Proc. Natl. Acad. Sci., India, Sect. B Biol. Sci. (July–Sept 2019) 89(3):1079–1086
https://doi.org/10.1007/s40011-018-1026-5

RESEARCH ARTICLE

Characterization of *Bacillus* Species with Keratinase and Cellulase Properties Isolated from Feather Dumping Soil and Cockroach Gut

Sonika Sharma[1] · Rajesh Kumar Prasad[1] · Soumya Chatterjee[1] · Angkita Sharma[1,2] · Mohan G. Vairale[1] · Kamlesh Kumar Yadav[1,3]

Received: 17 January 2018 / Revised: 25 June 2018 / Accepted: 28 August 2018 / Published online: 10 September 2018

Abstract The present study was conducted with an objective to isolate and screen bacteria with dual characteristics of cellulase and keratinase production from soil of feather dumping site and from the gut of cockroaches which inhabit these sites. By using standard microbiological protocols and techniques, 13 bacteria (K1–K13) were isolated from soil samples and 18 isolates from cockroach gut (K14–K31), and further these isolates were screened for keratinase activity. The positive isolates with keratinase activity were subsequently screened for cellulase production. The enzymatic activity of both keratinase and cellulase was quantified followed by characterization and identification of these positive isolates through 16S rRNA genetic region sequencing. The isolate showing maximum keratinase and cellulase enzyme activity was selected and tested for degradation potential using animal skin with hairs as a natural keratin. The present study demonstrates the waste management ability of *Bacillus* sp., with dual characteristics of cellulase and keratinase production isolated from cockroach gut.

Keywords Cellulase activity · Hydrolysis capacities · Waste management · 16S rDNA · Enzyme

Sonika Sharma and Rajesh Kumar Prasad have contributed equally.

Significant Statement The work describes isolation and characterization of culturable bacteria from soil of feather dumping site and from cockroach gut inhabiting at feather dumping site. The present finding suggests the potential ability of *Bacillus* sp., with dual characteristics of cellulase and keratinase in waste management.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s40011-018-1026-5) contains supplementary material, which is available to authorized users.

✉ Sonika Sharma
Sonika.snk07@gmail.com

[1] Defence Research Laboratory, DRDO, Tezpur, Assam 784001, India

[2] Present Address: Centre for Cellular and Molecular Biology, Amity Institute of Biotechnology, Amity University, Noida, U.P, India

[3] Present Address: Gangabishan Bhikulal Investment and Trading Limited (GBIT), Jalna-Aurangabad Road, Jalna 431 203, Maharashtra State, India

Introduction

Cellulose and keratin are the two most abundant biopolymers in nature, providing structural stability to living creatures. While origin of cellulose is mainly from plants, keratin is of animal origin. Cellulose, a linear polysaccharide having thousands of β-D-anhydroglucopyranose units linked by β(1,4)-glycosidic bonds, is chiral and insoluble in water, whereas keratin is a fibrous and recalcitrant protein constituting major structural component of feather, horns, hooves, skin, hair, etc [1]. Common protease cannot degrade keratin due to its intense cross-linking of cysteine disulfide bonds, hydrogen bonds and hydrophobic interactions [2]. However, being highly abundant components, accumulation of cellulose and keratin-based organic wastes is common due to natural and anthropogenic activities. Pollution and related health problems are thus apparent in the areas like feather dumping sites (slaughter houses). Further, urban garbage dumping grounds receive a mixture of both cellulose- and keratin-containing wastes. Therefore, microbial application to biodegrade such composite material through microbial enzymes is a challenge to the scientific community as typically same bacteria does not act upon both the polymers. Physical separation is a

cumbersome task for the cellulose–keratin mixed wastes and conventional methods for degrading them involve combustion and chemical treatments [3]. Approximately 20% of total insects harbor intracellular endosymbiotic bacteria in their gut, and in turn, these endosymbionts help in the digestion of food and supply essential nutrients to their host [4]. Both cellulose- and keratin-degrading bacteria are reported to be present naturally in the gut of different organisms [5, 6]. According to the feeding habits, gut of different insects like moths (Tineidae), chewing lice (Mallophaga), *Dermestidae trogidae* and Scarabaeidae beetles harbor keratin-degrading bacteria [7]. Furthermore, cockroaches (*Periplaneta* sp. order: Blattaria or Blattodea) are significant omnivores and have diverse hindgut microbiota encompassing hundreds of microbial species. [8, 9]. Moreover, due to its unique niche, the gut of cockroach is likely to be the habitat for unique bacteria, degrading different substrates like cellulose and keratin. The present study deals with the screening and characterization of bacteria collected from the gut of cockroach inhabiting a feather dumping site at Tezpur area, Assam, India. Associated soil samples were also collected from the same feather dumping site where cockroaches were collected, to assess the possibility of finding bacteria having dual nature of both cellulose and keratin degradation properties. All the strains were assessed for their caseinase and keratinase activities and filter paper assay (for total cellulase activity and endoglucanase activity). Additionally, dehairing properties of keratinase positive isolates were also studied. The present work will be helpful in developing microbial enzyme-based ecofriendly strategy for management of complex urban waste.

Material and Methods

Sample Collection and Isolation of Bacteria from Collected Sample

Soil samples from feather dumping site (slaughter houses) at Tezpur, Assam (26°39′4.3848″N and 92°47′1.7268″E), were collected from the top surface (up to 3 cm) having feather waste. Simultaneously, cockroach specimens (*Periplaneta americana*) from the same site were collected in live condition. Subsequently, insect specimens were knocked out by chloroform and surface-sterilized by ethanol, gut was dissected out, and isolation of culturable bacteria was done according to method described previously [10, 11]. On the other hand, soil sample (1.0 g) was suspended in 9 ml sterilized sterile PBS, and culturable bacterial colonies were isolated in nutrient agar [12].

Protease Activity and Caseinase Activity (Primary Screening)

Protease-secreting bacteria were screened by using skim milk agar medium (HiMedia, M530-500G) from collected samples. The index of hydrolysis of clear zone was recorded and calculated as ratio of diameter of clearing zone and colony [11, 12]. In addition, caseinase activity was determined by modified protocol of El-Ayouty et al. [13]. Further, the amino acid released due to breakdown of casein was estimated by the method of Lowry et al. [14] with bovine serum albumin as a standard. One unit of caseinase activity was defined as the amount of enzyme that liberates 1 µM of tyrosine per hour under optimal assay conditions.

Secondary Screening of Keratinolytic Bacteria and Keratinase Estimation

Proteolytic activity positive isolates were then secondary-screened for keratinolytic activity. For this, feathers were collected from dumping site and processed according to Sekar et al. [12]. Bacterial preparations (2×10^7 cells/ml) were incubated with single feather (carbon and nitrogen source for keratinase production) in test tubes containing 10 ml of minimal media (gm/l) modified basal salt medium according to Burtt and Ichida [15], at 37 °C on a rotary shaker at 160 rpm for 7 days. Degradation of feather was examined visually. After incubation, enzyme extraction was performed by centrifugation of the media at 2000 *g* for 20 min at 4 °C and the supernatant was used to estimate keratinase activity. For keratinase estimation, keratin powder was used according to Mazotto et al. [16]. One unit (U/ml) of keratinase activity was defined as the amount of enzyme required to produce an absorbance increase of 0.01 under the described assay conditions.

Determination of Feather Degradation

For feather degradation assay, chicken feather was washed with distilled water, surface-sterilized with 70% ethanol and rinsed twice with distilled water, followed by drying at 45 °C for 24 h in circulating hot air oven. Feather samples were then incubated with isolates (2×10^7 cells/ml) at 37 °C. After 7 days, the total culture was filtered through Whatman No. 3 filter paper, washed twice with distilled water and dried to a constant weight at 105 °C. The percentage degradation was calculated according to the method described in Kani et al. [17].

Dehairing Function of Keratinolytic Positive Isolate

Dehairing capability of the bacterial isolates was performed according to Jaouadi et al. [3] with slight

modification. Sterilized bovine, goat and rat skin samples were inoculated with 2×10^7 cells/ml in modified basal salt medium containing: (g/l) 0.25, Na_2HPO_4; 0.5, NaH_2PO_4; 0.5, NaCl; 0.01, $MnSO_4$; 0.01, $FeSO_4$; 0.5, $MgSO_4$; 2.0, gelatine; and 1 l distilled water, pH adjusted to 7.0), and the results were recorded according to Jaouadi et al. [3].

Screening of Cellulose-Degrading Bacteria (CMCase Activity Assay)

Qualitative estimation for cellulase positive bacterial isolates was conducted using carboxymethyl cellulose media (CMC) plates, according to Sharma et al. [10]. Isolates capable of degrading CMC were indicated by a clear zone on CMC plates, and the enzyme activity was indexed by measuring diameter of clear zone. Further, hydrolysis activities (HC) of the positive isolates were calculated according to Hendricks et al. [18].

Filter Paper Assay (Total Cellulase) Activity and Endoglucanase Activity

The cellulase activity was estimated according to protocol described by International Union of Pure and Applied Chemistry (IUPAC) [19]. Cellulase activity was expressed in filter paper unit (FPU) per ml of undiluted culture filtrate. One FPU is defined as the quantity (in mg) of reducing sugar liberated in one hour by undiluted enzyme under the standard assay conditions and calculated according to Ghose [19]. Endoglucanase (β 1-4 endoglucanase-EC 3.2.1.4) activity was assayed by measuring the amount of reducing sugar from CMC. Enzyme activity was estimated in the cellulase positive isolates according to Ghose [19]. One unit of enzymatic activity is defined as the amount of enzyme that releases 1 µmol reducing sugars (measured as glucose) per ml per minute.

Carbohydrate Utilization Pattern

Carbohydrate utilization pattern was performed using the HiCarbo test kit (HiMedia, India). Briefly, 50 µl of freshly prepared culture was added to the test modules, followed by incubation at 37 °C for 24 h, and interpretation of results was done on the basis of color change, as described in the manufacturer's instruction.

Amplification and Identification of Bacterial Isolates by 16S rRNA Gene Analysis

Total bacterial genomic DNA from each positive isolate was extracted and 16S rRNA genetic region was amplified using universal primers—16S1 5′-gagtttgatcctggctca-3′ (forward) and 16S2 5′-cggctaccttgttacgactt-3′ (reverse) [20]. Amplicons were purified (Sigma-Aldrich, MO, USA) and sent for sequencing in a commercial facility (Sci-Genom Labs, Kochi, India). The obtained sequences were submitted to the NCBI GenBank and are available under the accession nos. KY619843 through KY619856.

Results and Discussion

A total of 31 culturable bacterial isolates from feather dumping site and cockroach gut were screened for the dual characteristics of keratinase and cellulase production. Out of the studied isolates, 14 showed proteolytic activity on skimmed milk agar. Isolate numbers 4K, 7K, 9K, 11K,

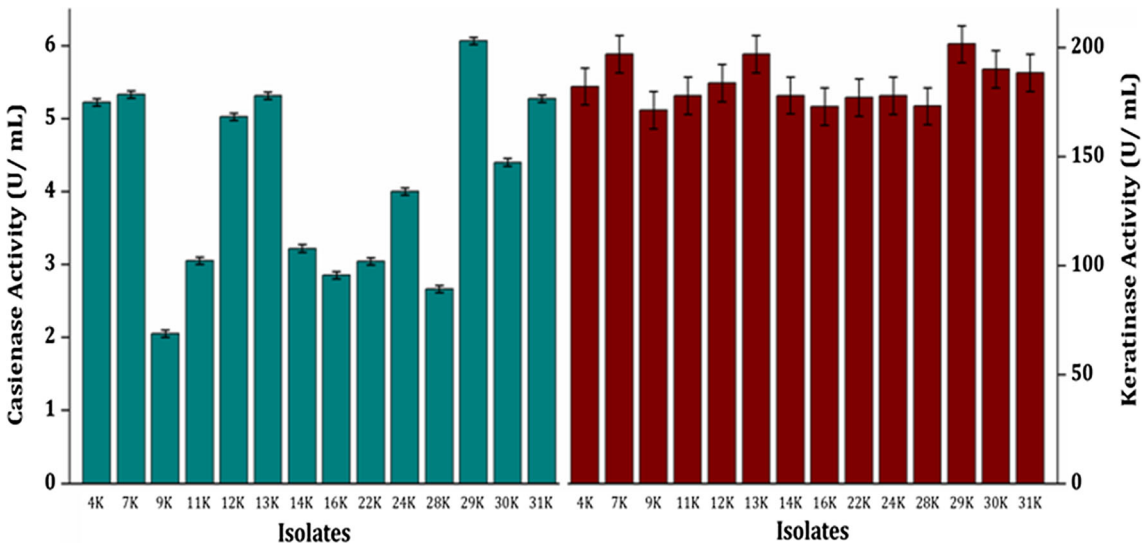

Fig. 1 Caseinase activity and keratinase activity estimated in different isolates

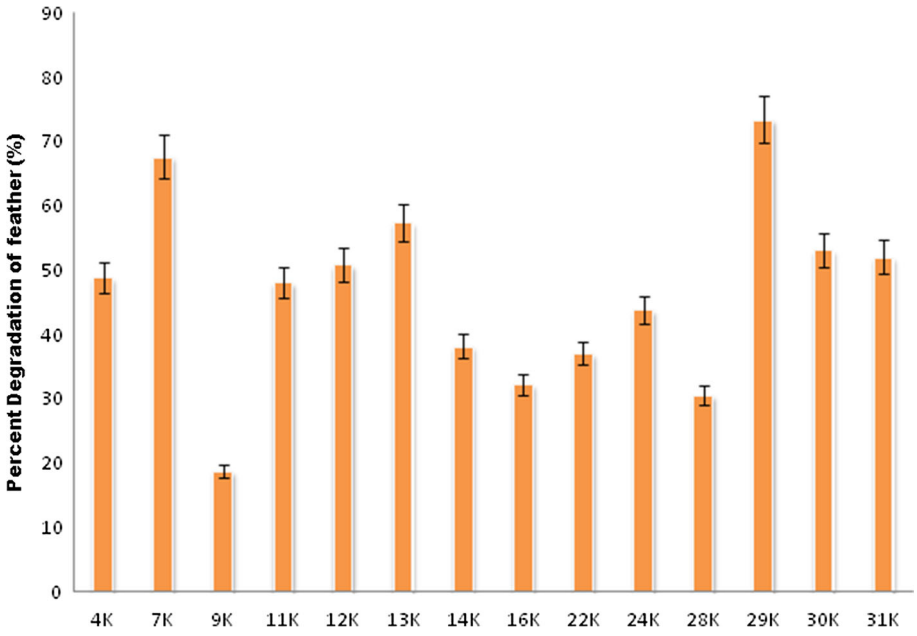

Fig. 2 Percent degradation of feather by different keratinase positive isolates

12K, 13K, 14K, 16K, 22K, 24K, 28K, 29K, 30K, 31K showed breakdown of casein into constituent peptides and amino acids in the presence of exoenzyme caseinase, as evident by disappearance of the white color of skim milk media (Fig. S1 and Fig. S2). Varying levels of caseinase activity were exhibited by different isolates, ranging from 2.05 U/ml (isolate 9K) to 6.06 U/ml (isolate 29K) (Fig. 1). Similarly, primary screening of keratin-degrading bacteria of *Bacillus* species (*Bacillus megaterium* F7-1 and *Bacillus* sp. CL33A) was carried out on skim milk agar plates [21, 22]. Besides, caseinase positive isolates were analyzed for keratin degradation by keratinase production and proteolytic activity on feather degradation. Keratinase activity was estimated in different isolates, and the highest activity (201.4 U/ml) was documented in isolate 29K from cockroach gut, 197 U/ml in isolate 13K from soil and 171.1 U/ml in isolate from soil 9K (Fig. 1). Feather degradation potential was also analyzed by calculating percent degradation by different isolates. Nearly complete degradation of feather was visible in 7 days (Figs. 2, 3), and maximum degradation efficiency was reported in isolate 29K with 73.4% degradation; however, the feather, barbules and rachises were found completely degraded and settled at the bottom (Fig. 3). Similar keratinolytic activity

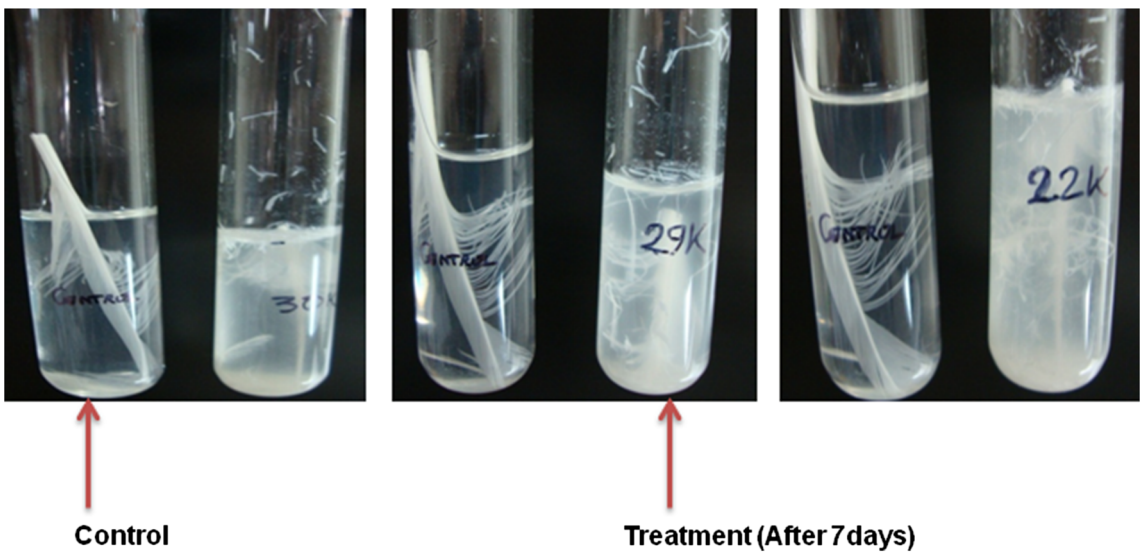

Fig. 3 Feather degradation by keratinolytic bacteria after 7 days of incubation

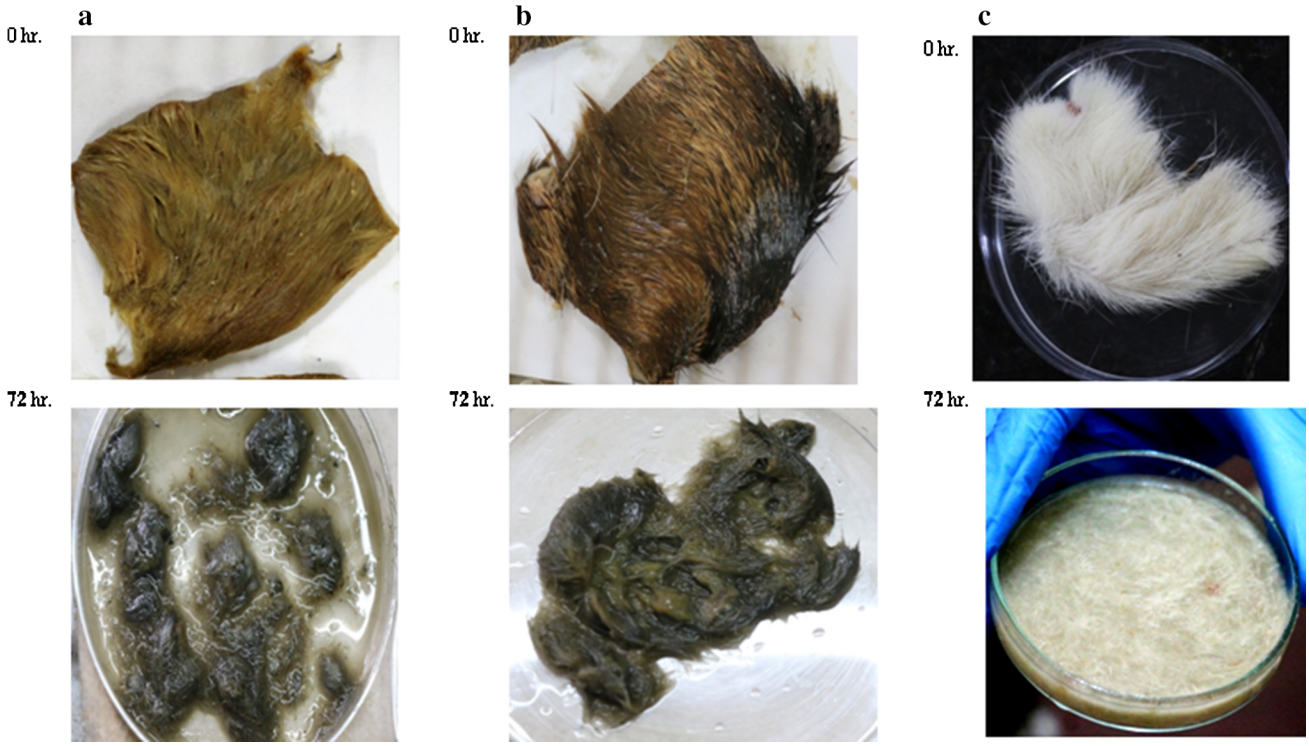

Fig. 4 Dehairing activity and degradation potential of 29K after 72 h. **a** Bovine skin. **b** Goat skin. **c** Rat skin

in bacterial isolates from feather dumping sites has been reported earlier [12, 23]. Recently, researchers have reported feather degradation by *Vibrio* sp. Strain kr2 [23], *Bacillus* sp. Strain kr16 [24] and *Chryseobacterium* sp. Strain kr6 [25].

Based on the keratinase enzyme activity, degradation potential of 29K was evaluated using natural keratin source including bovine, goat and rat skin with hairs. Incubation with isolate 29K (*Bacillus* sp.) resulted in notable digestion of skin. After 24 h of incubation, dislodging of hairs from rat and goat skin was observed, whereas dehairing of bovine skin took 48 h (Fig. 4). Nevertheless, after 72 h of incubation, all the treatments resulted in the total digestion of skin with partial degradation of hairs. Comparatively similar result on dehairing capacity of *B. pumilus* has been reported by Hunag et al. [26]. Remarkably, 16S rRNA gene sequences revealed keratinase positive isolates as different species of bacteria belonging to the genus *Bacillus*. Interestingly, isolates 22K and 24K showed maximum sequence homology with *Bacillus cereus* strain G5 while isolates 30K and 31K with *Bacillus kochii* strain You8, although the enzyme activities were diverse.

In addition to keratin waste, cellulose waste equally creates aesthetic nuisance due to its slow degradation in nature [27]. To find an enzyme-based approach, keratinolytic positive isolates were further screened for cellulase production and among them 8 isolates (4K, 7K, 12K, 13K, 28K, 29K, 30K, 31K) were found positive on CMC media plates (Fig. S3). Maximum hydrolysis capacity was noted in isolate 29K (3.6 mm), whereas isolates 9K, 11K, 14K, 16K, 22K, 24K did not show notable activity (Figures S3, S4). For isolate 29K, enzyme activity for total cellulase activity was recorded to be 0.11 U/ml, while endoglucanase activity was assayed to be 0.39 U/ml (Fig. 5). Cellulolytic bacteria in our ecosystem play a significant role to degrade cellulose based waste [28]. The present observations are similar to a previous study reporting isolation of cellulolytic bacteria from termite mid-gut [10]. Carbohydrate utilization is one of the important metabolic activities attributed to the cellulase producing bacteria. In the present study, cellulase positive bacteria showed

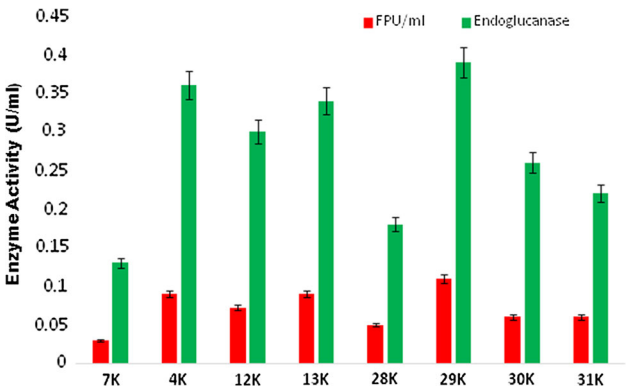

Fig. 5 Filter paper assay (total cellulase) activity and endoglucanase activity of cellulase positive isolates

Table 1 Carbohydrate utilization pattern of cellulase positive isolates from soil of feather dumping site and cockroach from same site

S. No	Test	4K	7K	12K	13K	28K	29K	30K	31K
1	Lactose								
2	Xylose								
3	Maltose								
4	Fructose								
5	Dextrose								
6	Galactose								
7	Raffinose								
8	Trehalose								
9	Melibiose								
10	Sucrose								
11	L-Arabinose								
12	Mannose								
13	Inulin								
14	Sodium gluconate								
15	Glycerol								
16	Salicin								
17	Dulcitol								
18	Inositol								
19	Sorbitol								
20	Mannitol								
21	Adonitol								
22	Arabitol								
23	Erythritol								
24	α-Methyl-D-glucoside								
25	Rhamnose								
26	Cellobiose								
27	Melezitose								
28	α-Methyl-D-mannoside								
29	Xylitol								
30	ONPG								
31	Esculin hydrolysis								
32	D-Arabinose								
33	Citrate utilization								
34	Malonate utilization								
35	Sorbose								

Red represents negative reaction

Green represents positive reaction

response to different carbohydrates, which are listed in Table 1. All the cellulase positive isolates shared similar biochemical traits for the utilization of dextrose, salicin, mannitol and esculin sugars; however, inability to utilize α-methyl-D-mannoside was observed in all the isolates. All were able to utilize D-cellobiose except 12K, to ferment glucose. Despite sharing common genus, the isolates have shown different biochemical profile. The differences in substrate utilization suggest species diversity among the same genus. Previous studies have shown that *Bacillus* species having potential hydrolytic activity with high degradation index isolated from different sources [3, 10, 11, 25, 29, 30].

Conclusion

The present results show that the feather dumping site and cockroach gut are an excellent source of microbes having potent keratinase and cellulase activities, which can be exploited for various biotechnological applications. Remarkably, in this study, eight isolates from cockroach gut show positive keratinolytic activity, which corroborates with their extremely wide range of food habit, as well its adaptability in extreme environments. The present findings further demonstrate the catabolic activity of isolate 29K (*Bacillus* spp.) against keratin and cellulose and thus represent a potent candidate species for application in management of wastes disposed from slaughter houses and tanneries. In conclusion, formulation of different potential hydrolytic bacteria having cellulase and keratinase activities may provide an efficient ecofriendly solution to the global problem of waste management.

Acknowledgements The authors thankfully acknowledge the support of Defence Research & Development Organization, Ministry of Defence, for intramural research funds. They also extend their sincere thanks to Dr. Sibnarayan Datta, Scientist, DRL and Dr. Yangchen Bhutia, Scientist, DRL, Dr. Narendra Kumar, DAVV & Director Defence Research Laboratory, Tezpur, for their kind support and help in manuscript preparation.

Compliance with Ethical Standards

Conflict of interest The authors declare no conflict of interest to publish this manuscript.

References

1. Russell JB, Muck RE, Weimer PJ (2008) Quantitative analysis of cellulose degradation and growth of cellulolytic bacteria in the rumen. FEMS Microbiol Ecol 67:183–197
2. Williams CM, Richter CS, Mackenzie JM, Jason JR, Shih CH (1990) Isolation, identification and characterization of a feather-degrading bacterium. Appl Environ Microbiol 56:1509–1515
3. Jaouadi NZ, Rekik H, Badis A, Trabelsi S, Belhoul M et al (2013) Biochemical and molecular characterization of a serine keratinase from *Brevibacillus brevis* us575 with promising keratin-biodegradation and hide-dehairing activities. PLoS ONE 8:e76722. https://doi.org/10.1371/journal.pone.0076722
4. Feldhaar H, Straka J, Krischke M, Berthold K, Stoll S, Mueller MJ, Roy G (2007) Nutritional upgrading for omnivorous carpenter ants by the endosymbiont Blochmannia. BMC Biol 5:48
5. Kim D, Kim SN, Baik KS, Park SC, Lim CH, Kim JO, Shin TS, Oh MJ, Seong CN (2011) Screening and characterization of a cellulase gene from the gut microflora of abalone using metagenomic library. J Microbiol 49:141–145
6. Han M, Luo W, Gu Q, Yu X (2012) Isolation and characterization of a keratinolytic protease from a feather-degrading bacterium *Pseudomonas aeruginosa* C11. Afr J Microbiol Res 6:2211–2222
7. Lima TA, Pontual EV, Dornelles LP, Amorim PK, Araújo SR, Coelho LCBB, Napoleão TH, Paiva PMG (2014) Digestive enzymes from workers and soldiers of termite *Nasutitermes corniger*. Comput Biochem Physiol B 176:1–8
8. Chamavit P, Sahaisook P, Niamnuy N (2011) The majority of cockroaches from the Samutprakarn province of Thailand are carriers of parasitic organisms. EXCLI J 10:218–222
9. Tinker KA, Ottesen EA (2016) The core gut microbiome of the American cockroach, *Periplaneta americana*, is stable and resilient to dietary shifts. Appl Environ Microbiol 82:6603–6610
10. Sharma S, Chatterjee S, Datta S, Prasad RK, Sharma A, Dubey D, Vairale MG, Veer V (2015) Isolation and characterization of cellulose degrading bacteria of termite gut from north eastern region of India. South Asian J Exp Biol 5:283–290
11. Gupta P, Samant K, Sahu A (2012) Isolation of cellulose-degrading bacteria and determination of their cellulolytic potential. Int J Microbiol. https://doi.org/10.1155/2012/578925
12. Sekar V, Kannan M, Ganesan R, Dheeba B, Sivakumar N, Kannan K (2014) Isolation and screening of keratinolytic bacteria from feather dumping soil in and around Cuddalore and Villupuram, Tamil Nadu. Proc Natl Acad Sci India Sect B Biol Sci 86:567–575
13. El-Ayouty YM, EL-said A, Salama AM (2012) Purification and characterization of a keratinase from the feather-degrading cultures of *Aspergillus flavipes*. Afr J Biotechnol 11:2313–2319
14. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the folin phenol reagent. J Biol Chem 193:265–275
15. Burtt EH, Ichida M (1999) Occurrence of feather degrading Bacilli in the plumage of birds. Auk 116:364–372
16. Mazotto AM, de Melo ACN, Macrae A, Rosado AS, Peixoto R, Cedrola SML, Couri S et al (2011) Biodegradation of feather waste by extracellular keratinases and gelatinases from *Bacillus* spp. World J Microbiol Biotechnol 27:1355–1365
17. Kani PT, Subha K, Madhanraj P, Senthilkumar G, Panneerselvam A (2012) Degradation of chicken feathers by *Leuconostoc* sp. and *Pseudomonas microphilus*. Euro J Exp Biol 2:358–362
18. Hendricks CW, Doyle JD, Hugley B (1995) A new solid medium for enumerating cellulose-utilizing bacteria in soil. Appl Environ Microbiol 61:2016–2019
19. Ghose TK (1987) Measurement of cellulase activities. Pure Appl Chem 59:257–268
20. Alam SI, Dixit A, Reddy GSN, Dube S, Palit M, Shivaji S, Singh L (2006) *Clostridium schirmacherense* sp. nov., an obligately anaerobic, proteolytic, psychrophilic bacterium isolated from lake sediment of Schirmacher Oasis, Antarctica. Int J Syst Evol Microbiol 56:715–720
21. Son HJ, Kim YG, Park YK (2004) Isolation and identification of feather-degrading bacteria for biotechnological applications of keratinaceous protein waste. J Life Sci 14:229–234

22. de Oliveira CT, Pellenz L, Pereira JQ, Brandelli A, Daroitet DJ (2016) Screening of bacteria for protease production and feather degradation. Waste Biomass Valor 7:447–453
23. Grazziotin A, Pimentel FA, Sangali S, de Jong EV, Brandelli A (2007) Production of feather protein hydrolysate by keratinolytic bacterium *Vibrio* sp. kr2. Bioresour Technol 98:3172–3175
24. Werlang PO, Brandelli A (2005) Characterization of a novel feather-degrading *Bacillus* sp. strain. Appl Biochem Biotechnol 120:71–79
25. Riffel A, Lucas F, Heeb P, Brandelli A (2003) Characterization of a new keratinolytic bacterium that completely degrades native feather keratin. Arch Microbiol 179:258–265
26. Huang Q, Peng Y, Li X, Wang H, Zhang Y (2003) Purification and characterization of an extracellular alkaline serine protease with dehairing function from *Bacillus pumilus*. Curr Microbiol 46:169–173
27. Chatterjee S, Sharma S, Prasad RK, Datta S, Dubey D, Meghvansi MK, Vairale MG, Veer V (2015) Cellulase enzyme based biodegradation of cellulosic materials: an overview. South Asian J Exp Biol 5:271–282
28. Zhao XH, Wang W, Wei DZ (2013) Identification of *Petriella setifera* LH and characterization of its crude carboxymethyl cellulase for application in denim biostoning. J Microbiol 51:82–87
29. Jaouadi NZ, Jaouadi B, Aghajari N, Bejar S (2012) The overexpression of the SAPB of *Bacillus pumilus* CBS and mutated sapB-L31I/T33S/N99Y alkaline proteases in *Bacillus subtilis* DB430: new attractive properties for the mutant enzyme. Bioresour Technol 105:142–151
30. Cavello I, Urbieta MS, Segretin AB, Giaveno A, Cavalitto S, Donati ER (2018) Assessment of keratinase and other hydrolytic enzymes in thermophilic bacteria isolated from geothermal areas in Patagonia Argentina. Geomicrobiol J 35:156–165