Microbial dynamics of different casing materials in the production of white button mushrooms (*Agaricus bisporus*)

N.A. SIYOUM
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By

Nazareth Afewerki Siyoum

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DECLARATION

I declare that the thesis which I hereby submit is my own original work and has not previously been submitted by me for a degree at this or any other tertiary institution.

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Nazareth A. Siyoum

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Microbial dynamics of different casing materials in the production of white button mushrooms (*Agaricus bisporus* (Lange) Imbach)

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Nazareth Afewerki Siyoum

Supervisor: Prof Lise Korsten
Co-supervisor: Dr Karen Surridge-Talbot
Department: Microbiology and Plant Pathology
Degree: Philosophiae Doctor

Summary

Peat is a suitable casing layer most commonly used as a substrate in commercial production of white button mushrooms. However, further exploitation of this substrate in South Africa is restricted due the environmental status of this scarce and protected natural resource. Therefore, finding an easily available and economically viable casing material that can either partially or completely replace peat is important for sustainable mushroom production. In the process of finding a replacement material, information regarding the microbiological properties of peat and alternative casing materials is required to profile a natural stable ecosystem since the presence of bacteria in the casing layer is important for pinning and fruit body formation. Pseudomonad bacteria in the casing layer are known to play a role in the fructification of mushroom mycelium. The aim of this study was to investigate the microbial dynamics and profiles of peat, industrial by-products and peat-based mixtures with by-products using cultural and a culture-independent molecular technique such as denaturing gradient gel electrophoresis (DGGE). Other aims of this study were to investigate the overall microbial profiles of compost, casing and mushrooms in a commercial mushroom production cycle and to evaluate effects of isolated micro-organisms on mycelial growth, mushroom yield and *in vitro* disease control. Total bacterial population densities in peat-based mixtures at pinning were comparable to that of peat alone and pseudomonads constituted part of bacterial populations in these mixtures. Other bacteria in peat and the mixtures included *Ensifer* spp., *Sinorhizobium* spp., *Bacillus* spp., *Sporosarcina* spp., *Microbacterium* spp. *Arthrobacter* spp. and *Sphingobacterium* spp. Bacterial profiles of peat at pinning also showed dominant bacteria other than pseudomonads including *Bacteroidetes*, *alpha-*

Proteobacterium, *beta-Proteobacterium*, *gamma-Proteobacterium*, *delta-Proteobacterium* and uncultured species. In viewing the overall microbial profile of compost, casing and harvested mushrooms, bacterial profiles of mushrooms were more similar to those of casing than to compost and were dominated by pseudomonads. Fungal profiles of compost, casing at pinning and freshly harvested and stored mushrooms were completely dominated by *Agaricus bisporus*. Bacterial and yeast populations increased significantly during mushroom storage at 4°C while fungal populations remained low. *Pseudomonas* spp. and an *Arthrobactor* sp. isolated from casing resulted in 100% *in vitro* growth stimulation of *A. bisporus*. Several bacteria and yeasts isolated from compost, casing and mushrooms inhibited *in vitro* growth of the fungal mushroom pathogens *Verticillium fungicola* var *aleophilum*, *V. psalliotae*, *Mycogone perniciosa*, *Cladobotryum dendroides*, *C. mycophilum*, *Trichoderma aggressivum* f. *europaeum*, *T. aggressivum* f. *aggressivum* and *T. harizianum*. In conclusion, peat-based casing mixtures with industrial by-products harbour bacteria important in *A. bisporus* fructification. Bacteria in these casings are also important in other aspects of mushroom production such as growth stimulation. Based on results in this study, mushroom bacterial profiles are similar to those of the casing, suggesting postharvest quality may be controlled by manipulating casing bacteria.

CONTENTS

CHAPTER ONE

GENERAL INTRODUCTION

The white button mushroom [*Agaricus bisporus* (Lang) Imbach] is the foremost cultivated species of mushroom (Foulongne-Oriol *et al*., 2009; Chikthimmah, 2006; Moore *et al*., 2001), which showed tremendous growth in terms of production over the later decades of the 20th century (Shen *et al*., 2004; van Griensven and van Roestel, 2004). Among the major producing countries are China, the United States of America (USA), Netherlands, Poland, Spain, France, Italy, Ireland, Canada, the United Kingdom (UK), Japan, Germany, Indonesia, India, Belgium, Australia, Korea, Iran, Hungary (van Griensven and van Roestel, 2004) and South Africa (Richardson, 2010; personal communication with Van Greuning). Total production in the above mentioned countries ranges from 16 000 (Hungary) to 637 000 tonnes (China) annually. South Africa produces 18 000 tonnes of mushrooms per annum (Richardson, 2010; personal communication with Van Greuning). In South Africa, white button mushrooms comprise 98% of total mushroom production (Richardson, 2010; personal communication with Van Greuning).

Commercial production of white button mushrooms depends on two main substrates for growth. These substrates are compost and casing medium. Compost is prepared from two major components: plant material and animal manure. Generally, plant materials include wheat straw, hay, corncobs, cotton seed and switch grass, whereas animal manure is obtained from horse and poultry wastes (Ingratta and Blom, 1979; Royse and Chalupa, 2009; Sanchez and Royse, 2009). Plant material serves as source of carbohydrate, mainly cellulose and lignin, in compost (Iiyama *et. al*., 1994). Animal manure is a source of nitrogen during the composting process, to which other supplements may also be added (Ingratta and Blom, 1979). In South Africa, compost is produced from wheat straw and chicken manure. The process of compost production is done artificially through the manipulation of environmental conditions.

Composting has three phases. In Phase I animal manure and plant materials are moistened and mixed to initiate the process (Ingratta and Blom, 1979; Noble and Gaze, 1998). During Phase II, compost is heated in order to eliminate pathogens and to promote activity of beneficial bacteria (Ingratta and Blom, 1979; Iiyama *et al*., 1994; Colak, 2004). Mushroom mycelium, grown on sterile grains, and called "spawn", is added to Phase II to make Phase III compost (Ingratta and Blom, 1979). When Phase III compost is fully colonised by the mycelium of *A. bisporus*, it becomes ready for the next step in mushroom production, which is casing.

Casing is the application of a top layer of mineral or organic material to Phase III compost in order to attain the quantity of mushrooms needed for commercial production (Dawson, 1977). This layer plays two major roles in the production process. Firstly, the casing layer is a water reservoir for developing fruit body of *A. bisporus*. Secondly, this layer is the source of beneficial bacteria for fruit body initiation in the mycelium.

Peat is the most common casing material used throughout the world (Pardo *et al*., 2003). Peat is a soil made during partial decomposition of dead plant materials in the absence of oxygen and is slowly deposited, mostly in waterlogged areas (Gorham, 1957). Soil in general is a dynamic biological system that harbours diverse micro-organisms (Nannipieri *et al*., 2003; Garbeva *et al*., 2004; Janssen, 2006). These diverse micro-organisms are characterised by complex interactions among each other and with their environment (Zak *et al*., 1994; Lipson *et al*., 1999; Kent and Triplett, 2002; Carney and Matson, 2006; Houlden *et al*., 2008). Micro-organisms are responsible for most processes in soils, resulting in the different functions of soil ecosystems (Nannipieri *et al*., 2003; Garbeva *et al*., 2004). Peat soil, widely accepted and used as a casing layer in the production of white button mushrooms, is known to harbour micro-organisms, specifically bacteria, associated with the process of mushroom fruit body formation (Hayes *et al*., 1969).

Concerns are arising regarding massive commercial peat mining. Continuous and immense peat mining could result in resource depletion and ecological imbalances, especially for countries with minimum natural reserves. In South Africa, the wetlands

where peat is mined are sources for pure water (Grundling *et al*., 1999; Grobler *et al*., 2004) and ecologically important biodiversity (Cowan, 1999; Kotze and Connor, 2000). In addition, the natural formation of peat is very slow, which can range between 0.02 to 10cm per year depending on the environment (Smuts, 1992). Owing to resource depletion and consequent environmental issues, peat mining in South Africa is now restricted by law – the National Environmental Management: Protected Areas Act, 2003 No. 57 (Government Gazette, 2009). Therefore, the search for easily available alternative materials that can replace peat in the commercial production of white button mushrooms has become crucial to the South African mushroom industry.

In order to find a replacement for peat, its physical, chemical and microbial properties need to be well understood. Many investigations have been done on the physical and chemical properties of peat and other materials (Nair *et al*., 1974; Galvin, 1976; Dasberg and Neuman, 1977; Dawson, 1977; Lohr *et al*., 1984; Levanon *et al*., 1986; Kalberer, 1987; Vijay *et al*., 1987; Awad and Nair, 1989; Eicker and Van Greuning, 1989; Valt *et al*., 1991; Border, 1993; Meerow, 1994; Ralph and Kurtzman, 1995; Wuest and Beyer, 1996; Prasad, 1997; Noble *et al*., 1999; Sharma *et al*., 1999; Singh *et al*., 2000; Huerta *et al*., 2001; Abad *et al*., 2002; Schwärzel *et al*., 2002; Pardo *et al*., 2003). However, relatively few studies on microbial properties of casing materials have been done. Total bacterial and fungal counts, as well as identification of fungal species from different casing materials, including South African topogenous peat, were done by Eicker and Van Greuning (1989). However, an elaborative study on bacterial profiles and dynamics is not available.

The major objectives of the current study were to investigate:

- The bacterial populations in casing materials at different stages in mushroom production;
- The bacterial profiles of peat and other potential replacement casing materials;
- The overall microbial dynamics of compost, casing and harvested mushrooms;
- The relationships between identified casing bacteria and mycelial growth and yield; and,
- The effect of isolated bacteria and yeasts on fungal pathogens of *A. bisporus*.

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CHAPTER TWO

LITRATURE REVIEW

1. Introduction

Up to 10 000 known mushroom species exist, of which about half are edible (Kües and Liu, 2000). Many of these species are wild and collected from forests around the world. About 25 species of the edible mushrooms are widely accepted and only a few of these are commercially produced (Daba *et al*., 2009).

Mushroom cultivation has a long history dating back to about 600 A.D. in China with *Auricularia auricular* (Kües Liu, 2000). Today commercial mushroom cultivation has expanded worldwide. Commercially cultivated mushrooms include various *Agaricus* spp., *Lentinus edodes* (Berkeley) Pegler (shiitake), *Pleurotus ostreatus* (Jacquin ex Fries) Kummer (oyster mushroom), *Auricularia* spp. (Bull.: Fr.) Wettst (woody ear or Jew's ear), *Volvariella volvacea* (Bulliard: Fries) Singer (straw mushroom), *Flammulina velutipes* (Curtis ex Fries) Singer (winter mushroom), *Hypholoma capnoides* (Fries) Quelet (brown-gilled woodlover), *Stropharia rugoso-annulata* (King Stropharia), and *Coprinus comatus* (Muller: Fries) S. F. Gray (shaggy mane), *Pholiota nameko* T. Ito S. Ito et Imai in Imai (Nameko), *Tremella* spp. (silver ear or white jelly fungus), *Grifola* spp., *Hypsizygus* spp. and *Tricholoma* spp. (Chang and Miles, 1984; Kües Liu, 2000; Shen *et al*., 2004).

China is the leading mushroom producing country accounting for more than 70% of total world production (Hu and Zhang, 2004). Oyster mushroom (*Pleurotus ostreatus*) is predominantly produced followed by shiitake, woody ear, white button mushroom and winter mushroom. Outside China, white button mushroom is the most important and popular commercially grown species (Sanchez and Royse, 2009).

2. Description of White Button Mushrooms [*Agaricus bisporus* (Lange) Imbach]

White button mushrooms, *A*garicus *bisporus*, belong to the kingdom *Myceteae*, division *Amastigomycota*, subdivision *Basidiomycotina*, class *Basidiomycete*, subclass

Holobasidiomycetidae II *Hymenomycetidae* II, order *Agaricales*, family *Agaricaceae* (Alexopoulos and Mims, 1979). Four main types of white button mushrooms exist and their classification is based on cap morphology and hybridity (Moor *et al*., 2001). The types are smooth white, rough off-white, hybrid and derivatives, and browns with pigment.

3. White Button Mushroom Production

According to the most recent global statistics, the world supply of white button mushrooms was 1, 956, 000 tonnes in 1997, which represents 31.8% of total mushroom production (Daba *et al*., 2009). Between 2006-2007, white button production increased to 40% of the total world mushroom supply (Sanchez and Royse, 2009). Major white button mushroom producing countries besides China include the United States of America, Netherlands, France, Italy, Spain, Poland, England, Ireland, Germany, Belgium, Canada, Australia (Chang *et al*., 1984; Griensven and Roestel, 2004), and South Africa (personal communication with van Greuning, 2009). In South Africa, white button mushrooms account for 98% of total mushroom production in the country.

4. Cultivation of White Button Mushrooms

White button mushroom cultivation involves two main substrates. These substrates are the compost and the casing medium. The process of preparing compost, called composting, is first described briefly and later more attention will be given to the casing medium and its microbiological properties.

4.1 Composting

White button mushrooms are commercially grown on compost, which is prepared from a mixture of materials undergoing microbiological processes. Common materials used are wheat straw, horse or chicken manure, and gypsum (Straatsma *et al*., 1994; Colak, 2004). Other materials include hay, corncobs, cotton seed and switch grass (Ingratta and Blom, 1979; Royse and Chalupa, 2009; Sanchez and Royse, 2009). Compost is prepared in three different phases. To begin the composting process, the mixture is pre-wetted for 3-10 days and periodically mixed or turned in an open air compartment (Iiyama *et al*.,

1994; Noble and Gaze, 1996). In phase I, this mixture is stacked and left in the open air to decompose for 7-14 days (Iiyama *et al*., 1994). Microbial activity is high in phase I and subsequently there is a high level of oxygen utilisation (Miller *et al*., 1989).

In phase II, a controlled aerobic composting for 6-9 days is conducted in a closed room (Iiyama *et al*., 1994; Colak, 2004). During this phase, the compost is first pasteurised at 60^{o}C to eliminate pathogenic organisms and then conditioned at 45-50^{o}C to create a selective environment for microbes beneficial to *A. bisporus* (Straatsma *et al*. 1994, Colak, 2004). At this point, micro-organisms degrade about 40% of the organic material (Straatsma *et al*., 1994). At the conclusion of Phase II, the compost has a nutritional content selective for *A. bisporus* (Straatsma *et al*., 1994).

Phase II compost is inoculated or seeded with spawn randomly at 0.5% (v/w) and incubated for 12 days to allow it to be fully colonised by *A. bisporus* mycelium (Straatsma *et al*., 1991). The spawn is an inoculum of *A. bisporus* produced by growing the mycelium on grains such as rye or millet under sterile conditions (Antonio, 1971). Compost colonised by *A. bisporus* is commonly called spawn-run compost or Phase III compost. After complete colonisation by *A. bisporus* mycelium, Phase III compost is packed into beds and covered (cased) with a 3-5 cm casing layer. Following sections will deal with this layer.

4.2 The Casing

During the commercial production of white button mushrooms, colonised compost is covered by a layer of nutrient poor material, the casing layer. The major functions of this casing layer are retention of moisture and initiation of mushroom fruit bodies. It is well established that beneficial bacteria found within the casing initiate the transformation of vegetatively growing mushroom mycelium to the reproductive phase, which results the formation of fruit bodies (Hayes *et al*., 1969; Hayes, 1979). Therefore, microbiological properties of the material used as a casing layer are of significance in the production of mushrooms.

5. Microbiological Properties of Casing Soil

5.1 Functions of Soil Micro-organisms

Soil micro-organisms are very diverse in nature and different biochemical processes occurring in soil are functions of these microbes (Garbeva *et al*., 2004). Among such processes are organic matter decomposition, nutrient cycling, removal of pollutants, plant growth stimulation and soilborne disease suppression. In relation to plants growing in soil, micro-organisms can be grouped as commensalistic, mutualistic and pathogenic (Beattle, 2007). Organisms in the first group use nutrients on plant surfaces, without affecting the plant health. Mutualistic organisms benefit the plant directly or indirectly while also making use of it. Pathogenic organisms are those that affect plant health negatively. Soil used to case mushroom beds in commercial production of white button mushrooms is known to naturally harbour beneficial micro-organisms and also to be contaminated with harmful micro-organisms.

5.2 Casing Bacteria and Mushroom Fruit Body Formation

The transformation process of mycelial *A. bisporus* to the reproductive phase is induced by bacteria within the casing soil. This concept was proposed because sterile casing could not induce fruiting (Eger 1963 cited in Miller *et al*., 1995). Later, bacteria related to *Pseudomonas putida* were isolated from casing material and found to induce fruiting when inoculated into sterile casing material (Hayes *et al*., 1969). Studies show that highest bacterial activity occurred consistently at the onset of fruit body formation (Hayes, 1979). Bacterial population peaks were evident at two growth stages of *A. bisporus*, onset of fruit body formation and third break (Hayes, 1979). Other researchers added a mixture of six bacteria and *P. putida* alone to sterile casing and found that these bacteria stimulate fruit body formation in equal amounts to that found in non-sterile casing (Fermor *et al*., 2000).

5.2.1 Mechanism of Fruit Body Formation

The mushroom fungus at its vegetative stage grows and colonises the compost as mycelium. Once this mycelium reaches the top of the casing, a sudden change in growth state occurs. This change is reflected in mycelia that cease growing forward and start branching abundantly to form thick round tips referred to as pin heads (Sinden, 1982). The pin heads are the initial visible fruit bodies and the stage of their formation is called pinning.

There are different theories in terms of how bacteria may be involved in mushroom fruit body formation. It was stated that metabolic products from micro-organisms in the casing material create a change in lipid metabolism of the mycelium, which may result in initiation of fruit bodies (Ingratta and Blom, 1979). It was also suggested that specific bacterial populations naturally found in casing soils produce a substance inhibiting vegetative growth of the fungus (Sinden, 1982). Another explanation is that bacteria may play a role in removing substances preventing fruit body formation, which are produced by the mycelium itself (Flegg, 1989). The fact that sterile casings of activated charcoal and lignite allowed fruit body formation (Rainey and Cole 1986 cited in Miller *et al*., 1995; Fermor *et al*., 2000), possibly due to their absorbing ability, supports the latter theory; that bacteria are involved in removing inhibitory substances from the mycelium. Noble *et al.* (2009) provided evidence for this theory. They identified substances inhibiting fruit body formation, which were 1-octen-3-ol and 2-ethyl-1-hexanol compounds. These eight-carbon compounds were produced by the mycelium and its substrate, respectively. They also reported that these compounds were metabolised by pseudomonads and adsorbed by activated charcoal.

5.2.2 Species Associated with Formation of Fruit Bodies

Several bacterial species associated with casing material and fruiting of *A. bisporus*, have been reported. Pseudomonads were found to be the dominant bacteria within the casing layer (Cresswell and Hayes, 1979, cited in Miller *et al*., 1995). The proportion of fluorescent pseudomonads was reported at different levels ranging from 50% (Samson *et*

al., 1986 cited in Miller *et al*., 1995) to 2% (Doores *et al*., 1986 cited in Miller *et al*, 1995). Miller *et al*. (1995) found that fluorescent pseudomonads constitute a small proportion (14%) of total casing microflora during the vegetative phase but these increased to 41% during the reproductive phase. It was also demonstrated by other researchers that fluorescent pseudomonads such as *Pseudomonas putida* were associated with fruit body formation (Hayes *et al*., 1969; Noble *et al*., 2003). Noble *et al.* (2003) reported that a strain of these bacteria inoculated to sterile casing, resulted in an equivalent number of pins compared with those in non-sterile casing.

Bacteria from the genera *Xanthomonas* and *Alkaligenes* were also found to be dominant along with pseudomonads throughout the growing stages (Hayes, 1979). Others found that rod and cocci shaped bacteria were associated with *Agaricus* hyphae (Masaphy *et al*., 1987). Electron microscopic studies revealed rod-like bacteria attached mostly to young hyphae. It was also reported that bacteria belonging to *Corynbacteriaceae* and *Bacillus* spp. were dominant in dry coir (Fermor *et al.*, 2000).

6. The Common Casing Material – Peat

During the commercial production of white button mushrooms, peat is the most common casing material used worldwide. Peat has a unique characteristic of absorbing and releasing water, which makes it widely acceptable as a casing material (Hayes, 1991). Peat is also considered generally free of pests and pathogens (Ingratta and Blom, 1979) unlike previously used materials such as top soil (Flegg, 1991). Due to the absence of pests and pathogens, in most cases, pasteurisation of peat is not required, reducing energy requirements. From a microbiological point of view, peat also naturally harbours beneficial bacteria known to be useful for fructification of the mushroom mycelium (Ingratta and Blom, 1979).

6.1 Peat - A Natural Resource

Peat is naturally formed during partial decomposition of plant matter over long period of time. Peat accumulation is therefore a slow process that develops between 0.02-10cm per year depending on environmental conditions (Smuts, 1992). During the formation of

peat, plant tissues containing lignin are degraded partially by complex microbial activities (Kuder *et al*., 1998). Different types of peat exist based on vegetation material, climate and other environmental factors such as water table (Kuder *et al*., 1998; Bourdon *et al*., 2000).

Peat lands are generally acidic, having a pH varying betwee 3 to 6 (Eakin, 1969), and low in nutrients resulting in low levels of decay (Peat Alert). Peat contains more than 50% partially decayed organic matter (Eakin, 1969). Abad *et al.* (1989) reported that amounts of available nutrients such as mineral nitrogen and phosphorus were low in a highly decomposed sedge peat. Organic matter (OM) in peat is mostly composed of lignin, hemicellulose and cellulose owing to terrestrial plants forming peat (Bourdon *et al*., 2000).

6.2 Peat Conservation and the Challenge to Mushroom Growers

Mushroom producers in South Africa traditionally used topogenous peat (or reed-sedge peat) as a casing medium to produce white button mushrooms. However, the wetlands where peat is mined represent sources of pure water (Grundling and Dada, 1999) and are ecologically sensitive areas protected under the National Environmental Management Act. Especially in the Highveld area, peat lands serve a very important ecological role in filtering pollutants from industries and mines. Consequently, peat extraction (mining) in South Africa was banned by law, National Environmental Management: Protected Areas Act 2003 No. 57 (Government Gazette, 2009).

Currently, South African mushroom producers import peat at high cost from countries with adequate supplies. For cost effective and sustainable mushroom production, however, South African mushroom farmers are funding projects that research alternative materials to replace or reduce the use of peat. The search for alternative casing materials is therefore of importance in places where peat resources are scarce or limited. Materials that can potentially replace peat as a casing should provide comparable yields at a competitive cost, be free from pests and diseases and easy to handle (Border, 1993). Using industrial waste materials as casing alternatives has a dual function of conserving

peat and recycling wastes that would otherwise be disposed of in the environment. Several researchers have investigated the suitability of various alternative materials for the production of mushrooms.

7. Research on Alternative Materials to Peat

7.1 Coir

Coir dust is a by-product from the coconut fibre industry. Kemp (1990) reported that coir showed higher water absorption, retention and release properties than peat and could potentially be an effective alternative casing material. Pardo *et al.* (2003) reported that coir showed low bulk density, high porosity, water holding capacity and hydraulic conductivity values, better structure and more uniform particle size than sphagnum peat. In his study, Kemp (1990) observed that coir vs. peat provided a comparable number of sporophores.

7.2 Wood Bark

Paper pulp (a fibrous waste material from the paper mill industry) resulted in cleaner mushrooms than control peat, however yields from the third and later flushes were lower (Eicker and Greuning, 1989). The main problem Eicker and Greuning reported from this material was weed moulds such as *Chromelosporium fulvum* (Link) McGinty, Hennebert & Korf, which could not be eliminated through pasteurisation and leaching.

7.3 Sugar Cane Bagasse and Filter Cake

According to FAO (2007) statistics, the annual world production of sugar cane for 20 major producing countries was about 1.5 billion MT, of this more than 20 million MT was produced by South Africa. During sugar cane production, the industry produces waste materials mainly bagasse and filter cake.

Bagasse is a fibrous material, by-product residue, remaining after juice is extracted from sugar cane (Pandey *et al*., 2000). The chemical composition of bagasse is mainly cellulose, hemicellulose and lignin (Okano *et al*., 2006). Use of sugarcane bagasse as a

compost substrate in growing mushrooms is mentioned in several studies (Suguimoto *et al*., 2001; Singh *et al*., 2005; Liang *et al*. 2008). Liang *et al*. (2008) used composted bagasse as a casing material for *Agaricus brasiliensis* and found lower yield compared with peat.

Filter cake is the settled residue after filtration of the juice from sugar cane (Ossom *et al*., 2009). Various research has been done on sugar cane filter cake connected to its use as a potential soil ameliorant (Van Antwerpen *et al*., 2003; Meunchang *et al*., 2005; Rasul *et al*., 2006; Rasul *et al*., 2008; Ossom *et al*., 2009). However, there is no record of filter cake in relation to mushroom production.

7.4 Spent Mushroom Substrate

Spent mushroom substrate (SMS) was investigated as a mushroom casing material by several researchers. Eicker and Greuning (1989) reported that decreased mushroom yield was observed using relatively fresh spent compost as a casing compared with a peat control. Mushroom yield increased when artificially and naturally leached spent compost was used but it was still lower when compared with the control and had a high electrical conductivity. Sharma *et al.* (1999) used SMS after treating it with chelating agents and pasteurising to eliminate pathogens. Treatment with the chelating agent EDTA reduced the electrical conductivity of SMS and resulted in the highest mushroom yield next to the control.

7.5 Tea Waste

Tea manufacturing waste containing dried straw and fibre of tea leaves was examined as a casing for mushrooms (Gülser and Peksen, 2003). It was reported that when used with peat in a 1:1 ratio it resulted in as good a yield as peat alone. However, yield was unacceptably low when used alone.

7.6 Other Materials

Wuest and Beyer (1996) reported on the performance of five alternative casing materials. These materials were recycled news paper (RPC+), domestic waste, a coal combustion by-product (called fly ash), manufactured Capogro and Peatwool (made from molten rock) products. RPC+ performed better than peat both in terms of mushroom quality and production cost. Capogro and fly ash blended with peat moss also produced satisfactory results.

Vijay *et al.* (1987) researched casing materials prepared from different mixtures of farmyard manure, garden soil, dry moss (*Funaria* spp.), spent compost and forest litter. The mixture of soil and moss produced the best yield as a result of its high water holding capacity, porosity, pH and low electrical conductivity.

8. Pathogens of White Button Mushrooms

White button mushrooms are infected by various bacterial, fungal and viral pathogens. A significant loss in yield and quality occurs following severe infections (Soković and van Griensven, 2006). Disease incidences are generally attributed to poor hygiene and management. Different pathogens causing common diseases are briefly described below.

8.1 Bacterial Diseases

Bacterial pathogens of white button mushrooms are mostly from the genus *Pseudomonas*. These diseases mainly occur under wet conditions.

8.1.1 Brown Blotch

Brown blotch disease of *A. bisporus* is caused by *Pseudomonas tolaasi* (Rainey *et al.*, 1992 cited in Jolivet *et al.*, 1998). A characteristic symptom of this disease is the appearance of superficial dark brown spots on the surface of caps and stalks (Gill, 1995; Oliver *et al.*, 1978 cited in Soler-Rivas *et al.*, 2000). As member of fluorescent pseudomonads, *P. tolaasi* is a saprophytic micro-organism in mushroom beds (Lilliott *et al.*, 1966 cited in Gill, 1995). Under favourable conditions, it produces a toxic substance

(tolaasin) as a metabolic by-product (Lilliott *et al*., 1966 cited in Gill, 1995). This substance activates enzymatic oxidation and polymerisation reactions of phenolic substances to melanins (Jolivet *et al*., 1998; Soler-Rivas *et al*., 2000). Mushroom surfaces are discoloured as a result of the formation of this dark pigment.

Brown blotch bacteria are commonly found in soils and this disease is associated with high relative humidity (>85%) and higher temperatures above 20 ^{0}C (Pest management, 2009). To control disease development, moist conditions especially on the mushroom surface should be prevented through proper ventilation and air circulation (Pest management, 2009). Once established, this disease can spread through watering and materials in cross-contact with healthy and diseased mushrooms, therefore suitable hygiene practices are important.

8.1.2 Ginger Blotch

Ginger blotch disease is caused by *Pseudomonas gingeri* and the initial symptom caused is a pale yellowish-red brown discoloration which eventually turns to reddish ginger (Wong *et al*., 1982). This pathogen causes superficial lesions that do not distort the cap and is of less economic importance than *P. tolaasi* (Gill, 1995). Godfrey *et al.* (2001) did phylogenetic analysis on the causative agent of ginger blotch and suggested that other pseudomonads may also cause the disease. Information as to the epidemiology and disease control is limited; however general hygiene and sanitation practices are important for prevention of bacterial diseases.

8.1.3 Drippy Gill

Drippy gill is caused by *Pseudomonas agarici* and the symptoms of this disease include bacterial ooze from the fruit body (Young, 1970 and Gill, 1994 cited in Gill, 1995), pale brown discoloration (Soler-Rivas *et al*., 2000), dark-brown lesions at the sides of the gills (Fett *et al*., 1995) and distorted mushrooms and delayed development (Cantore and Iacobellis, 2004). In later stages of infection, necrosis appears as a result of brown or black mucoid substance or secondary infection, such as soft rot (Gill, 1995). Good cultural practices, hygiene and sanitation may help to manage the disease.

8.1.4 Mummy

Mummy disease is caused by several bacterial species belonging to the sub-group *Pseudomonas fluorescens* (Gill, 1995; Pest management, 2009). Symptoms are delayed pinning, elongated, spindly and shriveled stems with tilted caps and retarded pileal development (Gill, 1995). The base of the stem also becomes thick and is covered by fluffy mycelium (Pest management, 2009). The disease is transmitted to healthy mushrooms via infected mycelium (Gill, 1995). In order to prevent this disease, proper compost moisture management and good sanitation and hygiene in compost preparation are important (Pest management, 2009). Spread of the disease from infected mushrooms to healthy ones can be stopped by digging a trench across the area of growing medium supporting diseased mushrooms.

8.2 Fungal Diseases

Casing soil can be contaminated with fungal pathogens to white button mushrooms that cause serious reduction in yield and quality. The following are known to have adverse effect on mushroom production.

8.2.1 Wet Bubble

Wet bubble disease is caused by *Mycogone perniciosa* (Magnus) Delacr. This disease is one of the most important diseases of *A. bisporus* that can cause a significant loss in quality and yield of mushrooms (Glamoclija *et al*., 2008). Characteristic symptoms are malformed mushrooms, swollen stems, masses of undifferentiated and distorted mushroom tissue, and amber to brown drops on a white fluffy surface under humid conditions (Pest management, 2009). The fungicide benomyl was found to control this disease effectively (Fletcher, 1975). However, benomyl is not registered in South Africa and the best control measure remains good cultural sanitation and hygiene practices (Pest management, 2009).

8.2.2 Dry Bubble

Dry bubble is one of the most important diseases of *A. bisporus* in the commercial production of this mushroom and is caused by *Verticillium fungicola* (Calonje *et al.*, 2000). The symptoms are dry bubble, necrotic lesions, brown spots or streaks on the stem and caps and bent or split stipes (Calonje *et al.*, 2000; Dragt *et al.*, 1996 cited in Soler-Rivas *et al.*, 2000; Largeteau *et al.*, 2007). The fungus infects fruit bodies but not the vegetative mycelium of *A. bisporus* (Fletcher *et al.*, 1986 cited in Calonje *et al.*, 2000). Bravo is used to control the disease (Chlorothalonil) (Pest management, 2009) but it is not a registered fungicide in South Africa. Other control measures are cultural and sanitary practices (Calonje *et al.*, 2000; Bernardo *et al.*, 2004).

8.2.3 Green Mould

Several moulds affect either mushroom quality or yield. Of these moulds, green mould is caused by *Trichoderma* species (Savoie and Mata, 2003). *T. viride* Persoon: Fries and *T. koningii* Oudemans are known to affect commercial mushroom production (Chen *et al.*, 1999). Biotypes of *T. harzianum* Rifai , Th2 and Th4, were also responsible for the onset of green mould epidemics in Europe and North America respectively (Chen *et al.*, 1999). Later, these biotypes have been renamed as *T. aggressivum* (Savoie and Mata, 2003). Green mould epidemic outbreaks in several parts of the world have been recorded (Hatvani *et al.*, 2007). Infestation by green mould is characterised by big areas of dense green spores colonising compost or casing surfaces (Anderson *et al.*, 2001). *Trichoderma harzianum* also causes brownish lesions on the stem (Soler-Rivas *et al.*, 2000). Applied control measures are sanitation and treatment of spawn grain with the fungicide benomyl (Anderson *et al.*, 2001) in places where the fungicide is allowed.

8.2.4 Cobweb

Cobweb disease can affect yield and quality of *A. bisporus* and is found in mushroom growing countries worldwide (Adie *et al.*, 2006). This disease is caused by several *Cladobotryum* spp. and the characteristic appearances are a mycelial mat surrounding mushrooms and brown spots on mushroom caps as a result of landed conidial spores (Adie *et al.*, 2006; Grogan, 2006). Since cobweb spores are highly airborne, avoiding

ventilation during watering could minimize disease spread in a cobweb contaminated room. Control measures include use of prochloraz-based (allowed in South Africa) and benzimidazole fungicides as well as hygiene practices (Grogan, 2006).

8.2.5 Cinnamon Brown Mould

This weed mould is caused by the *Chromelosporium fulvum* telemorph *Peziza ostraderma* syn. *Plicaria fulva* Schneider (Allan and Clift 2009). This mould is recognised by a dense mat of brown spores that resemble brown cinnamon powder (Ingratta and Blom, 1979). When dense spores occur, the mould can deplete nutrients in the compost, may discolour the mushrooms and slightly delay cropping (Allan and Clift, 2009). Disease dissemination occurs via airborne spores and growth of this weed mould is encouraged in overheated compost, very wet compost, over-pasteurised casing, or where the mushroom mycelium is killed by virus or green mould (Pest management, 2009). Proper cultural practices, sanitation and hygiene can prevent spread of this mould.

8.3 Viruses

Viruses infecting white button mushrooms can cause severe crop losses. The most common ones are mentioned below.

8.3.1 La France

Several virus-like particles were reported to be associated with diseased mushrooms and especially the La France disease (Harmsen *et al*., 1989). Harmsen *et al*. reported 10 major double stranded RNA (dsRNA) molecules that were always associated with mushrooms affected with this disease. The typical symptom of this disease is die-back of mycelium leading to crop loss. Infected mushrooms may appear normal or show symptoms such as delayed pinning, loose attachment to casing, small caps on normal size stems, and slightly bent elongated stems (Pest management, 2009). Transmission of virus particles to mushroom mycelium occurs through hyphal fusion (anastomosis) and germination of infected spores in the casing (Nair, 1979; Pest management, 2009).

Spread of the disease is therefore controlled by farm sanitation and strict hygiene (Pest management, 2009).

8.3.2 Virus X

Grogan *et al*. (2003) reported 26 dsRNA elements identified from *A*. *bisporus* fruit bodies exhibiting various symptoms. These symptoms, described as mushroom virus X (MVX), include crop delay, bare areas, premature veil opening, off- or brown-colored mushrooms, sporophore malformations and yield loss. Infected mushroom material or spores are sources of new infection (Pest management, 2009). Spread of the disease can be controlled by implementing strict hygiene practices (Pest management, 2009) and minimizing spread of basidiospores (Grogan *et al*., 2003).

9. Biological Control (Biocontrol) Potential of Bacteria in the Casing

Biocontrol agents are natural enemies used to control pests and disease-causing organisms through different mechanisms that include parasitism, pathogenesis and predation. Several fungi, yeasts and bacteria were reported as either commercial or potential biocontrol agents (Lewis and Papavizas, 1991; Lemanceau *et al*., 1992; O'Sullivan and O'Gara, 1992; Schelkle and Peterson, 1996; Bacon *et al*., 2001; Barka *et al*., 2002; Fredlund *et al*, 2002; Druvefors, 2004; Santos *et al*., 2004; Compant *et al*., 2005; El-Tarabily and Sivasithamparam, 2006; Vespermann *et al*, 2007; Sharma *et al*., 2009).

Some micro-organisms have been reported as biocontrol agents of white button mushroom diseases. Tautorus and Townsley (1983) reported that a *Bacillus* sp. isolated from phase I compost showed significant control on olive green mold pathogen (*Chaetomium olivaceum* Cooke and Ellis). Other studies have been done investigating biocontrol agents against the pathogen *Pseudomonas tolaasii* that causes brown blotch disease of white button mushrooms. Pseudomonads, potentially antagonistic to *P*. *tolaasii* were isolated from mushroom farms and from soil and plant materials (Fermor and Lynch, 1988). Fermor *et al*. (1991) reported that the disease was significantly controlled by fluorescent *Pseudomonas* spp. Another Gram-positive organism isolated

from *Pleurotus ostreatus* inactivated the toxin (tolaasin) produced by *P. tolaasii* (Tsukamoto *et al*., 1998). Tsukamoto *et al*. (2002) reported that bacteria isolated from wild mushrooms, namely *Mycetocola tolaasinivorans*, *M. lacteus*, *Sphingobacterium multivorum*, *Bacillus pumilus* and *Pedobacter* sp., controlled brown blotch by detoxifying tolaasin. All the above isolations were made from sources other than the casing. However, some researchers have isolated bacteria from casing mixtures (Singh *et al*., 2000) and healthy mushroom caps (Bora and Özaktan, 2000) and tested them against fungal pathogens such as *M. perniciosa* (Magnus) Delacr, *V. fungicola* (Preuss) Hassebr, *T. harzianum* Tul. and *C. dendroides* (Bulliard: Fries) Gams & Hoozemans and the bacterial pathogen *P. tolaasii*. Some of the isolates were reported to suppress growth of the pathogens. To the best of our knowledge, a study on biological control of mushroom diseases using yeasts was not reported.

10. Methods of Studying Soil Micro-organisms

Methods to study soil micro-organisms can be classified in two major categories. These categories are biochemical and molecular techniques (Kirk *et al*., 2004). The first group includes culturing methods, sole carbon source utilisation patterns and fatty acid analysis. The culturing method will be dealt below. The second group is based on the nucleic acids of micro-organisms. Several techniques of this group exist. One of these techniques, the denaturing gradient gel electrophoresis (DGGE), is described here after the culturing method.

10.1 Culturing Method

Methods of culturing micro-organisms are based on their artificial growth on various nutrient rich selective or non selective sources. Different techniques are employed to isolate, purify, preserve and study the organisms. Culturing methods include dilution plating for bacteria and fungi, soil plating for mainly fungi, immersion methods for *in situ* active hyphal growth and direct hyphal plating (Parkinson and Coleman, 1991). These cultural techniques are generally inexpensive and provide information on viable culturally or active populations (Kirk *et al*., 2004). Population richness of the cultivated

organisms is also determined by total microbial counts. Cultivation can also be targeted to specific groups of micro-organisms by using selective media.

10.2 Selective Media

Selective media are artificial media that allow only specific types of micro-organisms to grow. These media contain favourable substances and factors to select specific organisms and inhibiting substances and factors to prevent growth of undesirable organisms. In the preparation of these selective media different carbon and nitrogen sources are added. Examples of these energy sources to the micro-organisms are dextrose, peptone, yeast extract, casamino acids, glucose, sucrose and asparagine (Cuppels and Kelman, 1974; Nesmith and Jenkins, 1978; Gould *et al*., 1985; Hagedorn *et al*., 1987). Antimicrobial substances added include penicillin, tyrothricin, chloromycetin, 2, 3, 5 triphenyl tetrazolium chloride, polymyxin, vancomycin, bacitracin, benomyl, chloroneb, cycloheximide, pentachloronitrobenzene, pimaricin, dichloran, chloramphenicol, chlorothalonil, anisomycin, crystal violet, trimethoprim, nystatin, orthophenylphenol, streptomycin, fradiomycin and kanamycin (Cuppels and Kelman, 1974; Nesmith and Jenkins, 1978; Chen and Echandi, 1981; Edelstein, 1982; Gould *et al*., 1985; Hagedorn *et al*., 1987; Dietrich and Lamar, 1990; Otoguro *et al*., 2001). Growing conditions such as temperature, pH and incubation periods are also manipulated to increase the selectivity of media (Hagedorn *et al*., 1987; Dietrich and Lamar, 1990; Stevenson *et al*., 2004).

However, culturing methods are limited to laboratory growth conditions and by the selective nature of artificial nutrients used for culturable, fast growing and dominant organisms (Ranjard *et al*., 2000; Kirk *et al*., 2004; Bing-Ru *et al*., 2006). Therefore, for more reliable results, culturing methods should be used in combination with other techniques.

10.3 Molecular Techniques

Molecular techniques are based on nucleic acid sequence or genetic makeup of micro-organisms. Genetic material from these micro-organisms can be extracted directly from

the sample materials of different origins, i.e. aquatic, food, clinical and soil samples (Picard *et al*., 1992). Several techniques have been developed to detect and characterise micro-organisms using extracted genetic material (DNA). Probes that detect a unique sequence of a target organism can be designed and have been used successfully (Pickup, 1991). Restriction fragment length polymorphism (RFLP) involves enzymes that digest restricted fragments of DNA followed by the attachment of probes (Karp *et al*., 1996). However, one of the limitations for RFLP is that a large amount of quality DNA is required. To overcome this limitation in the case of low amounts of DNA, methods that amplify DNA were developed. Such methods are the randomly amplified polymorphic DNA (RAPD), arbitrary primed PCR (AP-PCR), DNA amplification fingerprinting (DAF) and amplified fragment length polymorphism (AFLP) (Karp *et al*., 1996). Results from RFLP, AFLP, RAPD, AP-PCR and DAF are used in detection and diversity studies (Karp *et al*., 1996; Powell *et al*., 1996; Suzuki *et al*., 1997).

One of the most commonly used nucleic acid techniques in analysis of microbial diversity is the determination of sequences of 16S ribosomal RNA (rRNA) genes encoded by rDNA (Hill *et al*., 2000). The 16S molecule is suitable for such studies because it is universal, conserved, easily amplified and rapidly sequenced (Hill *et al*., 2000). Methods that use16S-rRNA gene sequence analysis for microbial diversity studies include cloning and sequencing, denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), dot blots, single strand conformational polymorphism (SSCP), and terminal restriction fragment length polymorphism (T-RFLP) (Rondon *et al*., 1999). The DGGE method is described below.

10.4 Denaturing Gradient Gel Electrophoresis

Denaturing gradient gel electrophoresis (DGGE) was originally developed for detection of mutations in DNA sequences. Later, Muyzer *et al.* (1993) applied DGGE to determine genetic diversity of complex microbial populations. Amplicons (amplified DNA fragments) in a PCR product are separated using DGGE on the basis of their sequence composition. Therefore, DNA fragments with varying base-pair sequences, hence finger prints of individual organisms, are separated using DGGE.

The banding pattern from DGGE exhibits a profile of populations found in the analysed sample. This pattern provides qualitative and semi-quantitative information on the individual species in complex populations (Muyzer *et al*., 1993). Theoretically, the relative intensity of each band represents the relative abundance of a species. The species represented by a particular band can be characterised by excising the band, re-amplifying and sequencing the product.

DGGE can provide a detection capacity of 50% of the total individuals present (Muyzer and Smalla, 1998). The detection capacity can be increased close to 100% by attaching a GC-rich sequence (GC-clamp) to one side of the DNA fragment during PCR amplification (Muyzer and Smalla, 1998). The purpose of the GC-clamp is to increase the resolution of the bands and amplicon separation by increasing the melting temperature of the fragments. This technique is now widely used among microbiologists in the analysis of microbial communities and diversities.

Table 1. Common bacterial and fungal pathogens and diseases of white button mushrooms

Mushroom Disease	Pathogen	Symptom	Inoculum source	Yield and quality effect	Control measure	Reported in	Reference
Brown blotch	*Pseudomonas tolaasi*	Dark brown, wet and sunken lesions on caps and stalks, sporocarp distortion, cap splitting, necrotic lesions on stipes	Contaminated casing, dispersal through flies, mites, dust, people and tools	Up to 20% and quality deterioration	Good hygiene, ventilation and air circulation, chlorine in irrigation water	France, England, California, Belgium	Goor *et al*., 1986; Gill, 1995; Kobayashi and Crouch, 2009; Pest management, 2009
Ginger blotch	*Pseudomonas gingeri*	Small, pale yellowish brown flecks that develop to redish ginger, superficial lesions	-	Deteriorated quality	-	Australia, New Zealand, UK, Pennsylvania	Wong *et al*., 1982; Gill, 1995; Godfrey *et al*., 2001; Kobayashi and Crouch, 2009

Table 1 (*Continued*)

Mushroom Disease	Pathogen	Symptom	Inoculum source	Yield and quality effect	Control measure	Reported in	Reference
Drippy gill	*Pseudomonas agarici*	Bacterial ooze from the hymenial lamellae, necrosis of crusty light brown or black mucoid appearance, longitudinal splits on the stipes	-	Unmarketable mushrooms	-	England, Berkshire, Ireland, New Zealand, Australia, Eire, Italy, Netherlands	Gill, 1995, Fett *et al*., 1995; Cantore and Iacobellis, 2004
Soft rot	*Pseudomonas gladioli* pv *agaricicola*	Rapid browning and soft rot of sporocarp, deep oozing lesions from the cap and the stipe	-	Significant crop loss	-	England, NewZealand, France, Japan	Gill, 1995; Lincoln *et al*., 1999; Chowdhury and Heinemann, 2006
Mummy	*Pseudomonas fluorescens* or *P. aeruginosa*	Spindly and shrivelled stipes, retarded pileal development, tilted cap, dry and leathery mummy appearance, hard and abortive gills	Infected mycelium	unmarketable	Trenching infected area,	USA, Canada	Gill, 1995; Kobayashi and Crouch, 2009; Pest management, 2009

Table 1 (*Continued*)

Mushroom Disease	Pathogen	Symptom	Inoculum source	Yield and quality effect	Control measure	Reported in	Reference
Mummy	*Pseudomonas fluorescens* or *P. aeruginosa*	Spindly and shrivelled stipes, retarded pileal development, tilted cap, dry and leathery mummy appearance, hard and abortive gills	Infected mycelium	unmarketable	Trenching infected area,	USA, Canada	Gill, 1995; Kobayashi and Crouch, 2009; Pest management, 2009
Cobweb	*Cladobotryum* spp., syn. *C. mycophilum*, *Dactylium dendroides*	Circular mycelial colonies on the casing soil sporulating to masses of dry spores, brown spots	Airborne spores in the casing, spread on watering	Up to 40%	Hygiene, Steaming growing rooms, carbendazim and prochloraz	UK, Ireland	Grogan, 2006; Adie *et al*., 2006

Table 1 (*Continued*)

Mushroom Disease	Pathogen	Symptom	Inoculum source	Yield and quality effect	Control measure	Reported in	Reference
Dry bubble	*Verticillium fungicola*	Cinnamon brown spots on caps, dry bubble, split stipe	Infected casing, debris, and dissiminated by flies, tools, dust, watering and people	Can cause severe loss	Good sanitation and hygiene, the fungicide Bravo	USA, Canada, Pennsylvania, UK, France, Germany, Spain, Netherlands	Largeteau *et al*., 2006; Largeteau and Savoie, 2008; Pest management, 2009
Wet bubble	*Mycogone perniciosa*	Malformed caps and mushrooms, swollen stipes, undifferentiated tissue	Contaminated casing, floors, dust, tools, people	Significant crop loss	Good sanitation and hygiene, pasteurisation of casing soil, controlled movement of soil and dust	Canada, Serbia, Bosnia, Herzegovina, Netherlands	Glamočlija *et al*., 2008; Pest management, 2009

Table 1 (*Continued*)

Mushroom Disease	Pathogen	Symptom	Inoculum source	Yield and quality effect	Control measure	Reported in	Reference
Green mould	*Trichoderma* spp.	Dense, fluffy mycelium producing to green spores	Infected compost, casing, spread on dust, through water, flies and people	Yield reduction by the aggressive species	Sanitation and hygiene	Canada, USA, Hungary, Pennsylvania,	Chen *et al*., 1999; Anderson *et al*., 2001; Hatvani *et al*., 2007; Pest management, 2009
La France	Virus	Delayed pinning, small caps, loose attachment, elongated and bent stipes, die back	Infected mushroom spores, mycelium	Great crop loss from early infection at spawning	Strict hygiene	Canada, UK, Netherlands	Harmsen *et al*., 1989; Grogan *et al*., 2003; Pest management, 2009
Virus X	virus	Crop delay and bare areas, brown mushrooms	Infected mushroom spores, mycelium	Deteriorated quality, low production	Strict hygiene	Netherlands, Ireland, UK, Canada	Pest management, 2009

Table 2. Summary of some traditional and molecular methods to study soil micro-organisms

No.	Method of study	Micro-organism	Advantage	Disadvantage	Reference
1	Soil dilution plate	Bacteria Fungi	Viable counts	Not representative of total counts Selective for spore forming	Brierley *et al*., 1928 cited in Parkinson and Coleman, 1991
2	Soil plate method	Fungi	Fast	Selective for spore forming	Warcup, 1950 cited in Parkinson and Coleman, 1991
3	Immersion method (performed at site)	Fungi	For active growing hyphae	Microenvironmental alteration by apparatus, selection due to interspecific competition and contamination from soil invertebrates	Chesters, 1940 cited in Parkinson and Coleman, 1991
4	Direct hyphal isolation	Fungi	For specific studies	Requires great care and time consuming	Warcup, 1955 cited in Parkinson and Coleman, 1991
5	Sequential washing and plating soil particles	Fungi	Efficient with sandy soils	Difficult for clay and humus soils	Simmonds, 1930; Kübis, 1937; Harley and Waid, 1955 cited in Parkinson and Coleman, 1991

Table 2 (*Continued*)

No.	Method of study	Micro-organism	Advantage	Disadvantage	Reference
6	Direct isolation (using pre-stained soil films)	Bacteria	-	Time consuming, requires skill and not feasible for large scale studies	Casida 1962 cited in Parkinson and Coleman, 1991
7	Direct observation on buried slides, soil-agar films	Fungi	Measurement of hyphal lengths and types	Over growth and inaccurate quantification	Rossi *et al*., 1936; Jones and Mollison, 1948; Thomas *et al*., 1965 cited in Parkinson and Coleman, 1991
8	Direct observation under microscope	Bacteria	Greater numbers than plate counts	Impossible to determine live cells	Conn, 1918; Winogradsky, 1925; Jones and Mollison, 1948 cited in Parkinson and Coleman, 1991
9	Fluorescence Microscopy	Fungi and bacteria	Direct counting	Unable to differentiate live organisms	Trolldenier, 1973; Schmidt, 1973
	New fluorescence microscopy	Fungi and bacteria	Determination of living cells	Does not distinguish between cell types	Tsuji *et al*., 1995
10	BIOLOG system (based on utilization of carbon sources)	Bacteria	Microbial community diversity	Initial microbial density need to be standardized, unknown carbon utilization ability by different groups in a sample and artificial substrate	Winding, 1994, Lehman *et al*., 1995, Garland, 1996 cited in Hill *et al*., 2000

Table 2 (*Continued*)

No.	Method of study	Micro-organism	Advantage	Disadvantage	Reference
11	Fatty acid analysis	Fungi and bacteria	Active microbial biomass, does not require culturing	Cannot determine species, unusual variation in signature compounds results false community estimates	Hill *et al*., 2000; Kirk *et al*., 2004
12	Cloning PCR fragment and sequencing	Fungi and bacteria	Measures diversity	Time consuming, expensive and PCR bias	Ranjard *et al*., 2000
13	DGGE/TGGE	Fungi and bacteria	Reliable, reproducible, relatively inexpensive	PCR biases, laborious sample handling, variable DNA extraction efficiency	Macrae, 2000; Ranjard *et al*., 2000; Kirk *et al*., 2004; Bing-Ru *et al*., 2006
14	GC (guanine-cytosine) content	Fungi and bacteria	Not influenced by PCR biases	Rough determination	Ranjard *et al*., 2000; Kirk *et al*., 2004
15	DNA reassociation and hybridization	Fungi and bacteria	Qualitative and quantitative	Less sensitive	Ranjard *et al*., 2000; Kirk *et al*., 2004; Bing-Ru *et al*., 2006
16	DNA microarrays	Fungi and bacteria	No PCR biases, many target gene sequences	Works for abundant species	Kirk *et al*., 2004

Table 2 (*Continued*)

No.	Method of study	Micro-organism	Advantage	Disadvantage	Reference
17	Single stranded conformation polymorphism	Fungi and bacteria	No GC clamp, no gradient gels	PCR biases, laborious sample handling, variable DNA extraction efficiency	Kirk *et al*., 2004; Bing-Ru *et al*., 2006
18	Restriction fragment length polymorphism (RFLP)	Fungi and bacteria	Community structure	Does not measure community diversity	Kirk *et al*., 2004; Bing-Ru *et al*., 2006
19	Terminal restriction fragment length polymorphism (T-RFLP)	Fungi and bacteria	Measures community structure and diversity, can be automated, highly reproducible	Variable DNA extraction, PCR biases, existing universal primers not representative for all micro-organisms	Ranjard *et al*., 2000; Kirk *et al*., 2004; Bing-Ru *et al*., 2006
20	Ribosomal intergenic spacer analysi(RISA)	Fungi and bacteria	Highly reproducible	Time consuming, large DNA quantities	Ranjard *et al*., 2000; Kirk *et al*., 2004; Bing-Ru *et al*., 2006
21	Highly repeated sequence characterization/microsat ellite regions	Fungi and bacteria	Differentiation to the species and strain level	Not for complex communities, sequence of region should be known	Kirk *et al*., 2004

Table 3. Different examples of studies done using denaturing gradient gel electrophoresis (DGGE)

Environment applied	Nature of Investigation	Method combination	Reference
Agricultural soil	Microbial community changes	Sequencing	Øvreås and Torsvik, 1998
Activated sludge plants	Total microbial diversities	Individuals not characterized	Curtis and Craine, 1998 in Muyzer, 1999
Biofilm	Microbial diversity	Cloning	Gillan *et al*., 1998 in Muyzer, 1999
Sand dunes	Distribution of ammonia-oxidizing bacteria	Sequencing	Kowalchuk *et al*., 1997 in Muyzer, 1999
Marine sediments	Species composition	Hybridization analysis with probes	McCaig *et al*.,1999 in Muyzer, 1999
Lake	Distribution of bacterioplankton	-	Øvreås *et al*., 1997 in Muyzer, 1999
Marine water	Diversity of picoeukaryotes	Sequencing	Díez *et al*., 2001
Grassland and rhizosphere soils	Detection of Burkholderia spp.	sequencing	Salles *et al*., 2002
Organic soil	Diversity of methanotrophic bacteria	Sequencing	Fjellbirkeland *et al*., 2001
Arable soils	Structural diversity of bacterial communities	-	Kozdrój and van Elsas, 2001

Table 3 (*Continued*)

Environment applied	**Nature of Investigation**	**Method combination**	**Reference**
Activated sludge, Estuary sediment and agricultural soil	Diversity of ammonia-oxidizing bacteria	Sequencing	Nicolaisen and Ramsing, 2002
Manure treated agroecosystem	bacterial community structure and diversity	Cloning and sequencing	Sun *et al*., 2004
Heavy metal contaminated soils	Shift in microbial community diversity	-	Li *et al*., 2006a
Marine sponges	Bacterial community diversity	Sequencing	Li *et al*., 2006b
Clinical	Bacterial diversity in wounds	Cloning and sequencing	Dowd *et al*., 2008

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CHAPTER THREE

MICROBIAL DYNAMICS OF DIFFERENT CASING MATERIALS IN THE PRODUCTION OF WHITE BUTTON MUSHROOMS [*AGARICUS BISPORUS* (LANGE) IMBACH]

Nazareth. A. Siyoum and Lise Korsten
Department of Microbiology and Plant Pathology, Faculty of Natural and Agricultural Sciences, University of Pretoria
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Abstract

Commercial production of white button mushrooms requires peat substrate as casing medium. However, the concern about depletion of peat as a natural resource is increasing, especially in places where this resource is limited. In our study, bacterial population dynamics on peat and peat-based casing mixtures of coir, wattle bark, sugarcane bagasse and filter cake were assessed, as bacteria are necessary for the initiation of fruit bodies. Casing mixtures were examined at three growth stages; i.e. at casing, pinning, and end of the second break of mushrooms. The effect of pasteurisation on bacteria and total soluble phenolic compounds in casing materials was also investigated. The spread-plating technique was used to examine total counts and dominant population densities in casing materials and mixtures. Total bacterial counts of peat and the mixtures increased from casing to pinning time and either increased or plateaued at the end of the second break. Populations of *Pseudomonas* spp. showed a significant increase at pinning in all peat-based casing mixtures. Pasteurisation reduced total bacterial counts and increased total soluble phenolic compounds in all materials except wattle bark, in which it increased total bacterial counts and reduced total soluble phenolic compounds. DNA of dominant representative bacteria isolated from the casing mixtures was amplified and sequenced. The bacteria were identified as close relatives to *Ensifer* spp., *Sinorhizobium* spp., *Pseudomonas* spp., *Bacillus* spp., *Sporosarcina* spp., *Microbacterium* spp. *Arthrobacter* spp. and *Sphingobacterium* spp.

Introduction

Peat is an important natural resource, which has environmental and economical values. Peat lands are primarily sources of sustainable pure water and biodiversity. Besides its ecological value, peat is also mined for commercial purposes such as an amendment in green house and horticultural media (Abad *et al*., 1989), as an energy source (Sjörs, 1980) and as a casing medium in mushroom production. However, continuous peat mining for commercial purposes has contributed to faster depletion of this natural resource. Peat is formed from partially decomposed plant material at a very slow rate of development, ranging from 0.02 to 10 cm per year depending on environmental conditions (Smuts, 1992). Therefore, in places with a small area of total peat land reserves, finding alternative materials that can accomplish the different commercial purposes of peat is crucial. Assessing alternative materials to partially or completely replace peat as a casing medium in mushroom production is thus important.

The presence of micro-organisms in the casing layer is important for mushroom mycelium transformation from its vegetative stage to its sexual stage. The result of this transformation is the development of mushroom fruit bodies. Research has shown that some micro-organisms in the casing layer are associated with fruit body initiation (Hayes *et al*., 1969; Hayes, 1979). It has been suggested that some bacteria might play a role in this process by metabolising substances that inhibit initiation of fruit bodies (Miller *et al*., 1995). Recently, other researchers have found that volatile C8 compounds, such as 1-octen-3-ol, produced by mushroom mycelium have inhibited this initiation process (Noble *et al*., 2009). Noble *et al*. (2009) observed higher bacterial density, specifically pseudomonads, in the presence of these inhibitory compounds. These compounds were metabolised by populations of pseudomonads.

Other compounds, such as phenolics, have antimicrobial activity (Michalak, 2006; Silva *et al*., 2010) and are synthesised and accumulated in plants as part of their defence mechanism (Ascensao and Dubery, 2003; Mng'omba *et al*., 2008). The industrial by-products in the present research originate from plants and the total amount of soluble phenolic compounds required investigation.

Heat treatment (pasteurisation) of local peat has been practised by mushroom farmers in South Africa to eliminate pathogenic micro-organisms, such as *Mycogone perniciosa* (Magnus) Delacroix. Alternative materials to peat also harbour some pests and pathogens of mushrooms (Jaarsveld, 2010). Therefore, pasteurisation of casing materials that are not free of pathogens is necessary before use.

In this study, the microbial properties of peat, industrial by-products, and mixtures of peat with industrial by-products were investigated. The main objectives were to 1) determine total bacterial counts and population densities of representative dominant bacteria in peat and different casing mixtures at three different growing stages; i.e. casing, pinning, and end of second break (harvesting); 2) investigate the effect of pasteurisation on the microbial properties of by-products and peat; and 3) determine the amount of total soluble phenolic compounds in peat and industrial by-products before and after pasteurisation.

Materials and Methods

Mushroom Growing Unit

A semi-commercial mushroom growing unit was constructed at the University of Pretoria (Van Jaarsveld, 2010). The unit was built in such a way to have growing conditions comparable to commercial mushroom production systems. The unit has a humidifying system that can maintain up to 100% relative air humidity, and an adjustable heating and cooling systems. The room also has air tight plastic curtains inside a sealed door.

Growing Mushrooms

Phase III compost, known as spawn run compost, was obtained from Highveld Mushrooms (Pty) Ltd and Country Mushrooms (Pty) Ltd (Gauteng Province). The compost was transferred in to metal baskets (39 x 36 x 17.5 cm^3) covered with double plastic bags on the farm before being transported to the University of Pretoria. The baskets were placed in the growing unit, where the air temperature was set at 22^oC. Compost was then cased with peat and peat-based casing mixtures with coir, wattle bark, sugarcane bagasse and filter cake. Baskets were watered for four days and more

depending on the moisture of the casing. Mushroom fruit bodies (primordia) were initiated by altering growing conditions: temperature and CO_2. The CO_2 level was lowered by allowing fresh air into the room, and air temperature was reduced to 16^{o}C. Pinning was visible after four days and mature fruit bodies developed within seven days after pinning. Mushrooms were harvested in two flushes at ten days interval.

Casing Materials

South African peat and the industrial by-products coir, wattle bark, bagasse and filter cake were used to prepare the casing media in the trials. Commercial peat was collected from Highveld Mushroom Farm. Coir is a by-product from the coconut industry and was obtained from Galuku Africa, Sri-Lanka in a 30 x 30 x 15 cm^3 compressed brick form. Wattle bark is produced by Black Wattle trees (*Acacia mearnsii*), which are mainly used for extraction of tannins and charcoal. This material was obtained from Paulpietersburg, South Africa. Bagasse and filter cake are by-products of the sugar industry and were collected from Sezela Sugar Mill in KwaZulu Natal, South Africa.

The control casing medium was 100% farm peat neutralised using calcitic lime ($CaCO_3$). Other casing media were prepared from mixtures of peat and industrial by-products, in different ratios (individual materials without peat were not used to case mushroom beds because the materials were tested before and were poor compared to peat in terms of .mushroom quality, yield or management). The ratios of the mixtures were: 7:3 (peat : coir), 1:1 (peat : coir), 7:3 (peat : wattle), 1:1 (peat : wattle), 7:3 (peat : bagasse), 1:1 (peat : bagasse), 7:3 (peat : filter cake), 1:1 (peat : filter cake). All materials were pasteurised except wattle bark. Wattle bark was not pasteurised due to the development of cinnamon brown mould (*C. fulvum*). Materials were pasteurised at 65^{o}C for six to eight hours at Highveld Farm. All casing media were replicated in seven baskets.

Sampling

Samples of casing material were taken at three different stages of mushroom growth: at casing, pinning and the end of the second break. A sample of 500-1000 g was

taken for each unmixed material. Samples of 200 g casing media were collected in six replicates at the different growing stages.

Plating and Enumeration of Bacteria

Standard I Nutrient Agar medium (STD I) (Merck, Johannesburg, South Africa) was used to isolate bacteria. Ten ml of 0.1% cycloheximide (Sigma, Johannesburg, South Africa) was added to a litre of the medium to inhibit fungal growth. A ten gram casing sample was suspended in 90 ml of 0.1% agar solution (aqueous). A dilution series was prepared and a triplicate of 0.1 ml of each three final dilutions was plated. Plates were incubated in an incubator at 25^{o}C for seven days. Total colony counts were recorded and representative colonies were isolated. General groups of dominant bacteria based on colour and size of colonies were also counted and recorded. At least five colonies from each group were used for identification.

Isolation of Bacteria and DNA Extraction

Colonies of representative bacteria from each of the dominant representative groups were isolated on STD I. Pure cultures were grown from single colonies. DNA was extracted from pure cultures of these colonies using a ZR Fungal/Bacterial DNA Kit (Inqaba, Pretoria, South Africa) according to the manufacturer's instruction. To increase DNA yield, tissue was disrupted using FastPrep (Bio 101 Thermo Electron Corporation, Milford, USA) at 5 m/sec for 20 sec. DNA was finally eluted from the filter using 100 µl of ddH_2O.

PCR Amplification and Sequencing

Enzymatic amplification of the 16S rDNA region was performed using the primers Prun518r 5'ATT-ACC-GCG-GCT-GCT-GG3' and PA8f 5'GAG-AGT-TTG-ATC-CTG-GCT-CAG3' designed by Øvereås *et al*. (1997) and Fjellbirkelard *et al*. (2001) respectively. The reaction consisted of a total volume of 20 µl containing the following reagents: 10.8 µl of double sterilised distilled water, 2.5 µl of PCR buffer, 2 µl of $MgCl_2$ (10x), 2 µl of dNTPs (2.5 µM), 1 µl of each primer (10 pM), 0.2 µl of Taq DNA polymerase (5 U/µl) and 0.5 µl (~ 25 ng/µl) of sample DNA. PCR amplification was performed in an Eppendorf (Merck) thermal cycler starting with 10

min denaturation at 95°C followed by 35 cycles of 30 sec denaturation at 94°C, 30 sec annealing at 58°C, 1 min extension at 72°C, and a final 10 min extension at 72°C. The amplicon was visualised on a 1% agarose gel stained with 0.01% ethidium bromide in a Vilber Lourmat (Omni-Science CC, Randburg, South Africa) gel imaging system.

PCR products were purified with the PCR Purification Kit (250) (QIAquick, Cape Town, South Africa) according to the manufacturer's instructions and sequenced at the University of Pretoria's sequencing unit.

Phenol Extraction and Quantification

A solvent mixture of methanol/acetone/water (7:7:1 v:v) was used as a suspension solution for extraction of soluble phenolic compounds (Regnier and Macheix, 1996). A sample of 50 mg was suspended in 1ml solvent. The suspension was mixed using a VM-300 vortex (Labotec, Johannesburg, South Africa) and placed in a rotary shaker (Stuart Scientific, United Kingdom) for 30 min at 150 rpm. Supernatant was obtained by centrifuging suspensions at 8000 rpm for 5 min in a micro-centrifuge (Sigma). Extraction from the pellet was repeated twice. Supernatant obtained from the three extractions was mixed and concentrated to 1 ml by allowing it to evaporate.

The total soluble phenolic compounds were determined using the Folin-Ciocalteu's reagent method (Bray and Thorpe, 1954). In each well of a 96 wells ELISA-plate (Merck), 175 µl of distilled water was added. A 5 µl aliquot of the sample supernatant and distilled water for control was added to the wells. Twenty-five microliter of Folin-Ciocalteu's reagent (Sigma) was added to the wells. Next, 50 µl of 20% sodium carbonate (Merck) was added and mixed with the contents in each well. ELISA-plates were incubated at 40°C for 30 min. Absorbance values of samples were measured using ELISA reader version 1.3.1 (Multiscan Ascent VI. 24 354-0973, Finland). The absorbance of control wells was subtracted from all sample measurements. The absorbance values were used to calculate gallic acid equivalent in µg g^{-1} using the standard curve y = 1.3527x - 0.0109 (Regnier and Macheix, 1996).

Experimental Design and Statistical Analysis

Experiments were conducted in a complete randomised design. Six replicates from each casing medium were included. Trials in the growing room were repeated three

times. Results were analysed using the statistical package GenStat Discovery Edition. Treatments were compared using analysis of variance (ANOVA).

Results

Identification of Bacteria

The dominant representative bacteria were identified as close relatives to *Ensifer* spp., *Sinorhizobium* spp., *Pseudomonas* spp., *Bacillus* spp., *Sporosarcina* spp., *Microbacterium* spp. *Arthrobacter* spp. and *Sphingobacterium* spp.

Enumeration of Bacteria in Unmixed Materials

Peat and filter cake had similar total bacterial population densities that were significantly higher than the rest of the materials (coir, wattle bark and bagasse). Population densities of the bacteria identified as *Ensifer*/*Sinorhizobium* spp. were significantly higher in peat compared to the other test materials. Similarly, population densities of *Microbacterium* spp. *Arthrobacter* spp. and *Sphingobacterium* spp. were significantly higher in peat, bagasse and filter cake compared with coir and wattle bark. However, *Pseudomonas* spp., *Bacillus* spp. and *Sporosarcina* spp. had similar abundance in all the materials (Table 1).

Table 1 Bacterial populations of unpasteurised peat and the industrial by-products: coconut coir, wattle bark, sugar cane bagasse and filter cake

Material	Log_{10} value of bacterial colony forming units (cfu's)/g			
	Total	Representative dominant groups consisting of		
		Ensifer/ *Sinorhizobium* spp.	*Pseudomonas*, *Bacillus* and *Sporosarcina* spp.	*Microbacterium* *Arthrobacter* and *Sphingobacterium* spp.
Peat	6.258[a]	5.853[a]	4.95[a]	5.31[a]
Coir	5.462[b]	4.755[b]	4.45[a]	3.53[b]
Wattle bark	4.798[c]	4.200[c]	4.43[a]	3.05[b]
Bagasse	5.651[b]	5.069[d]	3.74[a]	5.16[a]
Filter cake	6.127[a]	5.653[e]	4.53[a]	5.62[a]
LSD	0.2053	0.1932	0.991	0.818
F pr.	<.001	<.001	<.2	<.001

In each column, values with different letters are significantly different.

After pasteurisation total bacterial populations in peat as well as in the by-products decreased except for wattle bark, in which the populations increased. Compared with

peat, total bacterial population densities were significantly lower in coir but higher in filter cake. For total bacteria and *Ensifer*/*Sinorhizobium* spp. peat had similar population densities as wattle bark and bagasse (Table 2).

Table 2 Bacterial populations of pasteurised peat and the industrial by-products: coconut coir, wattle bark, sugar cane bagasse and filter cake

Material	Log_{10} value of bacterial colony forming units (cfu's)/g			
	Total	Representative dominant groups consisting of		
		Ensifer/ *Sinorhizobium* spp.	*Pseudomonas* , *Bacillus* and *Sporosarcina* spp.	*Microbacterium Arthrobacter* and *Sphingobacterium* spp.
Peat	4.962^{ac}	4.593^{a}	3.79^{a}	3.34^{a}
Coir	3.881^{b}	3.048^{b}	3.73^{a}	1.44^{b}
Wattle bark	5.146^{a}	4.294^{a}	4.99^{b}	2.98^{a}
Bagasse	4.768^{c}	4.311^{a}	3.29^{a}	1.86^{b}
Filter cake	5.502^{d}	5.103^{c}	5.01^{b}	3.30^{a}
LSD	0.2679	0.3163	0.659	0.923
F pr.	<.001	<.001	<.001	<.001

In each column, values with different letters are significantly different.

Peat, coir, bagasse and filter cake exhibited significantly lower total bacterial populations after pasteurisation. However, that of wattle bark increased after pasteurisation (Figure 1).

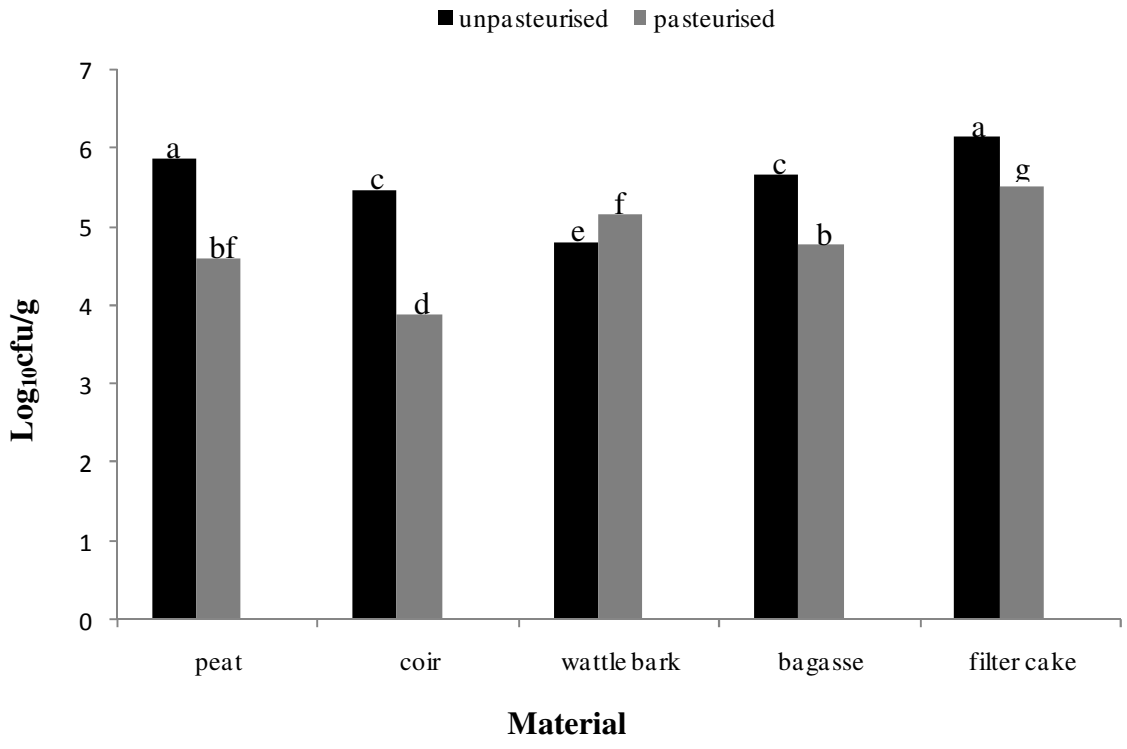

Figure 1. Effect of pasteurisation on total bacterial populations of peat and industrial by-products: coir, wattle bark, bagasse and filter cake (Different letters on bars of the same material indicate significant difference).

Quantification of Total Soluble Phenolic Compounds

The levels of total soluble phenolic compounds in peat, coir and filter cake were not significantly different before pasteurisation. Bagasse, followed by wattle bark had significantly higher values than the other materials (Table 3). The effect of pasteurisation on total soluble phenolic compounds of the materials varied (Figure 2). Pasteurisation resulted in a significant increase of soluble phenolic compounds in coir and filter cake. However, pasteurisation resulted in a significant reduction of these compounds in wattle bark.

Table 3 Measurements of total soluble phenolic compounds in peat and industrial by-products: coir, wattle bark, bagasse and filter cake before and after pasteurisation

Material	**Total soluble phenolic compounds in equivalent mg gallic acid/g of material**	
	Unpasteurised	Pasteurised
Peat	0.606[a]	0.714[a]
Coir	0.998[a]	1.558[a]
Wattle bark	7.612[b]	3.124[b]
Bagasse	23.156[c]	24.148[c]
Filter cake	1.256[a]	3.518[b]
LSD	1.1904	0.9526
F pr.	<0.001	<0.001

In each column, values with different letters are significantly different.

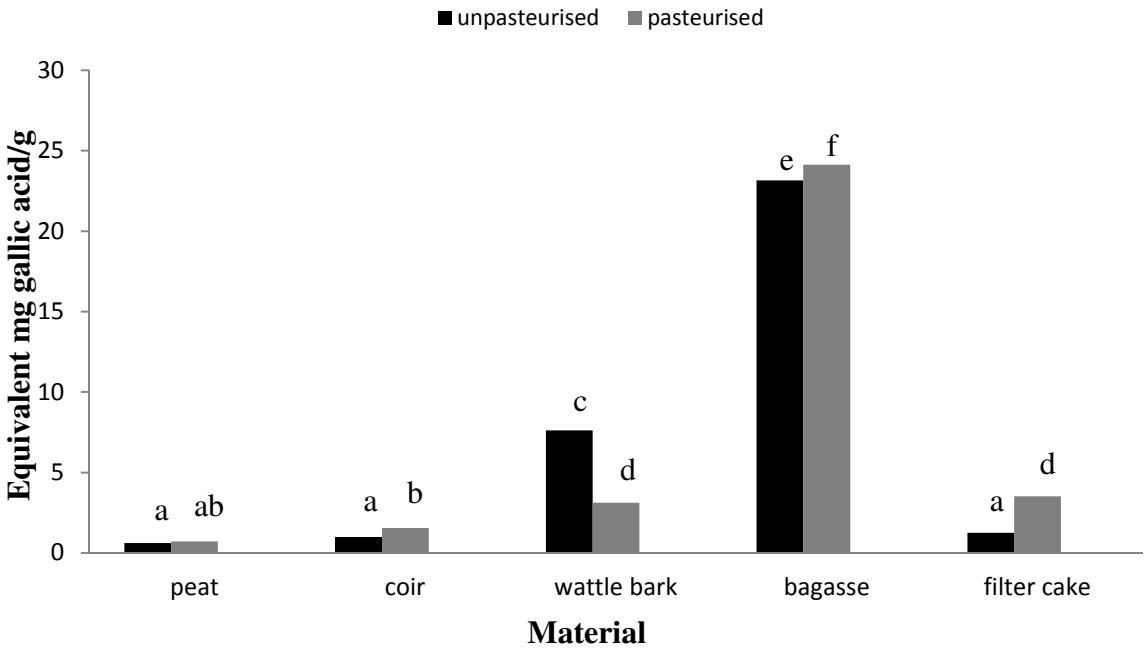

Figure 2. Effect of pasteurisation on total soluble phenolic compounds of peat and industrial by-products: coir, wattle bark, bagasse and filter cake (Different letters on bars of the same material indicate significant difference).

Enumeration of Bacteria in Casing Media (Peat and Mixtures)

Materials used as casing media were peat and peat-based mixtures of the industrial by-products: coir, wattle bark, bagasse and filter cake. At casing stage, all the mixtures had significantly higher total bacterial counts than peat (Table 4). At this stage, most of the mixtures in comparison to peat had higher counts of the main categories of dominant bacteria. At the pinning stage, the coir mixtures and 7:3 peat/wattle total bacterial counts were in similar abundance as in peat. The rest of the mixtures had significantly higher total bacterial counts than peat. However, for bacteria consisting *Pseudomonas*, *Bacillus* and *Sporosarcina* spp. similar abundance was observed in all the mixtures except 1:1 peat/wattle. At harvesting stage (end of second break), most of the mixtures had a similar abundance in total and the dominant categories of bacteria as in peat.

Table 4 Total viable bacterial spp. in different casing mixtures and peat at three different mushroom production stages

Stage	Casing medium	Log_{10} value of bacterial colony forming units (cfu's)/g			
		Total	Representative dominant groups consisting of		
			Ensifer/ Sinorhizobium spp.	*Pseudomonas*, *Bacillus* and *Sporosarcina* spp.	*Microbacterium Arthrobacter* and *Sphingobacterium* spp.
Casing	Cont/peat	4.769[a]	4.507[a]	3.190[a]	2.94[a]
	1:1peat/coir	5.534[b]	5.076[b]	5.092[b]	4.27[b]
	7:3peat/coir	5.159[b]	4.643[a]	4.873[b]	3.32[a]
	1:1peat/wattle	6.197[c]	5.668[c]	5.796[c]	5.24[b]
	7:3peat/wattle	5.984[c]	5.540[c]	5.561[c]	4.66[b]
	1:1peat/bagasse	5.268[b]	4.901[b]	4.824[b]	1.26[d]
	7:3peat/bagasse	5.471[b]	5.136[b]	4.837[b]	0.94[d]
	1:1peat/filter cake	5.820[bc]	5.341[b]	5.399[c]	2.92[a]
	7:3peat/filter cake	5.002[b]	4.618[abd]	4.244[d]	1.55[d]
	LSD	0.3610	0.3504	0.5618	1.039
	F pr.	<.001	<.001	<.001	<.001
Pinning	Cont/peat	6.593[a]	6.154[a]	5.733[a]	6.002[a]
	1:1peat/coir	6.624[ac]	6.181[ac]	5.855[a]	5.929[a]
	7:3peat/coir	6.591[a]	6.076[a]	5.997[a]	5.866[a]
	1:1peat/wattle	7.739[b]	7.199[b]	7.193[b]	7.161[b]
	7:3peat/wattle	6.652[ac]	6.168[a]	6.138[a]	6.003[a]
	1:1peat/bagasse	6.883[cd]	6.450[cd]	5.698[a]	6.382[c]
	7:3peat/bagasse	6.875[cd]	6.474[d]	6.033[a]	6.322[ac]
	1:1peat/filter cake	6.986[d]	6.618[d]	5.882[a]	6.472[c]
	7:3peat/filter cake	7.012[d]	6.620[d]	6.182[a]	6.464[c]
	LSD	0.2690	0.2756	0.5295	0.3326
	F pr.	<.001	<.001	<.001	<.001
Harvesting	Cont/peat	7.130[a]	6.508[a]	6.067[a]	6.825[ac]
	1:1peat/coir	7.115[a]	6.519[a]	6.369[b]	6.650[ab]
	7:3peat/coir	6.765[b]	6.073[b]	5.944[a]	6.374[b]
	1:1peat/wattle	7.607[c]	7.093[c]	6.866[c]	7.098[c]
	7:3peat/wattle	7.118[a]	6.601[ad]	6.316[ab]	6.600[a]
	1:1peat/bagasse	7.166[a]	6.673[ad]	6.042[ad]	6.822[ac]
	7:3peat/bagasse	7.159[a]	6.610[ad]	6.016[ad]	6.800[ac]
	1:1peat/filter	7.274[a]	6.833[cd]	6.239[ad]	6.922[ac]

	cake				
	7:3peat/filter cake	7.207[a]	6.754[ad]	6.290[ad]	6.772[a]
	LSD	0.2935	0.3094	0.2767	0.3227
	F pr.	<.001	<.001	<.001	<.003

In each column, values with different letters are significantly different.

Peat and each casing mixture showed significant increase of total cfu's and all three groups of bacteria from casing stage to pinning and harvesting.

Discussion

Unmixed and unpasteurised materials revealed that all the by-products besides filter cake had lower total bacterial populations than peat. However, the populations of bacteria consisting of *Pseudomonas*, *Bacillus* and *Sporosarcina* spp. were in similar abundance in all the by-products as well as in peat. It is well established that pseudomonads are associated with fruit body initiation in white button mushrooms (Hayes *et al*., 1969; Masaphy *et al*., 1987; Noble *et al*., 2003). Therefore, it might be desirable for potential casing media to harbour pseudomonads, as in the above mentioned by-products.

In the present study, pasteurisation significantly reduced the total bacterial populations of peat, coir, bagasse and filter cake as expected. Silva *et al*., (2009) reported similar results in which a reduction of total microbial populations in bagasse-based compost was observed after pasteurisation. Pasteurisation also has a significant effect in removing pathogenic micro-organisms from casing materials (Sharma *et al*., 1999). Mushroom farmers in South Africa used to pasteurise local peat before casing white button mushroom beds to eliminate mushroom pathogens, such as *M. perniciosa*. However, in our study, for wattle bark, an increase in total bacterial populations was observed after pasteurisation. Pasteurised wattle bark casing mixtures were also previously reported to support profuse growth of *Chromelosporium fulvum* (unpublished data). Analysis of total soluble phenolic compounds in wattle revealed significant reduction of these compounds after pasteurisation, unlike the other by-products. Phenolic compounds have antimicrobial properties as part of a plant's defence mechanism against diseases (Michalak, 2006; Silva *et al*., 2010). The increase in bacterial populations in wattle bark after

pasteurisation could be explained by reduced anti-microbial stress as a result of a decrease in phenolic compounds.

Each of the above mentioned by-products was used as a mixture with peat to improve their physical structure, and all mixtures except those with wattle bark were pasteurised before casing for the reasons mentioned above. Initially, at the time of casing, an increase in total bacterial populations was observed in the mixtures compared to individual materials (peat and by-products). This increase could indicate a synergetic effect of the bacteria from peat and the by-products. Or mixing the substrates could affect growth of bacterial populations in them positively as in the work by Franco *et al*., (2004) who reported that the addition of organic substrates to a contaminated soil increased microbial biomass.

Our results show that at pinning, a significant increase in total bacterial populations was observed in all the test materials. The group consisting of the *Pseudomonas* spp. was found to have increased and was in similar abundance in peat and in all the mixtures except the 1:1 peat/wattle (in which case it was larger). This increase was in agreement with findings by Miller *et al*. (1995) who reported large increases of total bacteria and fluorescent pseudomonads in peat casing media at the onset of fruiting. Therefore, the mixtures showed results comparable with those for peat and can be as suitable as peat alone in terms of beneficial bacteria for pinning.

Both bagasse and filter cake were by-products from the processing of sugarcane and exhibited some similar trends in bacterial populations. At the time of pinning, mixtures of these by-products had a significantly higher bacterial population density of total as well as the dominant categories consisting of *Ensifer*/*Sinorhizobium* spp., *Microbacterium*, *Arthrobacter* and *Sphingobacterium* spp. than that of peat. Our results agree with Silva *et al*. (2009) who reported high bacterial population density in bagasse compost.

In conclusion, although the industrial by-product materials had different bacterial richness from peat, these materials harboured the group of bacteria important for pinning. At pinning, peat-based mixtures of the by-products had increased total bacterial populations, and the group consisting of pseudomonads was equal or higher in population density than that of peat. Therefore, peat-based mixtures of coir, wattle

bark, bagasse and filter cake can be productive casing media as they consist of bacteria important in initiation of fruit bodies in *A. bisporus*.

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CHAPTER FOUR

BACTERIAL PROFILING OF CASING MATERIALS FOR WHITE BUTTON MUSHROOM [*AGARICUS BISPORUS* (LANGE) IMBACH] PRODUCTION USING DENATURING GRADIENT GEL ELECTROPHORESIS

[1]Nazareth. A. Siyoum , [2]Karen Surridge, and [1]Lise Korsten
[1] *Department of Microbiology and Plant Pathology, Faculty of Natural and Agricultural Sciences, University of Pretoria*
[2]*Department of Plant Production and Soil Science, Faculty of Natural and Agricultural Sciences, University of Pretoria*
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Abstract

In the commercial production of the white button mushrooms [*Agaricus bisporus*], a casing material is required to cover the spawn run compost to stimulate the reproductive stage. It is known that certain bacteria play an important role in this process. The dynamics of bacterial populations (bacterial richness and diversity) within peat and peat-based casing mixtures using alternative materials from industrial wastes (i.e. coir, wattle bark, bagasse and filter cake) were examined using denaturing gradient gel electrophoresis (DGGE). Casing materials were assessed at three different mushroom growth stages, viz casing, pinning and harvesting. Results from DGGE confirmed that higher bacterial species richness occurs at pinning and then harvesting compared to the casing stage. Increase in bacterial population density at pinning was higher in peat-based mixtures of alternative materials than in peat alone. All peat mixtures with the alternative materials provided favourable substrates for bacterial growth. The DGGE patterns after pasteurisation reflected the ability of the casing material to rapidly re-establish the original bacterial community structure reflecting homeostasis. The dominant bacteria in casing materials found during pinning were closely related to *Pseudomonas*, *Bacteroidetes*, *alpha-Proteobacterium*, *beta-Proteobacterium*, *gamma-Proteobacterium*, *delta-Proteobacterium* and uncultured species.

Introduction

In the commercial production of white button mushrooms (*Agaricus bisporus* (Lange) Imbach), spawn run compost is covered by a top layer of casing soil. This layer is necessary for the mushroom mycelium to transform from the vegetative to the reproductive stage. The growing mycelium suddenly stops its vegetative growth process and thickens to produce pinheads, which later develop into mushrooms (Sinden, 1982). Although the reason for this change is not completely understood, bacteria in the casing are often found to be associated with the process of pinhead initiation (pinning) (Ingratta and Blom, 1979; Sinden, 1982; Flegg, 1989).

Several researchers have reported the association of some pseudomonads with fruit body formation in *A. bisporus*. Umar and Van Griensven (1998) reported that the onset of fruit bodies occurred in casing soil which was naturally inhabited by *Pseudomonas*-like bacteria. Fluorescent *Pseudomonas* spp. consisting of *P. putida* were isolated from casing materials which resulted in primordial formation (Noble *et al*., 2003). Bacterial populations isolated from non-sterile casing and identified as close relatives to *P. putida* were found to stimulate fruit body formation when inoculated to pure cultures of *A. bisporus* (Hayes *et al*., 1969). Miller *et. al*. (1995) reported an increased proportion of fluorescent pseudomonads during the reproductive phase of *A. bisporus* and observed that these bacteria adhere rapidly and firmly to the walls of the mycelium. They suggested that the pseudomonads might have the ability to utilise oxalate crystals. It was pointed out that the sudden transformation of the fungus into its reproductive stage (spore production) could be a mechanism to survive stress, as is common for many fungi (Miller *et. al*., 1995).

Peat is a widely accepted casing material used in the commercial production of button mushrooms (Ingratta and Blom, 1979; Flegg, 1991). Peat is a nutrient poor material (Sinden, 1982, Abad *et al*., 1989, Smuts, 1992) which results from the partial decomposition of plant matter. Generally, peat is the preferred material due to its high water-holding capacity to provide adequate moisture for the growing mushroom mycelium. It has a slightly acidic pH which is neutralised by adding lime to make it suitable for mushroom production (pH 6.5-8.5) (Flegg, 1989). Although peat is considered to be generally free from pests and disease-causing organisms, it is known to harbour beneficial bacteria that are believed to play an important role during pinning (Ingratta and Blom, 1979).

The search for easily available alternative materials to replace or supplement peat in commercial production of white button mushrooms has become important for the mushroom industry in countries where this natural resource is scarce. In order to evaluate materials that could replace peat, certain physiological, chemical and microbiological criteria must be met. Certain bacteria associated with pinning could be used as an indicator for potential suitable alternative casing materials. Such materials should either be naturally inhabited by these beneficial bacteria or have the ability to support their growth once introduced.

It is speculated that only 20% of bacteria is culturable (Muyzer *et al*., 1993). This is mainly because selective growth media do not provide optimum growth conditions for micro-organisms obtained from natural environments. A biased profile towards culturable organisms will be reflected if only growth media are used to study microbial profiles of various environmental samples. Finger-printing molecular techniques, such as denaturing gradient gel electrophoresis (DGGE), provide more accurate and detailed results than cultural techniques.

Previously, microbial casing studies were done based on cultural methods (Chapter 3). In most casing studies, pseudomonads were found to be the prevalent bacteria in association with fruit body initiation and pinning (Hayes *et al*., 1969; Miller *et. al*., 1995; Umar and Van Griensven, 1998; Noble *et al*., 2003). In this study, the main objectives were to 1) develop bacterial profiles for casing materials in order to determine dominant bacterial species; and 2) examine bacterial community successions in the materials at different mushroom growing stages in the bacterial profiles established.

Materials and Methods

Casing Materials

The same materials as described in Chapter 3 were used to prepare mushroom casings, viz. South African peat, and industrial waste materials - coir, wattle bark, bagasse and filter cake. In addition, freshly mined peat from Potchefstroom was collected directly from the mining area and was mixed with lime on the experimental farm, University of Pretoria. Casing preparations for the trials were 100% peat, and industrial by-products mixed with peat at 7:3 and 1:1 ratios for coir and wattle bark (70% peat and 30% coir or wattle bark) and at 1:1 ratio for bagasse and filter cake. Each casing was replicated in three baskets each containing 10 kg

Phase III compost (spawn run compost that is colonised and ready for casing). The baskets were placed randomly in the mushroom growing unit maintained at the University of Pretoria, described in Chapter 3. For 100% peat, both pasteurised and unpasteurised casings were used. In the peat-based mixtures, materials were pasteurised except for the wattle bark. Pasteurised wattle bark was susceptible to cinnamon brown mould (*Chromelosporium fulvum*) contamination (unpublished data) and was therefore used unpasteurised. Of the three replicate baskets, two baskets were sampled (100 g) in duplicate for DNA extraction. Samples were taken directly after casing, at pinning and at harvesting of second break. The whole experiment was conducted twice.

DNA Extraction

DNA was extracted from duplicate samples of casing materials using the Soil Master Extraction Kit (Epicentre Biotechnologies, Madison, WI, USA), following the manufacturer's instructions with the following volume modifications: 100 mg of casing material sample was placed in a 2-ml screw cap micro centrifuge tube (Whitehead Scientific, Cape Town, South Africa) with two 0.25-inch ceramic beads; 375 µl of soil DNA extraction buffer was added; to increase DNA yield, tissue was disrupted using FastPrep at 5 m/sec for 20 sec; and 75 µl of soil lysis buffer was added. DNA was finally eluted from the filter using 100 µl of TE buffer.

PCR Amplification

Enzymatic amplification of the 16S rDNA region was performed using the primers Prun518r 5'ATT-ACC-GCG-GCT-GCT-GG3' and PA8f-GC 5'CGC-CCG-CCG-CGC-GCG-GCG-GGC-GGG-GCG-GGG-GCA-CGG-GGG-GAG-AGT-TTG-ATC-CTG-GCT-CAG3' designed for DGGE by Øvereås *et al.* (1997) and Fjellbirkelard *et al.* (2001) respectively. The reaction consisted of a total volume of 20 µl containing the following reagents: 10.8 µl of ddH_2O, 2.5 µl of PCR buffer, 2 µl of $MgCl_2$ (10x), 2 µl of dNTPs (2.5 µM), 1 µl of each primer (10 pM), 0.2 µl of *Taq* DNA polymerase (5 U/µl) and 0.5 µl (~ 25 ng/µl) of sample DNA. PCR amplification was performed in an Eppendorf (Merck, Johannesburg, South Africa) thermal cycler starting with 10 min denaturation at 95°C followed by 35 cycles of 30 sec denaturation at 94°C, 30 sec annealing at 58°C, 1 min extension at 72°C, and a final 10 min extension at 72°C. The amplicon was visualised on a 1% agarose gel stained with 0.01%

ethidium bromide in a Vilber Lourmat (Omni-Science CC, Randburg, South Africa) gel imaging system.

DGGE

The DGGE was performed using a D-Code (BIO-Rad, Johannesburg, South Africa) universal mutation detection system. PCR products of 10 µl, each mixed with 3 µl loading dye were loaded onto 40–55% denaturing gradient polyacrylamide (40%) gels. Electrophoresis was performed at 20 V for 10 min to allow gels to acclimatise and then at 70 V at a temperature of 60^{o}C for 17 hours. Next the gels were stained with 4 µl SYBR Gold (Whitehead Scientific, Johannesburg, South Africa) nucleic acid gel stain for one hour in the dark, then visualised and photographed under UV light in Vilber Lourmat gel imaging system.

Band Excision, Purification and Sequencing

Dominant bands from the resulting fingerprint pattern on the gel were excised under blue light on Jiffy Lites Blue Light Box (Inqba Biotech, Pretoria, South Africa) using a sterile scalpel. The excised acrylamide gel fragments containing the bands were placed in sterile 1.5-ml micro tubes, each containing 30 µl of sterile ddH_2O and stored at 4^{o}C for at least 24 h. A 0.5 µl aliquot of the liquid was then used as a template for PCR using the previously mentioned primers. The DNA yield was verified by visualising the amplicon on 1% agarose gel as mentioned previously.

PCR products were purified with PCR Purification Kit (250) (QIAquick, Cape Town, South Africa) according to the manufacturer's instructions and sequenced at the University of Pretoria's sequencing unit.

Gel Analysis and Phylogenetic Tree

Gel analysis was performed using Gel2k gel analysis software (Norland, 2004), where similarity between samples was compared using a Jaccard group average setting. This analysis is based on band intensity in the lanes.

Amplified DNA from the excised DGGE bands using the above primers was sequenced and produced tentative species identification. Partial sequences of the 16S region of the rDNA were obtained using the forward and reverse primers mentioned previously. Using BioEdit

(Hall, 1997-2005) a consensus sequence was created by aligning the forward and reverse partial sequences and electropherograms were edited using Chromas (Chromas Lite 2.01, 1998-2005 Technelysium Pty Ltd).

A BLAST search was done for each sequence on the GenBank database and matching hits, with highest percentage identity and e-values closest to 0.0 indicating a statistically acceptable match, were selected for alignment. Resulting sequences were edited using Contig express (Vector NTI advance 11.0, Invitrogen 2008), aligned with Clustal X (Thompson *et al*., 1994) and inserted gaps were treated as missing data. Analysis for Phylogenetic relationship was performed based on parsimony using PAUP 4.0b8 (Phylogenetic Analysis Using Parsimony) (Swofford, 2000). Random addition of sequences (100 replicates), tree bisection-reconnection, branch swapping, MULPAR-effective and MaxTrees were used to perform Heuristic searches. Tree length distributions over 100 randomly generated trees were evaluated to assess phylogenetic signal in the data sets. The consistency (CI) and retention indices (RI) were determined for all data sets. Phylogenetic trees were rooted with *Thermotoga maritima* as outgroup to the remaining taxa. In order to determine confidence in branching points (1000 replicates), bootstrap values were generated retaining groups with greater than 70% consistency.

Results

Bacterial Populations

The bacterial population density was very low during casing for all samples from casing mixtures. Therefore, sufficient amplification for samples at casing was obtained by doubling the amount of template DNA. PCR products run on a DGGE gel showed higher bacterial population densities at the pinning and harvesting stages, indicated by higher band intensities in the gel (Figure 1).

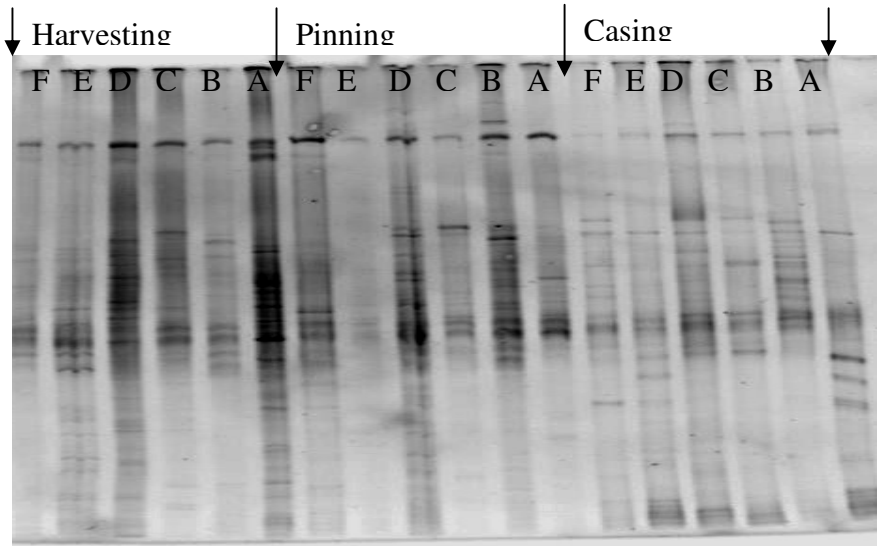

Figure 1. DGGE images of PCR products amplified of total DNA extracted from casing mixtures at three different mushroom growing stages: casing, pinning and harvesting (A 1:1 (peat: coir), B 1:1 (peat: wattle), C 1:1 (peat: bagasse), D 1:1 (peat: Filter cake), E 3:7 (peat: coir), F 3:7 (peat: wattle)), revealing higher bacterial numbers (more intense bands) at pinning and harvesting than at casing.

Bacterial populations at casing were higher in peat than in peat-based mixtures as well as the unmixed alternative materials. This was evidenced by the lower amount of DNA in the mixture samples compared to peat samples at casing. For the mixtures, to obtain PCR product that produced visible bands on the DGGE gel, the amount of template DNA had to be at least doubled compared to peat alone (optimization experiments not shown). However, at pinning there was a remarkable increase in bacterial population numbers in the mixtures than in peat alone. Visual analysis of the gels showed smaller increase in bacterial population sizes (from the time of casing to pinning) for peat than for the mixtures.

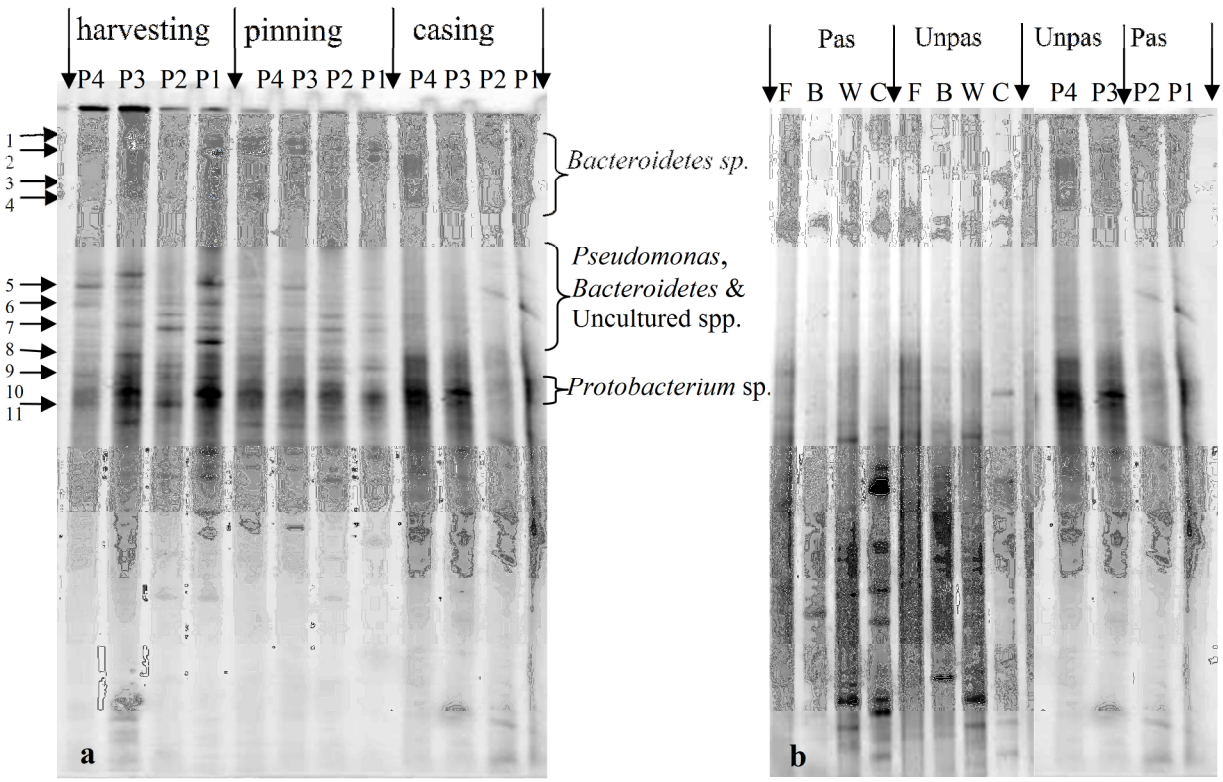

Figure 2. DGGE gel images of DNA from (a) peat samples at three mushroom growing stages: P1 and P2 represent replicate samples from pasteurised peat and P3 and P4 represent replicate samples from unpasteurised peat; (b) Pasteurised (pas) and unpasteurised (unpas) unmixed alternative materials and peat: C coir, W wattle bark, B bagasse and F filter cake, P1, P2, P3 and P4 as in (a).

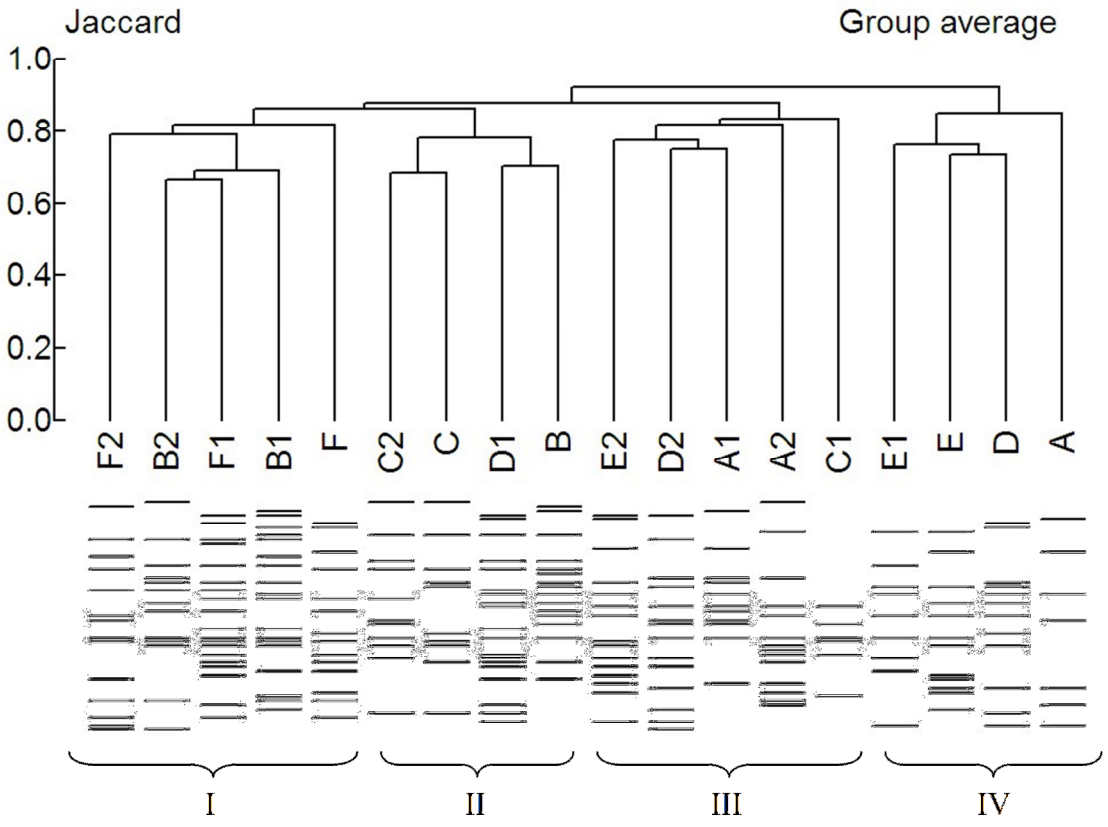

Figure 3. Cluster analysis dendrogram of similarities between bacterial populations of casing mixtures at three different mushroom growing stages. Letters and numbers identify samples and mushroom growth stages respectively (letters represent: A 1:1 (peat: coir), B 1:1 (peat: wattle), C 1:1 (peat: bagasse), D 1:1 (peat: Filter cake), E 3:7 (peat: coir), F 3:7 (peat: wattle); letter with no number is casing, 1 is pinning and 2 is harvesting).

Effect of Pasteurisation

Bacterial populations, as can be expected, were initially lower in the pasteurised peat samples than the unpasteurised samples (P1 & P2 vs P3 & P4 at casing in Figure 2a). However, at pinning, there was no difference in the population richness and diversity between pasteurised and unpasteurised samples. This is evidenced by the common bands (1-7 in Figure 2a) in both the pasteurised and unpasteurised samples.

Population Similarities

Jaccard group average analysis for casing mixtures resulted in four main clades (Figure 3). Most casing mixtures prepared from peat and wattle bark (B and F) fall into clade 1. Casing mixtures prepared from peat and coir (A and E) fall under two neighbouring clusters (III & IV). Casing mixtures prepared from the sugarcane by-products bagasse and filter cake (C and D respectively) were relatively scattered over clades II, III & IV. Generally, the clades tended to form as a result of the origin of the samples and not by the different mushroom growth stages. Jaccard group average analysis clustered pasteurised and unpasteurised samples of peat (from two sources) into five main clades (Figure 4). Peat samples did not tend to cluster by their source. All five clades are composed of samples from both sources.

Sequencing and Phylogeny

Sequencing of DNA from the DGGE gel bands indicated that the prevailing dominant species from pinning onwards were close relatives of *Bacteroidetes* spp., and *Pseudomonas* spp. Other dominant species included close relatives to *alpha-Proteobacterium*, *beta-Proteobacterium*, *gamma-Proteobacterium*, *delta-Proteobacterium* and uncultured species. Phylogenetic analysis showed that the bacteria that were dominant from pinning onwards were taxonomically diverse.

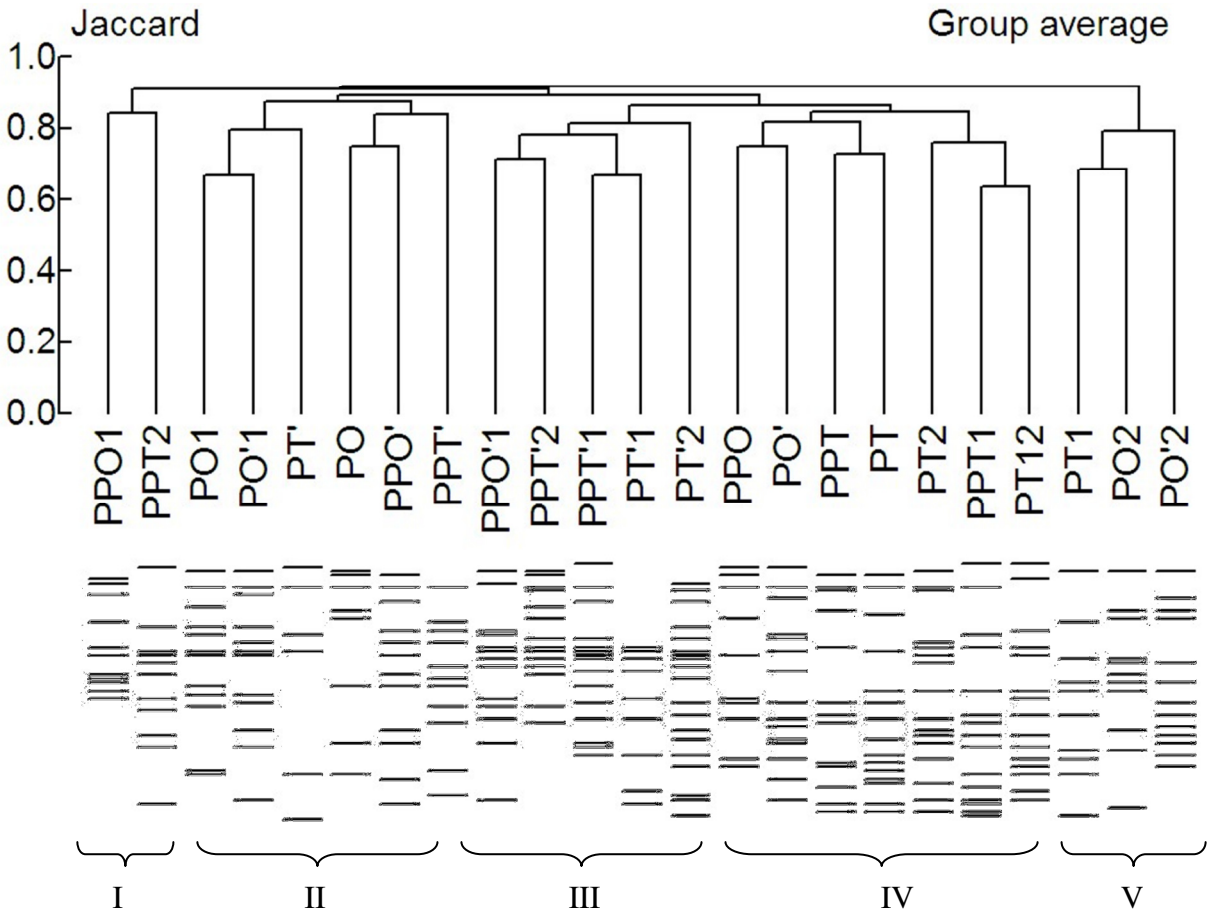

Figure 4. Cluster analysis dendrogram of similarities between peat samples (Pt: peat from mushroom farm and PO: peat from the mine; PP are replicates of respective samples) at three different mushroom growing stages. Letters and numbers identify samples and mushroom growth stages (letter with no number is casing, 1 is pinning and 2 is harvesting) respectively. Letters with "'" stand for pasteurised samples.

Sequences that originated from the brightest bands of samples from the time of pinning onwards are mainly in groups 2 and 3 of the phylogenetic tree (Figure 5), and their closest relatives are *Pseudomonas*, *Bacteroidetes* and uncultured spp. Group 4 also represents sequences of bands that remained high in population size at pinning and their closest relatives are from the *Proteobacteria* group, dominated by the *alpha-* and *beta-* groups. Sequences that originated from less bright bands of samples from pinning onwards and bright bands of samples at casing were grouped at the lowest part of the phylogenetic tree (Group 1).

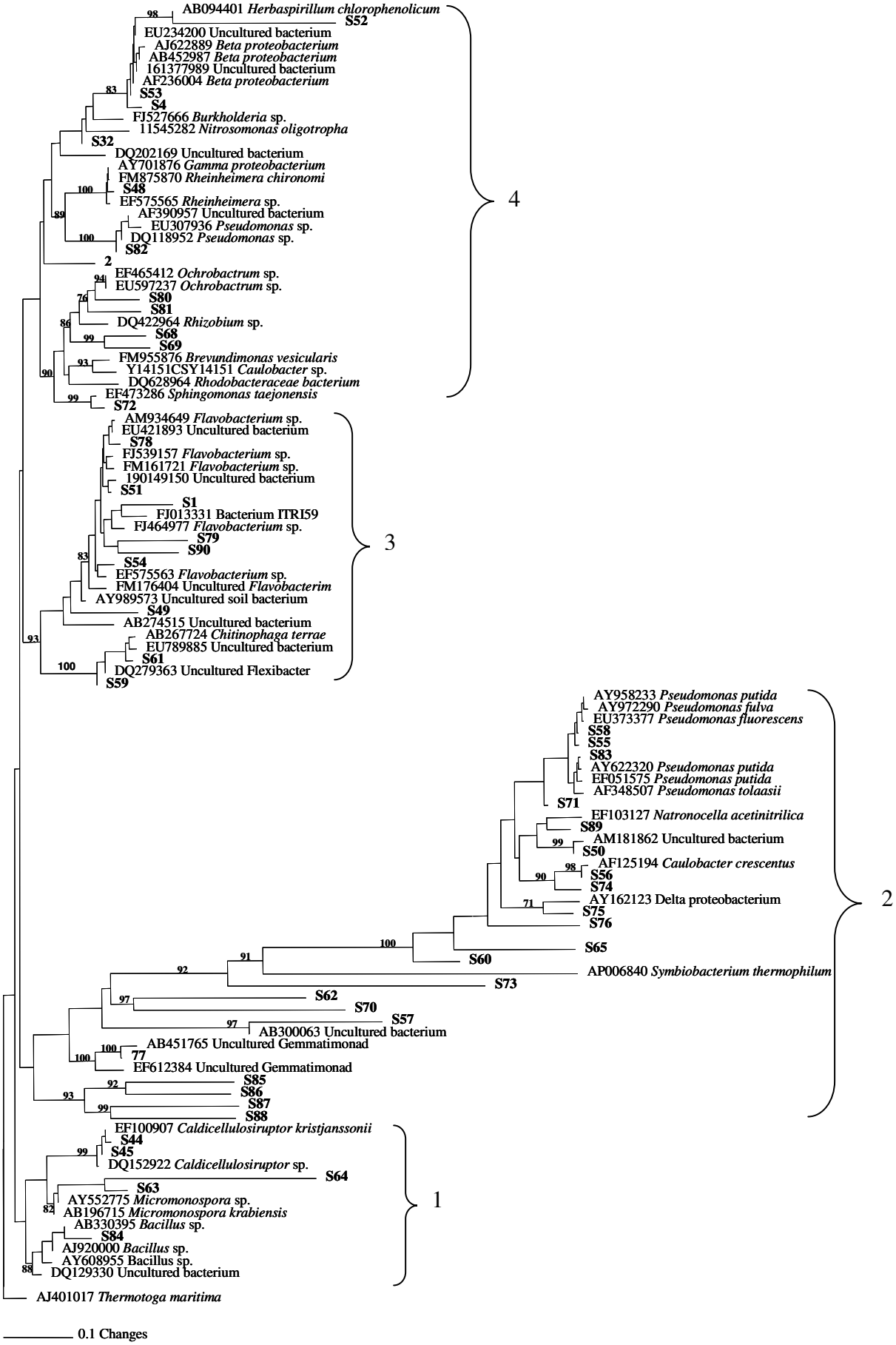

Figure 5. Rooted tree based on parsimony showing the phylogenetic relationships of dominant bacterial groups from DGGE gels of casing samples from peat and peat-based mixtures (with coir, wattle bark, baggase or filter cake) to most closely related sequences obtained from BLAST searches. Sequences of bands from DGGE gels are indicated as numbers preceded by "S" [Parsimony informative characters are 425, CI=0.4234, RI=0.8198, number of trees=100, tree length=963.74864 and g1=-0.633065].

Discussion

The relative intensity of a band in a DGGE gel is indicative of the relative abundance of a species in the population (Muyzer *et al*., 1993). Hence, intense bands depict high population numbers of that particular species. Based on the DGGE approach, it was found that bacterial populations belonging to *Pseudomonas*, *Bacteroidetes*, *alpha-Proteobacterium*, *beta-Proteobacterium*, *gamma-Proteobacterium*, *delta-Proteobacterium* and uncultured species increased in abundance from the pinning stage onwards, which underlines the importance of these populations in the pinning process. Bacteria associated with pinning are known to remove substances that inhibit fruit body formation (Noble *et al*., 2009). The dominant groups found in this study, namely the *Bacteroidetes* and *Pseudomonas* species, are known to have the ability to break down macromolecules such as protein, starch, cellulose, chitin and other compounds (Baar and Kinings, 2005; Hamann *et al*., 1999). Bacteria in these groups can also mineralize and assimilate several organic compounds (Madsen and Alexander, 1985; Hickey *et al*., 1993; Cottrell and Kirchman, 2000; Kirchman *et al*., 2003; Malmstrom *et al*., 2004; Elifantz *et al*., 2005). Several members of the *Proteobacteria* are reported to be associated with utilization and degradation of low- and high-molecular weight carbon compounds (Covert and Moran, 2001; Röling *et al*., 2002).

Lowering CO_2 levels by allowing fresh air into the growing room is a common practice that triggers or increases pinning of button mushrooms (Ingratta and Blom, 1979). Higher O_2 levels favour aerobic bacteria and results in this study show that aerobic bacteria constitute the bacterial profile of casing materials.

The position of a band in a DGGE profile most likely represents a particular species in the population (Muyzer *et al*., 1993). Thus, the similarity of band patterns (band positions in the gel) signifies species similarities in all peat-based casing mixtures. These patterns are similar to that of peat alone, which is the common material in all tested casing mixtures. According

to the band similarities, the bacteria that were prominent in peat casings alone also inhabited the casing materials prepared from peat-based mixtures supplemented with alternative industrial waste materials.

Smaller increases in population sizes of bacteria in peat alone compared with those in peat-based mixtures indicate that peat casings reflect relatively stabilized bacterial populations. On the other hand, mixtures of peat and the by-products used in this study were effective in supporting potentially beneficial bacteria to grow abundantly, which indicates a synergistic effect (Lü *et al*., 2009) of the by-products on bacterial growth. Our results agree with Raaijmakers and Weller (1998) who reported establishment of beneficial bacterial population densities above the threshold level and transfer of suppressiveness to conducive soils by adding both types of soils. The ability of the mixture materials to support abundant bacterial populations is important if specific bacteria selected from the profile data (with a well identified beneficial role at pinning) is to be inoculated into casing material to enhance pinning. Raaijmakers and Weller (1998) pointed out that soil disease suppression due to individual or selected groups of micro-organisms is transferable. Similarly, further investigations need to be done on selected beneficial bacteria in peat and transferability of their role at pinning.

Pasteurising casing materials is practiced by some growers for the purpose of eliminating disease causing organisms. From this research, using DGGE it is clear that pasteurisation did not eliminate the natural bacteria in the casing materials and allowed for a rapid re-establishment of these populations reflected by increased population densities at pinning stage. Our results are in agreement with Ryckeboer *et al*. (2003), who reported a decrease in number of mesophilic bacteria during heat treatment of compost followed by an increase during cooling stage. Klamer and Bååth (1998) also found that Gram-negative bacteria decreased during peak heat treatments of compost but increased again when temperature was lowered.

The Jaccard group average diversity analysis of samples from casing mixtures showed a general tendency of grouping samples based on its origin (i.e. the industrial by-product used to prepare the mixture), though not a very distinct grouping. This grouping reflects that bacterial populations in the industrial by-products constitute part of the mixtures' bacterial profiles. Although further investigations are needed, these results suggest that bacteria from the by-products might as well be involved in pinning as dynamic and diverse communities

can functionally be similar (Fernández *et al*., 1999; Ariesyady *et al*., 2007). The cluster analysis for peat samples, however, tends to group samples in relation to the mushroom growth stages. This reveals that the natural bacterial community structures in peat samples were not affected by pasteurisation or peat sources used. These results reflect resilience of bacterial populations in peat, which is the ability to recover to the original system after disturbance (pasteurisation in this case) as described by Nannipieri *et al*. (2003). Most peat samples at casing, irrespective of the source (from the farm or the mine), clustered together in two clades reflecting undisturbed bacterial communities during farm storage.

In conclusion, several species were indicated as part of the bacterial profile of the peat casing materials and as potential groups in the pinhead initiation process. Pasteurisation caused a reduction in bacterial population size in mushroom casing materials. However, by the time of pinning populations were re-established. Peat-based mixtures of alternative materials coir, wattle bark, bagasse and filter cake produced comparable profile to that of peat alone. Moreover, these materials are able to harbour high numbers of bacteria, and can successfully be used for casing mushrooms when mixed with peat.

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CHAPTER FIVE

EFFECT OF CASING BACTERIA ON GROWTH STIMULATION AND YIELD OF MUSHROOMS AND DISEASE SUPPRESSION BY BACTERIA AND YEASTS FROM CASING, COMPOST AND MUSHROOMS

Abstract

Casing soil in the production of white button mushrooms [*Agaricus bisporus*] provides an ideal microbial niche, especially for bacteria that are involved in fructification. Casing bacteria were investigated for other effects on *A. bisporus* both *in vitro* and by conducting production trials. Bacterial effects on mycelial growth of *A. bisporus* and mushroom yield were examined. Maximum *in vitro* growth stimulation by three *Pseudomonas* isolates, one *Arthrobacter* sp. and their mixture with fifteen other isolates was observed. A positive correlation was also found between total bacteria in different casing materials (peat-based mixtures of sugarcane bagasse and filter cake) and mushroom yield. *In vitro* disease suppression by bacteria and yeasts isolated from casing, compost and mushrooms against common fungal pathogens of the white button mushroom was also assessed. Different bacteria and yeasts showed inhibition effects on *in vitro* growth of *Verticillium fungicola* var *aleophilum*, *V. psalliotae*, *Mycogone perniciosa*, *Cladobotryum dendroides*, *C. mycophilum*, *Trichoderma aggressivum* f. *europaeum*, *T. aggressivum* f. *aggressivum* and *T. harizianum*.

Introduction

Casing layer plays an important role in white button mushroom [*Agaricus bisporus* (Lange) Imbach] commercial production. Application of a casing layer on top of compost colonised by mushroom mycelium is necessary for optimal fruiting of this mushroom species. This fructification process is related to the presence of some bacteria. These bacteria are involved in the transformation process of vegetative mushroom mycelium to its reproductive stage (Hayes *et al*., 1969; Miller *et al*., 1995; Fermor *et al*., 2000). This process, that is, fruit body initiation or its visible stage pinning, is important stage in the commercial mushroom production cycle. Several studies have been done in terms of the role of bacteria in this

process (Hayes *et al*. 1969; Miller *et al*., 1995; Fermor *et al*., 2000; Noble *et al*., 2003; Noble *et al*., 2009).

Bacteria can stimulate growth of different associated organisms. Fungal growth stimulation by bacteria is well reported (Sbrana *et al*., 2002; Wheatly, 2002; Hildebrandt *et al*., 2006; Kai *et al*., 2009). However, very few reports are available on mycelial stimulation of mushrooms by bacteria. Grewal and Hand (1992) reported a significant increase in mycelial growth of *A. bisporus* in the presence of *Pseudomonas* spp. that were not isolated from casing, but a nematode. Cochet *et al*. (1992) described a positive influence of casing bacteria on the growth of *A. bisporus*. Therefore, more studies on mycelial growth stimulation of *A. bisporus* needed to be done by screening dominant casing bacteria.

Yield of mushrooms is influenced by several factors such as temperature, water and nutrient supplements (Jodon *et al*., 1981; Kalberer, 1991; Seaby, 1999; Royse and Sanchez, 2008). However, there is paucity of information regarding the effect of bacteria on mushroom yield. Han (1999) reported that spraying suspensions of photosynthetic bacteria on to casing increased mushroom yield significantly. However, information on the effect of casing bacteria on mushroom yield is not available. Therefore, the relationship between bacteria in the casing and mushroom yield needs to be examined.

Disease suppression is another ecological role of certain soil micro-organisms. Bacteria and fungi that were isolated from compost, soil, plant material and mushroom farms have been reported as biocontrol agents of white button mushroom diseases. A *Bacillus* sp. was isolated from phase I compost and was found to control olive green mould pathogen (*Chaetomium olivaceum* Cooke and Ellis) (Tautorus and Townsley, 1983). Fermor *et al*. (1991) reported that fluorescent *Pseudomonas* spp. isolated from mushroom farms controlled the causal agent of brown blotch disease, *Pseudomonas tolaasii* Paine. Tsukamoto *et al*. (2002) reported that bacteria isolated from wild mushrooms, namely *Mycetocola tolaasinivorans* Tsukamoto *et al*., *M. lacteus* Tsukamoto *et al*., *Sphingobacterium multivorum* (Holmes *et al*.) Yabuuchi *et al*., *Bacillus pumilus* Meyer and Gottheil and *Pedobacter* sp., controlled brown blotch by detoxifying tolaasin. Bacteria isolated from casing mixtures (Singh *et al*., 2000) and healthy mushroom caps (Bora and Özaktan, 2000) were tested against fungal pathogens such as *Mycogone perniciosa* (Magnus) Delacr., *Verticillium fungicola* (Preuss) Hassebr, *Trichoderma harzianum* Tul., *Cladobotryum dendroides* (Bulliard: Fries) Gams & Hoozemans and *P. tolaasii*. Some of the isolates were reported to suppress the growth of

pathogens. To the best of my knowledge, studies on biological control of mushroom diseases using yeasts have not been reported.

In the present study, different bacteria isolated from the casing were examined for their effect on mycelial growth of *A. bisporus*. Mushroom yield assessments were done in relation to casing bacteria. *In-vitro* tests were done on antagonistic effect of different bacteria and yeasts isolated from casing, compost and mushrooms against fungal mushroom pathogens. In this chapter we therefore report on 1) effect of bacteria on mushroom yield and mycelial growth; and 2) antagonistic effect of bacteria and yeasts against fungal mushroom pathogens.

Materials and Methods

Bacteria for Growth Stimulation

Bacterial treatments were prepared from ninteen bacterial isolates previously isolated from casing media (chapter three). These isolates belonged to *Microbacterium* spp., *Arthrobacter* spp., *Pseudomonas* spp., *Bacillus* spp., *Ensifor* sp. and *Sphingobacterium* sp. Suspensions of each bacteria and their mixture were prepared from 24 h colonies grown on Standard I Nutrient Agar (STD I) (Merck, Johannesburg, South Africa) at 25^{o}C.

In Vitro Growth Stimulation

Spawn of two strains: the commercial strains A15 and A737 were obtained from Sylvan Africa (Pty) Ltd (Pretoria, South Africa). Sterile 90-mm Petri dishes containing malt extract agar (MEA) (Merck) were prepared and 3 g of autoclaved casing material, peat/filter cake (1:1), was placed on one side of each plate. Seven spawn seeds from each strain were placed in line in the centre of each prepared plate. Casing material was covered with 3 ml bacterial suspension of 10^{8} cfu's and with sterile water in the control plate. Duplicate plates were incubated in a 25 ^{o}C for seven days and the experiment was repeated. Growth percentage of mycelium on the casing was calculated from the radius of mycelial coverage.

Pot trial: Bacteria and Yield

Compost and Casing Preparations: Compost was collected from Highveld Mushroom Farm (Krugersdorp), filled in 18-cm diameter clean flower pots. Each pot contained 800 g of compost and was cased with 500 g of casing preparation. The different casings used were

peat inoculated with bacteria isolated from peat, 1:1 peat/bagasse, 1:1 peat/filter cake; 1:1 peat/bagasse inoculated with bacteria isolated from 1:1 peat/bagasse and 1:1 peat/filter cake inoculated with bacteria isolated from 1:1 peat/filter cake. Uninoculated casings were used as controls.

Inoculum Preparation and Application: Casing mixtures 1:1 peat/bagasse and 1:1 peat/filter cake were selected as sources of inocula. This selection was because of correlations between total bacteria in these mixtures and yield of mushrooms being observed previously (unpublished). An amount of 2 g of casing mixture was added to 200 ml of nutrient broth (Merck) in 500-ml Erlenmeyer flask. The mixture was incubated in a shaking incubator at 25^{o}C for 48 h and then centrifuged at 6000 rpm for 10 min and centrifuging was repeated with sterile water to wash the broth. Bacterial suspensions were prepared by adding bacterial pellets to 1 L of sterile distilled H_2O in an Erlenmeyer flask. To each pot, 140 ml of the suspension containing 10^8 cfu's was applied after eight days of casing, prior to pinning. Growing conditions were as described in chapter 3. First flash mushrooms were harvested and weight was recorded.

Semi-Commercial Growth Stimulation, Pinning and Yield Trial

Compost and Casing: Phase III compost was collected from Highveld Mushroom Farm in metal baskets containing 10 kg of compost and were transported to the semi-commercial growing unit at the University of Pretoria. Peat casing material was autoclaved twice at 75^{o}C for 24 h. Three kilograms were used to case each basket of compost. Baskets were in four replicates for each inoculum and one set for the control. Mushroom growing conditions were as described in chapter 3.

Inoculum Preparation and Inoculation: Six bacterial isolates previously isolated from casing media (chapter three) were used to prepare the different inocula. Three isolates belonged to *Pseudomonas* spp. and the remainder belonged to *Microbacterium* spp., *Arthrobacter* spp. and *Sphingobacterium* spp. Six inocula were prepared from the individual isolates and a seventh inoculum by mixing all six isolates. Cultures were grown on STD I (Merck) media for 24 h. Two loops full of colonies from these cultures were transferred into 1 L flasks containing 900 ml nutrient broth (Merck). The broth was shake incubated at 150 rpm at 25^{o}C for 48 h and centrifuged at 3500 rpm for 25 min in a High Performance Centrifuge (Avanti^{TM} J-25, Beckman, California). Pellets were washed with sterile Ringer's

solution (Merck) and centrifuged as above. A suspension of the pellets from the 900 ml of broth was prepared in 500 ml of sterile Ringer's solution. Two 900-ml flasks were used to obtain pellets for each isolate and a total of 1 L suspension was prepared from the pellets for each isolate. An inoculum of mixed bacteria was also prepared by mixing equal parts of suspensions of the six isolates. Finally, 200 ml of each inoculum suspension was added to 800 ml of sterile H_2O and drenched into the prepared casing just before casing. Bacterial inoculation treatments were done in four replicates. Four baskets of controls were also drenched with 1L of sterile H_2O.

Mycelial coverage of the casing was evaluated on a scale using scores 1-5, where 1 is no growth, 2 is ≤ 25%, 3 is 25-50%, 4 is 50-75% and 5 is 75-100% growth. when the mycelia reached the top of the casing. And finally, mushroom yield was determined for two flushes as described before.

***In Vitro* Biocontrol Analysis**

The antagonistic effect of different bacteria and yeasts against common fungal pathogens of the white button mushroom was determined *in vitro* using the dual culture technique (Boshoff, 2005). Fungal pathogens of mushrooms were obtained from Dr. Linda Meyer (Department of Microbiology and Plant Pathology, University of Pretoria). These pathogens were *Verticillium fungicola* var *aleophilum*, *V. psalliotae*, *Cladobotryum dendroides*, *Mycogone perniciosa*, *Trichoderma aggressivum* f. *europaeum*, *T. aggressivum* f. *aggressivum* and *T. harizianum*. Different bacteria and yeasts evaluated for potential antagonistic effect were isolated from compost, casing and mushrooms (chapters three and six). A 4-mm diameter agar disc colonised by the pathogenic fungus was placed on the centre of a 90-mm potato dextrose agar (PDA) (Merck) plate. A bacterial or yeast isolate was streaked on two opposite sides of the PDA plate at a 40-mm and 25-mm distance away from the centre for fast and slow growing fungi respectively. Control plates of each fungus were grown without bacteria or yeast. Plates were incubated at 25°C for 3-10 days. Growth diameters of the pathogens were measured between bacterial streaks and free sides. The difference between these diameters was used to analyse if there was significant growth inhibition by the bacteria or yeasts. This experiment was done in four replicates. Data was analysed using GenStat Discovery Edition 3 (VSN International Ltd). Growth inhibition of the pathogens by the different bacteria and yeasts was determined using one-way analysis of variance (ANOVA). Significant growth inhibition was considered at $P < 0.05$.

Results

Growth Stimulation

The degree of *in vitro* growth stimulation by the different isolates of bacteria varied from none to complete coverage of the casing material. The three *Pseudomonas* isolates, an *Arthrobacter* sp. and the mixed bacteria demonstrated maximum scores of growth stimulation. Two *Bacillus* spp. and another *Arthrobacter* sp. showed no growth stimulation at all, especially on the commercial *Agaricus* strain A15 (Table 1).

Table 1. *In vitro* growth stimulation of *Agaricus bisporus* by casing bacteria

Inoculated bacterial		Average growth stimulation percentage of *A. bisporus* isolates	
Isolate code	**Species**	**A15**	**737**
	Control	0	0
B1	*Microbacterium* sp.	22.5^{a}	45^{cd}
B2	*Microbacterium* sp.	67.5^{d}	72.5^{e}
B4	*Microbacterium* sp.	45^{bc}	50^{d}
B5	*Ensifor adhaerens*	22.5^{a}	15.5^{a}
B6	*Arthrobacter* sp.	72.5^{d}	50^{d}
B7	*Arthrobacter* sp.	100^{e}	97.5^{f}
B8	*Microbacterium* sp.	50^{c}	47.5^{d}
B9	*Microbacterium* sp.	100^{e}	50^{d}
B10	*Microbacterium* sp.	72.5^{d}	75^{e}
B11	*Microbacterium* sp.	17.5^{a}	22.5^{b}
B12	*Sphingobacterium* sp.	17.5^{a}	42.5^{c}
B13	*Arthrobacter* sp.	67.5^{d}	42.5^{c}
B14	*Arthrobacter* sp.	17.5^{a}	42.5^{c}
B15	*Sporosarcina* sp.	40.0^{b}	45^{cd}
B16	*Bacillus* sp.	0	0
B17	*Pseudomonas* sp..	100^{e}	100^{f}
B18	*Pseudomonas* putida	100^{e}	100^{f}
B19	*Bacillus* sp.	0	40^{c}
B20	*Pseudomonas* putida	100^{e}	100^{f}

Bmix	Mixed	100[e]	100[f]
LSD		5.785	5.558
F pr.		<0.001	<0.001

In each column, values with different letters are significantly different.

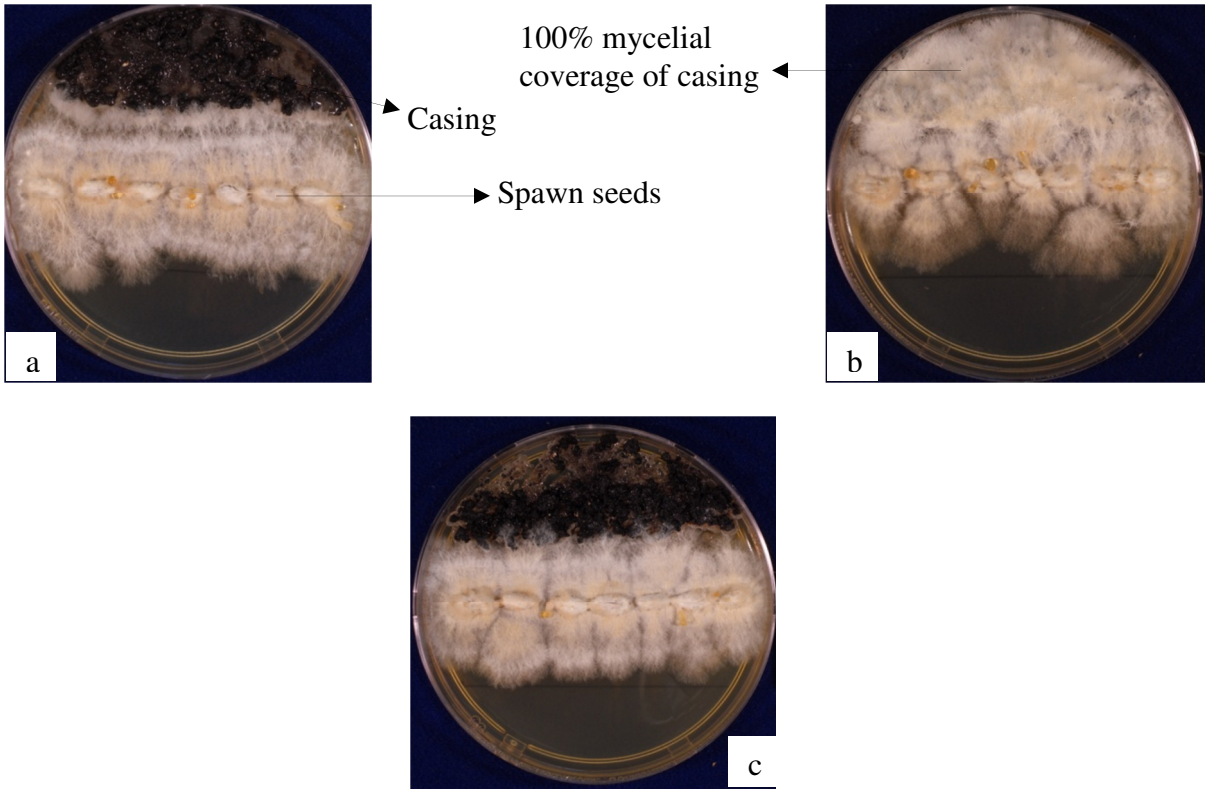
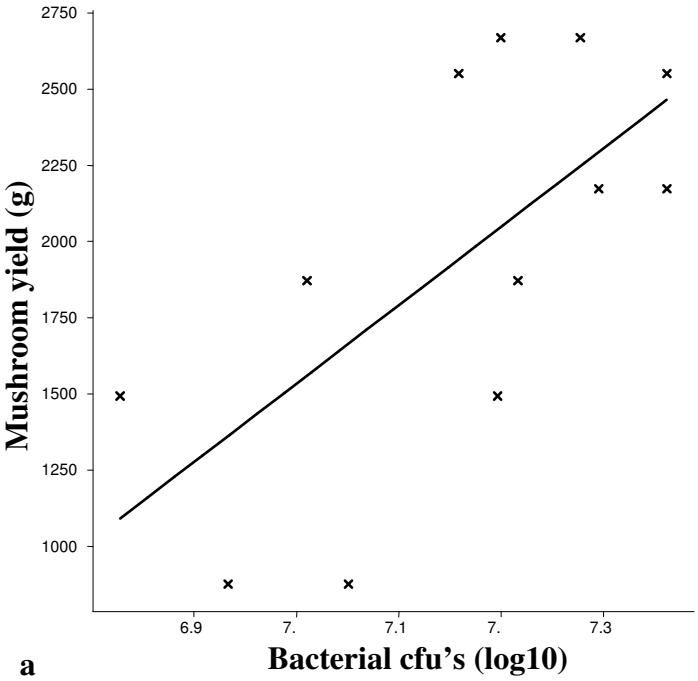


Figure 1. Growth stimulation of *Agaricus bisporus* on malt extract agar and casing soil inoculated with *Microbacterium* sp. (a), *Pseudomonas* sp. (b) and control (c).

Casing Bacteria and Mushroom Yield

In the mushroom growing trials in Chapter 3, correlations were observed between total bacteria in casing media and yield of mushrooms (Figure 2).



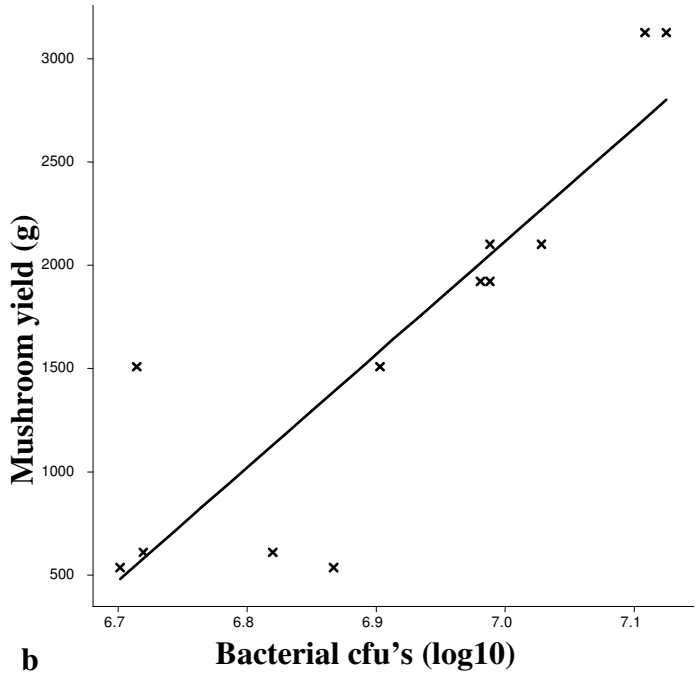
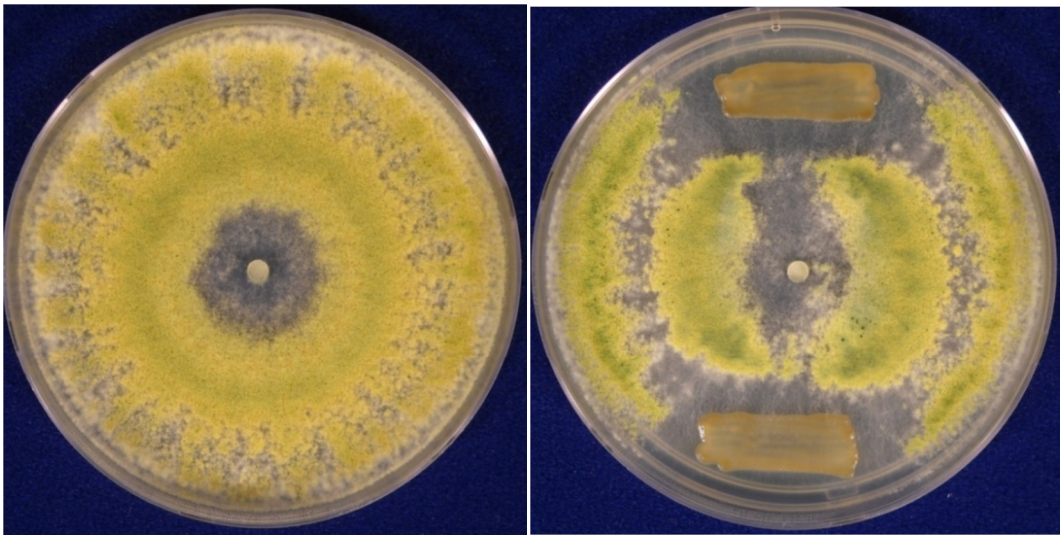


Figure 2. Correlations between mushroom yield and total bacteria in 1:1 peat/filter cake (r=0.674, F pr < 0.016) (a) and 1:1 peat/bagasse (r=0.875, F pr < 0.001) (b).

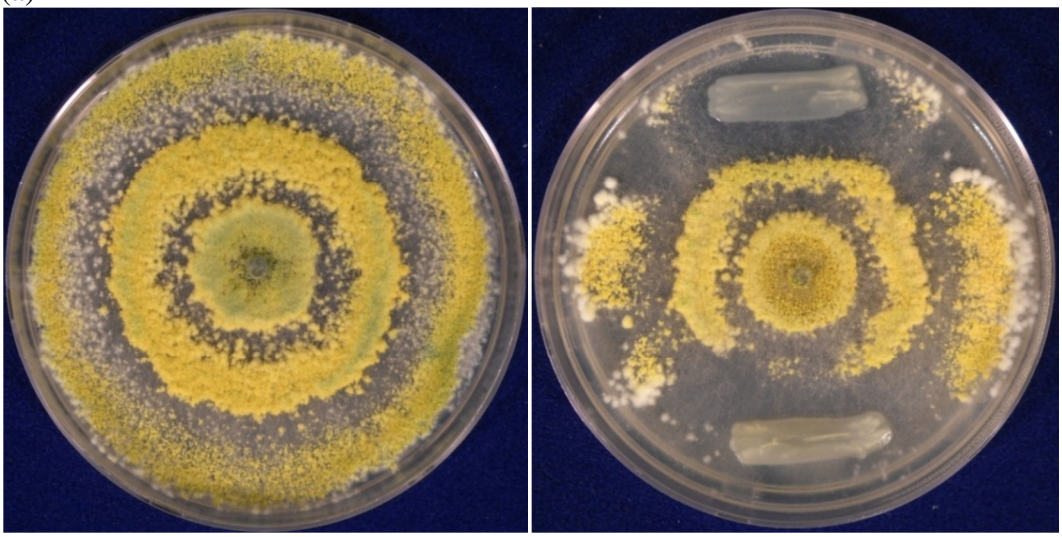
Yield results from inoculated casings, however, did not show significant difference from that of un-inoculated casing.

Semi-Commercial Trial of Growth Stimulation, Pinning and Yield

Results of mycelial growth stimulation and mushroom yield from the semi-commercial trial in the mushroom growing unit at the University of Pretoria did not show significant differences between bacterial treatments and non-inoculated controls. Different degrees of suppression were observed between 35 bacterial and 20 yeast isolates tested for antagonistic effect against fungal mushroom pathogens. Twenty four bacterial and 15 yeast isolates suppressed growth of the pathogens.



(a)



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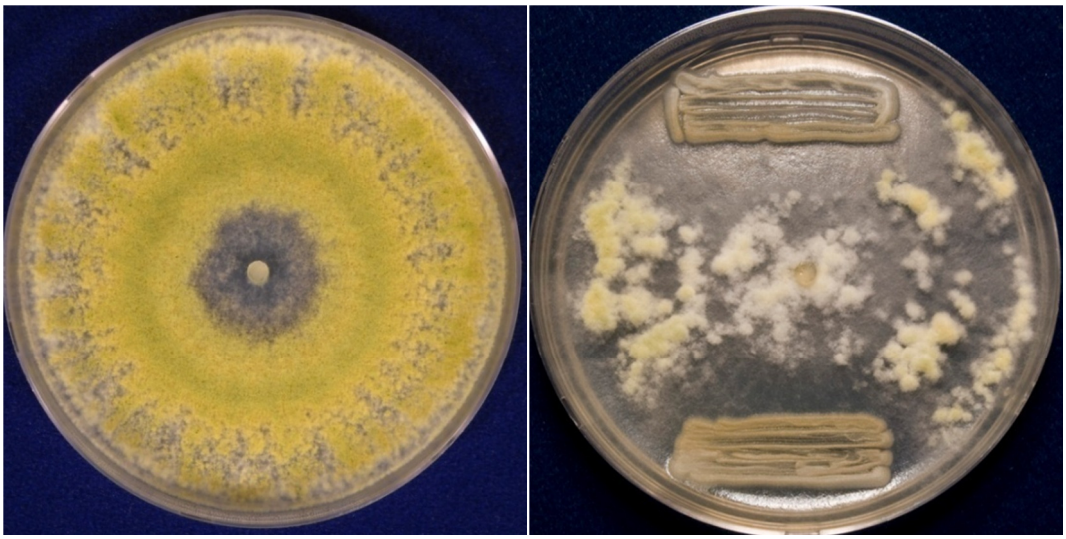
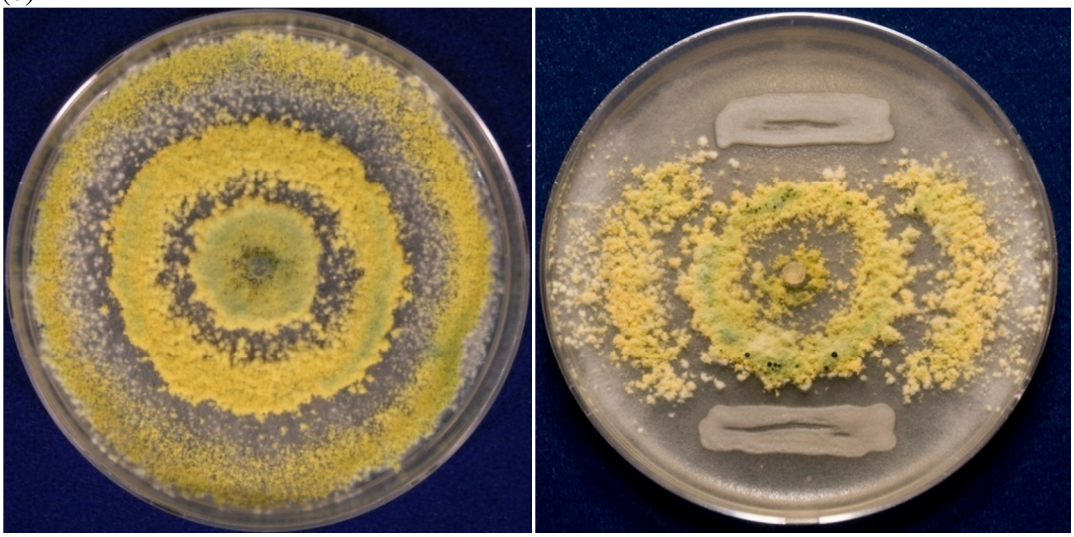
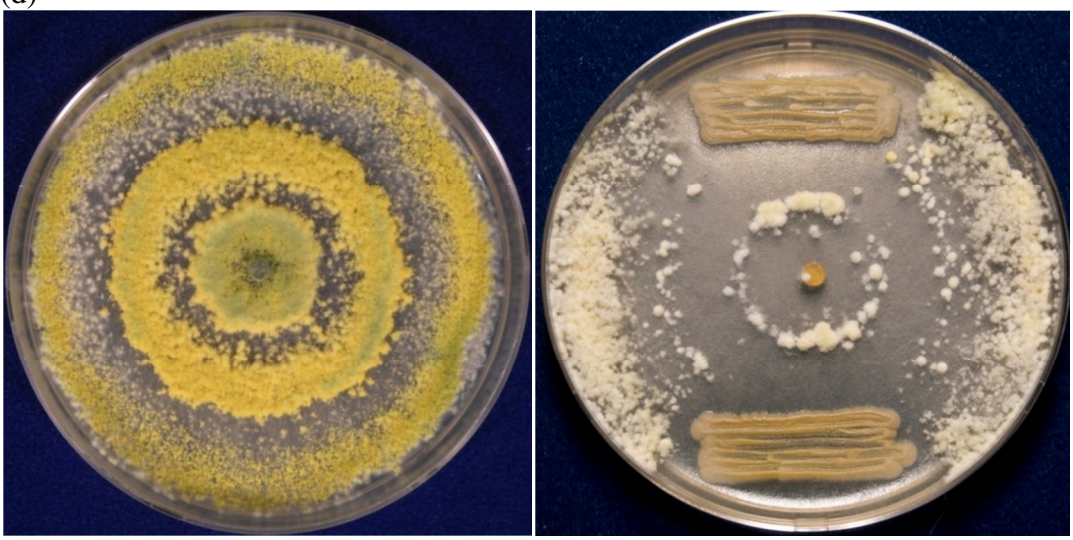


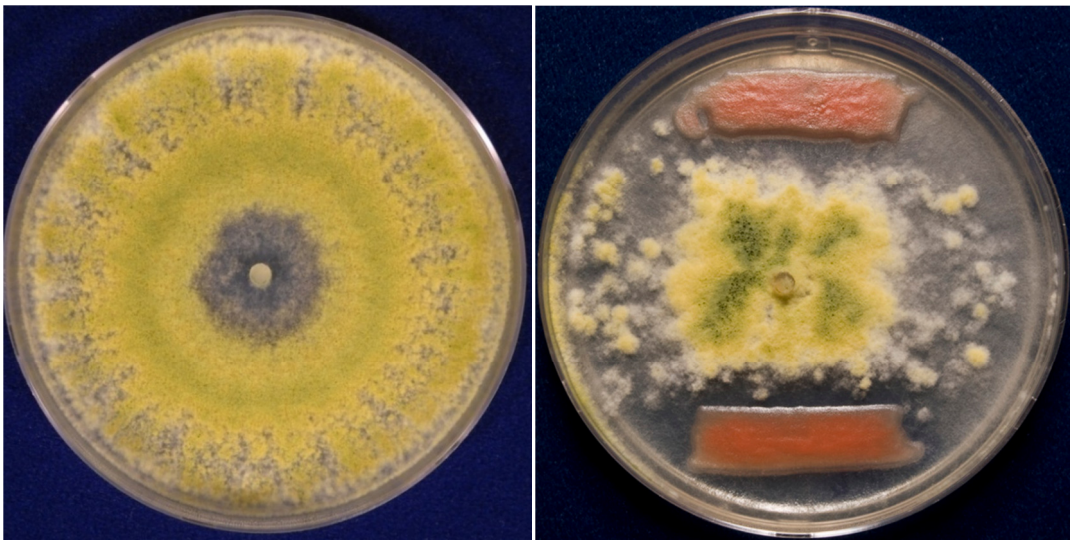
(c)



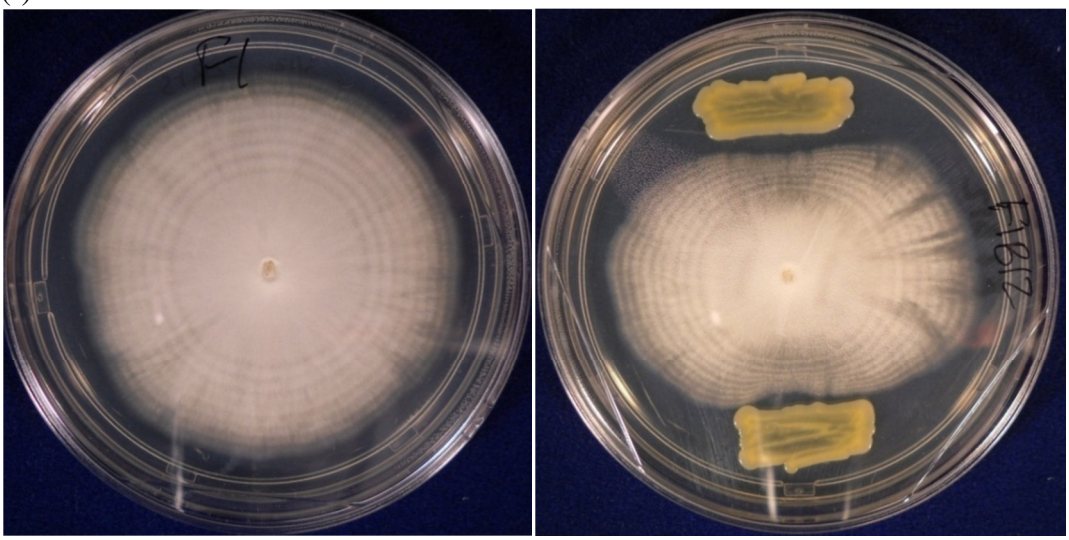
(d)



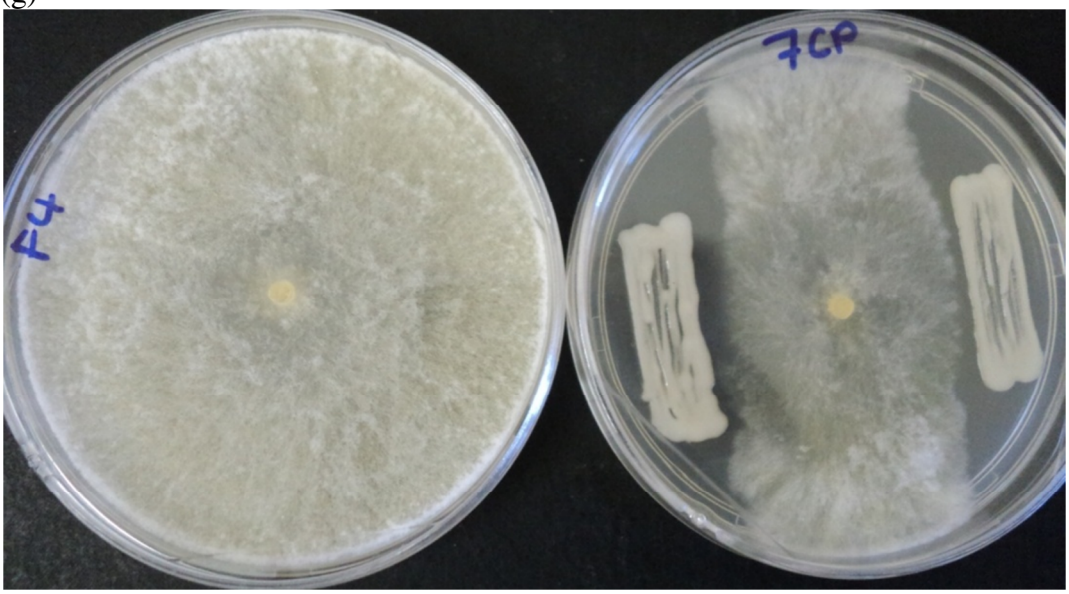
(e)



(f)



(g)



(h)

Figure 3. *In vitro* suppression of mushroom fungal pathogens by bacteria and yeasts: a) *Arthrobacter* sp. against *Trichoderma harizianum* b) *Microbacterium* sp. against *T. aggressivum* f. *aggressivum* c) against *Trichoderma harizianum* d) against *T. aggressivum* f. *aggressivum* e) against *T. aggressivum* f. *aggressivum* f) against *Trichoderma harizianum* g) *Arthrobacter* sp. against *Verticillium fungicola* h) *Trichosporon moniliiforme* against *Cladobotryum dendroides*.

Table 2. *In vitro* antagonistic effect of different bacterial isolates against common fungal pathogens of the white button mushroom

Bacteria	**Growth inhibition (mm) of pathogen**							
Closest relative to	*Verticillium psalliotae*	*V. fungicola* var *aleophilum*	*Cladobotryum dendroides*	*C. mycophilum*	*Mycogone perniciosa*	*Trichoderma harizianum*	*T. aggressivum* f. *europaeum*	*T. aggressivum* f. *aggressivum*
Control	0	0	0	0	0	0	0	0
***Enterobacter* sp**	13.25	13.25	40	-	25	15	-	-
***Bosea* sp**	10.25	6.25	-	-	40	45	30	20
***Acidovorax* sp**	15.75	4.25	-	-	-	-	-	-
***Acidovorax* sp**	14	6.25	-	-	-	45	20	-
***Enterobacter* sp**	9	9.75	40	-	30	-	-	-
***Citrobacter* sp**	13	12.5	32.5	-	-	40	-	40
Paracoccus koreensis	7.25	5.25	-	-	-	10	20	-
Sphingobacterium multivorum	14	17.5	48	-	31.5	-	-	-

Table 2 (continued)

Bacteria	**Growth inhibition (mm) of pathogen**							
Closest relative to	*Verticillium psalliotae*	*V. fungicola* var *aleophilum*	*Cladobotryum dendroides*	*C. mycophilum*	*Mycogone perniciosa*	*Trichoderma harizianum*	*T. aggressivum* f. *europaeum*	*T. aggressivum* f. *aggressivum*
***Arthrobacter* sp**	4	12.25	-	-	-	-	-	-
Microbacterium foliorum	5.75	-	-	-	-	-	-	-
Shinella kummerowiae	5	2.5	-	-	-	10	-	-
***Pseudaminobacter* sp**	3.25	-	-	-	-	10	-	-
***Arthrobacter* sp**	4.5	12.5	-	-	20	45	-	40
Serratia marcescens	8.75	15.25	15	-	30	-	-	-
***Arthrobacter* sp**	11	9.75	-	-	20	35	-	20
***Bosea* sp**	9	-	-	-	-	40	40	30

Table 2 (continued)

Bacteria	**Growth inhibition (mm) of pathogen**							
Closest relative to	*Verticillium psalliotae*	*V. fungicola* var *aleophilum*	*Cladobotryum dendroides*	*C. mycophilum*	*Mycogone perniciosa*	*Trichoderma harizianum*	*T. aggressivum* f. *europaeum*	*T. aggressivum* f. *aggressivum*
***Sphingobacterium* sp**	17	19.25	45	-	40	50	-	30
Microbacterium esteraromaticum	-	-	28	-	-	39.5	-	45.5
***Arthrobacter* sp.**	-	-	-	-	-	36	-	11.6
***Arthrobacter* sp.**	-	-	-	-	-	39.8	-	-
***Sphingobacterium* sp.**	3	-	25.8	38	26	37	53	35.8
***Arthrobacter* sp.**	-	-	-	-	-	37	-	0
***Arthrobacter* sp.**	-	13	-	-	-	35	-	0
Pseudomonas putida	-	5.8	-	-	-	0	-	31.5
LSD	3.1							
F pr.	<0.001							

NB. '-' represents 0 or non significant inhibition

Table 3. *In vitro* antagonistic effect of different yeast isolates against common fungal pathogens of the white button mushroom

Yeast	**Growth inhibition (mm) of pathogen**					
Closest relative to	*Verticillium fungicola* var *aleophilum*	*Cladobotryum dendroides*	*C. mycophilum*	*Mycogone perniciosa*	*Trichoderma harizianum*	*T. aggressivum* f. *aggressivum*
Control	0	0	0	0	0	0
Cystofilobasidium infirmominiatum	7.75	8.75	29.75	30	-	-
***Candida* sp**	5.5	8	17.75	43	60	45
Rhodotorula mucilaginosa	7	15.75	7.75	40	55	50
Trichosporon jirovecii	6.75	-	29.5	30	-	56
Cryptococcus macerans	6	14.75	9.5	40	-	-
Trichosporon cutaneum	7	11	24.25	40	-	-
Trichosporon moniliiforme	6	10	16.25	35	-	-
***Candida* sp**	4.5	4.75	14.5	43.75	59.5	45
***Candida* sp**	5.5	7	22.25	43	50	45

Table 3 (continued)

Yeast	**Growth inhibition (mm) of pathogen**					
Closest relative to	*Verticillium fungicola* var *aleophilum*	*Cladobotryum dendroides*	*C. mycophilum*	*Mycogone perniciosa*	*Trichoderma harizianum*	*T. aggressivum* f. *aggressivum*
Trichosporon moniliiforme	7.25	13.5	14	45	-	55
Trichosporon moniliiforme	6.75	6.75	23.75	37.5	53.5	50
Trichosporon moniliiforme	7.25	-	13.75	40	50	50
Candida glaebosa	8	18.75	8.75	50	60	58.75
Cystofilobasidium infirmominiatum	8	18.5	10.5	40	-	-
***Rhodotorula* sp.**	6.75	10.5	26	53.75	55	53.75
LSD	3.974					
F pr.	<0.001					

NB. '-' represents 0 or non significant inhibition

Discussion

In this study, variations in growth stimulation of *A. bisporus* by bacteria isolated from casing materials were found. Maximum growth stimulation was obtained by the *Pseudomonas* isolates, an *Arthrobacter* sp. and a mixture of 19 isolates, while the *Bacillus* isolates alone had no effect. Several researchers reported similar growth stimulation effect by these bacterial species. However, the bacteria were isolated from different sources or the research work was done on other mushroom species. Grewal and Hand (1992) reported that *Pseudomonas* spp. isolated from a nematode stimulated the mycelial growth of *A. bisporus*. A *Pseudomonas* sp. was also reported to promote mycelial growth of *Pleurotus eryngii* (Kim *et al*., 2008a). Kim *et al*. (2008b) reported a varying degree of *in vitro* growth inhibition on different mushroom species by *Bacillus* spp. Some of the *Bacillus* isolates had no effect on the mushroom species, which is in agreement with our results. A significant increase in mycelial growth of *Agaricus blazei* by an *Arthrobacter* sp. and *Pseudomonas* spp. was found by Young *et al*. (2012). They also reported that a *Bacillus* sp. had no significant effect on *A. blazei*'s mycelial growth stimulation, which is in accordance with results in this present work.

Our results demonstrated a positive correlation between mushroom yield and total bacteria in peat-based casing mixtures of sugarcane bagasse and filter cake. However, there was not any correlation between total bacteria in peat casing and mushroom yield. Therefore, effect of bacteria on mushroom yield might not be noticeable on common casing materials such as peat. This could be one reason for fewer studies on casing bacteria and mushroom yield compared to other growing conditions such as temperature, water and nutrient supplements (Jodon *et al*., 1981; Kalberer, 1991; Seaby, 1999; Royse and Sanchez, 2008). However, more investigations need to be done on casing bacteria and mushroom yield to examine whether the yield increase was as a result of increased bacterial populations or other reasons.

In the current study, bacteria and yeasts isolated from the casing, compost and mushrooms showed different degrees of suppressing fungal pathogens of *A. bisporus*. A greater number of bacterial isolates suppressed the growth of *Trichoderma* compared to

other fungal pathogens tested. This suppression of *Trichoderma* by the natural compost or casing microflora is important in controlling the disease, as it is reported that the success of *Trichoderma* strains was dependent on their competitive ability against indigenous bacteria (Naar & Kecskés, 1998). Savoie *et al*. (2001) investigated specifically the colonisation of mushroom compost by *T. aggressivum europaeum* (Th2) and found it was mainly due to the pathogen's ability to tolerate the inhibitory effect of compost bacteria. Our study indicates that *T. aggressivum europaeum* was least affected by bacterial isolates, compared to the other two *Trichoderma* species tested, and it was not affected by any of the yeasts tested. Although the presence of other *Trichoderma* species and *T. harzianum* in compost and casing materials (Eicker, 1980; Eicker and van Greuning, 1989) have been reported, a study on the presence and prevalence of *T. aggressivum europaeum* and *T. aggressivum aggressivum* in South African mushroom production is not available. *T. aggressivum europaeum* was originally reported in European mushroom farms (Kredics *et al*., 2010). Based on observations and research results obtained in this study, *T. aggressivum europaeum* can be more aggressive than the two *Trichoderma* species tested. Thus, further studies need to be done to confirm results as well as to investigate the presence and prevalence of this pathogen in South Arican mushroom farms.

Growth of fungal pathogens causing dry bubble, wet bubble and cobweb of mushrooms was also suppressed by bacterial and yeast isolates. On some of the pathogens, yeast isolates showed better control compared with bacterial isolates and on others vice versa. One of the dry bubble pathogens, *V. psalliotae*, was inhibited by 18 of the bacterial isolates but by none of the yeast isolates. On the other hand, *C. dendroides* was inhibited by only one bacterial isolate, whereas it was suppressed by 13 of the yeast isolates. As El-Tarabily and Sivasithamparam (2006) mentioned, compared with bacteria very little attention has been given to yeasts as biocontrol agents of soil borne fungal pathogens. However, yeasts should be given more attention as they have growth suppressive potential and on some pathogenic fungi show even significantly higher inhibition than bacteria.

In conclusion, soil is a microbiologically dynamic habitat and casing soil and compost used in the production of white button mushrooms harbour many micro-organisms that are involved in important processes in the overall production cycle. In this study, *in vitro* experiments showed that some bacteria naturally inhabiting the casing could stimulate mycelial growth of *A. bisporus*. Semi-commercial trials indicated a positive correlation between total bacteria in different casing materials and mushroom yield. Bacteria and yeasts isolated from casing soil and compost were found to suppress some fungal pathogens of white button mushrooms in *in vitro* dual culture studies, namely *V. fungicola* var *aleophilum*, *V. psalliotae*, *M. perniciosa*, *C. dendroides*, *C. mycophilum*, *T. aggressivum* f. *europaeum*, *T. aggressivum* f. *aggressivum* and *T. harizianum*. Further studies should be done investigating the effect of casing bacteria on mycelial growth stimulation of *A. bisporus* and mushroom yield in a semi-commercial scale. *In vivo* and semi-commercial studies should also be done on the antagonistic effect of the potential bacterial and yeast isolates in this study.

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CHAPTER SIX

ABUNDANCE, DIVERSITY AND PHYLOGENETIC PROFILES OF BACTERIA, FUNGI AND YEASTS IN COMPOST, CASING AND ON MUSHROOMS

[1]Nazareth. A. Siyoum , [2]Karen Surridge, and [1]Lise Korsten
[1] *Department of Microbiology and Plant Pathology, Faculty of Natural and Agricultural Sciences, University of Pretoria*
[2]*Department of Plant Production and Soil Science, Faculty of Natural and Agricultural Sciences, University of Pretoria*

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Abstract

Micro-organisms play important roles in the production of white button mushrooms. Abundance, diversity and phylogenetic profiles of bacteria, fungi and yeasts in production substrates and on mushrooms were investigated using basic plating techniques, denaturing gradient gel electrophoresis (DGGE) and sequencing the partial 16S and 18S rDNA of dominant representative organisms. Bacteria and fungi were more abundant in compost than casing and mushroom samples. Yeasts were also more abundant in compost than in casing and on fresh mushrooms, however, there was no significant difference between mushrooms stored for four, eight and twelve days. The plating method reflected a more diverse bacterial population in casing and on mushrooms than in compost while the DGGE method revealed higher diversity of compost bacteria. Yeasts were more diverse in compost than in casing or on mushroom samples while the genus *Penicillium* was more prevalent in all sample sources. Phylogenetic analysis revealed a general trend of species grouped from the same sources while there was some overlap of species between different sample sources. For compost and casing bacteria studied using DGGE there were several species, forming separate lineages, demonstrating highly diverse communities in these samples.

Introduction

The white button mushrooms [*Agaricus bisporus* (Lange) Imbach] is commercially produced using two main substrates - compost and casing. Production of mushrooms is dependent on different microbiological processes occurring in these substrates. Similarly, postharvest quality of mushrooms is affected by micro-organisms present on the harvested product. Therefore, understanding microbiological properties of the substrates and the mushroom is important to increase efficiency of production and quality of the product.

Composting is a microbial breakdown process of different substrates that allows for fungal mycelial development. Mushroom compost is commercially prepared from grain (wheat in South Africa) straw, animal (chicken in South Africa) manure and gypsum (Labuschagne, 1995). Phase I compost, where substrates are mixed and self heated after wetting, is characterised by high microbial activities (Miller *et al*., 1989). Phase II compost is the result of aerobic microbial activities under controlled temperatures (Straatsma *et al*., 1994). Several micro-organisms are known to be associated with general composting processes of organic materials and were extensively reviewed by Ryckeboer *et al*. (2003a).

Casing medium that covers phase III (spawn run) compost in mushroom beds harbours saprophytic micro-organisms. Although different fungi and bacteria were isolated from mushroom casing medium by several researchers (Hayes *et al*., 1969; Eicker and van Greuning, 1989; Fermor *et al*., 2000; Noble *et al*., 2003), much of the work done focussed on bacteria. The role of bacteria in the casing medium is of importance in the process of mushroom fruit body initiation as well as establishment of pinning. In this layer, pseudomonads are the most commonly found bacteria associated with mushroom fruit body initiation. Saprophytic pseudomonads isolated from casing medium include *P. fluorescens*, *P. putida*, *P. reactans*, *P. tolaasii*, and *P. gingeri* (Fett *et al*., 1995). Fungi isolated from peat casing medium include *Aspergillus* sp., *Penicillium* sp., *Trichoderma* sp., *Geotrichium* sp. and *Chrysonilia* sp. (Labuschagne, 1995).

Bacteria, mostly pseudomonads were also isolated from mushrooms. These bacteria are either saprophytic or pathogenic. Saprophytic groups include *P. reactans* (Fett *et al*., 1995). Pathogenic species cause discoloration and other symptoms on the cap of growing mushrooms. Pseudomonads isolated from symptomatic mushrooms include *P. tolaasii*, *P. gingeri* and *P. agarici* (Fett *et al*., 1995). The pseudomonads *P. tolaasii*, *P. reactans* and *P. agarici* have also been reported to be responsible for postharvest browning and decay of mushrooms (Wells *et al*., 1996).

As mushrooms are produced in close physical contact with the casing layer and the compost, and these substrates also have direct contact with each other, the microbial ecology in the substrates could be interrelated with the mushroom microflora. The microbial profile of mushroom substrates (compost and casing) and harvested mushrooms as a complete overview is not available to the best of our knowledge. The objectives of this study were to 1) investigate microbial species richness and -diversity of mushroom substrates and mushrooms; 2) determine dominant taxonomic groups; and 3) examine phylogenetic relationships between micro-organisms in substrates used for production and freshly harvested and stored mushroom product. This knowledge will be useful in manipulating casing micro-flora with regard to pinning and disease control. The knowledge will also be essential in understanding the origin of harvested mushroom micro-flora and thereby in extending shelf life. Techniques used in this study were a cultural method - dilution plating and a culture-independent method - denaturing gradient gel electrophoresis (DGGE) combined with DNA sequencing.

Materials and Methods

Compost, Casing and Mushroom Samples

This experiment was conducted by collecting samples of compost, casing and mushrooms from Highveld Mushroom Farm operating on standard commercial production practices. Compost and fresh casing samples were collected in a randomised design in sterile plastic bags aseptically on the day mushroom beds were cased. Another batch of casing samples was collected at pinning from the same trays as previously sampled. Mushroom samples

were obtained from first flush picking. Mushrooms weighing 250 g were picked from the same trays sampled for compost and casing. Mushrooms were commercially packed in punnets and wrapped with commercial plastic cling film and transported back to the laboratory for immediate processing. Mushrooms were analysed fresh and after cold storage at 4°C for four, eight and twelve days to simulate marketing conditions. All samples were collected in eight replicates (from eight mushroom trays). Each compost, casing and mushroom sample was 250 g.

Microbial Enumeration

Dilution plating was performed as described in chapter three. For mushroom samples, 25 g of mushrooms were blended in a sterile stomacher bag with 250 ml 0.1% peptone buffer (Merck, Johannesburg, South Africa) using a stomacher (® 400 Circulator, Lasec, Johannesburg). Each sample was stomached at 300 r.p.m. for 2 min. A series of dilutions were prepared from the buffer suspension and the appropriate dilutions were plated. Bacterial enumeration was performed using standard I nutrient agar (STD I) (Merck) amended with cycloheximide (0.1%, Sigma, Johannesburg, South Africa) (10 ml/L of media) was used. Fungi and yeasts were grown in malt extract agar (Merck) amended with chloroamphenicol (250 mg/L, CAPS Pharmaceuticals, Johannesburg). Plates were incubated at 25°C for 3-7 days. Colonies were counted and isolations were made for pure cultures of representative isolates. Bacteria and yeast cultures were preserved in their respective media at 4 °C and in 15% glycerol at -70°C. Fungi were preserved in double sterile distilled water and slant agar. From pure cultures, DNA was extracted using ZR Fungal/Bacterial DNA Kit (Inqaba, Pretoria, South Africa) and isolates were identified by sequencing. Cultures were preserved at -70°C

DNA Extraction

Extraction of pure culture DNA was done using ZR Fungal/Bacterial DNA Kit (Inqaba, Pretoria) according to the manufacturer's instruction. DNA was extracted from a loop full of pure colonies. Total microbial DNAs for DGGE were extracted from compost, casing and mushrooms using ZR Soil Microbe DNA Kit (Inqaba, Pretoria) according to

the manufacturer's instruction, with some modification. An amount of 150 ml of the above stomached mushroom suspension was filtered through sterile filter papers (0.2 µm pore size) (Sartorius Stedim Biotech, Goettingen, Germany). DNA was extracted from the filtrate by soaking the filter paper in lysis solution. To increase DNA yield of all samples, the lysis solutions were disrupted using FastPrep FP 120 (Bio 101 Thermo Electron Corporation, Milford, USA) at 5 m s^{-1} for 20 s. DNA was finally eluted using 100 µl ddH_2O. Samples from four replicate mushroom trays were used for molecular analysis.

PCR Amplification

Enzymatic amplification of the 16S rDNA region was performed using the primers in chapter four. The reaction consisted of a total volume of 25 µl containing the following reagents: 19.25 µl of ddH_2O, 2.5 µl of PCR buffer, 1 µl of $MgCl_2$ (10x), 1 µl of dNTPs (2.5 µ mol l^{-1}), 0.25 µl of each primer (10 p mol l^{-1}), 0.25 µl of *Taq* DNA polymerase (5 units μl^{-1}) and 0.5 µl (~ 25 ng μl^{-1}) of sample DNA. PCR amplification was performed in an Eppendorf (Merck) thermal cycler starting with 10 min denaturation at 95°C followed by 35 cycles of 30 s denaturation at 94°C, 30 s annealing at 58°C, 1 min extension at 72°C, and a final 10 min extension at 72°C. Fungal and yeast DNA was amplified using the primers ITS3 (5'-CGC-CCG-CCG-CGC-GCG-GCG-GGC-GGG-GCG-GGG-GCA-CGG-GGG-GCA-TCG-ATG-AAG-AAC-GCA-GC-3') and ITS4 (5'-TCC-TCC-GCT-TAT-TGA-TAT-GC-3') (White *et al*., 1990). PCR was performed as above for bacteria except that the annealing temperature was lowered to 55°C. The amplicons were visualized on a 1% agarose gel stained with 0.01% ethidium bromide in a Vilber Lourmat (Omni-Science CC, Randburg, South Africa) gel imaging system.

DGGE, Excision of Bands and Reamplification of DNA

DGGE was performed using a D-Code (BIO-Rad, Johannesburg) universal mutation detection system. PCR products of 10 µl, each mixed with 3 µl loading dye were loaded onto 40–55% and 30–60 denaturing gradient polyacrylamide (40%) gels for bacterial and fungal (or yeast) DNA respectively. Electrophoresis was performed at 20 V for 10 min to

allow gels to acclimatise and then at 70 V at a temperature of 60°C for 17 h. Next the gels were stained with 4 µl SYBR Gold (Whitehead Scientific, Johannesburg) nucleic acid gel stain for 1 hour in the dark, then visualized and photographed under UV light in a Vilber Lourmat gel imaging system.

Amplification of bacterial DNA for DGGE was done using the same primers and procedures as in chapter five. Fungal DNA was amplified using the primers ITS3 (5'-CGC-CCG-CCG-CGC-GCG-GCG-GGC-GGG-GCG-GGG-GCA-CGG-GGG-GCA-TCG-ATG-AAG-AAC-GCA-GC-3') and ITS4 (5'-TCC-TCC-GCT-TAT-TGA-TAT-GC-3') (White *et al*., 1990). Dominant bands of bacteria, fungi and yeast were excised under blue light as in chapter 5 and re-amplification of DNA was done using their respective primers. Amplified DNA was sequenced as in chapter 5.

Gel Analysis and Phylogenetic Tree

Gel analysis was performed using Gel2k gel analysis software, where similarity between sample populations was compared using a Jaccard group average setting. This analysis is based on number of bands in the lanes. Amplified DNA from excised DGGE bands using the respective primers was sequenced. Sequences and electropherograms were edited using Chromas (Chromas Lite 2.01, 1998-2005 Technelysium Pty Ltd).

A BLAST search was done for each sequence on the GenBank database and matching hits, with highest percentage identity and e-values closest to 0.0 indicating a statistically acceptable match, were selected for alignment. Resulting sequences were edited using Contig express (Vector NTI advance 11.0, Invitrogen 2008), aligned with Clustal X and inserted gaps were treated as missing data. Analysis for Phylogenetic relationship was performed based on parsimony using PAUP 4.0b10 (Swofford, 2001) (Phylogenetic Analysis Using Parsimony). Random addition of sequences (100 replicates), tree bisection-reconnection, branch swapping, MULPAR-effective and MaxTrees were used to perform Heuristic searches. Tree length distributions over 100 randomly generated trees were evaluated to assess phylogenetic signals in the data sets. The consistency (CI) and retention indices (RI) were determined for all data sets. Phylogenetic trees were

rooted with *Nitrospira* sp. and *Dipodascus capitatus* as outgroups to the remaining taxa of bacteria and yeasts respectively. In order to determine confidence in branching points (1000 replicates), bootstrap values were generated retaining groups with greater than 70% consistency.

Statistical Analysis

Microbial counts of samples of compost, casing and mushrooms were compared by performing general analysis of variance (ANOVA) using GenStat Discovery Edition 3 (VSN International Ltd). Significant difference was considered at $P < 0.05$.

Results

Total Counts of Bacteria, Fungi and Yeasts

Total bacterial counts differed significantly between mushroom growing substrates, compost and casing (Figure 1). Bacterial colony counts were significantly higher for compost samples than in casing and mushroom samples. Fungal colony counts were also significantly higher for compost samples than casing samples at pinning and on freshly harvested mushroom samples. At casing, no significant difference in fungal counts could be found between samples obtained from compost and casing material. Total colony counts of yeasts were significantly higher for compost compared with casing and fresh mushroom samples (Figure 1). There was a significant increase of bacterial and yeast colony counts on mushrooms from being freshly harvested to twelve days of storage while fungi remained constant except for a decrease on the fourth day of storage (Figure 2). Total bacterial, fungal and yeast counts on fresh mushrooms were 5.765, 3.333 and 4.065 log cfu/g respectively. During the twelve days of storage, bacterial, fungal and yeast counts reached 7.600, 2.957 and 6.176 log cfu/g respectively (appendix II).

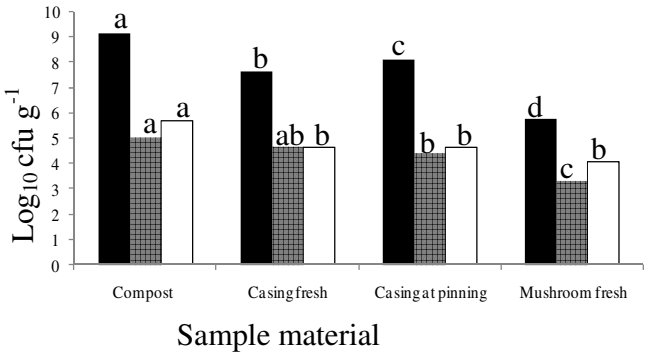


Figure 1 Total populations of bacteria (■), fungi (▩) and yeasts (▭) in compost, casing and on mushrooms during production (Different letters on same bars across different sample materials indicate significant difference at $P < 0.001$).

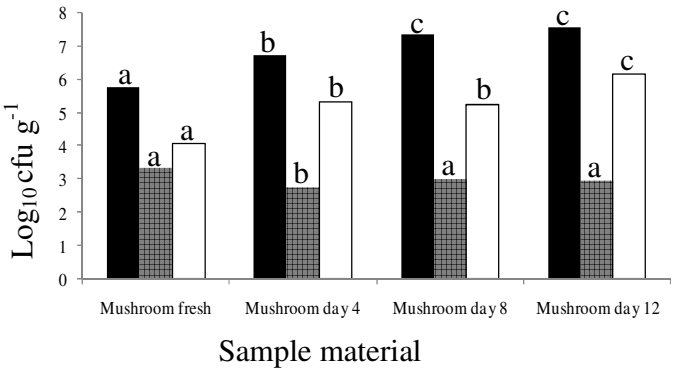


Figure 2 Total populations of bacteria (■), fungi (▩) and yeasts (▭) in fresh and stored mushrooms (Different letters on same bars across different sample materials indicate significant difference at $P < 0.001$).

Pure Culture Identification Using Sequencing

Across culturable bacteria, compost samples showed less diverse phyla than casing and mushroom samples. Casing samples were more diverse than mushroom samples. Bacteria isolated from compost samples were members of two phyla, *Actinobacteria* and *Proteobacteria*. Bacteria from casing samples were members of four phyla, *Actinobacteria*, *Proteobacteria*, *Firmicutes*, and *Bacteroidetes*. Bacteria from mushroom samples were members of the phyla *Actinobacteria*, *Proteobacteria*, and *Bacteroidetes*.

In all compost, casing and mushroom samples, the phylum *Proteobacteria* was more prevalent (Table 1). All isolated fungal cultures belonged to one phylum, *Ascomycota* and three classes i.e. *Pezizomycetes*, *Sordariomycetes* and *Eurotiomycetes*. From the class *Eurotiomycetes*, the genus *Penicillium* of the family *Trichocomaceae* was prevalent in compost, casing and mushroom samples (Table 2).

All bacterial DNA sequences of dominant DGGE gel bands matched with four phyla viz. *Bacteroidetes*, *Firmicutes*, *Actinobacteria* and *Proteobacteria*. Members of the first three phyla were extracted predominantly from compost samples while the last phylum, *Proteobacteria* originated mostly from casing and mushroom samples. From the three classes of this phylum, *Betaproteobacteria*, *Alphaproteobacteria* and *Gammaproteobacteria*, the latter was prevalent. The genus *Pseudomonas* of the family *Pseudomonadaceae* and order *Pseudomonadales* was the most dominant in mushroom samples (Table 3).

Table 1 Taxonomic identifications of bacteria isolated from compost, casing and mushrooms using DNA sequencing and tentative BLAST

Source	Closest relatives						
	Phylum	**Class**	**Order**	**Family**	**Genus**	**Species**	**Accession no.**
Compost	*Actinobacteria*	*Actinobacteria*	*Actinomycetales*	*Micrococcaceae*	*Arthrobacter*	*arilaitensis*	EU834260
	Proteobacteria	*Gammaproteobacteria*	*Alteromonadales*	*Pseudoalteromonadaceae*	*Pseudoalteromonas*	sp.	FJ876420
			Enterobacteriales	*Enterobacteriaceae*	*Enterobacter*	sp.	EU430754
					Citrobacter	*koseri*	EF059858
					Serratia	*marcescens*	EU302853
			Pseudomonadales	*Pseudomonadaceae*	*Pseudomonas*	*Koreensis,*	AB495129
						putida	EU931564
Casing	*Proteobacteria*	*Betaproteobacteria*	*Burkholderiales*	*Comamonadaceae*	*Acidovorax*	*temperans*	AB639116
		Alphaproteobacteria	*Rhodobacterales*	*Rhodobacteraceae*	*Paracoccus*	*koreensis*	NR_041238
			Rhizobiales	*Bradyrhizobiaceae*	*Bosea*	*thiooxidans*	EU730912
				Rhizobiaceae	*Shinella*	*kummerowiae*	NR_044066
				Hyphomicrobiaceae	*Devosia*	*hwasunensis*	AM393883
				Phyllobacteriaceae	*Aminobacter*	sp.	AB480343
				Rhizobiaceae	*Rhizobium*	sp.	AM084044
				Phyllobacteriaceae	*Pseudaminobacter*	*salicylatoxidan*	AJ294416
					Mesorhizobium	sp.	HQ540554
	Firmicutes	*Bacilli*	*Lactobacillales*	*Carnobacteriaceae*	*Trichococcus*	*collinsii*	EF111215
	Bacteroidetes	*Sphingobacteriia*	*Sphingobacteriales*	*Chitinophagaceae*	*Terrimonas*	sp.	JN848793
				Sphingobacteriaceae	*Sphingobacterium*	sp.	EF059711
	Actinobacteria	*Actinobacteria*	*Actinomycetales*	*Nocardiaceae*	*Rhodococcus*	sp.	
				Microbacteriaceae	*Microbacterium*	sp.	EU821337
					Leucobacter	sp.	AB514037

Table 1 (*Continued*)

Source	Closest relatives						
	Phylum	**Class**	**Order**	**Family**	**Genus**	**Species**	**Accession no.**
Mushroom	*Bacteroidetes*	*Sphingobacteriia*	*Sphingobacteriales*	*Sphingobacteriaceae*	*Sphingobacterium*	*multivorum*	HM355636
	Actinobacteria	*Actinobacteria*	*Actinomycetales*	*Microbacteriaceae*	*Microbacterium*	*foliorum*	JF303045
	Proteobacteria	*Betaproteobacteria*	*Burkholderiales*	*Alcaligenaceae*	*Alcaligenes*	*faecalis*	FJ959394
		Gammaproteobacteria	*Pseudomonadales*	*Pseudomonadaceae*	*Pseudomonas*	*Putida,*	JN228297
						fluorescens	EF602564
			Enterobacteriales	*Enterobacteriaceae*	*Ewingella*	*americana*	HE585223

Table 2 General taxonomic identifications of fungi and yeasts isolated from compost, casing and mushrooms using DNA sequencing and blasting ('C' represents compost, 'Cs' represents casing and 'M' represents mushrooms)

Source		Closest relatives						
		Phylum	**Class**	**Order**	**Family**	**Genus**	**Species**	**Accession no.**
Fungi	Cs	*Ascomycota*	*Pezizomycetes*	*Pezizales*	*Pezizaceae*	*Chromelosporium*		EF589890
	Cs		*Sordariomycetes*	*Hypocreales*	*Bionectriaceae*	*Bionectria*	sp.	AM944351
	Cs			*Microascales*	*Microascaceae*	*Pseudallescheria*	*boydii*	GU566282
	Cs			*Sordariales*	*Chaetomiaceae*	*Chaetomium*	sp.	AB506801
	Cs			*Hypocreales*	*Hypocreaceae*	*Trichoderma*	sp.	AY514867
	M		*Eurotiomycetes*	*Eurotiales*	*Trichocomaceae*	*Paecilomyces*	*sinensis*	FJ904847
	M,Cs					*Penicillium*	*raistrickii*	HM469416
	C,Cs,M					*Penicillium*	*chrysogenum*	GU565114
	C,Cs					*Penicillium*	*ochrochloron*	JF311909
	M					*Penicillium*	*meleagrinum*	HM469412
	C,Cs					*Penicillium*	*dipodomyicola*	FJ025172
	M,Cs					*Penicillium*	*brevicompactum*	HM469408
	Cs					*Penicillium*	*rolfsii*	HM043803

Table 2 (*Continued*)

Source		Closest relatives						
		Phylum	**Class**	**Order**	**Family**	**Genus**	**Species**	**Accession no.**
Yeast	C,M	*Basidiomycota*	*Tremellomycetes*	*Cystofilobasidiales*	*Cystofilobasidiaceae*	*Cystofilobasidium*	*infirmominiatum*	AY264716
	C,M		*Urediniomycetes*	*Sporidiobolales*	*Sporidiobolaceae*	*Rhodotorula*	*mucilaginosa*	EF174513
	C					*Rhodotorula*	*mucilaginosa*	EU149812
	C		*Tremellomycetes*	*Tremellales*	*Trichosporonaceae*	*Trichosporon*	*moniliiforme*	FR799471
	C					*Trichosporon*	*cutaneum*	FJ943422
	C	*Ascomycota*	*Saccharomycetes*	*Saccharomycetales*	*Saccharomycetaceae*	*Candida*	*glaebosa*	FJ153208
	Cs					*Candida*	*subhashii*	EU836707
	Cs					*Candida*	sp.	HQ623537
	Cs					*Candida*	sp.	JF895510
	Cs					*Pichia*		FJ153190

Table 3 General identifications of bacteria from dominant DGGE bands by sequencing DNA derived from compost, casing and mushroom samples ('C' represents compost, 'Cs' represents casing and 'M' represents mushrooms)

Source	Closest relatives						
	Phylum	**Class**	**Order**	**Family**	**Genus**	**Species**	**Accession no.**
C	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Chitinophagaceae	Flavisolibacter	sp.	HM130561
C	Bacteroidetes	Bacteroidetes Order II	Incertaesedis	Rhodothermaceae	Rhodothermus	obamensis	AF217493
C	Firmicutes	Bacilli	Bacillales	Bacillaceae	Bacillus	badius	JN624927
C				Cellulomonadaceae	Cellulomonas	sp.	JN181251
C				Streptosporangiaceae	Microbispora	sp.	AF131381
M, C				Geodermatophilaceae	Blastococcus	sp.	AB540018
C	Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	Bordetella	sp.	AB039335
Cs				Comamonadaceae	Curvibacter	sp.	HM357758

Table 3 (*Continued*)

Source	Closest relatives						
	Phylum	**Class**	**Order**	**Family**	**Genus**	**Species**	**Accession no**
Cs		Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae	Chelativorans	multitrophicus	FJ167676
Cs				Rhizobiaceae;	Rhizobium	sp.	DQ303370
Cs				Rhizobiaceae	Shinella	sp.	GQ246688
Cs			Sphingomonadales	Sphingomonadaceae	Sphingomonas	sp.	AJ009709
M							HQ331138
Cs					Novosphingobium	panipatense	JF459982
C		Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Luteimonas	sp.	AM932272
M			Enterobacteriales	Enterobacteriaceae	Ewingella	americana	HE585223
C			Pseudomonadales	Pseudomonadaceae	Pseudomonas	sp.	FM161512
M						sp.	AF408925
M						sp.	HQ224640
M						sp.	JF694807
M						fluorescens	JN907006
M						syringae	JN088486
M						migulae	DQ377758
M						migulae	AF501352
M, Cs						plecoglossicida	JN624752
M, Cs						putida	AF396076
M						putida	JN228297
M, Cs						tremae	HE647699
M, Cs						syncyanea	AB680130
M, Cs						reactans	JN662499
M, Cs						nitroreducens	JF513151

Microbial Profile of Compost, Casing and Mushroom Samples on DGGE

The compost, casing and mushroom samples displayed distinct bacterial profiles. Banding patterns depicted more diverse bacterial species in compost samples than in casing and mushroom samples. The bands also depicted that the bacterial profile of mushrooms showed greater similarity to that of the casing rather than to the compost. Mushroom samples had relatively less diverse but abundant bacterial species than those of the casing and the compost samples (Figure 3).

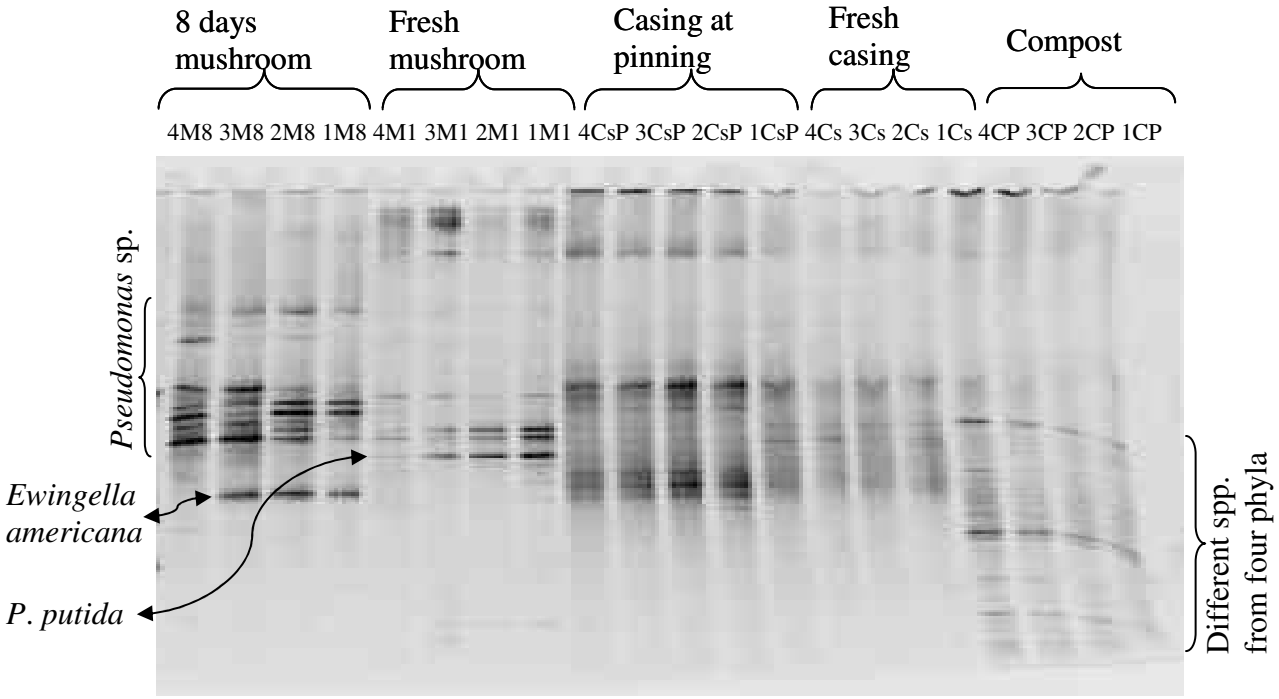


Figure 3. DGGE images of amplified bacterial DNA derived from compost, casing (fresh and at pinning) and mushrooms (fresh and after eight days of storage at 4°C).

DGGE gel of amplified fungal and yeast DNA showed a uniform pattern for compost, casing at pinning and both mushroom (fresh and eight days after storage) samples. All these samples were completely dominated by *A. bisporus*. Only fresh casing samples depicted a yeast and other fungal species.

Population Similarities Between Compost, Casing and Mushroom Ecology

For amplified fungal and yeast DNA, the uniformity of the compost, casing at pinning and both mushroom samples was clear on the DGGE gel (Figure 4). Only fresh casing was different from the other samples. For amplified bacterial DNA, the cluster analysis phylogram using Jaccard group average settings showed two main clades and three sub-clades. All casing and mushroom samples grouped in one big clade (I). Whereas, all compost samples clustered to a separate clade (II) (Figure 5).

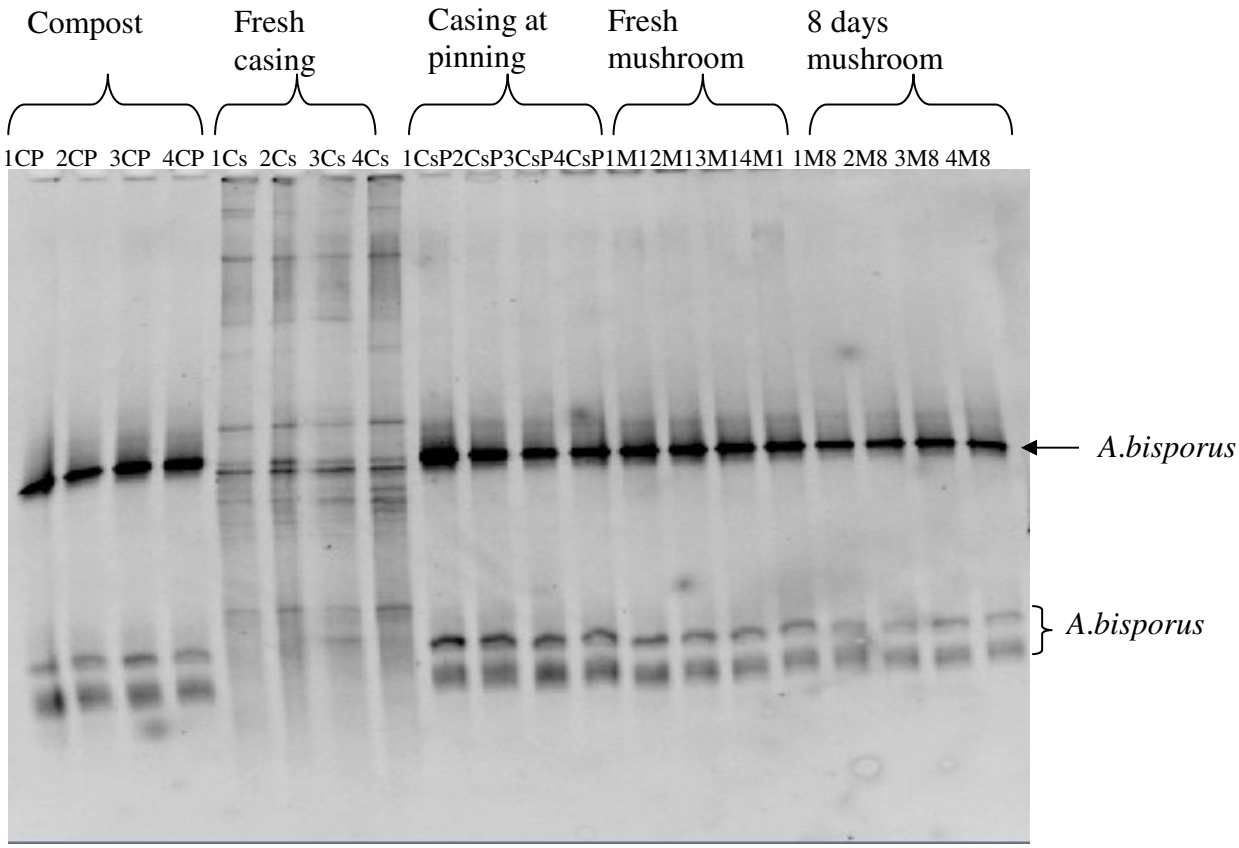


Figure 4. DGGE images of amplified fungal and yeast DNA derived from compost, casing (fresh and at pinning) and mushrooms (fresh and after eight days of storage at 4°C).

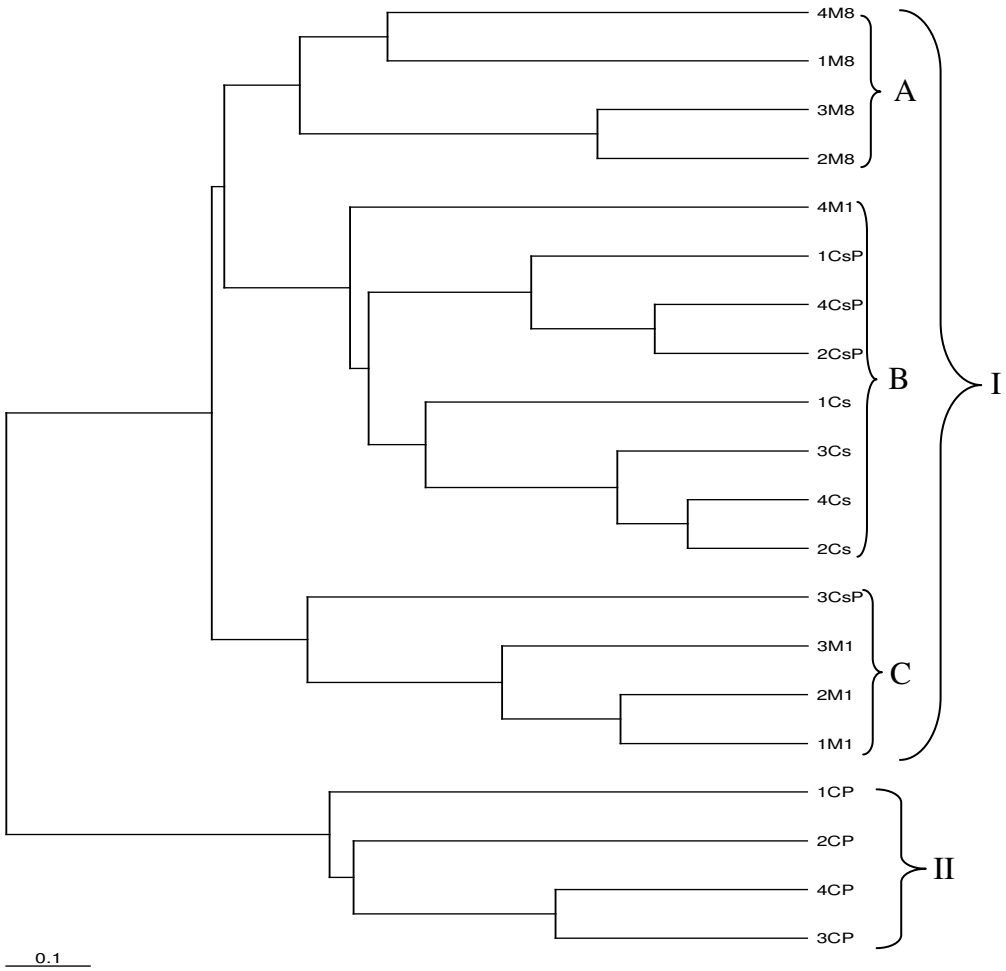
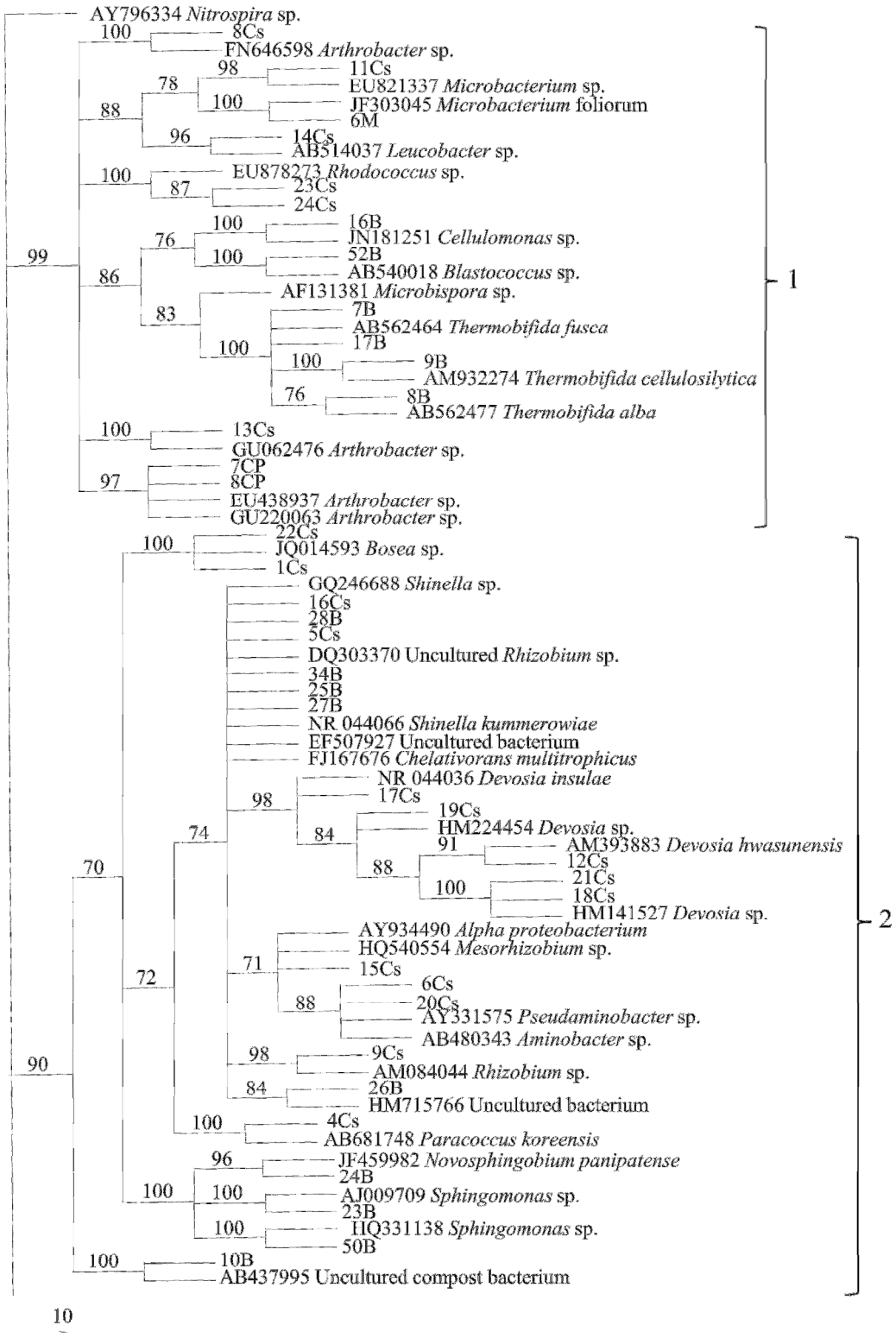


Figure 5. Cluster analysis phylogram of similarities between bacterial populations of compost, casing (fresh and at pinning) and mushroom (fresh and after eight days of storage at 4°C) samples. Letters and numbers indicate sample type: CP (compost), Cs (fresh casing), CsP (casing at pinning), M1 (fresh mushroom) and M8 (mushroom stored for eight days).

Phylogenetic Relationships of Cultured and Uncultured Micro-organisms from Compost, Casing and Mushrooms

Bacteria from mushrooms showed phylogenetic relationships both with those from casing and compost. The first four big clades of the phylogenetic tree (Figure 6) consisted of bacteria from the three sources (compost, casing and mushroom). Generally, bacterial sequences both from cultures and from amplified DNA of DGGE gel bands grouped together in the different clusters (clades 1-4). However, separate clades (5, 6, 8, and 9) of amplified DNA from DGGE gel bands were formed as well. Most of the *Pseudomonas* species in clade 3, originated from mushroom samples, specifically from amplified DNA of DGGE gel bands.

Almost all DNA sequences of yeasts and fungi were derived from pure culture isolates, since fungal (and yeast) DGGE profiles of samples were dominated by *A. bisporus*. *Penicillium* spp. were commonly isolated from compost, casing and mushroom samples. The phylogenetic tree of yeasts formed two clades (Figure 5). Clade 1 consisted of sequences predominantly from compost and mushrooms while clade 2 contained sequences from casing except one from compost.



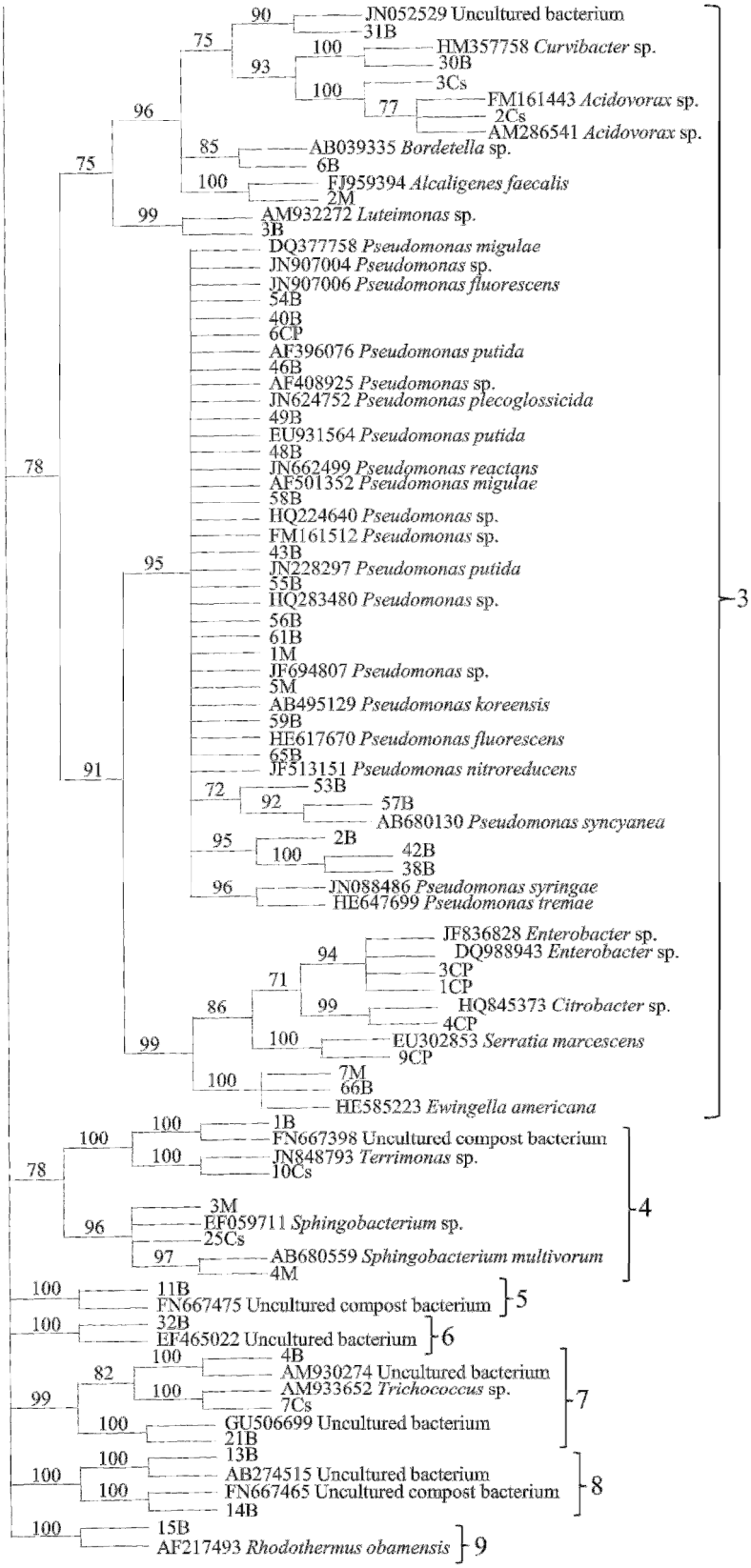


Figure 6. Rooted phylogenetic tree based on parsimony showing phylogenetic relationships of bacteria derived from compost, casing, mushroom samples and most closely related matching sequences obtained from BLAST searches. Sequences of isolates from compost, casing and mushrooms are suffixed by 'CP', 'Cs' and 'M' respectively. Sequences from amplified DNA of DGGE bands are represented by a number and the letter 'B' [Parsimony informative characters are 450, CI=0.3260, RI=0.7923, number of trees=100, tree length=765.56992 and g1=-0.265864].

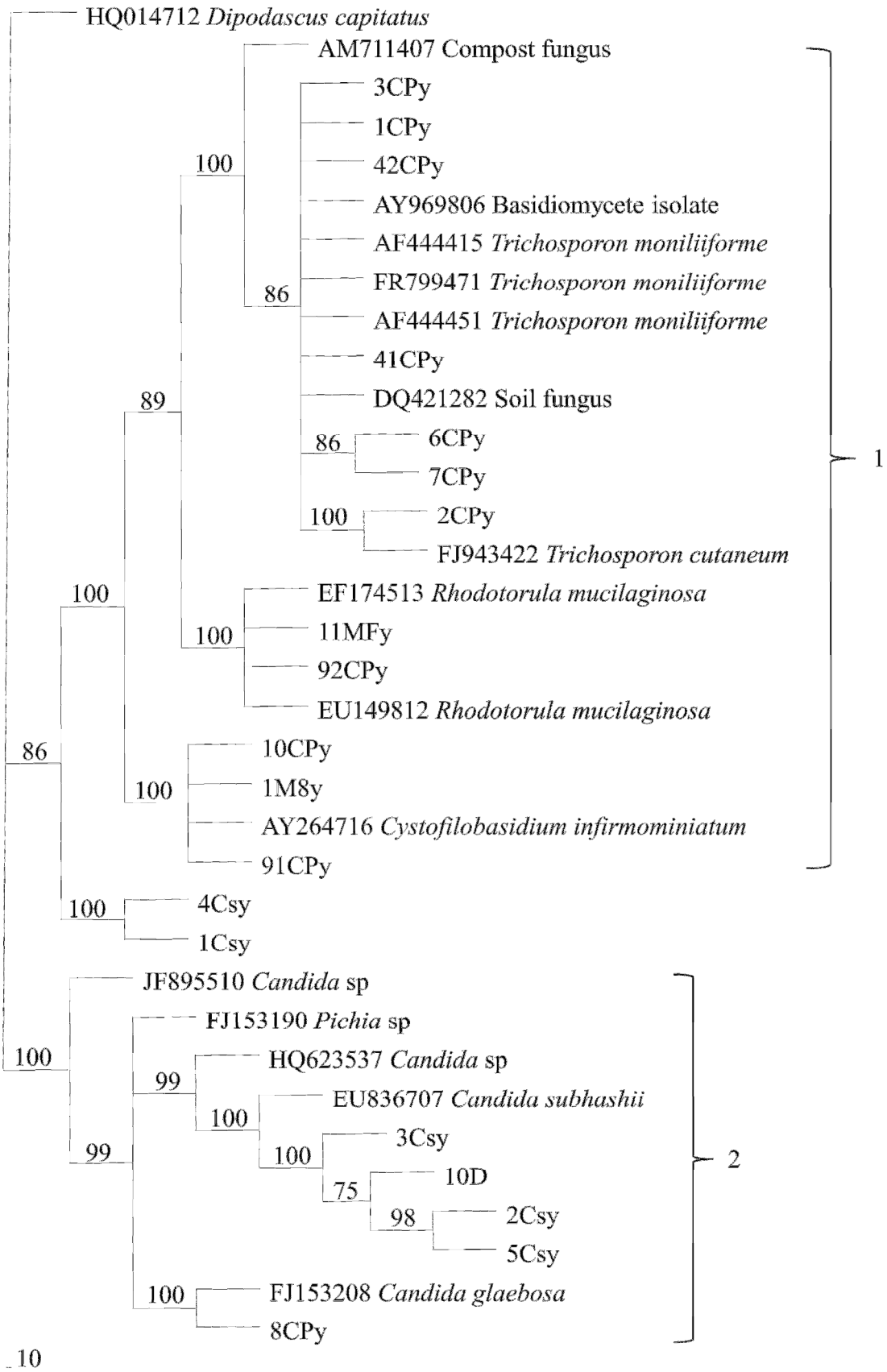


Figure 7. Rooted phylogenetic tree based on ITS region sequences of yeasts derived from compost, casing and mushroom samples and most closely related sequences obtained from BLAST searches. Sequences from compost, casing and mushrooms are suffixed by 'CPy', 'Csy' and 'My' respectively [Parsimony informative characters are 270, CI=0.7495, RI=0.9337, number of trees=100, tree length=470.22827 and g1=-0.379514].

Discussion

In this study, the plating method used demonstrated richer microbial population counts in compost compared with casing and mushroom samples. Especially, bacterial populations of compost samples were significantly higher than that of casing and mushroom samples. Bacterial populations at the end of Phase II composting create unfavourable conditions for competitive micro-organisms and provide selectivity to *A. bisporus* (Fordyce, 1970; Ross and Harris, 1983). Phase II composting involves conditioning, which is controlled heat treatment that selectively kills pathogenic micro-organisms. It is suggested that other non-pathogenic bacteria may remain static at this stage of composting and become active later when the temperature is lowered, which is in agreement with our results.

The casing layer in this study was prepared from peat, which is naturally formed from partially decomposed plant material. Unlike compost, peat is relatively poor in nutrients and decomposed organic matter (Eakin, 1969; Abad *et al.* 1989). Therefore, it is likely that due to these poorer nutrient conditions total bacterial colony counts were significantly lower in casing than in compost. Even so, total bacterial colony counts in casing increased significantly at the time of pinning although this count was still significantly lower than that of the compost. This significant increase of total bacteria from casing to pinning is consistent with previous work (Siyoum *et al.*, 2010). In the case of fungi, fresh casing samples had lower total colony counts than compost samples although the difference was not initially significant. However, at pinning, the total fungal colony count of the casing was reduced to a significantly lower level than that of the compost. According to Verhoeven (1986) fungi do not have a significant role in peat, unlike bacteria. Like bacteria and fungi, yeast populations were also significantly higher in compost samples than in the casing and fresh mushroom samples analysed. The overall lower microbial counts in casing compared to compost may be attributed to limited energy sources and availability of nutrients in peat soil (Verhoeven, 1986). However, after mushrooms were stored at 4^{o}C for four, eight and twelve days, there was a significant increase in yeast colonies. Information on spoilage of mushrooms caused

by yeasts is not common. Koorapati *et al*. (2004) and Chikthimmah (2006) reported on the control of microbial spoilage through irradiation. In their experiment, irradiation reduced all microbial counts including bacteria, yeasts and molds, and prolonged mushroom shelf life. However, it is not clear whether yeasts and moulds had also contributed to the spoilage.

In our study, there was a significant increase in total colony counts of both bacteria and yeasts on mushrooms from the time of harvest to 12 days after cold storage while the fungal colonies remained constant. A similar trend for bacteria, yeasts and molds was also reported by Chikthimmah. Postharvest spoilage of mushrooms caused by bacteria is well established (Burton and Noble, 1993; Fett *et al*., 1995; Wells *et al*., 1996). However, the role of yeasts in mushroom spoilage needs to be investigated, although the above results suggest a possible contribution.

Bacteria isolated from compost were less diverse at phylum and family level than those isolated from casing and mushrooms. Compost bacteria belonged to two phyla and three families only while casing bacteria belonging to four phyla and eleven families. The extent of diversity of bacteria isolated from mushrooms lay between that of the two substrates. Cultured bacteria of mushrooms belonged to three phyla and five families. Visual examination of bacterial plates (not shown) illustrated more uniform colonies for compost and mushroom than for casing samples, which could be attributed to the less diverse families. Our low diversity result of culturable compost bacteria is in agreement with Peters *et al*. (2000) although they investigated the thermophilic group. However, work by Ryckeboer *et al*. (2003b) is not consistent with our result. The reason for this could be the difference in the type of the compost material (vegetable, fruit and garden waste in their case). Cultured compost bacteria in the present work differ from those of Song *et al*. (2001) and Székely *et al*. (2009), which were dominated by the thermophilic groups. This can be explained by the high (50^{o}C and 45^{o}C respectively) incubation temperatures they used compared to ours (25^{o}C). Most of the microbiological studies on compost and composting were either not specific on materials for mushroom growing, or if so, they were on the phases before spawning.

Unlike in our studies, previous work on spawned mushroom compost did not provide information on microbial diversity. Fordyce (1970) reported a lower (10^{6} - 10^{7}) mesophilic bacterial count for a three week spawned compost, which is lower by two log units than the present experiment. A possible reason for this difference could be the lower temperature

(20°C) Fordyce used for the incubation of plates compared with the 25°C in the present work. Fermor and Wood (1981) also mentioned a reduction and stabilisation of bacterial numbers in spawned compost following the high number and activity of bacteria in Phase I and II compost. On both studies, there was no report on diversity.

However, results from DGGE analysis in this study, described a different picture of bacterial diversity in the three materials, compost, casing and mushrooms. Bacterial diversity was highest in compost samples. DNA sequences that were closely related to known cultured species belonged to four phyla for compost samples while only to one and two phyla for casing and mushroom samples respectively. The difference in diversity results of the culturing and the DGGE methods is understandable. It is known that bacteria that grow in an artificial medium represent only a small fraction (0.3% in soil habitat) of the total community in the sampled environment (Amann *et al*., 1995). Therefore, isolates obtained using culturing methods might not necessarily be the dominant ones in the habitat studied. Several researchers have reported that some cultured bacteria were not part of the dominant groups in in profiles obtained using DGGE/TGGE (Felske *et al*., 1999; Smit *et al*., 2001; Ellis *et al*., 2003) and other culture-independent molecular methods (Suzuki *et al*., 1997; Kaiser *et al*., 2001; Pearce *et al*., 2003). Therefore, in order to have a broader picture of microbial diversity, it is advisable to combine both culturing and non-culturing methods.

Similarly, fungi and yeasts other than *A. bisporus* could not be detected on the DGGE gel for compost, casing at pinning and mushroom samples. These samples were completely dominated by *A. bisporus*. In this case, the complete dominance of the mushroom fungal community by *A. bisporus* itself was likely due to the method used. DGGE reveals a broader picture of the members of a community. However, using the plating method, fungi and yeasts were detectable on MEA plates, although they were few and of low diversity dominated by the genus *Penicillium*. This low level of detectable fungi other than *A. bisporus* has been described, especially, in well prepared compost (Ross and Harris, 1983). This compost is produced in such a way that it selectively supports the growth of *A. bisporus* (Ross and Harris, 1982; Ross and Harris, 1983; Camp *et al*., 1990).

Comparing bacterial profiles of the compost, casing and mushroom samples using DGGE, compost populations were distinct from those of both the casing and mushrooms. In the cluster analysis phylogram using a Jaccard group average setting, compost samples grouped in one clade while the casing and mushroom samples grouped together in another big clade.

This result is in agreement with Reddy and Patrick (1990), who isolated similar bacteria from casing and mushroom mycelium colonising it. Their results showed that these bacteria were not similar to those isolated from compost. They also described that bacterial isolates from compost had no effect on formation of mushroom fruit bodies. Bacterial profiles for fresh casing and casing at pinning were similar except that population densities increased at pinning, which is consistent with previous work (Siyoum *et al*., 2010). Both fresh mushrooms and the ones stored for eight days had few but dominant bacterial species compared with the casing and compost. These profiles were similar to that of the casing as it was shown by the cluster analysis phylogram. Our results suggest that the mushroom surface could be selective to a specific group of bacteria dominated by *Pseudomonas* species. The whole profile also revealed that most bacteria on the mushroom surface originated very likely from the casing.

It is interesting that almost only *Pseudomonas* spp. were dominant on the mushroom profiles. On the casing profile, these species were less abundant (less intense bands) compared to the mushrooms. It was also clearly shown that certain *Pseudomons* spp. and *Ewingella americana* increased in abundance while a particular species, identified as a close relative of *P. putida*, could not be detected from mushrooms after the eight days storage. It is interesting that *P. putida* was abundant on fresh mushrooms but not on stored ones. This bacterium is well known for its role in fruit body initiation (Hayes *et al*., 1969; Noble *et al*., 2009) and our results indicate that it was not part of the postharvest spoilage syndrome associated with pseudomonads. The presence of pseudomonads and *E. americana* in mushrooms is in agreement with reports from different studies. Members of the genus *Pseudomonas* are well known as saprophytes and pathogens of mushrooms (Munsch and Alatossava, 2002; Sivanesan, 2003). The non-pseudomonad bacteria *E. americana* was also reported on mushrooms both as a pathogen (Inglis *et al*., 1996) and as a member of the indigenous micro-flora (Chowdhury and Heinemann, 2006).

Phylogenetic analysis of bacteria from different samples - compost, casing and mushroom - revealed that generally, species from the same sample source were the most closely related. Nevertheless, it was also noted that some species from all three different sources were phylogenetically related to each other. In addition, five minor separate lineages comprising of compost and casing inhabiting bacterial species were formed as indicated on the phylogenetic tree. These lineages indicate that species from these two sample sources were

diverse and several of them were not affiliated with known cultured bacteria. Ivors *et al*. (2000) reported a similar result in which several rDNA sequences from compost samples were unique and not related to previously identified species. Most of the sequences that were not affiliated with known cultured species in our study originated from excised DGGE bands of compost and casing samples. These results suggest that DGGE could reveal species that were not cultured previously, possibly novel.

The phylogenetic tree of yeasts from compost, casing and mushroom samples was relatively simple, consisting of two lineages. The first lineage consisted of sequences predominantly from compost samples. Two sequences from mushroom samples were also affiliated with this lineage. The second lineage contained samples from casing and one sequence from compost. The phylogenetic tree for yeasts consisted of sequences from cultured species only, except one DNA sequence obtained from DGGE band of fresh casing samples. Our results indicate that yeasts from compost samples were more diverse than those from casing. Four out of six yeast species from casing samples were closely related to *Candida* spp. The two species were closely related to each other but grouped with the lineage of yeasts from compost samples, although at a distant level. In the samples tested, yeast species originating from compost and casing samples were not closely related while yeasts from mushroom samples were closely related to those from compost samples. Although this result needs further investigation, it provides some background information on diversity and relationship of yeasts in compost, casings and mushroom production and storage. Future studies should focus on determining the role of yeasts on mushroom spoilage.

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CHAPTER SEVEN

GENERAL DISCUSSION AND CONCLUSION

Casing layer is an important substrate during commercial production of white button mushrooms (*Agaricus bisporus* (Lange) Imbach) and peat soil is the common material used for this purpose. However, vast mining of peat has resulted in environmental concerns leading to new legislation (Protected areas, Act No. 57 of 2003) (Government Gazette, 2009), restricting further exploitation of this natural resource. Easily available materials that can either partially or completely replace peat are necessary for sustainable mushroom production. Micro-organisms, especially bacteria are important in the biological ecology in casing contributing to fructification of mushroom mycelium by metabolising substances that inhibit this process (Noble *et al*., 2009). Microbiological properties of alternative casing materials therefore need to reflect a similar profile or at least be comparable to a healthy peat.

In this study, microbiological properties of peat and different alternative materials as partial replacements of peat were evaluated. The industrial waste materials coir, wattle bark, sugar cane bagasse and filter cake were mixed with peat at 1:1 and 3:7 ratios and used as casing to produce mushrooms. Bacterial abundance and diversity in peat-based mixtures of these materials were comparable with that of peat alone. At the time of pinning, bacterial populations increased significantly in peat-based mixtures of all industrial wastes tested as in peat. Dominant representative bacteria from these mixtures included *Pseudomonas* spp., which are well known bacteria in stimulating mushroom mycelium fructification (Hayes *et al*., 1969; Fermor *et al*., 2000; Noble *et al*., 2003).

A molecular finger printing technique, denaturing gradient gel electrophoresis (DGGE), revealed that bacterial profiles of peat and peat-based mixtures of coir, wattle, sugar cane bagasse and filter cake being similar. Thus, indicating that bacteria inhabiting peat casing were able to inhabit peat-based mixtures as well. Moreover, at pinning, bacterial population richness was greater in the mixtures than in peat alone. At pinning, bacteria that did not belong to any cultured species were also abundant; suggesting species other than pseudomonads may play a role in mushroom mycelium fructification.

Pasteurisation initially lowered bacterial population richness of casing materials; however, at the pinning stage populations in pasteurised and unpasteurised materials had a similar profile.

Thus, pasteurising casing materials appears not to affect mushroom mycelium fructification negatively.

Bacterial profiles of peat collected from farm and peat collected directly from a mining site were similar consisting of *Pseudomonas* spp. Therefore, if the need occurs, beneficial bacteria especially pseudomonads can be isolated and cultivated from mine peat when commercial peat is not available. Thus isolates of known beneficial bacteria can be inoculated into easily available alternative casing materials.

Bacteria are also associated with pre- and post-harvest mushroom quality deterioration. Bacterial profiles of mushrooms in this study were found to be more similar to those of casing than to compost. DGGE revealed that bacteria on mushrooms were less diverse and more abundant than those in casing and compost. In addition, bacterial profiles of mushrooms were dominated by *Pseudomonas* spp. These species except *Pseudomonas putida*, increased in population richness during storage of mushrooms at 4^{o}C for eight days. *Pseudomonas putida* was abundant on freshly harvested mushrooms but was not detected on stored mushrooms. It is interesting to note that this bacterium is not part of the post harvest mushroom quality deterioration group of micro-organisms but it is important in mushroom fructification.

The casing bacterial profiles were distinct from those of compost. The fact that transformation into fruit bodies occurs only after mushroom mycelium reaches the casing layer is possibly due to specific species of bacteria found only in the casing but not in the compost. The plating technique reflected lower level of diversity in compost bacteria compared with those of casing. However, DGGE revealed that compost inhabiting bacteria were more diverse than those of casing.

Fungal/yeast profiles of compost, casing at pinning and on mushrooms were similar and dominated by *A. bisporus*. However, plating showed yeasts and other fungi dominated by *Penicillium* spp. in all samples. Fungal population counts were low in all samples analysed and did not increase during mushroom storage while bacterial and yeast counts were higher and increased significantly during storage. These results suggest that yeasts possibly contribute to post harvest mushroom quality deterioration.

Micro-organisms affect growth and yield of different plants and other organisms in many ways. Some promote growth and yield while others either inhibit or have no effect on these parameters. Three *Pseudomonas* isolates, an *Arthrobacter* sp. and a mixture of these and 15 other isolates stimulated mycelial growth of *A. bisporus in vitro* at a 100% mycelial coverage. Total bacterial counts and mushroom yield for peat-based casing mixtures 1:1 peat/filter cake and peat/bagasse showed positive correlations.

Bacteria and yeast isolated from casing, compost and mushrooms inhibited *in vitro* growth of different fungal pathogens of white button mushrooms. Bacterial and yeast isolates belonging to the genera *Sphingobacterium* and *Candida* respectively, resulted in maximum growth inhibition of the tested pathogens.

Suggestions for Future Research

1. Development of growth media for culturing viable but non-culturable micro-organisms associated with casing medium;
2. Studying currently non-culturable casing bacteria possibly involved in the fructification of mushroom mycelium, growth stimulation and enhancement of mushroom yield;
3. More in depth study using molecular and cultural tools for bacteria involved in fructification and growth stimulation of mushroom mycelium to develop biological strategies;
4. Examination of different pseudomonads involved in fructification of mushroom mycelium and their role in post harvest mushroom spoilage. This information is useful if a casing medium needs to be inoculated with a particular pseudomonad;
5. Studies on different treatments of casing medium aimed at reducing or eradicating pseudomonads causing postharvest mushroom spoilage;
6. Investigation of the role of yeasts in mushroom mycelium fructification and postharvest mushroom quality deterioration; and
7. *In vivo* and semi-commercial trials of the antagonistic effect of different bacteria and yeast isolated from these casing studies that showed inhibition against various fungal pathogens of white button mushrooms.

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APPENDIX I

Populations of bacteria in peat and peat-based mixtures: 1:1 peat/coir, 7:3 peat/coir, 1:1 peat/wattle bark, 7:3 peat/wattle bark, 1:1 peat/bagasse, 7:3 peat/bagasse, 1:1 peat filter cake and 7:3 peat/filter cake at casing, pinning and end of second break (harvesting)

Casing material	Mushroom stage	Log_{10} value of colony forming units (cfu's)/g			
		Total bacteria	Representative dominant groups of		
			Ensifer/Sinorhizobium spp.	*Pseudomonas*, *Bacillus* and *Sporosarcina* spp.	*Microbacterium Arthrobacter* and *Sphingobacterium* spp.
Peat	Casing	4.769	4.507	3.19	2.94
	Pinning	6.593	6.154	5.73	6.00
	Harvesting	6.841	6.286	5.94	6.33
LSD		0.2267	0.2686	0.661	0.719
F pr		<0.001	<0.001	<0.001	<0.001
1:1 Peat/Coir	Casing	5.534	5.076	5.092	4.27
	Pinning	6.625	6.181	5.855	5.93
	Harvesting	6.878	6.357	6.086	6.34
LSD		0.3763	0.3839	0.2929	0.647
F pr		<0.001	<0.001	<0.001	<0.001
7:3 Peat/Coir	Casing	5.159	4.643	4.873	3.32
	Pinning	6.674	6.199	6.033	6.00
	Harvesting	6.765	6.073	5.944	6.37
LSD		0.1857	0.1831	0.2439	0.517
F pr		<0.001	<0.001	<0.001	<0.001
1:1 Peat/Wattle bark	Casing	6.197	5.668	5.796	5.244
	Pinning	7.739	7.199	7.193	7.161
	Harvesting	7.607	7.093	6.866	7.098
LSD		0.3696	0.4059	0.3544	0.3679
F pr		<0.001	<0.001	<0.001	<0.001
7:3 Peat/Wattle bark	Casing	5.984	5.540	5.561	4.66
	Pinning	6.652	6.168	6.138	6.00
	Harvesting	7.118	6.601	6.316	6.60
LSD		0.2824	0.2522	0.3938	0.510
F pr		<0.001	<0.001	<0.001	<0.001
1:1 Peat/Bagasse	Casing	5.268	4.901	4.82	1.26
	Pinning	6.883	6.450	5.70	6.38
	Harvesting	7.166	6.673	6.04	6.82
LSD		0.1873	0.2014	0.577	0.742
F pr		<0.001	<0.001	<0.001	<0.001

(Continued)

Casing mateial	Mushroom stage	Log_{10} value of colony forming units (cfu's)/g			
		Total bacteria	Dominant representative isolates		
			***Ensifer/Sinorhizobium* spp.**	***Pseudomonas*, *Bacillus* and *Sporosarcina* spp.**	***Microbacterium Arthrobacter* and *Sphingobacterium* spp.**
7:3 Peat/Bagasse	Casing	5.471	5.136	4.837	0.94
	Pinning	6.875	6.474	6.033	6.32
	Harvesting	7.159	6.610	6.016	6.80
LSD		0.2914	0.3340	0.2469	0.637
F pr		<0.001	<0.001	<0.001	<0.001
1:1 Peat/Filter cake	Casing	5.820	5.341	5.40	2.92
	Pinning	6.986	6.618	5.88	6.47
	Harvesting	7.274	6.833	6.24	6.92
LSD		0.1855	0.1994	0.575	0.974
F pr		<0.001	<0.001	<0.018	<0.001
7:3 Peat/Filter cake	Casing	5.002	4.618	4.244	1.55
	Pinning	7.012	6.620	6.182	6.46
	Harvesting	7.207	6.754	6.290	6.77
LSD		0.2078	0.2528	0.2128	0.736
F pr		<0.001	<0.001	<0.001	<0.001

APPENDIX II

Table 1 Total populations of bacteria, fungi and yeasts in compost, casing and on mushrooms during production and mushroom storage

Sample	Log_{10} value of colony forming units (cfu's)/g		
	Bacteria	**Fungi**	**Yeast**
Compost	9.184	5.045	5.676
Casing fresh	7.639	4.684	4.635
Casing at pinning	8.125	4.406	4.620
Mushroom fresh	5.765	3.333	4.065
LSD	0.3275	0.4696	0.668
F pr.	< 001	< 001	< 001
Mushroom fresh	5.765	3.333	4.065
Mushroom day 4	6.733	2.754	5.331
Mushroom day 8	7.367	3.011	5.235
Mushroom day 12	7.600	2.957	6.176
LSD	0.4018	0.5090	0.735
F pr.	< 001	< 0.159	< 001

APPENDIX III

DNA blast results of bacteria in three dominant categories isolated from peat and the industrial by-products (from GenBank data base)

Group 1

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
AB480327.1	Rhizobiales bacterium N21 gene for 16S ribosomal RNA, partial sequence, strain: N21	713	713	100%	0.0	100%
GQ214325.2	Ensifer adhaerens strain CNBb 16S ribosomal RNA gene, partial sequence	713	713	100%	0.0	100%
EU928872.1	Ensifer adhaerens strain LC04 16S ribosomal RNA gene, partial sequence	713	713	100%	0.0	100%
EU928871.1	Ensifer sp. T173 16S ribosomal RNA gene, partial sequence	713	713	100%	0.0	100%
FM173859.1	Ensifer sp. CL4.41 partial 16S rRNA gene, isolate CL4.41	713	713	100%	0.0	100%
FM173851.1	Ensifer sp. CL4.32 partial 16S rRNA gene, isolate CL4.32	713	713	100%	0.0	100%
FM173572.1	Ensifer sp. CL2.46 partial 16S rRNA gene, isolate CL2.46	713	713	100%	0.0	100%
FM174114.1	Ensifer sp. CL6.33 partial 16S rRNA gene, isolate CL6.33	713	713	100%	0.0	100%
AM922194.1	Ensifer adhaerens partial 16S rRNA gene, isolate Sulf-1426	713	713	100%	0.0	100%
EF599761.1	Sinorhizobium sp. IS-J5 16S ribosomal RNA gene, partial sequence	713	713	100%	0.0	100%
EF442029.1	Sinorhizobium sp. M14 16S ribosomal RNA gene, partial sequence	713	713	100%	0.0	100%
AM285019.1	Ensifer adhaerens partial 16S rRNA gene, isolate B04	713	713	100%	0.0	100%
AM181737.1	Sinorhizobium morelense partial 16S rRNA gene, strain LMG 21331	713	713	100%	0.0	100%

Group 2

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
DQ520823.1	Microbacteriaceae bacterium NR172 16S ribosomal RNA gene, partial sequence	806	806	100%	0.0	100%
EU714337.1	Microbacterium esteraromaticum strain 2122 16S ribosomal RNA gene, partial sequence	800	800	100%	0.0	99%
AB355700.1	Microbacterium esteraromaticum gene for 16S rRNA, partial sequence, strain: S45	800	800	100%	0.0	99%
AB099658.1	Microbacterium esteraromaticum gene for 16S rRNA, complete sequence, strain: S29	800	800	100%	0.0	99%
AB099657.1	Microbacterium esteraromaticum gene for 16S rRNA, complete sequence, strain: S51	800	800	100%	0.0	99%
AB099656.1	Microbacterium esteraromaticum gene for 16S rRNA, complete sequence, strain: S38	800	800	100%	0.0	99%
NR_026468.1	Microbacterium esteraromaticum strain DSM 8609 16S ribosomal RNA, partial sequence >emb|Y17231.1| Microbacterium esteraromaticum 16S rRNA gene	800	800	100%	0.0	99%
AY623899.1	Microbacterium sp. zzj4-1 16S ribosomal RNA gene, partial sequence	798	798	99%	0.0	100%
AY492094.1	Microbacterium sp. HZMB4-1 16S ribosomal RNA gene, partial sequence	798	798	99%	0.0	100%
FM173647.1	Sporosarcina sp. CL2.131 partial 16S rRNA gene, isolate CL2.131	846	846	100%	0.0	99%
FM173566.1	Sporosarcina sp. CL2.40 partial 16S rRNA gene, isolate CL2.40	846	846	100%	0.0	99%
FM173543.1	Sporosarcina sp. CL2.16 partial 16S rRNA gene, isolate CL2.16	846	846	100%	0.0	99%
FM174069.1	Sporosarcina sp. CL5.123 partial 16S rRNA gene, isolate CL5.123	846	846	100%	0.0	99%
FM174022.1	Sporosarcina sp. CL5.65 partial 16S rRNA gene, isolate CL5.65	846	846	100%	0.0	99%
FM174021.1	Sporosarcina sp. CL5.64 partial 16S rRNA gene, isolate CL5.64	846	846	100%	0.0	99%
FM174017.1	Sporosarcina sp. CL5.60 partial 16S rRNA gene, isolate CL5.60	846	846	100%	0.0	99%
FM173961.1	Sporosarcina sp. CL5.129 partial 16S rRNA gene, isolate CL5.129	846	846	100%	0.0	99%
FJ005056.1	Sporosarcina sp. enrichment culture clone Guo2 16S ribosomal RNA gene, partial sequence	841	841	100%	0.0	99%

Group 2 (Continued)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
FM173609.1	Sporosarcina sp. CL2.91 partial 16S rRNA gene, isolate CL2.91	841	841	100%	0.0	99%
FM173979.1	Sporosarcina sp. CL5.15 partial 16S rRNA gene, isolate CL5.15	841	841	100%	0.0	99%
DQ154361.1	Uncultured soil bacterium clone RFS-C33 16S ribosomal RNA gene, partial sequence	841	841	100%	0.0	99%
DQ333897.1	Paenisporosarcina quisquiliarum strain SK 55 16S ribosomal RNA gene, partial sequence	841	841	100%	0.0	99%
FJ472859.1	Pseudomonas putida strain JM7 16S ribosomal RNA gene, partial sequence	841	841	100%	0.0	100%
EU781731.1	Pseudomonas sp. VET-3 16S ribosomal RNA gene, partial sequence	841	841	100%	0.0	100%
CP000949.1	Pseudomonas putida W619, complete genome	841	5883	100%	0.0	100%
GU902299.1	Pseudomonas putida strain M2P3 16S ribosomal RNA gene, partial sequence	835	835	100%	0.0	99%
AM259176.1	Pseudomonas putida partial 16S rRNA gene, isolate GerR	835	835	100%	0.0	99%
AY972231.1	Pseudomonas plecoglossicida strain P9 16S ribosomal RNA gene, partial sequence	835	835	100%	0.0	99%
AY972197.1	Pseudomonas plecoglossicida strain P4 16S ribosomal RNA gene, partial sequence	835	835	100%	0.0	99%
AY972165.1	Pseudomonas plecoglossicida strain P1 16S ribosomal RNA gene, partial sequence	835	835	100%	0.0	99%
AY395005.1	Pseudomonas putida 16S ribosomal RNA gene, partial sequence	835	835	100%	0.0	99%
AF511436.1	Pseudomonas alcaligenes 16S ribosomal RNA gene, partial sequence	835	835	100%	0.0	99%
AF511433.1	Pseudomonas fluorescens 16S ribosomal RNA gene, partial sequence	835	835	100%	0.0	99%
AF325153.1	Pseudomonas sp. AU 16S ribosomal RNA gene, partial sequence	835	835	100%	0.0	99%
AY014810.1	Pseudomonas sp. NZ047 16S ribosomal RNA gene, partial sequence	835	835	100%	0.0	99%
AF408884.1	Pseudomonas sp. NZPN5 16S ribosomal RNA gene, partial sequence	835	835	100%	0.0	99%
FN668427.1	Pseudomonas alcaliphila partial 16S rRNA gene, strain HSD-7110	833	833	99%	0.0	99%
EF100617.1	Pseudomonas putida strain HS-N24 16S ribosomal RNA gene, partial sequence	833	833	100%	0.0	99%

Group 2 (Continued)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
AM937257.1	Pseudomonas sp. HpsW1 partial 16S rRNA gene, strain HpsW1	832	832	100%	0.0	99%
AY014809.1	Pseudomonas sp. NZ043 16S ribosomal RNA gene, partial sequence	832	832	100%	0.0	99%
FM173317.1	Pseudomonas sp. BF.70 partial 16S rRNA gene, isolate BF.70	830	830	100%	0.0	99%
AY972219.1	Pseudomonas plecoglossicida strain P6 16S ribosomal RNA gene, partial sequence	830	830	100%	0.0	99%
GQ199767.1	Bacillus sp. 210_65 16S ribosomal	826	826	99%	0.0	100%
FJ175031.1	RNA gene, partial sequence	826	826	100%	0.0	99%
	Uncultured bacterium clone sbrh_64 16S ribosomal RNA gene, partial sequence					
	Bacillus sp. E2(2008) 16S ribosomal RNA gene, partial sequence	826	826	100%	0.0	99%
GQ199770.1	Bacillus sp. 210_68 16S ribosomal RNA gene, partial sequence	821	821	99%	0.0	99%
FJ535469.1	Bacillus sp. E1(2008) 16S ribosomal RNA gene, partial sequence	821	821	99%	0.0	99%
FJ217196.1	Bacillus sp. BQAL7-01 16S ribosomal RNA gene, partial sequence	815	815	99%	0.0	99%
AB519003.1	Bacillus sp. MB95 gene for 16S ribosomal RNA, partial sequence	813	813	99%	0.0	99%
FJ605362.1	Bacillus sp. EL-1 16S ribosomal RNA gene, partial sequence	813	813	100%	0.0	99%
FJ535470.1	Bacillus sp. C3(2008) 16S ribosomal RNA gene, partial sequence	811	811	98%	0.0	99%
DQ122256.1	Curtobacterium sp. iMTIII1 16S ribosomal RNA gene, partial sequence	811	811	99%	0.0	99%
DQ122249.1	Bacillus sp. iMTIII11 16S ribosomal RNA gene, partial sequence	811	811	99%	0.0	99%
FJ605392.1	Bacillus sp. BL-1 16S ribosomal RNA gene, partial sequence	809	809	100%	0.0	98%
AY907562.1	Bacterium OVA12 16S ribosomal RNA gene, partial sequence	809	809	98%	0.0	99%
GQ844976.1	Bacillus idriensis strain REG180 16S ribosomal RNA gene, partial sequence	808	808	99%	0.0	99%
FJ605390.1	Bacillus sp. BB-1 16S ribosomal RNA gene, partial sequence	806	806	99%	0.0	98%
FJ605396.1	Bacillus sp. BR-3 16S ribosomal RNA gene, partial sequence	804	804	99%	0.0	99%
AF128759.1	Soil bacterium is11 16S ribosomal RNA, partial sequence	797	797	99%	0.0	98%
DQ122227.1	Bacillus sp. iMCIII11 16S ribosomal RNA gene, partial sequence	791	791	96%	0.0	99%

Group 2 (Continued)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
EU182839.1	Bacillus sp. MH10(2007b) 16S ribosomal RNA gene, partial sequence	773	773	92%	0.0	100%
AJ878859.2	Bacillus sp. NHTS-47 partial 16S rRNA gene, strain NHTS-47	773	773	92%	0.0	100%
GU726566.1	Bacillus sp. ISL3 16S ribosomal RNA gene, partial sequence	767	767	92%	0.0	99%
EU373529.1	Bacillus idriensis strain FR1_96 16S ribosomal RNA gene, partial sequence	767	767	92%	0.0	99%
FJ977614.1	Bacillus sp. SF242 16S ribosomal RNA (rrnE) gene, partial sequence	767	767	92%	0.0	99%
EU810836.1	Firmicutes bacterium 00YJ4 16S ribosomal RNA gene, partial sequence	767	767	92%	0.0	99%
EU734600.1	Bacillus sp. VC-YC6631 16S ribosomal RNA gene, partial sequence	767	767	92%	0.0	99%
AM950301.1	Bacillus sp. AS7 HS-2008 partial 16S rRNA gene, isolate AS7 HS-2008	767	767	92%	0.0	99%
EF101988.1	Bacillus sp. EEZMo-2 16S ribosomal RNA gene, partial sequence	767	767	92%	0.0	99%
EF010751.1	Bacillus koguryoae strain 091-4.2-LH-A-09 16S ribosomal RNA gene, partial sequence	767	767	92%	0.0	99%
DQ180949.1	Bacillus sp. MI-67a2 16S ribosomal RNA gene, partial sequence	767	767	92%	0.0	99%
AY904033.1	Bacillus idriensis strain SMC 4352-2 16S ribosomal RNA gene, partial sequence	767	767	92%	0.0	99%
DQ296006.1	Bacillus sp. Y4 16S ribosomal RNA gene, partial sequence	767	767	92%	00	99%
AM934692.1	Bacillus sp. BF149 partial 16S rRNA gene, strain BF149	839	839	100%	0.0	99%
AJ316313.1	Bacillus sp. LMG 20241 partial 16S rRNA gene, strain LMG 20241	839	839	100%	0.0	99%
AM983495.1	Bacillus sp. T53Y partial 16S rRNA gene, isolate T53Y	835	835	100%	0.0	98%
AM934695.1	Bacillus sp. CL1.120 partial 16S rRNA gene, strain CL1.120	835	835	100%	0.0	98%
GQ980245.1	Bacillus niacini strain NBK36 16S ribosomal RNA gene, partial sequence	833	833	100%	0.0	98%
AM983471.1	Bacillus sp. SC6T partial 16S rRNA gene, isolate SC6T	833	833	100%	0.0	98%
EU221375.1	Bacillus niacini strain Y2S5 16S ribosomal RNA gene, partial sequence	833	833	100%	0.0	98%
EU221338.1	Bacillus niacini strain YM1C7 16S ribosomal RNA gene, partial sequence	833	833	100%	0.0	98%
AY635874.1	Bacillus sp. BM-11_0 16S ribosomal RNA gene, partial sequence	833	833	100%	0.0	98%

Group 2 (Continued)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
FJ560473.1	Bacillus sp. W1-17 16S ribosomal RNA gene, partial sequence	830	830	99%	0.0	98%
EU221335.1	Bacillus niacini strain BM1C4 16S ribosomal RNA gene, partial sequence	830	830	100%	0.0	98%
AM983483.1	Bacillus sp. SA15T partial 16S rRNA gene, isolate SA15T	828	828	100%	0.0	98%
EU221359.1	Bacillus niacini strain J2S5 16S ribosomal RNA gene, partial sequence	828	828	100%	0.0	98%
GQ199756.1	Bacillus sp. 210_54 16S ribosomal RNA gene, partial sequence	824	824	98%	0.0	98%
AM983517.1	Bacillus sp. K5T partial 16S rRNA gene, isolate K5T	824	824	100%	0.0	98%
DQ416567.1	Bacterium S1cc4 16S ribosomal RNA gene, partial sequence	822	822	100%	0.0	98%
AY211186.1	Bacillus sp. 'Mali 342' 16S ribosomal RNA gene, partial sequence	822	822	100%	0.0	98%
NR_024695.1	Bacillus niacini strain IFO15566 16S ribosomal RNA, partial sequence >dbj|AB021194.1| Bacillus niacini gene for 16S ribosomal RNA	822	822	100%	0.0	98%
FJ973528.1	Bacillus niacini strain NBPP53 16S ribosomal RNA gene, partial sequence	817	817	100%	0.0	98%
FM174044.1	Bacillaceae bacterium CL5.91 partial 16S rRNA gene, isolate CL5.91	817	817	100%	0.0	98%
AM983530.1	Bacillus sp. K35T partial 16S rRNA gene, isolate K35T	817	817	100%	0.0	98%
FM173603.1	Bacillaceae bacterium CL2.84 partial 16S rRNA gene, isolate CL2.84	813	813	100%	0.0	98%
AM983473.1	Bacillus sp. SC10T partial 16S rRNA gene, isolate SC10T	811	811	100%	0.0	98%
AJ563533.1	Bacillus sp. IDA0663 partial 16S rRNA gene, isolate IDA0663	811	811	100%	0.0	98%
AJ563532.1	Bacillus sp. IDA0253 partial 16S rRNA gene, isolate IDA0253	811	811	100%	0.0	98%
AF128749.1	Soil bacterium ic12 16S ribosomal RNA, partial sequence	808	808	99%	0.0	97%
AJ563501.1	Bacillus sp. IDA1790 partial 16S rRNA gene, isolate IDA1790	808	808	100%	0.0	97%
AJ563503.1	Bacillus sp. IDA1851 partial 16S rRNA gene, isolate IDA1851	806	806	100%	0.0	97%
FM174201.1	Bacillaceae bacterium CL7.33 partial 16S rRNA gene, isolate CL7.33	800	800	100%	0.0	97%
FJ263036.1	Bacillus sp. RC33 16S ribosomal RNA gene, partial sequence	795	795	98%	0.0	98%
FM174075.1	Bacillaceae bacterium CL5.134 partial 16S rRNA gene, isolate CL5.134	795	795	100%	0.0	97%

Group 3

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
EU373400.1	Microbacterium oxydans strain TPR04 16S ribosomal RNA gene, partial sequence	774	774	100%	0.0	99%
EU373379.1	Microbacterium oxydans strain TPL09 16S ribosomal RNA gene, partial sequence	774	774	100%	0.0	99%
EU373335.1	Microbacterium oxydans strain SSR09 16S ribosomal RNA gene, partial sequence	774	774	100%	0.0	99%
EU373332.1	Microbacterium oxydans strain HNL03 16S ribosomal RNA gene, partial sequence	774	774	100%	0.0	99%
EU373321.1	Microbacterium oxydans strain SSL09 16S ribosomal RNA gene, partial sequence	774	774	100%	0.0	99%
DQ358653.1	Microbacterium sp. YIM KMY13 16S ribosomal RNA gene, partial sequence	774	774	100%	0.0	99%
AY275515.1	Microbacterium sp. MSB2098 16S ribosomal RNA gene, partial sequence	774	774	100%	0.0	99%
AY039502.1	Soil bacterium S86D1 16S ribosomal RNA gene, partial sequence	774	774	100%	0.0	99%
AM234160.1	Microbacterium sp. S15-M4 partial 16S rRNA gene, isolate S15-M4	774	774	100%	0.0	99%
AJ391205.1	Microbacterium sp. AS-44, partial 16S rRNA gene for 16S ribosomal RNA	774	774	100%	0.0	99%
GU726572.1	Microbacterium sp. IRH4 16S ribosomal RNA gene, partial sequence	769	769	100%	0.0	99%
GU726563.1	Microbacterium sp. IP22C 16S ribosomal RNA gene, partial sequence	769	769	100%	0.0	99%
GU726558.1	Microbacterium sp. IP19 16S ribosomal RNA gene, partial sequence	769	769	100%	0.0	99%
GU726556.1	Microbacterium sp. IP17 16S ribosomal RNA gene, partial sequence	769	769	100%	0.0	99%
GU726555.1	Microbacterium sp. IP16 16S ribosomal RNA gene, partial sequence	769	769	100%	0.0	99%
GU726554.1	Microbacterium sp. IP15 16S ribosomal RNA gene, partial sequence	769	769	100%	0.0	99%
GU726552.1	Microbacterium sp. IP12 16S ribosomal RNA gene, partial sequence	769	769	100%	0.0	99%
GU726551.1	Microbacterium sp. IP11 16S ribosomal RNA gene, partial sequence	769	769	100%	0.0	99%
GU726550.1	Microbacterium sp. IP10 16S ribosomal RNA gene, partial sequence	769	769	100%	0.0	99%
GU726548.1	Microbacterium sp. IP7 16S ribosomal RNA gene, partial sequence	769	769	100%	0.0	99%

Group 3 (Continued)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
GU726538.1	Microbacterium sp. IR64 16S ribosomal RNA gene, partial sequence	769	769	100%	0.0	99%
GU726535.1	Microbacterium sp. IR61 16S ribosomal RNA gene, partial sequence	769	769	100%	0.0	99%
AB461649.1	Stenotrophomonas sp. M113 gene for 16S rRNA, partial sequence, strain: M113	848	848	100%	0.0	100%
EU362178.1	Microbacterium sp. C5 16S ribosomal RNA gene, partial sequence	643	643	100%	0.0	97%
AY635868.1	Microbacterium sp. BM-25 16S ribosomal RNA gene, partial sequence	643	643	100%	0.0	97%
EU438937.1	Arthrobacter sp. VTT E-073079 16S ribosomal RNA gene, partial sequence	795	795	100%	0.0	100%
EU668003.1	Arthrobacter sp. BF-2-2 16S ribosomal RNA gene, partial sequence	795	795	100%	0.0	100%
AY911182.1	Marine sediment bacterium ISA-7256 16S ribosomal RNA gene, partial sequence	785	785	100%	0.0	99%
DQ015981.1	Arthrobacter nicotianae strain BCT-1 16S ribosomal RNA gene, partial sequence	774	774	99%	0.0	99%
GU220063.1	Arthrobacter sp. YM-M-25 16S ribosomal RNA gene, partial sequence	771	771	97%	0.0	99%
EU927410.1	Arthrobacter sp. 18-N-12 16S ribosomal RNA gene, partial sequence	771	771	97%	0.0	99%
AJ576423.1	uncultured bacterium partial 16S rRNA gene, clone PeM69	771	771	100%	0.0	98%
DQ279378.1	Arthrobacter sp. TM4_1 16S ribosomal RNA gene, partial sequence	767	767	100%	0.0	98%
FJ774965.1	Proteobacterium symbiont of Nilaparvata lugens clone TM85-114 16S ribosomal RNA gene, partial sequence	761	761	100%	0.0	98%
EU747702.1	Arthrobacter sp. S14 16S ribosomal RNA gene, partial sequence	761	761	100%	0.0	98%
AB259962.1	Arthrobacter sp. PO-08 gene for 16S rRNA, partial sequence	761	761	100%	0.0	98%
DQ279377.1	Arthrobacter sp. TM5_1 16S ribosomal RNA gene, partial sequence	756	756	100%	0.0	98%
AY940423.1	Arthrobacter sp. GOL01 16S ribosomal RNA gene, partial sequence	756	756	100%	0.0	98%
AY039503.1	Soil bacterium S86D3 16S ribosomal RNA gene, partial sequence	752	752	99%	0.0	98%
EU232734.1	Arthrobacter sp. NOB18 16S ribosomal RNA gene, partial sequence	745	745	95%	0.0	99%

Group 3 (Continued))

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
EU232729.1	Arthrobacter sp. NOB10 16S ribosomal RNA gene, partial sequence	745	745	95%	0.0	99%
DQ365556.1	Arthrobacter sp. GH01 16S ribosomal RNA gene, partial sequence	739	739	100%	0.0	97%
AY731366.1	Arthrobacter sp. GWS-BW-H86 16S ribosomal RNA gene, partial sequence	739	739	100%	0.0	97%
FJ626619.1	Arthrobacter sp. MH127 16S ribosomal RNA gene, partial sequence	737	737	98%	0.0	98%
EU834260.1	Arthrobacter arilaitensis strain DS37 16S ribosomal RNA gene, partial sequence	737	737	98%	0.0	98%
GU124493.1	Arthrobacter sp. endosymbiont of Nilaparvata lugens clone A300 16S ribosomal RNA gene, partial sequence	728	728	100%	0.0	97%
EU439409.1	Arthrobacter sp. DB-11 16S ribosomal RNA gene, partial sequence	728	728	100%	0.0	97%
EU344921.1	Uncultured Arthrobacter sp. clone Hg5-11 16S ribosomal RNA gene, partial sequence	728	728	100%	0.0	97%
EU339930.1	Arthrobacter sp. W1 16S ribosomal RNA gene, partial sequence	728	728	100%	0.0	97%
EU327526.1	Arthrobacter sp. ArthroaeroA3 16S ribosomal RNA gene, partial sequence	728	728	100%	0.0	97%
EU264108.1	Arthrobacter sp. A-1 16S ribosomal RNA gene, complete sequence	728	728	100%	0.0	97%
EF468656.1	Arthrobacter sp. TD4 16S ribosomal RNA gene, partial sequence	728	728	100%	0.0	97%
EF468655.1	Arthrobacter sp. TD3 16S ribosomal RNA gene, partial sequence	728	728	100%	0.0	97%
EF379937.1	Arthrobacter sp. TD2 16S ribosomal RNA gene, partial sequence	728	728	100%	0.0	97%
AY370618.1	Arthrobacter sp. GWS-BW-H45M 16S ribosomal RNA gene, partial sequence	728	728	100%	0.0	97%
DQ112533.1	Arthrobacter nicotianae strain Y3-2 16S ribosomal RNA gene, partial sequence	728	728	100%	0.0	97%
DQ112532.1	Arthrobacter nicotianae strain Y3-1 16S ribosomal RNA gene, partial sequence	728	728	100%	0.0	97%
DQ359933.1	Arthrobacter sp. LZX11 16S ribosomal RNA gene, partial sequence	728	728	100%	0.0	97%
DQ359930.1	Arthrobacter sp. LZX08 16S ribosomal RNA gene, partial sequence	728	728	100%	0.0	97%
DQ359927.1	Arthrobacter sp. LZX05 16S ribosomal RNA gene, partial sequence	728	728	100%	0.0	97%

Group 3 (Continued)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
NR_026190.1	Arthrobacter nicotianae strain DSM 20123 16S ribosomal RNA, partial sequence >emb|X80739.1| A.nicotianae 16S rDNA	728	728	100%	0.0	97%
AY635865.1	Arthrobacter sp. BM-3 16S ribosomal RNA gene, partial sequence	726	726	100%	0.0	97%
EU402968.1	Arthrobacter nicotianae strain MCCB 104 16S ribosomal RNA gene, partial sequence	717	717	100%	0.0	97%
EU246788.1	Bacterium RSW1-1 16S ribosomal RNA gene, partial sequence	717	717	100%	0.0	97%
EF376011.1	Arthrobacter sp. HWW-22 16S ribosomal RNA gene, partial sequence	717	717	100%	0.0	97%
EF376010.1	Arthrobacter sp. TD-1 16S ribosomal RNA gene, partial sequence	717	717	100%	0.0	97%
AM398213.1	Arthrobacter sp. EP04 partial 16S rRNA gene, strain EP04	717	717	100%	0.0	97%
AM260537.1	Arthrobacter sp. AE05102002_1 partial 16S rRNA gene, isolate AE05102002_1	717	774	100%	0.0	100%
NR_025611.1	Arthrobacter arilaitensis strain Re117 16S ribosomal RNA, partial sequence >emb|AJ609628.1| Arthrobacter arilaiti partial 16S rRNA gene, strain CIP 108037	717	717	100%	0.0	97%
AJ609627.1	Arthrobacter arilaiti partial 16S rRNA gene, isolate Po102	717	717	100%	0.0	97%
AJ609626.1	Arthrobacter arilaiti partial 16S rRNA gene, isolate Po101	717	717	100%	0.0	97%
AJ609625.1	Arthrobacter arilaiti partial 16S rRNA gene, isolate Epo104	717	717	100%	0.0	97%
AJ609624.1	Arthrobacter arilaiti partial 16S rRNA gene, isolate Cou102	717	717	100%	0.0	97%
AJ609623.1	Arthrobacter arilaiti partial 16S rRNA gene, isolate Bres102	717	717	100%	0.0	97%
AJ609622.1	Arthrobacter arilaiti partial 16S rRNA gene, isolate Ma107	717	717	100%	0.0	97%
AJ967024.1	Arthrobacter sp. R-23188 16S rRNA gene, strain R-23188	717	717	100%	0.0	97%
AJ315492.1	Arthrobacter nicotianae 16S rRNA gene, strain SB42	717	717	100%	0.0	97%
AY370617.1	Arthrobacter sp. GWS-BW-H53M 16S ribosomal RNA gene, partial sequence	715	715	100%	0.0	97%
GQ923776.1	Microbacterium oxydans strain L2 16S ribosomal RNA gene, partial sequence	795	795	100%	0.0	100%

Group 3 (Continued)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
GQ279110.1	Microbacterium oxydans strain XH0903 16S ribosomal RNA gene, complete sequence	795	795	100%	0.0	100%
GQ152132.1	Microbacterium oxydans strain WT141 16S ribosomal RNA gene, partial sequence	795	795	100%	0.0	100%
EU438942.1	Microbacterium sp. VTT E-073038 16S ribosomal RNA gene, partial sequence	795	795	100%	0.0	100%
EU918731.1	Microbacterium oxydans strain PBCC6 16S ribosomal RNA gene, partial sequence	795	795	100%	0.0	100%
EU794389.1	Microbacterium sp. A2 16S ribosomal RNA gene, partial sequence	795	795	100%	0.0	100%
FM173274.1	Micrococcineae bacterium BF.4 partial 16S rRNA gene, isolate BF.4	795	795	100%	0.0	100%
EU714374.1	Microbacterium oxydans strain 698 16S ribosomal RNA gene, partial sequence	795	795	100%	0.0	100%
EU714369.1	Microbacterium oxydans strain 407 16S ribosomal RNA gene, partial sequence	795	795	100%	0.0	100%
EU714357.1	Microbacterium oxydans strain 3227 16S ribosomal RNA gene, partial sequence	795	795	100%	0.0	100%
EU714348.1	Microbacterium oxydans strain 297 16S ribosomal RNA gene, partial sequence	795	795	100%	0.0	100%
EU714347.1	Microbacterium oxydans strain 2841 16S ribosomal RNA gene, partial sequence	795	795	100%	0.0	100%
EU714344.1	Microbacterium oxydans strain 2704 16S ribosomal RNA gene, partial sequence	795	795	100%	0.0	100%
EU714335.1	Microbacterium oxydans strain 2083 16S ribosomal RNA gene, partial sequence	795	795	100%	0.0	100%
EU446133.1	Microbacterium sp. SMCC G887 16S ribosomal RNA gene, partial sequence	795	795	100%	0.0	100%
EU086800.1	Microbacterium oxydans strain 448 16S ribosomal RNA gene, partial sequence	795	795	100%	0.0	100%
AB365061.1	Microbacterium oxydans gene for 16S rRNA, partial sequence	795	795	100%	0.0	100%
AM403723.1	Microbacterium sp. EP32 16S rRNA gene	795	795	100%	0.0	100%
AM403722.1	Microbacterium sp. EP31 16S rRNA gene	795	795	100%	0.0	100%
DQ417333.1	Microbacterium oxydans strain 15E 16S ribosomal RNA gene, partial sequence	795	795	100%	0.0	100%

Group 3 (Continued)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
AJ864858.1	Microbacterium sp. J72 partial 16S rRNA gene, isolate J72	795	795	100%	0.0	100%
AJ879106.1	Microbacterium sp. R1_3_cr partial 16S rRNA gene, strain R1_3_cr	795	795	100%	0.0	100%
AJ879099.1	Microbacterium sp. B3_13_cr partial 16S rRNA gene, strain B3_13_cr	795	795	100%	0.0	100%
AJ969168.1	Microbacterium sp. R-23195 16S rRNA gene, strain R-23195	795	795	100%	0.0	100%
AJ717358.1	Microbacterium oxydans 16S rRNA gene, isolate CV71a	795	795	100%	0.0	100%
AJ717357.1	Microbacterium oxydans 16S rRNA gene, isolate AC44	795	795	100%	0.0	100%
AY017057.1	Microbacterium sp. A8-2 16S ribosomal RNA, 5'-partial sequence	795	795	100%	0.0	100%
AM234158.1	Microbacterium oxydans partial 16S rRNA gene, isolate S15-M2	795	795	100%	0.0	100%
AM234157.1	Microbacterium oxydans partial 16S rRNA gene, isolate SW366-KB-3	795	795	100%	0.0	100%
AM181506.1	Microbacterium maritypicum partial 16S rRNA gene, type strain DSM 12512	795	795	100%	0.0	100%
AJ853910.1	Microbacterium maritypicum partial 16S rRNA gene, strain DSM 12512T	795	795	100%	0.0	100%
AJ576066.1	Microbacterium oxydans partial 16S rRNA gene, clone MR32Y	795	795	100%	0.0	100%
Y17227.1	Microbacterium oxydans 16S rRNA gene	795	795	100%	0.0	100%
NR_026162.1	Microbacterium liquefaciens strain DSM 20638 16S ribosomal RNA, partial sequence >emb|X77444.1| A.liquefaciens (DSM 20638) 16S rRNA gene	795	795	100%	0.0	100%
DQ063077.1	Actinobacterium BAL137 16S ribosomal RNA gene, partial sequence	791	791	99%	0.0	100%
AJ716148.1	Microbacterium sp. AJ115 partial 16S rRNA gene	791	791	99%	0.0	100%
EU821338.1	Microbacterium oxydans 16S ribosomal RNA gene, partial sequence	822	822	100%	0.0	99%
DQ417926.1	Microbacterium sp. ZD-M2 16S ribosomal RNA gene, partial sequence	822	822	100%	0.0	99%
AJ717356.1	Microbacterium oxydans 16S rRNA gene, isolate AC94	819	819	100%	0.0	98%
AM234159.1	Microbacterium oxydans partial 16S rRNA gene, isolate S15-M5	819	819	100%	0.0	98%
EU723126.1	Actinobacterium kmd_116 16S ribosomal RNA gene, partial sequence	817	817	99%	0.0	98%

Group 3 (Continued)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
EF204430.1	Microbacterium oxydans strain H190 16S ribosomal RNA gene, partial sequence	817	817	99%	0.0	98%
EF204429.1	Microbacterium oxydans strain H185 16S ribosomal RNA gene, partial sequence	817	817	99%	0.0	98%
EF204428.1	Microbacterium oxydans strain H173 16S ribosomal RNA gene, partial sequence	817	817	99%	0.0	98%
FJ009389.1	Microbacterium oxydans strain D6 16S ribosomal RNA gene, partial sequence	815	815	99%	0.0	99%
EU196564.1	Microbacterium sp. F10a 16S ribosomal RNA gene, partial sequence	815	815	99%	0.0	99%
EU036699.1	Microbacterium sp. JYC17 16S ribosomal RNA gene, partial sequence	815	815	99%	0.0	99%
FJ608797.1	Microbacterium sp. ENB-8 16S ribosomal RNA gene, partial sequence	813	813	100%	0.0	98%
FJ531669.1	Microbacterium sp. JQ-3 16S ribosomal RNA gene, partial sequence	813	813	100%	0.0	98%
EU714349.1	Microbacterium oxydans strain 3043 16S ribosomal RNA gene, partial sequence	813	813	100%	0.0	98%
AB098594.1	Sphingobacterium sp. TUT1026 gene for 16S rRNA, partial sequence	789	789	99%	0.0	95%
AY556417.1	Sphingobacterium sp. MG2 16S ribosomal RNA gene, partial sequence	789	789	99%	0.0	95%
AB100738.1	Sphingobacterium multivorum gene for 16S rRNA, partial sequence	787	787	96%	0.0	96%
FJ868219.1	Sphingobacterium sp. No.6 16S ribosomal RNA gene, partial sequence	785	785	98%	0.0	96%
AF380159.1	Sphingobacterium sp. F1 16S ribosomal RNA gene, partial sequence	780	780	95%	0.0	96%
FJ179538.1	Sphingobacterium sp. BHN1 16S ribosomal RNA gene, partial sequence	778	778	98%	0.0	95%
EU216022.1	Sphingobacterium sp. QMT3-2 16S ribosomal RNA gene, partial sequence	776	776	95%	0.0	96%
EU710554.1	Arthrobacter sp. AD38 16S ribosomal RNA gene, partial sequence	808	808	100%	0.0	100%
EU672425.1	Arthrobacter sp. AD37 16S ribosomal RNA gene, partial sequence	808	808	100%	0.0	100%
EF623831.1	Arthrobacter sp. AD26 16S ribosomal RNA gene, partial sequence	808	808	100%	0.0	100%
EF488200.1	Arthrobacter sp. ADX10 16S ribosomal RNA gene, partial sequence	808	808	100%	0.0	100%
EF373977.1	Arthrobacter sp. ADH-2 16S ribosomal RNA gene, partial sequence	808	808	100%	0.0	100%

Group 3 (Continued)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
EF017808.1	Arthrobacter sp. pnp-3 16S ribosomal RNA gene, partial sequence	808	808	100%	0.0	100%
AY628691.1	Arthrobacter sp. AD25 16S ribosomal RNA gene, partial sequence	808	808	100%	0.0	100%
AY628690.1	Arthrobacter sp. AD12 16S ribosomal RNA gene, partial sequence	808	808	100%	0.0	100%
AY628689.1	Arthrobacter sp. AD3 16S ribosomal RNA gene, partial sequence	808	808	100%	0.0	100%
AY611647.1	Actinomycetales bacterium AD3 16S ribosomal RNA gene, partial sequence	808	808	100%	0.0	100%
AF543695.1	Arthrobacter sp. AD1 16S ribosomal RNA gene, partial sequence	808	940	100%	0.0	100%
DQ117533.1	Bacterium #WM-A3 16S ribosomal RNA gene, partial sequence	808	808	100%	0.0	100%
AM113709.1	Arthrobacter sp. FR3 partial 16S rRNA gene, strain FR3	808	808	100%	0.0	100%
FJ477386.1	Arthrobacter sp. HY2 16S ribosomal RNA gene, partial sequence	802	802	100%	0.0	99%
EU373310.1	Arthrobacter sp. SSL02 16S ribosomal RNA gene, partial sequence	802	802	100%	0.0	99%
FJ851358.1	Arthrobacter sp. JDC32 16S ribosomal RNA gene, partial sequence	800	800	100%	0.0	99%
NR_029281.1	Arthrobacter ureafaciens strain NC 16S ribosomal RNA, partial sequence	800	800	100%	0.0	99%
X80744.1	A.ureafaciens 16S rDNA	800	800	100%	0.0	99%
FJ189784.1	Arthrobacter sp. CSB07 16S ribosomal RNA gene, partial sequence	797	797	100%	0.0	99%
EU854147.1	Arthrobacter sp. ATLJ-1 16S ribosomal RNA gene, partial sequence	797	797	98%	0.0	100%
AB262078.1	Arthrobacter sp. KI72 gene for 16S ribosomal RNA, partial sequence	797	797	100%	0.0	99%
FN433020.1	Arthrobacter ureafaciens partial 16S rRNA gene, isolate CCM12B	791	791	100%	0.0	99%
GQ921948.1	Arthrobacter sp. V810A1 16S ribosomal RNA gene, partial sequence	789	789	97%	0.0	100%
GQ921947.1	Arthrobacter sp. T10A20 16S ribosomal RNA gene, partial sequence	789	789	97%	0.0	100%
FJ378034.1	Arthrobacter sp. JDC-8 16S ribosomal RNA gene, partial sequence	789	789	100%	0.0	99%
DQ656488.1	Arthrobacter sp. WY 16S ribosomal RNA gene, partial sequence	789	789	100%	0.0	99%
DQ860088.1	Arthrobacter sp. PDS-9 16S ribosomal RNA gene, partial sequence	787	787	97%	0.0	100%
EU589417.1	Arthrobacter sp. M060824-10 16S ribosomal RNA gene, partial sequence	785	785	98%	0.0	99%

Group 3 (Continued)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
EU589412.1	Arthrobacter sp. M060824-3 16S ribosomal RNA gene, partial sequence	785	785	98%	0.0	99%
AY600962.1	Arthrobacter sp. MCMB-435 16S ribosomal RNA gene, partial sequence	785	910	99%	0.0	98%
FJ378035.1	Arthrobacter sp. JDC-9 16S ribosomal RNA gene, partial sequence	784	784	97%	0.0	100%
EU600208.1	Arthrobacter sp. AMD1 16S ribosomal RNA gene, partial sequence	784	784	99%	0.0	99%
GQ921946.1	Arthrobacter sp. T12B12 16S ribosomal RNA gene, partial sequence	776	776	96%	0.0	99%
GU459072.1	Arthrobacter sp. HB-5 16S ribosomal RNA gene, partial sequence	773	773	97%	0.0	99%
FJ538216.1	Arthrobacter ureafaciens strain BPSr55 16S ribosomal RNA gene, partial sequence	773	773	97%	0.0	99%
FN794230.1	Arthrobacter sp. V3M41 partial 16S rRNA gene, strain V3M41	771	771	95%	0.0	100%
AF256196.1	Arthrobacter keyseri 12B 16S ribosomal RNA gene, partial sequence	771	771	100%	0.0	98%
AY729888.1	Arthrobacter sp. RKJ4 16S ribosomal RNA gene, partial sequence	771	771	100%	0.0	98%
EF078488.1	Arthrobacter sp. JS443 16S ribosomal RNA gene, partial sequence	765	765	100%	0.0	98%
AY577525.1	Arthrobacter protophormiae 16S ribosomal RNA gene, partial sequence	765	765	100%	0.0	98%
AB127968.1	Micrococcus sp. YGJ1 gene for 16S rRNA, partial sequence	765	765	100%	0.0	98%
FJ538217.1	Arthrobacter ureafaciens strain BPSr57 16S ribosomal RNA gene, partial sequence	761	761	97%	0.0	98%
FJ975126.1	Arthrobacter sp. JDC-19 16S ribosomal RNA gene, partial sequence	760	760	94%	0.0	100%
FJ455452.1	Arthrobacter ureafaciens 16S ribosomal RNA gene, partial sequence	758	758	93%	0.0	100%
DQ118406.1	Arthrobacter sp. HL-2005 16S ribosomal RNA gene, partial sequence	758	758	96%	0.0	99%
EU600212.1	Arthrobacter sp. AMD5 16S ribosomal RNA gene, partial sequence	749	749	100%	0.0	97%
DQ129714.1	Arthrobacter sp. HPC379 16S ribosomal RNA gene, partial sequence	747	747	98%	0.0	97%
EF177362.1	Arthrobacter ureafaciens isolate JN2-2 16S ribosomal RNA gene, partial sequence	743	743	96%	0.0	98%
FN600407.1	Arthrobacter sp. DK2009-3b partial 16S rRNA gene, isolate 3b	737	737	100%	0.0	97%

Group 3 (Continued)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
EF050052.1	Arthrobacter keyseri 16S ribosomal RNA gene, partial sequence	736	736	100%	0.0	97%
DQ113888.1	Bacterium mgm18 16S ribosomal RNA gene, partial sequence	734	734	92%	0.0	99%
EU839221.1	Soil bacterium 6V-10 16S ribosomal RNA gene, partial sequence	728	728	92%	0.0	99%
AY039448.1	Earthworm burrow bacterium B1D1 16S ribosomal RNA gene, partial sequence	728	728	92%	0.0	99%
AB098573.1	Arthrobacter globiformis gene for 16S rRNA, partial sequence	728	728	92%	0.0	99%
M23411.1	A.globiformis small subunit ribosomal RNA	728	728	92%	0.0	99%
EU001326.1	Arthrobacter sp. VA3 16S ribosomal RNA gene, partial sequence	726	726	92%	0.0	99%
EU839069.1	Soil bacterium 2V-11 16S ribosomal RNA gene, partial sequence	739	739	93%	0.0	99%
AB461786.1	Arthrobacter sp. M448 gene for 16S rRNA, partial sequence, strain: M448	739	739	93%	0.0	99%
FJ539153.1	Arthrobacter sp. AS3.04 16S ribosomal RNA gene, partial sequence	739	739	93%	0.0	99%
AB363933.1	Arthrobacter nicotinovorans gene for 16S rRNA, partial sequence, strain: Y-1	739	739	93%	0.0	99%
EU149249.1	Bacterium EAB-F2.01 16S ribosomal RNA gene, partial sequence	739	739	93%	0.0	99%
AY714235.1	Arthrobacter sp. HSL-2 16S ribosomal RNA gene, partial sequence	739	739	93%	0.0	99%
EF028242.1	Arthrobacter sp. PZC6 16S ribosomal RNA gene, partial sequence	739	739	93%	0.0	99%
DQ530163.1	Arthrobacter sp. CI60 16S ribosomal RNA gene, partial sequence	739	739	93%	0.0	99%
DQ530146.1	Arthrobacter sp. RI52 16S ribosomal RNA gene, partial sequence	739	739	93%	0.0	99%
DQ530131.1	Arthrobacter sp. RI43 16S ribosomal RNA gene, partial sequence	739	739	93%	0.0	99%
DQ530101.1	Arthrobacter sp. CI34 16S ribosomal RNA gene, partial sequence	739	739	93%	0.0	99%
AY371237.1	Arthrobacter sp. BS14 16S ribosomal RNA gene, partial sequence	739	739	98%	0.0	97%
AY234695.1	Bacterium Ellin6043 16S ribosomal RNA gene, partial sequence	739	739	93%	0.0	99%
AY247743.1	Arthrobacter sp. IBN110 16S ribosomal RNA gene, partial sequence	739	739	93%	0.0	99%
AY822560.1	Bacterium PSB-1-14 16S ribosomal RNA gene, partial sequence	739	739	93%	0.0	99%

Group 3 (Continued)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
AY822528.1	Bacterium RSB-3-9 16S ribosomal RNA gene, partial sequence	739	739	93%	0.0	99%
AY822484.1	Bacterium RBA-1-7 16S ribosomal RNA gene, partial sequence	739	739	93%	0.0	99%
AF501360.1	Arthrobacter histidinolovorans strain HAMBI2383 16S ribosomal RNA gene, partial sequence	739	739	93%	0.0	99%
AF501359.1	Arthrobacter histidinolovorans strain HAMBI2379 16S ribosomal RNA gene, partial sequence	739	739	93%	0.0	99%
AF501358.1	Arthrobacter histidinolovorans strain HAMBI2372 16S ribosomal RNA gene, partial sequence	739	739	93%	0.0	99%
AF501356.1	Arthrobacter histidinolovorans strain HAMBI2381 16S ribosomal RNA gene, partial sequence	739	739	93%	0.0	99%
AY911073.1	Marine sediment bacterium ISA-7335 16S ribosomal RNA gene, partial sequence	739	739	93%	0.0	99%
AJ785759.1	Arthrobacter sp. B2-6 16S rRNA gene, strain B2-6	739	739	93%	0.0	99%
AM179862.1	Arthrobacter sp. A22 A22 A22 partial 16S rRNA gene, isolate A22	739	739	93%	0.0	99%
AY833098.1	Arthrobacter nicotinovorans isolate Ph13 16S ribosomal RNA gene, partial sequence	737	737	93%	0.0	99%