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Bacterial diversity in a bagasse-based compost prepared for the cultivation of edible mushrooms *Agaricus bisporus*

Agrawal Pavan Kumar[1*], Chaudhary Devendra kumar[2], Prakash Anil[3], and Johri B.N.[3]

[1]Sai institute of paramedical and allied sciences Affiliated to H.N.B. Garhwal University Dehradoon, Uttrakhand., India.
[2]Modi institute of Technology and Science Lakshmangarh, Sikar, rajsthan. India.
[3]Deparment of Biotechnology and Bioinformatics Centre, Barkatullah University, Bhopal - 462 026 M.P., India.

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Edible mushrooms are renowned for their nutritional and medicinal properties and are thus of considerable commercial importance. Mushroom production depends on the chemical composition of the basic substrates and additional supplements employed in the compost as well as on the method of composting. In order to minimize the cost of mushroom production, considerable interest has been shown in the use of agro-industrial residues in the preparation of alternative compost mixtures. However, the interaction of the natural microbiota present in agricultural residues during the composting process greatly influences the subsequent colonization by the mushroom. The relative numbers of microorganisms associated with compost during mushroom production were studied by the dilution plate method. The aim of the present study was to isolate and identify the bacteria present in a agricultural wastes like wheat straw, paddy straw and sugarcane bagasse, compost prepared for the production of *Agaricus bisporus*. Bacteria, (mainly *Bacillus*) were predominant micro-organisms presented throughout the composting process.

Key words: mushroom production; Bacterial diversity; wheat straw, paddy straw and sugarcane bagasse, *Bacillus sp*.,

Introduction

The mushroom species *A. bisporus* (Champignon), *Pleurotus sajor-caju* (Oyste rmushroom) and *Lentinula edodes* (Shiitake) are of important economical value on account of their exquisite flavour and proven medicinal properties (Sánchez, 2004). Moreover, the organoleptic, nutritional and medicinal characteristics of *A. bisporus* have been recognised worldwide and

* Corresponding author: Agrawal Pavan Kumar; e-mail: p_k_agarwal@rediffmail.com

have assured a prime position for this species on the international market (Angrish *et al.,* 2003). In addition they have a varied range of applications in bioremediation of soil, bioconversion of waste water, medicine and agricultural waste disposal (Vinciguera *et al.,* 1995, Magingo *et al.,* 2005). In India, the culture of *A. bisporus* is relatively recent, whilst productivity is variable and depends mainly on the type of substrate employed (Romaine & Schlagnhanfer, 1992). Wheat straw, straw-bedded horse manure, chicken manure and gypsum (Have *et al.,* 2003) are typically employed as substrates in the commercial culture of mushrooms, there is considerable interest in finding cheaper and more readily available materials for compost production in which the C:N ratio is between 25:1 and 50:1. In this context, the agricultural waste materials sugar-cane bagasse and wheat straw are considered to be potential substrates for the cultivation of *A. bisporus in India*.

The composting process comprises two stages: in stage I the organic residues are wetted, distributed in layers and mixed periodically for up 2 weeks, while in the second stage the resulting compost is pasteurised and conditioned (Chang, and Miles, 2004). Although, the final quality of the compost is determined by the overall composting process, success in mushroom production depends largely on stage II (Sánchez, 2004). During pasteurization, microbial activity produces large quantities of heat that serve to eliminate many pests, pathogens and competing micro-organisms. In the conditioning stage, the thermophilic micro-organisms (especially the Actinomycetes) multiply and convert free ammonia into microbial protein. These micro-organisms perform a crucial role in the preparation of an appropriate mushroom compost since residual ammonia is highly toxic and hinders mycelia growth of *Agaricus* species, while the synthesized protein is important for the nutrition of the cultivated fungi (Chang and Miles, 2004, Colak, 2004, Straatsma *et al.,*1994). In view of the pivotal importance of the Bacterial isolates that multiply and develop during stage I and act during stage II of composting, we undertook the task of evaluating the bacterial diversity that occurs during the preparation of a compost (based on sugar-cane bagasse and wheat straw) and during stages of mushroom composting .

Material and methods

Sampling Site

A total of six different compost samples from different stages of mushroom compost viz., pre-wetting I (PWI), end of phase I compost (Filling), peak heat stage of phase II compost (Peak heat), conditioning stage of phase II compost (Con), end of phase II compost (Spawn) and end of cropping sequence

(Drench) were collected from Flex Food, Dehradun, Uttrakhand. Samples were removed at three different locations and depths in the composting pile (lower, upper, and median point), mixed and an aliquot of 10 g was processed further.

Bacteriological analysis

A suspension using 10 g compost samples was prepared in 90 ml of sterile distilled water. This mixture was incubated at 30^0C, 120 rpm in an orbital shaker for 30 min to homogenize the sample. Bacterial population was enumerated using 10-fold serial dilutions and is expressed as CFU g^{-1} dry compost; serially diluted compost samples (up to 10^{-5}) were plated on nutrient agar medium. Plates were incubated in triplicates at 30^0C and counts (CFU) were recorded after 72 h. A total of 50 different morphotypes were detected and categorized on the basis of the composting stage. Purified and stored in 20% glycerol at - 80^0C for subsequent identification.

Phenetic characterization of bacterial isolates

Recovered bacterial isolates were phenotypically (morphotypic and functional) characterized. A total of 50 isolates were thus randomly selected morphologically from all the six stages of mushroom composting. Colony morphology of isolates was studied under a stereoscope microscope (Leica). This included shape, edge, elevation, surface and pigmentation. Cellular morphology was based upon cell shape and Gram staining (Leica fluorescent microscope).

Data analysis and Evaluation of Bacterial Diversity

Various indices viz., Shannon's index (H') (Shannon & weaver, 1949), Simpson's index (λ) (Simpson, 1949), Margalef's Richness index (R1) (Margalef, 1958) and Menhinick's Richness index (R2) (Ludwing and Reynolds, 1988), evenness index (E) were calculated based on the determination of morphotypic analysis of bacteria.

Sequencing of bacterial isolates

Bacteria identification was carried out on the basis of 16 S r DNA sequencing Table 2.The 16S rDNA sequence of bacterial isolates was determined by dideoxy chain termination method using bdt V3 ready reaction cycle sequencing kit on the applied Biosystems DNA sequencer Model 310.

Results and discussion

Compost is an interesting example of man-made ecosystem that harbours a complete spectrum of microbial diversity. The microbial abundance, composition and activity changes substantially during the composting process and are correlated with high microbial diversity and low activity in matured compost.

Microbial community succession during composting is a classical example of how the growth and activity of one group of organisms can create conditions necessary for the growth of others. Several generations of microorganisms succeed each other during composting wherein each crop of microbial form utilizes the available material in the substrate as also the cellular components of its predecessors for growth, spread and sustenance. The study of community structure and diversity by various workers (Bilai, 1984; Straatsma *et al*., 1994, Beffa *et al*., 1996 (a, b); Peters *et al*., 2000) has been instrumental in manipulating the compost environment in order to quicken the composting process and to improve the compost quality.

Taxonomic status of the bacterial diversity was assessed in mushroom compost. At the initial stage, a total 50 isolates were used from the departmental culture collection. These isolates were recovered from different stages (PWI, Filling, Pasteurizing, conditioning, spawning and drenching) of mushroom composting and temperature. A total 19 different bacterial morphotypes were selected on the basis of colour, morphological characteristics viz., colony morphology (shape, margin, elevation and surface) and cell morphology (Gram's reaction, cell shape and arrangement) and were studied in detail (Fig. 1).

Bacteria exhibited wide morphological variation. Maximum variation in colony shape viz., irregular and circular was exhibited by bacteria recovered at PWI, Fill, Con, Spawn and Drench stages while those from Past stage showed mostly irregular shape. Drench and Con microflora exhibited maximum variation in the margin, from entire to undulate. Fill and Spawn microflora varied from lobate to filamentous; bacteria recovered from Fill were entire, lobate or/with serrate margin whereas those belonging to past stage exhibited filamentous and serrate margin. Fill, Spawn and Drench microflora exhibited considerable variation in the colony elevation (flat, raised and convex). Raised and convex elevation was also observed in microflora representing stages PWI, Past and Con. Microflora derived from Con stage, exhibited varied colony surface from smooth to dry. Fill and Past stage flora was smooth, wrinkled and dry (Table 1). PWI, Spawn and Drench bacteria exhibited surface variation from smooth to glistening. Both Gram positive and Gram negative microflora

were present at all stages except Con which was dominated by the Gram negative forms.

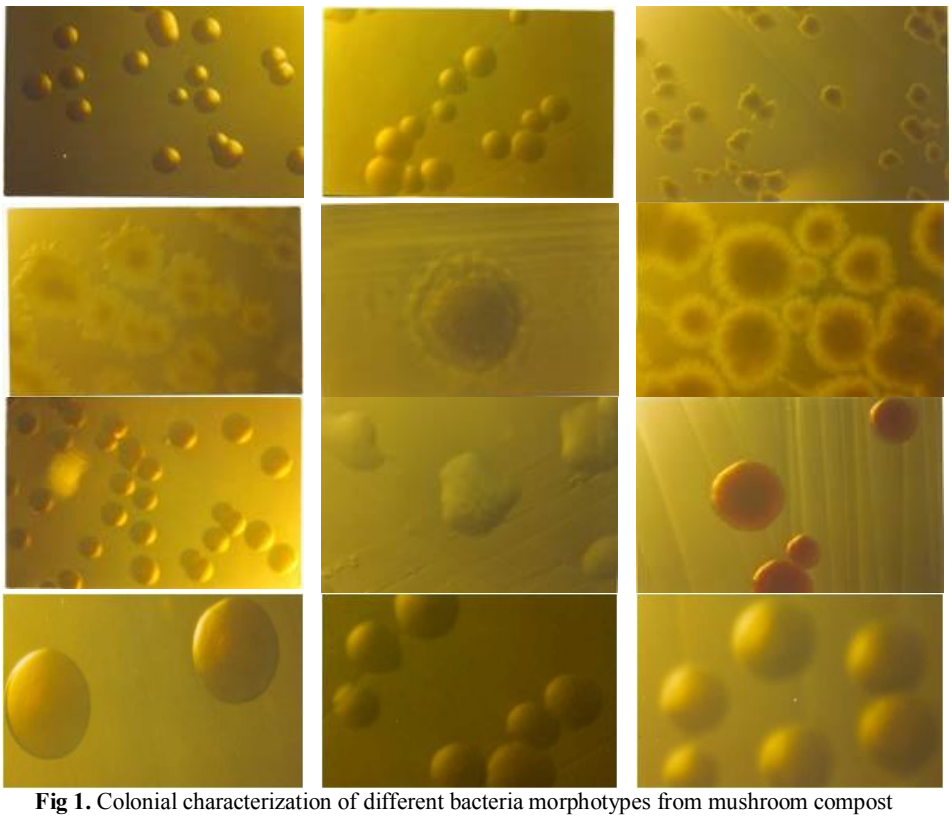

Fig 1. Colonial characterization of different bacteria morphotypes from mushroom compost

Table 1. Morphotypic characterization of Mesophilic bacteria isolated at 30°C from mushroom compost.

Origin	Name of isolates	Gram reaction	Colour of colony	Edge / Margin of colony	Elevation of colony	Surface of colony	Form of colony
Prewetting stage	PW1 A	-Ve, short rod	Creamish	Serrate	Convex	Glistening	Irregular
	PW1 B	+Ve, Rod	Creamish white	Entire	Convex	Glistening	Irregular
Filling stage	Fill D	+Ve, Thick Rod	Pale	Lobate	Convex	Wrinkle	Irregular
Pasteurising stage	Past A	+Ve, Thick rod	Whitish	Undulate	Raised	Smooth	Irregular
	Past C	+Ve, Thick rod	Creamish white	Undulate	Raised	Wrinkle	Irregular
	Past E	+Ve, Thick rod	Whitish	Serrate	Convex	Smooth	Irregular
	Past F	+Ve, Thick rod	Creamish white	Undulate	Convex	Dry	Irregular
Conditioning stage	Con A	-Ve, Very short rod	Creamish white	Undulate	Convex	Glistening	Irregular
	Con D	-Ve, short rod	Creamish	Undulate	Raised	Wrinkle	Irregular
	Con H	+Ve, rod	Creamish	Lobate	Raised	Wrinkle	Irregular
Spawning stage	Spawn B	+Ve, rod	Pale yellow	Entire	Convex	Glistening	Circular
	Spawn D	+Ve, thin rod	Creamish	Lobate	Flate	smooth	Irregular
	Spawn H	+Ve, rod	Creamish	Entire	Convex	Glistening	Circular
Drenching stage	Drench A	+Ve, rod	Creamish white	Entire	Convex	Smooth	Circular
	Drench B	+Ve, rod	Creamish white	Entire	Convex	Smooth	Circular
	Drench C	-Ve, rod	Creamish	Lobate	Flate	Dry	Irregular
	Drench D	+Ve, rod	Creamish	Entire	Convex	Glistening	Circular
	Drench F	+Ve, rod	Creamish	Lobate	Raised	Smooth	Irregular
	Drench G	+Ve, rod	Creamish	Lobate	Flate	Smooth	Irregular

Predominantly repeated bacteria species were isolated different stage of mushroom compost used in this study. They were characterized as PW1 30A (*Alcaligens sp.*), PW1 30 B (*Bacillus subtilis*), Fill 30 D (*Bacillus subtilis*), Past 30A (*Bacillus subtilis*), Past 30C *(Bacillus cereus)*, Past 30E (*Bacillus cereus*), Past 30F(*Bacillus cereus*), Con 30A , Con 30D (*Ochrobactrum sp*), Con 30H , Spawn 30B (*Arthrobacter arilaiti*), Spawn 30D (*Bacillus subtilis*), Spawn 30H (*Arthrobacter arilaiti*), Drench 30A (*Bacillus pumilus*) , Drench 30B (*Bacillus pumilus*), Drench 30C (*Stenotrophomonas maltophilia*) , Drench 30D (*Bacillus pumilus*), Drench 30F (*Bacillus pumilus*) and Drench 30G (*Ochrobactrum sp*) (Table. 2). These identifications were based on 16 S r DNA sequencing.

Table 2. List of bacterial isolates sequenced and their identity along with their NCBI accession numbers used in present study.

Isolates	Origin	Similarity (%)	Identitification	Accession No
BNJ_PKC_1 (PW1 30A)	Compost, Prewetting	99%	*Alcaligens sp.*	AY871052
BNJ_PKC_2 (PW1 30B)	,,	97%	*Bacillus subtilis*	AY940671
BNJ_PKC_3 (Fill 30D)	Compost, Filling	99%	*Bacillus subtilis*	AY871053
BNJ_PKC_4 (Past 30A)	Compost, Pasteurising	97%	*Bacillus subtilis*	AY871054
BNJ_PKC_5 (Past 30C)	,,	97%	*Bacillus cereus*	AY871055
BNJ_PKC_6 (Past 30E)	,,	985	*Bacillus cereus*	AY871056
BNJ_PKC_7 (Past 30F)	,,	98%	*Bacillus cereus*	AY871057
BNJ_PKC_8 (Con 30A)	Compost, Conditioning		*Could not identify*	-
BNJ_PKC_9 (Con 30D)	,,	96%	*Ochrobactrum sp*	AY940671
BNJ_PKC_10 (Con 30H)	,,		*Could not identify*	-
BNJ_PKC_11 (Spawn 30B)	Compost, Spawning	98%	*Arthrobacter arilaiti*	AY871058
BNJ_PKC_12 (Spawn 30D)	,,	97%	*Bacillus subtilis*	AY871059
BNJ_PKC_13 (Spawn 30H)	,,	98%	*Arthrobacter arilaiti*	AY944465
BNJ_PKC_14 (Drench 30A)	Compost, Drenching	97%	*Bacillus pumilus*	AY940672
BNJ_PKC_15 (Drench 30B)	,,	99%	*Bacillus pumilus*	AY944466
BNJ_PKC_16 (Drench 30C)	,,	98%	*Stenotrophomonas maltophilia*	AY871060
BNJ_PKC_17 (Drench 30D)	,,	98%	*Bacillus pumilus*	AY871061
BNJ_PKC_18 (Drench 30F)	,,	98%	*Bacillus pumilus*	AY864923
BNJ_PKC_19 (Drench 30G)	,,	96%	*Ochrobactrum sp.*	AY940673

Maximum variation in cell shape and arrangement was observed in Past bacterial flora. Amongst mesophilic bacterial morphotypes, bacteria derived from Con, exhibited maximum structural diversity (H`=5.02) followed by Drench (H`=4.84), Fill (H`=3.729), Past (H`=3.14), PWI (H`=2.90) and Spawn (H`=1.83). Morphotypes from PWI showed high Simpson's index (λ=4.87) followed by Spawn (λ=4.38), Con (λ=3.85), Fill (λ= 3.55), Drench (λ=3.35) and Past (λ=2.86). Maximum species richness represented by Margalef (R_1=4.57) and Menhinick's index (R_2=2.66) was noted for Con stage bacteria.

Maximum evenness value was reported for PWI stage (E= 5.70) and minimum for spawn bacteria (Fig 2).

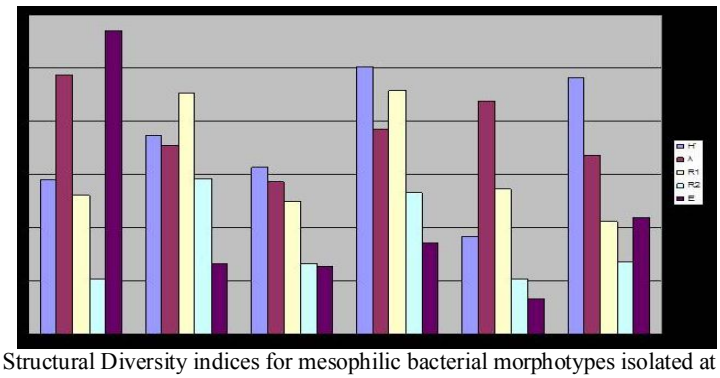

Fig 2. Total Structural Diversity indices for mesophilic bacterial morphotypes isolated at 28±2ºC from different stage of mushroom compost

Mesophilic bacterial counts (log $_{10}$ cfu) in mushroom compost were found to vary from 9.34 (Prewetting stage) to 6.55 (drench) and thermophilic bacteria from 6.68 to 5.50, respectively; pseudomonads count varies from 8.40 to 6.53. Maximum mesophilic bacterial diversity was observed at first Con compost (Shannon's index H' = 5.022). Isolates identical to *Bacillus badius* dominated the entire composting process of mushroom cultivation. The other organisms with high similarities belonged to the γ-*Proteobacteria* and were related to *Azotobacter salinestris* and *Pseudomonas stutzeri*. SSCP (Single stranded conformation polymorphism) profiles and sequencing of subcloned products has revealed the presence of lactobacilli at the early stage of composting whereas during the heating phase, 5 of 12 molecular isolates were closely related to *Bacillus* and one to *Clostridium* (Peters *et al*., 2000). The occurrence of bacteria, and especially of species of *Bacillus*, was observed in the bagasse and wheat straw mix during all of the composting stage, with an average population density of 3 x 108 CFU/g. Indeed, throughout composting, the bacteria formed the most predominant and numerous groups of micro-organisms present, followed by the Actinomycetes and then the filamentous fungi (Fig.1). Similar results have been reported in the composting rice straw (Cahyani, *et al.,* 2003) in which prokaryotes together with a few fungi predominated during the earliest days of composting, whilst the Actinomycetes prevailed during the final period. In the present study, 19 bacteria were identified in the substrate during composting and after pasteurisation (Table 1). Species of *Bacillus* and *Stenotrophomonas*, as well as members of the Actinomycetes, are believed to play important roles in the assimilation and transformation of ammonia (Ryckeboer *et al.,* 2003).

The bacterial population altered notably during composting since, from the first day of mixing and onwards, other species of *Bacillus* were detected including *B. subtilis, B. pumilus, and B. cerus,* (Gbolagade 2006) also identified various species of *Bacillus* in compost prepared for the culture of *Pleurotus tuber-regium* and *Lentinus squarrosulus*. This author has suggested that *Bacillus* bacteria may stimulate the growth of cellulolytic thermophilic Actinomycetes, since the latter appeared in the substrate after the 2nd day of composting when the diversity of *Bacillus* was greatest. Species of *Paenibacillus* and *Bacillus* were also reported to be the most predominant Gram-positive bacteria in wheat straw-based compost employed in the culture of *Pleurotus ostreatus* (Velázquez-Cedeño, *et al.,* 2008). Pasteurizations does not diminish the viability of these micro-organisms since the bacterial population was larger following this procedure than at any other point during the composting, and this is important for the maintenance of the natural microbiota of the compost.

In conclusion, compost based on sugar cane bagasse and wheat straw supported a diverse bacterial population, consisting mainly of Bacillus spp, followed by *Arthrobacter arilaiti*, *Ochrobactrum sp*, *Stenotrophomonas maltophilia* which are responsible for the degradation of fibres and for the physical and chemical characteristics of the final compost. These species were the most persistent micro-organisms during stage I of composting, the most thermostable during pasteurization and also after mushroom production. Low cost substrates, such as solid agro- industrial residues, are an economical alternative mainly because they are constantly available irrespective of the season, and are readily converted into an adequate mushroom substrate without requiring refined technology.

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