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Production and characterization of feather degrading keratinase from *Bacillus* sp. JB 99

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An inducible keratinase is produced by *Bacillus* sp. JB 99 in a feather medium. Under submerged fermentation condition with continuous agitation (180 rpm), high level of keratinase production occurred at 45°C after 36 h at *p*H 10. The presence of carbon source in feather medium suppressed the enzyme production, while 0.1% yeast extract enhanced the production. The purified enzyme showed maximum keratinase activity at temperature 65°C and *p*H 10. The enzyme was monomeric and has a mol wt of approximately 66 kDa (SDS-PAGE). The enzyme may belong to serine protease group as completely inhibited by PMSF. Presence of metal ions, such as, Ca^{2+}, Mg^{2+}, Co^{2+} and Ba^{2+} stimulated the enzyme activity, while Hg^{2+}, Pb^{2+}, $Zn^{2+}$and Fe^{2+} decreased the activity. Present results indicate that *Bacillus* sp. JB 99 can be a highly useful organism for feather meal production and in leather industry.

Keywords: *Bacillus* sp. JB 99, feather degradation, feather meal, keratin, keratinase

Introduction

Keratin occurs in nature mainly in the form of hair, horn, nails and cornified tissue[1]. Keratin by virtue of its insolubility and resistance to proteolytic enzymes is not attacked by most of living organisms. Nevertheless, keratin does not accumulate in the nature and, therefore, biological agencies can be presumed to accomplish its removal[2]. Keratin utilization has been reported in variety of organisms including non-filamentous and filamentous bacteria, water moulds and filamentous fungi[3]. Alongwith bacteria and fungi, some insects, including cloth mouth larvae, carpet beetles and chewing lice, are known to digest keratin[4]. They hydrolyse the keratin by synthesizing specific class of extracellular enzymes called keratinases, which degrade keratin into small peptides that can be utilized by the cell. Several feather-degrading bacteria have been isolated from soil, poultry wastes, hair debries and animal skin, and most of these isolates were confined to genera *Streptomyces* and *Bacillus*. However, novel Gram-positive, feather-degrading bacteria have been identified as *Arthrobacter* sp.[5], *Kocuria rosea*[6] and *Microbacterium* sp[7].

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Feather contains over 80% of protein and conversion of the feathers into feed by keratinolytic microbes is inexpensive. However, this feed is relatively superior to other protein supplement feeds like soyabean meal. The keratinolytic microorganisms and technologies developed for feather degradation not only remove the waste feathers efficiently from the nature but also make the by-products of the process as a valuable protein supplement. The protein rich, concentrated feather meal can also be used for organic farming as semi-slow release, nitrogen fertilizer[8,9]. Use of keratinase enzymes in leather industry was known long back in dehairing process as an alternative to chemical processing. The present paper reports on the optimization of methodology for keratinase production and its characterization using locally isolated *Bacillus* sp. JB 99, a thermotolerent bacterium.

Materials and Methods

Microorganism and Culture Conditions

The strain *Bacillus* sp. JB 99 used in the study was isolated in the department[10] and the stock culture was maintained at 0°C on nutrient agar medium. The organism was grown in basal salt medium (g/L): $CaCl_2.H_2O$, 0.2; K_2HPO_4, 5.0; $MgSO_4$, $7H_2O$, 0.4; NaCl, 10.0; $NaNO_3$, 10.0; $NaCO_3$, 10.0; yeast extract, 5.0; and feathers, 10.0. Sodium carbonate

was separately autoclaved and added to the medium after cooling. For submerged fermentation, 24 h grown seed culture was used at 3% (v/v) concentration. The cultivation was performed at 45°C at 180 rpm on a rotary incubator. After 2 d incubation, the culture was centrifuged at 10,000 rpm for 15 min .The supernatant was used as crude enzyme source.

Optimal cultural conditions for keratinase productions was studied in cells grown in basal salt medium supplemented with different carbon and organic nitrogen sources. Other parameters, such as, initial *p*H of the medium (*p*H 6-12), incubation time (12-72 h) and different concentration of feather (0.5-3%) were also investigated.

Source of Keratin

Chicken feathers (whole feathers) were collected from chicken shop. Feathers were extensively washed in tap water and finally with double distilled water. Feathers were dried under sunlight and then in hot air oven at 60°C for 48 h. They were stored at 5°C until used.

Keratinase Enzyme Assay

Keratinase activity was measured by using 1% (w/v) keratin substrate in 25 m*M* glycine-NaOH buffer (*p*H 10) with some modification[11]. The reaction mixture was incubated for 20 min at 65°C and the reaction were terminated by adding TCA (10% w/v). The Folin Ciocalteau reagent is used as colouring reagent and the absorbance was measured at 660 nm. One unit of enzyme is defined as the amount of enzyme required to liberate 1 μg of tyrosine per minute under optimal experimental conditions.

Purification and Characterization of Keratinase

All the purification procedures were performed at 4°C. Ammonium sulphate was added to 250 mL of culture filtrate at 20, 30, 40, 50, 60, 70 and 80% saturation. Each precipitate was dissolved in particular quantity of glycine-NaOH buffer (*p*H 10.0) and dialysed against same buffer with change of buffer 3 times. The dialysed sample was applied to sephadex G-75 column (1.5 × 90 cm) previously equilibrated with 25 m*M* glycine-NaOH buffer (*p*H 10.0) and column was eluted with the same buffer. Aliquots of volume 2 mL fractions were collected with flow rate 20 mL/h. Keratinase activity and protein concentration was measured[12].

Mol Wt Dtermination and Zymogram Analysis of Keratinase

The mol wt determination and homogeneity test were carried out by SDS-PAGE using 10% polyacrylamide gel according to Laemmli method and stained with Coomassie brilliant blue R 250[13]. Zymogram was carried out on vertical slab gel according to Laemmli method, in the absence of β-mercaptoethanol, with modification[13]. Samples containing 10 μg of protein in 20 μL of enzyme were mixed with sample buffer containing 0.003% bromophenol blue (w/v), 10% glycerol (w/v) in 0.063 *M* Tris-HCl (*p*H 6.8). The enzyme samples were subjected to electrophoresis on 12% separating gel at 100 V with electrode buffer (*p*H 8.3), containing 0.025 *M* Tris-HCl and 0.192 *M* glycine, and the stacking gel containing 5% polyacrylamide in 1.5 *M* Tris-HCl (*p*H 6.8). After electrophoresis, the gel was soaked in 1% (w/v) Triton-X-100 for 15 min and then in glycine-NaOH buffer (*p*H 10) for 30 min at 50°C. The gel was now over layered on 0.8% agarose plate containing 0.5% keratin in 50 m*M* glycine-NaOH buffer (pH-10) for 3 h at 45°C and stained to visualize hydrolysed clear band.

Effect of pH and Temperature on Keratinase Activity

Keratinolytic activity of purified enzyme was measured in the range of *p*H 5 to 13 using following buffers: citrate phosphate buffer (*p*H 5 to 6), sodium phosphate buffer (*p*H 7.0), Tris-HCl buffer (*p*H 8.0) and glycine-NaoH buffer (pH 9 to 13). The optimum temperature was determined by incubating reaction mixture at different temperature range from 40 to 80°C for 20 min.

Effect of Organic and Inorganic Compounds on Keratinase Activity

Purified keratinase solution was pre-incubated at 40°C for 45 min with different chemical agents and the residual activity was measured. The chemicals tested were: DTT, glutathione, β-mercaptoethanol, sodium sulfite, cysteine, DMSO, PMSF, EDTA, $CaCl_2$, $BaCl_2$, $MnCl_2$, $PbCl_2$, $CuSO_4$, $MgCl_2$, $FeCl_3$, $ZnCl_2$, $CoCl_2$ and $HgCl_2$.

Results and Discussion

The organism, *Bacillus* sp. JB 99 grew well upto 50°C in presence of 15% NaCl, indicating that the organism is a thermophilic and halotolerant. It grew optimally at *p*H 10 but also showed good growth at neutral *p*H, showing that the organism can be of facultative alkalophile. The organism degraded

the feathers efficiently (Fig. 1). The results on optimization of nutritional conditions and other parameters for production and characterization of keratinase by *Bacillus* sp. JB 99 are presented below.

Effect of Carbon and Nitrogen Sources on Keratinase Production

The results presented in Table 1 shows that keratinase production was most inhibited in the presence of dextrose, followed by citric acid and glucose. This indicated that bacteria had catabolic repression regulatory mechanism. Previous studies also showed inhibitory effect of carbohydrates on keratinase production[14]. In *B. licheniformis* PWD-1, glucose totally suppressed the keratinase secretion. In case of *Bacillus* sp. MIR-99, beside glucose, glycerol and sucrose were also shown to suppress the enzyme secretion[15].

Fig. 1—Showing organism degrading feathers efficiently

Table 1—Effect of carbon source on production of keratinase from *Bacillus* sp. JB 99

Carbon Source 1% (w/v)	Activity µg/mL/min	% reduction
Control (only feather)	32.0 (±2.5)	0.00
Maltose	26.0 (±2.0)	18.75
Starch	27.0 (±1.1)	15.26
Dextrose	15.0 (±1.4)	53.12
Lactose	25.0 (±2.7)	21.87
Citric acid	19.5 (±1.8)	39.06
Glucose	23.0 (±1.6)	34.37

Feather medium supplemented with 0.1% (w/v) yeast extract as an external organic nitrogen source showed maximum production of keratinase by *Bacillus* sp. JB 99, but enzyme production was decreased when yeast extract concentration was increased to 1% (w/v) (Table 2). Followed by yeast extract, casein, soyabean meal, peptone, tryptone and gelatin had the stimulating effect on enzyme activity in decreasing order. In the presence of two different substrates, one which is structurally more compact and resistant (feather) and other which is more accessible and small protein supplement, the bacteria may preferentially use the latter. This would explain the comparative lower enhancement of keratinase activity measured in the presence of external nitrogen sources[16]. A different result is reported in *B. licheniformis* strain K-508, where casein increased the enzyme secretion in feather medium[17].

Effect of *p*H and Inducer (Feather) Concentration of Medium on Keratinase Production

The effect of *p*H on the enzyme production is depicted in Table 3. The results indicate that *Bacillus* sp. JB 99 is an alkalophilic bacterium with broad *p*H range for enzyme production (*p*H 6 to 12). The maximum enzyme production occurred at *p*H 10, which was also reported optimum *p*H in

Table 2—Effect of organic nitrogen sources on production of keratinase from *Bacillus* sp. JB 99

Organic nitrogen source 1% (w/v)	Activity (µg/mL/min)	% stimulation
Control	8.0 (±1.4)	0.00
Peptone	27.0 (±.1.8)	237.5
Yeast extract	35.0 (±1.2)	337.5
Tryptone	19.0 (±1.9)	137.5
Gelatin	16.5 (±1.0)	106.25
Soyabean meal	22.0 (±2.2)	175.0
Casein	29.0 (±2.0)	262.5
Yeast extract (0.1%)	53.0(±1.1)	562.5

Table 3—Effect of *p*H of the medium on production of keratinase from *Bacillus* sp. JB 99

*p*H	Activity (µg/mL/min)
6	13.2 (±1.6)
7	28.0 (±3.5)
8	40.5 (±2.6)
9	46.5 (±1.8)
10	52.0 (±1.2)
11	43.5 (±0.8)
12	36.0 (±2.0)

cases of *Bacillus* sp. FK46[18] and *B. licheniformis*[17]. However, in *Fervidobacterium pennavorans*, optimal keratinase production was reported at *p*H 6.3[19].

The present study on production of keratinase by *Bacillus* sp. JB 99 indicated that major regulatory mechanism is inductive (Table 4). The keratinase achieved its maximum production in presence of the 1% feather in the medium. The increased concentration of feather in the medium decreased the enzyme production. High substrate concentration may cause the substrate inhibition or repression of keratinase production. Production of keratinase by *Chryseobacterium* sp. kr6 is repressed by higher percent of inducer in medium[14]. Some studies have shown the constitutive or partially inducible nature of keratinase production[20]. *B. lichenformis* K-508 produced a constitutive keratinase[21].

Effect of Incubation Period on Keratinase Production

Bacillus sp. JB 99 took considerably less period of incubation (36 h) for maximum enzyme production (Table 5). After 48 h of incubation, keratinase production was decreased. Production was maximal in 3 d in *Bacillus* sp. KH 28[16], while *Bacillus* sp. FK-46 produced higher amount of enzyme in 5 d[18].

Table 4—Effect of inducer concentration on production of keratinase from *Bacillus sp.* JB 99

Inducer (%)	Activity (μg/mL/min)
Control	4.0 (±1.2)
0.5	46.5 (± 1.1)
1.0	52.4 (±1.0)
1.5	45.5(±1.8)
2.0	42.0 (± 1.3)
2.5	41.0 (±2.0)
3.0	34.0(±1.0)

Table 5—Effect of incubation period on the production of Keratinase from *Bacillus* sp. JB 99

Incubation period (h)	Activity (μg/mL/min)
12	17.0 (±1.6)
24	31.2 (±1.0)
36	52.6 (±1.0)
48	48.0 (±1.2)
60	36.0 (±1.0)
72	28.0 (±2.4)

Characterization of Keratinase from *Bacillus* sp. JB 99

Mol Wt Determination of Keratinase and Zymogram Analysis

SDS-PAGE of purified keratinase sample having 320 μg/mL/min of enzyme activity and specific activity of 2370 U/mg with 0.135 μg/mL of protein was carried out and stained with coomassie brilliant blue. A single band of approximately 66 kDa was observed (Fig. 2). It indicates that purification of keratinase was achieved to homogeneity. Zymogram showed a clear zone of hydrolysis, at the same place where a single band was observed on SDS-PAGE gel. *Cryseobacterium* sp. kr6 produced a keratinase of 64 kDa[22]. The mol wt of keratinase from *B. pumilus* was 65 kDa[23]. A small molecular mass keratinase of 18 kDa was also reported in *Streptomyces albidoflavus*[24].

Effect of pH and Temperature on Keratinase Activity

The purified enzyme proved to be active over a broad range of *p*H values (Fig. 3) and temperature (Fig. 4). The optimal *p*H and temperature values were 10 and 65°C, respectively. In case of *B. pseudofirmus*, optimum *p*H range 8.3-10.3 and temperature 60°C were reported[25]. Keratinases active at 90°C have also been reported in the literature[26]. The keratinase of *Bacillus* sp. JB 99 retained its complete activity after 1 h pre-incubation at 50°C but completely inactivated at 80°C. However, a keratinase produced by *F. pennavorans* showed maximum activity at 80°C[27].

Effect of Chemicals

The effects of various inhibitors, solvent, reductants and metal ions on the keratinase activity are summarized in Table 6. Keratinase from *Bacillus* sp. JB 99 irreversibly lost its keratinolytic activity

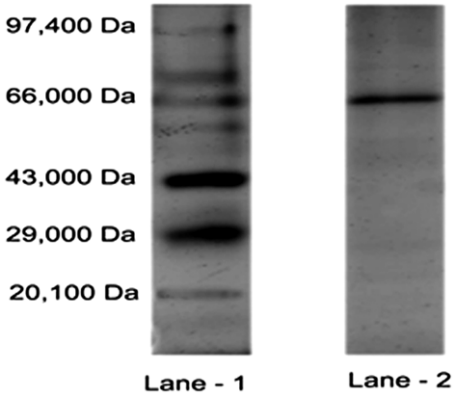

Fig. 2—SDS-PAGE of purified keratinase sample: Lane 1, Markers; & Lane 2, Enzyme having 66 kDa mol wt

in presence of PMSF. Similar results were also observed for *Kocuria rosea* LPB-3[6] and *Vibrio* sp. kr2[28]. However, metalloprotease inhibitor EDTA had a marginal inhibitory effect on activity of the enzyme. This inhibition pattern is similar to the classification of this keratinase as serine protease. DMSO either had no effect or slightly reduced the enzyme activity.

The reducing agents stimulated the hydrolysis of keratin by keratinase of *Bacillus* sp. JB 99. DTT and glutathione enhanced the enzyme activity maximally; while β-mercaptoethanol, cysteine and sodium sulfite were less stimulatory in this order (Table 6). The keratinase of *B. subtilis* KS-1 also showed increased keratin degradation in presence of reductants[29]. The keratin degradation by keratinases *in vitro*, therefore, accompanied by simultaneous reduction in the disulfide bond or cysteine bonds.

Many divalent metal ions activated the keratinase of *Bacillus* sp. JB 99 (Table 7). Especially, Ca^{2+} Mg^{2+}, Co^{2+} and Mn^{2+} increased the enzyme activity, which is a common phenomenon for serine protease.

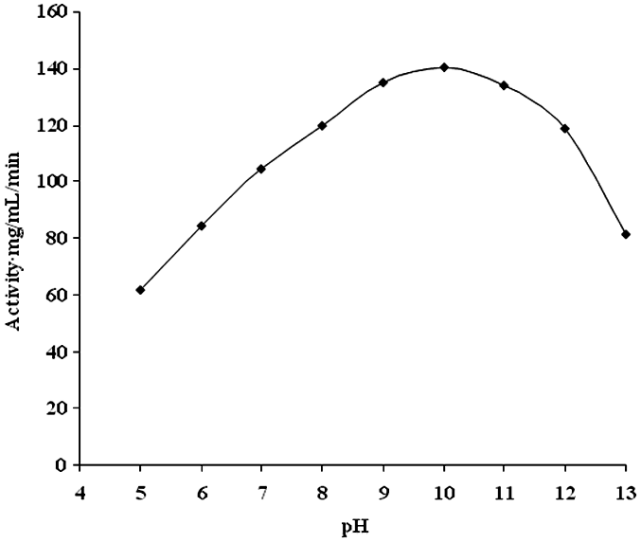

Fig. 3—Effect of *p*H on activity of keratinase from *Bacillus* sp. JB 99

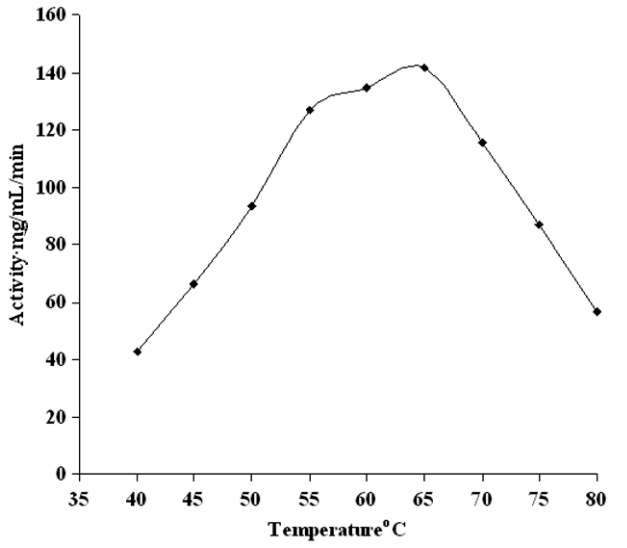

Fig. 4—Effect of temperature on activity of keratinase from *Bacillus* sp. JB 99

Table 6—Effect of reductants, organic solvents, inhibitors on keratinase activity

Reductants	Concentration (%/m*M*)	Residual activity (%)
Control	-	100
DTT	0.1 [a]	159
	0.5[a]	180
Glutathione	0.1[a]	139
	0.5[a]	142
Sodium sulfite	0.1[a]	117
	0.5[a]	131
β-Mercaptoethanol	0.1[b]	127
	0.5[b]	141
Cysteine	0.1[a]	122
	0.5[a]	137
DMSO	1.0[b]	100
	2.5[b]	92
PMSF	1.0 m*M*	2.14
	2.5 m*M*	000
EDTA	1.0 m*M*	95.00
	2.5 m*M*	81.42

a: w/v; b: v/v

Table 7—Effect of metal ions on keratinase activity

Metal ion/inhibitor	Concentration (mM)	Residual activity (%)
Control	-	100
$CaCl_2$	1.0	111.42
	2.5	124.28
$BaCl_2$	1.0	105.7
	1.5	84.28
$MnCl_2$	1.0	112.00
	2.5	109.24
$PbCl_2$	1.0	65.73
	2.5	57.85
$CuSO_4$	1.0	107.14
	2.5	94.28
$MgCl_2$	1.0	108.57
	2.5	103.00
$FeCl_3$	1.0	76.42
	2.5	62.85
$ZnCl_2$	1.0	82.85
	2.5	52.80
$CoCl_2$	1.0	115.00
	2.5	118.87
$HgCl_2$	1.0	66.40
	2.5	52.85

However, in presence of $PbCl_2$, $FeCl_3$, $ZnCl_2$ and $HgCl_2$, the keratinase activity was decreased. Ba^{2+} and Cu^{2+} had a concentration-dependent effect on the keratinize activity; at lower concentration, they are slightly stimulatory and at higher concentration they are slightly inhibitory. Mg^{2+} was also reported to increase the keratinase activity of *Thermoanaerobacter keratinophilus* sp. nov.[30]; whereas the enzyme activity was decreased in the presence of Hg^{2+} in case of *Microbacterium* sp.[7].

The keratinase reported in this communication is different from alkaline protease reported in our earlier paper[10]. The alkaline protease had a mol wt 43 kDa and an optimum *p*H and temperature 11 and 70°C, respectively; whereas the keratinase reported in the present communication has 66 kDa mol wt and optimum *p*H and temperature of 10 and 65°C, respectively. The presence of different carbon source in defined medium enhanced the alkaline protease production, where as the presence of carbon source in feather medium suppressed the enzyme production of keratinase.

Conclusion

The objective of the present investigation was to optimize conditions for keratinase production and its characterization. It is evident from the results that the *Bacillus* sp. JB 99 is a good producer of keratinase. The maximum extracellular, active alkaline serine keratinase production was achieved after 36 h of incubation at 40°C and *p*H 10.0, at 180 rpm in a 1% feather medium. In addition, the enzyme was found active over a wide range of *p*H and was relatively stable upto 70°C. These results show that enzyme may be useful in industrial applications.

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