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Isolation and molecular characterization of keratinase producing Bacillus species from soil

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Abstract

Bacillus species present in soil contain enzymes which show proteolytic properties to decompose proteins. Soil proteases are mainly provided by soil bacteria. Bacillus species have various industrial uses. The aim of this study was to isolate and molecular characterization of keratinase producing Bacillus species from soil. Bacterial strains have been isolated from soil. Biochemical and molecular characterization have been performed using different biochemical tests (Catalase, Nitrate, simmon citrate, Triple sugar iron tests) and 16S rDNA sequence analysis respectively. Moreover, keratinase test has been performed to check the presence of keratinase enzyme in isolated species. In this study, five different bacterial strains have been isolated from soil. Out of five strains, four were Bacilli and one strain is *Citrobacter farmeri*. Sequencing results showed that four strains were Gram positive, Bacilli and one strain was Gram negative, *Citrobacter farmeri*. A keratinase test was performed to see the presence of keratinase enzyme in all strains. The results showed that keratinase was present in *Bacillus cereus* and *Bacillus thuringiensis*. This study concluded the significant role of Bacillus species in the production of keratinase enzyme. A keratinase enzyme is very important in leather industry for dehairing. Keratinase enzyme is non pollutant and economical.

Introduction

Bacillus species present in soil have enzymes, which show proteolytic properties to decompose proteins. Proteolytic enzymes not only have an important role in industrial uses, but these enzymes also contribute to nitrogen cycle. Soil proteases are mainly provided by soil bacteria [1]. Proteases have been significantly described as a resourceful group of enzymes with vast industrial applications. Keratinases can degrade the insoluble structure that is involved in formation of keratin substrates. They belong to class of serine and metalloproteases [2].

Bacillus species are rod shaped Gram-positive bacteria which form endospores and are chemoheterotrophic in nature. They are aerobic and sometimes, facultative anaerobic, catalase positive and have peritrichous flagella for movement. Bacillus species are capable of being survived in the soil environment as they form spores [3]. Bacillus species have a range of physiological characteristics which help in the growth of organism under various environmental conditions and provide a competitive superiority over the other microorganisms within the same environment. The heat and cold stable metabolites are produced because of their capacity to form spores [4]. Bacillus species have many industrial uses. Enzymes produced by bacillus species are used in textile, starch, dairy, baking, beverages, and leather industry. Amylase and protease have vast industrial production [5]. In the poultry industry, keratin waste is being produced in large amount and spread in environment. The proteolytic enzymes degrade the keratinous waste into soluble form; decompose the keratinous waste into useful form with the help of keratinase which is a type of protease [6].

The correct identification of microbial species is an important task for clinical microbiological laboratories. The universal distribution of 16S ribosomal DNA (rDNA) among bacteria and the presence of species dependent variable regions make the molecular identification of microbes possible. The bacterial phylogeny which leads to making and sustenance of large bacterial strain databases can be created by using the above-mentioned molecular approach. The created databases can be used as an application to identify different bacterial strains including unusual and unique isolates which may be clinical and environmental uncultured microorganisms [7]. Keratinases got the special interest because of their several potent uses related to the hydrolysis of keratinous substrates and other industrial applications [8]. In a previous study researcher worked on the comparative analysis of *Bacillus mycoides, Bacillus anthracis* and *Bacillus thuringiensis* based on sequencing of 16S rRNAs. Researchers investigated that the primary structure of *Bacillus thuringiensis, Bacillus cereus, Bacillus mycoides* and *Bacillus anthrac*is was determined on the basis of reverse transcriptase. They investigated that 16S rRNA sequence of *Bacillus thuringiensis* differed from the *Bacillus mycoides* and sequence of *Bacillus cereus* from *Bacillus anthracis* by four to nine nucleotide [9].

In a study *Streptomyces gulbargensis* was isolated. It was investigated that this new isolated strain is thermostable and has ability to produce keratinase enzyme. This strain has ability to degrade feather. The greatest activity of enzyme was seen at pH 9.0 and at 45℃ [10]. Schallmey *et al*., (2004) worked on the Bacillus species and investigated that the capacity of selected isolated Bacillus strains has the capacity to produce and secrete greater quantities (20–25 g/L) of extracellular enzymes which placed them among the most important industrial enzyme producers. A wide variety of commercial enzyme products has been developed with desired characteristics including pH, temperature and solubility characteristics to deal with the specific areas of application [11]. In a study, new strain was isolated which has high feather degradation activity at room temperature. They identified that this strain has greater uses in biotechnological processes. They isolated mesophilic feather degrading bacteria which were comparatively less energy consuming from thermophilic strains [12].

Materials and Methods

Sample processing

Soil samples were taken from three different areas of Lahore including soil near poultry farm, sewerage and crop field and transferred in sterile plastic bags. Soil samples were diluted in 0.85% normal saline solution. 10g of each soil sample was dissolved in 10mL saline solution. Five serial dilutions were prepared such as 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}. Sterilized basal media such as nutrient agar (28g/1000mL) was used for the growth of soil bacteria. All serial dilutions were spread on nutrient agar plates separately and plates were incubated at 37℃ for 24 hours. Bacterial colonies were purified on nutrient agar to obtain isolated colonies. The colony characteristics like size, color, shape, margin and elevation were assessed by growth pattern appearing on the agar surface by examining the plates.

Isolation and biochemical characterization of Bacillus species

Gram staining was used for the differentiation of Gram-positive and Gram-negative bacteria. Spore staining was used to identify spore forming Bacillus species. Gram-positive rod-shaped spore forming pure colonies were further grown on selective and differential media such as mannitol salt agar (MSA) and polymyxin pyruvate egg-yolk mannitol bromothymol blue agar (PEMBA) by streaking method. The bacterial isolates were further characterized biochemically by catalase test, nitrate test, Simon citrate test and carbohydrate fermentation test (glucose, sucrose and lactose) through the standard protocols [13].

DNA extraction

Single bacterial colonies were cultured in nutrient broth and cells were taken by centrifugation (5,000rpm) for 4 minutes for DNA isolation by phenol-chloroform method [14].

16S rRNA gene sequence (universal primers)

PCR method was used for the confirmation of Bacillus species. 16S ribosomal RNA gene sequencing was carried out for the identification of the Bacillus species. The universal primers for 16S ribosomal gene amplification were used. The sequence, length and melting temperature (Tm) of universal primers are given in the **Table 1**.

Table 1: Sequence and melting temperature (Tm) of primers

Primers	Sequence	Length	Tm
1F	AGAGTTTGATCCTGGCTCAG	20	54.3°C
1R	TACGGTTACCTTGTTACGACTT	22	58.4°C

The conditions for 16S PCR amplification (initial denaturation 95°C for 1min, 35 cycles of denaturation at 94°C for 30 sec, annealing 53°C for 1min and extension at 72°C for 45 sec and hence final extension 72°C for 1.5min) were used. DNA fragments were visualized using 1% agarose gel electrophoresis.

Enzyme assay for keratinase

Minimal medium was prepared to see the presence of keratinase enzyme in previously characterized Bacillus species. Keratin was used as substrate for the enzyme. 1% keratin was added into sterilized nutrient agar medium. The media was poured in plates, when the media got solidified, 3 wells in the media gel were formed with sterile tips on each petri plate. 25µL inoculum of each Bacillus species was poured in each well. The plates were incubated at 37°C for 2 days. After 2 days plates were stained with Coomassie brilliant blue and de-staining was performed afterwards. Light blue patterns or clear zone around the wells showed the presence of keratinase enzyme [12].

Results

Five bacterial isolates were obtained from three different regions of Lahore (Poultry farm soil, Field and nearby area of sewerage). Bacterial strains produced on nutrient agar plate were collected and incubated at 37°C for 24 hours. Isolated bacteria were characterized on the basis of morphology and grow on different media plates. Gram staining performed and media like Nutrient agar, EMB (Eosin methylene blue), MSA (mannitol salt agar) and MacConkey agar used for culture characterization.

After purification of the bacterial strains, bacteria were identified by biochemical tests including, catalase, nitrate reduction test, Simon citrate, triple sugar iron (TSI). Isolated bacteria were characterized and identified according to the criteria as given in **Table 2**. The analysis of biochemical tests was made to confirm the expected isolates. According to biochemical tests performed Isolate A is *Bacillus cereus*, isolate B is *Bacillus thuringiensis*, isolate C is *Bacillus mycoides*, isolate D is *Bacillus subtilis*, isolate E is *Citrobacter farmeri* (**Table 3**).

DNA isolation from bacterial isolates

DNA of different bacterial strains was isolated. The results of 1% agarose gel electrophoresis with positive genomic DNA are given in **Fig. 1**.

PCR amplification of DNA

PCR has been performed by using 16S ribosomal RNA Primers. Gene was amplified from genomic DNA (**Fig. 2**).

After the sequences obtained by Sanger sequencing, isolates were further confirmed by BLAST alignment tool. BLAST result of sample sequence 1 shows the 98% similarity with *Bacillus cereus*. BLAST result of sample sequence 2 shows the 98% similarity with *Bacillus thuringiensis*. BLAST result of sample sequence 3 shows the 99% similarity with *Bacillus mycoides*. BLAST result of sample sequence 4 shows the 90% similarity with *Bacillus subtilis.* BLAST

Table 2: Morphology and culture characterization of isolated bacteria

Sr. No.	Nutrient agar	Gram staining	Spore staining	MSA	PEMBA	EMB	MacConkey agar
A	Large, cream color, dull texture	Purple rod	Diplobacillus, central endospore	NG	Good growth; peacock blue colonies with precipitate	NG	NG
B	Irregular margins	Purple rod	Straight or slightly curved slender bacilli with square ends singly or in short chains.	NG	Blue color with crystalline formation	NG	NG
C	Forming rhizoid filaments, spreading colonies	Purple rod	Rod bacilli as chains of cells	NG	Blue colonies with rhizoidal and filamentous growth	NG	NG
D	large, cream color, spreading,	Purple rod	Diplo-bacillus, sub-terminal endospore	NG	Growth; straw colored colonies	NG	NG
E	Small, Circular, irregular, lightly curved	Pink long rod	NG	Turn pink media to yellow	NG	Colorless growth	Peach color growth

Note: MSA: Mannitol Salt Agar, PEMBA: Polymyxin Pyruvate Egg yolk Mannitol, EMB: Eosin methylene blue, NG: No growth

Table 3: Biochemical characterization of bacterial strains

Test	*Bacillus cereus*	*Bacillus thuringiensis*	*Bacillus mycoides*	*Bacillus subtilis*	*Citrobacter farmeri*
Catalase Test	+	+	+	+	+
Simmon citrate test	+	+	-	+	+
Nitrate reduction test	+	+	+	+	-
Triple sugar iron test	K\A Red slant, yellow butt No gas, No H_2S	K\A Red slant, yellow butt No gas, No H_2S	K\A Red slant, yellow butt No gas, No H_2S	K\A, Red slant, yellow butt Gas produce, No H_2S	K\A Red slant, Yellow butt, No Gas, No H_2S

Note: +: Positive, **–**: Negative, K/**A**: alkaline slant/acid butt, H_2S: hydrogen peroxide

result of sample sequence 5 shows the 97% similarity with *Citrobacter farmer.*

Keratinase test

Light blue circles around wells present in *Bacillus cereus* and *Bacillus thuringiensis*, which shows that keratinase enzyme is present in *Bacillus cereus* and *Bacillus thuringiensis*. Keratinase enzymes present in both strains hydrolyzes the keratin and light blue circles around wells shows the hydrolysis of keratin. No enzyme activity shown in *Bacillus mycoides* and *Bacillus subtilis* (**Fig. 3**).

Discussion

In this study, five strains of bacteria have been isolated from soil, out of which four were Bacillus strain. The samples were cultured on nutrient agar media for 24 hrs at 37°C. The biochemical and morphological characteristics showed that the isolates were *Bacillus cereus, Bacillus mycoides, Bacillus thuringiensis, Bacillus subtilis* and *Citrobacter farmeri*. In a study, 6 different soil sample were collected from which 40 strain were isolated. Biochemical test showed that out of forty strains 24 were *Bacillus subtilis, Bacillus megaterium, Bacillus firmus, Bacillus sphaericus* and *Bacillus thuringiensis* [15]. Keratinase producing bacterial strains were identified by gram staining and spore staining [16].

Gram staining technique has been performed for further confirmation which showed that all strains were Gram positive except one strain which was Gram negative. DNA of each strain was isolated, further gene amplification was done by PCR. 16S rRNA gene sequencing was performed for further identification. Phylogenetic investigations are integral to many areas of research in biological fields and commonly include the identifying homologous sequence; their related multiple alignments, the phylogenetic recreation and the graphical representation of derived phylogenetic tree [17]. In a previous study *Bacillus* species were identified by 16S rRNA gene sequencing. Results showed the differences of *Bacillus anthracis* from *Bacillus cereus* and *Bacillus thuringiensis* [18].

It was observed that the concentration and purity of the DNA samples was pretty enough to go for sequencing. The Sanger method was used to carry out sequencing in the Laboratories in Pakistan. After two weeks, results were obtained. The DNA sequences

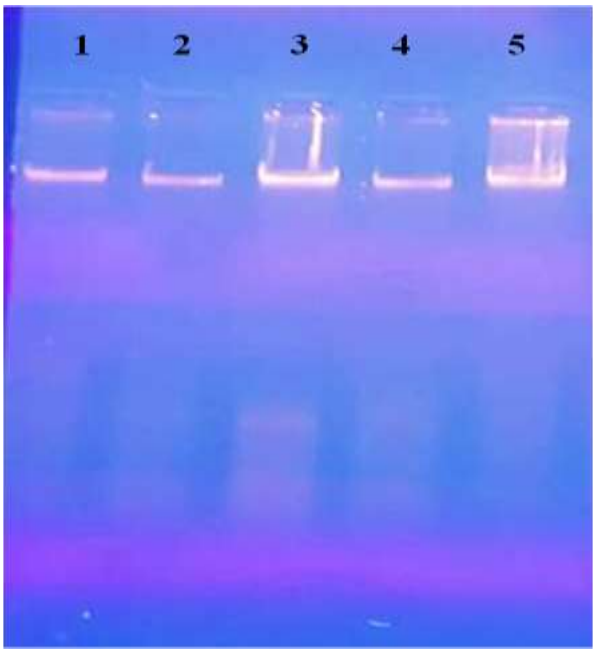

Fig. 1: Lane 1: Genomic DNA of *Bacillus cereus*; Lane 2: DNA of *Bacillus thuringiensis*; Lane 3: DNA of *Bacillus mycoides*; Lane 4: DNA of *Bacillus subtilis*; Lane 5: DNA of *Citrobacter farmeri*.

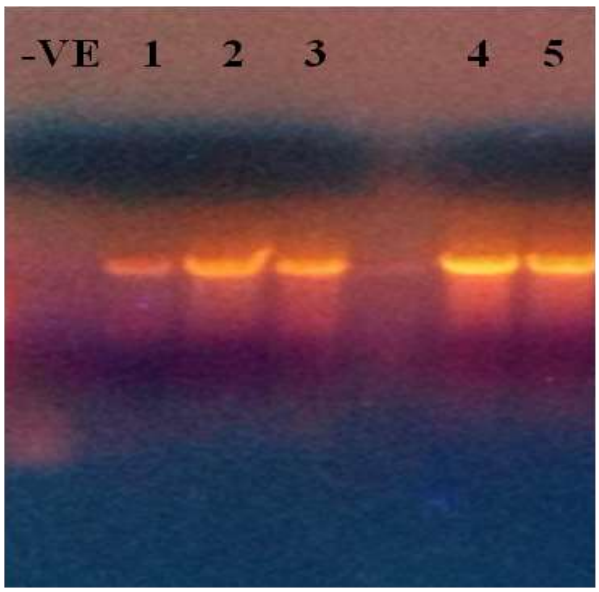

Fig. 2: Agarose gel electrophoresis analysis shows the PCR amplification of gene. Lane 1: Amplified gene of *Bacillus cereus*. Lane 2: Amplified gene of *Bacillus thuringiensis.* Lane 3: Amplified gene of *Bacillus mycoides*. Lane 4: Amplified gene of *Bacillus subtilis.* Lane 5: Amplified gene of *Citrobacter farmeri.*

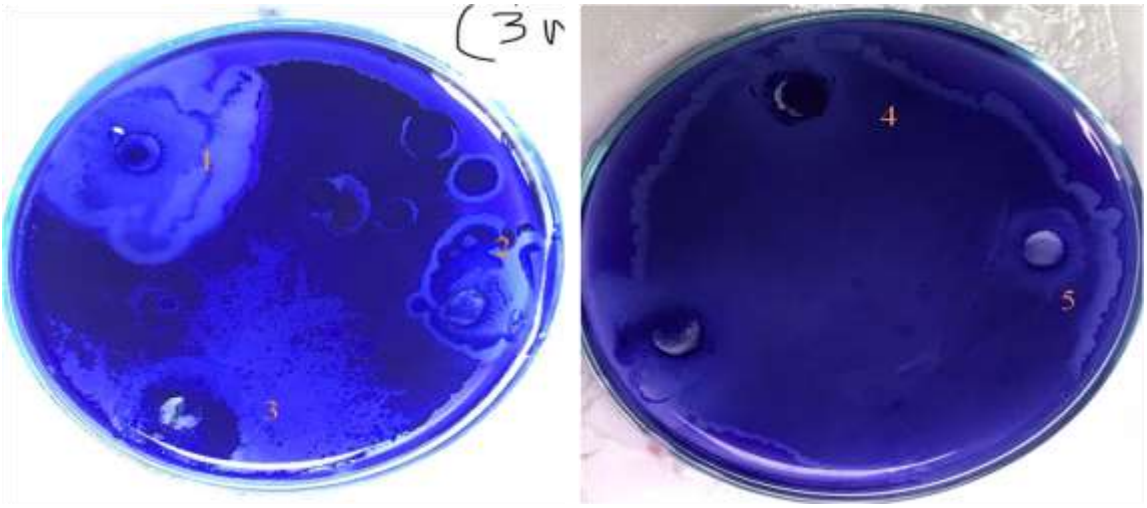

Fig. 3: Test to check keratinase production bacterial isolates

were then opened using online tool Basic Logical Alignment Search Tool (BLAST). However, it is important to clean the DNA sequence by omitting and replacing unsuitable bases which result due to probable errors during the method. The already reported sequences which are present in NCBI were further used to compare the DNA sequences using nBLAST so that the isolates could be confirmed and to check their further reactions towards the production of enzyme keratinase. Sequencing results showed that four strains were Gram positive, Bacilli and one strain was Gram negative, *Citrobacter farmeri*.

Enzyme assay for keratinase was performed to see the presence of keratinase in all strains. Keratin was used as a substrate. 1% keratin was added into nutrient agar medium. The media was autoclaved at 121°C for 20 min. at 15psi. The media was poured in plates, when the media got solidified, 3 wells in the media gel were formed with sterile tips on each petri plates. 25µL of each strain was added in separate well. The plates

were incubated at 37°C for 2 days. After 2 days plates were stained with Coomassie brilliant blue and de-staining was performed afterwards. Keratinase producing strains were isolated and enzyme assay was performed. Light blue patterns or clear zones around the wells were seen in *Bacillus cereus* and *Bacillus thuringiensis* strain which show that keratinase enzyme was present in these strains which hydrolyze the keratin. Another more or less same study was conducted in which the screening of protease producing bacteria was carried out. Skimmed milk media was added in culture plates and clear zones were seen in plates which show the hydrolysis of protein by protease [19]. Keratinase enzyme is used in leather industry for dehairing purpose and it has different industrial applications. In leather industry, sodium sulfide chemical is used for dehairing which is very dangerous for health and causes pollution so as an alternative keratinase enzyme is better option because it does not cause pollution and is relatively safe for health. Bacterial strains contain the keratinase enzyme. The keratinase enzyme can be produced from different strains in larger amount economically which is a cheaper and easier way.

Conclusion

Present study concludes the significant role of *Bacillus* species in the production of keratinase enzyme. In this study, five different bacterial strains have been isolated from soil. Out of five *Bacillus cereus* and *Bacillus thuringienesis* contain keratinase enzyme. A Keratinase enzyme is very important in leather industry for dehairing. Keratinase enzyme is non pollutant and economical.

Conflict of interest

The authors declare no conflict of interest.

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