J. Phytol. Res. **29** (1 & 2) : 17-30, 2016 **ISSN 0970-5767**

BACTERIAL KERATINASE CATALYZED BIOREMEDIATION OF KERATIN RICH WASTES FOR POTENTIAL AGRICULTURAL AND OTHER APPLICATIONS

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Microbial keratinase mediated biodegradation of keratin rich wastes such as chicken feathers, nails, hairs and wool, is an environment friendly biotechnological process, which converts this abundant waste into low-cost, nutrient-rich animal feed. Keratinase mediated hydrolysis of feather meal/raw feather significantly enhances the level of essential amino acids in the product. Optimization of various physical and nutritional parameters showed that maximum protease production of 4250 U/l from *Bacillus* S-2 strain occurred in 36 h at pH 7, temperature 37 ºC, agitation rate 200 rpm, in a medium having lactose as carbon source, proteose peptone as nitrogen source and 1 % feather meal as inducer. The isolated enzyme efficiently degraded raw feathers to soluble form in 48 h of incubation at 37 ºC and 150 rpm. The enzyme has the potential for application in the manufacturing of organic manure and production of nutrient rich animal feed. The enzyme was compatible with detergents and removed stains of blood, egg white and coffee more efficiently than the detergent alone, so can also be used as a detergent aid.

Key words: Animal feed; Feather waste bioremediation; Keratinase; Organic manure; Plant growth promotion; Process optimization; Wash performance.

Introduction

Keratin is a highly stable, water-insoluble protein molecule, having very high steadiness and low degradation rate. It is mainly found in hair, feather, nails, wool and horns. High protein content of keratin waste can be used as a good source of protein and amino acids by systemic recycling. This provides a cheap and alternative protein (amino acids) feed stuff for animals and for many other purposes[1,2]. However, poor digestibility of keratin is a problem in recycling[3].

Keratin can be degraded by keratinases (EC.3.4.99.11) which are serine proteases. They are extracellular microbial proteases produced in an environment rich in keratinous substrates such as feathers and hairs. Keratinases have applications in traditional industrial sectors including feed,

detergent, medicine, cosmetics and leather manufacture[4]. The crude enzyme can also serve as a nutraceutical product, leading to significant improvement in broiler performance[5]. They also find application in more recent fields such as prion killing for treatment of the dreaded mad cow disease[6], biodegradable plastic manufacture and feather meal production[7]. Bioresource International's (BRI) has been producing keratinase enzyme with the Trade name *Versazyme* for feather meal production[8].

Keratianses are produced by many microbial groups such as Fungi (*Doratomyces microsporus, Alternaria radicina, Trichurus spiralis, Aspergillus* sp. etc.)[9], Actinomycetes (*Streptomyces pactum, S. albus, Thermoactinomyces candidus* etc.)[10] and several bacterial species *Flavobacterium pennavorans*, *Bacillus* sp.[11], *Stenotrophomonas* sp.[12], *Bacillus licheniformis* and *B. pumilus*[13].

In the present study we focused on the isolation of an extracellular keratinase producing bacteria from the soil of Poultry farm of Veterinary University, Bikaner, India. Production of keratinase from the selected isolate was optimized and the extracellular keratinase was used for various applications such as feather degradation, and washing of protein stained cloths either solely or in combination with detergents.

Materials and Methods

All the chemicals used were of AR grade. Dehydrated culture media bases were purchased from Himedia (Mumbai, India), other media components and chemicals were purchased from Central Drug House (New Delhi, India). Soil samples for isolation of keratinase producing bacteria were collected from different feather accumulating areas in Bikaner (India).

Isolation of bacteria: Isolation of bacteria was performed by serial dilution and plating method on skimmed milk agar (SMA) plates having composition (g/l): peptone, 5.0; yeast extract, 3.0; dextrose, 1.0; skim milk powder, 10.0, agar 15.0 and pH was adjusted to 7.2±0.2. The medium was autoclaved at 10 psi for 20 min[14].

Various dilutions of the soil samples were made by serial dilution method. 100 µl of the diluted samples from marked dilutions as 10^{-3}, 10^{-5} and 10^{-7} were inoculated on the SMA plates and uniformly spread all over the agar surface with the help of sterile glass spreaders. Plates were incubated at 37ºC for 24 h. The bacterial isolates were further sub cultured on SMA plates by quadrate streaking to obtain pure cultures. Pure cultures were maintained on nutrient agar (NA) slants at 4ºC for further studies.

Enzyme Production: Following steps were followed for enzyme production:

a) Preparation of seed culture- The bacterial isolates were inoculated in Erlenmeyer flasks (250ml capacity) containing 50ml of nutrient broth (NB). The inoculated NB flasks were incubated at 37 ºC and 150 rpm for 24 h. These 24 h old cultures were used as seeds or inoculums for inoculation of the flasks of basal production medium as described below.

b) Inoculation of production medium: One % of the seed culture of every bacterial isolate was inoculated in the respective flask having the modified feather meal medium with composition (g/l): NH_4Cl, 0.5; NaCl, 0.5; $MgSO_4.7H_2O$, 0.1; K_2HPO_4, 0.3; yeast extract, 0.1; chicken feathers, 10. The medium pH was adjusted to 7.5±0.2[15]. The inoculated medium flasks were incubated in a rotary shaker at a speed of 150 rpm at 37°C for 72 h.

c) Biomass removal and enzyme recovery: After incubation, the biomass of every culture was removed by centrifugation at 10,000 rpm for 10 min and the supernatants

were collected and examined for enzyme activity. The isolate exhibiting the maximum enzyme activity was selected for further studies.

Enzyme assay

a) Qualitative assay: Wells of around 1 cm diameter were made on the agar surface of SMA plates, using a sterile gel borer. Then 100µl of supernatants from each culture was transferred to the respective wells using a micropipette. The plates were incubated at 37ºC for 12 h and then observed for zone of hydrolysis.

b) Quantitative assay: Five ml of casein solution (6.5 mg/ml in phosphate buffer of pH 7.5) was primed at 37 ºC for 5 min in a water bath. To this 1ml of enzyme solution (supernatant of the fermentation broth) was added and the mixture was incubated at 37 ºC for 10 min. The reaction was stopped with 5ml of 110mM trichloroacetic acid (TCA). Whole reaction mixture was further incubated at 37 ºC for 30 min. Then 5 ml of sodium carbonate (500mM) and 1 ml of Folin's phenol reagent (1:1 with distilled water) was added and incubated at 37 ºC for 1 h. The amino acids liberated were measured at an absorbance of 660 nm against a reagent blank and the quantity was determined from a standard tyrosine curve prepared in the range of 1-100µg/ml using a spectrophotometer[16].

Enzyme Unit: One unit (U) of enzyme activity is defined as the amount of the enzyme that catalyzes the conversion of one microgram of substrate per minute under the standard assay conditions.

Screening of keratinase activity of bacterial isolates

a) Qualitative screening: For qualitative screening 100 µl of supernatant from each bacterial culture broth was inoculated in wells bored on SMA plates. The inoculated plates were incubated at 37 ºC for 24 h and then observed for zone of hydrolysis.

b) Quantitative screening: For quantitative screening the supernatants from each bacterial isolates were examined for enzyme units produced per milliliter of the fermentation broth by the colorimetric method as described above.

Characterization of keratinolytic bacterium

The selected bacterial isolate was characterized for morphological, physiological and biochemical characteristics *viz*. bacterial cell shape, Gram reaction, sporulation, anaerobic growth, sugar fermentation, indole test, methyl red test, Voges Proskauer test, citrate test, catalase test, urease, caseinase and gelatinase acivity.

Medium Optimization

Optimization of keratinase production from the selected bacterial isolate was done by evaluating the effects of following parameters on enzyme production.

a) Effect of medium: pH- Four sets of the feather meal medium with different pHs *viz.* 6, 7, 8 and 9 were prepared to study the effect of pH on keratinase production. The selected bacterial isolate was grown in the seed medium (nutrient broth) for 12 h. 1 ml of this seed culture was inoculated into the 50ml production medium (contained in 250 ml Erlenmeyer flask) and was incubated in a rotary shaker at an agitation rate of 150 rpm at 37ºC for 48 h.

b) Effect of temperature: Effect of temperature on keratinase production was studied by incubating the inoculated medium flasks at respective temperatures: 25 ºC, 30 ºC, 37 ºC, and 42 ºC at an agitation rate of 150 rpm for 48 h.

c) Effect of sugars: Effect of sugars on keratinase production was studied by preparing production medium sets each having a different carbon source (glucose,

Table 1. Results of morphological, physiological, cultural and biochemical characteristics of *Bacillus* sp. S-2

CHARACTERISTICS	OBSERVATION
Physiological characteristics	
Endospore formation	+
Motility	Motile
Anaerobic growth	-
Morphological characteristics	
Shape of bacteria	Rod shaped
Colony growth	Fast
Colony shape	Circular
Colony surface	Smooth & shiny
Colony margin	Entire
Colony colour	Creamy
Colony elevation	Convex
Colony opacity	Opaque
Biochemical characteristics	
Glucose catabolism	-/-
Lactose catabolism	-/-
Mannitol	-/-
Indole production	-
Methyl red test	-
Voges-Proskaure test	-
Citrate catabolism	-
Gelatinase	-
Caesinase	+

lactose, fructose, mannitol or starch) at 0.2% (w/v) concentration. The inoculated flasks were incubated in a rotary shaker at 150 rpm and 37ºC for 48 h.

c) Effect of nitrogen source: Both organic and inorganic nitrogen sources were evaluated to find the best nitrogen source for optimal enzyme production. Following nitrogen sources at 0.1% (w/v) concentration were evaluated: proteose peptone, beef extract, urea and ammonium chloride. Other production conditions were same as above.

d) Effect of feather concentration: To find the optimal concentration of feather for maximum enzyme production following concentrations of feather viz. 1%, 2%, 3% and 4% (w/v) were used in the production medium. Other conditions were same as above

f) Effect of agitation rate: Agitation and aeration plays an important role in enzyme yield. Therefore, enzyme production was carried out at different agitation rates *viz.* 100, 150, 200 and 250 rpm at 37ºC for 48 h.

g) Effect of incubation period: Enzyme production from the selected bacterial isolate was investigated after 12, 24, 36, 48, 60 and 72 h.

Applications

a) Bioremediation of feather waste: For feather degradation 250 ml of optimized production medium was sterilized in 1000ml

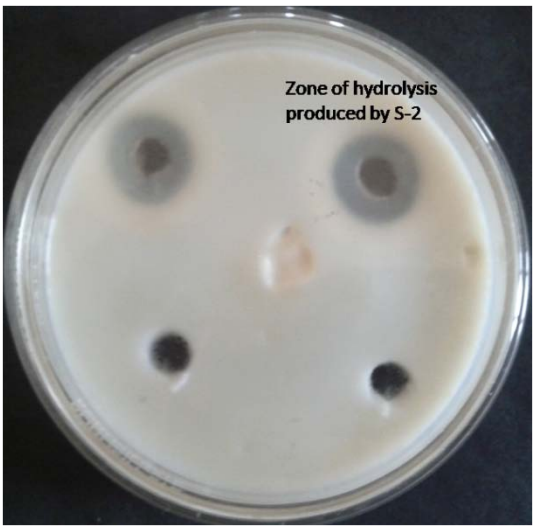


Fig.1. Qualitative screening: Agar diffusion assay of culture supernatants (S-2 and others) on SMA plate

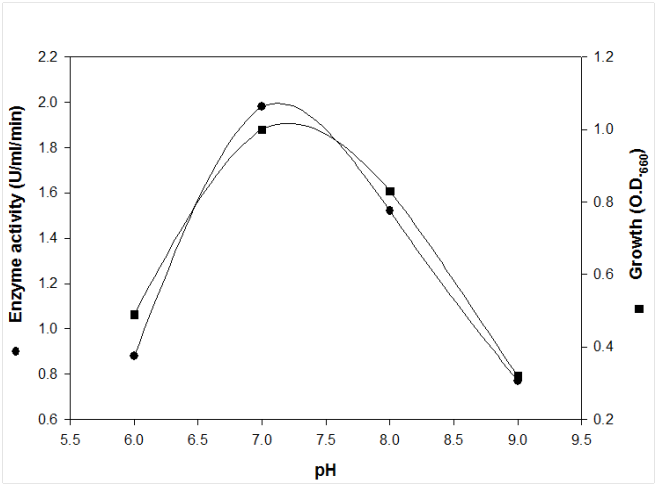


Fig. 2. Effect of medium pH on enzyme production by *Bacillus* sp. S-2

Erlenmeyer flask. Then 1% seed culture was inoculated to the sterilized medium which was incubated at 37 ºC and 150 rpm for 36 h. After 36 h, the culture broth was centrifuged to get cell-free supernatant containing the enzyme. This enzyme (200ml of supernatant) was transferred to another Erlenmeyer flask of 500ml capacity containing 5 g of sterilized chicken feathers and the flask was incubated at 37 ºC and 150 rpm for 72 h. The flask was observed after every 12 h to observe the complete degradation of feathers. Also samples were taken after every 12 h till 72 h of incubation and were tested for protein content by Lowry's method in order to assess the increase in protein content due to keratinase activity of the bacterial enzyme.

b) Organic manure: The protein content of the degraded feathers were determined by the Lowry's method in order to assess its potential for application as a amino acid and peptide rich manure for enhancing the growth of plants.

c) Animal feed: The degraded products of feather meal are rich in certain essential amino acids like methionine, lysine and arginine. Therefore, in the present study the degraded products of feathers were also analyzed for amino acid profile by thin layer chromatography (TLC).

d) Detergent aid: Detergent compatible proteases are used as washing aids which help in removal of proteinacious stains from the cloths. Therefore, the protease from *Bacillus* S-2 was investigated for removal of various stains *viz*. blood, egg and coffee stains from the cotton cloths. Experimentally, muslin cloths of 2×2 inches were taken 4 in number for each stain. Blood, egg and coffee stains were applied on cloth pieces and were dried by blowing hot air through hair dryer on them. After drying the four pieces of each stain *viz*. blood, egg and coffee were taken and washed in following way: one piece with water alone, another with detergent solution, another with enzyme alone and last one with detergent + enzyme solution and the cloth pieces were observed for cleanliness.

Results and discussion

Keratin is a well-built protein present in skin, hair, nails and horns. Keratin is very difficult to break up because of the presence of cysteine disulfide that can form disulfide bridges. These disulfide bridges create a tremendously strong helix shape. Microorganisms can degrade the keratin by the production of keratinase (an extracellular enzyme).

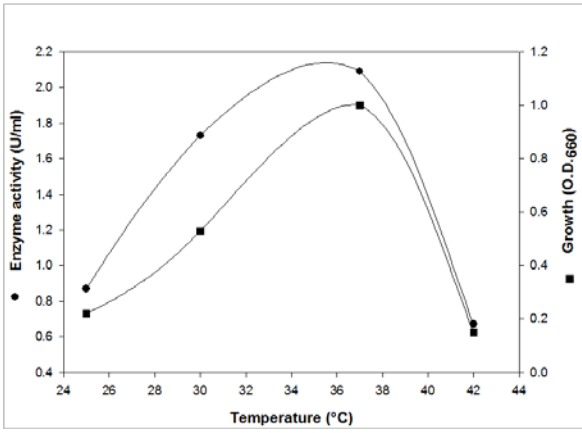


Fig. 3. Effect of incubation temperature on enzyme production by *Bacillus* sp. S-2

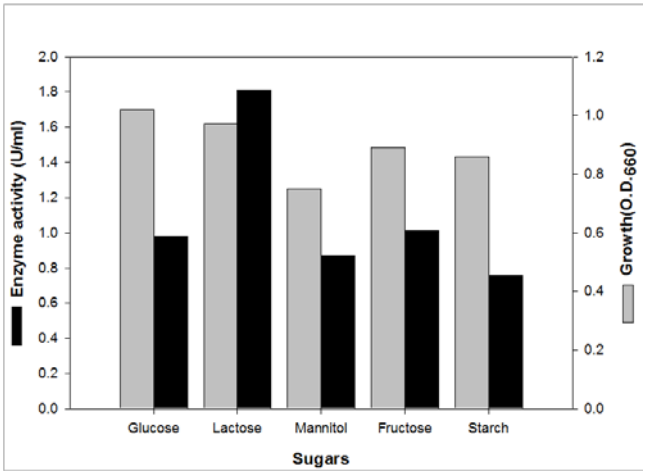


Fig. 4. Effect of different sugars on enzyme production by *Bacillus* sp. S-2

Screening

a) Qualitative screening: A total of 28 bacterial isolates were examined for protease activity on SMA plates. Results showed that out 28 isolates isolate S-2 produced protease (keratinase) in the feather meal medium, which was evident in the form of a 12 mm zone of hydrolysis in 24h of incubation on SMA plate (Fig 1). On the other side other bacterial isolates showed negligible or no protease production in the feather meal medium.

b) Quantitative screening: Out of 28 bacterial isolates which were evaluated for quantitative production of protease, only isolate S-2 exhibited protease (keratinase) activity of 2 U/ml, whereas, other isolates exhibited negligible protease activity. Isolate S-2 was therefore selected for further studies.

Culture Characteristics

The results of morphological, physiological and biochemical characteristics are presented in Table 2. These characters clearly show that theisolate S-2 belongs to the genus *Bacillus* (Table 1).

Previous studies conducted for the isolation of keratinolytic organism from soil and other natural sources, reports *Bacillus* sp. as a potential keratinolytic organism and its possible use in field studies for biodegradation of feather in feather processing[17,18].

Medium Optimization

a) Effect of medium: pH- The isolate S-2 exhibited maximum enzyme production (1.98 U/ml) at pH 7. There were steep declines in enzyme production below and above pH 7 (Fig. 2). The growth profile was similar to enzyme production profile. Enzyme production was growth associated with respect to pH. So the optimum pH for protease production was 7. The optimum pH for enzyme production varies from organism to organism, for instance *Bacillus cereus, B. subtilis* and *B. pumilus* produced maximum

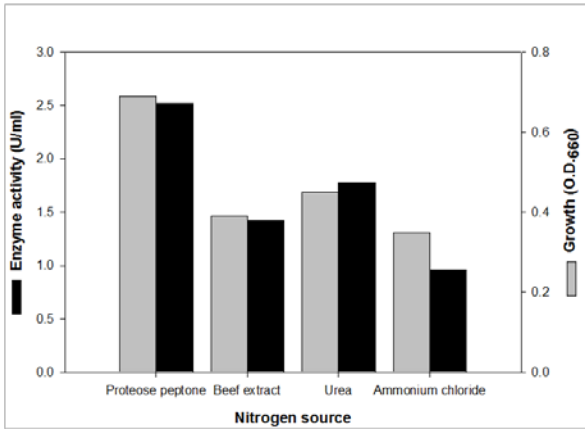


Fig. 5. Effect of different nitrogen sources on enzyme production by *Bacillus* sp. S-2

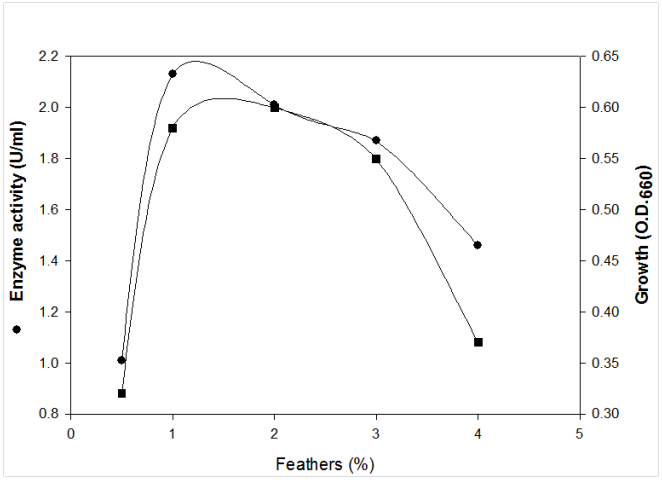


Fig. 6. Effect of different concentrations of feathers on enzyme production by *Bacillus* sp. S-2

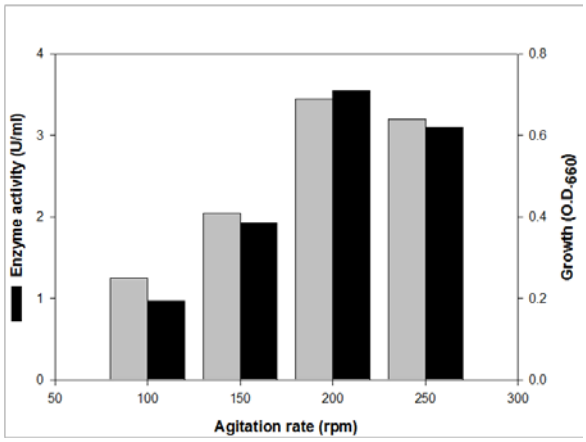


Fig. 7. Effect of different agitation rates on enzyme production by *Bacillus* sp. S-2

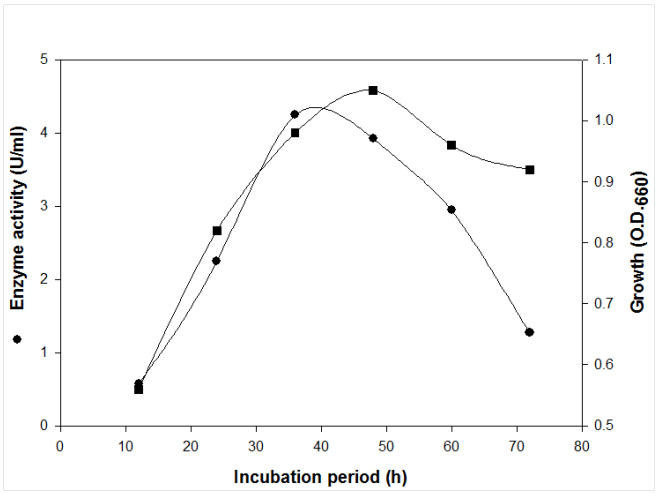


Fig. 8. Effect of incubation period on enzyme production by *Bacillus* sp. S-2

keratinase at pH 7, 5.9 and 5.6 respectively[19].

b) Effect of temperature: Incubation temperature plays an important role in enzyme production by a microorganism. The *Bacillus* S-2 showed maximum protease production (2.09 U/ml) at 37 ºC in 48 h of incubation. However, similar to pH profile above and below 37 ºC there was decline in enzyme production (Fig. 3). Therefore, the optimum temperature for proteaseproduction was 37 ºC. The growth profile was similar to enzyme production profile. Optimal temperature of 40 ºC for protease production has been reported for *Bacillus subtilis* and *B. pumilus*[20]. Temperature optima of 20-30 ºC has been reported for other bacteria[21]. On the other side, higher temperature of 50 ºC has been reported as optimal for keratinase production from *Bacillus cereus*[19].

c) Effect of sugars: Addition of sugars to the production medium did not have any positive effect on enzyme production by the selected bacterial *Bacillus* sp. S-2 (Fig. 4). Sugars actually decreased protease production. Except for lactose other sugars like glucose, mannitol and starch substantially decreased protease production. Negative effect of glucose on protease production has been reported[12,22,23]. On the contrary, enhanced protease production in the presence of (sugars, maximum being in the presence of starch) has been reported for *Bacillus cereus*[19].

Table 2. Total soluble protein (keratin) content in the aqueous mixture of keratinase and chicken feathers incubated at 37 ºC and 150 rpm for different incubation periods.

Incubation period (h)	Keratin (mg/ml)	Keratin %
12	4.8	21
24	9.9	44
36	14.8	66
48	19.8	88
60	20.1	89
72	20.4	90

Fig. 9. Feather degradation by S-2 protease after 48 h of incubation at 37 °C and 150rpm

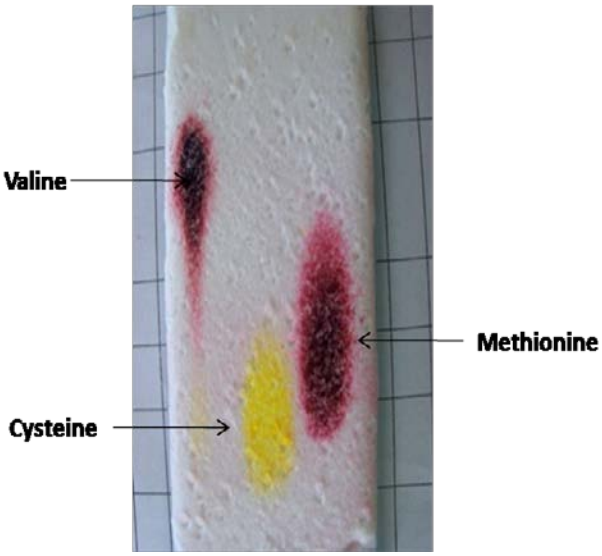


Fig. 10. TLC showing the spots of amino acids released from the degraded feathers after 44 h incubation at 37 °C and 150rpm

d) Effect of nitrogen source: Nitrogen plays an important role in growth and enzyme production by an organisms. Among various nitrogen sources investigated proteose peptone showed highest protease production (2.52 U/ml) as well as good growth (Fig. 5).

Effect of nitrogen sources on keratinase production also varies[19]. Peptone has been reported to induce maximum protease production by *Bacillus pumilus*[19]. Similarly, supplementation of medium with organic nitrogen source like yeast extract, enhanced keratinase production by *Stenotrophomonas* sp. and *Bacillus* spp.[12,19]. Soy meal has been reported to induce enzyme production[24].

e) Effect of feather concentration: Feathers are rich source of keratin protein. Therefore, their optimal concentration in the medium is necessary for the maximum induction of protease with keratinase activity. Feathers at a concentration of 1 % were most suitable for enzyme production (Fig. 6). Higher concentration resulted in similar growth but decreased enzyme production. Importance of feathers for keratinase production has been reported by a number of researchers[19,25-28].

f) Effect of agitation rate: Aeration plays an important role in microbial growth and metabolism. Maximum enzyme production of 3.55 U/ml was observed at an agitation rate of 200 rpm (Fig. 7). Enzyme production at 100 rpm was substantially low. On the other side there was no further enhancement in enzyme production above 200 rpm. Although effect of different agitation rates has not been investigated by other researchers but all have carried out protease production under shaking conditions *viz*. 150 rpm[27], 200 rpm[26], and 120 rpm[19].

g) Effect of incubation period; Maximum enzyme production of 4.25 U/ml) was achieved after 36h of incubation (Fig. 8). Thereafter enzyme activity started to decline. Optimal incubation period for keratinase production from *Bacillus cereus* has been reported to be 72 h[19]. Microbial enzyme production in general is affected by incubation period[29,30].

Applications

a) Bioremediation of feather waste: The protease from *Bacillus* S-2 completely degraded the feathers to soluble form in 48 h of incubation at 37 °C (Fig. 9). Here it is important to mention that under natural conditions the degradation process may take several weeks to complete. Whereas, the protease from *Bacillus* S-2 has completed this process in 48h, this is a significant achievement. Proteases with keratinase activity have been applied for bioremediation of feather wastes by different researchers[5,15,25-28,31].

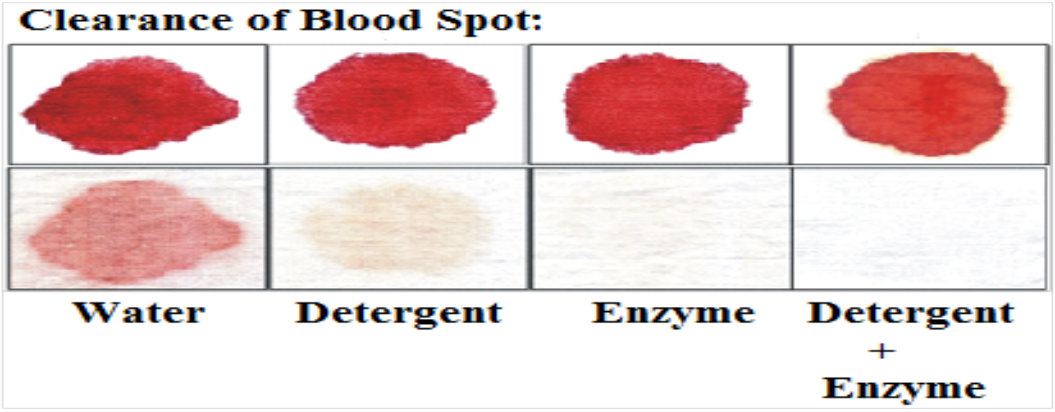
b) Organic manure: It is evident from the results presented in table 2, that protein content of as high as 19.8 mg/ml (~90 % keratin content) has been achieved by the protease of *Bacillus sp. S-2*S-2 strain in 48 h of incubation (Table 2). The degraded protein products viz. amino acids and peptides can be applied to the soil for enhanced growth and productivity of the plants. It has been reported that the degraded products of proteinacious compounds may have plant growth promoting activity[32,33]. The amino acids present in the degraded meal can easily be absorbed by the plants roots and through foliage[34,35]. They have the capacity to enhance nitrogen use efficiency of the plants and further stimulate photosynthesis and plant growth[36]. Their effects on carbon and nitrogen metabolism, and plant primary and secondary metabolism have been reported[37,38]. Hormone-like-activity including Auxin-like-activity and Gibberellin-like-activity has also been attributed to amino acids and peptides[37].

c) Animal feed: TLC analysis of the products of feather degradation showed the presence of amino acids cysteine, methionine and valine (Fig. 10). This was determined on the basis of rf values. As the

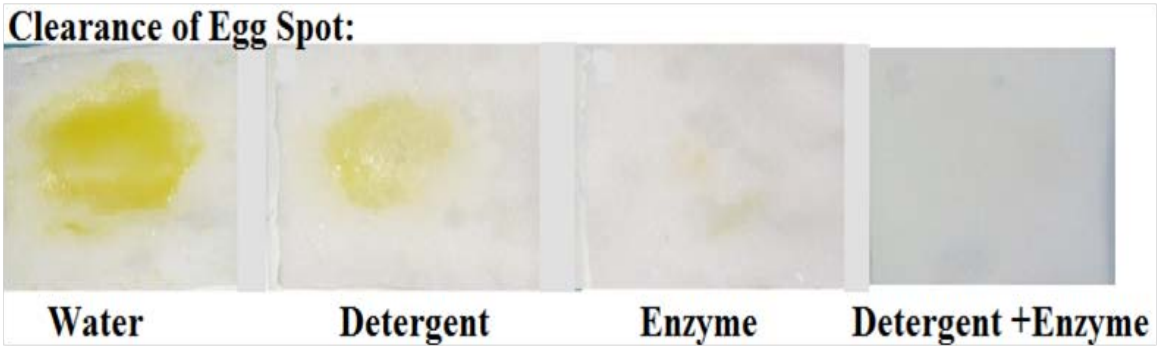
degraded feather meal is rich in essential amino acid methionine it can very well be used as an animal feed. Use of feather meal as animal feed has been reported[26,39,40].

*d) Detergent aid:*The results of wash performance showed that the protease from *Bacillus* S-2 efficiently removed the stains the results were better than the application of detergent alone (Fig. 11a,b & c). Application of microbial proteases as washing aid has been reported[41] earlier; however, this may be the first report, wherein a keratinase with wash performance activity is being reported.

(a) Cleaning of blood stain



(b) Cleaning of egg stain



(c) Cleaning of coffee stain

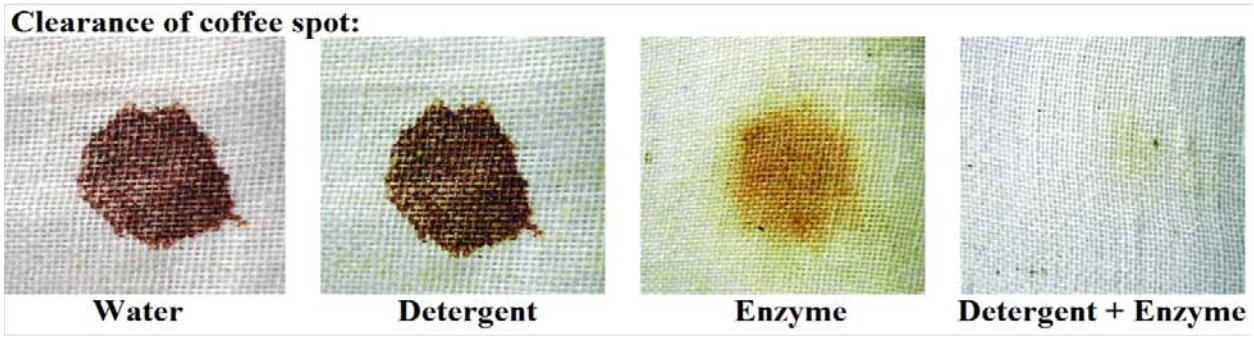


Fig. 11. Wash performance analysis of protease from *Bacillus* sp. S-2

Conclusion

The bacterial isolate *Bacillus* sp. S-2 produced a protease enzyme with keratinolytic activity with applications of industrial and environment importance. Under unoptimized conditions the isolate produced around 2 U/ml/min. After process (fermentation) optimization of both physical and nutritional parameters more than 2 fold increase in enzyme production i.e. 4.25 U/ml/min was achieved. So the enzyme productivity increased from an initial value of 2.5 U/ml/h to 7 U/ml/h which is a significant enhancement. Nitrogen source, agitation rate and incubation period played important roles in enhancing the enzyme production. The enzyme efficiently bioremediated the feather waste converting it to a complete soluble form in 48 h. Bioremediation resulted in enrichment of important amino acids like methionine, cysteine and valine in the end product, which can be used for formulation of organic manure and as animal feed. The enzyme was compatible with commercial washing detergents and efficiently removed the stains of blood, egg and coffee from cloths when used along with detergent. The results were better than those obtained with detergent alone.

Acknowledgement

Authors sincerely acknowledge the financial support provided by Department of Science and Technology (DST), Government of Rajasthan for carrying out this important research work.

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