Isolation and selection of cellulose-degrading microorganisms for utilization along with earthworms in efficient conversion of municipality waste mix to compost

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Municipality biowaste (MBW) was decomposed alone or in mixture with rice straw (RS) and cow dung (CD) using cultures of *Streptomyces xanthochomogens* (CDM9) and *Eisenia fetida*. Cellulose degrading microorganisms (CDMs) were isolated from six source materials which contained CDM population in the range 5.4–8.5 log cfu/gdb and their cellulolytic activity ranged from 0.0 to 0.431 IU/min. CDM9 showed consistently high cellulase activity in cellulose and MBW substrate, and its co-inoculation with earthworm enhanced decomposition of the MBW mix. A positive correlation between pH of the composting feed mix and N loss suggested N loss in form of NH_3. Overall, mixing of municipality biowaste with rice straw and cow dung and inoculation with the two decomposer agents resulted in better quality compost in all respects.

Keywords: Cellulose degrading microorganism, municipality biowaste, nitrogen loss, pathogenic and beneficial bacteria.

DIVERSE types of solid waste, including waste of municipality, agriculture and farm animals pose both problems and prospects in terms of disposal and conversion to useful bioinputs in agriculture[1–5]. Crop residues and animal waste mix are commonly composted for application during crop production[6–10]. However, substantial amount of nutrients in the mix may be lost during composting[11–13]. Reports indicate that N loss to the extent of 45.36–79.61%, P to the extent of 11.8–28.2% and K to the extent of 8–16% can occur during composting of organic residues[9,14,15]. Organic materials of biological origin in the municipality solid waste (MSW) are also important sources of compost, biogas and biofuel[16,17]. It is estimated that MSW is composed of 40–50% cellulose, 9–12% hemicelluloses and 10–15% lignin on a dry weight basis[18,19]. In many cities, much of the cellulosic waste biomass is often disposed-off by burning or landfill dumping, but such unscientific disposal causes an adverse impact on all components of the environment and human health[20]. Municipality biowaste (MBW) can also be converted to nutrient-rich compost[21,22]. Use of inocula of cellulose degrading microorganisms (CDMs) can convert cellulose-rich MBW to compost rapidly[23] and increase nutrient content in the final compost by reducing its loss during composting. Owing to dumping of this waste in the open, MBW and compost from it are likely to contain high levels of pathogenic microorganisms. On the other hand, crop residues including plant leaves and roots in organic solid waste of MSW may also contain beneficial microorganisms. Data on content of pathogenic bacteria (PB) in MBW-generated compost are limited[24,25], and there is no information on the level of beneficial bacteria (BB) in MBW compost. In general, MBW is alkaline in nature[19] and under conditions of high pH, loss of N may occur by the process of volatilization during composting[10,26]. Imphal city generates considerable amount of municipality waste[27,28] and rice fields around the city generate about 3×10^5 tonnes of rice residue annually[29,30]. Currently, the rice residue is burnt and municipality waste is disposed in the open leading to environmental problems. The objectives of this research were to (1) study decomposition of MBW alone and in mixture with RS and CD using inocula of superior CDMs with earthworm (EW), and (2) determine N loss from MBW, load of PB and BB and quantity of different size fractions in the final compost.

Materials and methods

Sample collection

In this study we wanted to use inoculum of efficient CDM isolates occurring naturally in the region in aerobic

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decomposition of MBW. Therefore, different organic materials, namely herbivore faeces such as cow dung, horse dung, goat dung, elephant dung and omnivore faeces such as cat dung, pig dung, plant sources like sugarcane trash, rice straw, shoot and root of water hyacinth, and field soils such as uncultivated upland soil, cultivated corn fields and swampy soil were collected from different areas (Nagamapal, Wahengleikai and Dharmasala) of Imphal city (24°44′0″N, 93°58′0″E). The elephant dung used was collected from Kaziranga National Park (26°33′N, 93°36′E), Assam, India.

Isolation of CDMs and selection of efficient cellulose-degrading microbial strains

CDMs were isolated by serial dilution of 1 g of the sample and plating appropriate dilutions on Omeliansky agar medium composed of 1.0 g KH_2PO_4, 1.0 g $MgSO_4$, 5.0 g cellulose, 2.0 g $CaCO_3$, 0.1 g NaCl, 15 g agar, l litre distilled water and at pH 7.18. The plates were incubated at 30°C for 48–72 h and colonies with distinct halo zone of clearance were purified and preserved in cryovial at –20°C for further study. This method isolated only aerobic CDMs.

Screening of efficient strains of the isolated cellulolytic microorganisms

Initially, cellulose degrading ability of the isolates was confirmed by qualitative test on carboxy methyl cellulose (CMC) agar with the following composition: 1.0 g $NH_4H_2PO_4$, 0.2 g KCl, 1.0 g $MgSO_4{\cdot}7H_2O$, 2 g cellulose, 15 g agar, 0.2 g congo red, 1 litre distilled water and at pH 6.8–7.2 (ref. 31). A loopful from individual slants of the pure culture was inoculated at the centre of the CMC plates and the plates were incubated at 30°C until substantial growth was observed. The petri plates were flooded with Congo red solution (0.1%), which was discarded after 5 min, and the plates were washed with 1 M NaCl solution and allowed to stand for 15–20 min. The clearing zone was observed around the colony when the enzyme had utilized the cellulose. Hydrolysis capacity (HC), which is the ratio of the diameter of clearing zone and colony was calculated.

Subsequently, efficient strains were screened based on quantitative assay of cellulolytic activity of the isolates determined by 3,5-dinitro salicylic acid (DNS) reagent method[32]. Inoculum level for each isolate was standardized by taking variable volume of broth cultures of isolates based on optical density into Omeliansky broth supplemented with filter paper strip as carbon source. For cellulolytic fungal isolate, quantity of inoculum was taken arbitrarily.

Reference strain MTCC23 (*Cellulomonas cellulans*) was procured from the Institute of Microbial Technology (IMTECH), Chandigarh, India. Along with the reference strain, a positive control component of HiMedia celluase enzyme (RM3331) as well as negative control broth (without culture) were maintained during the cellulase activity test. All culture broths were incubated at two temperatures 30°C and 40°C, in triplicates each. Sampling was done on the third, fifth and ninth day and cellulase activity was determined by the DNS reagent method. Finally, efficient strains were selected.

Subsequently, DNS assay-based efficient CDM isolates were screened depending on cellulase activity and CO_2 evolution using solid substrate and broth cultures of the isolates in a conical flask reactor[33]. This method is described below.

Conical flask reactor experiment: In the flask reactors, substrates (7 g in case of cellulose powder or 30 g in case of municipality waste) were added. Cellulose powder was in dried form, whereas MBW contained 50% moisture and therefore 15 g dry MBW contained ~7 g cellulose on the basis of 45% cellulose content on dry basis[18,19]. Five CDM cultures (CDM2, CDM6, CDM7, CDM9, CDM10) were selected for this experiment based on their cellulase activity (CA) in quantitative assay and cellulose hydrolysis capacity (CHC) determined in previous experiments. CDM2 showed low CA and CHC, CDM6 negligible CA and CHC, CDM7 medium CA and negligible CHC, CDM9 highest CA and highest CHC and CDM10 showed the highest CA and low CHC. CDM9 was used in both cellulose and MBW substitute, and the other cultures only in cellulose substrate. Then 15 ml from each of 48-h-old five CDM isolates (CDM2, CDM6, CDM7, CDM9, CDM10) and reference strain (*C. cellulans*, MTCC164) cultures was inoculated to saturate the materials in the respective reactors. Aeration of the conical flask reactors, connected by three-way connectors, was done every day at 2 pm using vacuum pump (Supplementary Figure 1). At weekly intervals up to the fifth week, 1 g sample was removed from the flask. Then 0.5 g of the sample was dried and weighed, and 0.5 g was added in 2.5 ml sterile distilled water so that crude enzyme extract could be prepared using syringe filter. Enzyme activity in the extract was determined by DNS reagent method as described above.

Measurement of trapped CO_2: The test tube containing 10 ml of 0.1 N $Ba(OH)_2$ solution was placed inside each reactor. The solution absorbs the CO_2 evolved due to the decomposition of organic materials by action of the respective isolates and native microflora in MBW. At weekly intervals up to the fifth week, $Ba(OH)_2$ solution was replaced by a fresh solution and the amount of C in terms of CO_2 was determined by titrating the removed solution with standardized 0.02 N H_2SO_4 solution.

Air treatment: In order to remove CO_2 from the reactor flask present in the incoming air, the air was passed

through 300 ml of 0.1 N NaOH with the help of a vacuum pump during which CO_2 was trapped in NaOH solution.

Characterization of CDM isolates by classical and molecular techniques

For morphological, physiological and biochemical characterization, CDM isolates were sent to IMTECH, Chandigarh. The isolates were also characterized by molecular method at the Institute of Bioresources and Sustainable Development (IBSD), Imphal, based on the 16S rRNA gene sequence and ITS region. Isolates were grown in the respective culture broths at 37°C for an incubation time of 24 h for bacteria and 72 h for actinomycetes based on appearance of colonies on CDM plates within 72 h after incubation during their first isolation. DNA was extracted using CTAB method for bacteria[34] and actinomycetes[35].

For bacteria and actinomycetes, PCR was performed using universal 16S rRNA gene primers FD1: 5′-GAGTTTTGATCCTGGCTCAG-3′ and RD1: 5′-AAGG AGTGATCCAGCC-3′. Each 25 µl PCR reaction mixture contained 50 ng of DNA in the cell-free DNA lysate, 2.5 µl of 10× PCR reaction buffer (105876, GeNei), 25 mM $MgCl_2$ (105881, GeNei), each dNTP (1054876, GeNei) at a final concentration of 200 µM, each primer (Sigma) at a final concentration of 0.25 pmol/µl and 0.03 U of *Taq* DNA polymerase (M300Bs, Promega). Amplification was performed in a thermal cycler (ICycler, Biorad, USA) and the cycling programme began with an initial denaturation at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 1 min and extension at 72°C for 30 s. The PCR ended with a final extension at 72°C for 7 min and the amplified product was stored at 4°C. A control PCR without DNA was set up to check for nonspecific amplification. The amplified 16S rDNA fragment size of 1500 bp was analysed by applying 5 µl of the PCR product in 0.8% agarose gel (V3125, Promega). The amplified PCR products of the ten efficient CDM isolates were sent to Banglore Genei, Bengaluru for partial sequencing of the 16S rRNA gene sequence, whereas the first two highest activity exhibiting isolates, CDM2 and CDM9, were sent to IMTECH, Chandigarh for full-length sequencing of 16S rRNA gene.

Production of compost from MBW in mixture with RS and CD

An experiment was conducted to study the effect of inoculation of the efficient cellulose degrading strain, CDM9, on decomposition trend and compost quality of vegetal MBW alone, or in mixture with RS and CD. The fresh MBW was collected from Imphal municipality waste dump site. It comprised of stem, leaf, fruit, bud, root, etc. of various vegetables.

Experimental set-up

Decomposition of total 10 kg feed materials in each treatment was carried in specially designed plastic bins of $19 \times 14 \times 16$ cm^3 dimension (Supplementary Figure 2) under six treatments and four replicates each from 22 August to 4 December 2009. The mixture of MBW, RS and CD was in the ratio 7 : 2 : 1. The bottom of the bins had 30% slope and a groove to allow accumulation of the liquid produced during decomposition (Supplementary Figure 2).

Inoculation of CDM9 and earthworm mix and record of different parameters

CDM9 inoculated treatment received 100 ml of 72-h-old culture having 8.86 log cfu/ml in nutrient broth (NB). NB which is commonly used for bacterial culture also supports actinomycetes growth and at pH 7, 2.32×10^6 cfu/ml (OD 0.40) has been reported earlier[36]. CDM9 was found to be a fast-growing actinomycetes. The uninoculated treatment received equal amount of uninoculated NB. Treatment with earthworms alone received 100 adults of *Eisenia fetida*, weighing 14.1–14.7 g. *E. fetida* is a well-adapted exotic species which carries out decomposition at a compost heap temperature range of 8–30°C. Earthworms were added into the mix on the tenth day when the temperature of the composting mix was below 30°C. During decomposition water and CO_2 were produced, and the water was drained out through the groove of the bin. The quantity of water generated was measured on a daily basis and stored in a big container for each treatment replicate. Adult earthworms, cocoons and juveniles were counted at three different intervals and biomass of juveniles and adult earthworms were measured. The earthworms individuals were returned to the composting mix after counting and weighing on the 30th and 60th day but not on the 105th day. Gain in earthworm biomass was calculated by subtracting the weight of the inoculated earthworms of the respective treatments from the weight of juveniles and adult earthworms at maturity. At maturity (C : N ratio <20) of the compost, different size fractions of the final compost, i.e. fine fraction (<2.0 mm), moderate size fraction (2.0–4.0 mm) and coarse size fraction (>4.0 mm) were determined using a sieve of appropriate pore diameter.

Determination of nutrient content, pH, PB and BB

Representative samples of initial MBW, final compost and sediment obtained after evaporation of all the drained-out liquid under different treatments individually

were collected, stored separately and analysed for chemical characteristics and population of both PB and BB; however, PB and BB were not determined in the sediments. The pH of dried initial mix and final compost was measured in 1 : 10 suspension using a pH meter and total organic carbon (C) and total nitrogen (N) estimated by CHNSO analyser (Vario MICRO V2.2.0, Elementer Analysensysteme GmbH). Five milligram of sample measured in a silver foil was combusted in the furnance connected to helium and oxygen gas, and carbon converted to carbon dioxide and nitrogen to nitrogen gas were measured through a thermal conductivity detector. For analysis of other nutrients in the MBW, feed mix and final compost, 0.5 g was digested with 10 ml of triple acid (nitric acid : perchloric acid : sulphuric acid) in the ratio 10 : 4 : 1 in a Kjeldahl digestion block at 650°C till clear fume was produced. Then, the digested sample was made up to 50 ml with distilled water and filtered through Whatman filter paper No. 42. Total P content in the sample was determined by vanadomolybdophosphoric yellow colour method using 2,4-dinitrophenol indicator in 5 ml of the 50 times diluted digest solution and absorbance were taken at 440 nm in a spectrophotometer[37]. For potassium (K), magnesium (Mg), calcium (Ca), aluminium (Al), sodium (Na), iron (Fe) and zinc (Zn), different dilutions were prepared to ensure that absorption readings of the samples fell within the range of the standard graphs drawn for each nutrients using an atomic adsorption spectrophotometer (analyst, version 6, Perkin Elmer Inc.). Microbial populations of both PB and BB in the initial feed as well as in the final compost were determined by serial dilution of 1 g of the sample and plating appropriate dilutions on the respective selective media. The plates were incubated at 30°C for 48–72 h and distinct colonies having typical morphological appearance were counted. The PBs determined were *Salmonella* spp., *Shigella* spp., *Enterobacter* spp. and *Micrococcus* spp. on their selective media, namely *Salmonella* agar, *Shigella* agar, MacConkey agar and trypton glucose extract agar respectively. The BBs determined were phosphate solubilizer, *Azotobacter* spp., *Azospirillum* spp. and cellulose degraders isolating on their selective media, namely Pikovskaya agar, Ashby mannitol agar, Rhozo Congo agar and Omeliansky agar medium.

Statistical analysis

All statistical analyses were performed using PASW Statistics 18.0.0. 2014 (ref. 38). For every parameter reported in this study, the different treatments were analysed for differences among mean ($P < 0.05$) by performing ANOVA to test the quantity of group variance and least significant difference (LSD) test at $P < 0.05$. Standard deviations were calculated for the earthworm parameters. Sequences of efficient CDM isolates were compared to those present in the databases using BLAST search program at the National Centre for Biotechnology Information (NCBI) website. Sequences were aligned using Clustal W (1.81) as implemented. The phylogenetic tree of the aligned sequences was constructed with 1000-fold bootstrap analysis using the neighbor joining method with the MEGA 4.1 (molecular evolutionary genetics analysis, MEGA) software[39].

Results

CDMs and selection of superior cellulase activity exhibiting isolates

Altogether, 5.4–8.5 log cfu of aerobic CDMs/g dry biomass were obtained from different source materials and population of the CDM isolates in different source materials was statistically different (Table 1). Among the herbivores, horse dung contained highest population of CDMs (8.54 log cfu/g dry biomass) followed by elephant dung, CD and goat dung. Among the omnivores, cat dung contained the lowest CDM population (5.72 log cfu/g dry biomass). For vegetative organic waste, sugarcane trash contained the highest CDM population (8.35 log cfu/g dry biomass) followed by water hyacinth shoot and root. Both cultivated and swampy field soil had low CDM populations (Table 1). Based on the size, shape and colour of the colonies, altogether 46 representative colonies were selected for characterization. Selection of isolates for MBW decomposition were selected on the basis of clearing zone detected around the colonies by Congo red assay. Twenty-nine cultures were bacteria and 16 were actinomycetes. Originally, six cellulose degrading fungal isolates were observed by plating serial dilution of the samples which were subsequently purified and stored in mineral oil[40]. After one month, while carrying out other experiments, the fungal isolates did not revive, except one.

The isolates varied in their cellulose degrading ability in the CMC agar medium. The isolates originated from sugarcane trash substrate and those obtained from the herbivore excreta did not show appreciable cellulase activity. HC of 10 CDM isolates (eight bacteria, one actinomycetes and one fungi) ranged from 0.1 to 3.44 (Figure 1 *c*), and the remaining isolates did not produce distinct clearing zone on CMC agar plate. The cellulase activity of the isolates ranged from 0 to 0.276 IU/min at 30°C, and 0 to 0.431 IU/min at 40°C based on quantitative DNS method (Figure 1 *a* and *b*). Although the isolates showed a general tendency of high cellulase activity at 40°C than at 30°C, the trend was inconsistent with respect to individual isolates. Similarly, time trend of cellulase activity was also inconsistent. In general, three patterns of cellulase activity were visible for the three determinations at 30°C (Figure 1 *a*). In case of CDM1,

Table 1. Cellulose degrading microorganisms (CDMs) obtained by plating serial dilution of different types of source materials on Omeliansky agar

CDMs (N) cfu/gdb													
Herbivores				Omnivores		Plant samples				Soil samples			
Cow dung	Horse dung	Elephant dung	Goat dung	Pig dung	Cat dung	Rice straw	Sugarcane trash	Water hyacinth shoot	Water hyacinth root	Uncultivated upland soil	Cultivated corn field	Swampy soil	LSD ($P = 0.05$)
7.79	8.54	8.41	7.48	6.21	5.72	6.32	8.35	7.34	7.24	5.71	5.46	5.98	1.03

All values are mean of four replicates. cfu/gdb, Colony forming unit/gram dry biomass.

CDM2, CDM7, CDM9 and CDM10, cellulase activity increased up to the fifth day after incubation and for other isolates it decreased after the third day. At 40°C, cellulase activity of all isolates, except CDM1 and CDM3 increased up to the fifth day and thereafter remained constant or decreased up to the ninth day (Figure 1 *b*). Cellulase activity of CDM1 and CDM3 increased continuously up to the ninth day. The reference strain, *C. cellulans* showed increase in activity at 30°C on the fifth day. CDM9 showed highest level of cellulase activity up to the ninth day at 30°C, compared to the other high cellulase activity exhibiting isolate – CDM10, the cellulase activity of which strikingly dropped by the ninth day. Furthermore, CDM9 also exhibited very high level of cellulose hydrolysis (clearing zone, Figure 1 *a* and *c*).

Characterization of CDM isolates by classical and molecular techniques

Figure 2 shows the 16S rRNA gene sequence-based identity of the six bacterial and three actinobacterial isolates and their phylogenetic relationship. These isolates have been deposited in the Microbial Repository Centre (MRC), IBSD, Imphal, and their accession numbers obtained. Based on full-length sequence, high cellulase activity producers CDM9 and CDM2 were found to be 100% similar to *Streptomyces xanthochromogenes* and *Sphingobacterium multivorum* of the NCBI database respectively. CDM 5 (*Aspergillus versicolor*) did not fit in the phylogenetic tree of bacteria and actinomycetes (Figure 2).

Selection of the best CDM based on cellulase activity and CO_2 evolution in reactor flask

Figure 3 shows cellulase activity and quantity of CO_2 evolved due to inoculation of the isolates in cellulose paper and MBW at three time intervals. The magnitude of cellulase activity (0.325–5.961 IU/min) and CO_2 evolution (0.442–1.158 mg) was higher at the seventh day and cellulase activity gradually declined at 14th and 21st day. Evolution of CO_2 during these days remained similar. There was no any statistically significant correlation between the two parameters. Cellulase activity of CDM9 was highest among the isolates at the 7th and 14th day and at par with those of other isolates on the 21st day. Cellulase activity of CDM9 in MBW-added reactor flask was strikingly higher up to the 14th day after inoculation than that in filter paper-added flask (Figure 3). By the 21st day, cellulase activity in the MBW-added flask also decreased, but was more than the activity in the filter paper substrate. Although inoculation of CDM9 increased cellulase activity in MBW, it had no effect on CO_2 evolution at the 7th day and the inoculation effect was clearly visible up to the 14th day (Figure 3). Based on the hydrolysis and cellulase activity at ambient temperature, CDM9 and CDM10 were superior isolates for testing in MBW decomposition experiment (Figure 1 *a*). However, cellulose hydrolysis capacity (Figure 1 *c*) and cellulase activity at three time intervals (Figure 3) showed the activity of CDM9 to be consistent and therefore, it was considered as the best isolate for co-inoculation with earthworms in MBW decomposition.

Drained-out liquid, C, N, C : N ratio, P and K content

During composting, substantial quantity of liquid escaped along the angle bottom of the pot, which varied in different treatments. Cumulative quantity of drained-out liquid ranged from 817.3 to 4297.2 ml (Table 2). The amount of drained-out liquid was much higher in MBW alone treatment compared to treatment containing RS and CD in mixture with MBW. Inoculation with EW and/or CDM9 appeared to increase the quantity of drained-out liquid. However, increase in liquid production was not related to biomass loss from composting mix under different treatments (Table 2).

The pH of the composting mix during composting ranged from 6.4 to 9.0 and of the drained-out liquid under different treatments ranged from 8.13 to 9.18. There was a positive correlation between cumulative N loss during composting and pH of the composting mix (Figure 4).

Table 3 shows the content of N, C, P, K and C/N ratio of the individual raw feeds used and the composts produced under different treatments. The amount of N in the initial individual feeds ranged from 0.88% to 1.53% and

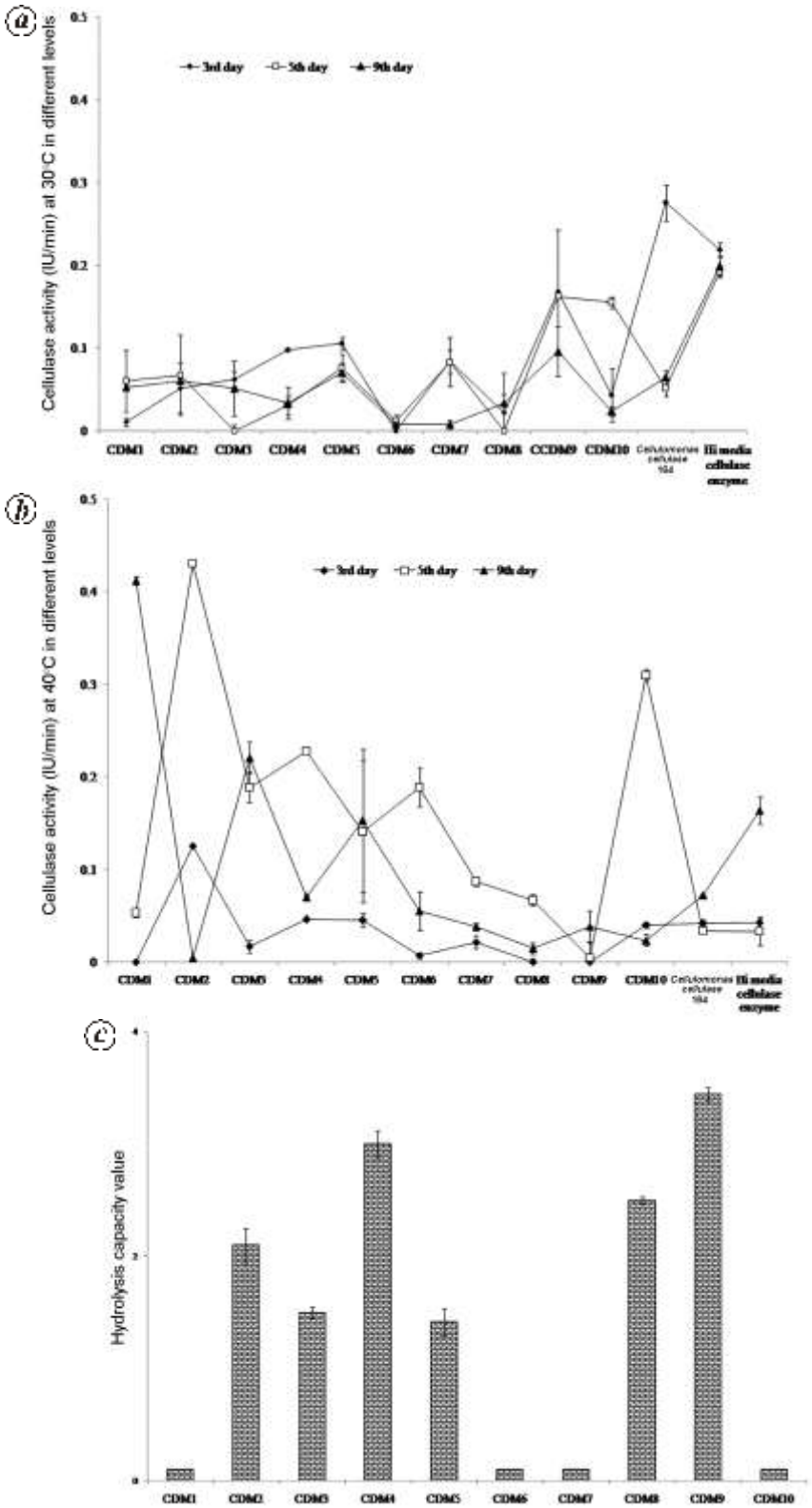

Figure 1. Cellulase activity determined in the culture extract at (***a***) 30°C and (***b***) 40°C. ***c***, Hydrolysis capacity (clearing zone) of the cellulose degrading microorganism (CDM) isolates determined by qualitative method at 30°C. Bar indicates standard deviation. CDM1, *Stenotrophomonas* spp. (KF986629); CDM2, *Sphingobacterium* spp. (KF986630); CDM3, *Klebsiella pneumoniae* (KF986631); CDM4, *K. pneumoniae* (KF986632); CDM5, *Aspergillus versicolor* (KF986638); CDM6, *Bacillus benzoevorans* (KF986633); CDM7, *Stenotrophomonas maltophilia* (KF986634); CDM8, *Pseudomonas fluorescens* (KF986635); CDM9, *Streptomyces xanthochromogene* (KF986636); CDM10 and *Bacillus barbaricus* (KF986637). Data within parenthesis indicate accession numbers obtained from NCBI database.

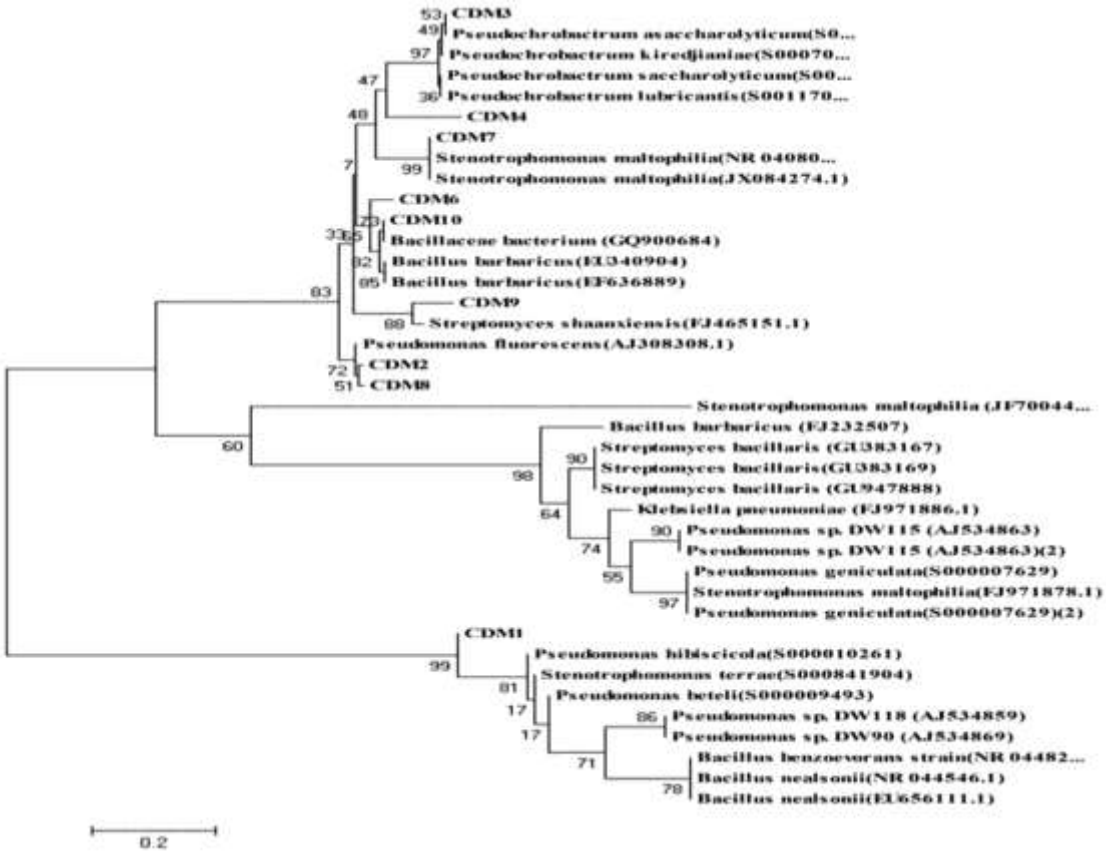

Figure 2. Phylogenetic dendrogram showing the relationship between 16S rRNA gene sequences retrieved from efficient cellulose degrading bacteria and actinomycetes with reference to sequences in GenBank.

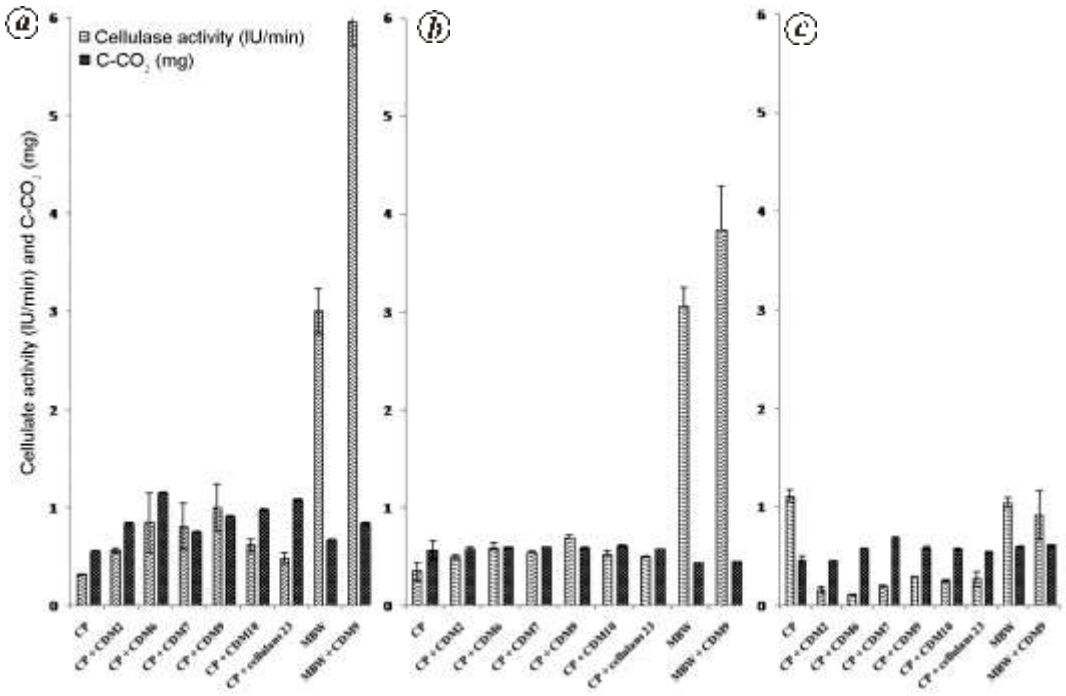

Figure 3. Cellulase activity (IU/min) and C–CO_2 evolution (mg) due to inoculation of different CDM isolates separately in two substrates (cellulose filter paper and municipality biowaste) in microcosm determined in different intervals for (***a***) 7th day, (***b***) 14th day and (***c***) 21st day.

Table 2. Estimated loss of biomass, quantity of drained-out liquid from composting mix and quantity of fine, medium and coarse fractions of the final composts under different treatments

Treatment	Amount of initial dried composting feed mix (g)	Amount of final dried compost** (g)	Final compost (g) in different size fractions			Cumulative amount of drained out liquid (ml)	% Loss of composting biomass
			Fine compost (<2.0 mm)	Medium-size compost (2.0–4.0) mm	Coarse size compost (> 4.0 mm)		
MBW + NB	10,000 (1853)*	476	84 (17.62)***	52 (10.92)***	340 (71.44)***	3767.2 ± 96	74.31
MBW + EW + NB	10,000 (1853)	463	94 (20.30)	51 (11.01)	317 (68.690	3777.5 ± 59	75.01
MBW + CDM9	10,000 (1853)	520	115 (22.11)	48 (9.23)	357 (62.65)	4000.0 ± 36	71.93
MBW + EW + CDM9	10,000 (1853)	484	128 (26.44)	53 (10.95)	303 (62.61)	4297.2 ± 261	73.88
MBW + RS + CD + EW + NB	10,000 (2871)	1796	642 (35.75)	682 (37.97)	472 (26.28)	817.3 ± 12	37.44
MBW + RS + CD + EW + CDM9	10,000 (2871)	1850	738 (39.89)	612 (33.08)	500 (27.03)	2265.7 ± 16	35.56
LSD (P = 0.05)	–	21	22	17	NS	–	–

All values are mean of four replicates. *Data within parenthesis are dry biomass out of 10 kg composting feed mix under each treatment. **Compost was dried to constant weight. ***Data within parenthesis represents percentage of total final dried compost.

Table 3. C, N, P and K content in original feeds, their treatment mix, final compost, sediment and difference in N, P and K content in initial feed, and final compost with water accumulated sediment under different treatments

Treatment	Initial composting feed mix		Final compost		Initial composting feed mix			Nutrient content (g) final compost			Sediment			% Loss of major nutrients		
	C (%)	C : N	C (%)	C : N	N	P	K	N	P	K	N	P	K	N	P	K
MBW + NB	32.1	36.5	23.2	9.9	16.0 (0.88)	0.85 (0.05)	3.89 (0.21)	11.19 (2.35)	0.76 (0.16)	3.86 (0.81)	0.96	0.08	0.01	24.3	1.8	7
MBW + EW + NB	32.1	36.5	23.2	10.6	16.03 (0.88)	0.85 (0.05)	3.89 (0.21)	10.14 (2.19)	0.69 (0.15)	3.24 (0.70)	0.94	0.13	0.09	30.9	3.5	16
MBW + CDM9	32.1	36.5	22.3	11.0	16.03 (0.88)	0.85 (0.05)	3.89 (0.21)	12.58 (2.03)	1.01 (0.16)	3.81 (0.74)	0.54	0.09	0.03	30.0	0.0	2.05
MBW + EW + CDM9	32.1	36.5	20.8	12.8	16.03 (0.88)	0.85 (0.05)	3.89 (0.21)	7.89 (1.63)	0.76 (0.16)	3.24 (0.67)	1.03	0.02	0.11	44.4	8.2	16.4
MBW + RS + CD + EW + NB	28.8	18.8	26.9	17.4	35.67 (1.5)	2.03 (0.08)	6.68 (0.28)	27.75 (1.55)	2.85 (0.16)	6.25 (0.33)	0.11	0.03	0.01	21.9	12.3	11.1
MBW + RS + CD + EW + CDM9	28.8	18.8	26.0	14.3	35.67 (1.5)	2.03 (0.09)	6.68 (0.28)	27.88 (1.82)	2.76 (0.15)	7.20 (0.39)	0.16	0.09	0.01	21.3	12.3	0
LSD (P = 0.05)	–	–	1.46	1.76	–	0.02	0.01	0.82	0.02	0.01	0.42	–	–	0.19	0.21	1.51

All values are mean of four replicates. MBW, Municipality biowaste; NB, Nutrient broth; EW, Earthworm; CDM9, Cellulose degrading actinomycetes in NB; RS, Rice straw; CD, Cow dung. Data within parenthesis are percentage content of the major nutrients in the initial composting feed mix and final compost.

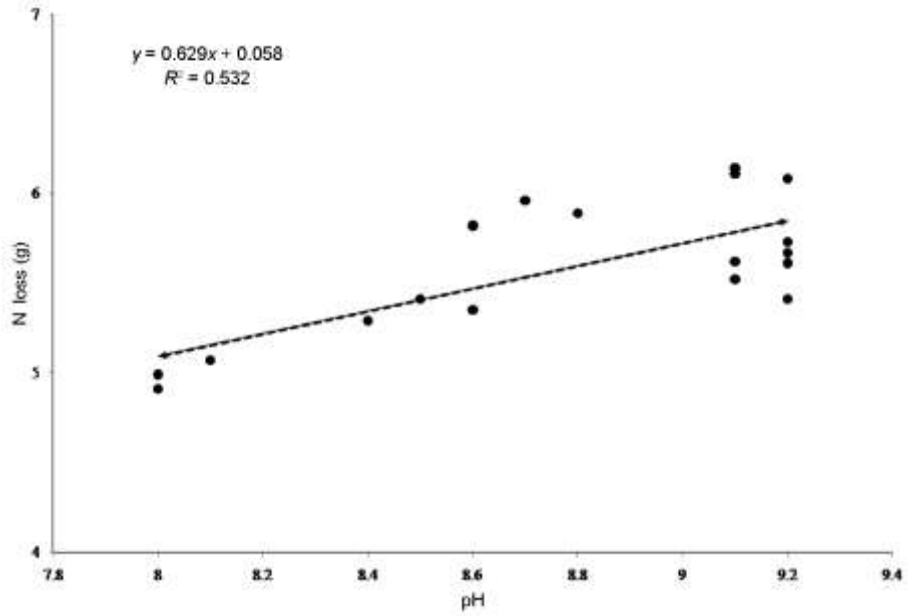

Figure 4. Correlation of N loss (g) from the initial feed during decomposition and pH of the final composts produced under different treatments.

C : N ratio ranged from 18.82 to 36.48. N content in the final composts under different treatments ranged from 1.55% to 2.35% and C content ranged from 20.8% to 26.9%. The C/N ratio in the final compost ranged from 9.9 to 17.4 (Table 3). Total N content in the final compost was less than that in the initial feed, irrespective of the treatment used. The total N content in the sediments of different treatments ranged from 0.11 to 1.03 g, showing the highest value in the sediments collected from MBW + EW + CDM9 treatment. The percentage of N loss from the mix during composting was least (21.3) in case of MBW + RS + CD + EW + CDM9 treatment (Table 3).

P content in the initial feed mixture ranged from 0.85 to 2.03 g and in the final compost it ranged from 0.69 to 2.85 g. The loss of P from the initial composting mix ranged from 0% to 12.3%. Similarly, the total K content in the initial composting mixture ranged from 3.89 g to 6.68 g and the amount in the final compost ranged from 3.24 g to 7.20 g. The loss of K from the initial composting mix ranged from 0% to 16.4% (Table 3).

Quantity of different size fractions of compost under different treatments

The quantity of finer material (<2.0 mm) in the final compost was found to be highest (39.89%) among the three fractions in case of MBW + RS + CD + EW + CDM9 treatment followed by MBW + RS + CD + EW treatment (35.75%) (Table 2). Similarly, the quantity of medium sized fraction (2.0–4.0 mm) of MBW + RS + CD mix compost was also higher than other treatments. There was also a trend in increase of smallest size fractions due to inoculation with the decomposer agents. The quality of compost in >4.0 mm size fraction was lower in the two RS and CD mixed treatments than in only MBW treatment. In these two treatments, loss of composting biomass was much lower than that under other treatments. Cumulative quantity of drained-out liquid was also less under these two treatments (Table 2).

Load of pathogenic and beneficial bacteria and earthworms

The population of PB in the final compost ranged from 5.56 to 9.25 log cfu/g dry biomass (Table 4) and in the initial composting mix, it ranged from 6.3 to 9.5 log cfu/g dry biomass. Among the four types of PB, population of *Micrococcus* spp. was found to be very high in the final compost under all the treatments. In general, the population of *Salmonella* spp. and *Shigella* spp. was less in MBW + RS + CD + EW + CDM9 treatment than in other treatments (Table 4). In case of BB, the population in the final compost ranged from 7.6 to 9.15 log cfu/g dry biomass and in the initial composting mix it ranged from 8.2 to 9.2 log cfu/g dry biomass. In general, the population of CDM9 or CDM9 + EW inoculated treatment was higher than in uninoculated treatments. The population of phosphate solubilizer and *Azotobacter* spp. was highest in MBW + RS + CD + EW + CDM9 treatment. Population of *Azospirillum* spp. and cellulose degrader was found to be higher in MBW + RS + CD + EW and MBW + RS + CD + EW + CDM9 treatments than in other treatments (Table 4). However, there was no statistically significant

Table 4. Population of pathogenic bacteria (PB) and beneficial bacteria (BB) in the final composts under different treatments of MBW feed mix composting

Treatment	Population of PB (log cfu/gdb)				Population of BB (log cfu/gdb)			
	Salmonella spp.	*Shigella* spp.	*Enterobacter* spp.	*Micrococcus* spp.	Phosphorus solubilizer	*Azotobacter* spp.	*Azospirillum* spp.	Cellulose degraders
MBW + NB	8.6	8.8	8.5	8.3	7.7	7.6	7.9	7.8
MBW + EW + NB	8.4	8.6	7.5	8.6	8.3	8.2	7.9	8.3
MBW + CDM9	7.5	8.7	7.6	8.0	8.7	8.8	8.2	8.5
MBW + EW + CDM9	7.5	8.5	7.9	8.7	8.7	8.7	8.1	8.2
MBW + RS + CD + EW + NB	7.0	8.7	7.3	9.3	8.7	8.8	8.9	8.7
MBW + RS + CD + EW + CDM9	5.9	5.6	7.7	7.9	9.1	9.2	8.7	8.7
LSD (P = 0.05)	0.41	1.73	NS	NS	0.05	NS	NS	NS

All values are mean of four replicates.

Table 5. Earthworm, cocoon, juvenile, adult and their biomass under different treatments at different time intervals

Treatment	Days after inoculation									
	0	30th		60th		105th				
	No. of adult EWs	No. of adult EWs	No. of cocoons	No. of adults	No. of cocoons	No. of adult EWs	No. of cocoons	No. of juveniles	Total biomass of worms (g)	Biomass gain (g)
MBW + NB			0	0	0	0	0	0	0	0
MBW + EW + NB	100 (14.2)	49 ± 16.5 (21.5)	41 ± 27.8	24.5 ± 12.5 (13.1)	46.5 ± 9.9	47.6 ± 11.9 (19.2)	59 ± 7.7	37.33 ± 19.2 (0.43)	19.6 ± 2.1	5.5 ± 1.3
MBW + CDM	0	0	0	0	0	0	0	0	0	0
MBW + EW + CDM	100 (14.7)	93 ± 10.2 (45.3)	152.3 ± 15.7	88 ± 9.8 (43.4)	282 ± 21.3	91 ± 9.5 (38.8)	127 ± 12.5 (0.46)	45.67 ± 12.1	39.3 ± 4.3	24.6 ± 4.8
MBW + RS + CD + EW	100 (14.7)	99 ± 2.6 (48.7)	306 ± 16.2	98 ± 1.2 (51.3)	347 ± 23.1	100.3 ± 2.5 (52)	136 ± 26.0	284 ± 9.4 (7.8)	59.8 ± 5.7	45.1 ± 6.3
MBW + RS + CD + EW + CDM	100 (14.1)	100 ± 0.0 (52)	298.7 ± 18.9	100 ± 0.5 (54.1)	353 ± 16.2	102.6 ± 4.6 (54.4)	156 ± 38.7	742.5 ± 10.0 (13.5)	67.9 ± 2.3	53.8 ± 3.3

All values are mean of four replicates. CDM9, *Streptomyces xanthochomogens*; Data within parenthesis are biomass (g) of worm.

difference in population of BB in compost under different treatments, except the population of phosphate solubilizers. The number of cocoons and juveniles, and earthworm biomass gain were higher in the case of EW + CDM9 inoculated treatment than the earthworm inoculated treatment alone. In the EW + CDM9 inoculated mix of MBW + RS + CD, values of earthworm parameters were much higher compared to those in EW + CDM9 inoculated with only MBW (Table 5).

Discussion

MBW is a rich source of plant nutrients and its decomposition can be accomplished efficiently using inocula of efficient CDMs and earthwarms as well as by mixing with RS and CD. MBW is alkaline (pH 7.5) and our search for efficient CDMs for use as inoculum included excreta of herbivores and municipality biowaste which were also alkaline (pH 7.2 to 8.1). CDM population in different materials ranged from 5.46 to 8.54 log cfu/g dry biomass, with higher level of population in herbivore faecal material and lower level in omnivore faecal material and soil (Table 1). The population of CDM found in this study was lower than those found in ruminant content (10^9–10^{10} cfu/ml)[41] and much higher than in poultry dropping (6.7×10^4–7.2×10^4 cfu/g)[42]. Among different materials, sugarcane trash with 40%–50% cellulose contained 8.35 log cfu/g dry biomass[19,43,44].

Although a large number of CDM isolates were obtained by plating on cellulose powder supplemented medium[32], the number of isolates with considerable level of cellulase activity was less. Cellulase activity of these isolates was comparable with those of efficient CDMs reported by other researchers[42,45,46]. Efficient CDMs were selected based on either qualitative cellulose hydrolysis method (clearing zone diameter) or quantitative DNS method which determines cellulase enzyme activity in the culture filtrate of three-day-old culture broth. Initially, we evaluated cellulase activity using both methods and found

that only CDM9 showed consistently high levels of cellulase activity at 30°C up to the ninth day. CDM9 also showed highest clearing zone in the hydrolysis method. Cellulase activity of the other isolates was not consistent when determined by the two methods. For example, CDM10 showed high activity at 30°C (Figure 1 *a*) only up to the fifth day and its hydrolysis ability was less (Figure 1). Studies have also reported inconsistency between results of cellulase activity determined by qualitative and quantitative methods[19]. This inconsistency in cellulase activity determined by *in vitro*-based methods can lead to confusion in selection of the right kind of efficient strain for inocula development. Nevertheless, based on the results obtained using the two methods, we selected ten isolates with considerable cellulase activity. Nine isolates (bacteria and actinomycetes) were identified based on 16S rRNA and ITS region. The sequence similarity based on 16S rRNA or full length ranged from 86% to 100% for the nine isolates. The isolates were identified as *Stenotrophomonas* spp. (CDM1), *Sphingobacterium* spp. (CDM2), *Klebsiella pneumoniae* (CDM3), *Klebsiella pneumonia* (CDM4), *Bacillus benzoevorans* (CDM6), *Stenotrophomonas maltophilia* (CDM7), *Pseudomonas fluorescens* (CDM8), *S. xanthochromogene* (CDM9) and *Bacillus barbaricus* (CDM10). The fungal isolate (CDM5) was identified as *A. versicolor*.

We also screened five selected CDM isolates and reference strain by inoculation in sterilized solid cellulose substrate and unsterilized MBW in a reactor flask for cellulase activity and CO_2 production. The cellulase activity of the five efficient isolates and the positive control, *C. cellulans* at the seventh day after incubation (Figure 1) was different compared to those determined in the culture filtrates of liquid broths at the third, the fifth and the ninth day after inoculation (Figure 1). The cellulase activity of culture filtrate of solid cellulose substrate treatment was highest at seventh day after inoculation and thereafter decreased which might be due to drop in average ambient maximum and minimum temperature from the first week (26.5°–17.6°C) to the third week (24.1°–15.2°C). Production of CO_2 as a result of metabolism of glucose inside the flask was not correlated with cellulase activity. Among the isolates, *S. xanthochromogene* (CDM9) produced highest cellulase activity in the sterilized substrate or in the unsterilized MBW in the presence of native cellulose degrading microorganism, as evident from the data of MBW-added reaction flask (Figure 3). Cellulase activity in MBW due to CDM9 inoculation was 5.961 IU/min on the 7th day and 3.845 IU/min on the 14th day after inoculation. However, these data do not indicate whether glucose release increased by high cellulase activity or glucose was released but not metabolized by the decomposer microorganism. Oxygen was not a limiting factor inside the reaction flask as it was aerated. Glucose estimation inside the flask would have thrown more light on the process, but unfortunately could not be done. Furthermore, only CDM9 showed consistency in cellulase activity determined by the three methods, but for the other stains activity changed when the method of estimation was changed. Thus, results of this experiment also showed that among the tested isolates, CDM9 possessed the highest cellulase activity determined by the three different methods. This strain was originally isolated from sugarcane trash waste and also found to be capable of producing high cellulase activity even in the presence of indigenous CDMs. Hence, it was a logical choice for use in decomposition experiments subsequently. However, it was interesting to observe that CDM6, despite its low cellulose activity in cellulose substrate and very low cellulose HC showed cellulase activity at par with CDM9 (Figure 2). This lends to a speculation that CDM6 might have shown higher cellulase activity in MBW of conical flask as well. Therefore, it will be worth investigating CDM6 in future experiments.

Inoculation of CDM9 in MBW or in MBW + RS + CD mix had different effects on decomposition, nitrogen loss and final compost quality. In this study, cumulative amount of drained-out liquid from the composting mix was taken as an indication of the extent of decomposition, and the quantity was higher for CDM inoculated than in the uninoculated mix (Table 2). CDM inoculation in the presence of earthworms further enhanced the quantity of drained-out liquid. Water drained out naturally along the 30% slope bottom through the groove and accumulated in the container below. Decomposition of MBW was more compared to that of MBW + RS + CD, as evident from higher quantity of drained-out liquid from MBW and lower C : N ratio in the final compost (Table 3). The quantity of drained-out liquid indicates the extent of decomposition of the mix under different treatments, which in turn seems to have reduced C : N ratios in the final composts (Table 3). For example, MBW alone decomposed faster as higher quantity of drained-out liquid and loss of original compost mix was produced in this treatment compared to that of MBW + RS + CD treatment (Table 2). Mixing of MBW with RS and CD resulted in lowering of C : N ratio. The per cent content of lignin and cellulose in MBW, RS and CD was 3.9 and 36.4, 4.87 and 33.6 and 5.2 and 23% respectively. The high carbon content in the form of less complex organic compound (lignin) and high C : N ratio in MBW might have increased the decomposition and produced more water. Inoculation of the mix either with earthworm alone or earthworm + CDM9 increased water production (Table 2). Thus, this study shows that *S. xanthochromogene* (CDM9) is an effective decomposer of MBW, which is comprised of mostly vegetable waste mix and indigenous decomposer microflora. A strain *Streptomycess hundungensis* sp. Nov. from limestone quarry at Hundung, Manipur is reported to be 99.6% similar to *S. xanthochromogene* NRRL B-5410^T. This study also revealed

that *S. xanthochromogene* possessed anti-fungal and plant growth promoting ability[47]. Thus, compost produced by this strain may have additional advantage of plant growth promotion, which needs to be investigated in future studies. Previous studies reported that vegetal biomass rich in MBW decomposed faster[48,49] and we have shown that inoculation of CDM culture can further enhance decomposition. Previous workers also showed that inoculation of earthworm and CDM cultures resulted in shorter time of composting and lower C : N ratio of compost from plant biomass[50]. However, in this study, higher decomposition did not result in lower C : N ratios (Tables 2 and 3).

Total N, P and K content in final compost under different treatments was less than that in the initial composting mix (Table 3). In MBW treatment, the difference in N content was 24.3% to 44.3% and in MBW + RS + CD, it was 21.3% to 21.9%; this difference was actually loss of N during composting. N loss was 5.7–20.1% more from MBW when it was inoculated with CDM and/or EW (Table 2). Mixing of MBW with RS and CD could reduce N loss by about 100%. In MBW + CDM9 + EW treatment, loss of N was 44.4% whereas in the MBW + RS + CD + CDM9 + EW treatment, loss was 21.3%. Difference in total content of P and K between the initial composting mix and final compost was 0–12.3% and 0–16.4%. This can be accounted from their content in the sediment derived after evaporation of the drained-out liquid (Table 3). However, such loss in case of N could not be accounted from the content of N in the sediment. These data suggest that substantial amount of N might have escaped through volatilization. A positive co-relationship was obtained between pH of the composting mix under different treatments and total N loss from the compost calculated from N content at initial and final composting mix. Although the *r* value was not statistically significant (Figure 4), this result indicates that highly alkaline environment of the MBW mix promotes substantial volatilization loss of N as NH_3.

Volatilization loss of N might have occurred either directly from the composting mix, or from the drained-out liquid with which N as NH_4 or NO_3 might have escaped from the composting mix or from the drained-out liquid. The pH of the drained-out liquid varied from 8.1 to 9.2 and after collection, this was kept exposed for evaporation to obtain the sediments. Volatilization loss of N occurs through NH_3 under alkaline environment. N loss data in the present study are only an estimate and cannot confirm in which form N might have escaped. Earlier research showed that 46.64–64.43% of N in the form of NH_3, N_2 and N_2O may be lost during composting of household waste and sludge from slaughtered pigs[15,51,52]. These studies also found losses of total nitrogen from chicken litter compost to the extent of 10.2–64.2%, and largely attributed to volatilization of ammonia (NH_3), when the pile temperature and pH were above 33°C and 7.7 respectively. Our estimate of N loss is 21.3–44.4%. We could reduce the loss by mixing MBW with RS and CD, and also increase the amount of final compost and thereby increase the total content of N in the compost. N loss in aerobic composting process could be reduced using sawdust which contains high lignin content[53].

There was a substantial gain in earthworm biomass over 105 days as reflected in the difference in biomass of earthworms at the start and end of composting. Earthworm biomass gain was more in CDM9 + EM inoculated MBW + RS + CD mix treatment compared to MBW treatment (Table 5). Previous studies documented increase in microbial activity inside the gut of soil earthworm, *Lumbricus terrastis*[54] and cellulase activity in the gut of epigeic earthworm, *E. fetida*[55]. We observed a distinct increase in cocoons, juveniles and total biomass of earthworm due to inoculation with CDM9. Cocoons, juveniles and adults of earthworm were removed at the harvest of the compost and thus a portion of the nutrients, including N in the original composting mix assimilated by earthworms during composting was also removed. The N loss during this study cannot be accounted as actual loss, although it has also contributed to the difference between N only in initial and final composting mix. Adult *E. fetida* biomass contains 4.8% N and a biomass gain of 3.5 g on dry basis (80% moisture) suggests that N loss under MBW + RS + CD + CDM9 + EW treatment is 11.4% with earthworm biomass considered as part of the final compost instead of 21.3% (Table 3). Thus, this study shows that CDM9 and earthworms are useful in efficient decomposition of MBW + RS + CD and nutrient conservation in the compost.

A distinct effect of inoculum of CDM9 and earthworms on compost quality in terms of the different size fractions of the final compost was also visible. In general, quantity of compost in fine-sized fraction (<2.0 mm) was statistically higher in the compost produced under CDM9 alone or CDM9 + EW inoculated treatment. It is also interesting to note that mixing of MBW with RS and CD resulted not only in high amount of final compost, but also larger percentage of it in fine and medium-sized fractions. This indicates that decomposer agents result in the production of higher amounts of fine grain compost. Finer size fractions of compost provide larger surface area for better interaction on the application for crop production. Earlier, in a pilot-scale study, we documented that only earthworms inoculation resulted in finer-sized vermicompost production from MBW with reduced load of pathogenic microorganisms[55]. This study had systematically selected an efficient cellulose degrading actinomycetes for co-inoculation with earthworms to convert MBW to compost.

Occurrence of pathogenic microorganisms in MBW decomposition is a major concern against its application to increase soil fertility and crop yield. We found log cfu of four major PB in the range 5.6–9.3/g dry compost. That colonies on the selective medium were representatives

of PB species of the four groups, was ascertained by comparing colonies of their type strains obtained from MTCC, IMTECH, Chandigarh. An earlier study reported the occurrence of PB in the range which was below the US PAS 100 and APEX limits of ≤1000 cfu/g dry biomass in MBW-derived compost sold in the market[56,57]. The colony forming units of PB in market samples of compost sold in Imphal city was found in the range 5.35–8.37 log cfu/g dry biomass (data not shown); this suggests that PB are common in compost. However, we found that use of CDM and earthworm cultures and mixing of MBW with RS and CD were somewhat effective in reducing the level of PB, except *Micrococcus* spp. In fact, *Salmonella* and *Shigella* species were significantly reduced when MBW was composted in mixture with RS and CD and inoculated with the two agents. In view of the occurrence of a large number of cfu of PB, it is necessary to carry out further systematic study to establish that PB strains that occur in the final compost are actually virulent and can cause disease, and also the extent of occurrence of such virulent strains. The present study also indicates that there may be some biocidal factor produced by RS and CD in the presence of CDM and earthworms, which can reduce the PB population. We suggest testing the effect of biomass of abundant medicinal herbs available in Manipur, on the PB population in compost.

Interestingly, four groups of BB in the final compost under different treatments were found to be in more number due to inoculation of either the decomposer agents or mixture of MBW with RS and CD, and then inoculation with CDM9 and earthworms. The log cfu data of phosphate solubilizers and *Azotobacter* spp. in compost obtained from MBW + RS + CD + CDM9 + EW treatment were statistically higher than those in the MBW treatment alone. The source of BB observed in compost may be phyllosphere and rhizosphere bacteria associated with the plant biomass component of MBW, and also RS and CD in the MBW + RS + CD compost. To the best of our knowledge, no data are available on population of BB in the MBW-derived compost.

Conclusion

S. xanthochromogenes CDM9 is an efficient CDM for decomposition of alkaline MBW. Compost produced from MBW alone contained low levels of N with 24.3% to 44.4% loss during composting. By mixing MBW with RS and CD and inoculation with CDM and earthworms, compost yield had enhanced and N loss reduced by more than twofold. N and P contents also increased in the final compost. Inoculation of the mix with CDM9 and earthworms can be a useful technique for the production of compost with significantly reduced populations of *Salmonella* and *Shigella* species, and increased population of phosphate solubilizes and *Azotobacter* spp. from MBW.

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ACKNOWLEDGEMENTS. We thank the Department of Biotechnology and Department of Science and Technology, Government of India, for financial assistance (Sanction No. SR/WOS-A/LS-55/2008 dated 22/09/2008), and IBSD, Imphal, for providing infrastructure support.

Received 4 January 2017; revised accepted 22 September 2017

doi: 10.18520/cs/v114/i06/1261-1274