Characterization of functional activity in composted casing amendments used in cultivation of *Agaricus bisporus* (Lange) Imbach

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In cultivation of button mushroom [*Agaricus bisporus* (Lange) Imbach], casing layer that is nutritionally deficient to compost is believed to trigger the fruit body formation and this is conducted by the bacterial community residing in casing layer. The change in nutritional status of the casing is highly correlated with microbial flora. Therefore, an attempt was made to characterize the bacterial flora in casing layer, i.e., Farm Yard Manure and Spent Mushroom Substrate (FYM+SMS, 3:1) and Farm Yard Manure and Vermi Compost (FYM+VC, 3:1), employing phenetic approaches. Morphotypically different and functionally characterized bacterial isolates were identified by partial 16S rDNA gene fragment sequencing. Available data showed a significant variety of organisms that included *Acinetobacter* and *Pseudomonas* of the \(\gamma\)-proteobacteria, that were most frequently encountered genera. Amongst Gram-positive bacteria, *Bacillus* was the most highly represented genus that was derived from the agaric fruit bodies. In addition, FYM+SMS was found to be a high yielding casing mixture, which took minimum case run period together with superior fruit body quality as compared to FYM+VC.

**Keywords:** *Acinetobacter*, *Bacillus*, casing, endotrophs, phenetic approach, *Pseudomonas*

**Introduction**

Mushroom science is a discipline concerned with the principles and practices of mushroom cultivation. Consistent production of successful mushroom crops is built upon scientific knowledge and practical experience. *Agaricus bisporus* (Lange) Imbach is the most widely cultivated species of edible mushroom and it is the most popular cultivar among the artificially grown fungi of the world that contributes about 31.8% to the global mushroom cultivation and 85% of the total produce in India.\(^1\) The triple advantage of growing mushroom is that mushroom culture is a biotechnological process that recycles lignino-cellulosic wastes, mushrooms are soma of health food for human consumption and the spent mushroom substrates can be utilized profitably in different ways.\(^2\) *A. bisporus* requires two different substrates to form the fruit bodies, i.e., the compost for nutrition on which it grows vegetatively and the nutrient deficient casing soil in which the suitable physicochemical/biological conditions stimulate the initiation process of pin head formation for fruit body production.\(^3\) Inspite of being nutritionally deficient medium, casing layer plays an important role in the productivity of button mushroom.

The casing layer is one of the important growing parameter and source of variation in production, quality and uniformity of commercial cropping. A variety of casing materials have been used the world over. Among these, use of farm yard manure (FYM) as a casing medium for mushroom cultivation has been in vogue in Indian subcontinent because of its easy availability and non-availability of peat moss generally used for casing in Europe and USA. The advantages of using FYM as a casing material over other agro-industrial wastes have been highlighted in a recent report.\(^4\)\(^5\)

Spent mushroom substrate (SMS) is the remainant of compost from which mushrooms have been produced and for which several important roles have been described.\(^6\) Despite evaluation of large number of agro-industrial wastes for their use as casing material in *A. bisporus* cultivation, scant attention has been given to the importance of biological properties of the casing layer.\(^7\) Bacteria present in casing layer considerably influence the growth and morphogenesis...
of A. bisporus production. It supports beneficial microbial populations that release growth stimulating substances, which are reportedly involved in stimulating the initiation of pin heads. Several reports are available on the beneficial effects of casing soil microbes, especially Pseudomonas putida and Alcaligenes faecalis, on A. bisporus\(^8\). Not much information is available in the literature about the role of associated microflora in the casing layer and how the resident microflora in casing layer interacts with the vegetative mycelium of A. bisporus. There are two production related issues requiring attention of researchers in casing soil microbiology, viz., (i) the composition of microorganisms present in casing soil, and (ii) their influence on mushroom production in situ. Several methods and approaches are now available to generate information on microorganisms that reside in casing layer, which allow better assessment of microbial flora, wherein molecular tools for the identification of microorganisms are now in common use, and 16S rRNA gene analysis is intensively used in phylogenetic investigations. Among the 16S rRNA gene analysis method, amplified ribosomal DNA restriction analysis (ARDRA) is commonly used to estimate phylogenetic relationship among different microbial isolates or rDNA clones recovered from the environment\(^9,10\). Furthermore, 16S rRNA sequence typing approach now permits identification of the surviving and culturable bacterial species, based on employing highly conserved 16S rDNA oligonucleotide primers for the eubacteria with an intervening hypervariable gene sequence, which could be used as signature sequence to aid in species identification\(^11,12\).

The aim of the present study was to investigate the cultivable bacterial flora of casing layer and its impact on quality of casing amendments.

**Materials and Methods**

**Sampling Site**

Casing substrates for present study included Spent Mushroom Substrate (SMS), and Vermi Compost (VC). Both the substrates in the stages were amended with Farm Yard Manure (FYM) in the ratio of 3:1. These casing substrates were collected from Mushroom Research and Training Centre (MRTC), GB Pant University of Agriculture and Technology, Pantnagar, India. Samples were drawn from successive stages of each casing, namely, 0 day casing (when casing was applied over the mycelium impregnated compost, i.e., at the time of casing), mycelium impregnated stage (MIS) of casing, casing at primordial stage (PS), and harvesting stage (HS) casing.

**Physico-chemical and Nutrient Analysis of Casing Mixtures**

Processed casings (formalin treated, 2% v/v) were used for physico-chemical analysis using methods suggested by various workers: Bulk density\(^13\), water holding capacity\(^14\), porosity\(^15\), electrical conductivity and pH\(^16\) (TPS Smartchem Laboratory Analyzer), total organic matter and C\(^17\), available N\(^18\), available P\(^19\) and available K\(^20\). For macro and micronutrients (Ca, Mg, Cu, Zn, Mn & Fe) analysis procedure of Tandon\(^21\) was employed.

**Isolation of Bacterial Isolates**

Casing sample (10 g) was suspended in 90 mL of 0.85% normal saline (pH 7.0) and shaken vigorously at 150 rpm at 18°C for 1 h. The resulting slurry was serially diluted (100 µL) to 900 µL of 0.85% normal saline in each Eppendorf tube and appropriate dilution (10\(^{-4}\)) of this suspension (0.1 mL or 100 µL) was spread plated in triplicate on King’s B medium\(^22\). Cultures were incubated at 20°C±2 for 2 d. For experimental use, isolates were transferred when needed to King’s B medium that was stored at 4°C.

**Phenetic Characterization**

Recovered bacterial isolates were phenotypically (morphotypic, physiological & functional) and genotypically characterized.

**Morphological**

Isolates were checked for fluorescence under UV light (312 nm) (Geldocmega, Biosystematica) and distinct colonies were picked up. A total of 50 isolates were thus randomly selected morphologically from all the four successive stages of two types of casing soil, namely, 0 day casing, mycelium-impregnated casing, casing with initials (primordial) and harvesting stage (cropping). Colony morphology of isolates was studied under a stereo microscope (Leica). This included shape, edge, elevation, surface and pigmentation. Cellular morphology was based upon cell shape and Gram staining (Leica fluorescent microscope).

**Physiological**

Morphotypically different bacterial isolates isolated from various stages of two type of casing mixtures were further characterized based on their substrate utilization and enzyme secretion. These included: catalase and oxidase enzyme substrates, gelatin
hydrolysis, citrate utilization test, nitrate reductase (NO$_3^-$ → NO$_2^-$ → N$_2$)/denitrification test and trehalose utilization in substrate utilization tests.

**Functional**

Assessment of functional diversity among isolates was studied by qualitative plate assay for amylolytic, proteolytic, cellulolytic, xylanolytic, pectinolytic and laminarinolytic activity, phosphorus solubilization (inorganic phosphates, and organic phosphates), and siderophore production. Minimal medium was used for plate assay wherein isolates were spot-inoculated with sterile tooth-pick on solid medium and incubated at 20±2°C. The zone of diameter of clearing, if any, of all +ve isolates were measured.

**Amylase (Starch Diastase)**—All isolates were spot inoculated on functional minimal medium amended with 1% (w/v) starch and incubated for 24-72 h at 20±2°C for growth. Plates were then flooded with Lugol’s iodine for 10 min. Then iodine was drained off and +ve isolates exhibit a zone of clearance against dark blue background.

**Protease**—The minimal medium amended with skimmed milk (20 mL L$^{-1}$) was spot inoculated with isolates. Plates were incubated for 24-72 h at 20±2°C for growth. Formation of a clear halo around the bacterial colony was considered as +ve 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 in an incubator for 72 h at 20±2°C for growth and flooded with Congo red solution (0.2% w/v) for 30 min. Excess reagent was discarded after destaining with 1 M NaCl solution for 30 min. Zone of clearance around bacterial colonies was considered as +ve result for this test.

**Pectinase**—All isolates were spot inoculated on minimal medium amended with 1% (w/v) pectin and plates were incubated for 3-5 d at 20±2°C for growth. Activity of β-1,3-glucanase was determined by the ability of bacteria to grow on laminarin as the sole carbon source.

**Siderophore Production**—Chromeazurol ‘S’ agar plates$^{23}$ were spot inoculated with bacterial isolates, and incubated for 48-72 h at 20±2°C for growth. Formation of an orange halo around bacterial colony was considered as +ve result.

**Inorganic ‘P’ Solubilization**—Bacterial isolates were spot inoculated on Pikovaskya’s agar and incubated for 3-5 d at 20±2°C for growth. Formation of a clear zone around bacterial colonies was considered as +ve result.

**Organic ‘P’ Solubilization (Phosphatase)**—Bacterial isolates were spot inoculated on tryptose phosphate agar supplemented with methyl green (0.05 mg mL$^{-1}$) as indicator dye. Plates were incubated for 3-5 d at 20±2°C for growth. Development of green colour by bacterial colonies was considered as +ve result.

**Genotypic Characterization**

**Recovery of Genomic DNA**

Total DNA from bacterial isolates was prepared following the procedure outlined by Bazzicalupo and Fani$^{24}$ with the exception that for Gram-negative bacteria, no lysozyme was used.

**Quantification and Detection of Purity of Extracted DNA**

Extracted genomic DNA was run in 0.8% agarose gel at 80 V for 45 min with qualitative marker in one lane (Low DNA Mass Ladder, MBI Fermentas). Gel was visualized under UV transilluminator. 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. The concentration of DNA was calculated as:

\[
[DNA] = A_{260} \times 100 \times \text{Dilution factor (µg mL}^{-1})\]

**REP-PCR (Box Element)**

REP-PCR fingerprints were obtained using the following BOX-AIR sequence as primer$^{25}$: 5’ GAT CGG CAA GGC GAC GCT GAC G 3’. The amplification was carried out in 30 µL reaction mixture containing following working concentration: Milli Q water, Taq DNA Pol buffer + MgCl$_2$ 15 mM, 1.0 ×; dNTP (10 mM), 0.3 mM; Box Primer (10 µM), 0.25 µM; Taq DNA Polymerase (3 U), 1.0 U/Rxn;
and Target DNA, 20-100 ng. One positive control
with standard DNA template and one negative control
without DNA were maintained to check reaction mixture preparation and contamination. The reaction
conditions were: Initial denaturation at 95°C for 5
min, followed by 40 cycles of denaturation at 95°C
for 1 min, annealing at 60°C for 1 min, extension at
72°C for 1 min, and final annealing at 60°C for 5 min
and extension at 72°C for 5 min. The reaction was
performed in a PTC-100 Thermal cycler (MJ
Research). Amplified product was analyzed by agarose gel (2.0%) electrophoresis in 1× TAE buffer
at 70 V for 4 h. The amplification profile was
analyzed using NTSYS pc version 2.02i. The
clustering was done using Jaccard’s similarity
coefficient based on presence and absence of band
ignoring their intensities. Only sharp and consistent
bands were considered for analysis.

**PCR Amplification of 16S rDNA**

The amplified 16S rRNA gene was obtained from
each bacterial isolate by PCR amplification employing
the eubacterial universal primers\(^26\) fDI (5′-
AGAGTTTGATCCTGG -3′) and rP2 (5′-
TACCTTGTTACGACTT -3′) which were targeted at
universally conserved regions and permitted
amplification of ~1,500-bp fragment. PCR
amplification was carried out in a PTC-100
thermocycler (MJ Research). Amplified product was
analyzed by agarose gel (2.0%) electrophoresis in 1× TAE buffer
at 70 V for 4 h. The amplification profile was
analyzed using NTSYS pc version 2.02i. The
clustering was done using Jaccard’s similarity
coefficient based on presence and absence of band
ignoring their intensities. Only sharp and consistent
bands were considered for analysis.

**Partial Sequencing of 16S rDNA**

PCR products obtained from 33 bacterial strains
were purified with an EXO-SAP. Components were
supplemented with gold buffer (Applied Biosystem)
and sequenced on an Applied Biosystem 310 Genetic
analyzer (ABI Prism 310 Genetic analyzer), using big
dye terminator cycle sequencing Ready Kit (Lab
India). The partial sequences amplified by the f DI
primer were used to determine the similarities.
Homology tree for the data sets were inferred from
the Fast Alignment method by employing software
DNAMAN version 4.0, Lynnon Biosoft, USA.
Several accessions (borrowed from NCBI database)
were employed for making relationship among recovered bacterial isolates (present study) from
different stages of two casing materials under
evaluation.

**Nucleotide Sequence Accession Numbers**

The sequences determined in this study have been
deposited in the GenBank database of NCBI and
discussed under the accession numbers: AY961042-
AY961047, AY961049-AY961053, AY961055-
AY961059, AY961061, AY967718-AY967722,
AY967724, DQ074746, DQ074751-DQ074752,
DQ074754-DQ074757.

**Cultivation of Button Mushroom**

The experimental trial was conducted seasonally
for the year (2003-04) under controlled conditions
(temperature 18-25°C and relative humidity 70-80%)
in crop room at the Mushroom Research and Training
Centre, Pantnagar. FYM (2-yr-old), SMS and VC
(1-yr-old) based (3:1 v/v), chemically treated (4% formalin) casing material was used for cultivation of
button mushroom. Mushroom cultivation was carried

out in polybags of 45 × 60 cm<sup>2</sup> size each containing 10 kg of wheat straw-chicken manure based pasteurized compost. The spawn of strain X-13 of <i>A. bisporus</i> was mixed with the compost @ 0.75% and spawn run done at 22-25°C. After complete spawn run, the bags were cased uniformly with above casing mixtures (4.0 cm thickness). Five replicates of each treatment were arranged in completely randomized design (CRD). The yield data were obtained for six weeks of cropping and were subjected to Analysis of Variance (ANOVA) in terms of kg mushrooms/100 kg compost. The quality parameters of the mushrooms harvested from casing, FYM+SMS (3:1) and FYM+VC (3:1) were assessed from first flush, which included: protein content (mg g<sup>−1</sup> of fresh wt), whole mushroom wt (g), whole mushroom length (cm), pileus diameter (cm), pileus thickness (cm), stipe length (cm), stipe diameter (cm), pileus wt (g), stipe wt (g), whiteness (%) and dry weight of mushroom (g).

### Results

**Physico-chemical Properties of Casing Samples**

The casing sample FYM+SMS (3:1) had minimum bulk density (BD) (0.60 g/cm<sup>3</sup>), while the casing FYM+VC (3:1) showed a BD value of 0.68 g/cm<sup>3</sup>. The porosity was significantly high (92.0%) for FYM+SMS. Water holding capacity appears directly related to porosity and bulk density and may directly affect microbial build up and yield of <i>A. bisporus</i>. It was high (191.19%) for FYM+SMS. A significant increase in electrical conductivity (EC) was observed for FYM+VC (570.33 deci-simen<sup>−1</sup>) whereas low value (398.00 deci-simen<sup>−1</sup>) was obtained for FYM+SMS (Table 1). The high value of EC is harmful for fructification of button mushroom.

**Macro and Micronutrients Analysis of Casing**

<i>A. bisporus</i> was grown on wheat straw based mushroom compost (pasteurized/long method). Two casing mixtures prepared from three casing materials, FYM, SMS and VC were evaluated for their nutritional status. In order to assess the nutritional status of casing mixtures (FYM + SMS, FYM + VC) during various stages of cropping, status of availability macro and micronutrients were analyzed (Table 2). Maximum level of organic matter was recorded from the two casing mixtures (28.91% ± 0.2 & 26.42% ± 0.08) collected at mycelium-impregnated stage, followed by the pinning stage (27.76% ± 0.2 & 25.82 % ± 0.3). Minimum organic matter for FYM+SMS and FYM+VC was recorded in samples collected at the harvesting stage (26.06 % ± 0.08 & 25.02 % ± 0.6); 0 day stage casing soil mixtures showed comparatively lesser organic matter content, i.e., 25.38 % ± 0.11 and 24.81 % ± 0.2. A significant variation was recorded in FYM+SMS casing preparation, which showed different nutritional status than FYM+VC. Micronutrients estimation (ppm) included Cu, Zn, Mn and Fe. The relative order of nutritional status (macro and micronutrients) for various stages of casing mixture was, mycelium-impregnated stage > pinning state > harvesting stage > 0 day stage (Table 2).

**Population Dynamics of Recovered Isolates**

There was little variation (6.02 ± 0.81 to 5.87 ± 0.81) in total bacterial population counts (log<sub>10</sub> CFU) among casing layers FYM+SMS and FYM+VC tried at successive stages of cropping (Fig. 1). The casing layer FYM+VC from the mycelium-impregnated stage showed slightly higher population (6.02 ± 0.81) compared to FYM+SMS (5.92 ± 1.69). The lowest population was observed at pinning stage in FYM+SC (5.87 ± 0.81).

The endotrophic mesophilic bacterial population recovered from tissues at primordial stage and button stage showed little variation. Bacterial population was higher at button stage (5.52±1.69) compared to primordial stage (5.34±1.24) (Fig. 2).

**Physiological and Functional Characterization**

Considerable differences were observed among isolates recovered from successive stages of casing soils, including endorphils, in their nutritional/physiological characteristics. Most of the isolates utilized citrate and exhibited NO<sub>3</sub>− reductase.

### Table 1—Physico-chemical properties of casing mixtures

<table>
<thead>
<tr>
<th>No.</th>
<th>Casing soil</th>
<th>Properties</th>
<th>Bulk density (g/cm&lt;sup&gt;3&lt;/sup&gt;)</th>
<th>Porosity (%)</th>
<th>Water holding capacity (%)</th>
<th>pH</th>
<th>Electrical conductivity (deci-simen&lt;sup&gt;−1&lt;/sup&gt;)</th>
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<tbody>
<tr>
<td>1</td>
<td>FYM+SC; 3:1</td>
<td></td>
<td>0.60</td>
<td>92.00</td>
<td>191.19</td>
<td>7.21</td>
<td>398.00</td>
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<tr>
<td>2</td>
<td>FYM+VC; 3:1</td>
<td></td>
<td>0.68</td>
<td>82.00</td>
<td>95.97</td>
<td>7.13</td>
<td>570.33</td>
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</tbody>
</table>

FYM= Farm Yard Manure, SMS= Spent Mushroom Substrate, VC= Vermi Compost
and catalase activity. Bacteria recovered from primordial and button stages showed oxidase, catalase activity and utilized trehalose as sole carbon-source. None of the isolates hydrolyzed gelatin (Table 3).

Bacterial morphotypes isolated from FYM+VC at mycelium-impregnated stage showed protease, cellulase, siderophore and ‘P’-solubilizing activity. However, none of the isolates recovered from this casing mixture at 0 d, pinning and harvesting stages exhibited protease, cellulase, amylase, pectinase, xylanase and siderophore activity; but exhibited ‘P’-solubilization activity which was maximum for 0 day stage morphotypes. Endotrophic bacterial morphotypes isolated from primordial and button stages of cropping showed good amylase activity. Amylase +ve diversity was much higher for primordial stage morphotypes compared to button stage.

### Table 2—Chemical characteristics with respect to macro and micronutrients for casing mixtures recovered during the successive stages of button mushroom

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>FYM + SC (3:1)</th>
<th>FYM + VC (3:1)</th>
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<tr>
<td></td>
<td>0 d stage</td>
<td>Mycelium-impregnated stage</td>
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<tr>
<td>Organic matter (%)</td>
<td>25.38 ± 0.11</td>
<td>28.91 ± 0.20</td>
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<td>Organic C (%)</td>
<td>14.10 ± 0.14</td>
<td>16.06 ± 0.16</td>
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<td>N (%)</td>
<td>0.92 ± 0.02</td>
<td>1.41 ± 0.05</td>
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<td>P (%)</td>
<td>0.78 ± 0.01</td>
<td>1.20 ± 0.09</td>
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<td>K (%)</td>
<td>0.90 ± 0.02</td>
<td>1.21 ± 0.01</td>
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<td>Ca (ppm)</td>
<td>11.51 ± 0.18</td>
<td>13.72 ± 0.08</td>
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<td>Mg (ppm)</td>
<td>5.23 ± 0.02</td>
<td>5.01 ± 0.02</td>
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<td>Cu (ppm)</td>
<td>0.025 ± 1.65</td>
<td>0.037 ± 1.03</td>
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<td>Zn (ppm)</td>
<td>0.128 ± 0.04</td>
<td>0.416 ± 0.01</td>
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<td>Mn (ppm)</td>
<td>1.01 ± 0.01</td>
<td>1.2 ± 0.02</td>
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<td>Fe (ppm)</td>
<td>0.059 ± 0.01</td>
<td>0.088 ± 0.02</td>
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FYM= Farm Yard Manure, SMD= Spent Mushroom Substrate, VC= Vermi Compost
Table 3—Physiological and nutritional characteristics of bacterial isolates

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*Bacterial isolates: strains USC1-8, isolated from 0 d casing of FYM+SMS (3:1); strains USC9-15, isolated from 0 day casing of FYM+VC (3:1); strains USC23-32, isolated from mycelium-impregnated stage (MIS) of casing FYM+SMS (3:1); strains UVC1-5&8, isolated from mycelium-impregnated stage (MIS) of casing FYM+VC (3:1); strains CSC1-CSC2, isolated from harvesting stage (HS) of FYM+SMS (3:1); strains TSC1-TSC2, TVC3 & SFB, isolated from pinning stage (PS) of FYM+SMS (3:1); strains CVIC1-CVIC2, TVC1-TVC2, isolated from pinning stage (PS) of FYM+VC (3:1); strains TFB &TPH, isolated from harvesting stage (HS) of FYM+VC (3:1); strains PH1-PH4, isolated from primordia of A. bisporus; strains FB1-FB3, isolated from fruiting body of A. bisporus

(FYM= Farm Yard Manure, SMS= Spent Mushroom Substrate, VC= Vermi Compost)
Morphotypes isolated from FYM+SMS at mycelium-impregnated stage showed higher diversity of protease and cellulase. Low potential of cellulolytic bacteria was observed in FYM+SMS casing morphotypes from the harvesting stage. Morphotypes isolated from different stages of cropping from FYM + SMS casing layer also showed siderophore and ‘P’-solubilization activity; harvesting stage morphotypes were an exception where none of the isolates showed ‘P’-solubilization. A significant variation in siderophore secreting diversity was observed in FYM+SMS morphotypes from mycelium-impregnated stage. Maximum ‘P’-solubilization diversity was observed in FYM+SMS layer morphotypes derived from the mycelium-impregnated stage and least in those recovered at the pinning stage of FYM+SMS (3:1); none of the isolates recovered from successive stages of cropping exhibited amylase, xylanase and pectinase producing morphotypes (Table 3).

**Genotypic Characterization**

**REP-PCR (Box Element)**

Distinctive banding pattern was observed in the Box element. The dendrogram of banding patterns based on UPGMA showed significant discriminatory relationship among the isolates. Isolates placed within 8 clusters showed 100% similarity within any single group. Isolates CVC2, TCV1 and TVC2 exhibited 100% similarity with each other and made single group. Likewise, isolates USC11-USC14, UVC2-UVC4 made another single group with 100% similarity. Isolates USC12, USC15 and USC25-USC32 made single largest group wherein 10 isolates showed 100% similarity with each other, whereas smallest groups made by isolates UVC5, UVC8 and PH4, FB1 wherein 2 isolates exhibited 100% similarity with each other (Fig. 3).

**ARDRA Analysis of Bacterial Isolates**

Fifty casing soils isolates, representing different cropping stages/environmental niche (source location) including endotrophs, were selected based on colony morphology, and were subjected to ARDRA analysis by digestion of the amplified 16S rRNA gene with *Taq*1, *Msp*1, and *Alu*1. The dendrogram of banding patterns was obtained after combination of the three independent digestions. The similarity value ranged widely between 50-100%. The first group was represented by reference strains, viz., biovar of *P. fluorescens* I-V that showed 100% similarity, whereas reference strain *P. chlororaphis* showed ~60% similarity with biovars of *P. fluorescens*. Most groups showed 90-100% similarity with some groups delineated at 80-90% similarity level. Isolates USC5 and USC7-USC10 made single largest group and exhibited 100% similarity with each other, whereas isolates USC3-USC4 and USC13-USC14 made two smallest groups with 100% similarity. Other isolates exhibited discriminatory relationship with each other (Fig. 4).

**16S rRNA Gene Partial Sequencing**

Partial 16S rDNA sequences were determined for 33 isolates and were compared with the corresponding sequences of standard strains obtained from the GenBank database to evaluate the phylogenetic diversity. Most strains were grouped together and were close to the genus *Pseudomonas* and *Acinetobacter* of the family Pseudomonadaceae and Moraxellaceae, respectively. Four strains recovered from basidiocarp of *A. bisporus*, i.e., FB1 (DQ074754), PH4 (DQ074755), PH1 (DQ074756), and PH3 (DQ 074757), exhibited high level of similarity with reference strain *Bacillus* (AY842872) and they showed 55% similarity with reference strain *Sphingobacterium* (AY881645) (Fig. 5).

**Family Pseudomonadaceae**—Of 33 strains, 11 exhibited close proximity with the genus *Pseudomonas*. All the 11 strains showed 95-99% similarity with reference strain *Pseudomonas putida* (AY785244).

**Family Moraxellaceae**—Large numbers of strains were distributed in family Moraxellaceae and they exhibited close proximity to genus *Acinetobacter*. Largest group of this family compromised of 5 strains (UVC4, TSC2, UVC3, USC2 & USC9), which exhibited close proximity (95-100%) with reference strains *Acinetobacter* sp. (AY362002) and *A. calcoaceticus* (AJ888983).

**Yield and Quality of Fruit Body of Button Mushroom**

The data on effect of casing treatments on fruit body yield and quality are presented in Table 4. The bags cased with casing mixture of FYM+SMS (3:1) took a minimum of 11 d for case run during cropping, while FYM and VC (3:1) took a maximum of 14 d. The yield data for 6 wk of cropping showed that FYM+SMS favoured development of a higher number (1202.3) of sporophores. On the basis of yield performance, it was concluded that the casing material prepared by FYM+SMS in was the superior over FYM+VC (Table 4).
Discussion
Considering the significance of mushroom production in the country and yet limited availability of information on casing soil microflora, an exhibitive analysis of bacterial diversity of this unique material was undertaken. A significant variation in bacterial flora was observed at various stages of cropping from the casing soils. The different morphotypes were screened and quantified based on colony and cellular morphology. Microbial populations and communities have been described in terms of a wide range of phenotypic traits, many of which are related to the practical interest in the habitat studied.

The casing material was so selected that its nutritional status was very low compared to that of compost and was thus expected to create conditions of nutritional

Fig. 3—Composite UPGMA dendrogram of bacterial isolates based on REP-PCR (Box). 1-50 in dendrogram represent strains USC1-USC15, USC23-USC32, UVC1-UVC5, UVC8, CSC1-CSC2, TSC1-TSC2, CVC1-CVC2, TVC1-TVC3, SFB, TFB, TPH, PH1-PH4 and FB1-FB3, respectively.

Table 4—Effect of casing treatments on mushroom yield and fruit body quality

<table>
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<tr>
<th>Casing treatment</th>
<th>Case run period (d)</th>
<th>Yield wt (kg/100kg compost)</th>
<th>Soluble protein (mg g⁻¹)</th>
<th>Mushroom wt (g)</th>
<th>Mushroom length (cm)</th>
<th>Pileus diam (cm)</th>
<th>Pileus thickness (cm)</th>
<th>Stipe length (cm)</th>
<th>Stipe diam (cm)</th>
<th>Pileus wt (g)</th>
<th>Stipe wt (g)</th>
<th>Whiteness (%)</th>
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<tr>
<td>FYM+SMS (3:1)</td>
<td>11</td>
<td>1202.3</td>
<td>14.70</td>
<td>8.12</td>
<td>14.98</td>
<td>4.8</td>
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<td>9.89</td>
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<td>14</td>
<td>1089.6</td>
<td>12.28</td>
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FYM= Farm Yard Manure, SMS= Spent Mushroom Substrate, VC= Vermi Compost
*Fruit body quality: All readings are mean of 10 values
stress. If the casing material is rich in organic matter, it is likely to disturb the selectivity of compost and thus provide continued support to the vegetative growth of the fungus and thus discourage fruiting. Characteristically, microorganisms bring about physical and chemical changes to their habitats. Water holding capacity appears directly related to porosity and bulk density. These factors are directly affecting microbial build up and yield of *A. bisporus* and was maximum (191.19%) for FYM+SMS (3:1). In general, increase in electrical conductivity (EC) is almost proportional to decrease in number of pinheads. Data of the present investigation on EC (Table 1) and mushroom yield (Table 4) exhibited that the higher EC of the casing FYM+VC (3:1) resulted in lower yield. The results indicate that the EC plays an important role in the production of button mushroom but it is not the sole controlling factor. These findings are in agreement with the finding of Bhatt *et al*\(^2^9\). Carbon/nitrogen content seemed to be directly affecting the mushroom yield\(^3^0\). In addition, physico-chemical properties of Indian casing materials have also been reported by Singh *et al*\(^3^1\). The macro and micronutrients levels increased and decreased correspondingly.

Molecular tools for the identification of casing soil bacteria were used and 16S rRNA gene analysis was intensively used to understand the phylogenetic relationships. Among the 16S rRNA gene analysis, amplified ribosomal DNA restriction analysis (ARDRA) was performed. This molecular technique has been successfully used for bacterial community analysis in a great variety of environments. This study examined the cultivable bacterial community comprising successive stages of casing soils and endotrophs using ARDRA and 16S rRNA gene fragment sequencing. Results show considerable
diversity of bacterial community in casing soils based on the large number of ARDRA patterns obtained. Phylogenetic analysis revealed that 85% of the bacterial isolates belonged to γ-proteobacteria group and other isolates were bacilli. A single isolate in this study was found to belong to the genus

Fig. 5—Homology tree of 33 bacterial isolates recovered from casing soils including endotrophs and their comparison with sequences (AB020205, AY842872, AY881645, AJ888983, AY362002, and AY785244) borrowed from the GenBank. The numbers at the branching points are the percentage of occurrence of in 1000 bootstrapped tree. Tree was made by Fast Alignment Method of Software DYNAMAN ver 4.0 of Lynnon Biosoft.
Sphingobacterium. Two genera, Acinetobacter and Pseudomonas were dominant and were sole representative of γ-proteobacteria. Dominance of the genus Acinetobacter was of significance since this has not earlier been reported from the mushroom casing ecosystem.

Bacterial community of casing layers used in mushroom production and bacteria recovered from pinheads and fruiting bodies of A. bisporus were assessed in this study. Morphotypes recovered at various stages of cropping and those from the fruit bodies were different compared to reference strains, P. fluorescens and P. chlororaphis. Box element (rep-PCR) enabled discrimination amongst bacterial isolates recovered from successive stages of casing soils and endotrophic population. Most isolates showed 100% similarity among themselves based on the presence of palindromic unit sequence. To our knowledge, no 16S rDNA sequences from microorganisms in the casing layer at successive stages of cropping including endotrophs of A. bisporus have been reported. Partial sequencing of bacterial isolates showed a 62-100% overlap with several other bacterial isolates. However, comparison of the 16S rDNA region could be used to build up more complicated phylogenetic affiliations, e.g., our sequence analysis indicated that there is a cluster that incorporates only endotrophs and another cluster incorporates bacterial sequences recovered from mycelium-impregnated stage of FYM-VC (Fig. 5). In this preliminary study, 16S rDNA PCR and direct automatic sequencing of the amplicons was only carried out on cultivable organisms isolated on the nonselective King’s medium B (KMB). In conclusion, this is the first preliminary report on the microbial diversity of casing soils and demonstrates the presence of Acinetobacter sp. that has not been previously described in casing material.

Acknowledgement

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References


19. Olsen S R, Estimation of available phosphorus in soil by
extraction with sodium bicarbonate (US Dept Agric, Washington) 1954, 939.


21 Tandon H L S, Methods of analysis soils, plants, water and fertilizers (Fertilizers Development and Consultant Organization, New Delhi) 1993, 144.


