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ORIGINAL ARTICLE

Phylogenetic profiling of culturable bacteria associated with early phase of mushroom composting assessed by amplified rDNA restriction analysis

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Abstract The edible mushroom *Agaricus bisporus* is grown commercially on composted manure/straw mixtures. Mushroom composting is a fermentation process in which various groups of microorganisms play important roles at different stages of composting. The present study was conducted to explore the mesophilic bacterial diversity in the early phase of mushroom composting. Morphologically all the isolated bacteria were either Gram-positive rods, cocci or Gram-negative rods. The functional diversity of the bacterial isolates was examined by plate enzyme assays, siderophore production and antagonistic property. Good enzymatic activity for amylase, cellulase, xylanase and protease was reported for different bacterial isolates. Nine bacterial isolates showed siderophore production activity. During antibiosis assay, most of the isolates inhibited growth of *Verticillium fungicola* and *Mycogone perniciosa*. However, a consortium of selected bacterial isolates produced good amounts of lytic enzymes (amylase, cellulase, xylanase and protease) in solid state fermentation experiments that might help in enhancing the composting process. Amplified 16S-rDNA restriction analysis (ARDRA) of bacterial isolates indicated that four groups of nine bacterial isolates had 100% similarity in all the restriction profiles. However, other isolates exhibited discriminatory relationships with each other. The present study reveals culturable mesophilic bacterial diversity and community succession in the early phase of mushroom composting process as well as emphasizing the application of a bacterial consortium to enhance the composting process.

Keywords Mushroom composting · *Agaricus bisporus* · ARDRA · 16S rDNA · Phylogenetic profiling

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Introduction

The white button mushroom 'Imbatch' is the most widely cultivated and popular species of edible mushroom among the artificially grown fungi of the world that contributes about 31.8% of the global mushroom cultivation and 85% of the total production in India. The triple advantages of growing this mushroom is that its cultivation is a biotechnological process that recycles lingo-cellulosic wastes, mushrooms are protein-rich food for human consumption and the spent mushroom substrate can be utilized profitably in different ways (Masaphy et al. 1987). The white button mushroom (*Agaricus bisporus*) is cultivated on a substrate consisting of a composted mixture of straw-bedded horse manure, wheat straw, chicken manure and gypsum. Conventionally, two phases of mushroom composting are distinguished (Fermor and MaCauley 1991). After mixing and moistening, the ingredients are subjected to a phase I composting process. Mixed ingredients are stacked in windrow in the open air for uncontrolled self-heating for 1–2 weeks. The temperature in the windrow range is from ambient to 80°C. During phase I, ammonia and foul-smelling compounds are

emitted, causing environmental problems. Phase II is an aerobic process carried out by maintaining the compost at 45°C.

Composting is an aerobic process driven by microorganisms that by degrading the organic matter cause an increase of temperature. The initial microflora is mesophilic and utilizes the soluble organic carbohydrates and nitrogen. This is followed by an increase in growth of mesophilic microflora and release of carbon dioxide, ammonia and considerable heat. At the later stage of composting, the temperature rises whereby thermophilic microorganisms dominate the compost material (Derikx et al. 1989; Wiegant et al. 1992). However, thermophilic microorganisms present in the compost are mainly responsible for the indoor pasteurization. Thus, while the composition of the microflora varies with the stage of composting, the spectrum of successional cycle is determined by the prevailing temperatures.

It is possible to characterize the microorganisms present in the compost without the culturing step. The edge gained by culturing is the availability of biomass in pure culture to be used as the starter for further applications. Several methods and approaches are now available to generate information on microorganisms that reside in mushroom compost, which allows a better assessment of the microbial flora, wherein molecular tools for the identification of microorganisms are now in common use, and 16S rDNA gene analysis is intensively used in phylogenetic investigations. Among the 16S rDNA gene analysis methods, amplified ribosomal DNA restriction analysis (ARDRA) is used to estimate the phylogenetic relationships among different microbial isolates or rDNA clones recovered from the environment (Amann et al. 1995; Massol-Deya et al. 1995).

The aim of the present study was to investigate the morphological, functional characterization and phylogenetic relationship of the culturable mesophilic bacterial population associated with the early phase of mushroom composting. This finding is helpful for the monitoring of phylogenetic profiling of culturable bacterial diversity in the early phase of mushroom composting and beneficial for the biotechnologically and industrially relevant process in reference to the enzyme secretion property of the bacterial isolates.

Materials and methods

Isolation of bacterial isolates

Twenty bacterial isolates were used in this study, belonging to different turning stages of phase I mushroom composting and originally isolated from the Mushroom Research and Training Centre (MRTC), and collected from the Department of Microbiology culture collection, College of Basic Sciences and Humanities, Govind Ballabh Pant University of Agriculture and Technology, Pantnagar, Uttarakhand, India. The compost samples were collected from five different steps of the phase I mushroom composting process. The first sample was collected on the 2nd day from the first step of wet straw stack formation, while the other samples were collected from four turning steps of compost preparation on days 5, 9, 11 and 13. Dilutions (10^{-6}, 10^{-7}, 10^{-8}) of the compost samples were plated on nutrient agar (NA: beef extract, 3 g; peptone, 5 g; agar agar, 20 g; distilled water, 1,000 ml; pH, 7.0±2) medium. Three plates of nutrient agar medium were kept for each dilution and incubated at 28±2°C for 3 days in BOD incubator. Morphologically distinct and isolated colonies were transferred to the respective media and purified thereafter following standard protocols (Holt et al. 1994; Silva et al. 2009). All the bacterial isolates were maintained on nutrient agar slants at 4°C for regular use and in 15% glycerol stocks for long-time preservation at −80°C.

Morphological characterization

Colony morphology of the isolates was studied under a stereoscope microscope (Olympus, SZH 10). This included shape, edge, elevation, surface and chromogenicity. The cellular morphology was based upon cell shape and Gram staining (Leica fluorescent microscope).

Functional characterization

Assessment of functional diversity among the isolates was carried out by qualitative plate assay for amylolytic, proteolytic, cellulolytic, xylanolytic activity and siderophore production. Minimal medium was used for plate assay, wherein isolates were spot-inoculated with sterile tooth-picks on solid medium and incubated at 28±2°C. The diameter of the zone of clearing, if any, for all positive isolates was measured.

Amylase (Starch diastase)

All the overnight-grown bacterial cultures were spot-inoculated on amylase-producing minimal medium amended with 1% (w/v) starch and incubated for 24–72 h at 28±2°C for growth. Plates were flooded with Lugol's iodine for 10 min. Then, iodine was drained off and positive isolates exhibited a zone of clearance against a dark blue background.

Protease

The protease-producing medium amended with skimmed milk (20 ml l^{-1}) was spot-inoculated with overnight-grown

bacterial isolates. Plates were incubated for 24–72 h at 28± 2°C for growth. Formation of a clear halo zone around the bacterial colony was considered as a positive result for this test.

Cellulase and xylanase

The minimal medium was supplemented with 1% (w/v) birch wood xylan and carboxymethyl cellulose (CMC) and spot-inoculated with bacterial isolates. Plates were incubated for 72 h at 28±2°C for growth and flooded Congo red solution (0.2% w/v) for 30 min. Excess reagent was discarded after destaining with 1 M NaCl solution for 30 min. A zone of clearance around bacterial colonies was considered as a positive result.

Siderophore production

Chromeazurol 'S' agar plates (Schwyn and Neilands 1987) were spot-inoculated with bacterial isolates, and incubated for 48–72 h at 28±2°C for growth. Formation of an orange halo around bacterial colony was considered as a positive result.

Solid state fermentation

For the solid state fermentation, nine potential bacterial isolates were selected on the basis of qualitative enzymes assays and the mixture of the isolates were inoculated on '0' day mushroom compost as substrate. The 20 g of substrate was dispended in a 250-ml flask and 50 ml of sterilized distilled water was added to achieve a moisture level of 2 ml g^{-1} solid. The experiment was carried out in two sets of treatments, set I consisted of inoculated autoclaved substrate and un-inoculated autoclaved substrate as a control, while set II consisted of inoculated un-autoclaved substrate and un-inoculated un-autoclaved substrate as a control. Flasks were aseptically inoculated with 5 ml (2.1×10^8 CFU ml^{-1}) of mixed bacterial suspension in triplicate and incubated at room temperature for 7 days.

Enzyme assays

After 7 days of incubation, 100 ml phosphate buffer (0.1 M, pH 7.0) was added to the fermented mixture for enzyme extraction. Flasks were placed on a rotary shaker for 1 h at 180 rpm and then left overnight under refrigeration for the release of any bound enzyme. The contents were centrifuged at 8,000 rpm for 15 min and the supernatant was used as crude enzyme.

α-Amylase activity was determined according to Chadha et al. (1997) by incubating 1 ml reaction mixture containing 0.1 ml culture filtrate, 0.4 ml 50 mM sodium acetate buffer (pH 5.0) and 0.5 ml of 1% starch solution, at 45°C for 5 min. The reaction was terminated by adding 1 ml of 0.5 M HCl, and 0.5 M iodine reagent (0.25% I_2+0.5% KI) was added to develop the color and absorbance was recorded against reagents as blank at 540 nm.

Xylanase and cellulase activity were assayed by the dinitrosalicylic acid (DNS) method (Miller 1959) using a calibration curve of D-xylose and D-glucose, respectively. Xylanase activity was determined by incubating 0.5 ml of culture filtrate and 0.5 ml of 1% xylan solution in 0.1 M phosphate buffer (pH 6.0), while the cellulase activity was determined by adding 0.5 ml of culture filtrate and 0.7% carboxy methyl cellulose solution in 0.1 M phosphate buffer (pH 6.0). Both mixtures were incubated at 30°C for 30 min. The reaction was terminated by adding 1 ml of dinitrosalicylic acid (DNS) and boiling for 10 min in a water bath. The contents were cooled at room temperature and then 400 μl of 33% Na-K tartrate were added to develop the color, and absorbance was recorded against reagents as blank at 540 nm.

Protease activity was measured following the method of Upton and Fogarty (1977). Briefly, casein was used as substrate and dissolved (0.5%) in 0.05 M glycine HCl buffer (pH 3.0). For assay, 1 ml casein was added to 1 ml culture filtrate and the reaction mixture incubated at 37°C for 30 min. The reaction was terminated by the addition of 2.0 ml of 0.44 M trichloroacetic acid (TCA). The precipitated protein was filtered through Whatman No.1 filter paper. Next, 1 ml of TCA filtrate was added to 5 ml of 0.44 M Na_2CO_3 followed by 1 ml of Folin's reagent (1: 1 v/v). The liberated protein was estimated according to protein estimation method of Lowry et al. (1951).

Enzyme activity is expressed in terms of International Unit (IU). It is defined as the amylase, xylanase and cellulase enzymes that hydrolyze 1 mg of starch, xylose and glucose per minute, while the protease activity is expressed as 1 mg of tyrosine released by 1 ml of enzyme solution in 30 min. The amount of enzyme activity was read from the standard curves of starch, xylose, glucose and tyrosine, respectively.

Antibiosis assay

All the isolates were tested for antagonism against pathogenic fungi of mushroom, *Verticillium fungicola* and *Mycogone perniciosa*. The actively grown fungal culture (5 mm disc) was placed in the center and the bacterial culture spotted towards the periphery of the culture plate, which was incubated at 20±2°C for 7 days. Inhibition of growth of pathogenic fungi around the bacterial colony was considered as a positive result.

Genotypic characterization

Recovery of genomic DNA

Total DNA from bacterial isolates was prepared following the procedure outlined by Bazzicalupo and Fani (1994) with the

exception that, for Gram-negative bacteria, no lysozyme was used. Extracted genomic DNA was run in 0.8% agarose gel at 80 V for 45 min. DNA was quantified spectrophotometrically by measuring OD at 260 nm and 280 nm. Purity of DNA was checked measuring the extinction at A_{260}/A_{280} on a DU 640 B Beckman spectrophotometer.

PCR amplification of 16S rDNA

The PCR amplification of 16S rDNA for each of bacterial isolates was performed by using the eubacterial universal primers GM3f (5' AGAGTTTGATCMTGG 3') and GM4r (5' TACCTTGTTACGACTT 3') which were targeted at universally conserved regions and permitted amplification of ~1,500-bp fragment (Weisburg et al. 1991). PCR amplification was carried out in a PTC-200 thermocycler (MJ Research). Initial DNA-denaturation and enzyme activation steps were performed at 95°C for 7 min, followed by 25 cycles of denaturation at 94°C for 1 min, annealing at 51°C for 1 min and extension at 72°C for 1 min, and a final extension at 72°C for 10 min.

Amplified 16S rDNA restriction analysis

Amplified 16S rDNA was digested with three restriction endonucleases: *Msp1, Sau 3AI* and *Taq1*. Totals of 15 μl (120 μg) of amplified 16S rDNA were digested with each restriction endonucleases in the reaction mixture. For the preparation of 30 μl of reaction mixture, the following were added: restriction enzyme (10 U), 0.05 U (MBI Fermentas); restriction buffer (10X), 1.0X (MBI Fermentas); 16S rDNA amplicon 120 μg; and Milli Q water. The reaction mixture was incubated at 37°C for *Msp1* and *Sau 3AI*, and at 65°C for the *Taq 1* enzyme for 4 h. The restriction product was resolved on 2.5% agarose gel in 1X TBE at 70 V for 4 h, and the gel was stained with ethidium bromide (0.5 μg ml^{-1}) and visualized on a UV transilluminator (GelDocMega, Biosystematica). The restriction profile was analyzed using NTSYS PC v.2.02i. The clustering was done using Jaccard's similarity coefficient based on the presence and absence of bands ignoring their intensities.

Results and discussion

Morphological and functional characterization

During the study of morphological and functional characterization, considerable and significant differences were observed among the bacterial isolates recovered from the different turning stages of the phase I mushroom composting. During this microbial succession, each type of microorganism was present in one specific condition of short duration and only active in decomposing of the organic matter present

Table 1 Morphological characteristic of bacterial isolates

Bacterial isolate	Gram reaction	Cell shape	Cell size (μm)	Edge	Elevation	Surface	Chromogenicity
PW II B	+ve	Long rod	3.19	Curled	Convex	Rough	White
PW II C	+ve	Short rod	2.84	Entire	Pulvinate	Smooth	Yellow
PW II D	+ve	Cocci	3.12	Entire	Pulvinate	Smooth	White
PW II G	−ve	Short rod	3.12	Entire	Convex	Smooth	Yellow
PW II H	+ve	Short rod	3.55	Entire	Convex	Smooth	Yellow
T_1A	−ve	Short rod	2.13	Entire	Convex	Smooth	Cream
T_1D	+ve	Short rod	1.98	Entire	Flat	Smooth	Cream
T_1E	+ve	Short rod	2.45	Entire	Flat	Rough	Yellow
T_1F	−ve	Short rod	2.84	Entire	Convex	Smooth	Cream
T_2A	+ve	Cocci	2.13	Entire	Umbonate	Smooth	White
T_2E	+ve	Cocci	2.27	Entire	Convex	Smooth	Cream
T_2F	+ve	Long rod	3.55	Curled	Umbonate	Smooth	Cream
T_3A	−ve	Long rod	2.84	Curled	Pulvinate	Smooth	Cream
T_3B	−ve	Long rod	2.84	Curled	Pulvinate	Smooth	Cream
T_3E	+ve	Cocci	2.13	Entire	Umbonate	Smooth	Cream
T_4B	+ve	Cocci	2.84	Undulate	Convex	Rough	Cream
T_4C	+ve	Cocci	2.13	Undulate	Convex	Rough	Cream
T_4D	+ve	Cocci	3.12	Undulate	Raised	Rough	Cream
T_4E	+ve	Cocci	2.84	Undulate	Raised	Rough	Cream
T_4G	+ve	Cocci	2.13	Undulate	Umbonate	Rough	Cream

Bacterial isolates: PW II B–PW II H isolated from wet straw stacks of mixed compost. T_1A–T_1F, T_2A–T_2F, T_3A–T_3E and T_4B–T_4G isolated from I, II, III and IV turning stages of phase I mushroom compost, respectively

Table 2 Functional characteristic and antagonistic property of bacterial isolate

Bacterial isolate	Functional characteristic					Antibiosis assay	
	Amylase	Cellulase	Xylanase	Protease	Siderophore	*Verticillium fungicola*	*Mycogone perniciosa*
PW II B	+	−	−	−	−	−	+
PW II C	+	+	−	−	−	++	++
PW II D	+	+	+	−	−	+++	++
PW II G	++	+++	+++	+	+	+++	++
PW II H	+	++	+	−	+	++	++
T_1A	+	++	++	+	−	−	++
T_1D	−	+	++	−	−	++	++
T_1E	−	+	+	−	+	−	+
T_1F	+	+	+	+	−	−	++
T_2A	+	++	−	+	+	+	++
T_2E	+	++	−	−	−	+	+
T_2F	−	−	−	+	−	+	+
T_3A	+	+	+	−	−	++	+
T_3B	++	++	+++	+	−	++	+
T_3E	+	++	++	+	++	+	+
T_4B	+	++	++	+	+	+	++
T_4C	++	++	+++	−	++	++	+
T_4D	+	+	++	+	+	++	+
T_4E	++	+++	+++	+	+++	−	+
T_4G	+	+	++	−	−	+	++

− No activity, + lower activity, ++ moderate activity, +++ maximum activity

(Szekely et al. 2009). Out of 20 bacterial isolates, 5 were Gram-negative and the rest of the isolates were Gram-positive (Table 1). The majority of the bacterial isolates exhibited amylase, cellulase, and xylanase activity by producing clear halo zones on the plate. Amylase positive diversity was much higher compared to the others and the isolates of turning stage III and IV were more active and showed maximum activity (Table 2). However, the significant variation in enzyme and siderophore secreting diversity were observed among the isolates. Low potentiality of proteolytic and siderophore-producing bacteria was observed during the assay. But the isolates of turning stage IV had good potential of siderophore production except for one isolate, T_4G. These findings are in agreement with the findings of Choudhary et al. (2009) who described the functional activity of casing amendments used in the cultivation of *Agaricus bisporus*.

Antibiosis assay

In recent years, researchers have focused on various microorganisms, especially fluorescent *Pseudomonas*, for biocontrol of mushroom diseases. In this context, the antagonistic property of bacterial isolates against V*erticillium fungicola*

Table 3 Enzyme activity after mixed culture inoculation under solid state fermentation

	Un-autoclaved (UA)'0' day compost				Autoclaved (A) '0' day compost			
	Control		Inoculated		Control		Inoculated	
	Mean value[a]	SD	Mean value[a]	SD	Mean value[a]	SD	Mean value[a]	SD
Cellulase (IU/ml^{-1})	0.075	±0.04	0.145 (93.33)	±0.019	0.056	±0.006	0.075 (33.92)	±0.004
Xylanase (IU/ml^{-1})	0.196	±0.007	0.097 (−50.51)	±0.009	0.037	±0.005	0.043 (16.21)	±0.004
Amylase (IU/ml^{-1})	0.092	±0.017	0.157 (65.21)	±0.007	0.066	±0.005	0.143 (116.66)	±0.013
Protease (IU/ml^{-1})	0.241	±0.008	0.272 (12.86)	±0.003	0.184	±0.060	0.298 (61.95)	±0.013

Values in parentheses indicate percent increase over control

[a] Mean value of three replicates

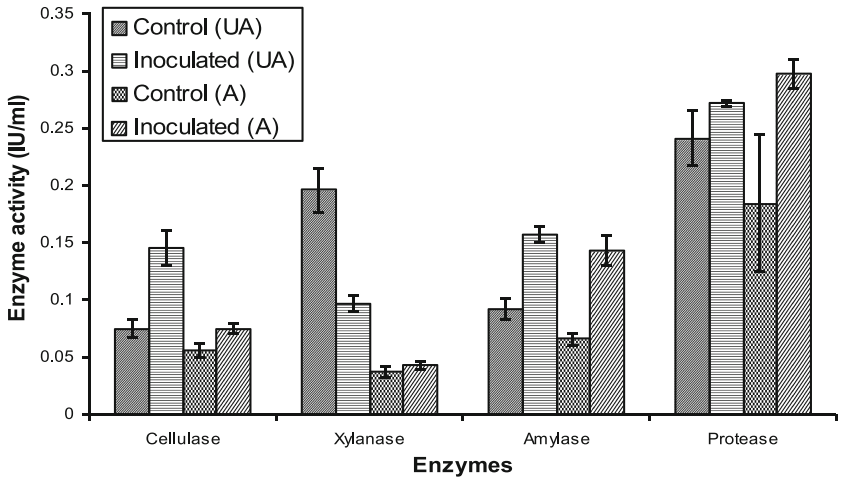

Fig. 1 Enzyme activity of mixed culture under solid state fermentation

and *Mycogone perniciosa* have been checked in a dual culture assay. The fungus *Mycogone perniciosa* is reported to cause wet bubble disease in *Agaricus bisporus* in all the major growing countries of the world (Umar et al. 2000), while V*erticillium fungicola* is a causal agent of dry bubble disease of mushroom and more severe than *Mycogone perniciosa*. Results of antibiosis assay indicated that all the isolates showed antagonism against *Mycogone perniciosa*, though the intensity varied with the isolates (Table 2), while 15 isolates showed antagonism against *Verticillium fungicola* (Table 2).

Solid state fermentation

White button mushroom (*Agaricus bisporus*) obtains nutrition from a selective, well-decomposed substrate prepared by a consortium of microorganisms. In addition, controlled degradation of specific metabolites were generated through microbial growth that determined the final quality of the compost (Johri and Rajni 1999). In this context, nine potential bacterial isolates were selected for solid state fermentation on the basis of qualitative enzymes assays and the mixture of the isolates was inoculated on '0' day mushroom compost for the quantitative enzyme assay of cellulase, xylanase, amylase and protease. During assay, maximum activity was recorded for the amylase enzyme, which was increased by 116.66 and 65.21% over control, respectively, under autoclaved and un-autoclaved condition (Table 3). The cellulase, xylanase and protease activity was also increased over control, except under un-autoclaved condition the xylanase activity was reported lower over control (Fig. 1). The enhancement of enzyme activity under solid state fermentation was mainly reported on the autoclaved '0' day compost and increased by 16.21, 116.66 and 61.95% over control, respectively, for xylanase, amylase and protease activity, while the maximum enhancement of cellulase by 93.33% was measured under un-autoclaved condition (Table 2). Chang and Hudson (1967)

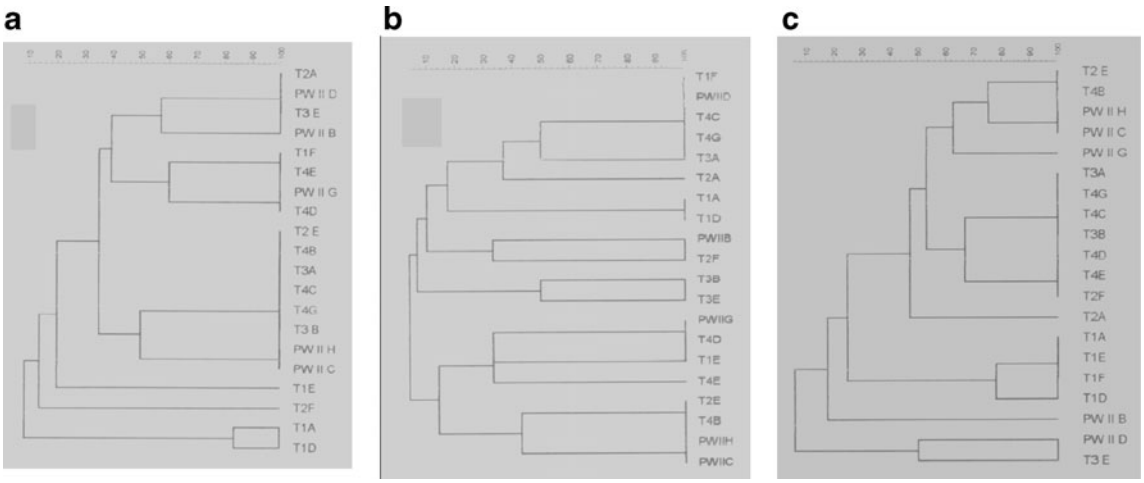

Fig. 2 Comparative UPGMA dendrogram analysis of bacterial isolates based on 16S rDNA restriction profile: **a** Digested with *Msp 1*, **b** with *Sau 3AI*, **c** with *Taq 1*

reported that solid state fermentation is a process of mushroom compost preparation which is brought about the succession of microorganisms.

ARDRA analysis

Considering the significance of mushroom cultivation in the country and the as yet limited information on mushroom composting bacterial flora and their phylogenecity, an exhibitive analysis of mesophilic bacterial diversity of this unique material was undertaken. Molecular tools for the identification of mushroom compost bacteria were used and 16S rDNA gene analysis was used intensively to understand the phylogenetic relationship. To analyze 16S rDNA, amplified ribosomal DNA restriction analysis (ARDRA) was performed. This molecular technique has been successfully used for community analysis in a great variety of environments (Lagace et al. 2004). In this study, 20 bacterial isolates, representing different turning stages of early phase of mushroom compost, were subjected to amplified rDNA restriction analysis by digestion of the amplified 16S rRNA gene with *Msp1, Sau 3AI* and *Taq1*. Three separate dendrograms of band pattern were obtained after three independent digestions. All the restriction profiles based on dendrograms had shown specific similarity value ranging widely between 50 and 100%. On the basis of comparative UPGMA dendrogram analysis, four groups of nine bacterial isolates have shown 100% similarity in all three restriction profiles. The first group consisted of PW II H and PW II C, the second group of T_2E and T_4B, the third group of T_1A and T_1D, and the fourth group of T_3A, T_4C and T_4G (Fig. 2). On the basis of restriction profiling, the grouping of the bacterial isolates has shown their successional profiles of different turning stages of mushroom compost. Some of the isolates also showed grouping, but not with all the restriction analyses; likewise, T_4D and T_4E, PW II D and T_3E showed 100% similarity with *Msp 1* and *Taq 1* but not with the *Sau 3AI* restriction profile. Other isolates also exhibited discriminatory relationships with each other (Fig. 2). These findings are also supported by the finding of Choudhary et al. (2009), who described the phylogenetic profiling of casing amendments used in the cultivation of *Agaricus bisporus* on the basis of amplified rDNA restriction analysis. In conclusion, this is the first preliminary report on the functional characterization of mesophilic bacteria for the early phase of mushroom composting.

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