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Isolation and Characterization of Polysaccharide-Degrading Microbes from Compost Samples

ABSTRACT

This study isolated and identified novel microbial polysaccharide degrading species involved in composting process. Bacteria were isolated from different composting samples from Lahore Compost Private Limited, Lahore, Pakistan. Physico-chemical analysis of compost samples made from municipal waste were collected at 15-day interval during the composting process. A total of 55 bacteria were isolated and identified using morphological and biochemical characteristics. Out of 55 isolates, 26 mesophilic and 14 thermophilic had cellulose degrading potential. Out of 26 mesophyllic bacterial isolates, 20 were found cellulolytic. The isolated bacteria and fungi were identified morphologically and biochemically. The highest potential to degrade cellulose was recorded from four Bacillus strains. Molecular characterization of potential cellulolytic bacteria by 16S rRNA was performed. Bacillus sp. were found as the most prevalent cellulolytic bacteria in composting process. Fungi were also isolated and characterized morphologically and microscopically following techniques. The potential cellulolytic fungal isolates were Aspergillus fumigatus, Mucor sp., Saccharomyces sp., and Aspergillus niger. The results of this study would be helpful in highlighting the potential role of different microbes involved in enhancement of the composting process. These microbes can be used for the preparation of microbial inoculum based on their polysaccharide cellulose, bacteria, composting degrading ability.

Keywords: *cellulose, bacteria, composting, fungi*

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INTRODUCTION

Due to the hazardous effects of chemical fertilizers that pose serious threats to human health, biofertilizers have gained attention during the recent years. Aside from their role in food safety, being environment-friendly and cost-effective are among the significant advantages of biofertilizers. Compost, a mixture of incompletely disintegrated carbon-based matter from plant and animal sources (*Piet et al. 1990*), is a type of biofertilizer.

Compost may be produced by bacteria and fungi that have the potential to consume cellulose as their energy source, hence, they can be utilized for the transformation of green waste into useful fertilizers. This microbial flora is the main component that facilitate the composting processes (*Taiwo and Oso 2004*).

Manufacturing of compost has been a significant strategy for the conversion of municipal solid waste. Much work is done on compost however, little is known about the microflora present during the different stages of composting. Monitoring of the microbial progression is important for the successful management of composting as microorganisms are essential in the process (*Ryckeboer et al. 2003*).

Composting process consists of three stages on the basis of different temperature, moisture content at different days, i.e., mesophilic stage, thermophilic stage and maturation stage. Microbial diversity varies with the change in temperature (*Ryckeboer et al. 2003*). Length of composting process depends on the composition or mixture of materials to be composted, kind of microorganisms present, temperature, moisture, composting containers, and room or any venue where composting should be done (*Palaniveloo et al., 2020*).

C N^{-1} ratio is an indicator of compost maturity. It has been reported that with an increase in composting time,

there is a decrease in C:N of composts (*Iqbal et al. 2010*). Bacteria and fungi utilize carbon as their sole energy source and nitrogen for the protein production. Litter consists of different compounds like carbohydrates, celluloses, hemicelluloses, fats, and proteins that are broken down into CO_2 and H_2O (*Ghanim et al. 2016*).

This decease in C:N is due to the fact that during the process of composting there is a decrease in carbohydte content and an inrease in protein content (*Hidayanti et al. 2013*). Molecular characterization by 16S rRNA is an effective method for identification of microorganisms present in several compost samples (*Muyzer and Smalla 1998*).

This study aimed to isolate bacteria and fungi that have the ability to degrade polysaccharides, the major constituent of waste. Hence, this study is a step forward in the isolation and identification of bacteria and fungi that are responsible for the degradation of cellulose present in the municipal and agriculture wastes.

MATERIALS AND METHODS

Municipal waste compost samples were collected from Lahore Compost Pvt. Ltd Pakistan. Samples were taken from 15 different windrows. One windrow as control in which the waste is present at day 1. Samples were collected from 7 windrows at mesophilic (1 month) and 7 at thermophilic (two months) stages of composting. Samples were taken in 3 replicates per sample. Samples were placed in polythene ziplock bags and stored in a refrigerator at 4ºC for isolation and identification.

Figure 1. Compost window for the collection of samples after 1 month composting.

Isolation and Screening of Bacteria

Compost samples were dissolved in autoclaved distilled water and the serial dilutions of the suspension spread on nutrient agar media. Carboxymethyl cellulose (CMC) medium was used as selective bacteriological media and supplemented with 0.1 g $NaNO_3$, 0.5 g CMC, 0.05 g $MgSO_4$, 0.1 g KCL, 0.1 g K_2HPO_4 , 1.5 g Agar and 0.05 g Yeast extract (*Khokhar et al. 2013*). All the ingredients were mixed in a sterilized flask and dissolved in 100 mL of distilled water (*Kasana et al. 2008*). The pH of the liquid medium was adjusted to pH 7 using 1N NaOH and 1M HCl. The liquid medium was sterilized in an autoclave set at 121 psi for 20 min. When the sterilized medium cooled down, this was poured into Petri plates in a laminar flow hood.

Colonies observed after 48 hours suspected to be cellulose-degrading bacteria were purified by re-streaking on Petri plates filled with CMC agar medium. Plates were incubated at 37ºC and 60ºC for 24 h. Isolated colonies were preserved on CMC slants, covered by paraffin oil for short term preservation, whereas for long term preservation glycerol stock was prepared and stored at 4ºC.

Screening of Cellulose Degrading Potential of Isolates

Each bacterial isolate was estimated for high cellulolytic activity by Congo red assay (*Sharrock 1988*). Colonies were streaked on CMC media and incubated at 37ºC for 48 h. Petri plates were filled with 0.1 % aqueous solution of Congo red reagent and left for 20 min. Plates were destained with 1 M NaCl solution and discarded after 15 min. The diameter of the clear zone was measured, which showed the cellulose hydrolyzing ability of each bacterium.

Identification of cellulose-degrading bacterial strain

Morphological characters of the bacterial isolate, such as colony shape, elevation, surface color, and colony count were observed and compared with Bergey's Manual of Determinative Bacteriology (*Buchanan and Gibbons 1986*). Microscopic characteristics of bacteria were also determined by Gram staining protocol and motility test. Biochemical characterization was performed by catalase and oxidase tests. Catalase test was performed by inoculating a bacterial colony from culture plate in hydrogen peroxide solution. Tetra-methyl-p-phenylenediamine dihydrochloride (TMPD) was reduced to deep purple color by the bacteria. Oxidase test was

performed by soaking filter paper with TMPD reagent. Then bacterial colony was inoculated with an inoculating loop onto the filter paper disc.

Isolation of Cellulolytic Fungi

Carboxymethyl cellulose medium was used as selective fungal media and supplemented with 0.03 g urea, 0.14 g $(NH_s)_2SO_4$, 0.2g KH_2PO_4, 0.03 g $CaCl_2$, 0.03 g $MgSO_4$, 0.025 g yeast extract and 0.075 g proteose peptone with 1 g of CMC and 1.75 g agar (*Mandels 1974*). All ingredients were dissolved in 100 mL of distilled water and autoclaved (*Khokhar et al. 2013*).

One week old fungal colony grown on potato dextrose agar (PDA) plate was cut along with media and inoculated in the center of the basal media plates. The plates were incubated at 37ºC for seven days. Cellulolytic fungal species were selected on the basis of the diameter of the hydrolysis zone surrounding the colonies.

Screening Cellulolytic-Degrading Fungi

Congo reagent assay was done to determine the cellulolytic activity of fungi. Fungal plates were stained with 1% Congo red dye for 30 min. Plates were de-stained with 1 M NaCl solution for 20 min. Clear zones were formed around colonies of potential cellulolytic fungal strains. The diameter of the clear zone was measured which showed the cellulose hydrolyzing ability of each fungus. Colonies with the largest diameter of clearance zone have the highest cellulolytic activity.

Identification of Fungi

The fungal isolates cultured on PDA were identified on the basis of colony morphology such as, color, texture and size, morphology of conidiophore, hyphae and shape of conidial head, as well as shape and size of spores (*Anastasi et al. 2005*). Fungi were identified microscopically after staining with methylene blue..

Molecular Characterization of Cellulolytic Bacteria

The bacterial strains which were showing highest cellulolytic activity were isolated and selected for molecular characterization by using 16S ribosomal DNA sequencing technique using forward 5′GTTTTCCCAGTCACGACGTTG 3′ and reverse 5′TGAGCGGATAACAATTTCACACAG 3′ primers. Bacterial genomic DNA was extracted by following Sambrook methodology where phenol chloroform method was used. The extracted DNA was analyzed on gel by using gel electrophoresis technique. Gel was observed under UV transilluminator. The genomic DNA was amplified by using polymerase chain reaction (PCR) machine (Thermo scientific) under optimized PCR conditions. The sequencing was achieved on automated genetic analyzer (Applied Biosystems; 3100 DNA Analyzer). Sequencing results were analyzed by using Chromas Lite version 2.1. The FASTA sequence was retrieved and BLAST was performed by inserting this sequence into query box using NCBI BLAST tool.

Determination of Physiochemical Properties of compost

The physicochemical properties of 15 compost samples, i.e., pH, temperature, moisture content and C N^{-1} ratio were, determined. Carbon content of different compost samples was determined by following combustion method. Nitrogen content of compost samples at day 30 and 60 was determined by Kjeldahl method (*Nahm 2001*). Oxygen and temperature of compost samples were determined by using Diemesta Oxygen Temperature meter (OT-21) with 1 m probe. The pH of compost was determined by Hanna pH meter by preparing dilution sample (1:10 compost to water).

Statistical Analysis

The data for physico-chemical characteristics were analyzed using SPSS version 9. Duncan Multiple range test was employed to find out significance.

RESULTS AND DISCUSSION

Compost acts as soil conditioner and is used to enhance the soil fertility and quality of crops (*Sarkar et al. 2016*). The identification and use of microbes which are appropriate for composting a particular substrate is a matter of concern to the scientists during the recent years (*Ryckeboer et al. 2003*).

Bacteria isolated from all compost samples cultured at 37ºC were mostly Gram-positive rods (**Figure 2**). Out of the 44 strains isolated, only 4% strains were Gram-positive cocci while 95% strains were Gram-positive rods. Only 40% strains were non-motile while 59% strains were highly motile. Composting process was facilitated by the presence of microbes (*Ryckeboer et al. 2003*).

Composting process consisted of three stages (*Bernal et al. 1998*). Many microbial communities prevail during different phases of composting in which all the communities dominate at their feasible temperature of growth. Microbial flora

present in the earlier stage of composting prepared substrates for microbial communities of secondary stage of composting (cross feeding) (*Davis et al. 1992*). The increase in temperature of composting involves shift of mesophilic organisms towards thermophilic stage (*Mckinley and Vestal 1984; Mckinley 1985; Ryckeboer et al. 2003*).

Screening for Cellulolytic Bacteria

Bacteria isolated at 37ºC and at 60ºC were screened based on the formed halo zone on CMC plates (**Figure 3**) where 77% bacteria were cellulolytic.

Molecular Characterization

The four bacterial strains with highest cellulolytic activity were isolated and selected for molecular characterizations by using 16S ribosomal RNA sequencing technique (**Figure 4**) and the amplified DNA was also presented (**Figure 5**). The product was sequenced and it was found that most prevalent cellulolytic strains belong to *Bacillus* sp. For the identification of the bacterial isolates, the technique is based on DNA sequence variations present in PCR-amplified 16S rRNA genes is of great importance (*Rastogi et al . 2011*).

Isolation of Cellulolytic Fungi from Different Compost Samples

The four different fungal colonies isolated and identified based on colony morphology and microscopic characteristics were *Aspergillus fumigatus*, *Mucor* sp., *Saccharomyces* sp. and *Aspergillus niger* (**Figure 6**). All the fungal strains were cellulolytic.

Physicochemical Analysis of Compost Samples

Different physicochemical characteristics of compost

Figure 2. Bacterial growth on nutrient agar media.

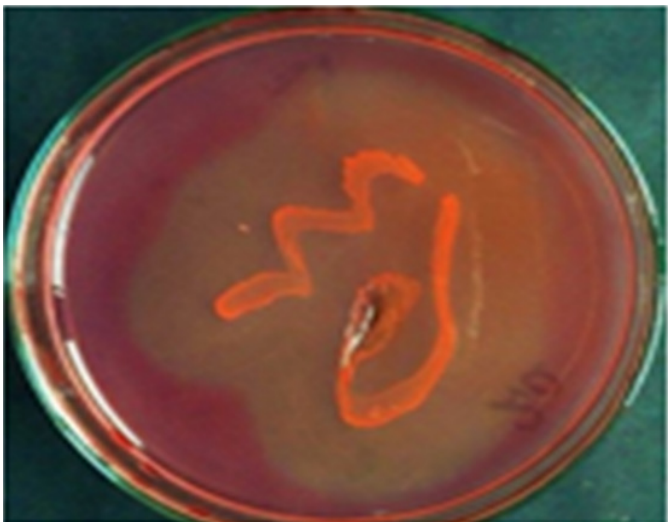

Figure 3. Halo zone formed by cellulolytic bacteria due to cellulysis showing isolates with the widest, medium, small and no clear zones.

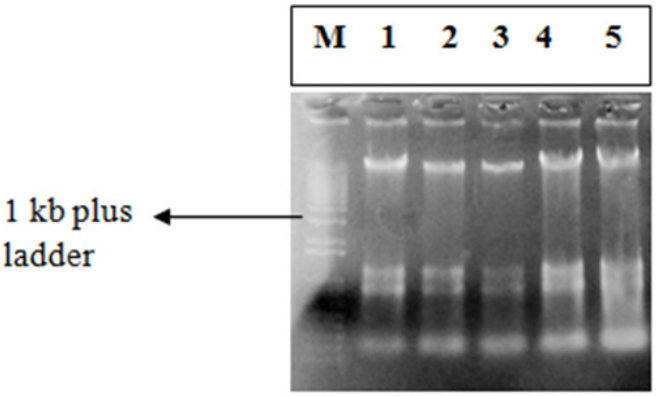

Figure 4. Agarose gel electrophoresis (1%) of bacterial genomic DNA. Lane M: 1 kb plus ladder. Lane 1 and 2: genomic DNA of most cellulytic bacteria.

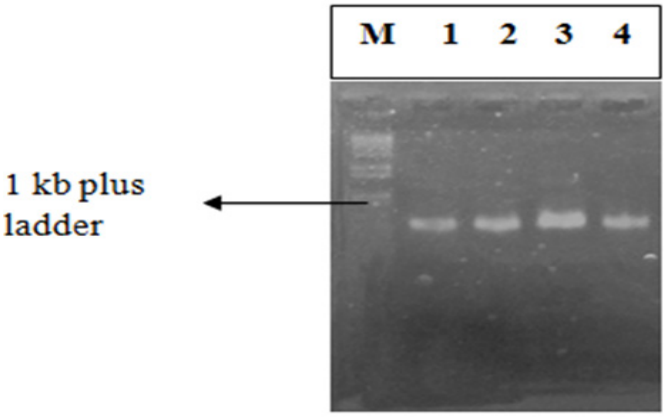

Figure 5. Agarose gel electrophoresis of (1%) Amplification of PCR product. Lane M:1 kb plus ladder. Lane 1, Lane2, Lane 3 Lane 4: PCR Product of most prevalent cellulose degrading bacteria.

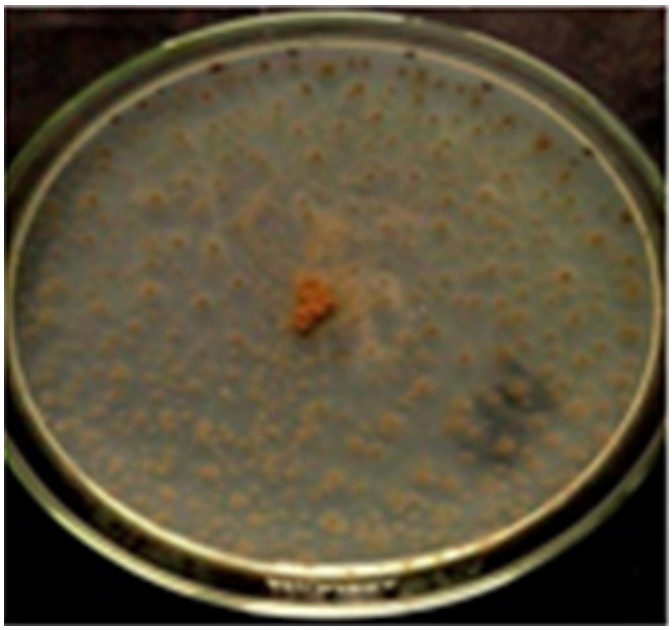

Figure 6. Fungal growth on cellulolytic media indicating cellulose-degrading potential.

samples were analyzed such as temperature, pH, organic content, carbon content, nitrogen content and C:N (**Table 1**).

The mean difference was significant at the level of 0.05 indicated the standard deviation among the three parallel replicates in each column. Values having different letters in the same column were significantly different ($P<0.05$) according to Duncan's multiple range test.

Comparison of Temperature of Composting Samples Collected at Different Stages

The highest temperature was recorded in sample 7 (56.6°C) while lowest temperature was recorded in sample 14 (43.9°C). The compost piles had variabletemperature at different stages. The samples collected at early stage (sample 8) of composting had a higher temperature while compost piles showed a decrease in temperature when reached maturity (*McKinley and Vestal 1984*).

Comparison of pH of Composting Samples Collected at Different Stages

There was a variation in mean pH values of the samples. The sample which exhibited lowest pH value was samples 1 and 5 which was 6.9. The highest pH value was recorded in sample 11 (7.9).

The pH of the composting samples remained neutral to slightly basic. The pH in the current experiment ranged from 6.9 to 7.9. As indicated in earlier reports, the pH becomes acidic at the end of composting (*Pan et al. 2013*).

Comparison of Organic Contents of Composting Samples Collected at Different Stages

At beginning of the composting process, the organic content was high, which showed a decline during the progress of composting process. The highest organic content was recorded in sample 1 (45.3) and the lowest was measured in sample 13 (40.8).

Organic contents of the composting samples were higher at the start and decreased gradually from 45.3 to 40.8. No absolute value of organic content is reported for best quality compost with values ranging from 30-70% (*Herity and Lorraine 2003*).

Table 1. Physicochemical characteristics of compost samples collected from windrows at different stages of composting.

Samples	Temperature (°C)	pH	Organic content	Carbon g 100 g^{-1}	Nitrogen g 100 g^{-1}	C/N ratio
1	35.22±0.3^{g}	5.43±0.05^{e}	25.3±0.2^{a}	25.3±0.1^{a}	0.93±0.05^{bc}	30.2±0.1^{a}
2	45.2±0.3^{g}	6.93±0.05^{e}	45.3±0.2^{a}	26.3±0.1^{a}	0.93±0.05^{bc}	29.2±0.1^{a}
3	49.9±0.1^{de}	7.03±0.05^{de}	43.9±0.05^{cd}	25.5±0.0^{cd}	0.93±0.05^{bc}	28.2±0.05^{b}
4	52.2±0.3^{bc}	7.00±0.1^{de}	44.3±0.5^{bc}	25.7±0.3^{bc}	0.93±0.05^{bc}	28.2±0.1^{b}
5	51.3±0.5^{c}	7.10±0.1^{cd}	44.1±0.2^{ab}	25.6±0.1^{ab}	0.93±0.05^{abc}	28.2±0.1^{c}
6	52.6±0.5^{b}	7.03±0.05^{de}	43.5±0.5^{de}	25.2±0.2^{de}	0.93±0.05^{a}	28.0±0.3^{de}
7	55.8±0.2^{a}	7.23±0.05^{c}	43.0±0.05^{ef}	25.0±0.001^{ef}	0.93±0.05^{bc}	27.7±0.05^{c}
8	56.6±0.5^{a}	7.13±0.05^{cd}	43.0±0.05^{ef}	25.0±0.001^{ef}	0.93±0.05^{bc}	27.7±0.05^{c}
9	55.6±0.5^{a}	6.93±0.05^{e}	42.7±0.1^{fg}	24.7±0.05^{fg}	0.90±0.001^{c}	27.4±0.05^{c}
10	50.3±0.5^{d}	7.06±0.05^{de}	42.8±0.2^{fg}	24.8±0.1^{fg}	0.90±0.001^{ab}	27.9±0.1^{bc}
11	49.1±0.1^{e}	7.53±0.05^{b}	41.1±0.05^{h}	23.8±0.05^{h}	0.90±0.001^{c}	26.5±0.1^{f}
12	48.9±0.1^{e}	7.93±0.05^{a}	42.2±0.1^{g}	24.4±0.05^{g}	0.90±0.001^{c}	27.2±0.05^{d}
13	47.7±0.3^{f}	7.06±0.05^{de}	41.0±0.1^{j}	23.5±0.4^{j}	0.93±0.05 ^{ab}	26.5±0.05^{f}
14	45.5±0.4^{g}	7.23±0.05^{c}	40.8±0.1^{i}	23.7±0.1^{i}	0.93±0.05 ^{ab}	26.7±0.05^{ef}
15	43.9±0.9^{h}	7.10±0.1^{cd}	41.1±0.2^{k}	23.8±0.1k	0.90±0.001^{c}	25.8±0.5^{bc}

Comparison of Carbon Content of Compost Samples at Different Composting Days

There was a variation in the means of carbon contents of all different compost samples. The highest carbon content was recorded in sample 1 (26.5 g 100 g^{-1} compost), while the lowest was carbon content was recorded in sample No.12 (23.5).

Comparison of Nitrogen Content of Compost Samples at Different Composting Days

The highest nitrogen content was recorded in sample 1, 2, 4, 5, 6, 7, 12 and 13, which was 1.0 g $100g^{-1}$ compost. The lowest nitrogen content was recorded in sample 8, 9, 10, 11 and 14, which was 0.9 . It was evident that nitrogen content of compost varies randomly during whole composting process. It ranged from 0.9 to 1.0. Earlier research have reported that nitrogen is an important component for plant growth. The normal range of nitrogen for good quality compost is 1.0-3.0% (*Barker 1997*).

Comparison of C: N Ratio of Compost Samples at Different Composting Days

There was variation in the means of C:N of all different compost samples. The highest C:N was recorded in sample 1 (29.2). The lowest C:N was recorded in sample 10 and 13 (26.5). C:N of compost samples was greater at the start of composting process. It ranged from 25.8 to 29.2. It is reported from earlier research work that C:N is not a test itself but it is the estimation of organically bound carbon with total nitrogen so these results indicate a variation in the C:N ratio during composting process (*Pan et al. 2013*).

CONCLUSIONS AND RECOMMENDATIONS

Based on this study, *Bacillus* sp. are the most prevalent polysaccharide-degrading bacteria. Among the fungal isolates, *Aspergillus niger*, *Saccharomyces*, *Aspergillus fumigatus*, *Mucor* sp. were potential cellulolytic strains. As polysaccharides, including cellulose, are abundant in the agricultural and household waste, the study was important to highlight the potential role of different microbes involved in the composting process. These microbes can be used for the preparation of microbial inoculum to enhance the process of composting that may have diverse uses, such as compost inoculants, enzyme production, and biological control agents.

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