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Characterization of Thermostable Keratinase from *Bacillus* sp.

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Abstract

Keratinase enzymes produced by two improved strains, *Bacillus subtilis* (MBF20) and *Bacillus cereus* (MBF45) were characterized to evaluate the application potential of the produced enzyme. Study of the physico-chemical characters of the enzyme showed that the isolated keratinase enzymes belonging to serine protease group were significantly stable at refrigerated conditions retaining >90% activity till one month while the activity reduced to 50% after two months. Further, the enzyme was active up to 48 hours at room temperature indicating that the enzyme had significantly higher stability than most of the reported keratinase enzymes. The enzyme was also themo-tolerant exhibiting optimum activity at 55°C though its activity range was between 28°C - 55°C. With optimum activity at alkaline pH of 8.5 and only marginal inhibition in presence of surfactants, organic solvents and considerable enhancement of activity in presence of reducing agents, the scope and versatility of the application potential of the isolated keratinase enzymes is significantly high.

Keywords: Bacillus, keratinase, Purification, characterization

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Introduction

Feather, though very rich in protein, has not been considered as serious source of dietary supplement traditionally. This is because of its poor digestibility and low nutritive value (Eggum, 1970; Zang *et al*., 2014). In recent years with the isolation of keratinase producing microorganisms, a lot of interest is generated to use keratinase treated feather as animal feed supplement / organic manure (Brandelli *et al*., 2010; Tiwari and Gupta, 2012). In spite of several organisms degrading keratin being characterized worldwide todate, only the non-pathogenic organisms can be utilized commercially among which *Bacillus sp.* are prominent. Further, only limited number of indigenous microorganisms with application potential are characterized from India or their enzymes characterized (Pushpa Lata and Naik, 2010; Ponnusamykonar *et al*., 2011; Sivakumar *et al*., 2012). Viability of the commercial enzyme application depends to a large extent on cost of enzyme production and the downstream operations required for extraction and purification of enzyme. Most of the applications of keratinase including bioconversion of feather into animal feed, leather processing *etc*., employ crude or partially purified enzyme (Gupta and Ramnani, 2006). In order to make the process of utilization of keratin waste economical, which is hitherto, is underutilized, it is essential to characterize keratinase enzymes from indigenous isolates producing neutral to alkaline keratinases that are thermostable.

Two indigenous *Bacillus* isolates producing keratinase isolated in our studies were developed to yield >300 units/ml of enzyme by optimizing parameters of keratinase production (Jeevana Lakshmi, 2007). The enzyme produced by the isolates was partially purified and characterized. Present work is focused on the characterization of partially purified keratinase enzyme from two isolates to ascertain its application potential. In view of high stability and tolerance to surfactants and organic solvents the characterized keratinases can have good application.

Materials and methods

Keratinase enzyme from the two MBF isolates was purified and characterized to determine the nature and the type of enzyme produced. The molecular weight and their sensitivity to metals, solvents, protein inhibitors, reducing agents *etc*. was also determined to evaluate the application potential of the enzyme in feather degradation. The fermentative production of enzyme from two improved *Bacillus* isolates, MBF20 and MBF45 was carried out in 500ml volumes of production medium in two liter flasks for 5 days for partial purification of keratinase (Jeevana Lakshmi, 2007). The residual biomass was separated by centrifugation at 5000 rpm at 4°C. As the enzyme is extracellular, the culture filtrate was pooled from the 2l flasks and partial purification of the keratinase enzyme was carried out using ammonium sulphate precipitation (Mukhopadhyay and Chandra, 1990). Protein precipitate at 40%, 60% and 80% ammonium sulphate saturation was collected and washed twice with 0.1M Tris HCl buffer (pH 7.5). The samples were dialyzed for 18 hours at 4°C against the same buffer. The fractions showing highest keratinase activity were pooled and loaded on DEAE cellulose columns equilibrated with Tris- HCl buffer. 5ml sample the columns were collected after successively washing with buffer containing gradient salt concentration in the range of 0.1M -0.5M. Keratinase activity of the collected samples was determined using azokeratin assay method of Riffel *et al.*, 2003. The specific activity of the enzyme was calculated by determining total protein of the fractions (Lowry *et al*., 1951) using bovine serum albumin as a standard and expressed as KU per mg of the estimated protein. Stability of the PPK enzyme was determined by checking the activity at 4ºC and room temperature every 24 hours for a week and consequently every month for a period of 4 months.

Table 1: Partial purification of keratinase enzyme from MBF isolates with Ammonium sulphate fractionation

Isolates	Culture filtrate			Ammonium sulphate fractions								
				40%			60%			80%		
	Total Protein (mg)	Total units (KU)	Specific activity (KU/mg)	Total Protein (mg)	Total units (KU)	Specific activity (KU/mg)	Total Protein (mg)	Total units (KU)	Specific activity (KU/mg)	Total Protein (mg)	Total units (KU)	Specific activity (KU/mg)
MBF20	319.4	3452	10.8	6.2	186	30.0	4.3	423.12	98.4	10.8	1890	173.5
MBF45	215.2	1920	8.9	4.0	12.5	31.3	3.8	254.2	66.9	7.8	1219	155.4

Effect of physical and chemical parameters on PPK activity

The effect of pH (4.5-10.5), temperature of incubation (4 -75 °C) and substrate concentration (5mg to 20mg) on enzyme activity were determined to evaluate the optimum parameters for enzyme activity. The influence of metal ions was determined by treating the PPK with various metal ions at two concentrations (3 and 5mM) for 15 minutes prior to assay of keratinase. The resultant activity was expressed as percentage of residual activity as compared with the solvent controls. Sample without any treatment was maintained as control where the activity was taken as 100%. The influence of organic solvents like DMSO, isopropanol, acetone *etc*, was determined in concentration range between 1 to 10%. The effect of surfactants, and reducing agents like- Mercaptoethanol(ME), Dithiothreitol (DTE), Dithioerythreitol (DTT), sodium sulphide, urea *etc.* in the range of 0.1 and 0.5% concentrations `on the activity of the PPK was also determined.

Characterization of the produced keratinase

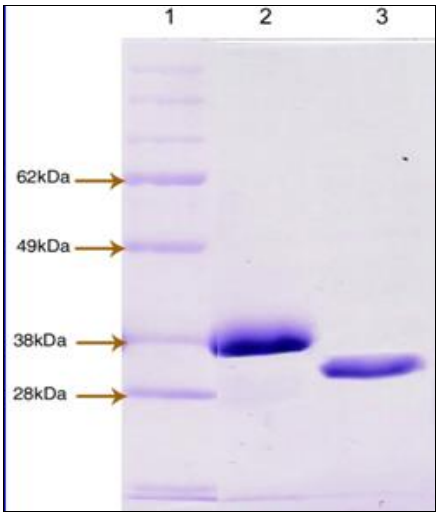
Depending on the nature of active site, proteases are classified into different groups namely serine, aspartic, thiol and metallo proteinases. Keratinases produced by different microorganisms have been reported to belong to all these different categories groups. Hence the type of keratinases produced by MBF20 and MBF45 was determined by comparing sensitivity to various protein inhibitors, along with metals and reducing agents (Rao and Deshpande, 1998). The molecular weight was determined on SDS PAGE as per the method of Laemmli, 1970, using protein marker from invitrogen. Enzymatic activity of keratinase on various keratin and non-keratin substrates like hair, feather, wool, nails, silk, casein, BSA and gelatin *etc.* was tested to ascertain the substrate range application potential. 1% of different substrates were added to basal medium and keratinase activity was measured using azokeratin assay and the degradation pattern was observed.

Results and Discussion

Keratinase activity of the culture filtrate and the cell free extract showed that the keratinase activity was found mainly in culture filtrate (> 300 KU/ml) for both the isolates, whereas negligible enzyme activity was observed in the cell free extracts (only 0.11-0.13KU/ml). This indicated that the keratinase was primarily extracellular and thus was secreted into the medium. Maximum activity was observed at 80% ammonium sulfate saturated fraction which was used for partial purification of enzyme. The specific activity of the PPK enzyme was 155-174KU/mg protein when compared to that of culture filtrate which was about 8.9KU/mg to10.8KU/mg (Table1).

Thus, fractionation resulted in 16-18 fold purification. Specific activity of 85-86 units/mg protein was observed for keratinase enzyme purified from several *Bacillus* species by salt precipitation in earlier studies (Lin *et al*., 1992; Nam *et al*., 2002; Riffel *et al*., 2003; Kanchana and Parvathy, 2010). Our results indicated that PPK from MBF isolates exhibited higher

specific activity compared to several earlier reports. On the 10%SDS gels, PPK enzyme was observed as single prominent band in both the cases indicating homogenous nature of enzyme (Fig.1)



1-Marker, 2-MBF20, 3-MBF45
Fig 1: SDS gel of the PPK of MBF isolates

The molecular weight of keratinase enzyme from MBF20 was 38kD whereas that of MBF45 was 32kD.

Stability of PPK: Stability of the PPK enzyme from MBF isolates was determined between 0º C and room temperature and the results are given in Fig. 2.

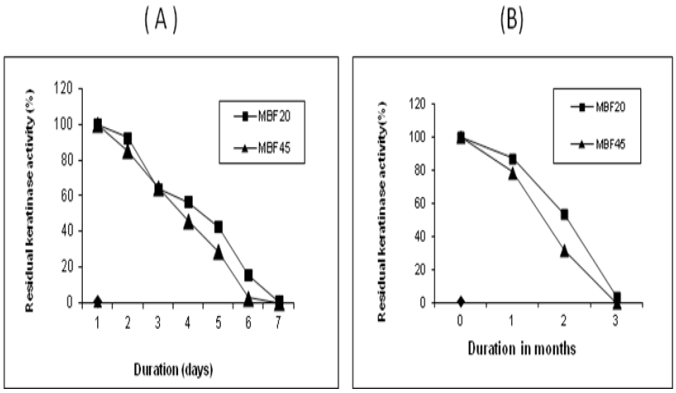


Fig 2: Residual keratinase activity of PPKs after storage (A) at room temperature and (B) at 0°C

The enzyme retained >90% activity after storage for one month at 0 ºC and 50% activity even after two months. At room temperature on the other hand, the enzyme was stable for 24 hours retaining majority of the activity. There was a 50% reduction in activity in 3-4 days and by day seven most of the enzyme activity was lost. Keratinases reported till date, have been observed to have varied stabilities with very few reports of good stability. When stored at 1-5°C, no detectable decrease in activity was observed by Young and Smith, 1975 till 8 days whereas activity was lost within for 3-6 hours at 50°C. The keratinase from *B. licheniformis* was reported to lose 2% of activity when stored at -20°C, 10% at 40ºC and 20% when stored at 20°C within a days time. Autolysis is suggested to account for major loss in enzyme activity in *Bacillus sp*. on storage (Lin *et al*., 1992). Keratinase from the MBF isolates which have significantly higher stability at 0°C for more than a month and at least 48 hours at room temperature have a definite advantage in terms of their application potential.

There was a gradual increase in keratinase activity of PPK with increase in pH from 4.5 when the effect of pH in the range of 4.5-10.5 was tested. Though enzyme was stable up to pH 10.5, optimum keratinase activity was observed at pH 8.5 for both MBF isolates (Fig. 3).

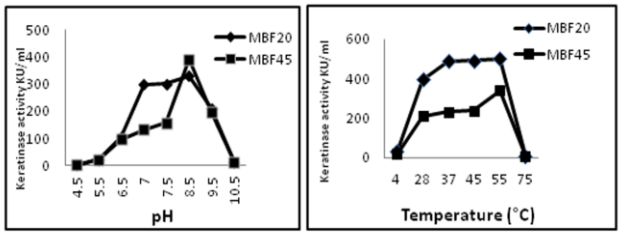


Fig 3: Effect of pH and temperature on partially purified keratinase

Several keratinases reported are active over a range of pH between 5-13. Keratinase from *Streptomyces pactum* DSM40530 (Bockle *et al*., 1995) has the least reported optimum value of pH 5 whereas the highest optima of pH 13 was observed for *Bacillus halodurans* AH-101(Takami *et al*., 1999). However, most of the other keratinases isolated have optimum activity in neutral to alkaline range (Bockle *et al*., 1995; Bressoller *et al*, 1999; Dozie *et al*., 1994; Farag and Hassan, 2004; Nam *et al*., 2002). Enzyme with optimum activity at alkaline pH, has definite advantage in application both in degradation of feather as well as in leather industry as significant increase in pH are found associated in these processes. Optimum activity of PPKs from MBF cultures was recorded at 55°C (398-635KU/ml) indicating thermo-tolerant nature of the obtained enzymes. However, good activity was observed between range 37°C - 55°C indicating versatility of application (Fig 3). Optimum temperature of activity from majority of the microorganisms producing keratinase has been observed to be between 28-45°C (Malviya *et al*., 1992; Bockle *et al*.,1995; Kunert, 1998; Alpress *et al*., 2002; Nam *et al*., 2002). Keratinases with an optimum temperature of 60-75°C were isolated from *Thermoactinomyces sp., S. pactum, Fervidobacterium sp.* (Bockle *et al*., 1995; Friedrich and Anthranikian, 1996; Ignatova *et al*., 1999). The enzyme isolated from *Chrysosporium keratinophilum* and *Fervidobacterium islandicum* AW-1 showed exceptionally higher optima of 90 - 100°C, but with half-life of only 30 - 90 minutes (Dozie *et al*., 1994; Nam *et al*., 2002). The keratinases produced by MBF isolates showed better stability at the desired optimum conditions.

Effect of chemicals on enzyme activity

Influence of surfactants, organic solvents, reducing agents, metal ions and on the activity of keratinase enzyme was studied

which also determines their application potential. Further, sensitivity to different groups of protease inhibitors was used to characterize the enzyme. In the present study, effect of 11 metal ions, four reducing agents three solvents and several protease inhibitors was determined to ascertain the stability and activity of the produced keratinase enzyme (Table 2).

Among the metal ions tested only Ca^{+2} showed 10% enhancement of the enzyme activity at 3mM and 30% at 5mM concentration (Table 2). Fe^{+2} and Al^{+2} salts showed less than 10% inhibition, followed moderate inhibition by Zn^{+2}, Ba^{+2}, Mg^{+2} (< 50% inhibition). Mg^{+2}, Mn^{+2} and Co^{+2} ions showed <40% inhibition at both the concentrations tested. At 5mM concentration Hg^{+2}, Pb^{+2}, and Co^{+2} exhibited maximum inhibition of 60-80% of enzyme activity while Ag^{+2} ions exhibiting a maximum 90% inhibition. Fe^{2+}, Ba^{+2}, Zn^{2+} have shown varied effect *i.e.* low inhibition or Keratinase from both the isolates was strongly inhibited in the presence of serine protease inhibitor PMSF, where as there was only a partial inhibitory effect with EDTA (Table 3). Benzimidine hydrochloride also exhibited a significant inhibition of 43-48%. The inhibition pattern with PMSF and EDTA corresponds with reports demonstrating keratinase belonging to serine protease group. This indicated that the keratinase produced by both MBF 20 and 45 isolates was a serine protease (Giongo *et al*., 2007; Riffel *et al*., 2003; Suntornsuk *et al*., 2004; Lin and Yin, 2010; Venkata Saibabu, 2013).

An enhancement in the keratinase activity was observed with all the reducing agents tested (Table 4).

A two fold increase in keratinase activity was observed with DTE followed by urea where a 1.5 fold increase was observed. Other reducing agents tested also showed a 20-40% enhancement in the keratinase activity. Reducing agents like ME, DTE, DTT *etc.*, have been shown to alter keratinolytic activity (Mukhopadyay and chandra, 1990; Lin *et al*., 1992; Kobayashi *et al*., 1995; Bockle and Muller, 1997; Lee *et al*., 2002; Mitsuiki *et al*., 2004). The thiol activation has been proposed as a mechanism for the observed enhancement in sulphitolysis (Onifade *et al*., 1998). In several studies presence of organism and its biomass was proposed to be contributing to the reducing environment and was found essential for degradation of feather whereas partially purified enzyme showed much low activity in degradation of feather *in vitro* (Ignatova *et al*., 1999; Bresoller *et al*., 1992; Bockle *et al*., 1995; Suh and Lee, 2001; Nam *et al*., 2002; Ramnani and Gupta, 2004; Ramnani *et al*., 2005). However, the PPK isolated from MBF strains was efficient in degradation of feather completely in 48 hours even in absence of biomass. The activity was specific against feather where as there was a very low activity with other substrates (Table 5) of indicating its specificity and potential application for degradation of feather.

The nutritive value of the feather meal produced by treatment of keratinases and their application as feed supplement is under study.

Table 2: Effect of metal ions on keratinase activity

Metal ions	Concentration of metal ions	Isolates	
		MBF20	MBF45
		Residual keratinase activity (%)	
Control	None	100	100
Al^{+2}	3mM	91.4	98.1
	5mM	66.7	97.2
Ca^{+2}	3mM	122.9	109.9
	5mM	130.7	131.6
Hg^{+2}	3mM	36.3	44.7
	5mM	0.1	19.8
Pb^{+2}	3mM	35.6	60.9
	5mM	0.4	30.5
Fe^{+2}	3mM	97.3	100.0
	5mM	95.4	97.7
Zn^{+2}	3mM	77.3	90.2
	5mM	36.9	80.5
Mg^{+2}	3mM	72.3	78.6
	5mM	58.1	61.6
Mn^{+2}	3mM	76.6	62.9
	5mM	58.0	39.1
Ba^{+2}	3mM	73.1	81.2
	5mM	45.2	59.0
Co^{+2}	3mM	72.7	78.5
	5mM	57.5	39.4
Ag^{+2}	3mM	48.9	38.6
	5mM	11.2	10.2

Conclusion

The characters like thermo-stability, resistance to metal ions, organic solvents and chemicals make keratinases from MBF20 and MBF45 ideal for biodegradation and utilization of feather waste as value added product.

Table 3: Effect of organic solvents and surfactants on keratinase activity

Test		Concentration (mM)	Isolates	
			MBF11	MBF21
			Residual Keratinase activity (%)	
Organic Solvents	DMSO	1%	85.4	89.2
		5%	84.8	88.4
		10%	84.6	84.4
	Isopropanol	1%	98.8	97.8
		5%	95.2	85.8
		10%	86.9	86.5
	Acetone	1%	96.7	97.9
		5%	95.0	88.8
		10%	89.2	86.1
Surfactants	SDS	0.1%	97.3	99.4
		0.5%	96.1	98.7
	Triton x 100	0.1%	95.4	101.9
		0.5%	102.2	106.7

Table 4: Effect of reducing agents and inhibitors on keratinase activity

Test		Concentration (mM)	Isolates	
			MBF11	MBF21
			Residual Keratinase activity (%)	
Reducing agents	None	Control	100	100
	β-mercaptoethanol	0.1	119.4	111.6
		0.5	136.4	138.4
	DTT	0.1	111.7	121.0
		0.5	142.2	159.7
	Sodium tetrathionate	0.1	112.4	114.6
		0.5	139.6	148.2
	Sodium sulfite	0.1	107.8	119.1
		0.5	131.4	139.2
	Urea	0.1	121.1	112.1
		0.5	141.6	151.1
	DTE	0.1	152.8	181.1
		0.5	214.7	222.2
Inhibitors	EDTA	1	89.9	90.8
		5	63.8	60.7
	PMSF	1	9.5	10.9
		5	0.2	2.6
	Benzimidine hydrochloride	1	52.7	43.8
		5	31.1	49.6

Table 5: Activity sof PPK enzyme on various substrates

Keratinous substrates	Isolates	
	MBF20	MBF45
	Keratinase activity (KU/ml)	
Feather	400.7	201.7
Hair	17.4	9.6
Silk	40.4	35.8
Wool	13.2	6.2
Nail	8.4	8.2
Casein	7.2	6.8
Gelatin	2.3	2.1
BSA	2.1	1.6

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