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Production and characterization of alkaline thermostable protease from newly isolated *Bacillus sp.*

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Abstract

An inducible protease is produced by *Bacillus sp*. in undefined feather medium. Under submerged fermentation condition high level of protease production occurred at 45^{o}C after 36 h at pH 10, with continuous agitation (180 rpm). 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 protease activity at temperature 65^{0}C and at pH-10.The enzyme was monomeric and has molecular weight of approximately 66 kDa (SDS-PAGE).The enzyme may belong to serine protease group as it is completely inhibited by PMSF. Presence of metal ions such as Ca^{2+}, Mg^{2+}, Co^{2+}, Ba^{2+} stimulated, while Hg^{2+}, Pb^{2+}, Zn^{2+}, Fe^{2+} decreased the activity. The results indicate *Bacillus sp*. is a highly useful organism for feather meal production and in leather industry.

Keywords: *Bacillus sp*., Protease, feather degradation, Thermostable.

Introduction

Keratin occurs in nature mainly in the form of hair, horn, nails and cornified tissue [14]. 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 may be presumed to accomplish its removal [13]. Keratin utilization has been reported in variety of organisms including non filamentous and filamentous bacteria, water moulds and filamentous fungi [28] Along with bacteria and fungi, some insects, including cloth mouth larvae, carpet beetles and chewing lice are known to digest keratin [22]. They hydrolyse the keratin by synthesizing specific class of extracellular enzymes called alkaline thermostable Proteases which degrade keratin into small peptides that can utilized by cell. Several feather-degrading bacteria have been isolated from soil, poultry wastes, hair debries and animal skin, and most of the isolates were confined to genera *Streptomyces* and *Bacillus*. In Gram-positive bacteria, novel feather-degrading bacteria have been identified as *Arthrobacter sp.* [18], *Kocuria rosea* [1,2] and *Microbacterium sp.* [32].

Feather represent over 80% of protein, conversion of these feathers into feed by keratinolytic microbes is inexpensive. 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 valuable protein supplement. The protein rich, concentrated feather meal can also be used for organic farming as semi-slow release, nitrogen fertilizer [11, 5]. Use of alkaline thermostable Protease enzymes in leather industry was long back known in dehairing process as an alternative to chemical processing. The present studies reports on the optimization of methodology for alkaline thermostable Protease production and its characterization using locally isolated *Bacillus sp*., a thermotolerent bacterium.

Materials and methods

Microorganism and culture conditions

The strain *Bacillus sp*. was previously isolated in our department; the stock culture of the organism 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; feathers, 10.0. Sodium carbonate was

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

Optimal cultural conditions for alkaline thermostable Protease productions was studied in cells grown in basal salt medium supplemented with different carbon and organic nitrogen sources. Other parameters such as initial pH of the medium (pH-6-12), incubation time (12-72 hrs) 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, finally with double distilled water. Feathers were then dried under sunlight and then in hot air oven at 60°C for 48hrs. They were stored at 5°C until used.

Alkaline thermostable Protease enzyme assay

Alkaline thermostable Protease activity was measured by using 1% (w/v) keratin substrate in 25 mM Glycine -NaOH buffer (pH-10) with some modification [31]. 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 1µg of tyrosine per minute under optimal experimental conditions.

Purification and characterization of alkaline thermostable Protease

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. The precipitate obtained was dissolved in particular quantity of Glycine - NaOH buffer (pH-10.0) and dialysed against same buffer with change of buffer 3 times. The dialysed samples were applied to sephadex G -75 columns (1.5 X 90 cm) previously equilibrated with 25 mM Glycine NaOH buffer (pH-10.0) and column was eluted with same buffer. Aliquots of volume 2 ml fractions were collected with flow rate 20 ml/h. alkaline thermostable Protease activity and protein concentration was measured [17].

Molecular weight determination and zymogram analysis of alkaline thermostable Protease

The molecular weight 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 [15, 16]. Zymogram was carried out on vertical slab gel according to Laemmli method in the absence of β-mercaptoethanol, with modification [16]. 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 (pH 6.8). The enzyme samples were subjected to electrophoresis on 12% separating gel at 100 v with electrode buffer (pH-8.3) containing 0.025 M Tris-HCl and 0.192 M glycine and the stacking gel contains 5% polyacrylamide in 1.5 M Tris-HCl (pH-6.8). After electrophoresis, the gel was soaked in 1% (w/v) Triton-X-100 for 15 min and then in glycine-NaOH buffer (pH-10) for 30 min at 50^0C. The gel was now over layered on 0.8% agarose plate containing 0.5% keratin in 50 mM glycine-NaOH buffer (pH-10) for 3 hrs at 45^0C and stained to visualize hydrolysed clear band.

Effect of pH and temperature on alkaline thermostable Protease activity

Keratinolytic activity of purified enzyme was measured in the pH range from pH 5 to 13 using following buffers: Citrate phosphate buffer (pH-5 to 6), Sodium phosphate buffer (pH-7.0), Tris-HCl buffer (pH-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 alkaline thermostable Protease activity

Purified alkaline thermostable Protease 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 results are shown in Table no 1 to 7 and fig 1-3.*Bacillus sp.* grew well upto 50°C in presence of 15% indicating that the organism is a thermophilic and halotolerant. It grew optimally at pH-10, but also showed good growth at neutral pH indicating 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 alkaline thermostable Protease by Bacillus *sp.* are investigated.

Effect of carbon and nitrogen sources on the alkaline thermostable Protease production

The results presented in Table 1shows that inhibition of alkaline thermostable Protease production in presence of dextrose followed by citric acid was observed. This indicated that bacteria have catabolic repression regulatory mechanism. Previous studies also showed inhibitory effect of carbohydrates on alkaline thermostable Protease production [4]. In *Bacillus licheniformis* PWD-1 glucose totally suppressed the alkaline thermostable Protease secretion. In *Bacillus strain* MIR-99, beside glucose; glycerol and sucrose were also shown to suppress the enzyme secretion [23]. Feather medium supplemented with 0.1% (w/v) yeast extract as an external organic nitrogen source showed maximum production of alkaline thermostable Protease by *Bacillus sp*. but enzyme production was decreased when yeast extract concentration was increased to 1% (w/v). Followed by yeast extract, casein, soyabean meal, peptone, tryptone, gelatin the effect in decreasing order (Table 2). 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 lower alkaline thermostable Protease activity measured in the presence of external nitrogen sources. A different result is reported in *Bacillus licheniformis* strain at 508 K, where casein increased the enzyme secretion in feather medium [26].

Effect of pH and inducer (feather) concentration of the medium on the alkaline thermostable Protease production

The result of effects of pH on enzyme production is depicted in Table 3. The results indicate that *Bacillus sp* JB 99 is an alkalophilic bacteria with broad pH range for enzyme production (pH-6 to 12). Maximum enzyme production was occurred at pH-10 optimum in *Bacillus* sp.FK 46 [29] and *Bacillus licheniformis* respectively [26]. *Fervidobacterium pennavorans* produced alkaline thermostable Protease with optimum pH at 6.3 [7]. The present study on alkaline thermostable Protease synthesis by *Bacillus sp.* indicated that major regulatory mechanism is inductive. The alkaline thermostable Protease achieved its maximum production in presence at 1% feather in medium. The increased concentration of feather decreased the enzyme production (Table 4).High substrate concentration may cause the substrate inhibition or repression of alkaline thermostable Protease production. Production of alkaline thermostable Protease by *Chryseobacterium sp.* Kr6 is repressed by higher percent of inducer in medium [4]. Some studies have shown the constitutive or partially inducible nature of alkaline thermostable Protease production [6]. *Bacillus lichenformis* K-508 produced a constitutive alkaline thermostable Protease [19, 20].

Effect of incubation period on alkaline thermostable Protease production

Bacillus sp took considerably less time (36 h) of incubation period for maximum enzyme production. After 48 h of incubation, alkaline thermostable Protease was decreased (table-5). Production was maximum in 3

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days, using Bacillus *sp* KH 28 [32]. *Bacillus* FK-46 produced higher amount of enzyme in 5 days [29].

Characterization of alkaline thermostable Protease from *Bacillus sp.*

Molecular weight determination of alkaline thermostable Protease and zymogram analysis

SDS-PAGE of purified alkaline thermostable Protease sample having 320 µg/ml/min of enzyme activity and specific activity of 2370 units/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 alkaline thermostable Protease 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. *Cryseosporium sp Kr6* produced a alkaline thermostable Protease of 64 kDa [25]. The molecular weight of alkaline thermostable Protease from *Bacillus pumilus* was 65 kDa [9]. A small molecular mass alkaline thermostable Protease of 18 kDa in *Streptomyces* albidoflavus [3] was also reported.

Effect of some chemicals

The effects of various inhibitors, solvent, reductants and metal ions are summarized in Table-6. Alkaline thermostable Protease from *Bacillus sp.* irreversibly lost its keratinolytic activity in presence of PMSF. Similar results were observed in *Kocuria rosea* LPB-3. *Vibrio sp* Kr2 [1, 21, 27]. Metalloprotease inhibitor EDTA has a little inhibitory effect on activity of the enzyme. This inhibition pattern is similar to the classification of this alkaline thermostable Protease as serine protease. DMSO positively affected the enzyme activity. The reducing agents stimulated the hydrolysis of keratin by alkaline thermostable Protease of *Bacillus sp.*. DTT and glutathione enhanced activity maximally; β-mercaptoethanol, cysteine and sodium sulfite were less stimulatory in this order. The alkaline thermostable Protease of *Bacillus subtilis* KS-1 also showed increased keratin degradation in presence of reductants [30].The keratin degradation by alkaline thermostable Proteases *in-vitro* therefore accompanied by simultaneous reduction in the disulfide bond or cysteine bonds. Many divalent metal ions activated alkaline thermostable Protease of *Bacillus sp.* (Table.No-7). Especially Ca^{2+} Mg^{2+}, Mn^{2+} increased the activity, which is a common phenomenon for serine protease. In presence of $FeCl_3$, $ZnCl_2$, $HgCl_2$, the alkaline thermostable Protease activity was decreased. Mg^{2+} increased the alkaline thermostable Protease activity of *Thermoanaerobacter keratinophilus sp. Nov*[24]. The activity was decreased in presence of Hg^{2+} in *Microbacterium* species is reported [32].

Effect of pH and temperature on alkaline thermostable Protease activity

The purified enzyme proved to be active over a broad range of pH values and temperature (Fig-3).The optimal pH and temperature values were 10 and 65^0C respectively. *B. pseudofirmus* had optimum pH 8.3-10.3 and temperature 60^0C [21]. Alkaline thermostable Proteases which are active at 90°C also have been reported [8]. Alkaline thermostable Protease of *Bacillus sp* retained its complete activity after one hour pre incubation at 50^0C but inactivated at 80^0C. An alkaline thermostable Protease produced by *Fervidobacterium pennavorans* showed maximum activity at 80^0C [10].

Conclusion

The objective of the present investigation was to optimize conditions for alkaline thermostable Protease production and its characterization. It is evident from the results that the *Bacillus sp.* good producer of alkaline thermostable Protease. The greatest alkaline active, extracellular serine alkaline thermostable Protease production was achieved after 36 h incubation at 40^0C, pH -10.0, 180 rpm, 1% feather. In addition the enzyme was active over wide range of pH and relatively stable (upto 70^0C). These results may be useful in industrial applications of the enzyme.

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Table 1- Effect of carbon source on production of alkaline thermostable Protease from Bacillus sp.

Carbon Source 1% (w/V)	Activity µg /ml/min	% Reduction
Control (only feather)	32.0 (±2.5)	0
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

Table 2- Effect of organic nitrogen sources on production of alkaline thermostable Protease from Bacillus sp.

Organic Nitrogen 1% (w/v)	Activity Source (µg/ml/min)	% Stimulation
Control	8.0 (±1.4)	0
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
Casein	29.0 (±2.0)	262.5
Yeast extract (0.1%)	53.0(±1.1)	562.5

Table 3- Effect of pH of the medium on production of alkaline thermostable Protease from Bacillus sp.

pH	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)

Table 4-Effect of inducer concentration on production of alkaline thermostable Protease from Bacillus sp.

Inducer (%)	Activity (µg/ml/min)
Control	4.0 (±1.2)
0.5	46.5 (± 1.1)
1	52.4 (±1.0)
1.5	45.5(±1.8)
2	42.0 (± 1.3)
2.5	41.0 (±2.0)
3	34.0(±1.0)

Table 5- Effect of incubation period on the production of Alkaline thermostable Protease from Bacillus sp.

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)

Table.No-6: Effect of reductants, organic solvents, inhibitors on alkaline thermostable Protease activity

Reductants	Concentration (%) / mM	Residual activity (%)
Control	-	100
DTT	0.1a	159
	0.5a	180
Glutathione	0.1a	139
	0.5a	142
Sodium sulfite	0.1a	117
	0.5a	131
λοναητεοτπαχρεμ–β	0.1b	127
	0.5b	141
Cysteine	0.1a	122
	0.5a	137
DMSO	1.0b	100
	2.5b	92
PMSF	1.0mM	2.14
	2.5mM	0
EDTA	1.0mM	95
	2.5mM	81.42

a) Weight / Volume, b) Volume / Volume

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Table 7-Effect of metal ions on alkaline thermostable Protease activity

Metal ion/ Inhibitor	Concentration (mM)	Residual activity (%)
Control	-	100
CaCl2	1	111.42
	2.5	124.28
BaCl2	1	105.7
	1.5	84.28
MnCl2	1	112
	2.5	109.24
PbCl2	1	65.73
	2.5	57.85
CuSO4	1	107.14
	2.5	94.28
MgCl2	1	108.57
	2.5	103
FeCl3	1	76.42
	2.5	62.85
ZnCl2	1	82.85
	2.5	52.8
CoCl2	1	115
	2.5	118.87
HgCl2	1	66.4
	2.5	52.85

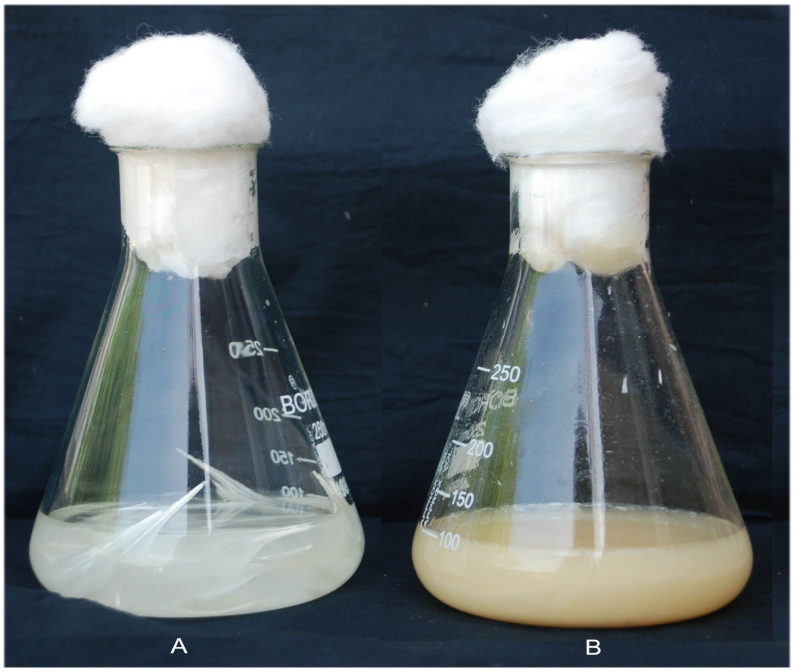

Fig. 1-conical flask showing organism degraded the feathers efficiently

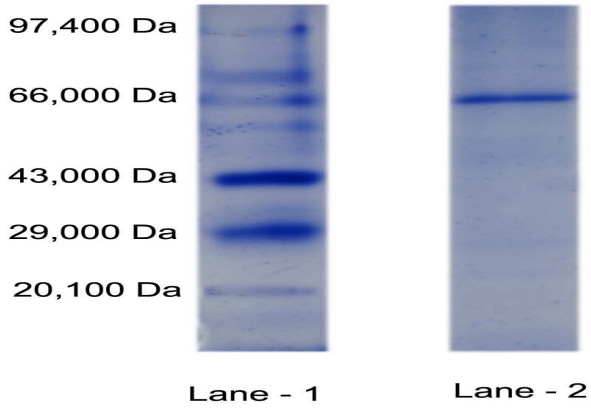

Fig. 2-Lane1: SDS PAGE Marker, Lane2.sample showing 66kDa Molecular weight

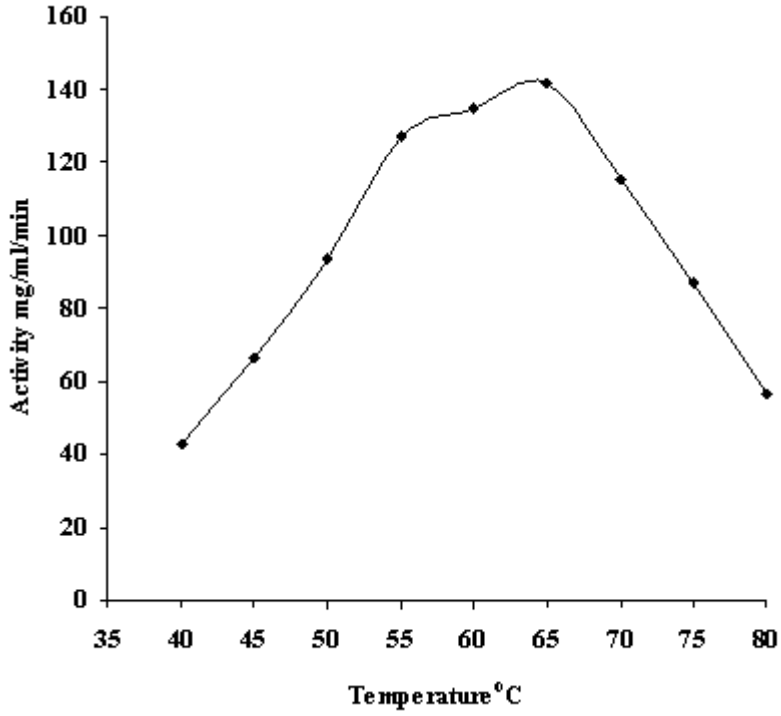

Fig. 3-Effect of temperature on activity of kertainase from *Bacillus sp.*JB 99