Characterization and Phylogenetic Diversity of Carboxymethyl Cellulase Producing *Bacillus* Species from a Landfill Ecosystem

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Abstract Total population of cellulose degrading bacteria was studied in a landfill ecosystem as a part of microbial diversity study. Samples were obtained from 3 and 5 feet depth of a local landfill being operated for past 10 years. Among many isolates, 22 bacterial strains were selected based on their capability to decompose carboxymethyl cellulose (CMC). These isolates were cultivated on agar medium with CMC as the carbon source. All isolates were Gram positive, endospore forming and alkalophilic bacteria with optimum growth pH 9–10. They were grouped based on the phenotypic and chemotaxonomic characters and representative strains of different groups along with high carboxymethyl cellulase (CMCase) producing strains were included for further characterization. Analysis of 16S rRNA gene indicated that these strains belong to different species of the genus *Bacillus*. Maximum CMCase activity of 4.8 U/ml at 50°C was obtained by strain LFC15. Results in the present study indicated the potential of waste land ecosystems such as landfill are potential source for isolation of industrially important microorganisms.

Keywords Landfill ecosystem · *Bacillus* · CMCase · 16S rRNA gene phylogeny

In recent years there is an increased interest in microbial diversity studies as little prokaryotic diversity is known from the total estimated $4\text{--}6 \times 10^{30}$ [1]. Among different ecosystems wasteland have become a subject of great

interest in recent past [2–4]. Landfills being the waste disposal area are considered to be more important due to their unique composition. Composition of landfill is differed with the kind of wastage added including grass, leaves and food waste. A typical landfill is constitute of approximately paper 40%, yard waste 17%, food waste 14%, glass 7%, metals 8%, plastics 8% and other refuse 12% [5]. However, Indian landfills differ in composition and the major ingredients found were plant litter, polythene and building waste material. Plant litter being major ingredient, is constituted of carbohydrate polymers such as cellulose and hemicellulose. Waste land soils were found to contain cellulolytic bacteria belonging to genera *Bacillus*, *Cellulomonas*, *Cytophaga*, *Pseudomonas*, *Sporocytophaga* and *Streptomyces* that were been isolated [6]. These cellulolytic bacteria had been reported under different conditions and isolated with potential activity on different forms of cellulose [7–9]. Although landfill contains different forms of cellulose, the microbial diversity of cellulolytic bacteria present in landfill ecosystem has not been much studied. In the present study we have made an attempt with an objective of quantifying cultivable cellulolytic bacteria and their phylogenetic diversity.

Samples were collected from a local landfill ecosystem from 3 and 5 feet subsurface environment. These were analyzed for aerobic and anaerobic carboxymethyl cellulase (CMCase) producing bacteria using CMC agar containing 0.5%, caboxymethyl cellulose; 0.1%, NaNO₃; 0.1%, K₂HPO₄; 0.1%, KCl; 0.05%, MgSO₄·7H₂O; 0.05%, yeast extract; 1.5% agar, pH 8.0 and for anaerobic cultivation 0.01% of resazurin was added in addition to the above and medium was boiled, purging with nitrogen gas. Colonies were screened with Congo red for CMCase production [10] and positive colonies were selected and preserved at –70°C. Strains were grown in nutrient broth with

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pH 8.0 to observe morphological features and biochemical tests [11]. Sodium carbonate and bicarbonate solutions were used to adjust pH of the medium to check the growth of strains in alkaline conditions. For fatty acid methyl esters extraction and analysis [12], cells were grown on Trypticase Soy Agar (TSA) medium at 30°C. Analysis was performed according to the instructions of the Microbial Identification (MIDI) system [13]. PCR amplification of the 16S rRNA gene was done using universal forward and reverse primers [14]. The sequencing was performed using an ABI PRISM Big Dye Terminator cycle sequencing kit (Applied Biosystems) and an automatic DNA sequencer (ABI PRISM model 3700). These sequences were compared with reference sequences available at NCBI GeneBank and aligned for phylogenetic analysis. The pair-wise evolutionary distances were computed using the DNADIST program with the Kimura 2-parameter model [15]. Neighbour-joining phylogenetic tree was constructed using TREECON (version 1.3b) [16]. The stability among the clades of a phylogenetic tree was assessed by taking 1,000

replicates. All sequences in the present study were submitted to NCBI GeneBank and the accession numbers are as follows: LFC2 (FN397904); LFC3 (FN397905); LFC9 (FN397906); LFC12 (FN397907); LFC15 (FN397908); LFC18 (FN397909). Enzyme production was done using isolation medium with 0.1% of peptone and 5% inoculum was used for enzyme production. A culture grown for 48 h was centrifuged at 10,000 rpm for 10 min at 4°C and the supernatant obtained was used as crude enzyme source. Activity was estimated as the amount of reducing sugar released using DNS method [17]. One unit of enzyme activity was defined as the amount of enzyme that released 1 µmol of glucose. To determine the temperature effect, crude CMCase enzyme was incubated at various temperatures range between 37 and 55°C along with substrate.

The total bacterial count was calculated in terms of number of CFU obtained and it was 5.4×10^6 and 6.4×10^5 cfu/g for 3 and 5 feet samples respectively. However, it was 11.5×10^4 and 20×10^4 cfu/g on CMC agar. Among the colonies obtained, 7 colonies from 3 feet

Table 1 Phenotypic characteristics of the landfill isolates from 3 feet and 5 feet samples

Property	Isolate																					
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Citrate utilization	+	–	–	–	+	+	–	+	+	–	+	+	–	–	+	+	–	–	+	–	–	+
Gelatin hydrolysis	+	+	+	–	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Esculin hydrolysis	+	+	–	–	–	+	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Starch hydrolysis	+	+	–	–	–	+	+	+	+	+	+	+	+	+	+	+	–	–	+	+	+	–
Casein hydrolysis	(+)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	+	+
Urea hydrolysis	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Indole	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Methyl red	+	+	+	+	+	–	+	(+)	–	–	–	–	–	–	+	+	+	+	+	+	+	+
Voges Proskaur	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Nitrate reduction	+	+	+	–	–	+	–	+	+	–	+	+	+	+	–	–	+	+	–	–	–	+
Growth at pH																						
8.0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
9.0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
10.0	+	+	(+)	+	+	+	+	(+)	+	–	+	+	–	–	+	+	+	+	+	–	–	+
11.0	+	+	–	+	+	+	+	–	+	–	–	–	–	–	+	–	+	+	+	–	–	+
Temperature (°C)																						
42	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
45	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	+
50	–	–	–	–	–	–	–	–	(+)	+	–	–	–	–	(+)					+	+	
NaCl (%)																						
2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4	+	+	(+)	+	+	(+)	+	(+)	(+)	+	–	+	+	+	+	+	+	(+)	+	+	+	+
6	+	+	–	(+)	(+)	–	–	–	–	+	–	+	+	+	+	(+)	(+)	(+)	(+)	(+)	+	+
8	+	+	–	(+)	(+)	–	–	–	–	+	–	+	+	+	+	(+)	(+)	(+)	(+)	(+)	(+)	(+)
10	(+)	(+)	–	–	–	–	–	–	–	+	–	+	(+)	+	+	–	–	–	–	–	–	(+)

+, positive; –, negative; (+), weak positive

samples and 15 colonies from 5 feet samples were selected. These 22 isolates were screened with Cong red for their CMCse enzyme activity. The results of phenotypic analysis indicated that all isolates were Gram-positive and sporulated with few differences in their characters (Table 1). Isolates from 5 feet sample were found to grow well at pH 9.0–10.0 than from 3 feet samples (Fig. 1). This may be due to adaptation of isolates to alkaline conditions that prevailed at 5 feet depth. All isolates found to be facultative anaerobic, their phenotypic characters and capability of spore formation affiliated them to genus *Bacillus*. These are found to be ubiquitous in nature as they are capable of surviving under stress conditions [18, 19] and had been observed in soils and wastelands [20, 21]. Isolates that exhibited identical phenotypic properties were grouped together and representative of each group was analyzed for their membrane fatty acid composition. Although they had ISOC15:0 and AIC15:0 as major fatty acids (Table 2), which are considered as FAME signatures of the genus *Bacillus*, they differed with nearest relatives in composition. Upon grouping the isolates further based on FAME analysis, six isolates including representative isolate from each group including high CMCse producers were subjected to 16S rRNA gene sequence analysis and enzyme studies. These six isolates exhibited optimum CMCse activity between 37 and 45°C, however, highest activity of 4.8 U/ml was observed at 50°C for the strain LFC15 (Fig. 2). Isolates from 5 feet samples showed enzyme activity at increased temperature.

Fig. 1 Growth of *Bacillus* isolates from landfill ecosystem at various pH conditions. All isolates were grown in CMC medium at 30°C temperature and shaking at 150 rpm for 24 h. Growth was measured spectrophotometrically at 600 nm. Strains LFC2 (filled diamond), LFC3 (filled small square), LFC9 (filled triangle), LFC12 (filled large square), LFC15 (six-spoked asterisk), LFC18 (filled circle)

Table 2 Total membrane fatty acid composition of the isolates

	14:0		15:0		16:0		17:0	
	Iso	14:0	Iso	Anteiso	Iso	16:0	Iso	Anteiso
LFC2	1.31	1.61	57.52	28.46	1.93	2.48	3.37	2.54
LFC3	5.15	2.86	34.57	38.19	1.77	7.73	2.33	3.37
LFC4	4.96	3.87	31.35	33.26	6.28	5.08	8.74	0.87
LFC7	4.59	2.04	39.67	37.31	1.35	4.23	2.65	3.50
LFC9	3.93	2.17	36.97	45.82	0.80	3.27	1.33	2.19
LFC10	–	–	20.46	41.62	–	7.36	13.94	16.62
LFC11	4.97	2.28	39.02	42.30	0.79	3.09	1.46	2.08
LFC12	–	–	19.50	39.19	5.35	5.26	13.99	16.71
LFC13	–	1.65	18.07	37.89	1.78	13.72	13.25	13.63
LFC15	–	–	19.32	38.53	5.39	5.38	13.77	17.60
LFC16	3.52	2.46	22.93	28.18	8.20	6.10	3.02	12.15
LFC18	3.51	–	40.64	41.33	–	6.97	3.25	4.31
LFC19	5.50	–	41.45	26.72	8.24	4.59	3.95	9.55
LFC20	1.46	2.01	53.97	22.96	3.04	6.01	7.00	3.54
LFC22	7.35	–	48.24	37.22	–	7.19	–	–

Fig. 2 Effect of temperature on crude CMCse activity produced by isolates. Strains LFC2 (filled diamond), LFC3 (filled small square), LFC9 (filled triangle), LFC12 (filled large square), LFC15 (six-spoked asterisk), LFC18 (filled circle)

The selected isolates were sequenced approximately 800 bp and further, BLAST analysis revealed that they belong to different species of genus *Bacillus* as many of them showed >99% similarity. Phylogenetic analysis assigned all isolates to various species of the genus *Bacillus*. Neighbour-joining phylogenetic tree indicated that isolates formed coherent clusters with the reference strains that were exhibited maximum similarity at 16S rRNA gene sequence (Fig. 3). Strain LFC15 had low similarity of 97.7% and exhibited differences in phenotypic properties with *B. pumilus*, indicating its probability of being novel species. *Bacillus* species were known to produce CMCse and reported to produce in 72 h of

Fig. 3 Neighbour-joining tree based on 16S rRNA gene (approx 800 bp) sequences showing the phylogenetic relationship between isolates and various species of genus *Bacillus*

incubation under optimal conditions [22, 23]. In contrast to this, in the present study our isolates produce the enzyme within 24–48 h of incubation in production medium with high activity at increased temperature. These findings indicate that ecosystems such as landfill are potential source for isolating industrially useful microbes.

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