechnol., 36: 875-883.

- Cheng, S.W., H.M. Hu, S.W. Shen, H. Takagi, M. Asano and Y.C. Tsai, 1995. Production and characterization of keratinase of a feather-degrading *Bacillus licheniformis* PWD-1. Biosci. Biotechnol. Biochem., 59: 2239-2243.
- De Toni, C.H., M.F. Richter, J.R. Chagas, J.A.P. Henriques and C. Termignoni, 2002. Purification and characterization of an alkaline serine endopeptidase from a feather-degrading *Xanthomonas maltophilia* strain. Can. J. Microbiol., 48: 342-348.
- El-Naghy, M.A., M.S. El-Ktatny, E.M. Fadl-Allah and W.W. Nazeer, 1998. Degradation of chicken feathers by *Chrysosporium georgiae*. Mycopathologia, 143: 77-84.
- Essien, J.P., A.A. Umoh, E.J. Akpan, S.I. Eduok and A. Umoiyoho, 2009. Growth, keratinolytic proteinase activity and thermotolerance of dermatophytes associated with alopecia in Uyo, Nigeria. Acta Microbiol. Et Immunol. Hungarica, 56: 61-69.
- Gessesse, A., R. Hatti-Kaul, B. Gashe and B. Mattasson, 2003. Novel alkaline proteases from alkaliphilic bacteria grown using chicken feather. Enz. Microbiol. Technol., 32: 519-524.
- Gradisar, H., S. Kern and J. Friedrich, 2000. Keratinase of *Doratomyces microsporus*. Applied Microbiol. Biotechnol., 53: 196-200.
- Gradisar, H., J. Friedrich, I. Krizaj and R. Jerala, 2005. Similarities and specificities of fungal keratinolytic proteases: Comparison of keratinases of *Paecilomyces marquandii* and *Doratomyces microsporus* to some known proteases. Appl. Environ. Microbiol., 71: 3420-3426.
- Holt, J.G., N.R. Krieg, P.H. Sneath, J.J. Stanley and S.T. Williams, 1994. Bergey's Manual of Determinative Bacteriology. Williams and Wilkins, Baltimore, USA.
- Hoq, M.M., K.A.Z. Siddiquee, H. Kawasaki and T. Seki, 2005. Keratinolytic activity of some newly isolated *Bacillus* species. J. Biol. Sci., 5: 193-200.
- Hossain, M.S., A.K. Azad, S.M. Abu Sayem, G. Mostafa and M. Mozammel Hoq, 2007. Production and partial characterization of feather-degrading keratinolytic serine protease from *Bacillus licheniformis* MZK-3. J. Boil. Sci., 7: 599-606.
- Ignatova, Z., A. Gousterova, G. Spassov and P. Nedkov, 1999. Isolation and partial characterisation of extracellular keratinase from a wool degrading thermophilic actinomycete strain *Thermoactinomyces candidus*. Can. J. Microbiol., 45: 217-222.
- Johnson, P. and L.B. Smillie, 1974. The amino acid sequence and predicted structure of *Streptomyces griseus* protease A. FEBS Lett., 47: 1-6.
- Joo, H.S., J.C. Kumar, G.C. Park, K.T. Kim, S.R. Paik and C.S. Chang, 2002. Optimization of the production of an extracellular alkaline protease from *Bacillus horikoshi*. Process. Biochem., 38: 155-159.
- Kim, J.M., W.J. Lim and H.J. Suh, 2001. Feather-degrading *Bacillus* species from poultry waste. Process. Biochem., 37: 287-291.
- Kim, J.S., L.D. Kluskens, W.M. de Vos, R. Huber and J. van der Oest, 2004. Crystal structure of fervidolysin from *Fervidobacterium pennivorans*, a ketatinolytic enzyme related to subtilisin. J. Mol. Biol., 335: 787-797.
- Korkmaz, H., M.N. Unaldi, B. Aslan, G. Coral, B. Arıkan, S. Dincer and O. Colak, 2003. Keratinolytic activity of *Streptomyces satrin* BA7 a new isolate from Turkey. Ann. Microbiol., 53: 85-93.
- Kumar, A.G., S. Swarnalatha, S. Gayathri, N. Nagesh and G. Sekaran, 2008. Characterization of an alkaline active-thiol forming extracellular serine keratinase by the newly isolated *Bacillus pumilus*. J. Appl. Microbiol., 104: 411-419.

- Kumar, C.G., M.P. Tiwari and K.D. Jany, 1999. Novel alkaline serine proteases from alkalophilic *Bacillus* spp: Purification and some properties. Proc. Biochem., 34: 441-449.
- Lal, S., R.C. Rajak and S.K. Hasija, 1999. *In vitro* degradation of keratin by two species of *Bacillus*. J. Gen. Appl. Microbiol., 45: 283-287.
- Letourneau, F., V. Soussotte, P. Bressoier, P. Brandland and V.V. Verneuil, 1998. Keratinolytic activity of *Streptomyces* sp. S.K-02: A new isolated strain. Lett. Applied Microbiol., 26: 77-80.
- Lundy, F.T., A.C. Magee, L.S. Blair and D.A. McDowell, 1995. A new method for the detection of proteolytic activity in *Pseudomonas lundensis* after sodium dodecyl sulfate polyacrylamide gel electrophoresis. Electrophoresis, 16: 43-45.
- Manchini, P., M. Foretina and C. Parini, 1998. Thermostable alkaline protease produced by *Bacillus thermorubber*: A new species of *Bacillus*. Appl. Microbiol. Biotechnol., 28: 409-413.
- Mohamedin, A.H., 1999. Isolation, identification and some cultural condition of a protease-producing thermophilic *Streptomyces* strain grown on chicken feather as a substrate. Int. Biodeter. Biodegr., 43: 13-21.
- Moritz, J.S. and J.D. Latshaw, 2001. Indicators of nutritional value of hydrolyzed feather meal. Poultry Sci., 80: 79-86.
- Nam, G.W., D.W. Lee, H.S. Lee, N.J. Lee and B.C. Kim *et al.*, 2002. Native feather degradation by *Fervidobacterium islandicum* AW-1, a newly isolated keratinase-producing thermophilic anaerobe. Arch. Microbiol., 178: 538-547.
- Onifade, A.A., N.A. Al-Shane, A.A. Al-Musallam and S. Al-Zarban, 1998. A review: Potentials for biotechnological applications of keratin-degrading microorganisms and their enzymes for nutritional improvement of feathers and other keratins as livestock feed resources. Bioresour. Technol., 66: 1-11.
- Pillai, P. and G. Archana, 2008. Hide depilation and feather disintegration studies with keratinolytic serine protease from a novel *Bacillus subtilis* isolate. Applied Microbiol. Biotechnol., 78: 643-650.
- Raju, K.C., U. Neogi, R. Saumya and N.R. Goud, 2007. Studies on extra cellular enzyme keratinase from dermatophyte *Microsporium gypseum*. Int. J. Biol. Chem., 1: 174-178.
- Rao, M.B., A.M. Tanksale, M.S. Ghatge and V.V. Deshpande, 1998. Molecular and biotechnological aspects of microbial proteases. Microbiol. Mol. Biol. Rev., 62: 597-635.
- Rozs, M., L. Manczinger, C. Vagvolgyi and F. Kevei, 2001. Secretion of a trypsin-like thiol protease by a new keratinolytic strain of *Bacillus licheniformis*. FEMS Microbiol. Lett., 205: 221-224.
- Saber, W.I.A., M.M. El-Metwally and M.S. El-Hersh, 2010. Keratinase production and biodegradation of some keratinous wastes by *Alternaria tenuissima* and *Aspergillus nidulans*. Res. J. Microbiol., 5: 21-35.
- Sangali, S. and A. Brandelli, 2000. Isolation and characterization of a novel feather degrading bacterial strain. Applied Biochem. Biotechnol., 87: 17-24.
- Sinha, U., S.A. Wolz and P.J. Lad, 1991. Two new extracellular serine proteases from *Streptomyces fradiae*. Int. J. Biochem., 23: 979-984.
- Sivaramakrishnan, S., D. Gangadharan, K.M. Nampoothiri, C.R. Soccol and A. Pandey, 2006. Alpha amylases from microbial sources: An overview on recent developments. Food Technol. Biotechnol., 44: 173-184.
- Soomro, I.H., 2000. Keratinophilic fungi: The destroyer of the wool fibres. Pak. J. Biol. Sci., 3: 1323-1325.

- Suh, H.J. and H.K. Lee, 2001. Characterization of a keratinolytic serine protease from *Bacillus subtilis* KS-1. *J. Protein Chem.*, 20: 165-169.
- Syed, D.G., J.C. Lee, W.J. Li, C.J. Kim and D. Agasar, 2009. Production, characterization and application of keratinase from *Streptomyces gulbargensis*. *Bioresour. Technol.*, 100: 1868-1871.
- Takami, H., T. Akiba and K. Hprikoshi, 1989. Production of extremely thermostable alkaline protease from *Bacillus* sp. No. AH-101. *Applied Microbiol. Biotechnol.*, 30: 120-124.
- Tamilmani, P., A. Umamaheswari, A. Vinayagam and B. Prakash, 2008. Production of an extra cellular feather degrading enzyme by *Bacillus licheniformis* isolated from poultry farm soil in Namakkal district (Tamilnadu). *Int. J. Poult. Sci.*, 7: 184-188.
- Tatineni, R., K.K. Doddapanem, R.C. Potumarthi, R.N. Vellanki, M.T. Kandathil, N. Kolli and L.N. Mangamoori, 2008. Purification and characterization of an alkaline keratinase from *Streptomyces* sp. *Bioresour. Technol.*, 99: 1596-1602.
- Tork, S., M.M. Aly and L. Nawar, 2010. Biochemical and molecular characterization of a new local keratinase producing *Pseudomonas* sp., MS21. *Asian J. Biotechnol.*, 2: 1-13.
- Valle, B.L. and D.D. Ulmer, 1972. Biochemical effects of mercury, cadmium and lead. *Annu. Rev. Biochem.*, 41: 91-128.
- Wang, J.J. and J.C.H. Shih, 1999. Fermentation production of keratinase from *Bacillus licheniformis* PWD-1 and a recombinant *B. subtilis* FDB-29. *J. Indus. Microbiol. Biotechnol.*, 22: 608-616.
- Xie, F., Y. Chao, X. Yang, J. Yang, Z. Xue, Y. Luo and S. Qian, 2010. Purification and characterization of four keratinases produced by *Streptomyces* sp. strain 16 in native human foot skin medium. *Bioresour. Technol.*, 101: 344-350.
- Yamamura, S., Y. Morita, Q. Hasan, K. Yokoyama, E. Tamiya, 2002. Keratin degradation: A cooperative action of two enzymes from *Stenotrophomonas* sp. *Biochem. Biophys. Res. Commun.*, 294: 1138-1143.