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Fungal Flora of Vermicompost and Organic Manure : A Case Study of Molecular Diversity of *Mucor racemosus* using RAPD Analysis

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In the present study seven samples were collected of which four were from vermicompost from Prof. T.S. Murthy Udyan, Obaidullahganj and 3 from organic manure pit of Ambari, Himmatpur & Gwalipur. Mucor racemosus strains were screened for the production of lipase, gelatinase, xylanase, amylase and caseinase enzymes which showed its biodegradable role in composting process. RAPD techniques was used for genetic diversity of Mucor isolated from vermicompost resulted 64.29% as polymorphism the *Mucor racemosus* indicating higher percentage of homogeneity among them.

Key words: *Mucor*, polymorphism, genetic diversity, RAPD & PCR.

Soil is a dynamic habitat for an enormous variety of life forms such as plants animals and microorganisms. All these forms of life interact with one another and with the soil to create continually changing conditions. This allows an on going evolution of soil habitat. Soil is also a complex environment colonized by an immense diversity of microorganism. Soil microbiology focuses on the soil viruses, bacteria, actinomycetes, fungi and protozoa. Microbial life with in the soil ecosystem is a fascinating aspect of soil biology (Jeewon & Hyde, 2007). The biological component of the soil is responsible for soil humus formation, cycling of nutrient and building soil tilth and structure along with many other functions. Microorganisms in a soil form part of the biomass and contribute to the reserve of soil nutrients that are generally referred to as the microbial biomass (Arslan *et al.*, 2008; Carroll & Wicklow, 1992).

Fungi are an important component of soil microbiota. They mediate important symbiotic relationship with plant and bacteria. Many Fungi are pathogenic and some may be useful in bio exploitation. A diverse range of fungi are present in soil ecosystem which includes ascomycetes, basidiomyetes, some being ectomycorrhizal fungi, anamorphic fungi and arbuscular mycorrhizal fungi (Jeewon and Hyde 2007; Cooke & Whipps, 993). Most fungi are classified as saprophytes because they live on dead or dying material and obtain energy by breaking down organic material present in dead plants and animals (Alexopoulos *et al.*, 1996). As a group the fungi exhibit a remarkable ability to utilize almost any carbon source as food. Different species do however have different nutritional requirement (Griffin, 1993).

Solid waste is the organic and inorganic waste material produced by different sources. It has been estimated that in India as a whole, as much as 25 million tons of urban and solid waste of diverse composition was generated per year. Most common particles of waste processing are uncontrolled dumping which cause mainly water and soil pollution. Sanitary and filling or dumping the final disposal of solid waste can be carried out

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by other methods like incineration and composing (Aalok *et al.* 2008).

Biological fertilizers have great importance then chemical fertilizers. They are not harmful to human being as well as very effective for the field. Organic manure and vermicompost are this type of biological fertilizers. The application of compost in the soil increases the percentage of organic matter, nutrient level (providing a slow filleterization action over a long period of time), microbial biomass and improved the soil physical properties like aeration, water holding capacity etc. (Arslan *et al.*, 2008). Compost obtained by composting process that uses naturally occurring microorganism to convert biodegradable organic matter into humus like product by reaching thermophilic temperature and reducing mass and volume, which makes compost suitable for agricultural application (Gea *et al.*, 2005; Kastner & Mahro, 1996).

Organic manure made from cattle dung, excreta of other animals, rural and urban compost, crop residues etc. are widely used as a biological fertilizer. Organic manure is an important source of plant nutrients, especially in organic cropping system, where mineral fertilizers are not used. The soil microbial decomposition of the applied organic manure is essential for the release of plant nutrient to the crop. Increased knowledge concerning which organism those are active in the decomposition might be important for improved nutrient management in organic farming. Physical properties of soil like soil porosity, water stable aggregates, water holding capacity, infiltration rates, hydraulic conductivity increase by organic manure. Various mineral provided by organic manure to plant are N, K, P, Mg, Co, Zn, Cu. Earth worm forming (vermiculture) is another biotechnique for converting the solid organic waste into compost. The vermiculture provides for the use of earthworm or natural bioreactors for cost effective and environmentally sound waste management (Aalok *et al.*, 2008). Vermicomposting involves the stabilization of organic solid waste through earthworm consumption which converts the material into worm castings. The process is faster then composting. Earthworm cast is rich in microbial activity and plant growth regulators and fortified with pest repellence attribute as well. In short earthworm through a type of biological alchemy, are capable of transforming garbage into gold (Azarmi *et al.*, 2008).

Due to advance in molecular biology technique, large number of highly informative DNA markers has been developed for the identification of genetic polymorphism. Random amplified polymorphic DNA (RAPD) technique based on the PCR technique is one of the most commonly used molecular techniques to develop DNA markers by which we can obtain genetic information. RAPD markers are ten-mer DNA fragment from PCR amplification of random segments of genomic DNA with single primer of arbitrary nucleotide sequence which are able to differentiate between genetically distinct individuals. Although RAPD analysis can be performed on any organism with no prior DNA sequence information is required. However it, is effective with tiny amount of DNA, low expense, efficiency in developing large Number of DNA markers in a short time and requirement for less sophisticated equipment has made the RAPD technique valuable. RAPD markers can be used for the characterization of microorganism and detections of microbial diversity in meta genome, without the need for culturing the some also. The present study utilize random markers to further characterize genetic diversity and population structure of mucor. This study also represent a unique opportunity to compare estimate of genetic diversity and habitat study of mucor.

MATERIALS AND METHODS

Sample Collection and Microbial Analysis

Four samples of vermicompost were collected from Prof. T.S. Murthy Udyan, Obaidullaganj at 15 days intervals and samples of organic manure were collected from organic manure pits at Ambari, Gwalipur and Himmatpur (Shivpuri, M.P.) under sterile condition in sample containers. Temperature of samples was noted with the help of mercury thermometer. Samples were collected with the help of sterile spatula and scoops. Sample was stored in sterile container at 4^0C for studying the genetic diversity of bacterial community at different stages of vermicompost. Fungus was isolated through serial dilution of samples and inoculated on Potato dextrose agar at 28 ^{0}C for 72 hrs identification of fungus was done from Agarkar Institute Pune, India. Identified *Mucor* were used in further studies like screening

of enzyme on solid media, Casein hydrolysis, amylase, xylose hydrolysis, lipase production and gelatine hydrolysis.

Genomic DNA isolation and Polymerase Chain Reaction

DNA was extracted using CTAB method as provided by (Janarthanan & Vincent, 2007). Quantification of analysis DNA was done by UV-Vis spectrophotometer ND-1000. Reaction mixture for RAPD amplification was prepared in 25µl, which contain sterile water- 11 µl, Red dye- 12.5? µl, RAPD Primer- 1 µl, DNA Template (50ng/µl) -0.5 µl. After preheating for 5 minutes at 94°C, PCR was run for 40 cycles. It consisted of a 94°C denaturation step (0.45 minutes), 37°C annealing step (1 minute) and 72°C elongation step (1.5 minutes) in a Thermal Cycler (Corbet Research, Australia). At the end of the run, a final extension period was appended (72°C, 7 minutes) and then stored at 4°C until the PCR product was analysed. PCThen the PCR tubes were kept in the thermal cycler. After the PCR was completed, PCR products were loaded and analyzed on a 1% Agarose gel run at 50-100V (5-6V/cm Gel). The gel was visualized under UV light using Gel Documentation System.

Analysis of Gel and DNA Fingerprinting

Staining with Ethidium Bromide (Et-Br) made the gel to visualize the DNA bands. This commonly used dye is intercalating and fluorescent in nature and fluorescent in nature which can be used before or after electrophoresis. Stock solution of Et-br (10µg/ml) was added in the gel before electrophoresis. After running the gel it was placed on Gel Documentation System (Alpha Innotech, USA) and was visualized by 302 nm at high intensity of UV light. Image was captured and analyzed by using Alpha-View Software. Molecular weight was also calculated by using the same software.

Biostatistical Analysis

Gel-Quest is software for the analysis of DNA fingerprints like AFLP, t-RFLP, RAPD. It works in a fast and efficient way with trace files (FSA files) as well as with gel image files (TIFF, JPG, BMP, etc.) and handles AFLP, T-RFLP or RAPD data. Tree-Mee is comprehensive Phylogenetic tree visualization and manipulation software for phylogenetics and research on evolution. The bands were analyzed and scored (0) for absence and (1) for the presence for all the primers. Similarity indices were prepared using "Cluster Vis" (SequentiX-Digital DNA Processing Germany) Software, using Jaccard's coefficient and UPGMA, Input data of mentioned software is Binary matrix. The similarity Indices were also calculated with each primer and a matrix was developed. Finally, all matrices were analyzed as an average. The graphic phenogram of the genetic relatedness among the four accessions was produced by means of UPGMA (Un-weighted pair group method with arithmetic average) cluster analysis of averaged similarity index.

RESULTS AND DISCUSSION

Seven samples were collected, 4 of vermicompost from T.S. Murthy Udyan, Obaidullahganj, Bhopal and 3 from Organic manure pit of Ambari, Himmatpur & Gwalipur respectively. The samples were serially diluted (10^{-1} to 10^{-10}). 0.1 ml of 10^{-4} and 10^{-5} dilutions of

Table 1. Growth of *Mucor* at various temperature and pH

Fungal	Temperature			Growth at various pH (measure in radius in cm)				
ID	28 °C	37 °C	48 °C	5.0	6.0	7.0	8.0	9.00
021	++	+	-	0.33	1.34	1.49	1.38	1.18
025	++	+	-	0.30	2.32	0.28	0.62	0.29
026	++	+	-	0.39	0.53	0.11	0.08	0.03
040	++	+	-	0.70	0.55	0.16	0.14	0.08
042	++	+	-	0.14	0.20	0.26	0.07	0.04
052	++	+	-	0.34	0.59	0.21	0.10	0.06
063	++	+	-	0.85	0.76	0.57	0.36	0.14

Note: ++ = Good growth, + = Moderate Growth, - = No Growth

samples were poured on PDA plates and incubated for 2 days at 28 ^{0}C.

Growth of Mucor isolate at different temperature

Temperature is one of the most important physical factor affecting fungi. Fungi are different from higher plants and animals in the lack of homeostatic mechanism and cannot regulate heat generated by metabolism and are therefore, directly and readily affected by temperature. Fungi can normally tolerate the range of temperature of the environment from which they are taken. High or low temperature may be insufficient to restore metabolic activity. *Mucor* is a fast growing fungus and its growth was observed at 28 °C, 37 °C & 48 ^{0}C. All the *Mucor* isolate show growth at 28 °C and 37 °C and no one show growth at 48 ºC (Table 1).

Growth of Mucor at different pH

Growth and survival of microorganisms are greatly influenced by the pH of the environment. Which limits the activity of enzymes with which on organism is able to synthesize new protoplasm. Each species has the ability to grow within a specific pH range, which may be broad or limited, with the most rapid growth occurring within a narrow optimum range. These specific pH needs reflect the organism's adaptations to there natural environment. Mucor can grow on wide range of pH (5-9). Fug 052 & Fug 063 grow best on pH 5. Fug 025, Fug 026 & Fug 042 Show best growth on pH 6 while Fug 021 & Fug 040 shows best growth in pH 7 and growth range of different *Mucor* isolates at different pH (Table 1). The similar observations were found by Jadhav *et al.* (1997). All the fungi produce a large number of enzymes to break down the complex materials for their growth. The secretion of enzymes to the extracellular environment might be an important adaptive mechanism during the life cycle of fungi. Fungi biodegrades the undesirable waste material into useful products such as compost by their efficient enzyme system.

Amylase production test

Amylase is an extracellular enzyme hydrolyze starch into shorter polysaccharide namely dextrin's and ultimately into maltose molecule. Screening of amylase was done at 28

Table 2. Growth of *Mucor* at various temperature and pH

Fungal	Amylase			Xylanase			Lipase			Gelatinase		
ID	28 °C	37 °C	48 °C	28 °C	37 °C	48 °C	28 °C	37 °C	48 °C	28 °C	37 °C	48 °C
021	6	2	NG	-	-	NG	5	9	NG	2*	1*	NG
025	2	3	NG	3	2	NG	5	5	NG	2*	2*	NG
026	3	8	NG	-	-	NG	6	NG	NG	1*	-	NG
040	3	3	NG	3	1	NG	7	11	NG	28	1*	NG
042	3	4	NG	-	-	NG	4	5	NG	-	2*	NG
052	2	4	NG	-	-	NG	6	NG	NG	5*	2*	NG
063	4	3	NG	2	1	NG	5	NG	NG	1*	-	NG

Note: * = RA activity in mm, NG= No growth, - = Negative result

Table 3. Pattern of polymorphism and uniqueness of 07 individuals of genus *Mucor*

Primer with accession Number	Total no. of bands	No. of monomorphic bands	No. of polymorphic bands	Monomorphism %	Polymorphism %
Rfu-2 (AM 911696)	24	6	18	25	75
Rfu-4 (AM773320)	28	9	19	32.14	67.86
Rfu-14 (AM 911703)	20	13	7	65	55
Rfu-15 (AM765823)	16	4	12	25	75
Rfu-20 (AM750056)	10	3	7	30	70
Total	98	35	63	35.71	64.29

°C, 37 °C & 48 °C. All fungi show positive result at 28 °C and 37 °C for the enzyme. At 48 °C none of fungi can grow. Maximum relative hydrolytic amylase activity (RA) was shown by Fug - 026 at 37 °C of 8mm. At 28 ^{0}C Fug 021 show maximum RA of 6mm (Table 2).

Casein hydrolysis

Fungi have the ability to degrade the protein casein by producing proteolytic exoenzyme, called proteinase (caseinase). Screening of caseinase was done at 28ºC, 37ºC & 48 °C. At 28 °C all the *Mucor* isolates showed positive casein hydrolysis test with maximum RA 3 mm except Fug 052. At 37 °C Fug 021, Fug 025, Fug 040 and Fug 063 show positive casein hydrolysis with maximum RA of 3mm. While at 48 °C none of fungi can grow (Table 2).

Xylose degradation test

Xylose also known as wood sugar is a constituent of xylane, which is present in high concentration in corn meal. Fungi release enzyme xylanase which degrade xylose to produce acid. Screening of xyalanase was done at 28 °C, 37 °C & 48 °C. In this test at 28 °C, Fug 025, Fug 052 and Fug 063 show positive result with maximum RA of 3mm while remaining fungi showed negative results, while at 48 °C none of fungi can grow (Table2).

Lipid hydrolysis

Lipase cleaves ester bonds in the lipid by the addition of water to form the building blocks glycerol and fatty acid. Tween 80 utilized as carbon source and stimulated lipase excretion by the fungus (Muhsin *et al.,* 1997). Screening of lipolitic activity was done at 28 °C all the *Mucor* isolates show positive result with maximum RA of 7mm at 37 °C Fug - 021, Fug 025, Fug 040 and Fug 063 show lipolytic activity with maximum RA 11mm while Fug 026, Fug 042 and Fug 052 do not show growth at 37 °C and none *Mucor* isolate can grow at 48 °C (Table 2).

Gelatin hydrolysis

Screening of gelatin hydrolysis was done at 28 °C, 37 °C & 48 °C at 28 °C all *Mucor* isolates except Fug 040 show positive result with maximum RA of 5mm. While at 37 °C, Fug- 021, Fug 025, Fug 040, Fug 042 and Fug 063 show positive result

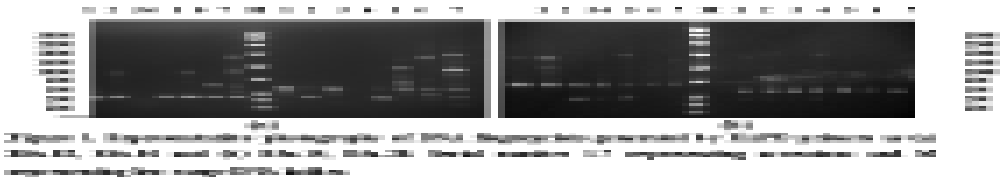

Fig. 1. Representatitve photographs of DNA fingerprints generated by RAPD primers as (a) Rfu-02, Rfu-04 and (b) Rfu-15, Rfu-20. Serial number 1-7 representing accessions and M representing low range DNA ladder

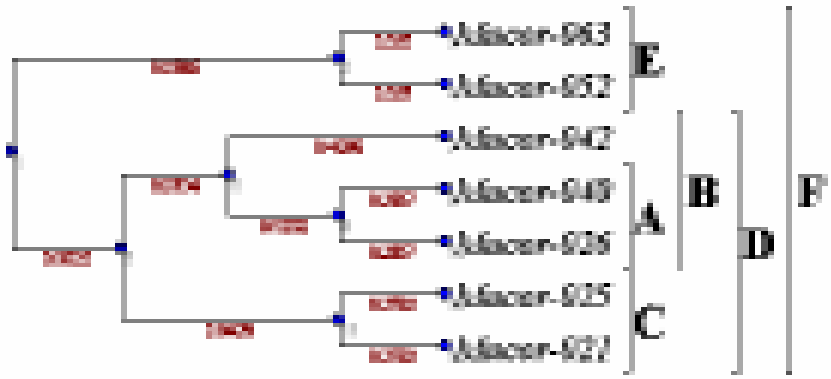

Fig. 2. UPGMA cluster analysis based on Jaccard's genetic distance matrixes using RAPD phenotypes

with maximum RA of 2mm remaining fungi show negative result while at 48 °C none of fungi can grow be grown (Table 2).

Isolation of genomic DNA from *Mucor* RAPD-PCR

DNA was isolated through CTAB method and quantitative and qualitative estimation was done by using ND-1000 Nanodrop UV-Vis spectrophotometer. The amplification of genomic DNA from the 07 fungal isolates of *Mucor* with the primer Rfu-2, 4, 14, 15 & 20 had show that these isolates have different RAPD profile. Table 3 showed different type of polymorphism such monomorphism, polymorphism etc. Rfu-2 primer generated 24 bands of which 18 were polymorphic giving 75% polymorphism, Ffu-4 showed 67.86%, Rfu-14 showed 55% polymorphism, Rfu-15 showed again 75% polymorphism and primer Rfu-20 produced total 10 bands of which seven were polymorphic representing 70% polymorphism. However, overall 98 bands were generated by seven primers used of which 63 were polymorphic and 35 were monomorphic representing 64.29 as overall polymorphism and 35.71 as monomorphism (Table 3).

In this study total 27 fungi were isolated from 4 samples of vermicompost and 3 sample of organic manure. Seven *Mucor racemosus* were growing at different temperature and pH to know how they exist at different temperature and pH during composting process. *Mucor racemosus grow* best at temperature 28 °C and poorly grow at 37 °C, while *Mucor racemosus* can grow on wide range of pH. It grows best from pH 5-7 but on 8 and 9 pH poorly grow or no growth. Different *Mucor racemosus* stains can produce lipase, gelatinase, xylanase, amylase and caseinase enzyme at different temperature which show its biodegradable role in composting process. *Mucor racemosus* were further studied for its gentic diversity through RAPD using different primers. The study shows 35.71% monomorphism and 64.29% polymorphism among the *Mucor racemosus* isolated from different samples, indicating higher percentage of homogeneity among them.

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REFERENCES

1. Aalok, A., Tripathi, A.K., Soni, P. Vermicomposting: A better option for organic solid waste management. *J. Hum. Ecol*. 2008; **24**: 59-64.
2. Alexopoulos, C.J., Mims, C.W., Blackwell M (ed.). Introductory Mycology 4th edn.John Wily and Sons. Toronto. 1996; pp869.
3. Arslan EI, Obek E, Kirbga S, Ipek U, Topal M. Determination of the effect of compost on soil microorganisms. *J. Sci. Tech*.2008; 3**:** 151-159.
4. Azarmi, R., Giglou, M.T., Taleshmikail, R.D. Influence of vermicompost on soil chemical and physical properties in tomato (*Lycopeusicum esculentum*) field. *Afr. J. Biotech*. 2008; **7**: 239-240.
5. Gea, T., Artola, A., Sanchez, A. (ed). Composting of De-linking sludge from the recycled paper manufacturing industry. *Bioresource Technol*. 2005; **96**: pp 1161-1167.
6. Griffin, D. (ed). Fungal physiology Wiley Liss. 2nd edn. New york. 1993; pp 458.
7. Janarthanan, S., Vincent, S. (ed). Practical Biotechnology, Methods and Protocols, University Press, India. 2007; pp 11-12.
8. Jeewon, R., Hyde, K.D.(ed). Detection and diversity of fungi from environmental samples: Traditional versus molecular approaches In. Advance Technique in Soil Microbiology. Springerverlag Berlin Heidelberg. 2007; pp 1-3.
9. Carroll, G.C., Wicklow, D.T., The fungal community: Its organization and role in the ecosystem . Marcel Deker, Inc, New York, 1992.
10. Cooke, R.C., Whipps, J.M. Ecophysiology of fungi. Blackwell scientific Pub, London. U.K. 1993; pp 113-115.
11. Kastner, M., Mahro, B. Microbial degradation of polcyclic aromatic hydrocarbons in soil affected by the organic matrix of compost. *Appl Microbio/ Biotech*.1996; **44**: 668-675.
12. Jadhav, A.D., Talashikar, S.C., Pawar, A.G.. Influence of the conjunctive use of Fym, vermicompost snd urea on growth and nutrient uptake in rice. J Maha. Agri. University., 1997; **22**: 249-250.