

Original Research Article

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## Characterization of Cellulolytic Microorganisms Associated with Naturally Decomposing Waste Material

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### ABSTRACT

Thirty four samples of naturally decomposing waste material were collected from rural and urban parts of Western Maharashtra from which 64 bacteria, 49 fungi and 63 actinomycetes strains were isolated. The isolates were screened for cellulolytic activity on Czapek's mineral salt agar media amended with carboxymethyl cellulose. Seventeen bacteria, thirty fungi and fifty seven actinomycetes isolates showed zone clearance indicating cellulase enzyme activity. Functional potential of most efficient cellulolytic microorganisms which include seventeen bacteria, twenty fungi and nineteen actinomycetes isolates was confirmed by endoglucanase assay. Among bacterial isolates, highest endoglucanase activity was recorded in isolate B-30 (0.130 U/ml), among fungal isolates the maximum activity was shown by isolate F-13 (0.099 U/ml) while among actinomycetes isolates maximum enzyme activity was shown by isolate A-41 (0.204 U/ml). The cellulolytic microorganisms were identified on the basis of characterization. Based on cultural, morphological and biochemical characteristics, bacterial isolates belonged to *Bacillus*, *Pseudomonas*, *Cellulomonas*, *Staphylococcus* and *Micrococcus* genera, where *Bacillus* was most predominant group. On the basis of colonial morphology and microscopic observations the cellulolytic fungi identified were *Aspergillus terreus*, *Aspergillus flavus* and *Aspergillus niger*, while cellulolytic actinomycetes were identified as *Streptomyces* spp. on the basis of spore chain morphology and microscopic observations.

#### Keywords

Cellulolytic microorganisms, Carboxymethyl cellulase, Endoglucanase, Characterization

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### Introduction

Microbial degradation of organic wastes plays a vital role in carbon recycling. For a long-range solution to resource problems of natural fertilizers, organic matter is available in large quantities in the form of agricultural wastes, forest residues and urban wastes. The undervalued organic waste can be converted to value-added products by using native cellulolytic microorganisms. Organic matter

decomposition is carried out by various microorganisms including bacteria, fungi and actinomycetes. Different communities of microorganisms predominate during the various composting phases. There is practically no substance existing in nature that is not used by one microorganism or another. It is therefore necessary to identify the microorganisms present in the different processes, as several different species of microbes are usually involved. These

microorganisms are also important to maintain nutrient flows from one system to another and to minimize ecological imbalance (Novinsak *et al.*, 2008). Initial decomposition is carried by mesophilic microorganisms, which rapidly biodegrade the soluble and easily degradable compounds. As temperature increases, thermophiles take over. Temperature during decomposition process follows a pattern of rapid increase to 49°C to 60°C within 24 to 72 hours and is maintained for several weeks.

This is the active phase of decomposition, in which easily degradable compounds and oxygen are consumed, pathogens *viz.*, *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis*, *Clostridium botulinum* and weed seeds are killed, and phytotoxins are eliminated. As the active composting phase subsides, temperature gradually declines to around 38°C and mesophilic microorganisms once again take over the other types of microorganisms and the curing phase begins (Fourti *et al.*, 2008). Microbes play a key role as degraders during the composting process, and the microbiology of composting has been studied for decades, but there are still open questions regarding the microbiota in composting processes. Microorganisms that populate substrates during composting reflect the evolution and the performance of the composting process. Their metabolic paths lead to significant changes in the physical and chemical parameters of the composting substrate, and that, in turn, leads to changes in the microbial community structure. Hence, research was undertaken to study the cellulolytic microorganisms associated with naturally decomposing wastes.

## **Materials and Methods**

### **Isolation of microorganisms**

A total of 34 samples of naturally decomposing wastes were collected in paper

bags from different places of 10 districts of Western Maharashtra. Isolation of bacteria, fungi and actinomycetes was carried out on specific medium i.e. nutrient agar medium, potato dextrose agar medium and starch casein agar medium, respectively by serial dilution and plating technique. Single isolated colonies of microorganisms were picked up, numbered and maintained on the respective agar medium for further studies after ascertaining their purity.

### **Screening of cellulolytic microorganisms**

A preliminary qualitative analysis for cellulolytic activity was conducted following the protocol of Hankin and Anagnostakis (1977).

The microorganisms were grown in duplicate on cellulose amended Czapek's mineral salt agar medium. To inoculate solid media, the microorganisms were suspended in 5 ml distilled water in test tubes. Drop of each suspension was applied to the surface of solidified test media. The inoculated plates were incubated at 28±2°C in BOD incubator for 6 days. At the end of incubation, to visualize the hydrolysis zone, the agar medium was flooded with an aqueous solution of Congo red (1% w/v) for 15 min. Congo red solution was then poured off, and the plates were further treated by flooding with 1M NaCl solution for 15 min. The ratio of the clear zone diameter to colony diameter was measured. The largest ratio was assumed to contain the highest cellulase activity.

### **Endoglucanase assay**

Cellulase activity of most efficient 17 bacteria, 20 fungi and 19 actinomycetes isolates was confirmed by endoglucanase assay as described by Miller *et al.*, (1960) and Mandels and Weber (1969). The selected isolates were grown in Czapek's mineral salt broth at 28 ± 2

°C in incubator with stirrer. The supernatant of culture broth centrifuged at 5000 rpm for 20 min. at 4 °C served as enzyme source. The culture filtrate 0.5 ml was added to 0.5 ml of 1% carboxymethyl cellulose sodium salt prepared in 0.05M sodium citrate buffer (pH 4.8) in a test tube.

The mixture was incubated at 50 °C for 30 min. Reaction was terminated by adding 3.0 ml dinitrosalicylic acid reagent subsequently placing the reagent tubes in water bath at 100 °C for 5 min. After boiling, the tubes were transferred immediately to a cold water bath. Distilled water 20 ml was added to the tubes and mixed by completely inverting the tubes several times.

Glucose standard solution was used instead of the sample simultaneously for preparing glucose standard curve. The colour so formed was measured against spectro zero at 540 nm. The enzyme activity was expressed in units as the amount of enzyme required to release 1 µmol of reducing sugar as glucose equivalent min/g of the enzyme sample.

### **Characterization and identification of cellulolytic microorganisms**

The selected seventeen cellulolytic bacterial isolates were identified on the basis of morphological and biochemical characterization as per the standard procedures and keys given by Bartholomew and Mittewer (1950), Cappuccino and Sherman (1992) and Anonymous (1957). Twenty cellulolytic fungal isolates were identified on the basis of cultural and microscopic features as described by Subramanian (1971), Barnett and Hunter (1972) and Aneja (2003). Nineteen cellulolytic actinomycetes were identified on the basis of morphological and biochemical characterization according to the procedures and keys by Waksman (1961), Lechevalier (1989) and Cappuccino and Sherman (1992).

## **Results and Discussion**

### **Isolation of microorganisms**

The population of bacteria, fungi and actinomycetes varied from 0.3 to 42.0 x 10<sup>5</sup>, 0.0 to 17.0 x 10<sup>5</sup>, 0.0 to 12.0 x 10<sup>5</sup> cfu/g of dry matter, respectively in different waste samples. Thirty four samples yielded 64 bacteria, 49 fungi and 63 actinomycetes isolates. The pure culture of isolates was stored at 4 °C in refrigerator and used as per necessity of work.

### **Screening of cellulolytic microorganisms**

Cellulolytic microorganisms create a clearing zone around the colony on cellulose amended media. A higher production of cellulase when carboxymethyl cellulose (CMC) served as substrate may be as a result of induction of the enzyme, since cellulase is known to be universal inducer of cellulose synthesis. Several research workers (Teather and wood, 1982; Hendricks *et al.*, 1995; Ariffin *et al.*, 2006) screened the microorganisms for cellulolytic activity on the basis of zone clearing ability.

Out of 64 bacterial isolates only 17 isolates showed zone clearing indicating cellulase enzyme activity. Among the cellulolytic bacteria maximum cellulase activity was in isolate B-30 (3.48 cm) followed by isolate B-28 (2.98 cm). These results are in conformity with the results of Lu *et al.*, (2005) who screened fifteen mesophilic bacteria which showed the ability to develop clearing zones around their colonies ranging from 2.55 to 6.40 cm. Similar results have also been reported by Ariffin *et al.*, (2006), Shankar *et al.*, (2011) and Majidi *et al.*, (2011).

Out of 49 fungal isolates tested, 30 isolates were able to hydrolyze cellulose. Maximum cellulase activity was shown by isolate F-13

and F-24 (1.18 cm). Ray and Rath ((2007) found that two fungi viz., *T. viride* and *T. harzianum* showed higher activity with 6.4 and 6.2 cm clearance zone, respectively. Gautam *et al.*, (2010) recorded cellulolytic activity of 87 fungal isolates ranging from 1.5 to 4.7 cm. The work of Shahriarinnour *et al.*, (2011) also supports the above results.

Sixty three actinomycetes isolates were screened for cellulase activity, out of which 57 isolates showed zone clearance ability. The cellulase activity of cellulolytic actinomycetes ranged from 1.05 to 7.78 cm. The maximum cellulase activity was shown by isolate A-41 (7.78 cm) followed by isolate A-40 (5.30 cm).

Jeffrey and Azrizal (2007) screened a total of 282 isolates of actinomycetes for cellulase activity, out of which 106 isolates gave positive results, with the ratio of halo zones to colony diameter between 3.00 and 4.60 cm. Similar results have been reported by El-Sersy *et al.*, (2010).

### **Endoglucanase assay**

The assay for endoglucanase activity is based on the ability of the cellulase enzyme produced by the strains to hydrolyze CMC to reducing sugars. Ezekiel *et al.*, (2010) revealed that CMC medium was a more favorable medium for the induction of endoglucanase enzyme in most of the isolates. The most efficient 17 bacteria, 20 fungi and 19 actinomycetes isolates were studied for endoglucanase activity (Table 1).

In the present studies, it was found that all the selected bacterial isolates were positive for endoglucanase assay. The endoglucanase activity ranged from 0.034 to 0.130 U/ml. Highest endoglucanase activity was found in isolate B-37 (0.130 U/ml) followed by B-28 (0.121 U/ml). Ariffin *et al.*, (2008) studied endoglucanase production of aerobic

bacterium *Bacillus pumilus* EB3 and activity recorded was 0.076 U/ml. Acharya and Chaudhary (2011) estimated endoglucanase activity of two bacteria *Bacillus licheniformis* and *Bacillus* sp. and recorded activity 0.388 and 0.342 U/ml, respectively. Gupta *et al.*, (2012) estimated endoglucanase activity of eight isolates of cellulose-degrading bacteria which ranged from 0.162 to 0.400 U/ml.

All the selected 20 fungal isolates were also found positive for endoglucanase activity. The endoglucanase activity shown by isolates ranged from 0.012 to 0.099 U/ml.

The maximum activity was shown by isolate F-13 (0.099 U/ml), followed by F-24 (0.087 U/ml). Present results are in conformity with the results of Jahangeer *et al.*, (2005) who studied endoglucanase assay of 6 *Aspergillus* isolates and reported that the activity ranged from 0.640 to 1.240 U/ml. Gautam *et al.*, (2010) estimated the endoglucanase activity of fungal strains where maximum activity recorded was 0.976 U/ml.

All the selected isolates of actinomycetes were positive for endoglucanase activity. The endoglucanase activity of isolates ranged from 0.068 to 0.204 U/ml. The maximum enzyme activity was shown by isolate A-41 (0.204 U/ml) followed by isolate A-40 (0.192 U/ml). Jaradat *et al.*, (2008) reported that *Streptomyces* sp. strain J2 showed highest endoglucanase activity of 0.432 U/ml. Golinska and Dahm (2011) analyzed endoglucanase activity of 20 strains of actinomycetes.

The results ranged from 0.007 to 0.128 U/ml. Singh and Kapoor (2013) reported that endoglucanase activity of efficient isolate of *Streptomyces* sp. MSC702 was 0.010 U/ml. The present results are thus, in conformity with the work done by earlier research workers.

**Table.1** Endoglucanase activity of selected cellulolytic microorganisms

Bacterial isolates		Fungal isolates		Actinomycetes isolates	
Isolate No.	Endoglucanase activity (U/ml)	Isolate No.	Endoglucanase activity (U/ml)	Isolate No.	Endoglucanase activity (U/ml)
B-4	0.088	F-3	0.036	A-4	0.145
B-11	0.060	F-4	0.050	A-15	0.167
B-13	0.056	F-9	0.025	A-17	0.160
B-15	0.055	F-12	0.022	A-18	0.126
B-24	0.075	F-13	0.099	A-20	0.090
B-26	0.106	F-14	0.030	A-22	0.165
B-27	0.108	F-16	0.044	A-24	0.172
B-28	0.121	F-17	0.027	A-29	0.137
B-30	0.130	F-18	0.017	A-35	0.179
B-37	0.034	F-19	0.022	A-38	0.132
B-38	0.085	F-24	0.087	A-40	0.192
B-42	0.044	F-25	0.030	A-41	0.204
B-46	0.067	F-27	0.039	A-42	0.103
B-51	0.079	F-31	0.047	A-46	0.140
B-53	0.049	F-32	0.060	A-47	0.068
B-57	0.040	F-33	0.051	A-48	0.124
B-64	0.105	F-34	0.054	A-52	0.133
		F-35	0.052	A-62	0.144
		F-37	0.027	A-63	0.103
		F-44	0.012		

**Table.3** Morphological and biochemical characteristics of cellulolytic actinomycetes

Isolate No.	Aerial mycelium Colour	Spore chain morphology	H <sub>2</sub> S production
A-4	Grey	Retinaculiaperti, spores in short chains and individuals	+
A-15	Grey	Rectiflexibles, spores in long chains	-
A-17	White	Rectiflexibles, spores in long chains	-
A-18	Whitish grey	Retinaculiaperti, spores in long chains with open loops	-
A-20	White	Retinaculiaperti, spores in long chains with open loops	-
A-22	Whitish grey	Rectiflexibles, spores in long chains	+
A-24	White	Rectiflexibles, spores in long chains	-
A-29	Grey	Retinaculiaperti, spores in long chains with open loops	-
A-35	Grey	Retinaculiaperti, spores in long chains with open loops	-
A-38	Grey	Retinaculiaperti, spores in long chains with open loops	-
A-40	Whitish grey	Retinaculiaperti, spores in short chains	-
A-41	Grey	Rectiflexibles, spores in short chains	+
A-42	Light grey	Retinaculiaperti, spores in long chains with loops	-
A-46	Grey	Rectiflexibles, spores in short chains	-
A-47	Grey	Retinaculiaperti, spores in long chains with loops	-
A-48	Grey	Retinaculiaperti, spores in long chains with loops	-
A-52	Yellowish white	Rectiflexibles, spores in long chains	+
A-62	Pinkish white	Retinaculiaperti, spores in long chains with loops	-
A-63	grey	Retinaculiaperti, spores in long chains with loops	-

All isolates were positive for gram reaction, catalase, oxidase and gelatin liquefaction tests while negative for acid fast staining

**Table.2** Morphological and biochemical characteristics of cellulolytic bacteria

Isolate No	Colony morphology			Gram reaction	Endospore staining	Cell shape	Size (µm)	Citrate test	Oxidase test	Motility test	Identification
	Colour	Shape	Elevation								
B-4	Creamy white	Punctiform	Raised	Positive	Negative	Oval to round	0.9-1.1	-	+	+	<i>Cellulomonas</i>
B-11	Light brown	Circular	Flat	Positive	Positive	Rod chain and pair	0.7-0.8 x 3.6-3.8	-	+	+	<i>Bacillus</i>
B-13	Light brown	Circular	Flat	Positive	Positive	Rod chain and pair	0.7-0.9 x 3.7-3.9	-	+	+	<i>Bacillus</i>
B-15	Light brown	Circular	Flat	Positive	Positive	Rod chain and pair	0.6-0.8 x 3.4-3.6	+	+	+	<i>Bacillus</i>
B-24	Yellow	Punctiform	Raised	Negative	Negative	Rod isolated	0.4-0.6 x 0.9-1.1	+	+	-	<i>Pseudomonas</i>
B-26	Yellow	Punctiform	Raised	Negative	Negative	Rod isolated	0.4-0.5 x 1.0-1.5	-	+	-	<i>Pseudomonas</i>
B-27	Light brown	Circular	Flat	Positive	Positive	Rod chain and pair	0.7-0.8 x 3.4-3.6	+	+	+	<i>Bacillus</i>
B-28	Light brown	Circular	Flat	Positive	Positive	Rod chain and pair	0.8-0.9 x 4.0-4.2	-	+	+	<i>Bacillus</i>
B-30	Creamy white	Circular	Raised	Positive	Negative	Cocci	1.0-1.2	+	-	-	<i>Staphylococcus</i>
B-37	Light brown	Circular	Flat	Positive	Positive	Rod chain and pair	0.6-0.8 x 3.3-3.6	-	+	+	<i>Bacillus</i>
B-38	Light brown	Circular	Flat	Positive	Positive	Rod chain and pair	0.7-0.9 x 3.4-3.6	-	+	+	<i>Bacillus</i>
B-42	Light brown	Circular	Raised	Positive	Positive	Rod chain and pair	0.8-0.9 x 4.0-4.2	-	+	+	<i>Bacillus</i>
B-46	Light brown	Circular	Flat	Positive	Positive	Rod chain and pair	0.5-0.7 x 3.2-3.6	-	+	+	<i>Bacillus</i>
B-51	Light brown	Circular	Umbonate	Positive	Positive	Rod chain and pair	0.6-0.8 x 3.0-3.2	-	+	+	<i>Bacillus</i>
B-53	Pale yellow	Punctiform	Flat	Positive	Negative	Cocci clusters	0.7-0.9	-	-	-	<i>Micrococcus</i>
B-57	Light brown	Circular	Flat	Positive	Positive	Rod chain and pair	0.6-0.9 x 4.3-4.6	-	+	+	<i>Bacillus</i>
B-64	Yellow	Punctiform	Raised	Negative	Negative	Rod chain and pair	0.5-0.6 x 1.0-1.4	-	+	-	<i>Pseudomonas</i>

All the isolates were negative for indole production, MR-VP, H<sub>2</sub>S and phenylalanine deaminase test, while positive for catalase test



### **Characterization and identification of cellulolytic microorganisms**

The distinct variation in cell morphology among the different bacterial isolates was noticed (Table 2). Out of the 17 isolates, 11 produced circular, light brown colored and flat colonies. These isolates were rod shaped in pairs and chains with size ranging from 0.5-0.7 x 3.2-3.6 to 0.8-0.9 x 4.0-4.2  $\mu\text{m}$  and were positive for motility test, oxidase test, catalase test, gram reaction and endospore staining. These isolates were identified as *Bacillus* spp and was the most predominant genera. Three colonies were yellowish, raised and punctiform in shape. These isolates were isolated and rod shaped with size ranging from 0.4-0.6 x 0.9-1.1 to 0.4-0.5 x 1.0-1.5  $\mu\text{m}$  and were negative for motility test, gram reaction and endospore staining. These isolates were identified as *Pseudomonas* spp. The colony of isolate identified as *Cellulomonas* sp. was creamy white, punctiform and with raised elevation. The isolate was oval to round in shape with size 0.9 to 1.1  $\mu\text{m}$  in diameter, positive for gram reaction, motility test, catalase test and oxidase test. The colony of isolate identified as *Staphylococcus* sp. was creamy white, circular and with raised elevation. The isolate was cocci with size 1.0 to 1.2  $\mu\text{m}$  in diameter, positive for gram reaction and catalase test, while negative for motility and oxidase test. The single colony of isolate identified as *Micrococcus* sp. was pale yellow, punctiform with flat elevation. The isolate was cocci in clusters with size 0.7 to 0.9  $\mu\text{m}$  in diameter, positive for gram reaction and catalase test, while negative for motility and oxidase test. All the 17 bacterial isolates were negative for indole production, methyl red test, Voges-Proskauer test, H<sub>2</sub>S test and phenyl-alanine deaminase test. Several workers (Shiva Reddy *et al.*, 2010; Mirdamadian *et al.*, 2011; Otajevwo *et al.*, 2011) reported similar characteristics.

Twenty fungal isolates were identified by microscopic observations and colony morphology. Three distinct groups of *Aspergillus* isolates were formed. The first group comprised of ten isolates producing Cinnamon-buff to sand brown colour colony on potato dextrose agar media. Hyphae were colourless while conidial heads compact, columnar and biseriate. These isolates were identified as *Aspergillus terreus*. Second group comprised of five isolates having yellow colony at first which quickly become bright to dark yellow green with age. Hyphae in this group were colourless, conidial head typically radiate, biseriate. These isolates were identified as *Aspergillus flavus*. Third group comprised of five isolates having velvety textured colony with white mycelium spreading rapidly with dense layer of dark brown to black spores. Hyphae were colourless, conidial head biseriate, large, globose, dark brown, becoming radiate and tend to split into several loose columns with age. This group belonged to *Aspergillus niger*. Several research workers (Jahangeer *et al.*, 2005; Rathnan *et al.*, 2012; Naveenkumar and Thippeswamy, 2013; Adeyemo *et al.*, 2013) identified *Aspergillus* on the basis of routine cultural and morphological characteristics and noted similar characters.

All the nineteen actinomycetes isolates studied were gram positive and non-acid fast in reaction (Table 3). There were 14 isolates having grey to whitish grey aerial mycelium colour, 3 isolates were whitish in colour while 2 isolates were yellowish white and pinkish white in colour. Spores of aerial mycelium and substrate mycelium in all the isolates were in chain and hence identified as *Streptomyces* spp. Twelve isolates were retinaculiaperti in spore chain morphology and 7 isolates were rectiflexibles. All the 19 isolates showed good to excellent growth on starch casein agar medium. The present results are in conformity with those of Oskay

*et al.*, (2004) and Reddy *et al.*, (2011) who reported that actinomycetes are filamentous rod shaped bacteria, gram positive but nonacid fast in reaction. The observations of several research workers (Krishna Kumari *et al.*, 2006; Nonoh *et al.*, 2010; Mythili and Ayyappa Das, 2011) supports the noted characteristics of *Streptomyces* sp.

Considerable population of bacteria, fungi and actinomycetes is associated with the naturally decomposing waste material. *Bacillus*, *Pseudomonas*, *Cellulomonas*, *Staphylococcus* and *Micrococcus* genera was found associated with decomposing material, where *Bacillus* was most predominant group while among fungi, *Aspergillus* spp. was predominant. *Streptomyces* spp was the predominant genera of actinomycetes associated with decomposition process. Microbes play a significant role in decomposition of organic waste, hence these cellulolytic microorganisms can be exploited for enhancing the decomposition rate in composting piles.

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