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Exploring the Keratin Degradation Potential of *Bacillus* Isolates of Indian Soil Sites

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Keratinous waste which comprises feathers, hairs, nails, skin, wool etc creates problem of solid waste management due to presence of highly recalcitrant keratin. Many workers reported about a myriad of microorganisms, mostly fungi and bacteria, to utilize keratin, thus removing it from environment. In the present study, ten strains of *Bacillus* genera were isolated from soil samples collected from different sites. These strains were identified on the basis of different biochemical tests. Feather degradation and keratinolytic activity of these strains were carried out and soluble protein release was measured. Among these strains, *Bacillus badius* RS1 showed remarkable keratinolytic and proteolytic activity with 93.3% of feather degradation in 48 hours of incubation with significant release of soluble protein (approximately 3150 μg/ml) in the keratinase production media. This strain may be used efficiently for keratinous waste management and production of industrially important protease having keratinolytic activity even at high temperature and pH, which would be extremely useful for biotechnological process involving keratin hydrolysis or in the leather industry.

Key words: Keratinase, Solid waste management, Feather degradation, Keratinolysis, Debarbulation.

Feathers, which are produced in large amounts as waste by poultry industry, are composed of over 90% protein having keratin as main component[11]. Keratins, a major class of hard to degrade animal proteins, are constituents of vertebrate skin, nail, hair, feather, wool etc are abundant in nature but have limited uses in practice. Since they are insoluble and resistant to degradation, creating a problem of solid waste management[13]. Worldwide poultry processing plants produce million of tons of feather as a waste product annually which consists of approximately 90 % keratin[14]. The keratin is largely responsible for their high degree of recalcitrance. Little attention is given to the utilization or recycling of these wastes. The accumulation of some of these wastes in nature is considered to be a serious source of pollution and health hazards. However, they also represent a potentially valuable source of protein as animal feed stock if keratinolysis can be achieved by the enzyme[15]. Keratinases are proteolytic enzymes able to hydrolyze keratins efficiently. These are produced by microorganisms including fungi, bacteria, actinomycetes etc. and are considered important for many biotechnological applications[6].

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Keratinolytic enzymes have been studied from a variety of fungi and, to a lesser extent, bacteria. However, much current research is centered on the potential use of keratinases of bacterial origin for the industrial treatment of keratin containing compounds, e.g. serine proteases produced by *Bacillus licheniformis* PWD-1[9] and *B. licheniformis* Carlsberg NCIMB 6816[5]. Such results from the broad substrate range of these bacterial enzymes, their rates of activity towards keratin-containing compounds and their thermostability.

This study describes isolation and screening of indigenous alkali-thermophilic bacterial isolates with known proteolytic activity for keratinolysis from different soil samples.

METHOD

Isolation and screening of protease producing organisms

Different soil samples were collected and serially diluted and plated onto skimmed milk agar plates (pH 8) and kept at 60°C. Colonies exhibiting protease activity were again transferred to fresh skimmed milk agar plates and kept at 60°C. Organisms showing maximum protease activity were further checked for protease and keratinase production in cultivation media.

Characterization of Isolates

The morphological and physiological characteristics of isolates were investigated by microscopic observations and different biochemical tests. The isolates were further compared and identified according to Bergey's Manual of Determinative Bacteriology.

Collection of feathers

Chicken feathers were provided by a local poultry farm at G. B. Pant University, Pantnagar campus. It was washed twice with distilled water and dried at 60°C overnight and kept at 4°C, until used.

Culture media

Protease screening medium contained (g/l) Peptone 1.0, NaCl 5.0, Skimmed milk 20.0 and Agar 20.0 at pH 8.0. Protease production medium contained (g/l) Peptone 7.5, Glucose 5.0, 50 ml salt solution containing $MgSO_4$ 0.5%, KH_2PO_4 0.5% and $FeSO_4$ 0.01%, at pH 8.0. Keratinase production medium contained (g/l) NaCl 0.05, KH_2PO_4 0.40, K_2HPO_4 0.40, $MgSO_4$ 0.04, $FeCl_3$ 0.01 in basal salt media and native chicken feathers 10, at pH 9.0.

Enzyme production

Isolates were cultivated in 100 ml conical flask containing 50 ml cultivation media at 55°C and 150 rpm for 4 days. The crude enzyme was prepared by centrifugation at 4°C, 10,000 rpm for 20 min.

Feather degrading activity

Residual feather in the culture broth of keratinase production media was harvested by filtration through whatmann no. 1 filter paper washed twice with buffer and dried at 65°C to constant weight. The feather degradation percentage was calculated from the difference in residual feather dry weight between control (Feather without bacterial inoculation) and treated sample.

Feather degradation studies

Pattern of detachment of feather fibrils were recorded by periodic observation of the treated feathers using compound light microscope at 10Xmagnification.

Protein determination

The protein content in culture filtrate and crude enzyme (supernatant) was determined by the method of Lowry *et al.*[10].

Keratinase assay

Keratinase activity was determined by modified method of Letourneau *et al*.[8] using keratin as substrate. The keratin was suspended in Tris buffer (0.05 M, pH 9) at concentration of 4 mg/ml. The reaction mixture contained 1 ml of enzyme and 1 ml of substrate solution. The sample was incubated at 60°C for 1 h with regular shaking. After incubation, the reaction was terminated with 2 ml of 10% trichloroacetic acid (TCA) followed by centrifugation at 5000 rpm for 15 min to remove unutilized substrate. The supernatant was measured for release of azo dyes at 595 nm. A control was kept with enzyme and buffer without substrate. One unit of keratinase (1KU) was defined as the amount of enzyme required to increase 0.01absorbance between sample and control at 595 nm in an hour under the specified conditions.

Protease Assay

Proteolytic activity was assayed by modified Anson method; using 0.5% casein as substrate dissolved in 50 mM Tris buffer pH 8.0.

The reaction mixture was incubated at 60°C for 30 minutes, and reaction was stopped using 10% TCA. Tyrosine released was estimated using Folin Ciocalteau's Reagent and absorbance taken at 670 nm. One unit of protease (1 PU) was defined as amount of enzyme required to release 1 µg of tyrosine under the assay condition when reaction was incubated for 1 min.

Statistical Analysis

The analysis of data was performed with Microsoft Excel 2007 (Window XP) for mean and standard deviation. All experiments were carried out in triplicate and the results are expressed as mean ± S.D.

RESULTS

Screening and Identification

The aim of present investigation was to isolate an alkali thermophilic bacterial strain that is adapted to grow and produce keratinolytic protease using feathers as sole carbon and nitrogen source. All isolates were initially checked for proteolytic activity at 60°C and pH 8.0. Among these, ten isolates showed strong protease activity on skimmed milk agar, were further checked for feather degradation and keratinolytic activity as well as protease activity.

On the basis of morphological and biochemical characteristics of isolates, these were identified to be of genus *Bacillus* and the results were summarized in Table 1 and 2.

Feather Degrading Activity

Among all the strains, *B. badius* RS1 showed feather degradation of 93.3% with better soluble protein content in culture filtrate i.e. 3150 µg/ml with in 48 hrs. of incubation (Figs. 1 & 2). Maximum degradation of feather was shown by *B. stearothermophilus*. VSAP2 i.e. 95.5% with soluble protein content in culture filtrate to be 2890 µg/ml (Table 3). Nagal *et al.*[11] reported 78.16 % degradation whereas El-Refai *et al.*[4] reported 87.2 % degradation in *Bacillus* strains previously in the same media. The use of enzymatic and or microbiological methods to hydrolyze chicken feathers is an attractive alternative when compared to currently used methods of feather meal preparation using high temperature and pressure treatments[2,15]. The ability of these strains to grow using feathers as sole nitrogen and carbon source could offer tremendous potential for the development of biotechnological methods for the hydrolysis of feathers.

Keratinase Production

The culture supernatant of keratinase production media after centrifugation was used as crude enzyme for keratinase assay. The highest keratinase activity was found in *B.badius* RM4 (29 U/ml) followed by *B. stearothermophilus* VSAP2 (27 U/ml) and RS1 (25 U/ml). All strains showed maximum keratinase activity in 48 h of incubation which was decreased when incubated further. The loss of enzyme activity beyond 48 h incubation was probably due to enzymatic autolysis or end product inhibition[16]. The production of extracellular keratinase during growth of keratinophilic microorganisms is well established[12]. The results were compiled in Table 3.

Protease Production

The culture supernatant of protease

Table 1. Bacterial strains screened for extracellular keratinolytic protease

Strain	Source
Bacillus badius RS1	Soil from compost dump, Rajasthan, India
Bacillus badius RS4	Soil from compost dump, Rajasthan, India
Bacillus badius RS6	Soil from compost dump, Rajasthan, India
Bacillus stearothermophilus RS11	Soil from compost dump, Rajasthan, India
Bacillus badius VSAP1	Soil from N E Borlaug CRC, Pantnagar, Uttrakhand, India
Bacillus stearothermophilus VSAP2	Soil from N E Borlaug CRC, Pantnagar, Uttrakhand, India
Bacillus megaterium VSAP7	Soil from N E Borlaug CRC, Pantnagar, Uttrakhand, India
Bacillus stearothermophilus LI1	Leather industry waste, Kanpur, India
Bacillus badius LI3	Leather industry waste, Kanpur, India
Bacillus badius RM4	Soil from rice mill, Kashipur, India

production media after centrifugation was checked for proteolytic activity, which showed highest proteolytic activity of 14.60 U/ml by *B. badius* RS4 followed by *B. badius* RS1 (12.46 U/ml) and *B. badius* RM4 (11.80 U/ml). Maximum proteolytic activity was observed after 48 hours of incubation with all strains. The results were shown in Table 4.

Table 2. Comparison of morphological and biochemical characters of *Bacillus* strains isolated from different sources

Characteristics	*Bacillus* strains									
	RS1	RS4	RS6	RS11	VSAP1	VSAP2	VSAP7	LI1	LI3	RM4
Morphology	rods	Rods	rods	rods	rods	rods	rods	rods	rods	rods
Gram staining	+	+	+	+	+	+	+	+	+	+
Endospores	+	+	+	+	+	+	+	+	+	+
Growth on MacConkey agar	-	-	-	-	-	-	-	-	-	-
Growth in Carbohydrates										
-Glucose	+	+	+	+	+	-	+	+	+	+
Arabinose	-	-	+	+	+	-	-	-	-	-
Adonitol	-	-	-	-	-		-	-	-	-
Lactose	-	-	-	-	-	-	-	-	-	-
Sorbitol	-	-	-	-	-	-	-	-	-	-
Citrate utilization	-	-	-	-	-	-	+	-	-	-
Lysine decarboxylase	+	+	+	+	+	+	+	+	+	+
Ornithine decarboxylase	+	+	+	+	+	+	+	+	+	+
Phenylalanine deamination	-	-	-	+	-	-	-	-	-	-
H2s production	+	+	-	-	-	-	-	-	-	-
Hydrolysis of-Starch	+	+	+	+	+	+	+	+	+	+
Gelatin	+	+	+	+	+	+	+	+	+	+
Tween20	+	+	+	+	+	+	+	+	+	+
Tween80	+	+	+	+	+	-	-	+	-	+
Catalase	+	+	+	+	+	+	+	+	+	+
Oxidase	+	+	-	-	-	-	+	+	+	-
Nitrate reduction	+	+	+	+	+	+	+	+	+	+
Urease	-	+	-	-	-	-	-	-	-	-
Methyl red	+	+	+	+	+	+	+	+	+	+
Voges-poskeur	-	-	-	-	-	-	-	-	-	-

Table 3. Keratinolytic activity of *Bacillus* strains isolated from soil samples

S. No.	Organism	Feather-degradation %	Final pH	Culture filtrate protein content (µg/ml)	Keratinase activity (U/ml)
1.	*Bacillus badius* RS1	93.3±0.12	8.93±0.02	3150±6.24	25±0.10
2.	*Bacillus badius* RS4	80.0±0.05	8.88±0.01	3200±2.00	22±0.35
3.	*Bacillus badius* RS6	77.0±0.04	8.87±0.01	2700±10.00	15±0.43
4.	*Bacillus stearothermophilus* RS11	84.0±0.03	8.84±0.01	2950±7.00	17±0.26
5.	*Bacillus badius* VSAP1	86.6±0.06	8.88±0.00	2880±7.21	21±0.26
6.	*Bacillus stearothermophilus* VSAP2	95.5±0.77	8.91±0.02	2890±2.64	27±0.17
7.	*Bacillus megaterium* VSAP7	73.3±0.05	8.76±0.01	2590±4.35	24±0.1
8.	*Bacillus stearothermophilus* LI1	68.8±0.04	8.65±0.01	2400±11.35	10±0.43
9.	*Bacillus badius* LI3	57.7±0.02	8.70±0.03	2380±7.81	21±0.52
10.	*Bacillus badius* RM4	86.6±0.04	8.98±0.03	2950±5.19	29±0.26

Table 4. Proteolytic activity of *Bacillus* strains isolated from soil samples

S. No.	Organism	Supernatant Protein Content (µg/ml)	Proteolytic unit (U/ml)
1	*Bacillus badius* RS1	1658±9.80	12.46±0.32
2	*Bacillus badius* RS4	2162±7.25	14.60±0.41
3	*Bacillus badius* RS6	2080±6.74	12.20±0.28
4	*Bacillus stearothermophilus* RS11	1560±3.45	10.16±0.24
5	*Bacillus badius* VSAP1	1680±6.50	10.20±0.36
6	*Bacillus stearothermophilus* VSAP2	1620±6.52	11.66±0.55
7	*Bacillus megaterium* VSAP7	1580±4.83	11.80±0.10
8	*Bacillus stearothermophilus* LI1	1480±7.80	10.86±0.64
9	*Bacillus badius* LI3	1520±6.22	9.60±0.31
10	*Bacillus badius* RM4	1560±7.23	11.80±0.20

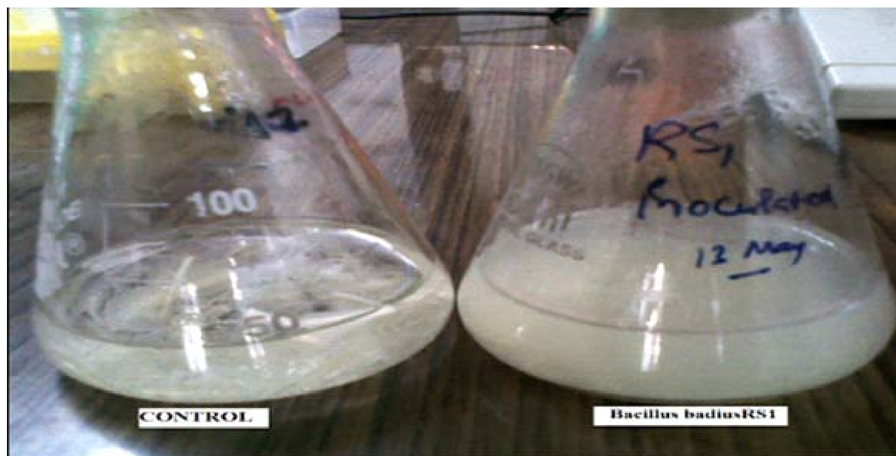

Fig. 1. Complete feather degradation by *Bacillus badius* RS1 with 48 hours incubation

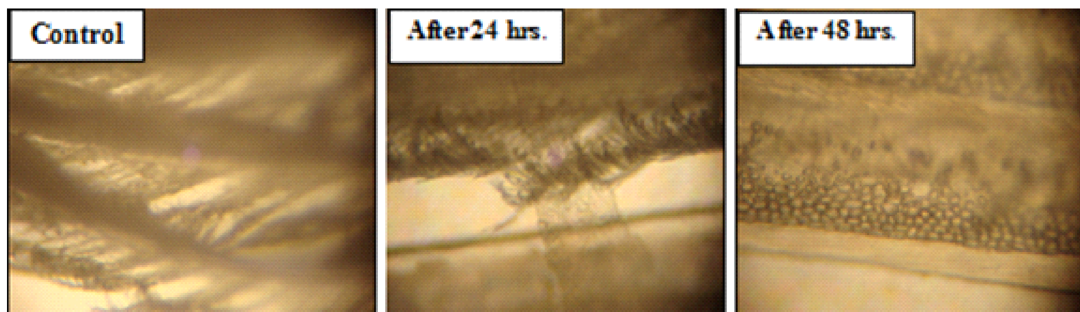

Fig. 2. Feather degradation as observed under 10x magnification. Disintegrated barbules after 24 hrs. and complete debarbulation after 48 hrs. can be clearly observed

CONCLUSION

Currently over 30% of total industrial enzyme market is accounted by proteases used for detergent, leather tanning, and food application[3]. In the production of industrial enzymes, upto 30-40% of the production cost is accounted for by the growth substrate used in the production media[7]. Therefore, the use of feather, a cheap and readily available substrate, could result in low cost of enzyme production. So these *Bacillus* strains are potential keratinolytic strains which are suitable for tackling hard to degrade keratin wastes. Protease having keratinolytic property from these strains are active at alkaline pH and high temperature which meets the requirement of various above discussed industries. Also, keratinase from *Bacillus* strains VSAP2, RS1, RM4 have considerable potential in biotreatments particularly for dehairing and bating in leather industry which causes sulphide pollution. Thus, the isolation and cloning of protease gene from these promising strains can also gives a way for better yield and improved versions of keratinase with desired quality to serve industrial requirements in future.

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