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ISOLATION, IDENTIFICATION AND MOLECULAR CHARACTERIZATION OF THE MOST PREVALENT BACILLUS BACTERIA IN DECOMPOSITION OF MUNICIPAL SOLID WASTE

Ayesha Ameen*[1,2], Neelma Munir[1], Jalil Ahmad[3] and Shahid Raza[2]

[1]Lahore College for Woman University, Lahore, Pakistan.
[2]University of South Asia, Lahore Pakistan.
[3]Lahore Compost Pvt Ltd, Punjab, Pakistan.

***Corresponding Author: Ayesha Ameen**
Lahore College for Woman University, Lahore, Pakistan.

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ABSTRACT
Many bacterial strains degrade the organic waste. Many strains have shown best degradation ability. This study was performed to check the most prevalent bacteria in the municipal solid waste degradation which is used to make compost. The identification and molecular characterization of the most prevalent specie was done and phylogenetic tree was constructed by using 16s ribotyping and cloning of 16S rRNA gene. It was concluded that the Bacillus specie is the most dominant specie in composting and Bacillus cereus strains were actively degraded the organic waste.

KEYWORD: 16s ribotyping and 16S rRNA gene.

INTRODUCTION

Microorganisms work in the world's ecosystem and are the most important contributors in the biogeochemical cycles. There is a variety of beneficial microorganisms present in soil, they take active part in degradation, breakdown and in remediation of pollutants in result give soil fertility and better crop productivity. (Dileep et al., 2005; Weyens et al., 2009; Sindhu et al., 2011) the most important and major contributors are the lignocellulytic bacteria, they rapidly degrade the agriculture residues. The addition of cellulytic bacteria or fungi artificially is done to rapid the composting process. They have hydrolytic enzymes. The properties of microbes can be used as an indicator for composting process, besides the characteristics of control the composting process through physiochemical activities. Many bacterial strains are responsible for the degradation of organic waste. To select strains of bacteria for decomposition of organic waste, change of colour of compost pile, volume and weight reduction, pH, temperature and odour and the length of time of process should be checked. Many strains have shown the best activity in terms of volume and weight loss of compost pile (Zaved et al., 2008). The most prevalent microbes that are involve in the composting process are Bacillus Cereus, Bacillus Subtilis and White rot fungi (Torsvic et al., 1996; Rondon et al., 1999; Dees and Ghiorse, 2001). Aerobic bacteria are the most important in many kinds of bacteria, they start the decomposition process. There are different strains of bacteria involved in composting process e.g. thermophilic bacteria works at high temperature, mesophilic bacteria works at moderate temperature and psychrophilic works at lower temperature.

Methodology

Samples of organic waste were collected from Lahore compost Pvt Ltd Pakistan at thermophilic stage. The samples were free from any inert material.

Isolation of bacteria

The bacteria were isolated from the collected sample by dissolving 1 g of sample in 10ml of normal saline which was sterilized properly. The 1ml sample was taken and transferred in 10ml of LB broth and 24 hours incubation was given. The bacterial growth was judged by turbidity in LB broth. The 0.5ml of turbid solution was taken and streaked on agar plates.

Identification

The colonies having different morphology were separately streaked on Nutrient Agar plate for the purification. Once the colonies were purified, they were inoculated to check their haemolytic pattern on blood agar plates. The identification was performed by using microscope and biochemical characteristics.

Gram Staining

After the purification of bacterial isolates on the basis of their colony characteristics, microscopic study was performed to study the morphology and staining

characteristics of the bacterial isolates. The protocol for the gram staining is given below:

The bacteria was fixed on the slide by gentle warming and air drying. The crystal violet dye was added on the slide to stain cell wall of bacteria for 1 minute. The slide was washed with distilled water after 1 minute. Few drops of mordant dye iodine was added on slide for 1 minute. The slide was washed with acetone. A counter stain safranin was added for 1.5 minute at the end to mark bacteria. It was washed with distilled water and left at room temperature for drying (Clause, 1992). The slides were observed under the 100X oil emersion lens.

Spore staining

Spore staining of the purified isolates was performed as the protocol below:

The bacteria was fixed on the slide by gentle warming and air drying. The malachite green dye was added on the slide and the slide was heated until the fumes start. The slide was washed with distilled water and a few drops of counter stain safranin was added for 2 minutes. The slide was washed with distilled water again and left at room temperature for drying (Bartholomew and Mittwer, 1950).

Biochemical characterization

The biochemical test of the isolated bacteria were performed by using API 20 kit (API® Biomerieus) according to the instruction and protocol mentioned on the catalogue.

Molecular characterization

To further identify and molecular characterized isolates, PCR was performed by using universal primers for 16S ribotyping (Hegde et al., 2002).

Genomic DNA extraction

The DNA was extracted by culturing bacteria on agar plates for 24 hours. The solution of normal saline was prepared and autoclaved properly to eliminate any contamination. 10ml of sterilized normal saline solution was added on cultured agar plates. The cells of bacteria were harvested by using loop. The suspension having bacteria was taken in an Eppendorf's. The extraction of DNA was done by using phenol chloroform DNA extraction method. (Cheng and Jiang, 2006)

Agarose gel electrophoresis

The analysis of the extracted DNA was performed by loading on 1% gel. The 1% gel was made in 0.5X TAE buffer from 50X TAE stock buffer then 2µl of ethidium bromide was added to the gel after dissolving it. The sample were prepared by adding 2µl of 6X bromophenol blue dye, 2µl of DNA and 2µl of distilled water. Prepared samples were loaded and run on the gel then gel was visualized under UV transiluminator.

PCR amplification for 16S rRNA gene

For the amplification of 16S rRNA gene, PCR was performed with final volume of 50ul. All the PCR components were mixed in the following concentration for 50ul reaction mixture: 1X buffer A 5µl, 4µl $MgCl_2$, 4µl dNTPs, 0.06µl Taq polymerase and 1µl forward and reverse primers. The volume was made up to 50µl by adding sterile water. The amplification of 16S rRNA gene was performed in Mycycler^{TM} thermal cycler (Williams JF, 1989).

PCR reaction mixed contained

Table 1: Reaction mixture of PCR for 16S rRNA amplification

Sr. No	Reagents	Stock	Required	Final concentration
1	PCR buffer	10X	1X	5µl
2	Mgcl2	25mM	2-2.5mM	4µl
3	Genomic DNA	50ng	25ng	2µl
4	Forward primer	10.0mM	0.24µM	1µl
5	Reverse primer	10.0mM	0.24µM	1µl
6	Taq polymerase	5 units/ul	0.15 units	0.06µl
7	Genomic DNA	50ng	25ng	2µl
8	dH2O	50µl	8µl	Final volume.25µl
9	dNTPs	25mM	2.5mM	4µl

Universal Primers to amplify 16S rRNA gene

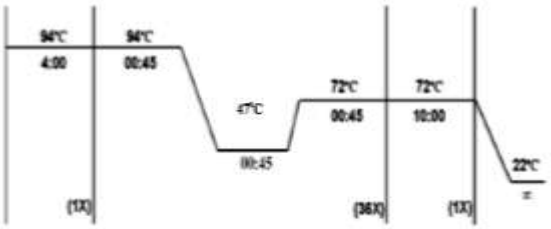
The following pair of universal primer were designed to amplify the 16S rRNA gene (Hedge et al., 2002).

Table 2: Primers used in PCR amplification

Sr.No	Gene Name	Primer Name	Nucleotide sequence
1.	16S rRNA	M13-F	5''GTTTTCCCAGTCACGACGTTG 3'
2.	16S rRNA	M13-R	5'TGAGCGGATAACAATTTCACACAG 3'

PCR profile for amplification of 16S rRNA gene

The denaturation initially was achieved at 94^0C for 4 minutes. The optimized profile of PCR had an annealing temperature less than 10^0C to melting temperature of the used primers. It was followed by 37 cycles of denaturation for 45 sec, the annealing was performed at 47^0C for 40 seconds and the extension time was given of 45 seconds at 72^0C so that the all unfinished products can be extended, the final extension at 72^0C for 10 minutes was given at the end.



Agarose Gel electrophoresis

1.5% agarose gel was prepared to analyse the PCR results. The 4µl of PCR product was mixed with 2µl of distilled water and 3µl of xylene cyanole loading dye (Johansson, 1972).

Purification of PCR product

PCR product was cut from the 1.5% agarose gel carefully and placed in eppendorfs. The purification was performed by using Thermo scientific Gene Jet Gel Extraction kit. The 700ul of binding buffer L3 was added in an eppendorf and put in the water bath at 50ºC for ten minutes along vortexing the eppendorfs in every 3 minutes to dissolve the gel.

The whole solution was transferred in to purification column and the column was centrifuged at 12000 rpm for 1 minute. The flow through was collected in the column and discarded. Then 500ul of W1 wash buffer was added in the column and again centrifuged for 1 minute at 12000 rpm. The flow through in the column was discarded. To remove any residue left of wash buffer, the column was centrifuged again. The column was placed into a new sterile eppendorf. The E1 Elution buffer 50ul was added in eppendorf and left at room temperature for 2 minutes and centrifuged at 12000 rpm for 1 minute. The flow through was saved containing pure gene.

To check the gene purification. The 1.5% gel was run and sample was prepared by taking 5ul of purified gene, 2µl Xylene cyanole loading dye and 2µl distilled water. The gel was visualized under UV transilluminator. The purified gene was stored at -20ºC.

Cloning of 16S rRNA gene

The purified gene was ligated in to TA vector and the vector was transformed into the DH5-α strain of E.coli to clone it. (Johnson et al., 2006)

Ligation Reaction

The ligation reaction was made by mixing purified gene product: 8µl, TA vector: 2µl, Ligase enzyme: 1µl and ligation buffer: 4µl. The total volume of ligation reaction was 20µl.

Table 3: ligation reaction mixture for the cloning purified gene

Sr.No	Reagents	Stock	Required
1	Ligase enzyme	T4	1µl
2	TA vector	25ng/µl	3µl
3	Ligation buffer	5X	4µl
4	PCR product	50ul	10µl
5	Distilled water	2µl	Made vol. 18µl

DH5-α competent cells preparation

A single colony of DH5-α was picked and inoculated in 10ml of LB broth. The flask was placed in incubator along with shaking for 24 hours. 1ml of inoculum from the 1oml culture was taken and added in 40ml of LB broth then the 40ml flask was placed in incubator for 1 hour at 37ºC till the adjusted to $O.D_{550}$ = 0.4.The inoculum was shifted in sterile falcon and placed on ice for 5 minutes. The falcon tube containing culture was centrifuged at 4ºC at 5000 rpm for 5 minutes. The Supernatant was collected in the laminar flow cabinet and discarded then 20ml of 50mM ice chilled $CaCl_2$ was added in to it. Pallet was dissolved. Incubated the falcon on ice for 40 minutes. Again centrifuged at 4ºC at 5000 rpm for 5 minutes, the supernatant was again collected and discarded. The pallet was re-suspended in 2 ml ice chilled $CaCl_2$. The prepared competent cells were stored on ice for 24 hours. (Chung et al., 1989)

Transformation of rRNA gene in DH_5-α

From the prepared competent cells 200µl was taken and added in the eppendorf containing 20µl of ligation mixture. The eppendorf was incubated on ice for 40 min and the mixture was then heat shocked in water bath for 2 min at 42^o C. The mixture was incubated again on ice for 5 min after heat shock. The 800ul of LB broth was taken in the Eppendorf contains ligation mixture and competent cells and incubated at 37ºC for 1.5 hours. The Ampicillin /IPTG/X-Gal/Agar plates were prepared and 50µl of transformation mixture was spreaded on plates. Then the plates were incubated at 37ºC for 24hours (Nakata et al., 1997)

Plasmid isolation of cloned colonies

Miniprep was performed by using PureLink Quick Plasmid Miniprep Kit by life technologies.

A single colony of transformed DH_5-α harboring TA plasmid was inoculated in 5ml LB broth having 50ug/ml kanamycin and was placed in shaking incubator at 37°C for overnight. The bacterial suspension was centrifuged to harvest the bacterial cells at 5000 rpm for 10 minutes at room temperature and the supernatant was discarded. 250µl resuspension buffer (R3) was added and vortexed to get homogenous mixture. Then the 250µl of lysis

buffer (L3) was added and mixed it by gently inverting the eppendorf. The mixture vial was incubated at room temperature for 5-10 minutes. After incubation 350µl of resuspension buffer (N4) was added and mixed thoroughly by inverting the tube. The suspension was centrifuged at 12000 rpm for 10 minutes. Supernatant was shifted to the spin column and centrifuged at 12000 rpm for 1 min and flow-throw was discarded. The (W10) 500µl wash buffer to column was added and incubated at room temperature for 1-2minutes. Centrifuged the column and discarded the flow-throw. Then 700ul wash buffer (W9) was added in column and centrifuged and decanted the flow-throw. Placed the spin column to a new sterile eppendorf tube. Plasmid was eluted by adding 75µl of TE buffer and incubated at room temperature for 1 min. centrifuged the column at 12000 rpm for 2 minutes. Removed column from eppendorf tube and stored it at -20ºC for further use. Visualized the isolated plasmid on 1.5% agarose gel. The plasmid having 16S RNA gene was sent to 1st base Malaysia for sequencing.

RESULTS AND DISCUSSIONS

The bacteria capable of degrading organic waste were isolated and characterized. The isolated bacterial strains were purified to identify the most prevalent specie of bacillus bacteria.

Isolation of bacteria

The sample collected from Lahore compost was used as a source of finding bacteria with efficient ability to degrade bio waste. The initial identification was based on colony characteristics, biochemical tests and molecular characterization. Fujio and kume, (1991) reported that the Bacillus bacteria is the most dominant in composting process in their study on sewage sludge compost. The Bacillus specie is identified as the most dominant specie in the degradation of organic waste.

Colony morphology

The morphology of colony was observed on simple nutrient agar plate. The bacterial colonies were irregular in shape. Colony morphology was observed on Nutrient agar. The colonies were further purified on agar by morphology. The bacterial isolates were irregular in form. The color of colonies was white (Figure 1). The bacterial isolates showed different hemolytic pattern on blood agar. One bacteria showed Beta hemolysis after 24 hours of incubation and the other one showed Beta hemolysis after 2-3 days for preservation. (Figure 2) the morphology characters of bacterial isolates are given in the table 1. Ohgiwari et al., (1992) isolated Bacillus by colony morphology and it was irregular in form and colony colour was also white.

Figure 1: Growth pattern of isolates on nutrient agar

Figure 2: Hemolytic pattern of isolates on blood agar

Microscopy

To check the staining characteristics, gram staining was performed. The results of gram staining showed rod shaped bacteria, gram positive. The bacteria also formed spore, the results of gram staining showed terminal spores (Figure 3 and 4). The microscopic characteristics are summarized in the table 1(Gregersen et al., 1978) identified gram positive rods that had terminal spore as Bacillus.

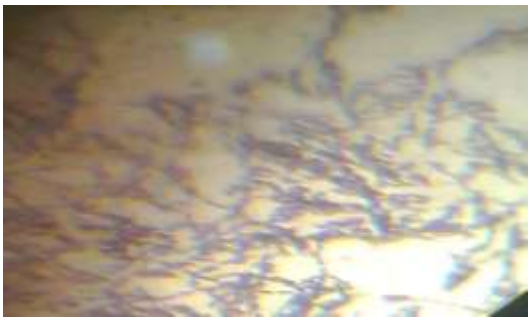


Figure 3: Gram staining of bacillus 1

Figure 4: Gram staining of bacillus 2

The macroscopic and microscopic characteristics of the Bacillus 1 and Bacillus 2 observed are summarized in the table below:

Table. No: 4 Characteristics of Bacillus 1 and 2

Characteristic	**Strains**	
	Bacillus 1	**Bacillus 2**
Colony Characteristics		
Surface	Smooth	smooth
Color	White	White
shape	irregular	irregular
Morphology		
Straight Rods	-	+
Rods in chain	+	-
Gram stain	Purple	purple
Spore Position	Terminal	Terminal
Hemolysis	Complete hemolysis when grow	Beta hemolytic but showed hemolysis when preserved in refrigerator

Biochemical characterization

The biochemical characteristics of the bacterial isolates were checked by using API 20 kit (API® Biomerieus)**.** The results of API 20 kit are given below in table 2.

Table. No: 5 Biochemical characterization of gram positive bacteria

Biochemical test	**Observation Bacillus 1**	**Observation Bacillus 2**
VP	-/+	+
Citrate	+	+
Catalase	+	+
Glucose	+	+
Lactose	-	-
Xylose	-	-
Arabinose	-	-

Molecular characterization

The isolates after morphological differentiation and purification were molecular characterized by DNA isolation, PCR amplification and sequencing of the 16S rRNA gene.

Extraction of genomic DNA and PCR amplification of 16S rRNA

The DNA was isolated from the two bacterial isolates by phenol chloroform extraction method (Catheriene et al., 2009) and their 16S rRNA gene were amplified by using universal primers. The amplified products were analysed using gel electrophoresis method. The products were run on 1.5% agarose gel and 400bp product size were obtained after amplification on the agarose gel. 1Kb plus DNA ladder (Invitrogen USA) was used with 250ug unit (Figure 6). The Purification of the PCR products was performed by using QIAquick® kit (Invitrogen USA). The purified PCR product was checked by using gel electrophoresis. The M13 universal primers used for amplification, the sequence of primers is given below (Hegde et al., 2002).

Primer Name	**Nucleotide sequence**	**PCR product Size**
M13-F	5’′GTTTTCCCAGTCACGACGTTG 3’	400bp
M13-R	5’TGAGCGGATAACAATTTCACACAG 3’	

Figure: 5 Shows the extracted DNA molecule on 1% agarose gel

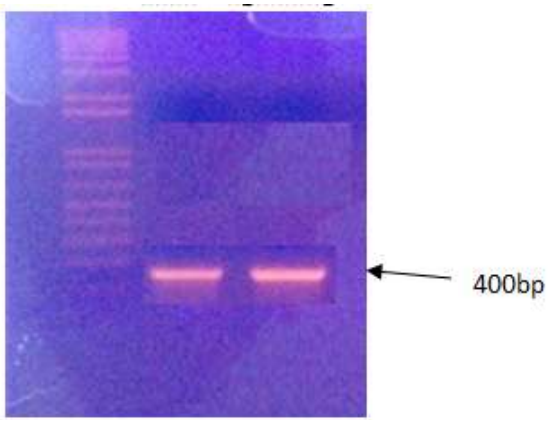


Figure: 6 Results of PCR Reaction: Lane 1: 1 Kb DNA Ladder, Lane 2: PCR product of 16s rRNA. (Invitrogen, cat. 10787-018)

Sequencing of amplified 16S rRNA

The sequencing of the isolates was performed by using BigDye® Terminator Sequencing Kit by 1st base sequencing Malaysia. The sequencing results were analysed by using chromaslite. These sequences were Blast by using NCBI nucleotide blast (Figure 4.7 and 4.8). The results showed both isolates bacteria belong to Bacillus cereus. These are two different strains of Bacillus Cereus. Phylogenetic tree analysis were performed by using T- Coffee software (figure 9) which showed the close lineage of the isolated bacteria with Bacillus cereus strains KJ833790.1 KR002672.1.

Figure 9 Phylogenetic tree of Bacillus 1 and 2

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