Chapter 8

Keratinolytic protease production by *Bacillus amyloliquefaciens* 6B using feather meal as substrate and application of feather hydrolysate as biocontrol (antifungal) as well as organic nitrogen input for agricultural soil

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8.1. Introduction

Low commercial value keratinous wastes of animal origin such as nails, hair, feathers, horns, hoofs, beak etc., which are generated in huge amount from poultry and cattle slaughter houses, are hard-to-degrade. Considering the fact that feathers constitute nearly 8.5% of the total weight of a chicken, the amount of solid waste generated annually in India from approximately 823.5 million chickens, with an average individual body weight of 2 kg, would be approximately 140 million kg (FAO 2002). Currently, the waste feathers are either disposed by dumping or incineration which can lead to serious health hazards and environmental pollution (Deydier et al 2005). Alternately, they are transformed into animal feed supplement by harsh hydrothermal and chemical treatment that result in loss of nutritionally essential amino acids, such as methionine, lysine, and tryptophan, and formation of non-nutritive amino acids, such as lysinoalanine and lanthionine (Dalev et al 1997). Consequently, microbial degradation of such wastes along with production of commercially important enzymes (especially keratinase) and other value-added products such as nutrient-rich animal feed or fertilizer (Onifade et al 1998, Brandelli 2008, Jeong et al 2010, Lateef et al 2010) is desirable.

Contemporary to this problem, is the development of new fertilizers using natural materials. In the same context, feather hydrolysate resulting from the bioconversion of keratinous wastes being rich in nitrogen and inexpensive could be used as a potential fertilizer for soil amendment (Gupta and Ramnani 2006), serving the dual purpose of improving plant growth and stimulating microbial activity in soil (Vasileva-Tonkova et al 2009). On the other hand, inadequate effectiveness of chemical control and increasing restrictions on the use of fungicide/pesticides have given increasing impetus to biological control as an alternative tool for disease management in agriculture (Raaijmakers et al 2002). Hence, microorganisms capable of degrading keratinous waste as well as exhibiting antagonism against soil borne plant pathogens hold prospects of dual benefit, waste utilization along with production of biocontrol metabolites. Most of investigations reported in literature on keratinolytic microorganisms have been directed toward keratinase production for increasing the digestibility of feathers as an animal feed (Onifade et al 1998, Odetallah et al 2003, Grazziotin et al 2006, Fakhfakh et al 2011). However, there is only one report on the isolation and characterization of keratinolytic Stenotrophomonas maltophilia R13 with
plant growth-promoting activity (Jeong et al 2010). To the best of our knowledge, there is no report on keratinolytic Bacillus sp. exhibiting biocontrol activity.

From this perspective, the poultry soil isolate Bacillus amyloliquefaciens 6B (BA6B) was investigated for: its ability to grow on and efficiently degrade whole feathers, production of keratinolytic protease and antifungal metabolites. Additionally, the enzyme and the antifungal metabolites produced by BA6B were characterized. Further, the fermentation broth with the bacterial cells and hydrolyzed feather was investigated as nitrogenous soil input.

8.2. Materials and Methods

8.2.1. Materials

Bushnell and Haas Medium (BHM), Luria Bertani Broth, gelatin, yeast extract and bovine serum albumin (BSA, protein standard) were obtained from Hi-Media, India. Hammerstein casein was procured from Sisco Research Laboratories, India. Chicken feathers were procured from a local poultry (Karamsad, Anand, Gujarat, India), washed with tap water and air dried. The feathers were then ground to get feather meal which was added to basal salt medium as a substrate for enzyme production. All other solvents and chemicals used during the experiment were of analytical grade. Phytopathogenic fungi namely, Aspergillus niger MTCC 16404, Aspergillus fumigatus MTCC 343, Aspergillus parasiticus NCIM 898, Trichosporon sp. NCIM 1110, Fusarium oxysporum NCIM 1072 were obtained from National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratory, Pune, India and Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh, India.

8.2.2. Isolation and identification of keratin hydrolysing bacterium

Feather degrading bacteria were isolated upon enrichment of the poultry soil (Karamsad, Gujarat) with whole feathers for 15 days. Upon incubation a 10% (w/v) soil suspension was prepared in sterile distilled water and 100 µL aliquots of serially diluted soil suspension were spread on 1% casein agar plates and incubated at 37°C for 48 to 72 h. Individual colonies exhibiting clear zones around them resulting from casein hydrolysis were picked and purified by repeated sub-culturing on Luria Bertani agar plate. The pure cultures were further screened for their ability to degrade feather on
Feather meal agar (BHM supplemented with 1% w/v feather meal; FMA) plates. The bacterial isolates exhibiting feather degradation on FMA plates were then investigated for native feather degradation. The isolate exhibiting the highest keratinolytic activity was then selected for further studies.

The identity of the selected bacterial isolate designated as 6B was determined on the basis of 16S rRNA gene sequence analysis. The genomic DNA was extracted as described by Ausubel et al (1997) and the 16S rRNA gene was amplified and sequenced as described in Chapter 2. Further, the sequence has been submitted to Genbank at NCBI.

8.2.3. Organic solvent tolerance of bacterial isolate 6B

The bacterium 6B was spot inoculated on casein agar plates using sterile tooth picks upon which each plate was flooded with 7 mL of different solvents (iso-octane, n-heptane, n-hexane, cyclohexane, xylene, toluene, benzene, chloroform, ethyl acetate and methanol) and incubated at 37°C in air tight canister in order to prevent the evaporation of solvent from the plates. Upon incubation, plates were observed for growth as well as clear zone around the colony in the presence of solvents.

The stability of keratinolytic protease in organic solvents was investigated by mixing equal volume of solvent and culture supernatant (crude enzyme) in screw cap test tubes and incubated in shaking water bath (50 rpm) at 37°C. After 24 h of incubation residual protease activity was assayed in the aqueous phase.

8.2.4. Profile of keratinolytic protease production by bacterial isolate 6B

The 500 mL Erlenmeyer flasks containing 200 mL feather meal broth (BHM amended with 0.2% w/v feather meal; FMB) was inoculated with an overnight grown culture of 6B to obtain an initial culture density (O.D.600nm) of 0.05 and incubated on orbital shaker (150 rpm) at 37°C. The 2 mL aliquots were withdrawn after 3, 6, 9, 12, 24, 36 and 48 h of incubation, and monitored for growth and protease activity.

8.2.5. Medium optimization for keratinolytic protease production by bacterial isolate 6B

Various sugars (fructose, galactose, glucose, lactose, maltose, mannitol, sucrose and xylose; 0.5% w/v) were supplemented individually to FMB, in order to investigate
their effect as co-carbon sources on the enzyme production by 6B. The influence of varying concentrations (0.1% to 2%, w/v) of feather meal on the keratinolytic enzyme production was also investigated.

8.2.6. Concentration of enzyme

The fermentation broth was harvested after 12 h of incubation and subjected to centrifugation at 4°C and 12,310 × g for 15 min. The supernatant was collected and subjected to 40-80% ammonium sulphate saturation and kept overnight at 20°C to allow protein precipitation. The precipitates were then harvested by centrifugation at 4°C and 27,540 × g for 20 min. The pellet thus obtained was resuspended in minimum volume of 50 mM Tris-Cl buffer (pH 8.0) to allow solubilization of proteins and dialyzed against same buffer overnight at 4°C.

The dialyzed enzyme preparation was then analyzed by Native and SDS-polyacrylamide gel (10%, w/v) electrophoresis according to the method of Laemmli (1970). One part of gel was subjected to activity staining in order to visualize protease band and the other part was subjected to silver staining (Sambrook and Russell 2001) in order to visualize protein bands. For activity staining, the native gel was transferred onto a casein agar slab (1% casein and 1% (w/v) agar in 50 mM Tris-Cl buffer pH, 8.0) and incubated at 37°C till transparent bands of protease activity appeared due to casein hydrolysis in the agar slab. In case of SDS-PAGE, after electrophoresis, SDS was removed by washing the gel for 15 min with 2.5% Triton X-100 and then twice for 10 min each with distilled water. Then, the gel was subjected to activity staining as described above. Relative molecular mass (Mr) was estimated by comparison with molecular mass standards (3.5 to 97.4 kDa) (Bangalore Genie, Bangalore, India).

8.2.7. Characterization of crude keratinolytic protease from isolate 6B

The keratinolytic protease activity was determined at different temperatures viz., 30, 40, 50, 60, 70, 80°C in presence of 50 mM Tris-Cl buffer, pH 8.0. The thermostability of enzyme was determined by pre-incubating the enzyme at 50 and 60°C in shaking water bath for varying time intervals upto 120 min and then assayed for residual activity at 50°C, which was expressed as percentage of initial activity.

The activity of keratinolytic protease was monitored over a pH range of 5.0 to 11.0 at 37°C. Sodium acetate buffer (50 mM) for pH 5.0-5.5, sodium phosphate buffer
(50 mM) for pH in range of 6.0 to 7.5, Tris-Cl buffer (50 mM) for pH 8.0 to 8.5 and glycine-NaOH (50 mM) for pH in range of 9.0 to 11.0 were used to adjust the pH of the reaction mixture. The pH stability of the enzyme was estimated by pre-incubating the enzyme in buffers of various pH for 1 h and then assayed for protease activity at optimum pH.

To determine the effect of various additives viz., metal ions (Ca$^{2+}$, Cu$^{2+}$, Fe$^{2+}$, Mg$^{2+}$, Mn$^{2+}$, Hg$^{2+}$, Zn$^{2+}$; 5 mM), enzyme inhibitors (EDTA and PMSF; 5 mM), surfactants (SDS, CTAB, Triton X-100, Tween 80; 1% w/v), and bleach-oxidant (H$_2$O$_2$; 1% v/v); the enzyme was pre-incubated with these additives for 1 h at 37$^\circ$C and then assayed using casein as a substrate. The enzyme activity was expressed as % activity relative to control (without any of the aforementioned additives) which was considered as 100%.

8.2.8. **In vitro screening for antifungal activity**

A co-culture technique (Trivedi et al 2008) was used to test the ability of bacterium 6B to inhibit the growth of several phytopathogenic fungi (A. niger MTCC 16404, A. parasiticus NCIM 898, A. fumigatus MTCC 343, Trichosporon sp. NCIM 1110 and F. oxysporum NCIM 1072). The bacterium was spot inoculated on one side of PDA plate and incubated at 37$^\circ$C for 48 h after which each fungal strain was inoculated individually, next to the bacterial culture and the plates were incubated at 27$^\circ$C up to 5 d for monitoring antifungal activity.

The antifungal activity of the culture supernatant harvested from stationary-phase culture of isolate 6B was bioassayed against the above mentioned fungi using agar well diffusion method. Briefly, the culture supernatant (100 µL) was inoculated in the center of PDA plates which had been inoculated previously with test fungus. After the plates were incubated at 28$^\circ$C for 5 d, the inhibition of fungal growth was observed around the well. The control plate was prepared by growing each test fungus with uninoculated feather medium.

Further the antifungal metabolites from culture broth were precipitated by lowering the pH of cell free supernatant upto 2.0 using (6M) HCl. The precipitate thus obtained was solubilized in methanol, which was then analyzed by ESI-MS. Mass spectra was recorded in positive ion mode using Esquire 3000 plus mass spectrometer (Bruker Daltonics, Germany) consisting of two octopole followed by an ion trap.
Helium was used as collision gas for collision induced dissociation (CID) experiment and the data obtained was processed using Esquire data analysis software, version 3.1.

8.2.9. **Use of the feather hydrolysate as a bioactive agricultural nitrogen input (30 d plant growth assay).**

Pot study was carried out in triplicates to compare the ability of feather hydrolysate and the reference commercial fertilizer as agriculture input and its effect on the growth of *Vigna radiate* var. meha (mung). The seeds (5 seeds/test pot) were planted in 6 liters plastic pots with 4 kg garden soil. The feather hydrolysate (concentrated five times using rotary evaporator at 60°C; 153 mL of feather hydrolysates/kg soil) and the fertilizer (625 mg of fertilizer/kg soil) were diluted with tap water and poured onto the top of the soil to obtain the desired concentration of soluble nitrogen (100 mg of N/kg soil). All the pots were watered regularly with tap water to replace the evaporation loss and after 30 days of sowing, all plants were carefully uprooted and washed. Various growth parameters such as plant height, plant fresh weight, root length and root fresh weight were recorded.

8.2.10. **Analytical procedures: enzyme activity**

The enzyme activity was determined as per the modified method of Khardenavis *et al* (2009). In brief, 2 mL of reaction mixture containing 1 mL of 1% (w/v) casein (acc. to Hammerstein) and 1 mL of appropriately diluted enzyme in 50 mM Tris-Cl buffer (pH 8.0) was incubated for 20 min at 50°C. The reaction was stopped by precipitation of the residual substrate with 2 mL 10% (w/v) trichloroacetic acid. A control was run in parallel in which the enzyme was added after addition of trichloroacetic acid. The reaction mixture was then centrifuged at 13,201 ×g for 10 min and supernatant was assayed for tyrosine by reading its absorbance was read at 280 nm (absorption maxima for tyrosine) against the assay blank, using UV-Visible spectrophotometer (Thermo Electron corporation, HELIOS α). The standard curve of tyrosine was prepared by reading absorbance of different dilutions of 500 µg tyrosine/mL stock. One unit of protease activity was defined as the amount of enzyme required to liberate 1 µg of tyrosine/min.mL under specified assay conditions. The total protein was estimated according to the method of Lowry *et al* (1951) using bovine serum albumin (fraction V) as standard.
Figure: Proteolytic activity of isolates 3B, 6B, 1B and 2A on (a) casein agar plate, (b) gelatin agar plate.

Figure: Initial screening for feather degradation by isolates 3B, 6B, 2A and 1B
8.1. Results and Discussion

8.1.1. Isolation and identification of keratin hydrolysing bacterium

Thirteen proteolytic bacterial strains isolated from the poultry farm soil sample were screened for feather degradation on FMA plates. The growth of bacterial isolates on FMA indicated their ability to utilize feather keratin as a sole carbon source for growth. Amongst all, four isolates designated as 1B, 2A, 3B and 6B were selected on the basis of their growth on FMA and were then screened for their ability to degrade native feather. Among the four isolates, 6B exhibited complete degradation of intact feather within 24 h of incubation at 37°C (Fig. 8.1).

![Figure 8.1 Feather degradation by isolate designated 6B](image)

The isolate 6B was found to be a Gram positive, spore-forming bacillus. The nucleotide sequence of 16S rRNA gene of this isolate exhibited 100% sequence homology with the partial sequence of *Bacillus amyloliquefaciens* DJFZ40 (GU568197) 16S rRNA gene. The 1,354 bp sequence of isolate 6B (Appendix 3) identified as *B. amyloliquefaciens* has been submitted to NCBI GenBank (JQ904625).

8.1.2. Organic solvent tolerance of *B. amyloliquefaciens* 6B (BA6B)

The solvent tolerance of BA6B was determined by its ability to grow on casein agar plates flooded with different solvents (*iso*-octane log $P_{ow} = 4.5$, *n*-heptane log $P_{ow} = 4.39$, *n*-hexane log $P_{ow} = 3.86$, cyclohexane log $P_{ow} = 3.2$, xylene log $P_{ow} = 3.1$, toluene log $P_{ow} = 2.64$, benzene log $P_{ow} = 2.13$, chloroform log $P_{ow} = 2.0$, ethyl acetate $P_{ow} = 0.68$ and methanol log $P_{ow} = -0.764$) during incubation. The BA6B exhibited growth as well as protease production in presence of all the solvents except ethyl acetate.
and methanol, with maximum zone of hydrolysis on plates flooded with iso-octane (Table 8.1).

Table 8.1: Organic solvent tolerance of B. amyloliquefaciens 6B and its protease.

<table>
<thead>
<tr>
<th>Organic solvents</th>
<th>log $P_{ow}$</th>
<th>Zone/Growth ratio</th>
<th>Relative enzyme activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (Control)</td>
<td>4.3</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>iso-Octane</td>
<td>4.5</td>
<td>4.5</td>
<td>99</td>
</tr>
<tr>
<td>n-Heptane</td>
<td>4.39</td>
<td>4.1</td>
<td>94</td>
</tr>
<tr>
<td>n-Hexane</td>
<td>3.86</td>
<td>3.6</td>
<td>85</td>
</tr>
<tr>
<td>Cyclohexane</td>
<td>3.2</td>
<td>2.6</td>
<td>82</td>
</tr>
<tr>
<td>Xylene</td>
<td>3.1</td>
<td>2.8</td>
<td>70</td>
</tr>
<tr>
<td>Toluene</td>
<td>2.64</td>
<td>2.4</td>
<td>46</td>
</tr>
<tr>
<td>Benene</td>
<td>2.13</td>
<td>1.6</td>
<td>39</td>
</tr>
<tr>
<td>Chloroform</td>
<td>2.0</td>
<td>1.4</td>
<td>22</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>0.68</td>
<td>ND</td>
<td>7</td>
</tr>
<tr>
<td>Methanol</td>
<td>-0.76</td>
<td>ND</td>
<td>4</td>
</tr>
</tbody>
</table>

In general, organic solvents with a log $P_{ow}$ between 1.5 and 4.0 are extremely toxic to microorganisms and other living cells because they partition preferentially in the cytoplasmic membrane, disorganizing its structure and impairing vital functions (Inoue and Horikoshi 1991). However, solvent toxicity not only depends on the inherent toxicity of the solvent but also on the intrinsic tolerance of the bacterial species and strains (Sikkema et al 1995). There exits few protease producing Bacillus sp. that can grow in presence of toxic organic solvents such as DMSO, methanol, toluene, xylene, cyclohexane, hexane and iso-octane (Gorbel et al 2003, Sareen and Mishra 2008, Fang et al 2009, Li et al 2009). The solvent tolerance in such microorganisms has been attributed to the alteration in cell morphology, composition of the membrane fatty acids and/or presence of active efflux pumps which maintain minimal sub toxic concentration of organic solvents within cells (Torres et al 2011).

The stability of crude enzyme from BA6B was determined in presence of all the above organic solvents at 50% (v/v) concentration upto 24 h (Table 8.1). The enzyme was found stable in presence of non-polar (high log $P_{ow}$ value) solvents, retaining more than 80% activity, whereas, the enzyme activity was significantly inhibited in presence of solvents with lower log $P_{ow}$ value such as chloroform, ethyl acetate and methanol. Low log $P_{ow}$ value corresponds to greater polarity and toxicity of the solvent to the enzyme (Inoue and Horikoshi 1991). Similar inactivation of keratinase from B. subtilis KD-N2 in presence of polar solvents was reported by Cai et al (2008). In contradiction to our observations, keratinase from Kocuria rosea (Bernal et al 2006),
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_Fervidobacterium islandicum_ (Nam _et al_ 2002) and _Chryseobacterium_ sp. kr6 (Riffle _et al_ 2003) have been reported to be stable in presence of even polar organic solvents.

### 8.1.3. Production profile of the extracellular keratinolytic protease by BA6B in batch culture

The isolate BA6B was able to produce keratinolytic protease in medium containing feather meal as sole carbon source. It was observed that the enzyme production by BA6B was growth associated and attained maxima (236.46 ± 12.5 U/mL) at the end of exponential growth phase (Fig. 8.2). The production of similar growth-associated keratinases has been reported by Brandelli and Riffel (2005) as well as Park and Son (2009). Daroit _et al_ (2011) also demonstrated growth associated keratinase production in optimized medium by an Amazonian _Bacillus_ sp. P45.

![Figure 8.2 Time course of keratinolytic protease production by Bacillus amyloliquefaciens 6B (Data are means of three replicates, error bars indicate S.E.)](image)

The keratinolytic protease production by BA6B was higher in comparison to those reported for _Serratia_ sp. HPC1383 (210 U/mL) (Khardenavis _et al_ 2009) and _B. licheniformis_ RG1 (161 U/mL) (Ramnnani and Gupta 2004).

Furthermore, keratinolytic protease production by BA6B was accompanied by rise in media pH from 7.2 to 8.4. It has been proposed that microbial degradation of keratinous substrate is a complex process and involves three steps, i.e. deamination, sulphitolysis and proteolysis. Such change in pH due to deamination of peptides and amino acids originating from keratin degradation, allows the substrate to swell and create an environment required for sulphitolysis and proteolysis (Kunert 2000). Any
further increase in pH was limited by the volatility of ammonia, which escapes from culture broth in gaseous form. Similar increase in medium pH from neutral to alkaline, have been reported during keratinase production by several other feather-degrading bacteria (Park and Son 2009, Lateef et al 2010) and fungi (Kaul and Sumbali 1999).

8.1.4. Optimization of media components and culture conditions for BA6B keratinolytic protease production

The isolate BA6B produced maximum keratinolytic protease (380.85 U/mL) in presence of xylose as co-substrate followed by lactose (286.16 U/mL) (Fig. 8.3). Contrary to most reports (Cai and Zheng 2009, Park and Son 2009, Jeong et al 2010), presence of mannitol, fructose, glucose, maltose and sucrose as co-carbon source in medium resulted in reduced enzyme production by BA6B. Such suppression in enzyme production has been attributed to catabolite repression, a phenomenon observed for biosynthesis of bacterial proteases including keratinases (Cheng et al 1995, Hossain et al 2007, Wang and Shih 1999).

![Figure 8.3 Effect of co-carbon source on keratinolytic protease production by B. amyloliquefaciens 6B. (Data are means of three replicates, error bars indicate S.E.)](image)

In subsequent experiment, the effect of varying concentrations of feather meal on keratinolytic protease production by BA6B was evaluated. The increase in BA6B protease production was observed with increase in feather meal concentration up to 0.5% (w/v) in fermentation medium and further increase in feather meal up to 1% (w/v) had no influence on protease production (Fig. 8.4). However, further increase in feather meal concentration above 1% (w/v) in medium resulted in lower protease production. Cheng et al (1995) reported highest yield of B. licheniformis PWD-1 keratinase in 1%
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(w/v) feather meal medium. The higher feather meal concentrations (2%, w/v and above) has been shown to increase medium viscosity, thereby affecting mass transfer and causing oxygen limitation for the growing culture, ultimately resulting in lower yields of keratinase (Park and Son 2009).

Figure 8.4 Effect of feather meal concentration on keratinolytic protease production by B. amyloliquefaciens 6B. (Data are means of three replicates, error bars indicate S.E.)

8.1.5. Characterization of BA6B keratinolytic protease

The extracellular keratinolytic protease from cell free supernatant of feather meal broth was concentrated upto 24-fold by employing 40 to 80% ammonium sulphate...
precipitation. The molecular weight of BA6B protease was found to be ~58 kDa based on its electrophoretic mobility in SDS-PAGE (Fig. 8.5). The molecular weight of keratinases from other Bacillus sp. has been reported in the range of 25 to 69 kDa (Lin et al. 1992, Kojima et al. 2006, Balaji et al. 2008, Cai et al. 2008). In contrast, Lysobacter NCIMB 9497 and Kocuria rosea have been reported to produce higher molecular mass keratinases (Allpress et al. 2002, Bernal et al. 2006).

The BA6B keratinolytic protease exhibited maximum activity at 50°C (Fig. 8.6a), which is similar to reported for keratinase activity from other Bacillus sp. (Lateef et al. 2010; Daroit et al. 2011). The half life of the enzyme was found to be 44 and 19 min at 50 and 60°C, respectively (Fig. 8.6b). Although the BA6B protease has lower thermal stability but its efficient feather degrading property suggests its potential application in feather waste management.

The BA6B protease (Fig. 8.7a) exhibited maximum activity at pH 8.0 which is similar to the previous reports from several other keratinolytic microorganisms (Riffel et al. 2003, Cai et al. 2008). Generally, bacterial keratinases are reported to exhibit neutral (Lateef et al. 2010, Daroit et al. 2011) or alkaline pH optima (Bernal et al. 2006, Prakash et al. 2010, Xie et al. 2010). Studies on pH stability revealed that the enzyme was stable in the pH range of 8.0 to 11.0 retaining more than 85% activity after 1 h (Fig. 8.7b).
The values of $K_m$ and $V_{max}$ of BA6B keratinolytic protease for caesin hydrolysis were found to be 1.14 mg/mL and 1.43 mg/mL.min, respectively (Fig. 8.8).

Amongst different metal ions, Ca$^{2+}$, Zn$^{2+}$ and Mn$^{2+}$ were found to stimulate enzyme activity while transition metal ions such as Cu$^{2+}$ and Fe$^{2+}$ strongly suppressed the enzyme activity (Table 8.2). Such stimulatory effect of divalent metal ions on enzyme activity has been attributed to structural alterations rather than catalytic effect (Glusker et al 1999). Several keratinases have been reported to exhibit higher activity in presence of Ca$^{2+}$ and Mn$^{2+}$ ions (Nam et al 2002, Riffel et al 2003, Corrêa et al 2010). However, in present study Zn$^{2+}$ was found to have stimulatory effect, which is contrary

Table 8.2 Effects of various metal ions, inhibitors, surfactants and bleach oxidant on B. amylooliquefaciens 6B protease activity

<table>
<thead>
<tr>
<th>Additives (concentration)</th>
<th>Residual enzyme activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
</tr>
<tr>
<td>Metal ions (5mM)</td>
<td></td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>130.94</td>
</tr>
<tr>
<td>Cu²⁺</td>
<td>34.52</td>
</tr>
<tr>
<td>Fe²⁺</td>
<td>17.04</td>
</tr>
<tr>
<td>Hg²⁺</td>
<td>105.82</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>99.94</td>
</tr>
<tr>
<td>Mn²⁺</td>
<td>195.96</td>
</tr>
<tr>
<td>Zn²⁺</td>
<td>167.24</td>
</tr>
<tr>
<td>Inhibitors (5 mM)</td>
<td></td>
</tr>
<tr>
<td>EDTA</td>
<td>106.27</td>
</tr>
<tr>
<td>PMSF</td>
<td>1.25</td>
</tr>
<tr>
<td>Surfactants (1%; w/v)</td>
<td></td>
</tr>
<tr>
<td>Triton X-100</td>
<td>110.12</td>
</tr>
<tr>
<td>Tween 80</td>
<td>91.53</td>
</tr>
<tr>
<td>SDS</td>
<td>82.35</td>
</tr>
<tr>
<td>CTAB</td>
<td>21.47</td>
</tr>
<tr>
<td>Oxidising agent (1%, w/v)</td>
<td></td>
</tr>
<tr>
<td>H₂O₂</td>
<td>94.66</td>
</tr>
</tbody>
</table>

Among the inhibitors tested, PMSF completely inhibited the enzyme activity (Table 8.2), suggesting it to be a serine hydrolase. Usually, EDTA has negative effects on keratinase activity, but we observed that the keratinolytic protease from 6B strain was slightly simulated by EDTA (5mM), similar to the keratinase from Fervidobacterium islandicum AW-1 (Nam et al 2002) and B. sublitis KD-N2 (Cai et al 2008). Furthermore, the enzyme remained quiet stable in presence of non-ionic (Triton X-100 and Tween 80) and anionic surfactant (SDS), whereas CTAB (cationic surfactant) exerted inhibitory effect (Table 8.2). Similar stability of B. halodurans PPKS-2 keratinase in presence of non-ionic and anionic surfactant has been reported (Prakash et al 2010). In general as per the literature, the proteases belonging to Bacillus genus are unstable against the bleech-oxidants (Anwar and Saleemuddin 2000). However, BA6B protease was found to be stable in presence of H₂O₂. Several researchers have engineered proteases for making them surfactant and bleach-stable (Tuschiya et al 1992, Wolff et al 1996), whereas the BA6B protease exhibited inherent stability towards surfactants and bleach-oxidant, which makes it suitable for use in the detergent industry.
8.1.6. In-vitro screening for antifungal activity

The genus *Bacillus* includes species considered particularly promising as biocontrol agents against plant pathogens (Ongena and Jacques 2008). Thus, the bacterium BA6B was investigated for its biocontrol potential.

![Figure 8.9 Antifungal activity of *Bacillus amyloliquefaciens* 6B against (a) *Aspergillus parasiticus* NCIM 898, (b) *Aspergillus niger* MTCC 16404, (c) *Aspergillus fumigatus* MTCC 343, (d) *Fusarium oxysporum* NCIM 1072, (d) *Trichosporon* sp. NCIM 1110 and (e) *Chrysosporium indicum*.

Table 8.3: Antifungal activity of *Bacillus amyloliquefaciens* 6B culture supernatant against fungal phytopathogens

<table>
<thead>
<tr>
<th>Test organism</th>
<th>Inhibition Zone (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (uninoculated broth)</td>
<td>-</td>
</tr>
<tr>
<td><em>Aspergillus niger</em> MTCC 16404</td>
<td>14</td>
</tr>
<tr>
<td><em>Aspergillus parasiticus</em> NCIM 898</td>
<td>19</td>
</tr>
<tr>
<td><em>Aspergillus fumigatus</em> MTCC 343</td>
<td>12</td>
</tr>
<tr>
<td><em>Fusarium oxysporum</em> NCIM 1072</td>
<td>21</td>
</tr>
<tr>
<td><em>Trichosporon</em> sp. NCIM 1110</td>
<td>17</td>
</tr>
</tbody>
</table>

In dual culture assays, the bacterium BA6B was found to inhibit all the test fungi (*A. niger* MTCC 16404, *A. fumigatus* MTCC 343, *A. parasiticus* NCIM 898, *Trichosporon* sp. NCIM 1110, *F. oxysporum* NCIM 1072) on PDA plates at 25°C (Fig. 8.9). Interestingly, BA6B grown in feather meal medium also inhibited mycelial growth of all the fungi tested (Table 8.3) with maximum antagonism against *F. oxysporum* (1072).
Figure 8.10 (a) ESI-MS spectrum of crude methanolic extract of *B. amyloliquefaciens* 6B, (b) ESI-MS/MS spectrum of [M+Na]^+ precursor ion at m/z 1058.6 and (c) sequence deduced on the basis of fragmentation pattern of [M+Na]^+ precursor ion.
Furthermore, to identify and characterize the bioactive compounds from BA6B, the crude extract of the fermentation broth was subjected to ESI-mass spectrometry. The peaks in ESI-MS spectra (Fig. 8.10a) at \( m/z \) 1030.5, 1044.5, 1058.6 and 1072.6 differ in mass by 14 Da and may be assigned as sodium adducts of C13-, C14-, C15- and C16-fatty acid chain length variants of a same surfactin group, respectively (Vater et al. 2002). The peak at \( m/z \) 1058.6 was further subjected to MS\(_2\) analysis and on the basis of fragment ions obtained, its sequence was deduced (Fig. 8.10b, c). The fragment ion at \( m/z \) 1030.5 with a mass difference of 28 Da from the parent ion (\( m/z \) 1058.6) revealed the carbonyl group coupled cleavage of lactone bond between fatty acid and C-terminal end of peptide leading to the linearization of surfactin cyclic lipopeptide ring (Cavelier et al. 1999). The peak at \( m/z \) 707.2 in MS\(_2\) spectrum of \( m/z \) 1058.6 could be assigned as sodiated form of internal fragment ion with molecular mass of 684 Da. The protonated from of this fragment ion (\( m/z \) 685) has been reported to be ubiquitously detected in MS\(_2\) spectra of surfactins \([\text{M+H}]^+\) and is considered as characteristic marker ion for identification of surfactins (Hue et al. 2001). The sequence derived from the fragmentation of \( m/z \) 1058.6 is: \( \beta \)-hydroxy fatty acid-Glu\(_1\)-Leu\(_2\)-Leu\(_3\)-Val\(_4\)-Asp\(_5\)-Leu\(_6\)-Leu\(_7\) (Fig. 8.10c). Similarly the peak at \( m/z \) 1036.5 and 1075.4 could be assigned as protonated \([\text{M+H}]^+\) form and potassium adduct \([\text{M+K}]^+\) of C15-surfactin with 1035 Da, respectively. The \( m/z \) 1080.5 may be attributed to sodium adduct of C15-Iturin A isoform (Pathak 2011).

Surfactin and iturin are the most commonly found antifungal lipopeptide belonging to *Bacillus* genus (Ongena and Jacques 2008). Both the lipopeptides are synthesized in a nonribosomal manner by large multienzyme complexes, exhibiting a wide antimicrobial spectrum and exceptional surfactant activities (Ongena and Jacques 2008). They share a common cyclic structure consisting of either a \( \beta \)-amino (for iturin) or \( \beta \)-hydroxy (for surfactin) fatty acid integrated into a peptide moiety. In addition, surfactins show a strong synergistic action in combination with iturin (Maget-Dana et al 1992). It has also been reported that the lipopeptides also play a role to enhance bacterial colonization on plant tissues and to induce plant resistance against pathogens (Ongena and Jacques, 2008). Consequently, a great deal of interest has centered on the use of such bioactive compounds in agriculture for eco-friendly and sustainable suppression of crop diseases (Ongena and Jacques, 2008). Thus, the feather
hydrolysates resulting from the microbial conversion of feather keratin by BA6B may represent a promising biocontrol agent against fungal phytopathogens.

8.1.7. Application of feather hydrolysate as nitrogenous soil input

The total nitrogen content of the amino acid rich feather hydrolysates resulting from the conversion of feather keratin by BA6B was found to be 0.131 g/l. Accordingly, the feather digest was concentrated five times and evaluated for its use as organic nitrogen input on the growth of V. radiate var. meha (mung). It was observed that the added feather hydrolysate exerted a beneficial effect on the germination and the growth of mung bean. Plant height, plant fresh weight, root length and root fresh weight was increased by 36%, 58%, 42%, 39%, respectively in the soil treated with feather hydrolysate over the untreated soil (Table 8.4). Further in comparison to the reference fertilizer in term of plant height and root length (Table 8.4), feather digest exhibited the same effect as that of the reference fertilizer. Similar effect of feather hydrolysate as nitrogen input has been reported by Kim et al (2005) for the growth of carrot and Chinese cabbage. Cao et al (2012) evaluated the feather fermentation broth as a leaf fertilizer and found 66 to 82% increase in pakchoi plant growth. Thus, the feather hydrolysate obtained upon decomposition of feathers by BA6B, holds potential to be applied as agriculture input (source of nitrogen and antifungal metabolites).

### Table 8.4 Effect of feather hydrolysate on growth of Vigna radiate var. meha (mung) as compared to the reference fertilizer.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Plant height (cm)</th>
<th>Plant fresh weight (g)</th>
<th>Root length (cm)</th>
<th>Root fresh weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (Control)</td>
<td>26.4 ± 0.94</td>
<td>8.7 ± 0.98</td>
<td>15.7 ± 0.41</td>
<td>4.6 ± 0.64</td>
</tr>
<tr>
<td>Feather hydrolysate</td>
<td>35.93 ± 0.69</td>
<td>13.8 ± 0.69</td>
<td>22.3 ± 0.86</td>
<td>6.4 ± 0.94</td>
</tr>
<tr>
<td>Fertilizer</td>
<td>37.32 ± 0.93</td>
<td>15.4 ± 0.52</td>
<td>23.8 ± 0.94</td>
<td>6.9 ± 0.78</td>
</tr>
</tbody>
</table>

Each value represents mean ± S.E. of 15 replicates per treatment.

8.2. Conclusion

In the present study, Bacillus amyloliquefaciens 6B isolated from feather dump soil sample was found to efficiently degrade native feather within 24 h of incubation. The rapid feather degrading ability of the bacterium makes this strain a potential tool for the development of processes suitable for conversion of feather to value added products such as feed or fertilizer. The keratinolytic protease production by B. amyloliquefaciens 6B was optimized using one-factor-at-a-time approach which resulted in 2.6-fold increase in enzyme yield. The enzyme produced was characterized and used for the
preparation of feather hydrolysate, which was evaluated as nitrogenous soil input for plant growth promotion. In addition, the strain was found to produce antifungal metabolites. Thus, the feather hydrolysate obtained upon degradation of feather by BA6B bacterial culture, may be useful as nitrogenous input for agricultural soils as well as biocontrol agent against fungal phytopathogens.
References


