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Review Article

EXPLORATION OF KERATINOLYTIC BACTERIA FROM DIFFERENT SOURCES

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A B S T R A C T

Keratinous waste is one of the major biological waste which is found in abundance in nature. At present this waste is managed by dumping or by physical or chemical treatment causing pollution in the environment and the waste remains underutilized. However, it can be utilized in an environment safe manner with the help of keratinolytic microorganisms. In the present study bacteria were isolated and screened from different sources for keratinase production using chicken feathers. Initially 265 bacterial isolates were obtained from 29 samples after enrichment. On the basis of zone of hydrolysis on skim milk agar 142 bacterial isolates were found to be proteolytic. But when tested for degradation of chicken feathers only 13 isolates could solubilise the feathers completely within 7 days. The K:C activity of all these 13 isolates was more than 0.5. The isolates were examined for morphological and biochemical characteristics. All the 13 isolates were found to be Gram positive, sporulating and belonging to genus *Bacillus*. Selected isolates shows great potential to utilise keratinous (white chicken feathers) waste, making them suitable candidate for keratinase production at industrial level.

INTRODUCTION

Keratin is a fibrous and recalcitrant structural protein, composed of a complex mixture of proteins, such as keratins, keratin filament-associated proteins and enzymes extracted from epithelia. A wide spectrum of animals e.g. mammals, fish, birds, and reptiles, has developed diversified keratin as a structural part of their outer protection. Keratin renders animals more robust against both abiotic and biotic stress. Such properties of keratin are attributed to highly crosslinked network structure with numerous disulfide and hydrogen bonds, as well as hydrophobic interactions, and tightly packed keratin micro-fibrils. Since microbial degradation of keratin is not widespread in nature, keratin can serve as an efficient defence even against microbial attack. (Meyers *et al*., 2008), (Bragulla and Homberger, 2009), McKittrick *et al*., 2012),(Lange *et al*., 2016). It is one of the most abundant biopolymer in nature and its availability is increasing especially due to increase in poultry waste, waste from leather and woolen industries. Globally chicken feathers are the most common keratinous waste product generally accumulated from poultry and slaughterhouses waste. Being keratinous these waste are generally rich in carbon, nitrogen, phosphorous and sulphur, which can be utilized to produce value added products. For example, feathers from the poultry industry can be utilized for the production of feedstuffs, fertilizers, biopolymers and keratinase enzyme.

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Keratinous waste creates nuisance in environment due to its limited utilization (Sharma and Gupta, 2016). Keratin can be degraded by keratinases produced by some species of saprophytic and parasitic fungi, actinomyces and bacteria. Keratinases from dermatophytic fungi have long been well known due to their notorious pathogenic nature. Therefore, the isolation of keratinases from non-pathogenic microorganisms especially from bacteria has recently gained industrial importance. (Sahni *et al*., 2015), (Lange *et al*., 2016).

Keratinases (E.C 3.4.21/24/99.11) are a special class of proteolytic enzymes that display the capability of degrading of both soluble and insoluble keratinous substrates. Substrate specificity is one of the major criteria used for designating any protease as 'Keratinase'. Keratinases are known to efficiently cleave materials that are high in keratin content like feather, nail, hair, wool (Onifade *et al*., 1998), (Gupta and Ramnani, 2006), (Evans *et al*., 2000), (McKittrick *et al*., 2012). Our main concern is to explore bacteria capable of producing keratinase utilizing poultry waste especially feathers (purest form of keratin) (1995; Onifade *et al*., 1998), (Gupta *et al*, 2012).

MATERIAL AND METHODS

All Chemicals and Reagents used were of analytical grade and purchased from HiMedia Laboratory.

Sample Collection and Enrichment: Different types of 29 samples were collected from various sources and sites of Kurukshetra (table 1), Haryana, India. These samples were incubated in the enrichment medium containing bird feathers

and sheep wool at 37 ^{o}C for 30 days under continuous shaking conditions (80 rpm).

Isolation: After incubation samples were diluted and inoculated on the skim milk agar (SMA) (HiMedia M763) by spread plate technique. The plates were incubated at 37 ^{o}C for 24-48 h. Colonies showing zone of clearance were picked, purified and maintained on nutrient agar (HiMedia M001).

Screening of isolates: Isolates were screened for keratinolytic activity by following steps given below:

Detection of Proteolytic activity*:* Individual isolates were screened primarily for proteolytic activity by spotting on SMA at 37 ^{o}C for 48 h. The clear zone of hydrolysis developed around the bacterial growth, was measured and isolates showing more than 2 cm zone (diameter) were considered as proteolytic and selected for testing keratinolytic activity.

Detection of Keratinolytic activity*:* Proteolytic isolates obtained after primary screening were incubated in test tubes containing a single white chicken feather in tap water and incubated at 37 ^{o}C for 7 days and observed visually everyday for complete solubilisation of feathers.

Isolates, which completely solubilize feathers were taken as keratinolytic isolates and analysed quantitatively for their keratinolytic activity on the basis of degradation at 37 ^{o}C and 50 ^{o}C and further K:C activity of their enzyme.

Production of Keratinase and degradation of feathers*:* Keratinolytic bacteria were incubated in 250 ml capacity flask containing 50 ml feather meal broth (Tork *et al*, 2010) at 37 ^{o}C and 50 ^{o}C for solubilisation of feathers for 72 h. A control without inoculation was also run simultaneously. Percent degradation of feathers was calculated on the basis of residues left after incubation. Broth of each isolate was centrifuged at 10,000 rpm for 10 minutes. Supernatant (crude keratinase enzyme) and residues were collected separately, washed with organic solvent (chloroform: acetone: methanol:: 4:1:3) to remove cell debris followed by washing with water. After washing, residues were collected on filter paper, dried at 50 ^{o}C for 48 h and weighed. To quantify solubilisation of feathers, their degradation (%) was determined as follows:

$$\text{Degradation (\%)} = \frac{C-R}{C} \times 100;$$

C-feather residues left in control, R-feather residues left in inoculated flask.

K:C (Keratinase: Caseinase)activity: Agar well diffusion method was used for determination of K:C activity of the keratinase enzyme of the keratinolytic bacteria. One hundred microliter of crude enzyme produced during degradation of feathers in the previous experiment of keratinolytic isolates was added to wells in keratin agar (g/l) with composition: soluble keratin -10 g, NaCl- 0.5 g, KH_2 PO_4 -0.7 g, K_2HPO_4 -1.4 g, agar-15 g at pH-7.2 ±0.2 and casein agar (g/l) with composition: casein-10 g, NaCl- 0.5 g, KH_2PO_4 -0.7 g, K_2HPO_4 -1.4 g, agar- 15 g at pH-7.2 ±0.2 plates for keratinase and caseinase respectively and incubated at 37 °C for 24 h. The zone of hydrolysis developed around the respective wells were measured and K:C activity were calculated as follows:

$$\text{K:C activity} = \frac{zone\ of\ hydrolysis\ on\ keratin\ agar}{zone\ of\ hydrolysis\ on\ casein\ agar}$$

Preparation of soluble keratin for keratin agar: Soluble keratin was prepared from white chicken feathers with some modifications in the method of Wawrzkiewicz *et al* (1987). Finely chopped feathers were soaked for defatting hem in an organic solvent containing mixture of chloroform: acetone: methanol in ratio 4:1:3 for 3 days and the solvent was replaced every day. The feathers were finally washed several times with tap water, to eliminate residual solvent and dried at 50 ^{o}C. Now 10 g of these feathers were dissolved in 500 ml of dimethyl sulfoxide followed by heating in a hot air oven at 100 °C for 2 h. Soluble keratin was then precipitated by addition of 1000 ml cold acetone at −18 °C for 6 h, followed by centrifugation at 10, 000×g for 10 min. The resulting precipitate was washed thrice with distilled water followed by heat dry at 50 °C and stored at 4 ^{o}C for further experiments.

Characterization of keratinolytic isolates*:* Cultural characteristics of all the keratinolytic isolates were studied by growing the isolates in nutrient broth and nutrient agar. Gram stained and endospore stained preparations of the isolates characteristics were examined microscopically. For morphological characteristics conventional microbiological methods were used for biochemical characterization of the isolates.

RESULTS AND DISCUSSION

Keratinaceous materials such as feather, hair and chicken wastes are water insoluble and resistant to degradation by common proteolytic enzymes & chemicals but keratinase enzyme is effective in their degradation. Keratin can converts keratin into soluble amino acids. The Keratin-degrading microorganisms thrive under different ecological and environmental conditions including soil, air, fodder, sand and different environmental wastes (Gupta and Ramnani 2006), (Kansoh *et al*., 2009), (McKittrick *et al*., 2012). In this study, we tried to isolate and identify bacterial capable of completely degrading keratin rich wastes.

Twenty nine samples from different sources and sites of Kurukshetra regions were collected and incubated in enrichment medium to enhance the growth of keratinolytic bacteria. Samples from enrichment media were then inoculated on to SMA plates by spread plate method for their proteolytic activity prior to evaluate them for their keratinolytic activity. It helped to narrow the number of isolates. Further, detection of proteolytic activity on SMA is very convenient, cheaper and less time consuming as compared to detect keratinolytic activity. Also, casein present in milk undergoes proteolysis with all the known proteolytic enzymes because of its complex composition and random structure without any necessity for prior denaturation. (Reimerdes and Klostermeyer 1976).

Initially 275 isolates were obtainedon SMA plates from different sources (table 1). All these isolates were assessed on the SMA for their proteolytic activity by spotting them individually on SMA again. Out of 275 isolates only 142 produced clear proteolytic zone more than 2 cm (diameter) after 48 h of incubation at 37 ^{o}C as shown in figure 1. Feathers represent one of the purest form of keratin as they contain approximately 90% keratin (Onifade *et al*. 1998), (El-Refai *et al*., 2005). Solubilisation of feathers by isolates can be easily visualized so used for initial screening of 142 isolates for their keratinolytic ability.

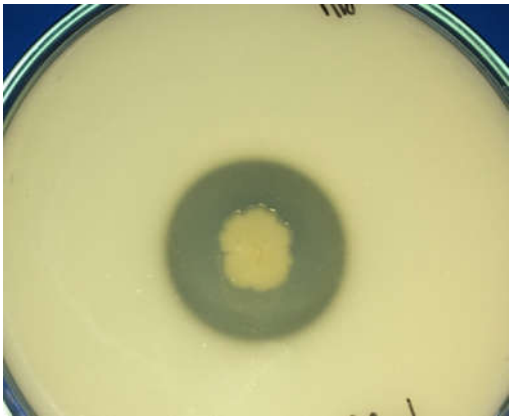

Figure 1 Zone of hydrolysis on skim milk agar by a proteolytic isolate.

higher than 60 ^{o}C in their studies Gessesse *et al*., (2003), Kumar *et al*., (2008), Benikar *et al*. (2013).

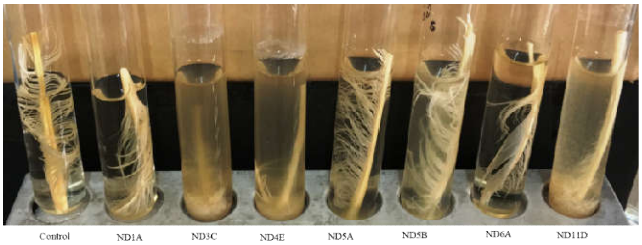

Figure 2 Solubilisation of feathers by some proteolytic isolates.

Isolates in the present research showed good growth and keratinolytic activity in the range of 37^{o}C and 50^{o}C, a few could even work at 55^{o}C.

Table 1 Isolation and screening of keratinolytic bacteria from different sources

Sample	Source/Site	No. of Samples	No of Isolates[a]	Screening	
				Proteolytic	Keratinolytic[b]
Bird droppings	Orchid	2	15	13	2
Chicken feathers	Local poultry farm	1	10	08	0
Pigeon feathers	Orchid	1	09	07	0
Hair	Human	2	13	08	1
Nails	Human	2	08	04	2
Pork	Pig Butcher House	1	12	09	0
Toes Scrapping	Human	1	07	04	0
Skin	Human	1	15	11	0
Soil	Garden, K.U	2	21	09	0
Soil	Dumpyard, K.U	2	35	14	0
Soil	Local dairy	2	21	12	0
Soil	Jyotisar	1	13	05	0
Waste	Local poultry farm	2	21	14	5
Waste	Hair saloon	1	15	04	0
Waste	Pig Butcher House	2	12	05	2
Waste	Chicken Butcher House	2	16	06	1
Water	Jyotisar	2	18	05	0
Water	Brahmsarovar	2	14	04	0
	Total	29	275	142	13

a-isolates from skim milk agar; b- isolates solubilize feather completely in 7 days

Table 2 Origin, degradation percent and K:C activity of keratinolytic bacterial isolates

Isolate Name	Sample	Source/site	Feather Degradation (%) at temperature		K:C activity
			37 °C	50 °C	
ND3C	Nails	Human(man)	25.00	19.10	3.00
ND3D	Nails	Human(man)	43.22	55.63	1.67
ND4E	Hairs	Human(woman)	25.61	10.23	1.83
ND5F	Waste	Local poultry farm	37.88	43.25	1.07
ND5G	Waste	Local poultry farm	35.66	49.49	1.64
ND5I	Waste	Local poultry farm	23.41	14.66	2.00
ND5J	Waste	Local poultry farm	44.65	53.16	0.86
ND5P	Waste	Local poultry farm	24.86	05.30	0.58
ND6D	Bird droppings	Orchid	46.20	60.16	3.50
ND6J	Bird droppings	Orchid	15.36	13.01	1.00
ND11D	Waste	Chicken butcher house	24.30	35.26	1.67
ND12A	Pork	Pig butcher house	39.60	48.25	0.59
ND12B	Pork	Pig butcher house	40.23	51.28	0.50

Feathers were solubilised (figure 2) by 54 proteolytic isolates out of 142, but only 13 isolates could solubilize 100% feathers in 7 days at 37 ^{o}C, so these were considered as efficient keratinolytic bacteria. These results indicated that all the proteolytic bacteria are not keratinolytic. Keratinolytic isolates were further evaluated by measuring % degradation of feathers at 37 °C and 50 °C by incubating them in feather broth meal. Generally, optimum temperature for the growth of keratinolytic microbes and the catalytic reaction temperature of the keratinase enzyme produced by them is reported above 35 ^{o}C and however many researchers have reported it even

Four isolates ND3D, ND5G, ND5J, ND6D, ND12B show more than 40 % degradation at 37 °C and more than 50 % at 50 °C after 72 h. Eight isolates ND3D, ND5F, ND5G, ND5I, ND6D, ND11D, ND12A, ND12B degraded feathers more efficiently at higher temperature i.e. 50 ^{o}C as compared to 37 ^{o}C .

To use K:C (keratinolytic: caseinolytic) activity as a parameter to judge the potential of an enzyme as keratinase was put forth by Evans *et al* (2000). So crude enzyme produced by each of the 13 keratinolytic isolates was analysed on the basis of ratio of zone of hydrolysis on keratin and casein agar. K:C ratio of all the keratinolytic isolates was more than 0.5 and highest being 3.5 found in ND6D isolate (table 2) indicating that keratinase produced by theisolates have potential to act on keratinous substrates. ND6D was found having maximum degradation percent of feathers and maximum K:C activity. However, other isolates with very high degradation percent of feathers have not necessarily high K:C activity. It might be due to high substrate specificity of the enzyme released by them. On further analysis, it was observed that source of all these 13 isolates was keratinaceous eitherin the form of abody part of vertebrate or their waste (table 1 & 2).

Morphological, biochemical and cultural characteristics of all the keratinolytic bacterial isolates were studied. All keratinolytic bacterial isolates were found to be Gram positive, sporulating, aerobic, motile rods, capable of growing at 50 ^{o}C

but not at temperature higherthan 60 ^{o}C, positive for casein hydrolysis, nitrate reduction, catalase test and negative for hydrogen sulphide reduction and indole test. Results of other tests were variable in different isolates and are given in table 3(a-c). Datagiven in table 3 (a-c) was analysed on the basis of Bergey's Manual of Systematic Bacteriology 2nd edition volume three, *The Firmicutes* for their tentative identification and all the isolates were found to be belonging to different species of genus *Bacillus*.

CONCLUSION

Keratinolytic microbes are ubiquitous in nature but sources/sites rich in vertebrates or their wastes are generally their hotspot.

Table 3 (a) Cultural characteristics of the keratinolytic bacterial isolates

Isolate Name	Colony morphology on the NA plates						On NA slants	In Nutrient broth (HiMedia M002)
	Color	Diameter (cm)	Consistency	Margin	Elevation	Form		
ND3C	White	0.5-0.7	Dry	Lobate	Flat	Circular	Effuse	Surface
ND3D	White	0.5-1.0	Mucoid	Undulate	Convex	Circular	Filamentous	Surface
ND4E	White	0.4-0.5	Dry	Filamentous	Flat	Irregular	Echinulate	Surface
ND5F	Off white	0.6-0.8	Dry	Undulate	Flat	Circular	Effuse	Surface
ND5G	Off white	0.7-1.0	Dry	Entire	Flat	Circular	Effuse	Surface
ND5I	Off white	0.9-1.2	Dry	Serrate	Flat	Circular	Effuse	Surface
ND5J	Off white	0.5-0.7	Dry	Serrate	Flat	Circular	Effuse	Surface
ND5P	White	0.8-1.2	Mucoid	Entire	Raised	Irregular	Arborescent	Surface
ND6D	Wheat	0.1-0.3	Dry	Serrate	Umbonate	Circular	Echinulate	Surface
ND6J	Cream	0.5-0.7	Moist	Entire	Flat	Circular	Echinulate	Diffuse
ND11D	Off white	0.1-0.3	Moist	Entire	Flat	Circular	Echinulate	Diffuse
ND12A	Off white	0.8-1.0	Moist	Entire	Flat	Circular	Effuse	Surface
ND12B	White	1.5-2.0	Mucoid	Undulate	Convex	Floral	Effuse	Surface

Table 3 (b) Biochemical characteristics of the keratinolytic bacterial isolates.

Isolate Name	7% NaCl Tolerance	Oxidase activity	Gelatin hydrolysis	Starch hydrolysis	Lipid hydrolysis	Urea hydrolysis	Cellulase activity	Methyl-Red test	Voges-Proskauer test	Citrate utilization	Utilization of different sugars*												
											D-Glucose	Fructose	D-Mannose	D-Mannitol	Galactose	D-Xylose	Trehalose	Sucrose	Lactose	Cellobiose	Maltose	D-Raffinose	Sorbitol
ND3C	+	+	+	+	+	(+)	+	-	-	+	+	+	-	+	+	-	+	+	+	+	+	+	-
ND3D	+	+	+	+	+	(+)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
ND4E	-	-	+	+	+	(+)	+	+	+	+	+	+	+	-	-	-	-	+	-	+	+	-	-
ND5F	+	-	+	+	+	(+)	-	-	+	+	+	+	+	-	-	-	+	+	-	+	+	-	-
ND5G	+	-	+	+	(+)	+	+	-	+	+	+	-	+	-	-	-	+	+	-	+	+	-	-
ND5I	+	-	+	(+)	+	-	-	-	+	+	+	-	+	-	+	+	+	+	-	-	+	-	-
ND5J	+	+	+	+	(+)	-	-	+	+	+	+	+	+	+	-	-	-	+	-	+	+	+	+
ND5P	+	+	-	(+)	+	+	+	-	+	+	+	+	+	+	+	-	+	+	+	+	+	-	+
ND6D	+	-	(+)	+	+	+	+	-	+	-	+	+	+	+	-	+	+	+	-	+	+	+	+
ND6J	-	+	-	-	-	-	-	-	-	-	+	-	-	-	+	-	+	+	+	+	+	+	+
ND11D	-	-	-	(+)	+	(+)	-	-	-	-	+	-	-	-	+	-	+	+	+	-	+	-	-
ND12A	+	+	+	(+)	+	-	+	-	-	+	+	+	-	+	+	-	+	+	+	+	+	+	+
ND12B	+	-	+	+	+	+	+	-	+	+	+	+	+	+	-	+	+	+	-	+	+	-	+

(+)-weak/slow reaction; *-Gas was not released in any case.

Table 3(c) Morphological characteristics of the keratinolytic bacterial isolates.

Isolate Name	Morphology of		Organism*
	Bacterial cells	Endospore	
ND3C	GP, Rod; occur singly, occasionally in chains	Oval, sub-terminal	*Bacillus megaterium*
ND3D	GP, Rod; occur singly	Oval, sub-terminal	*B. licheniformis*
ND4E	GP, Small rod; occur singly	Oval, terminally	*B. cereus*
ND5F	GP, Small rod; occur singly	Oval, terminally	*B. thuringiensis*
ND5G	GP, Rod; occur singly	Oval, sub-terminal	*B. thuringiensis*
ND5I	GP, Rod; occur singly	Oval, sub-terminal	*B. cereus*
ND5J	GP, Rod; occur singly	Oval, centrally located	*B. amyloliquefaciens*
ND5P	GP, Rod; occur in chains	Oval, sub-terminal	*B. licheniformis*
ND6D	GP, Rod; occur singly	Oval, sub-terminal	*Bacillus spp.*
ND6J	GP, Small rod (swollen); occur singly	oval, central	*Bacillus spp.*
ND11D	GP, Rod (swollen in one end); occur singly	oval, sub-terminal	*B. circulans*
ND12A	GP, Rod; occur singly	Oval, sub-terminal	*B. megaterium*
ND12B	GP, Rod; occur singly, occasionally in pairs	Oval, sub-terminal	*B. licheniformis*

GP- Gram positive; *-tentatively identified on the basis of Table 3 (a), (b) & (c)

However for confirmation of species, identification by molecular characterization is needed. According to review paper of Sharma and Devi (2018) keratinolytic bacteria generally belong to *Bacillus*.

Some of the keratinolyticbacteriaisolated in this study, are capable of degrading feathers at 50^{o} C temperature, so making them suitable candidate for the utilization of keratinaceous waste of agro-industries especially in tropical region. However for future application of an isolate, more detailed study on the production and characterization of itskeratinase enzyme is needed.

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