Purification and Characterization of Keratinase from Feather Degrading Bacillus sp.
J Peddu, C Chitturi, V Lakshmi

Abstract
Microorganisms producing keratinases are gaining prominence in recycling of poultry waste feather. Keratinase producing Bacillus sp. Isolated, were subjected to strain improvement along with optimisation of parameters of fermentation. Scaled up enzyme was partially purified and characterized to evaluate its application potential. Keratinase isolated from the bacillus species exhibited good stability with > 90% activity being retained for one month at refrigerated conditions and 50% activity was observed even after two months. At room temperature, the enzyme was active for 24 – 48 hours with optimum temperature of activity being 55°C. The activity range was between 28°C - 55°C and pH optima was 8.5 which matched with optima for production of the enzyme. ~38kDa keratinase produced by the isolates was categorized into subtilisin group of serine proteinase based on the sensitivity to protease inhibitors, Keratinase from the test isolates was only marginally affected by surfactants, organic solvents. A significant enhancement in activity was observed in presence of reducing agents indicating possible versatile application potential for this enzyme with high stability.

INTRODUCTION
Feather is generated in bulk quantities as a by-product of poultry industry. It is estimated that 400 million chickens are processed every week. Typically as each bird has up to 125gms of feather, the weekly worldwide production of feather waste is about 3000 tons. Piling up of this waste material result in accumulation of dumps. Disposal of this bulk waste is a global environmental problem accounting to pollution of land and underground water resources. Thus, feather in spite of being made up of almost pure keratin protein is neither profitable nor environmentally friendly forming a produce of high volume with low profit margin (Mc Govern 2000).

Various strategies are adopted to handle the volume of waste accumulating continuously. Feather waste generated by the member firms is disposed off to waste disposal sites, incinerated, sent to the feather meal industry or to gardeners/farmers. These methods are inefficient or expensive adding to the cost for the producer. Traditionally, feather is processed by mechanical or chemical treatment and converted to feedstuff, fertilizers, glues, foils etc. These methods of feather meal production lead to the destruction of heat labile amino acids like methionine, lysine and tryptophan, in addition to formation of non-nutritive amino acids like lysinoalanine, lanthionine etc. The process results in only a low nutritive value feed supplement thereby undermining the product value (Wang and Parsons 1997).

Keratinases are emerging to play a vital role in the degradation of keratin and its conversion into digestible animal feed of higher nutritive value. Keratinases are produced by several microorganisms of which Bacillus sp. have gained utmost prominence in the last decade. They are considered to be important for large scale production and commercialization of the product due to their effectiveness in feather degradation, non-pathogenic nature, availability of complete genome sequences of several Bacillus sp., amenability to genetic manipulation and adaptability to scaling up. Two feather degrading isolates BF11 and BF21 producing more than 10KU/ml were isolated and were characterized in our earlier studies as Bacillus subtilis and Bacillus cereus respectively. These were subjected to strain development to improve fermentative production of keratinase in our earlier studies. A significant 50 fold increase in keratinase yield was achieved by the improved strains (MBF11, MBF21) compared to native isolates. A cost effective fermentation media with starch as carbon source and soyabean meal as nitrogen source was designed along with optimisation of physical parameters of fermentation resulting a yield of >500KU/ml (Jeevana Lakshmi, 2007).
Viability of the enzyme production depends on the extent of downstream operations required for purification and extraction of enzyme, which is mainly determined by its application potential of the end product. Highly purified enzymes are required for applications in medicine and as pharmaceuticals. However, for most of the applications of keratinase including bioconversion of feather into animal feed, leather processing etc., crude or partially purified enzyme is sufficient. Hence the partial purification of keratinase from the MBF11 and MBF21 was carried out to determine characters and application potentials of the enzyme. The nature, stability and optimum condition for activity for partially purified keratinase enzyme (PPK) was determined along with determining the range of substrates it can attack.

**MATERIALS AND METHODS**

The fermentation of two improved Bacillus isolates was scaled up proportionately to 500ml volumes of media in two liter flasks in multiple numbers using production media (Jeevana Lakshmi, 2007). The fermentation was carried out for seven-day period in an orbital environmental shaker at 150rpm. The biomass of the organism and remnant substrate was separated by centrifugation at 5000rpm at 4°C. As keratinase produced was found to be primarily extracellular, pooled supernatant for each isolate was used for partial purification of the enzyme as per method of Mukhopadhyay and Chandra (1990). Partial purification of keratinase enzyme was carried by ammonium sulphate precipitation at 40%, 60% and 80% salt concentration. The collected protein fractions were washed twice with 0.1M Tris HCl buffer (pH 7.5) and dialyzed against the same buffer for 18 hours at 4°C. As keratinase produced was found to be primarily extracellular, pooled supernatant for each isolate was used for partial purification of the enzyme as per method of Mukhopadhyay and Chandra (1990). Partial purification of keratinase enzyme was carried by ammonium sulphate precipitation at 40%, 60% and 80% salt concentration. The collected protein fractions were washed twice with 0.1M Tris HCl buffer (pH 7.5) and dialyzed against the same buffer for 18 hours at 4°C. The dialysed fractions showing the highest keratinase activity were loaded on DEAE cellulose columns equilibrated with Tris- HCl buffer. The column was washed successively with buffers having 0.1M, 0.25M and 0.5M concentration of NaCl to elute the sample differentially. 2.5ml volumes of fractions were collected and keratinase activity of the samples was determined. The fractions with maximum keratinase activity were pooled, concentrated using of 100kDa and finally 10kDa cut offs were used for determining Keratinase activity using azokeratin assay method (Riffel et al. 2003). Total protein of the fractions was determined along with determining the range of substrates it can attack.

Stability of the keratinase enzyme: Stability of the partially purified keratinase enzyme was determined at room temperature and at 4°C. Activity of the keratinase enzyme was determined at 24 hour intervals for seven days for sample at room temperature and at 1 month intervals for a period of 4 months for enzyme stored at 4°C.

**RESULTS AND DISCUSSION**

Keratinase activity of the culture filtrate and the cell free extract was determined to find the localization of the enzyme. The activity of the culture filtrate was > 300 KU/ml for the isolates. On the other hand negligible enzyme activity was observed in the cell free extracts (only 0.04-0.07KU/ml) indicating that the keratinase was secreted into the medium and was thus primarily extracellular. The results of partial purification by ammonium sulfate fractionation showed that
maximum purification was observed at 80% saturation with specific activity of the partially purified keratinase enzyme being 173-193KU/mg protein compared to that of culture filtrate which was about 6.9KU/mg to 10.8KU/mg (Table 1). Thus fractionation resulted in 16-27 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; Riffel et al. 2003; Ramnani and Gutpa 2004). The results indicate that PPK from MBF isolates exhibited higher specific activity compared to several other keratinase producing Bacillus species reported. On the 10% SDS gels PPK enzyme was observed as single prominent band in both the cases with molecular weight of 38kDa indicating homogenous nature of enzyme (Fig. 1).

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

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Culture filtrate</th>
<th>40% Ammonium sulphate fractions</th>
<th>60% Ammonium sulphate fractions</th>
<th>80% Ammonium sulphate fractions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total Protein (mg)</td>
<td>Total activity (KU)</td>
<td>Specific activity (KU/mg)</td>
<td>Total Protein (mg)</td>
</tr>
<tr>
<td>MBF22</td>
<td>304.4</td>
<td>3402.8</td>
<td>10.4</td>
<td>6.2</td>
</tr>
<tr>
<td>MBF21</td>
<td>209.3</td>
<td>2014.5</td>
<td>9.6</td>
<td>4.1</td>
</tr>
</tbody>
</table>

Fig 1: SDS gel of the PPK of MBF isolates

1-Marker, 2-MBF11, 3-MBF21

Stability of PPK: Stability of the PPK enzyme from MBF isolates was determined at 0°C and room temperature and the results are given in Fig. 2. The enzyme retained >90% activity after storage for one month at 0°C 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 when protease 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 4°C and 20% when stored at 20°C with 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 present MBF isolates thus has significantly high stability at 0°C for more than a month whereas at room temperature retained activity for at least 24-48hrs. which is a definite
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advantage in terms of application potential.

**Figure 3**

Fig 2: Stability of partially purified keratinase enzyme at 0°C and at room temperature

Optimum physical parameters for PPK activity: The effect of pH on the activity of PPK in the range of 4.5-10.5 showed a gradual increase in keratinase activity with increase in pH from 4.5. Though enzyme was stable up to pH 10.5, optimum keratinase activity was observed at pH 8.5 for both MBF isolates (Table 2). Reported keratinases are active over a range of pH from 5-13. The least reported optimum value of pH 5 was found for keratinase from Streptomyces pactum DSM40530 (Bockle et al. 1995) and 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; Bressolier et al. 1999; Dozie et al. 1994; Farag and Hassan 2004; Nam et al. 2002; Ramnani and Gutpa 2004). 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 the processes.

**Figure 4**

Table 2: Effect of pH on PPK activity

<table>
<thead>
<tr>
<th>pH</th>
<th>MBF11</th>
<th>MBF21</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.5</td>
<td>3.02</td>
<td>2.01</td>
</tr>
<tr>
<td>5.5</td>
<td>34.90</td>
<td>22.50</td>
</tr>
<tr>
<td>6.5</td>
<td>138.50</td>
<td>111.05</td>
</tr>
<tr>
<td>7.0</td>
<td>435.75</td>
<td>212.00</td>
</tr>
<tr>
<td>7.5</td>
<td>444.75</td>
<td>214.50</td>
</tr>
<tr>
<td>8.5</td>
<td>468.25</td>
<td>330.75</td>
</tr>
<tr>
<td>9.5</td>
<td>222.50</td>
<td>271.25</td>
</tr>
<tr>
<td>10.5</td>
<td>19.75</td>
<td>11.75</td>
</tr>
</tbody>
</table>

PPKs had optimum activity 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 (Table 3). Further increase in the temperature resulted in decline in the enzyme activity. Optimum temperature of activity from majority of the mesophilic microorganisms producing keratinase has been observed to be between 28-45°C (Allpress et al. 2002; Bockle et al. 1995; Kunert 1988; Malviya et al. 1992; Mukhopadhyay and Chandra 1990; Ramnani et al. 2005). Keratinases with an optimum temperature of 60-75°C were isolated from Thermoactinomyces sp., S. pactum, Fervidobacterium sp.. (Bockle et al. 1995; Friedrich and Antranikian 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 had half-life of only 30 - 90 minutes (Dozie et al. 1994; Nam et al. 2002). Thus the keratinases produced by MBF isolates have better stability at the desired optimum conditions for activity.
Effect of chemicals on enzyme activity: Chemicals like metal ions, organic solvents, surfactants, reducing agents and inhibitors have been found to have varied effects on keratinase activity. The sensitivity to different groups is used for characterisation of the enzyme also. Among the 11 metal ions tested Hg\(^{2+}\), Pb\(^{2+}\) and Ag\(^{2+}\) showed maximum inhibition at 5mM concentration and Zn\(^{2+}\), Mg\(^{2+}\), Mn\(^{2+}\) and Co\(^{2+}\) showed moderate inhibition of the activity (Table 4). Fe\(^{2+}\) and Al\(^{3+}\) had least inhibitory effect. Ca\(^{2+}\) were the only divalent ion which exhibited marginal enhancement in the activity of the enzyme. Keratinase activity was observed to be strongly inhibited by transition and heavy metal ions like Hg\(^{2+}\), Ag\(^{2+}\), Pb\(^{2+}\), Co\(^{2+}\) Divalent metal ions like Ca\(^{2+}\), Mg\(^{2+}\) and Mn\(^{2+}\) were observed to stimulate the keratinase activity from various sources. Ca\(^{2+}\) ions also caused reversion of EDTA inhibition and restored keratinase activity. Metals like Ba\(^{2+}\), Zn\(^{2+}\), Fe\(^{3+}\) etc., have been reported to exhibit varied influence on the activity of keratinase ranging from no influence to moderate or strong inhibition or enhancing of enzyme activity in different reports (Lin et al. 1992; Lee et al. 2002; Nam et al. 2002; Riffel et al. 2003; Farag and Hassan 2004; Suneetha and Lakshmi 2005; Thys et al. 2004).

The reducing agents tested exhibited an enhancement in the keratinase activity invitro (Table 5). The increase was 1.6 – 2.1 folds with DTE followed by urea. Reducing agents like β-ME, DTE DTT etc. have been shown to alter keratinolytic activity at varying levels (Bockle and Muller 1997; Kobayashi et al. 1995; Lee et al. 2002; Lin et al. 1992; Mitsuiki et al. 2004; Mukhopadhyay and Chandra 1990). The possible mechanism for the observed enhancement is mainly attributed to thiol activation that contributes in sulphitolysis (Onifade et al. 1998). Purified keratinases alone...
have been reported not to degrade keratin completely invitro in several studies. Presence of organism was found essential and was proposed to be contributing reducing environment (Bockle et al. 1995; Bressolier et al. 1999; Ignatova et al. 1999; Ramnani and Gupta 2004; Suh and Lee 2001; Yamamura et al. 2002a & b). However, the PPK isolated from our strains was efficient alone in degradation of feather even in absence of biomass.

EDTA had a partial inhibitory effect on the activity of MBF enzymes whereas serine protease inhibitor PMSF strongly inhibited the activity (Table 5). There was significant inhibition of enzyme activity with benzimidine hydrochloride also. The results indicate that the keratinase produced by the MBF 11 and 21 isolates can be categorized as serine proteinase. Keratinase enzyme of serine protease group are also reported earlier to be strongly inhibited by inhibitor PMSF and benzimidine hydrochloride while EDTA exhibited only partial inhibition which were comparable with the results obtained in the presence study (Bockle et al. 1995; Bressolier et al. 1999; Giongo 2007; Ignatova et al. 1999; Riessen and Antranikian 2001; Riffel et al. 2003).

Solvents like DMSO, isopropanol and acetone, showed marginal inhibition of enzyme activity between 1-10% concentrations for MBF21. However, with PPK from MBF11, the enzyme activity was slightly enhanced with acetone at 5-10% concentration as compared to the controls (Table 5). Similar to our observation some of the keratinases characterized earlier are found to be quite stable in presence of organic solvents also (Mitsuiki et al. 2004; Nam et al. 2002; Riffel et al. 2003).

The response to SDS treatment varied with concentration. Similarly, slight enhancement in activity of the PPKs was observed on treatment with Triton X 100 at 0.5% concentration (Table 5). Proteins are known to be denatured by surfactants at concentrations higher than their critical micelle concentration (Henley and Sadana, 1985; Chaplin and Bucke, 1990; Rao and Deshpande, 1998).

### Figure 7

Table 5: Effect of chemicals on keratinase activity

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Range of PPK</th>
<th>Control</th>
<th>MBF11</th>
<th>MBF21</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>Control</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>β-mercaptoethanol</td>
<td>0.1</td>
<td>112.3</td>
<td>118.2</td>
<td>112.8</td>
</tr>
<tr>
<td>DTT</td>
<td>0.5</td>
<td>124.9</td>
<td>112.7</td>
<td>112.7</td>
</tr>
<tr>
<td>Sodium thiosulfate</td>
<td>0.5</td>
<td>124.1</td>
<td>111.2</td>
<td>111.2</td>
</tr>
<tr>
<td>Sodium sulfite</td>
<td>0.5</td>
<td>108.7</td>
<td>111.0</td>
<td>111.0</td>
</tr>
<tr>
<td>Urea</td>
<td>0.5</td>
<td>118.6</td>
<td>113.1</td>
<td>113.1</td>
</tr>
<tr>
<td>DTE</td>
<td>0.5</td>
<td>112.8</td>
<td>116.2</td>
<td>116.2</td>
</tr>
<tr>
<td>EDTA</td>
<td>0.5</td>
<td>149.0</td>
<td>121.9</td>
<td>121.9</td>
</tr>
<tr>
<td>DMSO</td>
<td>5%</td>
<td>77.3</td>
<td>72.2</td>
<td>72.2</td>
</tr>
<tr>
<td>Isopropanol</td>
<td>5%</td>
<td>0.8</td>
<td>18.9</td>
<td>18.9</td>
</tr>
<tr>
<td>Acetone</td>
<td>5%</td>
<td>0.3</td>
<td>31.1</td>
<td>31.1</td>
</tr>
<tr>
<td>Benimidine hydrochloride</td>
<td>5%</td>
<td>45.6</td>
<td>38.2</td>
<td>38.2</td>
</tr>
<tr>
<td>SDS</td>
<td>1%</td>
<td>99.8</td>
<td>92.8</td>
<td>92.8</td>
</tr>
<tr>
<td>Acetone</td>
<td>10%</td>
<td>82.8</td>
<td>89.1</td>
<td>89.1</td>
</tr>
<tr>
<td>SDS</td>
<td>0.5%</td>
<td>97.6</td>
<td>101.5</td>
<td>101.5</td>
</tr>
<tr>
<td>Triton X 100</td>
<td>0.5%</td>
<td>94.3</td>
<td>102.5</td>
<td>102.5</td>
</tr>
</tbody>
</table>

Substrates Range of PPK: The ability of the PPK to degrade different keratinous and non-keratinous protein substrates was determined to evaluate substrate specificity (Fig 3). Highest activity was observed against feather, followed by other keratinous substrates in the order hair > wool > nail. Other non-keratinous proteins like gelatin, casein and BSA were also attacked indicating their broad substrate specificity.

The results of the present study show that serine protease group of keratinase obtained from MBF isolates are robust with good stability and can have versatile application potential.
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