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The Potential Application of Keratinase from *Bacillus* sp. as Plant Growth Promotors

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Keratinolytic *Bacillus* sp. AJ4 and *Bacillus* sp. AJ9 isolated from feather dumped soil produced keratinase of 82 U/ml (specific activity of 37.3 U/mg) and 78 U/ml (specific activity of 34.2 U/mg), respectively at pH 7 and 10. Keratinase of *Bacillus* sp. AJ4 exhibited increased stability (82-94% and ~50%) in Triton X-100 and H_2O_2. *Bacillus* sp. AJ9 keratinase was 100% stable in SDS. The rice seeds treated with feather hydrolysate showed 30% increased vigour index. Significant stability towards detergents, improved feed conversion ratio and plant growth confirms the suitability of keratinase from *Bacillus* sp. AJ4 and AJ9 for agricultural applications.

Key words*: Bacillus* sp., Keratinase, Vigour index, PGPR.

Keratins are the fibrous insoluble structural proteins of feathers, wools, scales, hairs and stratum corneum. The main characteristic of keratin is its stability provided by disulfide, hydrogen bonds, salt linkages and other cross-linkings. Feathers are composed of over 90% keratin protein and constitute up to 10% of total chicken weight, reaching more than 7.7×10^8 kg/ year as a by-product of the poultry industry. Excessive keratinous materials are discarded also from slaughter house and leather industry. Accumulation of feathers will lead to environmental pollution and feather protein wastage. The feeding of biological waste to livestock is an accepted practice and has arisen because of the necessity to reduce costs both in terms of waste disposal and meat production from livestock (Grazziotin *et al.,* 2007). After hydrolysis, the feathers can be converted to feed-stuffs, fertilizer, glues and films or amino acids such as serine, cysteine and proline (Riffel *et al.,* 2003). For this purpose, destruction of the rigid keratin structure is necessary. Generally, the feather is steam pressure cooked or chemically treated before use results in the loss of nutritionally essential amino acids, such as methionine, lysine, and tryptophane. The keratin is resistant to common proteolytic enzymes such as trypsin, pepsin and papain. Hence, feather waste can be utilized on a limited basis as a dietary protein supplement for animal feedstuffs due to its poor digestibility (Suzuki *et al.,* 2006).

The microbial degradation of feather represents an alternative technology for

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bioconversion of keratinous wastes for the nutritional enhancement and application of eco-friendly technique to overcome environmental pollution. Currently, the most promising application of keratinolytic microorganisms is the production of nutritious, cost-effective, environmentally benign protein rich feather meal for poultry and also being applied for organic farming as a fertilizer (Onifade *et al.,* 1998). Keratinases have the ability to bind and hydrolyze solid substrates like feather. This is an important property of detergent enzymes as they are required to act on protein substrates attached to solid surfaces, making them attractive additives for hard-surface cleaners. They could also help in the removal of keratinous soils that are often encountered in the laundry (Gupta and Ramnani, 2006).

The environment is the main source for new microorganisms with potential industrial and commercial value. Many research works emphasis the detergent stability and nutritive values of keratinase for its application as laundry or as feed additive and for the synthesis of organic manure (Hadas and Kautsky, 1994; Nogueira *et al.,* 2006; Grazziotin *et al.,* 2007). However, only few keratinolytic strains have been explored and experimented for their multitude industrial and agricultural applications. In this paper we presents the isolation of robust keratinolytic bacterial strains and the potential utilization of the keratinase as feed additive, detergent additive and for promoting improved plant growth.

MATERIAL AND METHODS

Isolation of keratinolytic microorganisms

Chicken feather obtained from local hen house was washed with tap water, saline solution and finally with distilled water. The washed feathers were dried overnight at 50 ºC. The whole feathers were used as such for the basal screening and production medium. The keratinolytic microorganisms were isolated from feather waste dumped soil samples collected from local poultry farm at Sivakasi, India (9º 27' 0" North, 77º 49' 0" East). Samples were serial diluted, plated on nutrient agar (Hi media, Mumbai) plate and incubated at 37 ºC for 2 d. The colonies obtained were streaked onto nutrient agar plates and the purity of the isolates was checked by microscopic examination. The pure isolates were then screened for proteolytic activity on skim milk (1% w/v) agar (Hi media, Mumbai). The proteolytic strains were analyzed for keratinolytic activities on basal feather minimal medium that contained the following constituents (g/l): NaCl (0.5), KH_2PO_4 (0.7), K_2HPO_4 (1.4), Mg SO_4 (0.1) and feathers (10), pH 7-10 incubated at 37 ºC (Wang and Shih, 1999). The selected feather degrading isolates were identified based on morphological and biochemical tests given in Bergey's manual of systematic bacteriology (Sneath, 1986). Bacterial identification was also conducted using Biochemical kit (Hi media, Mumbai).

Keratinase production

The selected strains were grown in basal feather minimal medium. Aliquots (50 ml) of basal medium were dispensed in 250 Erlenmeyer flasks and sterilized at 121 ºC for 20 min. Each flask was inoculated with 1% (v/v) inoculum of 24 h old culture prepared in nutrient broth, centrifuged and suspended in saline. The flasks were kept under shaking condition (200 rpm) at 37 ºC for 2-5 d. The culture broth was centrifuged at 5,000 g for 5 min, and the supernatant was used as a crude enzyme solution. Samples were periodically withdrawn at regular intervals and analyzed for keratinase activity. The culture supernatant was passed through Whatman no. 42 filter then through a Millipore cellulose filter (0.45 µm). The feather hydrolysate consists of the supernatant fluid of 2 d growth cultivation broth of the strains *Bacillus* sp. in 10 g /l feather medium.

Synthesis of azokeratin

Feathers used in the preparation of azokeratin were grinded to fine powder. One gram of feather was suspended in 20 ml of deionized water and 2 ml of a 2 g of $NaHCO_3$ solution were added with continuous stirring. Simultaneously, 174 mg of sulfanilic acid was dissolved in 5 ml of 0.2N NaOH and 69 mg of $NaNO_2$ were added with stirring in a separate tube. This solution was mixed with the keratin suspension. The azokeratin was suspended in water and shaken at 50 ºC for 2 h and filtered. This wash cycle was repeated until the pH of the filtrate reaches 6 to 7. Finally, the wash cycles were repeated using 50 mM potassium phosphate buffer, pH 7.5. The

azokeratin was washed once again with water and dried overnight at 50 ºC (Lin *et al.,* 1992).

Keratinase assay

The keratinolytic activity was monitored using azokeratin as substrate. Five milli gram of azokeratin was added along with 0.8 ml of 50 mM potassium phosphate buffer, pH 7.5. This mixture was agitated until the azokeratin was completely suspended and a 0.2 ml aliquot of crude enzyme was added. The reaction mixture was incubated for 15 min at 50 ºC and the reaction was stopped by addition of trichloroacetic acid to reach a final concentration of 10% (w/v). After centrifugation at 10,000 g for 10 min the absorbance of the supernatant was measured using a spectrophotometer (Hitachi U-2000, Japan) at $OD_{450\ nm}$. One unit (U) of keratinase activity was the amount of enzyme that causes a change of absorbance of 0.01 at $OD_{450\ nm}$ in 15 min at 50 ºC (Lin *et al.,* 1992). The protein content of samples was estimated by the method of Lowry et al. (1951) using bovine serum albumin as the standard.

Keratinase for dietary treatment

Bird housing

The experiment was conducted on an animal house (Ayya Nadar Janaki Ammal college, Sivakasi). All experiments were performed with 8 d of age old broiler chicken. In every pen five broiler chickens were placed. Each treatment was replicated three times with control. The birds were housed in a room with controlled temperature ventilation and lighting (16 h light/8 h dark) (Odetallah *et al.,* 2003).

Short-term plant growth test

In this experiment rice seeds were sterilized by using 0.1% (w/v) $HgCl_2$ and 70% (v/v) alcohol and washed with distilled water. The feather hydrolysate was mixed with distilled water (1:1) prior to its application. The surface sterilized seeds were soaked in feather hydrolysate and water mixed solution for 12 h. Seed germination (%) refers to percentage of feather hydrolysate or water soaked seeds germinated after 24 h of incubation at room temperature. The pregerminated seeds were transferred on pot (12 cm × 15 cm) filled with different varieties of soil (Black soil, sandy soil, red soil in ratio 2:1:2. Plants were kept under natural photoperiod (16 h light/8 h dark) in environmental conditions and irrigated with sterilized distilled water or feather hydrolysate and water mixed solution. After 14 d of incubation, seedlings were harvested. Seed germination (%), shoot length (cm) and root length (cm) were determined and vigour index was calculated. Finally, the results were compared with the control treated only with water and increased vigour index was determined.

Vigour Index = Root length + Shoot length X Germination Percentage

$$\text{Increased Vigour Index (\%)} = \frac{\text{Vigour Index of the isolates - Vigour Index of the Control}}{\text{Vigour Index of the Control}} \times 100$$

RESULTS

Identification of keratinolytic bacteria

Screenings for feather-degrading microorganisms were carried out with feather dumped soil samples. Of the 300 bacterial strains tested, 193 isolates demonstrated proteolytic activity on skim milk agar. Ten of them were able to degrade the feather in the minimal medium with initial pH 6 to 11 on 2-5 d of incubation at 37 ºC (data not shown). Two isolates having higher keratinolytic activity with complete degradation of feather within 2 d of incubation at pH 7 and 10 were selected for identification and further characterization. Based on cell morphology, colony morphology and biochemical tests the selected isolates (AJ4 and AJ9) were identified as *Bacillus* sp. further, with the variation in their carbohydrate utilization (Table 1).

Keratinase production by Bacillus sp. AJ4 and AJ9

Keratinase activity was analyzed during the cultivation of *Bacillus* sp. AJ4 an AJ9 in whole feather basal minimal medium at 37 ºC and with initial pH 6 and 11 (Fig. 1). After 24 h of growth, a significant level of keratinase (38.2 U/ml) was detected in the crude culture supernatant.

Keratinase reached a maximum activity at 48 h, coinciding with the end of the exponential phase. The *Bacillus* strains AJ4 an AJ9 produced keratinase activity of 82 U/ml (specific activity of 37.3 U/mg) and 78 U/ml (specific activity of 34.2 U/mg), respectively. On further prolonged fermentation, both the *Bacillus* strains showed decreased keratinase activity and specific activity.

Washing performance on egg yolk and blood stain

Wash performance analysis of cotton cloth with blood and egg yolk stains were conducted and the efficacy of the keratinase for use as a detergent additive was assessed (Fig. 2). The results of de-staining experiment showed the complete removal of both blood and egg yolk stains in detergent solution (Rin®) supplemented with *Bacillus* sp. AJ9 keratinase and detergent solution with both *Bacillus* sp. AJ4 and AJ9 keratinase whereas, stain was not completely removed from cloth dipped only in detergent solution.

Table 1. Morphological and biochemical characteristics of keratinolytic bacterial strains AJ4 and AJ9

Characteristics	Selected Bacterial isolates	
	AJ4	AJ9
Colony property	On nutrient agar, colonies are circular, smooth round, waxy, slight yellow to white, mucoid produces no pigment.	
Gram's stain	Positive, rod	
Spores stain	Ellipsoidal and cylindrical, central subterminal, swelling the sporangium	
Motility	Motile	
Catalase	+	+
Indole production test	+	+
Methyl red test	+	+
Voges Proskauer test	+	+
Citrate utilization	–	+
Gelatin Liquefaction	+	+
Casienase	+	+
Lysine decarboxylase	+	+
Ornithine decarboxylase	+	+
Urease	–	–
Phenyl alanine deamination	+	+
Nitrate reduction	+	+
H2S production	–	–
Adonitol fermentation	–	+
Utilization of carbon sources		
Lactose	–	+
Xylose	–	+
Maltose	+	+
Fructose	–	–
Dextrose	+	+
Galactose	–	+
Raffinose	–	–
Trehalose	+	+
Mellibiose	–	–
Sucrose	–	+
Melezitose	+	–
Sorbitol	–	–
Arabinose	–	+
Inulin	–	+
Esculin	–	–

Table 2. Effect of keratinase supplementation of millet diets on performance of broiler chick raised to 30 d of age

Treatment/days[a]	BW (g)	FC (g)	FCR
Control/15 d	3.98±0.21	25.2±0.9	6.28±0.43
Test 1/ 15 d	12.8±0.84	29.5±1.1	2.26±0.12
Test 2/ 15 d	8.0±0.65	35.4±2.3	4.37±0.28
Test 3/ 15 d	19.0±0.53	42.3±2.8	2.16±0.15
Control/30 d	23.3±0.94	55.2±3.5	2.35±0.14
Test 1/ 30 d	30.0±1.6	62.6±3.7	2.07±0.11
Test 2/ 30 d	31.6±2.2	68.5±4.1	2.11±0.14
Test 3/ 30 d	44.4±2.7	89.3±6.1	2.02±0.12

[a] The birds were fed with millet seeds treated with keratinase of *Bacillus* sp. AJ4 (1), *Bacillus* sp. AJ9 (2) and both *Bacillus* sp. AJ4 and AJ9 (3). The millet seeds fed without keratinase supplementation were taken as control experiment. After 15 and 30 d intervals the body weight (BW) g, feed consumption (FC) g were recoded and feed conversion ratio (FCR) was determined.

DISCUSSION

Keratin is an insoluble protein which is found as a major constituent of hair, nail and feathers. Several bacterial strains were isolated from natural soil to find more robust and specific keratinolytic enzymes for variety of applications (Riffel *et al.,* 2003). Upon screening bacterial strains isolated from dumped soil two *Bacillus* sp. were identified as the potential keratinolytic strain. Similarly based on phenotypic and biochemical characterization alkaline protease producing *B. licheniformis* N-2 was isolated and identified from decaying organic soil (Nadeem *et al.,* 2007). Lucas *et al*. (2003) have reported the prevalence of *Bacillus* sp. among the isolated 13 feather degrading strains. *Bacillus* sp. showed complete degradation of native chicken feather within 2 d of incubation at 37 ºC. Giongo et al. (2007) reported that keratinolytic *Bacillus* sp. isolated from Brazilian Amazon basin shows considerable degradation after 72 h of incubation. Recently Ionata et al. (2008) isolated *Clostridium sporogenes* bv. *pennavorans* from solfataric muds which degraded native feathers in 7 d. Hence the isolates *Bacillus* sp. AJ4 and AJ9 can be considered as an efficient feather degrading strains.

The *Bacillus* sp. AJ4 (at pH 7) and AJ9 (at pH 9) exhibited higher keratinase activity in feather medium where whole feather was supplied as source of nutrient suggested the neutral and alkaline keratinolytic properties of these strains. Similarly, minimal medium supplemented with whole feather is used for keratinase production by *Vibrio* sp. kr2 and *Chryseobacterium* sp. kr6 (Sangali and Brandelli, 2000; Riffel *et al.,* 2003). In the present study, *Bacillus* sp. AJ4 and AJ9 produced maximum keratinase after 48 h of growth. Similarly Lin et al. (1999) observed maximum level of keratinase production between 48 and 60 h growth of *B. licheniformis* strains. *Microbacterium* sp. kr10 produces maximum keratinase activity in raw feather medium at 36 h coinciding with the end of exponential phase (Thys *et al.,* 2004).

For the possible commercial exploitation of enzyme in detergent industry, it should be compatibility with different detergents of common use. The significant stability of keratinase from *Bacillus* sp. AJ4 and AJ9 in the detergent confirms its suitability in detergent formulations. Protease from thermophilic *Bacillus* sp. retained more than 80% and 65% of its activity after 30 min incubation

Table 3. Effect of feather hydrolysate on rice seed germination and seedling growth

Treatment[a]	Seed Germination (%)	Root Length (cm)	Shoot Length (cm)	VI	IVI (%)
Control	60	4.1±0.84	17.5±1.18	1296	0
FH AJ4	80	4.5±0.73	17.0±1.08	1720	32.7
FH AJ9	85	5.64±0.54	19.0±0.70	2094.4	61.6
FH AJ4 & FH AJ9	90	6.54±1.17	21.5±1.58	2523.6	94.7

[a]The rice seeds treated with feather hydrolysate of *Bacillus* sp. AJ4 (FH AJ4), AJ9 (FH AJ9) and both (FH AJ4 & FH AJ9) were analyzed for seed germination (%), shoot length (cm) and root length (cm) and vigour index (VI) was calculated. The control treated only with water was taken as zero and increased vigour index (IVI) (%) was determined.

at 60 ºC in the presence of the detergent brands Tide® and Cheer®, respectively (do Nascimento and Martins, 2006). Oberoi et al. (2001) observed that *Bacillus* sp. RGR-14 alkaline protease acts synergistically with the detergent to efficiently remove 46% of grass stain and 34% of blood stain.

Supplementation of keratinase incorporated diet for broiler chick showed increased body weight and feed conversion rate. The results of this experiment revealed an interesting trend in the response for the keratinase treatment. Keratinase as feed additive apparently improves the utilization of amino acids by broilers fed diets (Wang *et al.,* 2006). Similarly, it has been shown that the enzymatic treatment of feathers with keratinase improved the *in vitro* digestibility of the feather meal product was confirmed with 0.1% of the crude preparation of keratinase in the diet indeed improved the body weight, the feed conversion rate (FCR) and the general health of the young birds (Odetallah *et al.,* 2003). *B. licheniformis* PWD-1 fermentation broth with incubated feathers, the resulted feather-lysate had a high digestibility and was able to replace soy bean meal up to 7% of dietary protein (Williams *et al.,* 1991).

The feather-degrading capability of the keratinolytic bacteria accelerate the composting of chickens feather waste and convert these organic materials into high-nitrogen fertilizers. In our study ccompare with the control the seeds treated with feather hydrolysate of *Bacillus* sp. AJ4 and AJ9 showed 30% increased vigour index. Grazziontin et al. (2007) have demonstrated the strains of *Vibrio* sp. kr2 produced hydrolysate rich in soluble protein (2.5 g/l) on growing in 40, 60 or 80 g/l feathers. Similarly, the germination percentage of ryegrass increased with increasing the amount of the added alkaline hydrolysate of sheep's wool waste reaching values of 80% on day 15 (10-fold higher than that in the control sample) (Nustorova *et al.,* 2005).

CONCLUSIONS

The keratinase of isolates *Bacillus sp*. AJ4 and AJ9 are stable and compatible with commercial detergent. The plant growth promoting ability confirms the effectiveness of utilization of keratin wastes as fertilizer which would establish an economical and environmental safe method of recycling these organic materials into high-nitrogen fertilizers. Hence the keratinase of *Bacillus sp*. AJ4 and AJ9 can be exploited for industrial and agricultural applications.

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