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3.1. QUANTIFICATION OF BIODEGRADABLE AND NON-BIODEGRADABLE WASTE MATERIALS OF IMPHAL CITY

In this research, waste generated in household, vegetable market and waste disposal site of Imphal were estimated through strategic sample. Type and quantity of waste generated in different cities vary greatly depending upon several factors such as seasons, population, industrial activity, food habits and food requirements. Since goal of this research is to convert organic waste of Imphal city into useful compost and its fortification, the first objective was to quantify organic portion of the municipality waste. But in the process, the quantity of other type of materials such as plastics, paper, glass, tin, cloth, were also quantified. The different types of waste generated in the city are dumped near the vegetable market and also near the city grocery markets. These wastes are dumped in open on daily basis and several loads are carried by truck for disposal under the authority of Imphal Municipality Corporation. At present, there is no permanent waste disposal site in Imphal city but the NGOs are dumping the waste at a site of 10 acres area at KRYPSA. The following methods were followed for estimation of various types of waste generated every day.

3.1.1. Biowaste generation of household of Imphal city

Quantification at domestic level was done by selecting five families each in three localities namely Nagamapal area, Thangal bazar area and Paona Bazar area, by collecting kitchen organic waste daily for 15 days. The families were asked to put their kitchen waste into plastic bucket provided and weight of the waste was taken in every morning. Samples were also drawn from this household waste to determine the microbial population load as well as to isolate cellulose degrading microorganisms.

3.1.2. Biowaste generation in vegetable market of Imphal city

In order to find out the amount of vegetable waste generated in the vegetable market daily, three sheds (No. 10, 11 & 12) of vegetable market centre at Nagamapal area were selected. In an individual shed, five sellers were selected and provided with containers to put the vegetable waste produced in a day by them. This was repeated for 7 days. In the vegetable sheds, only licensed sellers (1811) sell vegetables for whole day. As per statistics of the Municipality Corporation, for every 3 licensed sellers, there are five unlicensed sellers who sell for some period of the day as flying sellers and
quantity of waste generated by the non-licensed sellers to be half the quantity of those generated by licensed sellers. Every evening, weight of the waste in the container was recorded and amount of vegetable waste generated in the vegetable market was estimated. In order to find out the amount of waste generated daily, three sheds (No. 10, 11 & 12) of vegetable market centre at Nagamapal area were selected. In a shed, three women were selected and they were provided with containers for putting vegetables waste of each day during the sampling duration of three days. Weight of the vegetable waste was recorded. Based on the total number of licensed and non-licensed vegetable sellers and sample quantity, quantity of vegetable waste of market area of Imphal city was estimated.

3.1.3. Biowaste generation at the main disposal area (KRYPSA)

KRYPSA is about 4 Km away from Imphal City at 24°50'00"N, 93°55'00"E. From the records of Imphal Municipal Council as well as the present survey carried out (2009), total 29 loads of trucks (23 numbers of 407 tata trucks and 6 numbers of tata DI trucks) were engaged for disposal of waste daily. Five NGOs namely, Worker Union, Manipur (WUM), Centre for Research for Environmental Development (CRED), Security, Forum and Municipality carry out waste disposal activity in the city. Out of these 29 trucks, randomly two 407 tata truck and one DI Tata truck with full load were sampled for estimation of the waste at the KRYPSA site. For the convenience, a medium size bag was used to estimate how many bags of waste are carried in a 407 Tata truck and DI Tata truck, separately. The weight of the waste in one bag was taken and then this was repeated for 10 bags to obtain average wt. of waste in one bag. There were 158 bags in 407 Tata truck and 144 bags in DI Tata truck. Five bags each from two 407 trucks and five from one DI truck were chosen randomly for sorting out different fractions of waste such as partially decomposed tiny pieces of vegetables and others organic materials, fresh looking waste vegetable materials, cartoon, clothes, paper and plastics (Fig. 4). This was done for three consecutive days. From the data, the total waste generated from the Imphal city was estimated.
3.2. CHARACTERISATIONS OF MUNICIPALITY BIOWASTE OF IMPHAL CITY

The Imphal city biowaste as well as other waste like RS, CD and LP used for composting were analyzed for biochemical and microbiological properties. Representative samples were oven dried and powdered. They were analyzed by using standard procedures as given below.

3.2.1. Chemical characteristics of Imphal city biowaste

The different chemical parameters of biowaste determined are as follows:

3.2.2. pH

Dry and ground municipality waste was mixed with distilled water at 1:10 ratio and its pH was determined by a glass electrode using a digital pH meter (“Systronics” model 335).

3.2.3. Electrical conductivity

The EC of the waste was determined by using digital conductivity meter (Consort C933) by mixing with distilled water at 1:10 ratio.

3.2.4. Organic carbon

Total organic carbon is an important indicator of compost quality and its productivity. The well known method for determination of TOC is the Rapid
Dichromate Oxidation method (Walkey and Black, 1934). The recovery of organic 
carbon using Walkey and Black titration procedure ranged from 60 to 80%. To 
overcome the incomplete digestion of the organic matter, the procedure was modified in 
different ways.

5 mg of each sample in three replicates was taken in 50 ml volumetric flask. Then, 
10 ml of 1N K$_2$Cr$_2$O$_7$ was added. Mixed it properly and then 20 ml of conc. H$_2$SO$_4$ was 
added slowly. The solution was swirled and then kept at room temperature for 1 hour. 
Then, final volume was made up to 50 ml with distilled water. Cooled the solution and 
then absorbance was taken at 660 nm. Aliquots of glucose standards taking 1 mg, 5 mg, 
10 mg, 15 mg, 20 mg, 25 mg and 30 mg were also run in the same procedure. From the 
carbon standard curve, the conc. of C present in the samples was determined.

\[
\% \text{ of } C = \frac{\mu g \text{ of CX50X100}}{5000}
\]

3.2.5. Total Nitrogen

Nitrogen in the sample was estimated by following the Micro Kjeldhal method as 
outlined by Singh and Pradhan, 1981. Dried sample (0.5 g) was digested using 10 ml of 
concentrated sulphuric acid in presence of 0.3 g of catalytic mixture containing 
potassium sulphate and copper sulphate in the ratio of 5:1 in the Micro Kjeldhal 
digestion unit. The digested samples were diluted with distilled water and distilled after 
the addition of sufficient quantities (30 ml) of 40% NaOH to make the sample alkaline 
in the Micro Kjeldhal distillation unit. The ammonia evolved was trapped in 4% boric 
acid mixed indicator solution and titrated against standardised 0.05 N sulphuric acids. 
The amount of (%) of N content was calculated from the volume of acid consumed.

\[
\% \text{ N} = \frac{\text{TitreX N of } H_2SO4X0.014X \text{ Dilution factor}}{\text{Weight of the sample (g)X 100}}
\]

3.2.6. C: N

The C: N ratio was calculated by dividing percent of organic carbon by percent of total 
nitrogen.

3.2.7. CHNS (O) Analyser (Elementar Vario Micro Analysensysteme GmbH)

CHNS (O) Analyzer is an elemental analyzer dedicated to the simultaneous 
determination of the amount of (%) of C, H, N, S and O contained in organic, inorganic 
and polymeric materials and in substances of different nature and origin i.e. solid, liquid 
and gaseous samples. Technique used is DYNAMIC FLASH COMBUSTION and it 
involves combustion of test sample in an oxygen rich environment. The products of
combustion in a CHNS (O) analysis (CO$_2$, H$_2$O, N$_2$ and SO$_2$) are carried through the system by He carrier gas. The combustion products are measured quantitatively by means of a non-dispersive IR absorption detection system, except for the N$_2$ which is determined via a thermal conductivity detector.

The standardised sample weight of 10 mg housed in a tin capsule is dropped into a quartz tube at 1020°C with constant helium flow (carrier gas). A few seconds before the sample drops into the combustion tube, the stream is enriched with a measured amount of high purity oxygen to achieve a strong oxidizing environment which guarantees almost complete combustion/oxidation even of thermally resistant substances. The combustion gas mixture is driven through an oxidation catalyst zone, then through a subsequent copper zone which reduces nitrogen oxides and sulphuric anhydride eventually formed during combustion on catalyst reduction to elemental nitrogen and sulphurous anhydride and retains the oxygen excess. The resulting four components of the combustion mixture are detected by the thermal conductivity detector in the sequence N$_2$, CO$_2$, H$_2$O and SO$_2$. In case of oxygen which is analyzed separately, the sample undergoes immediate pyrolysis in a He stream which ensures quantitative conversion of organic oxygen into CO separated on a GC column packed with molecular sieves. Thus, the contents of N, C, H, S, C/N and C/H ratios of the sample can be detected.

### 3.2.8. Phosphorous

Total phosphorous (P) was estimated by Vanadomolybdophosphoric yellow color method (Koenig & Johnson, 1942). In this method, first 0.5 g sample was digested with 10 ml of mixture of triple acids namely concentrated nitric acid, perchloric acid and sulphuric acid in the ratio of 10:4:1 at 250°C in Kel-Plus digester till colourless fumes come out. The digested material was then made up to 50 ml with distilled water. From this 5 ml of aliquot was taken in a 25 ml volumetric flask and 2/3 drops of 2.5% dinitrophenol indicator was added. Add NH$_3$ solution till the yellow color developed and 6N HCl was added till the yellow color disappeared. Then, 10 ml of Vanadate – molybdate reagent was added and final volume was made up to 25 ml with distilled water and absorbance was taken at 440 nm. The concentration of P in the sample was obtained by comparing phosphorous standard curve. Then, using the following formula, percent of P was calculated.
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\[ P_2O_5 \, (\%) = \frac{\text{Graph ppm} \times \text{Volume of digest} \times \text{Dilution factor} \times 100}{\text{Weight of the sample}} \]

3.2.9. Secondary and Micronutrient analysis of samples by atomic absorption spectrophotometer

There are three steps in the nutrient analysis. The steps are:

1) Drying of the sample

The sample should be air dried. It can also be dried inside the net house. Then, the sample should be ground into powder.

2) Digestion of the sample

To detect the nutrient content of the samples, digestion of the sample should be done in Kel-Plus digester. For digestion, take 0.5 g of the ground sample and add 10 ml of the acid mixture which is in the ratio 10:4:1 of HNO₃:HClO₄:H₂SO₄. Digest it at 250°C in Kel-Plus till colourless fumes come out but at the end of the digestion, at least 2 ml of the suspension should be obtained. Remove the content by adding some water and dilute to 50 ml in volumetric flask to get 100 times dilution. Then, filter using Wattmann filter 42. This is the stock sample. In order to use in AAS, we should dilute the sample and the reading obtained from the AAS should be multiplied by the dilution factor.

3) For nutrient analysis done by atomic absorption spectrophotometer, calculation was done as

\[ \% \text{ of nutrient} = \frac{\text{Graph ppm} \times \text{Dilution factor} \times \text{Volume of sample} \times 100}{\text{Weight of the sample}} \]

3.3. MICROBIOLOGICAL CHARACTERISATION OF IMPHAL CITY BIOWASTE

3.3.1. Isolation or enumeration of pathogenic bacteria

The municipality biowaste and other organic waste which are used as raw material may contain pathogenic microorganisms such as *Salmonella* spp., *Shigella* spp., *Enterobacter* spp. and *Micrococcus* spp. It is important to determine these loads in waste and then device strategy for reduction so that the final product is safe for use. The PB was isolated in standard media used in study of pathogenic bacteria in medical pathology laboratory. These media are Salmonella agar for *Salmonella* spp., Shigella agar for *Shigella* spp., MacConkey agar for *Enterobacter* spp. and Trypton Glucose Extract agar for *Micrococcus* spp. However, these media were not specific and other
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Microorganisms may also grow on them and may lead erroneous results. We cross streaked the isolates of different media to ascertain that the method is valid. After cross streaking, the change in colony morphology was noticed.

3.3.2. Isolation or enumeration of beneficial bacteria

The determination of the load of the beneficial bacteria of the genus *Azospirillum* spp., *Azotobacter* spp. and *P. solubiliser* is important and was streaked using the selective media liked Rhozocongo agar for *Azospirillum* spp., Asbhys Mannitoal agar for *Azotobacter* spp. and Pikovskayas agar for *P. solubiliser*. In this case also, media were not specific and other microorganisms may also grow on them. To check the validity of the method, cross streaking was done to determine the change in colony morphology.

3.4. POPULATION OF MICROORGANISMS IN THE MUNICIPALITY BIOWASTE DUE TO DIFFERENT TREATMENTS

Microbial analysis is considered as an essential parameter to assess the safety of the compost product as city waste harbours different types of microorganisms some of which are harmful. Their population levels may be dependent on several factors. The harmful microorganisms in the degradable organic portion of the waste may be a risk for spreading disease in human and crop if their population in the final compost remain high. So, microbial analysis is considered as an essential parameter to assess both in the initial composting mix and at different stages of decomposition. Such assessment can also indicate effect of composting treatment for reducing population load of pathogenic bacteria (PB). Therefore, quantification of the population of PB and effect of pre-treatment on it was an important component of this research. Furthermore, along with the PB, the beneficial bacteria (BB) present in the waste were also determined. The population of *Salmonella* spp., *Shigella* spp., *Micrococcus* spp. and *Enterobacter* spp. in the city waste were determined in the sample drawn from KRYPSA disposal site. For each sample bag, known quantity of waste was drawn and made into a composite sample. Four replicated samples of 1.0 g each were serially diluted and 0.1 ml from appropriate dilution was plated on selective media for each of the PB and BB namely *Azospirillum* spp., *Azotobacter* spp., phosphate solubilising and cellulose degrading bacteria.
3.4.1. Population of PBs and BBs in the municipality biowaste due to exposure to different temperature and time interval regimes

Degradable organic portion of municipality waste was spread in ambient environment as well as under green house in equal quantity. The temperature in the green house was 30 - 50°C from 8 am to 4 pm and about 27 - 34°C from 4 pm to 8 am. Similarly, for ambient environment, temperature ranged from 27 - 34°C between 8 am to 4 pm and 20 - 25°C from 4 pm to 8 am. The materials were kept in 3 replicates and samples were drawn at 0, 24, 48, 72 and 120 hours from green house and at 0 and 120 hrs in case of materials kept in ambient environment. Serial dilutions were plated in selected media for PBs (Salmonella agar for *Salmonella* spp., Shigella agar for *Shigella* spp., MacConkey agar for *Enterobacter* spp., and Tryptone glucose extract agar for *Micrococcus* spp.) and BBs (Pikovskaya agar for *P. solubilizer*, Ashby’s Mannitol agar for *Azotobacter* spp., Rhizo Congo Agar for *Azospirillum* spp. and Omeliansky agar for cellulose degraders) for determining microbial population load. To confirm the occurrence of PB in compost heap, their morphology was compared by streaking reference strains of these PB (*Salmonella* MTCC 98, *Micrococcus* MTCC 2405, *Shigella* MTCC 2823) and BB (*Bacillus megaterium* MTCC 4126, *Azospirillum* amazonase MTCC 4128, *Azotobacter* RazoB spp.) in the respective selective media. The colony morphology of the PB and BB was also determined by cross streaking on the selective media.

3.4.2. Population of PBs and BBs due to heating at wide temperature range for short durations

In another set of experiment, the biodegradable organic portion of municipality waste was collected and chopped into small pieces. Then, in three replicates, the sample was exposed to 30 - 80°C for 10 hr. and 72 hr. and 30 - 100°C for 6 hr. and 72 hr. Population of both BB and PB were determined by serial dilution technique and cfu/gdb was determined. The difficulty of having continuous power supply at night compelled us to keep the method during day in the oven where temperature was either 80°C or 100°C and remaining hours without power supply was treated as ambient (30°C).
3.4.3. Population of PMs and BMs in biodegradable organic waste of disposal site at different duration after dumping

Three different heaps of biodegradable organic waste lying exposed for 3 different duration namely (a) 0 day i.e. just deposited (b) 4 days and (c) 15 days were selected for determination of level of PBs and BBs. Samples were drawn in triplicate, serial dilution was prepared and plated in selective media and incubated at 30°C. Colonies were counted after 48 hrs. of incubation.

3.4.4. Population of PBs and BBs in municipality biowaste exposed to direct UV light

The pre treatment of the municipality waste was also done by exposing to direct UV light. Sample was collected from the city waste disposal site and cut into small pieces. One set of 2 kg organic waste in three replicates was kept in the UV box containing two UV lamps kept around 1 ft above the material (Fig. 5) while the other set was kept in the box where there was no UV ray. Moisture was maintained by spraying water and mixing the materials uniformly. The whole system was covered by black plastic to prevent UV exposure. After exposure to UV light for different intervals, samples were collected and population of PB and BB were determined. The result was also compared with the samples kept in the box where there was no exposure of UV light.

Fig. 5. UV chamber for direct exposure of sample to UV light
3.5. DIVERSITY OF CELLULOSE DEGRADING MICROORGANISMS (CDMs) FROM CELLULOSE RICH MATERIALS OR CELLULOSE ENRICHED MATERIALS

Cellulose is the most abundant biopolymer and is recalcitrant by virtue of its crystalline nature and insolubility as well as complexation with lignin. Its efficient conversion is a critical phenomenon for maintaining the nutrient balance and energy flow in the earth biosphere. Only fewer microorganisms are found with the ability to utilize cellulose with their cellulase enzyme activity. We know that microorganisms provide the metabolic basis for nutrient cycling. So, isolation of cellulose degrading microorganisms (CDMs) from cellulose rich materials and inoculation of efficient strains into the waste materials for rapid decomposition of organic materials were important component of this research.

3.5.1. Morphological, physical and biochemical characterisation of the isolates
3.5.1.1. Isolation, quantification and purification of population of CDMs from different source materials

Different organic materials namely faeces of herbivorous animals like elephant, goat, cow, horse, omnivores like pig, cat, human, organic materials of plant origin like rice straw, azolla, water hyacinth shoot and root, and soil samples from uncultivated upland and cultivated corn field were used for isolation of CDMs by serial dilution techniques using Omeliansky agar medium. Their population counted by using a colony counter and representative colonies based on similarities of morphological features are purified by repeated streaking and maintained in glycerol stock.

An approach was also used for enrichment of CDMs in which sugarcane trash collected from Imphal city was mixed with garden soil and was incubated for 15 days at ambient environment. This mixture flourished the growth of cellulose degraders. Samplings were done on 5th, 10th and 16th day and were used for isolation of CDMs using Omeliansky agar medium maintained at pH - 5 and pH - 7.

3.5.1.2. Determination of cellulase activity

The cellulolytic activity of the isolates was determined based on the reducing power of sugar which is the end product of the enzymatic reaction of cellulase and the substrate (cellulose). Cellulase activities of the isolates were determined by DNS Reagent Method. In the method, the Rocelle salt was introduced to prevent the reagent
from dissolving oxygen; phenol to increase the amount of color produced and to balance the effect of phenol, and bisulfite to stabilize the color obtained in the presence of phenol. The alkali was added for the reducing action of glucose on dinitrosaliclyclic acid. Schematic presentation of the method is shown in fig. 6.

In cellulase quantification assay, colony growth was used to inoculate the Omeliansky broth supplemented with cellulose filter paper. Colonies were picked up based on visual examination so that approximately equal amount of colony growth were added in the equal quantity of broth and cfu was determined by serial dilution technique. Still it was difficult to get same cfu because some isolates were fast grower while some were slow grower. Therefore, for cellulase activity determination experiment, inoculum level for each isolates was standardized by taking variable volume of broth culture of isolates after determination of cell number in known volume of broth and inoculated into filter paper strip supplemented Omeliansky broth. Standardisation of the test was done by using different size filter paper, possibility of release of sugars from cellulose filter during autoclaving, different temperature, different incubation time, size of inoculums and cellulase enzyme of standard manufacturer.

a) Cellulase activity as affected by size of the inoculums

To have optimum enzyme activity, there should be proportionate relationship of binding sites of enzyme and substrate. So, standardisation of size of inoculum is very important. Different numbers of colony of CDM9 isolate from Omeliansky agar plate was inoculated in 10 ml of Omeliansky broth and incubated at 30°C. After 48 hr incubation, the crude enzyme taken by centrifugation was tested for cellulase activity by DNS method.

b) Cellulase activity as affected by pH and type of carbon source

Literature says that as the cellulose is crystalline, harsh conditions are needed to liberate glucose from these tightly associated chains. So, type of carbon source affected the liberation of glucose. Yields of glucose increase with temperature and acid concentration (Katzen et al., 1942; McParland et al., 1982; Brennan et al., 1986 and Lynd et al., 2002). So, for study of cellulase activity of the isolates, we used same carbon source i.e. Whatman filter paper 41, Cat No. 1441 125, cut into pieces having average wt. of 3.7 mg/piece.
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To check change of pH and amount of glucose released from the Omeliansky broth, four 10 ml broth supplemented with cellulose powder (Hi media Cat No. RM126) and cellulose paper (Whatman filter paper) as carbon source and maintained at different pH were taken. Then, autoclave at 121°C and 15 lb for 15 min. Then, the broths were centrifuged and glucose content in the supernatant was determined by DNS method.

c) Cellulase activity as affected by size of cellulose filter paper as carbon source

Finer the particle size of the carbon source, more is the amount of glucose released due to autoclaving. Since size of filter paper as carbon substrate may affect the ability of the CDM to degrade cellulose in determining the optimum size for maximum cellulase activity, different sizes were used. To the 10 ml Omeliansky broth, different size cellulose filter paper (powder, small size of 1.2 mg/piece and large size of 3.7 mg/piece) was added separately in triplicate and inoculated with CDM9 isolate and grow for 3 days at 30°C. The crude enzyme extract taken by centrifugation was tested for cellulase activity test determined by DNS method.

d) Cellulase activity as affected by autoclave

CDM9 was grown in triplicate in sterile Omeliansky broth supplemented with cellulose filter paper as carbon source. After 48 hr incubation at 30°C, the enzyme extract was taken by centrifugation. Then, it was divided into two parts. One part was again autoclave. Whatever enzyme present in the extract was denatured due to heat. Then, both autoclave and unautoclave extracts were tested for cellulase activity by DNS method. Whatever glucose released from the autoclaved extract was due to 1st autoclave for sterilisation of broth and 2nd autoclave of the extract but in case of unautoclave broth, whatever glucose released was due to 1st autoclave for sterilisation and enzyme activity of the CDM9 isolate.

e) Cellulase activity as affected by incubation temperature

CDM isolates were grown in triplicates in Omeliansky broth supplemented with cellulose filter paper as carbon source and incubated at two different temperatures, 30°C and 40°C. On third day, crude enzyme extract of each replicates of different isolates were tested for cellulase activity by DNS method.

f) Cellulase activity as affected by incubation time

CDM isolates were grown in triplicates of three sets for each isolates in Omeliansky broth supplemented with cellulose filter paper as carbon source and
incubated at 30°C. Crude enzyme extracts in triplicate of each isolates were taken on 3rd, 5th and 9th days of incubation from the three different sets of each isolates. Then, cellulase activity was determined by DNS reagent method.

g) Standardisation of Hi media cellulase enzyme

Hi media cellulase “ONOZUKAR – 10 and Cat. No. RM3331 having activity >= 1.0 u/mg solid at optimum temperature 40 - 50°C and optimum pH at 4 - 5, was used as positive control. To have optimum activity of the enzyme with 32 mg substrate used in DNS method, standardisation of optimum amount of enzyme was very essential. Different aliquots of enzyme solution was taken from 200 ppm enzyme stock and tested for cellulase activity by DNS Method.

h) Cellulase activity as affected by freeze storage

Positive control was very important to interpret our results. So, we had to check whether the activity of the Hi media cellulase changes due to storage or not. Two aliquots of enzyme solutions (22 µl from 200 ppm as standardised) were taken one from freshly prepared 200 ppm stock solution and another from 5 days old 200 ppm freeze stock solution and cellulase activity was determined by DNS method.

High cellulolytic activity strains Cellulomonas cellulans MTCC23, Trichoderma reesei MTCC164, and Bacillus subtilis MTCC2414) were run as reference strains. The negative control broth where there was no inoculum was also tested along with the test CDM isolates of our research collection for cellulase activity.
DETERMINATION OF CELLULASE ACTIVITY:

0.5ml crude enzyme extract

Incubation at 50 °C for 1 hr

5 min. incubation in boiling water bath

Immediate cooling in running tap water

Make up final volume to 15 ml with H2O

Centrifugation at 2500 rpm for 5 min.

Absorbance at 540nm

Calculation of glucose conc. of the sample from the Glucose standard graph

Fig. 6. Schematic representation of Cellulase activity by DNS Reagent method

3.5.2. Molecular characterisation of the isolates

3.5.2.1. Molecular characterisation of efficient isolates

The 10 efficient strains were characterised at molecular level based on the 16S rRNA gene sequence and ITS region. Isolates were grown at 37°C for 24 hr. for bacteria, 48 hr for actinomycetes and 120 hr for fungus.

a) Isolation of bacterial and actinomycetes genomic DNA by CTAB Method

The bacterial DNA extraction was done by modified CTAB method described by William S. and Helene Feil A. Copeland; 2012. The bacterial cells were grown in LB broth. The broth was centrifuged at 10000 rpm for 10 minutes. The supernatant was discarded. The cells were resuspended in 740 µl TE buffer. Lysozyme was added so that the final conc. be 2.7 mg/ml. It was incubated for 5 minute at room temperature. 40 µl of 10% SDS and proteinase K to a final conc. of 0.1mg/ml were added. It was incubated for 1 hrs. at 37°C. 100 µl of 5 M NaCl was added and it was mixed well. Then, 100 µl of CTAB was added (heated at 65°C). Incubate at 65°C for 10 min. 0.5 ml of phenol:
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chloroform: isoamyl alcohol (25:24:1) were added and mixed well. The mixture was spin at 10000 rpm for 10 minute at room temperature. Upper aqueous phase was transferred to clean eppendorf tube. 0.5 ml chloroform: isoamyl alcohol (24:1) was added to aqueous phase. This mixture was centrifuged at 10000 rpm for 10 minute at room temperature. Upper aqueous phase was transferred to clean eppendorf tube and add 0.6 volume of isopropanol (-20°C). It was incubate at room temperature for 30 minute. The spin was given at 12000 rpm for 15 minute. The pellet was washed with 70% ethanol and spin at 12000 rpm for 10 minute. The supernatant was discarded and let the pellet dry at room temperature for 5-10 minute. The pellet was resuspend in TE plus RNase (99 + 1) and kept at 80°C for 20 min. Run the DNA sample in 0.8% agarose gel for purity and quantified the DNA. Store it at -20°C for PCR.

b) Isolation of Fungal genomic DNA

Fungal DNA extraction was done by using extraction buffer as decribed by Guzeldag G., and Colak O., 2007 and Lecellier, G. and P. Silar, 1994. Fungal culture was grown in LB broth for 72 hr. the cell biomass was crushed into paste with 1ml extraction buffer (EB). 3 ml of EB was again added and paste was transferred to 2 ml tube. Dipped in liquid nitrogen for 30 seconds. Then, tube was thawed in water bath (60°C) for 30 minutes. Centrifuge at 12000 rcf for 15 minutes. Transferred the supernatant in a new tube. 1 µl of RNAas of 10 mg/ml was added and incubate at 37°C for 30 minutes. Phenol: chloroform: isoamylalcohol is added at 1:1 (v/v) and mix thoroughly. Centrifuged at 12000 rcf for 15 minutes. Upper layer was transferred to a new tube. Then, phenol: chloroform: isoamylalcohol treatment was repeated. To the upper layer, equal volume of chloroform: isoamylalcohol was added. Mix thoroughly by inverting and centrifuged at 12000 rcf for 15 minutes. Upper layer was transferred in a new tube and equal volume of ethanol was added. Incubate overnight at – 20°C. Centrifuge at 12000 rcf for 15 minutes. The supernatant was discarded and pellet was washed with 70% ethanol twice. Spin at 12000 rpm for 10 minute. The supernatant was discarded and let the pellet dry in laminar air flow for 5 - 10 minute. Then, dried pellets are dissolved in 50 µl TE buffer. Quantify the DNA in Nano spetetrophotometer and run in 0.8% agarose gel to check purity. Then, store it at -20°C for PCR.
c) Electrophoresis of DNA sample

Gel electrophoresis is a method for separation and analysis of macromolecules (DNA, RNA and proteins) and their fragments, based on their size and charge. In molecular biology, it is done to separate a mixed population of DNA and RNA fragments by length, to estimate the size of DNA and RNA fragments or to separate proteins by charge. Nucleic acid molecules are separated by applying an electric field to move the negatively charged molecules through an agarose matrix. Shorter molecules move faster and migrate farther than longer ones because shorter molecules migrate more easily through the pores of the gel. This phenomenon is called sieving. Gel electrophoresis can also be used for separation of nanoparticle. Gel electrophoresis uses a gel as an anticonvective medium and/or sieving medium during electrophoresis, the movement of a charged particle in an electrical field. Gels suppress the thermal convection caused by application of the electric field, and can also act as a sieving medium, retarding the passage of molecules; gels can also simply serve to maintain the finished separation, so that a post electrophoresis stain can be applied. DNA Gel electrophoresis is usually performed for analytical purposes. After isolation of genomic DNA, it was run on 0.8% agarose gel, in which ethidium bromide was added. Ethidium bromide is a fluorescent dye, which intercalates between the stacked bases. The fluorescent yield of the dye: DNA complex is much greater than the unbound dye. UV irradiation at 260 nm is absorbed by the DNA and transmitted to the dye and the bound dye itself absorbs radiation at 302 nm and 366 nm. This energy was retransmitted at 590 nm, the reddish-orange region of the visible spectrum.

d) Quantification of DNA sample using Nano spectrophotometer

The Nano Spectrophotometer is the perfect tool for kinetics, concentration measurement and estimation of purity of all kinds of UV/vis photometric applications in molecular biology, biochemistry and microbiology. Its spectrum ranges 190 – 1100 nm. Sample dilution is not required and concentration of undiluted DNA sample ranged 2 - 5,000 ng/µl. The Nanodrop will display an absorbance spectrum for each sample as it is being measured. DNA should have an absorbance peak centered at a wavelength of 260 nm. The ratio A260/A280 should be ~1.95. The presence of organic solvents (e.g. phenol) may lead to a spuriously high A260/A280 ratio (>2). Proteins will have a peak absorbance at 280 nm, so protein contamination will lower the A260/A280 ratio.
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Protein does not absorb as strongly as DNA so even a modest reduction in the A260/A280 ratio (e.g. 1.8) may represent a high level of contamination. The extinction coefficient is a factor that converts the peak absorbance to concentration. For DNA the extinction coefficient is 50 (ng/µL DNA) /260 nm. Nanodrop has a broad linear range. Accuracy drops off (error > 10%) for concentrations < 4 ng/µL and > 4000 ng/µL. Load 1 µl of 1XTE buffer on the pedestals (sensor) and select "Blank" and wipe pedestals. Then, load sample on the sensor and read the reading. Also check the peak of the graph.

e) Thermal cycler and amplification of 16S rRNA gene for bacteria and actinomycetes DNA and ITS for fungal DNA

The thermal cycler also known as a Thermocycler, PCR Machine or DNA Amplifier is a laboratory apparatus used to amplify segments of DNA via the polymerase chain reaction (PCR) process. The device has a thermal block with holes where tubes holding the PCR reaction mixtures can be inserted. The cycler then raises and lowers the temperature of the block in discrete, pre-programmed steps. Modern thermal cyclers are equipped with a heated lid, a heated plate that presses against the lids of the reaction tubes. This prevents condensation of water from the reaction mixtures on the insides of the lids. A basic PCR set up requires several components and reagents. These components include DNA template, Two primers that are complementary to the 3’ ends of each of the sense and anti-sense strand of the DNA target, Taq polymerase, deoxynucleoside triphosphates, buffer solution and MgCl$_2$ solution. PCR was performed using universal 16S rRNA gene primers FD1: 5’GAGTTTTGATCCTGGCTCAG3’ and RD1: 5’ AAGGAGTGATCCAGCC-3’. Each 25 µl PCR reaction mixture contained 50 ng of DNA in the cell free DNA lysate, 2.5 µl of 10XPCR 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). The amplification was performed in thermal cycler (ICycler, Biorad, USA) and the cycling program was started with an initial denaturation at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 58°C for 1 min and extension at 72°C for 30 s. The PCR was 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 analyzed by applying 5 μl of the PCR product in 0.8% agarose (V3125, Promega) gel.

For fungus, PCR was also performed using universal ITS1 and ITS4 primers having sequence of ITS1: 5’TCCGTAGGTGAACCTGCGG 3’ and ITS4: 5’TCCTCCGCTTATTGATATGC 3’. Each 25 μl PCR reaction mixture contained 50ng template DNA, 2.5 μl of 10XPCR reaction buffer with 1.5 mM MgCl₂, (105876, GeNei), each dNTP (1054876, GeNei) of 500 μM and 0.04 U of Taq DNA polymerase (M300Bs, Promega). The amplification was performed in thermal cycler (ICycler, Biorad, USA) and the cycling program was started with an initial denaturation at 95°C for 5 min followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 45 sec and extension at 72°C for 60 sec. The PCR was ended with a final extension at 72°C for 10 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 ITS rDNA fragment size of 500 - 1000 bp was analyzed by applying 5 μl of the PCR product in 0.8% agarose (V3125, Promega) gel.

The amplified PCR products of the 10 efficient CDM isolates were sent to Banglore genii for partial sequencing of the 16S rRNA gene sequence and the first two highest activity isolates, CDM2 and CDM9 were sent to MTCC, Chandigarh, for full length sequencing of 16S rRNA gene. The phylogenetic trees were constructed using neighbor-joining method by MEGA (Tamura et al., 2011). Bootstrap resampling analysis for 1000 replicates was performed to estimate the condense of tree topologies.

f) Gel DOC

Gel Doc, also known as Gel Documentation System, Gel Image System or Gel Imager, is widely used in molecular biology laboratories for the imaging and documentation of nucleic acid and protein polyacrylamide or agarose gels typically stained with ethidium bromide or other fluorophores such as SYBR Green. Generally, a Gel Doc is composed of an ultraviolet (UV) light transilluminator, a hood to shield external light sources and a camera for image capturing.

3.5.3. Preliminary screening of CDM based on cellulase activity in situ

In this expt., the screening of efficient CDM isolates was done on the basis of feasibility test of the isolate to decompose biowaste. As a result of biowaste decomposition by the action of microorganisms and in the presence of oxygen, the
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organic biowaste is converted to biomass, CO$_2$, residual polymer or oligomers and inorganic dissolved carbon. The principle of biodegradation is generally assessed either by oxygen consumption or by carbon dioxide production. In our experiment, the biodegradation of a material was measured in terms of carbon dioxide production. It was carried in a system as shown in Fig. 7.

1) Degradation reactor

In the reactors, substrates (7 g in case of cellulose powder or 30 g in case of city waste) were added and then 15 ml of 48 hr old 5 IBSD isolates (CDM2, CDM6, CDM7, CDM9, CDM10 and reference strain Cellulomonas cellulans MTCC164) were inoculated to saturate the materials in the respective reactors. Aeration of the reactors connected by three way connectors was done every day at 2 p.m. by using vacuum pump. A weekly interval up to 6$^{th}$ week, 1g sample was removed from the flask. Then, 0.5 g sample was kept for dry wt. while 0.5 g was dissolved in 2.5 ml sterile distilled water so that crude enzyme extract was prepared by using syringe filter. Then, enzyme activity in the extract was determined by DNS reagent method.

2) Carbon dioxide trapping measurement

Inside the reactor, 10 ml of 0.1N Ba (OH)$_2$ solution in big tube was kept in each set. The solution absorbs the CO$_2$ evolved due to the degradation of materials by the action of respective isolates. A weekly interval up to 5$^{th}$ week, solution of Ba (OH)$_2$ was replaced by fresh solution and the amount of C in terms of CO$_2$ was determined by titrating the removed solution with standardised 0.02 N H$_2$SO$_4$ solution.

3) Air treatment (production of CO$_2$ free air)

In order to remove the CO$_2$ present in the incoming air, the air was passed through 300 ml of 0.1 N NaOH with the help of vacuum pump.
3.6. DECOMPOSITION OF MUNICIPALITY WASTE

3.6.1. Decomposition of municipality waste either alone or mixture with other waste materials with or without earthworm

Biowaste was collected from vegetable market area. This comprised of stem, leaf, fruit, bud, root of various vegetables which includes Karif crops like Brassica oleracea var. capitata, Brassica oleracea var. botrytis, Pisum sativum, Daucus carota, Solanum melongena, Solanum tuberosum, Zea mays, Solanum lycopersicum, Nelumbo lutea, Lilium bulbiferum, Allium cepa, Coriandrum sativum, Brassica nigra, Beta vulgaris,
Brassica rapa, Allium odorum, Houttuynia cordata, Areca catechu, Musa acuminata, Bambusa vulgaris, Calocasia esculenta, Plantae vulgaris, Phaseolus lunatus, Solanum melongena, Abelmoschus esculentus, Lycopersicon esculentum, Solanum tuberosum, Coccinia grandis, Cucurbita maxima, Brassica juncea, Daucus carota, Brassica rapa, Beta vulgaris, Cucumis sativus, Malus domestica, Vitis vinifera, Citrus sinensis, Citrus limon, Nymphaea odorata, Nelumbo nucifera edible vegetables of grass family. The materials were chopped into small pieces. The agri waste materials used in mixture was rice straw (RS) and cow dung (CD). RS was made ready by cutting and CD by manual homogenization. The earthworm, Eisenia fetida was obtained from the culture reared in IBSD (Fig. 8).

Composting was conducted on the feed mixture as mentioned in table 15 in Bamboo bins of size 120X70X60 cm$^3$ dimension keeping inclined at 30$^\circ$ to allow the accumulation of the liquid produced during decomposition towards the sloppy end. Earthworm treatment received 500 adult earthworm of 153.6 g biomass. Composting was done from 22$^{nd}$ August to 6$^{th}$ December, 2009. Representative sample of biowaste was collected and analysed for chemical characteristics and population of both PB and BB at the start of decomposition. Temperature (°C) in the mixture (15 cm and 30 cm) and air above the bins were recorded everyday at 2 p.m. using a digital thermometer. Liquid produced in the three treatments was collected and measured at 2 days intervals and total amount of liquid produced from different treatments were compared. The parameter of earthworms in the decomposing mixture was determined by counting the number of adults, juveniles and cocoon at the time of maturity of the compost. At maturity of the compost, different size fractions of the final compost i.e. fine fraction < 0.2 mm, medium fraction 2.0 - 4.0 mm, coarse fraction > 4.0 mm were determined by sieving through sieve of appropriate pore diameter. Compost samples were analysed for pH in 1:10 suspension by pH meter, EC in 1:10 suspension by conductivity meter. The results of total organic carbon (C) determined by the rapid dichromate oxidation method.
Table 15. Details of treatments for composting of fresh MW without inoculums

<table>
<thead>
<tr>
<th>Type of study</th>
<th>Treatment</th>
<th>Composting mix details</th>
<th>Parameters obtained</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thermophilic decomposition of organic portion of municipality</td>
<td>T1 MW + RS + CD + EW</td>
<td>*Role of earthworm in increasing finer compost fractions</td>
<td></td>
</tr>
<tr>
<td></td>
<td>T2 MW + RS + CD</td>
<td>*Effect of rice straw and cow dung in decay of MW</td>
<td></td>
</tr>
<tr>
<td></td>
<td>T3 MW</td>
<td>*Beneficial and pathogen microbial populations load in the final compost</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>*Nutrient composition (C,N, P, K, pH, EC, C:N)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>*Micronutrient content (Cu, Al, Ca, Mg, Zn, Na, Fe, etc.)</td>
<td></td>
</tr>
</tbody>
</table>

(Walkey and Black, 1934), total nitrogen (N) determined by Kjeldahl method (Singh and Pradhan, 1981) and C:N ratio were compared with the results obtained from CHNS(O) analyser (Vario MICRO V2.2.0, Elementer Analysensysteme GmbH). Total phosphorous (P) was determined by Vanadomolybdophosphoric yellow color method (Koenig & Johnson, 1942), Potassium (K), Sodium (Na), Iron (Fe) and Zinc (Zn) by triple acid digestion and Atomic Adsorption Spectrophotometer (AAnalyst, Version 6, Perkin Elmer Inc.). Microbial population of both PB and BB in the final compost were determined by serial dilution method using selective media. The commercial compost available in the local market was also analysed to compare quality of the compost produced from biowaste of MSW.

3.6.2. Biodegradation of municipality organic biomass using CDM and EW

The role of CDM in waste mineralization is well documented in the literature (Khwairakpam and Bhargava, 2009; Suthar, 2010). CDM includes numbers of fungal strains and greater population of other microbes, such as bacteria, protozoa, nematodes, fungi and actinomycetes. The indigenous population plays an important role in organic matter decomposition by providing extra-cellular enzymes and further inoculation of
efficient strain of CDM into the composting mixture results rapid decomposition. Though organic waste harbours huge amount of microbes, only few microbes are having ability to degrade cellulose rich materials. Effect of efficient CDM and EW on the decomposition of city biowaste was examined in two sets of experiments.

In the first composting expt., efficient IBSD isolate, CDM9 and EW were treated in fresh municipality biomass which includes kharif crops like *Brassica oleraceavar. capitata*, *Brassica oleracea var. botrytis*, *Pisum sativum*, *Daucus carota*, *Solanum melongena*, *Solanum tuberosum*, *Zea mays*, *Solanum lycopersicum*, *Nelumbo lutea*, *Lilium bulbiferum*, *Allium cepa*, *Coriandrum sativum*, *Brassica nigra*, *Beta vulgaris*, *Brassica rapa*, *Allium odorum*, *Houttuynia cordata*, *Areca catechu*, *Musa acuminata*, *Bambusa vulgaris* etc. The biowaste collected from the Imphal city was chopped into small pieces. Then, by mixing MW with the bulky materials i.e. RS and CD, in 7:2:1 ratio, six feed mixtures (mixed biomass 10 kg) of three replicates each as mentioned in table 16 were prepared. EW treatment received 100 numbers of weighed adult worms and CDM treatment received 100 ml nutrient broth (NB) of 72 hr. old CDM9 culture having 8.86 log cfu/ml population.

All tests were carried out at ambient temperature. Every day the temperature in the mix and ambient air were recorded at 2 p.m. and the drained out liquid was measured in two days intervals for 39 days. 100 numbers of weighed adult earthworms were applied on 10th day when the temperature of the mix was below 30°C. Composting was started on 21-07-2011 and compost samples were harvested on 02-11-2011.
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Table 16. Details of treatments for composting of fresh MW with CDM and EW

<table>
<thead>
<tr>
<th>Type of study</th>
<th>Treatments</th>
<th>Testing details</th>
</tr>
</thead>
<tbody>
<tr>
<td>FT1</td>
<td>Fresh MW (10 kg) + 100 ml of nutrient broth</td>
<td></td>
</tr>
<tr>
<td>FT2</td>
<td>Fresh MW (10 kg) + 100 no. of adult EW + 100 ml of nutrient broth</td>
<td></td>
</tr>
<tr>
<td>FT3</td>
<td>Fresh MW (10 kg) + 100 ml of 72 hr. old CDM9 culture in nutrient broth</td>
<td></td>
</tr>
<tr>
<td>FT4</td>
<td>Fresh MW (10 kg) + 100 no. of adult EW + 100 ml of 72 hr. old CDM9 culture in nutrient broth</td>
<td></td>
</tr>
<tr>
<td>FT5</td>
<td>Fresh MW (7 kg) + CD (2 kg) + RS (1 kg) +100 no. of adult EW + 100 ml of nutrient broth</td>
<td></td>
</tr>
<tr>
<td>FT6</td>
<td>Fresh MW (7 kg) + CD (2 kg) + RS (1 kg) + 100 no. of adult EW + 100 ml of 72 hr. old CDM9 culture in nutrient broth</td>
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</tbody>
</table>

Decomposition of fresh municipality waste spiked with different consortia resulted in accumulation of large amount of liquid which was a by product of decomposition get drained out from the system. Along with liquid, a substantial amount of water soluble nutrients also leave the system. High pH of the liquid and of the composting vegetable waste mix could also lead to loss of N due to volatilisation. The time trend of production of liquid could also be indication of extent of decomposition of different biowaste mixture. Therefore, a 2\textsuperscript{nd} composting expt. was conducted on the completely dried biowaste in which moisture was maintained either at 70\% or 90\%. Fresh green biomass containing \textit{Brassica oleracea} var. \textit{capitata}, \textit{Brassica oleracea} var. \textit{botrytis}, \textit{Pisum sativum}, \textit{Daucus carota}, \textit{Solanum melongena}, \textit{Solanum tuberosum}, \textit{Zea mays}, \textit{Solanum lycopersicum}, \textit{Nelumbo lutea}, \textit{Lilium bulbiferum}, \textit{Allium cepa}, \textit{Coriandrum sativum}, \textit{Brassica nigra}, \textit{Beta vulgaris}, \textit{Brassica rapa}, \textit{Allium odorum}, \textit{Houttuynia cordata}, \textit{Areca catechu}, \textit{Musa acuminata}, \textit{Bambusa vulgaris} \textit{Calocasia esculenta}, \textit{Plantae vulgaris}, \textit{Phaseolus lunatus}, \textit{Solanum melongena}, \textit{Abelmoschus esculentus}, \textit{Lycopersicon esculentum}, \textit{Solanum tuberosum}, \textit{Coccinia grandis}, \textit{Cucurbita maxima}, \textit{Brassica juncea}, \textit{Daucus carota}, \textit{Brassica rapa}, \textit{Beta vulgaris}, \textit{Cucumis sativus}, \textit{Malus domestica}, \textit{Vitis vinifera}, \textit{Citrus sinensis}, \textit{Citrus limon}, \textit{Nymphaea odorata}, \textit{Nelumbo
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*nucifera* etc. collected from the Imphal city and chopped into finer pieces. Additional waste such as RS, CD and Loktak phumdhi (LP) were chopped into finer pieces and allowed to completely dry in the plastic house.

The moisture level at saturation point for different wastes like MW, CD, RS, LP and mixtures of the feeds i.e. mixture of MW + RS + CD and MW + LP of desired proportion were determined by taking 10 g of each sample in triplicates in funnel plugged with known wt. of filter paper and adding water drop by drop till a drop of liquid felt down from the tip of the funnel stem. Thus, the amount of water required at saturation point for different feeds was determined and using the result, the desire moisture level was maintained in different treatments.

Composting was done on 2 kg dry but finer pieces of MW in DT1 and DT2, 7:2:1 ratio mixture of MW, CD and RS in DT3 and DT4, 1:1 ratio of MW and LP in DT5 and DT6 while 2 kg of dry and chopped LP was used in DT7 treatment maintained moisture at different levels as indicated in table 17. Each treatment was replicated four times.

**Table 17. Details of treatments for composting of dry MW with CDM and EW**

<table>
<thead>
<tr>
<th>Type of study</th>
<th>Treatment Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Efficiency of CDM isolate</td>
<td>DT1: MW (2 kg) at 90% moisture with 100 ml nutrient broth</td>
</tr>
<tr>
<td></td>
<td>DT2: MW (2 kg) at 70% moisture with 100 ml nutrient broth</td>
</tr>
<tr>
<td></td>
<td>DT3: [MW (1.4 kg) + RS (0.4 kg) + CD (0.2 kg)] at 90% moisture with 100 ml nutrient broth</td>
</tr>
<tr>
<td></td>
<td>DT4: [MW (1.4 kg) + RS (0.4 kg) + CD (0.2 kg)] at 70% moisture with 100 ml of 72 hr. old CDM9 culture in nutrient broth + 100 no. of adult earthworm</td>
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<tr>
<td></td>
<td>DT5: [MW (1 kg) + LP (1 kg)] at 100% moisture with 100 ml nutrient broth,</td>
</tr>
<tr>
<td></td>
<td>DT6: [MW (1 kg) + LP (1 kg)] at 100% moisture with 100 ml of 72 hr. old CDM9 culture in nutrient broth + 100 no. of adult earthworm,</td>
</tr>
<tr>
<td></td>
<td>DT7: LP (2 kg) at 100% moisture with 100 ml nutrient broth.</td>
</tr>
</tbody>
</table>
100 ml of 72 hr. old CDM9 culture in NB with log cfu/ml of 7.48 and 100 numbers of weighed earthworms were inoculated into the respective treatments while the other treatments were also inoculated with equal amount of NB. Temperature of the composting mix and the ambient air was recorded everyday at 2 p.m. and drained out liquid was measured in 2 days intervals for 29 days.

Content of N and pH of the drained out liquid were also determined. The liquid sample drained out from a particular treatment, DT1, was divided into 5 fractions. The 1st fraction was kept in open, 2nd fraction in airtight container, 3rd fraction kept in open but maintained at high alkaline pH by adding by NH₄Cl, 4th fraction kept in air tight container but maintained at high alkaline pH by adding NH₄Cl, 5th fraction maintained at neutral pH by adding Tartaric acid. Available N was estimated at 0 hr, 2nd Day, 5th Day and 10th day by Kjeldhal method (Singh and Pradhan, 1981).Composting was started on 26-08-2012 and compost samples were harvested on 11-12-2012. Composts maturity was judged based on its physical appearance in both sets of composting. Dark brown to black colour with uniformly disintegrated structure of the composting mix was indication of maturity at which it was harvested. The time taken for composting and compost recovery were noted. The compost fractions i.e. fine (< 2 mm), medium (2.0 - 4.0 mm), coarse (> 4.0 mm) and undecomposed fractions of the biowaste were determined by sieving through different pore size sieves. The compost samples were analysed for pH in 1:10 suspension by pH meter and EC in 1:10 suspension by conductivity meter. The results of total organic carbon (C) determined by the rapid dichromate oxidation method (Walkey and Black, 1934), total nitrogen (N) determined by Kjeldahl method (Singh and Pradhan, 1981) and C:N ratio were compared with the results obtained from CHNS(O) analyser (Vario MICRO V2.2.0, Elementer Analysensysteme GmbH). Total phosphorous (P) was determined in triple acid digested sample by Vanadomolybdosphoric yellow color method (Koenig & Johnson,1942), Potassium (K), Calcium (Ca), Sodium (Na), Iron (Fe), Manganese (Mn), Copper (Cu) and Aluminium (Al) were determined by triple acid digestion and Atomic Adsorption Spectrophotometer (AAAnalyst 200, Version 6, Perkin Elmer,Inc.). To assess the quality and safety of the compost, microbial analysis of harvested compost was done based on number of BBs like *Azospirillum* spp., *P. solubilizer* and cellulose degrader
and PBs like *Salmonella* spp., *Shigella* spp and *Micrococcus* spp. determined by plating serial dilution of compost samples on selective media for these organisms.

### 3.6.2. 3. Decomposition of dry but ground Municipality biowaste

In the previous two sets of experiment, attempts were to determine effect of decomposer agents on composting speed and compost quality. Our observation on production of liquid in the composting mix led us to question if the liquid produced at different time interval can be a measure of extent of decomposition. However, results were erroneous which could be due to coarse size of the individual biowaste in the mix.

In this experiment, feed mixture of municipality biowaste containing different vegetable residues, rice straw, cowdung and Loktak phumdhi was ground and mix properly. Then, subjected to decomposition at 90% moisture level by adding required amount of H$_2$O including the 100 ml of NB with or without CDM9 culture of 72 hr old culture with population of 8.114 log cfu/ml. There were four treatments and four replicates of each treatment as details in table 18.

**Table 18. Treatment details for composting of dry but ground MW with inoculum**

<table>
<thead>
<tr>
<th>Type of study</th>
<th>Treatments</th>
<th>Testing details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Efficiency of CDM isolates</td>
<td><strong>GT1</strong> [MW (1.4 kg) + RS (4.0 kg) + CD (2.0 kg)] at 90% moisture with 100 ml nutrient broth</td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>GT2</strong> [MW (1.4 kg) + RS (4.0 kg) + CD (2.0 kg)] at 90% moisture with 100 ml of 72 hr. old CDM9 culture in nutrient broth + 100 no. of adult earthworm</td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>GT3</strong> [MW (1.0 kg) + LP (1.0 kg)] at 90% moisture with 100 ml nutrient broth,</td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>GT4</strong> [MW (1.0 kg) + LP (1.0 kg)] at 90% moisture with 100 ml of 72 hr. old CDM9 culture in nutrient broth + 100 no. of adult earthworm.</td>
<td></td>
</tr>
</tbody>
</table>

In GT2 and GT4, 100 numbers of weighed adult worms were added on 4$^{th}$ day. The temperature was recorded everyday one at 6.30 am and another at 2 pm and the amount of drained out liquid generated was recorded one at 6.30 am and another at 5 pm. The moisture level inside the bin was maintained by keeping 20 ml of water in container in
three replicates each in all the bins. Aeration was done in regular 5 days interval. Since composting was done on ground materials by inoculating earthworm and CDM9, it was completed only in 54 days from 13<sup>th</sup> August to 7<sup>th</sup> October, 2012.

When the composts were ready by its physical appearance in both fresh biomass composting as well as dry biomass composting, as judged by development of a dark brown to black colour with uniformly disintegrated structure, it was harvested. The time taken for composting and compost recovery were noted. The compost fractions i.e. fine (< 2 mm), medium (2.0 - 4.0 mm), coarse (> 4.0 mm) and undecomposed fractions of the biowaste were determined by sieving. The compost samples were analysed for pH in 1:10 suspension by pH meter and EC in 1:10 suspension by conductivity meter. The results of total organic carbon (C) determined by the rapid dichromate oxidation method (Walkey and Black, 1934), total nitrogen (N) determined by Kjeldahl method (Singh and Pradhan, 1981) and C:N ratio were compared with the results obtained from CHNS(O) analyser (Vario MICRO V2.2.0, Elementer Analysensysteme GmbH). Phosphorous (P) by Vanadomolybdophosphoric yellow color method (Koenig & Johnson, 1942), Potassium (K), Calcium (Ca), Sodium (Na), Iron (Fe), Manganese (Mn), Copper (Cu) and Aluminium (Al) by triple acid digestion and Atomic Adsorption Spectrophotometer (AAnalyst 200, Version 6, Perkin Elmer, Inc.). To assess the quality and safety of the compost, microbial analysis of harvested compost was essential and it was done based on the presence of BBs like Azospirillum, P. solubilizer and cellulose degrader and PBs like Salmonella spp., Shigella spp. and Micrococcus spp.

Using the composts obtained under fresh biomass composting processes in the proportion of 50 g compost with 3.450 kg of field soil in a pot, a test crop, Okra (Abelmoschus esculentus) was raised in a Randomized Block Design with six treatments and three replicates each while composts for moisture maintained biomass composting, another test crop, french bean (Phaseolus vulgaris) was raised in a Randomized Block Design with seven treatments and four replicates each. Biomeric observations were recorded at different stages of crop growth. The pots were maintained in fixed moisture content by spraying water and the temperature of the experimental area was recorded. On 26<sup>th</sup> day, the plants were uprooted, and dried to constant weight in an oven at 60°C, wt. of the dried Abelmoschus esculentus as well as Phaseolus vulgaris were taken.
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3.7. ENRICHMENT OF COMPOST BY BENEFICIAL BACTERIA AND BIOASSAY OF THE ENRICH COMPOST ON GROWTH OF PHASEOLUS VULGARIS

The manurial value of compost may be improved by increasing the load of BBs. It was done in two sets of composts obtained under different treatments.

In the first set of enrichment, 50 g of composts obtained from fresh municipality organic biomass treated with CDM and EW was inoculated with 5 ml each of 24 hr old Bacillus megaterium MTCC4126 of population 3.4X10<sup>8</sup> cfu/ml and Azospirillum amazonase MTCC4128 of 8.2X10<sup>8</sup>cfu/ml. The change in BB population was determined on 0 hr, 5<sup>th</sup> day and 10<sup>th</sup> day. The enriched compost was then analysed for BBs population as well as macro and micro nutrients contents. Then, using the enriched compost in the proportion of 50 g enriched compost with 3.450 kg of field soil, the test crop, Phaseolus vulgaris was raised in a Randomized Block Design with six treatments and three replicates including a control where there was no addition of compost. Biometric observations were recorded at different stages of crop growth. The pots were maintained in fixed moisture content by spraying water and the temperature of the experimental area was recorded. On 26<sup>th</sup> day, the plants were uprooted and biometric observations like shoot length, shoot wt., root wt. no. of branches and no. of fruits were recorded. Total wt. of the Phaseolus vulgaris was measured by drying to constant weight in an oven at 60°C.The results were compared with the control. Then, the soil samples used for growing plants were tested for C/N ratio by CHNS (O) analyser.

In the second set of enrichment, composts obtained from dry municipality organic biowaste mixed with RS and CD maintained at 90% moisture i.e. DT3 and DT4 were divided into three parts. Third part of each compost was made sterilised. Then, 5 ml each of the 24 hr old Bacillus megaterium MTCC4126 of 3.4X10<sup>8</sup>cfu/ml and Azospirillum amazonase MTCC4128 of 8.2X10<sup>8</sup>cfu/ml were inoculated in 50 g of each unsterilized and sterilized composts of both DT3 & DT4 treatments. The change in population was checked in 0 hr, 5<sup>th</sup> day and 10<sup>th</sup> day. Then, using unsterilized and sterilized enriched composts along with original composts of both DT3 and DT4, the test crop, Phaseolus vulgaris was raised in a Randomized Block Design with seven treatments and five replicates including two controls where one was with NB and another without NB. Biometric observations were recorded at different stages of crop
growth. The pots were maintained in fixed moisture content by spraying water and the temperature of the experimental area was recorded. On 46th day, the plants were uprooted and biometric observations like shoot length and wt., root wt., no. of branches and no. of fruits were recorded. Total wt. of the plant was determined by drying to constant weight in an oven at 60°C and compared the results with the controls.

3.8. DENATURING GRADIENT GEL ELECTROPHORESIS PROFILING OF ENVIRONMENTAL DNA

Denaturing gradient gel electrophoresis (DGGE) is a powerful molecular technique in which the PCR product was separated based on sequence differences (Fisher and Lermann, 1979). It is the mobility shift which can differentiate slightly different sequences.

The electrophoresis takes place in a vertically placed polyacrylamide gel in a gradient of denaturants. It is important that the buffer in the upper buffer compartment is circulated to prevent exhaustion of buffer components. In addition the gel should be kept at constant homogeneous temperature usually at 60°C. To accomplish this we place the gel in an aquarium containing the buffer. For stability the gel is placed in the plastic Protean II tank. Buffer is circulated by the thermostat pump to the upper buffer compartment and to the tank. Via overflow it is circulated to the aquarium in which the pump is hanging. To facilitate overflow from the upper compartment we have drilled two little holes in the upper part of the core. When applied to 16S rRNA genes, the method allows the dissection of microbial communities at the level of the phylogeny of their constituents. PCR applied to regions of this gene with conserved primers allows the generation of a mixture of amplicons which can be separated by DGGE. The technique was initially introduced into microbial ecology (Muyzer et al., 1993) and has been widely used since its inception (21). Muyzer and coworkers originally proposed a system based on the V3 region of the 16S rRNA gene. Using DGGE and simultaneously measure the enzymatic activity of cellulase, we could link genetic and functional diversity of the indigenous microbial communities in compost samples. Functional diversity of microbial populations in compost is important to achieve a better general understanding of the composting process (Haruta et al., 2005), and it could eventually become a tool to predict the quality of compost from the genetic make-up of the microbial populations present in it.
PCR - DGGE comprises three steps – extraction of total community DNA from the sample, PCR amplification and separation of PCR products using DGGE.

**a) Environmental DNA extraction**

The procedures for direct extraction of DNA or RNA from environmental samples have been developed from often laborious, manual methods, to commercially available kit-based protocols. Besides bead beating for disrupting the starting material, these procedures include various treatments with detergents for lysis of cell membranes, addition of solvents for separation of the nucleic acids from other cell components, and enzymatic treatment for recovery of the nucleic acid in purified form (Canon and Nicholl, 2002). Such extraction kits designed to obtain DNA from soils and sediments have been successfully used for compost samples. We used the MOBI O Power Max™ DNA Kit for obtaining DNA from the aged compost and enriched soil samples. Isolation was performed on 0.25 g compost/soil by following the manufactures instructions. DNA was isolated from replica I and II from each of the four composts. In all, a total of 24 extractions were made and, the DNA from replicate samples was pooled. The integrity of all 24 environmental DNA of the 4 different composts and 8 different soils with two replicates each was checked on 0.8% agarose Gel.

**b) Polymerase chain reaction (PCR)**

The extracted DNA is theoretically a mixture of genomic DNA from all microorganisms that were present in the compost. Using polymerase chain reaction (PCR), specific genes can be amplified in sufficient numbers for further analysis. Amplification and sequence analysis of the gene encoding 16S ribosomal RNA is one
such commonly used method. The primer design is crucial for obtaining PCR results that are unambiguous and readily interpretable, since it determines the specificity of the primers. Microbial diversity of the samples was determined by use of PCR-DGGE. Bacterial DNA was amplified using the universal bacterial primers 341f + GC (5’CGCCCGCCGCAGCGCCGCGCCGGGGCAGGCGCCGAGCGGAGCGTACGGGAA CGGAGCAG-3’ and 518r (5’-ATTACCGCGGCTGCTGG-3’) (Muyzer, de Waal, and Uitterlinden), which amplifies a 233 bp fragment of V3 region of 16S rDNA, including a 40 bp GC-clamp. PCR was performed using 2.5 µl 10XTaq buffer (15 mM Mg2+, 105876, GeNei), 1.5 µl MgCl2 (25 mM, 105881, GeNei), 0.5 µl 10 mM dNTP mixture (1054876, GeNei), 0.15 µl of 5 units/µlTaq polymerase (61060230005A, Genei), 0.25 µl of each primer (10 pmol/µl) (Sigma), 1 µl diluted template and H2O to a total of 25 µl. Touchdown PCR was performed with a Thermocycler (ICycler, Biorad, USA) using the following cycle conditions: an initial denaturization of 5 min at 95°C, 10 cycles of 1 min at 94°C, 1 min at 65°C (the temperature was decreased by 1°C every cycle until the touchdown temperature of 55°C was reached), 1 min at 72°C, 30 additional cycles with an annealing temperature of 55°C, and a final extension of 10 min at 72°C. All PCR products were stored at 4°C until used. Aliquots of PCR products were evaluated after separation on a 1.5% agarose gel (V3125, Promega) gel electrophoresis. The amount of DNA in each sample was estimated by image analysis using Gel documentation system (Biorad).

c) DGGE analysis

DGGE was performed using the D - GENE System (Bio-Rad) DGGE equipment. Equal amounts of DNA were loaded onto 8% (w/v) polyacrylamide gels (40% acrylamide/bis solution, 37.5:1, Bio-Rad) with denaturing gradients ranging from 30 - 50% for the bacterial DNA. The denaturing gradients were produced with 100% solution containing 7 M urea and 40% deionised formamide. The gel with bacterial DNA was run at 60°C for 17 hr. at 60 V. After electrophoresis, the gels were stained with ethidium bromide gel stain (1:10 dilution in 1XTAE buffer) putting on gel rocker for 30 min followed by destained 200 ml 1XTAE in darkness for 30 min each prior to UV transillumination. Digital images of the gels were obtained and analysed using image analysis (Quantity One 4.0.1, Bio-Rad).
CHAPTER 3
MATERIALS AND METHODS

ANALYSIS OF DATA

All statistical analysis was performed using AGRES (Agres statistical software, 1994) and the F-test of significance (Snedecor and Cochran, 1968). For every parameters reported in this investigation, the different treatments were analysed for differences among mean ($p < 0.05$) by performing one way analysis of variance (ANOVA) to test the quantity of group variance and the least significant difference test at $p < 0.05$. Paired t-test (SPSS Ver. 16.0) was done at different time interval to determine any significant difference among the treatments. The differences between all possible pairs of treatment means were compared to the CD value. If a difference was equal to or greater than the CD value, the difference is significant. Sequences of the efficient cellulose degrading isolates were compared to those present in the databases using BLAST search program at the NCBI web site. Sequences were aligned using Clustal W (1.81) as implemented. The phylogenetic tree of the aligned sequences was constructed with 1,000 - fold bootstrap analysis by using the neighborjoining method with the MEGA 4.1 (Molecular Evolutionary Genetics Analysis, MEGA) software (Tamura et al., 2011). Analysis of the DGGE profiles on the basis of presence or absence of bands at certain positions in each lane using NTSYS pc (ver. 2.20 pc) gel analysis software is done and binary similarity matrices were produced using the Dice coefficient which allowed construction of dendrograms using the unweighted pairwise grouping method with mathematical averages (UPGMA).