Identification and Characterization of Keratinolytic Bacterium Isolated from Poultry Waste

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ABSTRACT

Keratinases are modern proteases that can valorise poultry and textile industry wastes and may find applications in various biotechnological sectors like in feed, fertilizer, detergent, leather, and textile industries and for pharmaceutical and biomedical applications. A wide diversity of bacteria, actinomycetes and fungi are known to be keratin degraders. Among all the bacterial isolates due to the robust nature and its enzyme, Bacillus spp known to produce extracellular protease that degrade the highly recalcitrant chicken feathers . Therefore, in this article, we aimed to identify and characterize the bacterium isolated from chicken feather waste dumping site soil. Five efficient keratinase producing bacterial strains was identified based on the morphological, biochemical and cultural characteristics. In this report, we have identified bacterial strains using 16S rRNA gene sequence and performed bioinformatics analysis such as BLAST and phylogenetic tree. The isolated bacterial strains showed > 95 % similarity with Bacillus spp.

KEYWORDS: Keratinases, Chicken feather, Identification, Bacillus spp, 16s RNA

INTRODUCTION

Keratin is insoluble structural protein and difficult to degrade, however, some microorganisms are able to degrade it. Keratin can be degraded by some species of saprophytic and parasitic fungi (Nayaka et al., 2013; Allure et al., 2015 and Gurav et al., 2016), a few actinomycetes (Marcondes et al., 2008; Singh and Kushvaha et al., 2015) and *Bacillus* species (Priyanka, 2016; Manirujaman et al., 2016). The mechanical stability of keratin and its resistance to microbial degradation depend on the tight packing of the protein chain in α-helix (α -keratin) and β-sheet (β-keratin) structures and their linkage by cys-bridges due to high degree of cross-linkage by disulfide bonds, hydrogen bonding and hydrophobic interactions. Annually tons of feathers are discarded to the environment as a waste byproduct by poultry processing plants, causing serious problems as a pollutant and resulted in the outbreaks of serious health issues. Consequently a huge amount of keratinase waste accumulates in nature and imposes a great concern for the environment.

Keratinolytic enzymes are widely distributed in nature and are secreted by several microorganisms isolated from different environment niches including soils where keratinous rich materials i.e. feathers are dumped (Kaul and Sumbali 1997; Riffel and Brandelli 2006; Tork et al., 2010; Subugade et al., 2017) , agro industrial residues (Mazotto et al., 2011), alkaline mud (Gessesse et al., 2003), limestone habitat (Ningthoujam et al., 2016) and extreme environments (Rissen and Antranikian 2001; Nam et al., 2002) like hot, springs and soda soils etc. These are a specific type of proteolytic enzymes, primarily extracellular in nature. Keratinases of microbial origin are predominantly of the metallo, serine or serine

metallo type (Brandelli, 2008).on the basis of structure, keratins are classified as α-keratins (hair, hooves, nails, etc.) and β-keratins (feather, silk fibrion, β-amyloid) (Voet and Voet, 1995; Akhtar and Edwards, 1997). β keratins are more readily hydrolyzed than α-keratins (Ramnani and Gupta, 2004).

Bacterial keratinases especially *Bacillus spp* are of particular interest because of their the robust nature and its enzyme action on insoluble keratin substrates, for dehairing processes in the leather industry, to produce feather meal from discarded feathers, resulting in a low nutritional value product and also used for production of organic fertilizer. In the present, we have identified the five potential keratinolytic bacterial pure cultures colonies growth based on molecular method. Here, genomic DNA was extracted and performed PCR amplification using universal 16s rRNA pair of primers. Further, we sequenced and analyzed based on bioinformatics tools such as NCBI BLAST and CLUSTAL2 and neighbor-joining tree. After compilation of this data, we could assign the correct taxon to isolated sequence as a *Bacillus* sp.

MATERIALS AND METHODS

SELECTION OF SAMPLE COLLECTION SITES

On the basis of established Poultry Farm and Chicken hatchery in Mid-Hill regions of Himachal Pradesh, three different locations *i.e* Palampur (Distt. Kangra), Sunder Nagar (Distt. Mandi) and Nahan (Distt. Sirmour) were selected for the collection of chicken feathers and feathers dumped soil/ compost for further experiments. From Nahan chicken hatchery four sites were selected and from Palampur, Poultry Farm and Sundernagar, Breeding Farm and hatchery two sites were selected for sampling purpose.

COLLECTION OF SAMPLES

The eight soil/compost samples of chicken feathers dumping sites and cage surface soil in replica of two were taken from each selected sites and were mixed to make composite sample**.** The chicken feather dumping soil and cage surface soil sample was collected from a depth of 60-90 cm and 15-30 cm, respectively. All these samples were collected in sterilized polythene bags and were stored in refrigerator at 4°C for further processing.

ISOLATION OF MICROORGANISMS

Isolation of microbial population from feather dumped soil/compost was done by standard plate count technique (Wollum, 1982) and enrichment technique (Martinus, 1901) by employing different media for different groups of microorganisms. *viz.*, Nutrient Agar, Kenknight medium and Potato Dextrose Agar for enumeration of bacteria, actinomycetes and fungi, respectively.

QUALTITATIVE AND QUANTITATIVE ESTIMATION OF KERATINOLYTIC ACTIVITY

The above isolated microbes were screened for their keratinolytic activity on modified basal feather agar medium supplemented with native chicken feather, as source of β-keratin 0.5%, K_2HPO_4 0.03; KH_2PO4 0.04; NaCl 0.05; $MgCl_2$ 0.01 and Agar 2% were used to prepare the medium (Sahoo et al., 2012). Out of the all above screened isolates were further, screened for the quantitative estimation for the keratinase production as per method given by Gradisar et al., 2005.

IDENTIFICATION AND CHARACTERIZATION OF POTENTIAL BACTERIAL ISOLATES

1. MORPHOLOGICAL AND BIOCHEMICAL CHARACTERIZATION OF BACTERIAL ISOLATES

In total 193 microbial isolates were obtained from all the samples collected from different selected sites. Among these, 126 bacterial, 3 fungal and 9 actinomycetes isolates were obtained by Standard plate count technique while 38 bacterial, 2 fungal and 15 actinomycetes isolates were obtained by enrichment technique. All these isolates were screened for their keratinolytic potential qualitatively as well as quantitatively. Out of 126, only five bacterial isolates *viz.*N14, N27, N35, L2EN1 and DPE11, were found to be potent keratinase producers based on qualitative and quantitative screening. These isolates were identified based on their morphological and biochemical characteristics according to the standard methods described in Bergey's Manual of Systematic Bacteriology (Vos et al., 2009).

MOLECULAR CHARACTERIZATION OF POTENTIAL KERATINASE PRODUCERS

Identification of five potential keratinase producers was done based on morphological, physiological and biochemical characteristics. Furthermore, molecular characterization was done using 16S rRNA gene sequencing technique, which was carried out at the sequencing facility of National Centre for Microbial Resource (NCMR), National Centre for Cell Science, Pune. The phylogenetic analyses were carried out in MEGA 7.0 software program and the 16S rRNA gene sequences were submitted to GenBank, National Center for Biotechnology Information (NCBI), USA.

RESULTS AND DISCUSSION

ISOLATION OF MICRORGANISMS

In total 193 microbial isolates were obtained from all the samples collected from different selected feathers dumping sites. Among these, 126 bacterial, 3 fungal and 9 actinomycetes isolates were obtained by Standard plate count technique while 38 bacterial, 2 fungal and 15 actinomycetes isolates were obtained by enrichment technique. The isolation of extracellular keratinases from environmental sources such as feathers dumping sites has also been reported by other workers (Lee et al., 2004; Tork et al., 2010; Subugade et al., 2017). Through the strategy of isolation of keratinolytic microorganisms utilized in this work, bacteria presenting high keratinolytic activity were selected. Considering that feather protein has been showed to be an excellent source of metabolizable protein (Klemersrudet et al., 1998), and that microbial keratinases enhance the digestibility of feather keratin (Odetallah et al., 2003).

QUALTITATIVE AND QUANTITATIVE ESTIMATION OF KERATINOLYTIC ACTIVITY

Out of the above isolated all the bacterial strains were screened for their keratinolytic activity (plate assay) ranged from 2.00 to 27.67 per cent. Therefore, on the basis of qualitative estimation which showed more than 6.00 per cent keratinolytic activity were further screened for the quantitative assay or for the keratinase production. Hence, from all the screened bacterial isolates five potential bacterial strains *i.e* N14, N27, N35, L2EN1 and DPE11 showed maximum keratinase production. Jin et al.,(2017) and El-Ghonemy and Hamed (2017) also employed qualtitative and quantitative assay for isolation of efficient keratinases enzyme producers.

IDENTIFICATION AND CHARACTERIZATION OF POTENTIAL BACTERIAL ISOLATES

1. MORPHOLOGICAL AND BIOCHEMICAL CHARACTERIZATION OF BACTERIAL ISOLATES

Gram staining reaction of all the bacterial isolates revealed them Gram-positive rods. On the basis of biochemical tests these isolates were tentatively identified as *Bacillus* sp., results are depicted in the Table1.

Table1. Morphological and biochemical characteristics of the bacterial isolates

S. No	Isolate No.	Morphological characteristics					Biochemical characteristics					
		Colour	Form	Margin	Gram stain	Shape	Oxidase test	MR	VP	Citrate utilization	Catalase	Identifcation
1.	N14	Cream	Punctifom	Entire	+	Rod	+	+	-	-	+	*Bacillus sp.*
2.	N27	Cream	Irregular	Entire	+	Rod	+	+	-	-	+	*Bacillus sp.*
3.	N35	white	Punctifom	Lobate	+	Rod	+	-	+	-	+	*Bacillus sp.*
4.	L2EN1	White	Irregular	Undulate	+	Rod	+	-	+	-	+	*Bacillus sp.*
5.	DPE11	white	Irregular	Entire	+	Rod	+	-	+	+	+	*Bacillus sp.*

Our results are in confirmation with those of Ramnani et al., (2006), Godbole et al., (2017) and Jadhav, (2016) who also done the identification of bacterial isolates on the basis of morphological and biochemical characterization.

MOLECULAR CHARACTERIZATION OF POTENTIAL KERATINASE PRODUCERS

Based on partial 16S rRNA gene sequencing, these five potential keratinolytic bacterial strains *viz.* N14, N27, N35, L2EN1 and DPE11 were identified as *Bacillus cereus* N14 (MF355368), *Bacillus cereus* N27 (MF355367), *Bacillus megaterium* N35 (MF193346), *Bacillus halotolerans* DPE11 (MF193347) and *Bacillus halotolerans* L2EN1 (MF355366) that showed their similarity to different species of genus *Bacillus*. The isolate N35 showed maximum homology (99%) with *Bacillus tequilensis*-VITASMJ1, L2EN1 with *Bacillus halotolerans* ABS20 (99%), N27 with *Bacillus cereus* ST06 (99%), N14 with *Bacillus cereus* 1BS2 (99%) and isolate DPE11showed maximum homology (99.84%) with *Bacillus halotolerans* FJAT 47799 as depicted in Table 2. Unrooted phylogenetic tree, based on comparison of 16S rDNA sequence data of *Bacillus cereus* N14, *Bacillus cereus* N27, *Bacillus megaterium* N35, *Bacillus halotolerans* DPE11 and *Bacillus halotolerans* L2EN1, was constructed with their closest phylogenetic neighbours in the NCBI, GenBank, USA using Neighbour joining (NJ) method with the help of MEGA 7.0 software program (Figure 1). Numbers on the tree indicates percentage of bootstrap sampling derived from 1000 random samples.

Table 2. Molecular characterization of potential keratinolytic bacterial isolates

Sr. No.	Isolate	Identified as	Closest homology with	Identity (%)	Genbank Accession No.
1.	DPE11	*Bacillus halotelerans*	*Bacillus halotelerans*- FJAT-47799	99.0	**MF193347**
2.	N35	*Bacillus megaterium*	*Bacillus tequilensis*VITASMJ1	99.0	**MF193346**
3.	L2EN1	*Bacillus halotelerans*	*Bacillus halotelerans* ABS20	99.0	**MF355366**
4.	N27	*Bacillus cereus*	*Bacillus cereus* ST06	99.0	**MF355367**
5.	N14	*Bacillus cereus*	*Bacillus cereus* 1BS2	99.0	**MF355368**

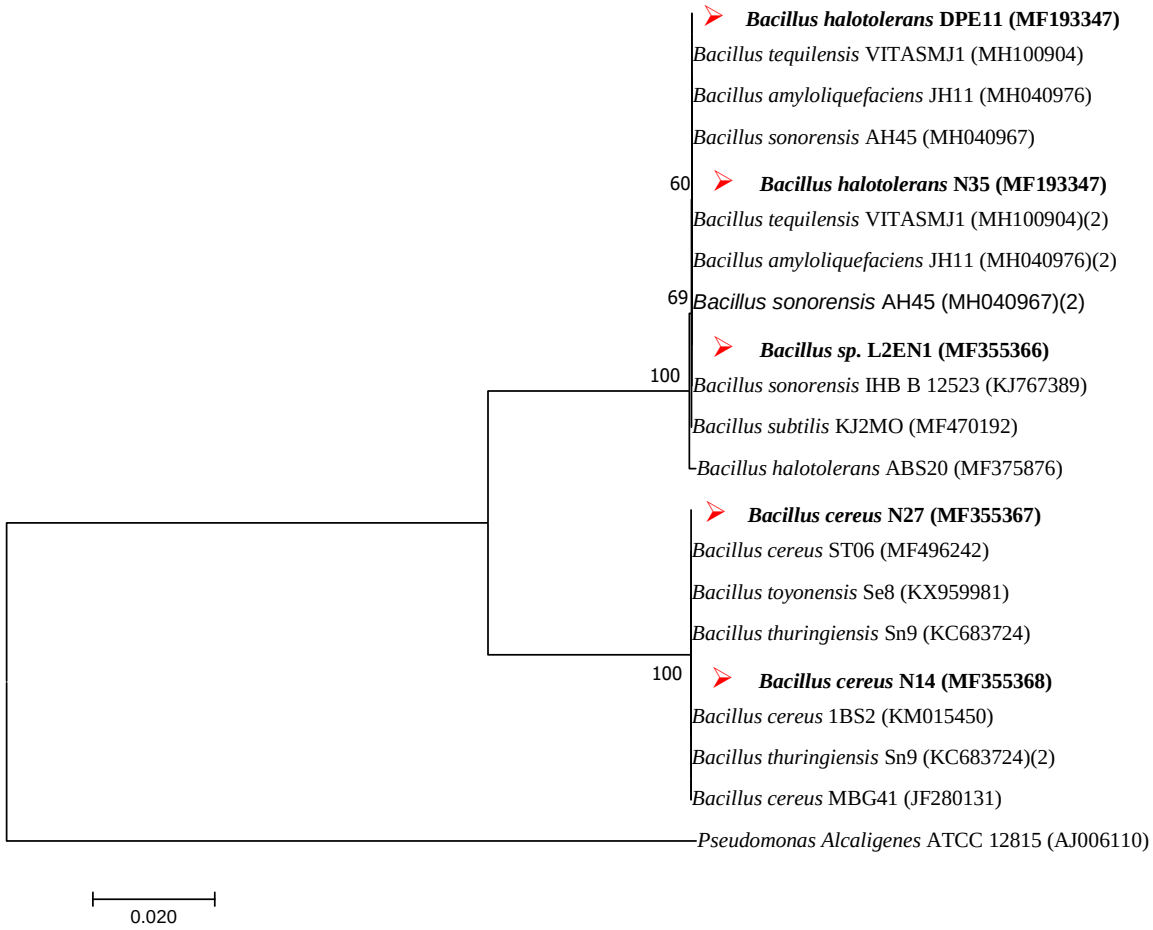

Fig1. Phylogenetic tree showing evolutionary relationship of potential keratinolytic strains isolated from chicken feathers dumping soil and its related taxa constructed using MEGA 7.0 software by neighbour joining method

The above identified five potential keratinolytic bacterial strains *viz.* N14 (*Bacillus cereus)*, N27(*Bacillus cereus*), N35 (*Bacillus megaterium*), L2EN1 *(Bacillus halotolerans* and DPE11 *(Bacillus halotolerans)* were also deposited in National Centre for Microbial Research (NCMR), Pune, Maharastra, India for public access purpose and were allocated with respective accession numbers *i.e.* N14 (MCC 3709*)*, N27(MCC3712), N35 (MCC 3776), L2EN1 (MCC 3711) and DPE11 (MCC 3710).

The morphological and biochemical characterization revealed the tentative identification of microorganisms upto genera level. Therefore in present study we have supplemented the molecular identification of bacteria on the basis of16S rRNA analysis. Similarly many researchers Lin et al., 1992; Kumar et al., 2005; Priyanka, 2016 and Manirujam et al., 2016) have also confirmed their identification of the potential keratinase producing bacterium as *Bacillus* sp. on the basis of16S rRNA analysis.

Conclusion

The results suggest that the potential keratinase producing bacterium isolated from feather waste belongs to *Bacillus* spp.

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