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NOTE

The Microbial Population in the Air of Cultivation Facility of Oyster Mushrooms

Se Chul Chun[1], Yu Na Ahn[1], Sajid Mohamad Khan[1], Il Min Chung[1], Hyang Yoen Won[2], Chang Sung Jhune[3], and Yool Jin Park[4]*

[1]Division of Applied Life Science, College of Life and Environmental Science, Konkuk University, Seoul 143-701, Republic of Korea
[2]The Center for National Genetic Resources, RDA, Suwon 441-853, Republic of Korea
[3]Department of Mushroom, Division Ginseng and Specialty Crop, National Institute of Horticulture and Specialty Crop Sciences, RDA, Suwon 441-707, Republic of Korea
[4]Division of Environmental and Resource, College of Environmental and Bioresource Sciences, Chonbuk National University, Iksan 570-752, Republic of Korea

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The microbial population in the air of mushroom cultivation facility was studied to understand the population structure and size depending on the cultivation methods and regions. The air contents of ten farmers' oyster mushroom cultivation facilities in Kyunggi province were sampled. The results indicated that there was no difference in population size depending on the regions of mushroom cultivation. In addition, the population size of bacteria in the growth room was bigger than that of the cooling room and outside of the mushroom house, but the fungal population was similar in size between cultivation stages. With regard to population structure, *Pseudomonas* and *Penicillium* species were most frequently isolated from the air of oyster mushroom cultivation facility.

Keywords: microbial population, oyster mushroom, *Penicillium*, *Pseudomonas*, *Trichoderma*

Worldwide mushroom cultivation is dominated by the production of *Agaricus bisporus* (button mushroom), *Lentinula edodes*, and *Pleurotus ostreatus* (oyster mushroom) (Chang, 1999). *Pleurotus ostreatus* is an important edible basidiomycete commonly known as oyster mushroom. This fungus is the third most commercially important edible mushroom worldwide (Chang, 1996). In addition, *P. ostreatus* is used for bioconversion of agricultural and industrial lignocellulose debris (Puniya *et al.*, 1996) and as a source of enzymes and other metabolites for industrial and medical applications (Gunde-Cimerman, 1999).

Mushroom quality declines rapidly in the bed due to the maturation or senescence process which is in fact the natural development of fruit bodies for the production of spores (Burton *et al.*, 1995). However, the most problematic reducer of quality comes from microbial colonization of mushroom caps (Soler-Rivas *et al.*, 1999). The disease has been detected and described all over the world and it affects not only the button mushroom markets in the USA (Tolaas, 1915), Australia (Nair, 1975), Israel (Bashan and Okon, 1981), and Turkey (Ozaktan and Bora, 1994), but also so-called 'exotic' mushroom (*Pleurotus*, *Lentinula*, *Flammulina*, etc.) markets in eastern and southern Asian countries such as Japan (Suyama and Fujii, 1993), Korea (Kim *et al.*, 1995), China (Cutri *et al.*, 1984), and India (Guleria, 1976).

Pseudomonas tolaasii is primarily known as the cause of brown blotch in the cultivated mushrooms *Agaricus bisporus*, *P. ostreatus*, and *Psalliota edulis* (Goor *et al.*, 1986; Rainey *et al.*, 1992). It was first described in St. Paul, MN, by Tolaas (1915). *P. tolaasii*, which is commonly isolated from mushrooms, is associated with growing media and is considered a normal constituent of microbiota (Fletcher *et al.*, 1986).

Trichoderma species are common contaminants of spawn, compost, and wood in commercial mushroom growing facilities (Castle *et al.*, 1998). *Trichoderma* has caused severe problems in many areas of North America including Ontario, British Columbia, and Pennsylvania (Rinker, 1993). This infection has come to be known as green mold disease (Castle *et al.*, 1998; Jhune, 2002). Since at least 1986, commercial production of mushrooms has been seriously affected by green mold epidemics. The first reported epidemic was in Ireland and the United Kingdom (Samuels *et al.*, 2002). Outbreaks of Trichoderma green mold have also been reported to seriously affect the commercial production of *Pleurotus ostreatus* in Korea (Park *et al.*, 2004) and Italy (Woo *et al.*, 2004). The incidence of Green mold and bacterial blotch disease in oyster mushrooms in Kyunggi province of Korea were under 10–20% in bottle and bed cultivation (Ahn *et al.*, 2009).

To understand the population structure and population size depending on the cultivation methods and regions, the air contents of ten farmers' oyster mushroom cultivation facilities were sampled from the cooling room, growth room

*For correspondence. E-mail: land@chonbuk.ac.kr; Tel.: +82-63-850-0783; Fax: +82-63-850-0735

Fig. 1. Sampling at the growth room (A) and outside (B) of mushroom facility.

and from the outside of the facilities of bottle mushroom cultivation facility, and growth room and the outside of the facilities of bed mushroom cultivation facility from Kwangju city, Kapyung city, and Yangpyung city of Kyunggi province.

Air was sampled in 100 L with an air sampler (MAS-100 Eco; Merck, Germany). The media for the air samplers were R2A agar (Difco, USA) amended with cycloheximide (0.25 g/L) for bacteria and rose bengal agar amended with streptomycin sulfate (0.3 g/L) for fungi (Fig. 1). The air samples for bacteria were incubated at 28°C for 48 h and for fungi at 28°C for 3–5 days and then the number of colonies (colony-forming unit) was determined.

The representative colonies were analyzed for determination of their genera on the basis of 16S rDNA PCR for bacteria. The single colonies were transferred from the plates and cultured in a 96 well plate with 300 µl R2A (Difco, USA) broth at 28°C, 10,000 rpm, for 2–3 days. In order to extract genomic DNA, colonies incubated in R2A broth were lysed with 5 times of shock-freezing in liquid nitrogen and thawing at 65°C.

Extracted genomic DNA was used for PCR amplification of the 16S rRNA gene using universal primers fD1 (5′-AG AGTTTGATCCTGGCTCAG-3′) as a forward primer and rP2 (5′-ACGGCTACCTTGTTACGACTT-3′) as a reverse primer (Weisburg *et al.*, 1991) to determine their genera. The 16S rRNA gene was amplified by PCR in a reaction mixture containing 100 pmol each of the primer fD1, rP2, 2.5 mM dNTP, and ×10 Taq polymerase buffer. The final volume of the PCR mixture was adjusted to 20 µl by adding 3rd dH_2O. 0.25 unit of Taq polymerase (Solgent Co., Ltd., Korea) was then added to the reaction solution. Thermal cycling was performed with Tgradient thermoblock (Biometra® GmbH, Germany). The PCR conditions consisted of one cycle at 95°C for 4 min, then 34 cycles at 95°C for 1 min, 58°C for 1 min, and 72°C for 1 min and finally one cycle at 72°C for 8 min. The PCR products were analyzed by electrophoresis on a 1% agarose gel and stained with ethidium bromide to detect for PCR-amplified DNA fragments.

PCR products were purified using Montage PCR 96 Cleanup kit (Montage, USA) according to the manufacturer's instructions and were directly sequenced (Hiraishi, 1992). The 16S rDNA sequences were determined with an Applied Biosystems 3100 sequencer (Applied Biosystems, USA) using a 783R primer (5′-GTGGACTACCAGGTATCTA-3′). DNA sequence analysis was performed using RDP (rdp. cme. msu. edu) and DNASTAR software program (Altschul *et al.*, 1997). The sequences were aligned together with those of representative members of selected genera by the CLUSTAL W program (Thompson *et al.*, 1994). The phylogenetic tree for the datasets was inferred from the neighbor-joining method (Saitou and Nei, 1987) using the MEGA version 3.1 (Kumar *et al.*, 2004). The stability of relationships was assessed by performing bootstrap analyses of the neighbor-joining data based on 1000 resamplings.

The fungi isolated as a single conidia grew on PDA and the areas of growing mycelial tip were observed with a stero-microscope and dissected for further observation under a light microscope. The morphology of conidiaphore, conidia and phialides were observed to identify the species (Barnett and Hunter, 1998). *Trichoderma* species was also identified according to Rifai (1969).

The population densities of bacteria and fungi, 7.40×10^3 CFU/m^3 and 8.09×10^3 CFU/m^3, respectively, from bottle cultivation of oyster mushrooms were significantly higher compared to those of bed cultivation (P=0.05) which were 2.69×10^3 CFU/m^3 for bacteria and 2.0×10^3 CFU/m^3 for fungi, respectively (Table 1). This might be attributed to the fact that farmers of bed cultivation practice steam sterilization or heat treatment up to 70°C for 2–3 days in the inside of the growth room facility before inoculation of mushroom fungi after the mushroom growth substrate was added into the beds. In contrast, the farmers of bottle cultivation by the automotive system do not have to practice this because the growth media in the bottle are already sterilized and inoculated with mushroom fungi. Thus, microbial population might be more built up in the air of the growth room in

Table 1. Microbial density in the air depending on cultivation methods

Sample location	Number of cultivation facility	CFU(×10³)/m³	
		Bacteria	Fungi
Bed cultivation (Growth room)	5	2.69a[a]	2.02b
Bottle cultivation (Growth room)	5	7.40a	8.09a
Outside of mushroom facilities	10	0.39b	1.01c

[a] The means followed by same letters within columns were not significantly different (LSD, P=0.05). Statistic analysis was conducted with log transformed data. Only growth rooms of bottle and bed cultivation facilities were compared.

Table 2. Microbial density in the air depending on growth stage of oyster mushroom house

Sample location	CFU(×10³)/m³	
	Bacteria	Fungi
Cooling room	0.43b[a]	4.30ab
Growth room	7.40a	8.09a
Outside of mushroom facilities	0.51b	1.94c

[a] The means followed by same letters within columns were not significantly different (LSD, P=0.05). Statistic analysis was conducted with log transformed data.

Table 3. Microbial population density in the air depending on area including bed and bottle cultivation of oyster mushroom house

Area	Number of houses	CFU(×10³)/m³	
		Bacteria	Fungi
Kapyung-gun	5	1.48a[a]	1.06a
Kwangju city	3	4.68a	4.34a
Yangpyung-gun	2	0.71a	5.43a

[a] The means followed by same letters within columns were not significantly different (LSD, P=0.05). Statistic analysis was conducted with log transformed data.

Table 4. Percent isolation from the air of growth room in the bed of cultivation facilities and cooling and growth room of bottle cultivation facilities of oyster mushroom

Genus[a]	Cultivation environment				Region of cultivation			
	Cooling room (%)	Growth room[b] (%)	Outside of mushroom house (%)	Mean (%)	Kapyung Gun (%)	Kwangju City (%)	Yangpyung Gun (%)	Mean (%)
Pseudomonas	23.8	30.7	12.4	22.3a[e]	8.9	15.6	57.6	27.4a
Pedobacter	11.9	10.0	12.5	11.5b	11.4	4.7	14.1	10.1a
Bacillus	7.1	6.4	16.7	10.1bc	10.6	9.4	4.4	8.1a
Arthrobacter	7.1	3.2	16.7	9.0bc	7.3	9.4	2.2	6.3a
Microbacterium	0.0	5.3	10.4	5.2bc	9.8	3.1	1.1	4.7a
Flavobacterium	4.8	5.8	0.0	3.5bc	8.9	3.1	0.0	4.0a
Chryseobacterium	2.4	3.7	4.2	3.4bc	4.9	0.0	4.4	3.1a
Herbaspirillum	4.8	3.7	0.0	2.8c	0.8	10.9	1.1	4.3a
Rhodococcus	0.0	3.7	2.1	1.9c	6.5	0.0	0.0	2.2a
Group 1[c]	38.1	27.5	25.0	30.2	30.9	43.8	15.2	30.0
Tota l(%)	100.00 (42)[d]	100.00 (189)	100.00 (48)	100.00 (279)	100.00 (123)[c]	100.00 (64)	100.00 (92)	100.00 (279)

[a] There were 279 of total isolated strains 16S rDNA analysed.
[b] Growth room indicates the air samples from bed and bottle cultivation together.
[c] Group 1 indicates the bacteria with very low percent isolation rates. This was not included for statistical analysis.
[d] Number in parenthesis indicates total number of bacteria isolated.
[e] The means followed by same letters are not significantly different (LSD, P=0.05).

bottle cultivation than in bed cultivation.

The population density of bacteria from the growth room, 7.40×10^3 CFU/m^3, was significantly higher compared to those of the cooling room (P=0.05) which was 0.43×10^3 CFU/m^3 for bacteria (Table 2), suggesting that the population density could be higher possibly due to higher temperature and higher humidity in the air compared to the cooling room. The population density of bacteria in the cooling room was similar to that of outside of the mushroom facilities which were 0.51×10^3 CFU/m^3 (Table 2). However, the population densities of fungi in the cooling room were not significantly different from the growth room, and outside of the mushroom facilities (P=0.05). This might be attributed to the characteristics that fungi can readily survive as spores in cool and harsh conditions compared to other common bacteria except for spore-forming bacteria. In general, fungal spores survive longer in harsh conditions than bacteria do.

Bacterial and fungal contaminants in the air of different regions' mushroom cultivation facilities were not significantly different (P=0.05) (Table 3). The microbial population densities of bacteria and fungi depending on the different regions of cultivation in Kapyung were 1.48×10^3 CFU/m^3 and 1.06×10^3 CFU/m^3, respectively. In Kwangju city, the population densities of bacteria and fungi were 4.68×10^3 CFU/m^3 and 4.34×10^3 CFU/m^3, respectively. In Yangpyung, the population densities of bacteria and fungi were 0.71×10^3 CFU/m^3 and 5.43×10^3 CFU/m^3, respectively (Table 3). There was no statistically significant difference in the microbial population densities of the mushroom cultivation facilities between the regions (P=0.05), implying that microbial population densities might be rather dependent on the individual house's sanitation conditions than regional conditions.

Percent isolation of *Psuedomonas* spp. were 22.3%, *Pedobacter* spp., 11.5% and *Bacillus* spp., 10.1% from all the indoor rooms and from outside of the mushroom cultivation facility (Table 4). The percent isolation from the cooling and growth rooms showed the same trend as the total percent isolation of 16S rDNA analysed from a total of 279 isolates (Table 4). *Psuedomonas* species was more frequently isolated with percent isolation of 30.7% from the growth

Table 5. Percent isolated proportion depending on the cultivation method of oyster mushroom

Genus	Cultivation method				Cultivation environment			
	Bed cultivation (%)	Bottle cultivation (%)	Outside of mushroom cultivation (%)	Mean (%)	Cooling room (%)	Growth room (%)	Outside of mushroom cultivation (%)	Mean (%)
Penicillium	83.1	79.3	25.0	62.4ab[b]	65.0	87.1	25.0	59.0ab
Cladosporium	9.2	11.1	51.0	23.8b	18.3	7.1	51.0	25.5b
Aspergillus	6.2	3.0	13.5	7.6bc	3.3	4.3	13.5	7.1bc
Trichoderma	1.5	3.7	4.2	3.1c	8.3	0.7	4.2	4.4c
Alternaria	0.0	0.0	5.2	1.7cd	0.0	0.0	5.2	1.7cd
Rhizopus	0.0	1.5	0.0	0.5d	3.3	0.0	0.0	1.1d
Absidia	0.0	0.7	0.0	0.2d	1.7	0.0	0.0	0.6d
Mucor	0.0	0.7	0.0	0.2d	0.0	0.7	0.0	0.2d
Ulocladium	0.0	0.0	1.0	0.3d	0.0	0.0	1.0	0.3d
Total (%)	100.00 (65)[a]	100.00 (135)	100.00 (96)	100.00 (296)	100.00 (60)[a]	100.00 (140)	100.00 (96)	100.00 (296)

[a] Number in parenthesis indicates total number of fungi isolated. The raw data are the same as the bed and bottle culvation but it is arranged depending on cooling room and growth room. Bed cultivation has only growth room. Thus, fungal isolates were included in only the growth room.
[b] The means followed by same letters are not significantly different (LSD, P=0.05).

Table 6. Percent isolated proportion depending on the regions of oyster mushroom cultivation facility

Genus	Kapyung province (%)	Kwangju city (%)	Yangpyung province (%)	Mean (%)
Penicillium	66.3	58.4	63.8	62.8a
Cladosporium	23.6	23.9	23.4	23.6b
Aspergillus	5.6	12.4	2.1	6.7c
Trichoderma	2.3	3.5	4.3	3.4c
Alternaria	2.3	0.9	2.1	1.8cd
Rhizopus	0.0	0.0	2.1	0.7d
Absidia	0.0	0.9	0.0	0.3d
Mucor	0.0	0.0	1.1	0.4d
Ulocladium	0.0	0.0	1.1	0.4d
Total (%)	100.00 (89)[a]	100.00 (113)	100.00 (94)	100.00 (296)

[a] Number in parenthesis indicates total number of fungi isolated. The raw data are the same as the table 5 but it is arranged depending on the regions of mushroom growth facility.

room than the cooling room with that of 23.8% (Table 4). *Psuedomonas* species which causes brown blotch disease in mushrooms was most frequently isolated from the air of oyster mushroom cultivation facility followed by *Pedobacter* and *Bacillus* species. Some of these *Psuedomonas* spp., *Pedobacter* spp., and *Bacillus* spp., showed hydrolytic activities against mycelium of oyster mushrooms (Ahn *et al*., 2010), suggesting that these bacteria could be pathogenic to oyster mushrooms.

Also, *Pseudomonas* spp. was most frequently isolated out of all the analyzed isolates from Yangpyung district with 57.6% compared to Kapyung district with 8.9% and Kwangju city with 15.6% (Table 4). Although these data are not strong enough to insist that *Psuedomonas* spp., *Pedobacter* spp., and *Bacillus* spp. are definitely the major components of bacteria in all the three regions, there are observable trends that these bacteria comprised of the major proportions of all the bacteria analyzed with 16S rDNA in all the three regions. Our study was intended to provide the types of various bacteria that could exist in mushroom growth facilities. Also, in our previous study, brown blotch disease occurred more frequently in the mushroom houses of Yangpyung province than the other two places (Ahn *et al*., 2009).

Penicillium species with the isolation rate of 62.4% was most commonly isolated from the air of cultivation facility of oyster mushrooms and outside of the mushroom cultivation facility, followed by *Cladosporium* spp. with 23.8%, *Aspergillus* spp. with 7.6% and *Trichoderma* spp. with 3.1% out of 296 isolates in total from all the air contents collected from the mushroom cultivation facilities and outside of the mushroom cultivation facilities (Table 5). It should be noted that *Penicillium* spp. was specifically most common in bed and bottle cultivations with the percent isolation of 83.1 and 79.3%, respectively. *Trichoderma* spp. was 1.5% and 3.7% in the air of bed and bottle cultivations, respectively (Table 5). *Alternaria*, *Rhizopus*, *Absidia*, *Mucor*, and *Ulocladium* were detected in very low proportions (Table 5).

Penicillium, *Trichoderma*, and *Aspergillus* spp., are all called green molds by farmers. *Penicillium* species is more common in the cooling room and growth room with the percent isolation of 65.0% and 87.1%, respectively, than outside of the facility. This may due to the build-up of fungal spores inside the facility than the outside (Table 5). Green molds are known to cause diseases in mushrooms (Jhune, 2002). *Cladosporium* spp. is a very common fungi in the air of most of crop farming places. However, these are not known to cause diseases in mushrooms.

Percent isolations of the *Trichoderma* species were 8.3% and 0.7% for the air of the cooling room and growth room, respectively (Table 5). However, these results may not be significant considering relatively low number of fungal isolates.

With regard to the isolation rate depending on regions, *Penicillium* was most common in all regions, showing 66.3%, 58.4%, and 63.8% percent isolation from Kapyung, Kwangju city, Yangpyung, respectively (Table 5). Percent isolations of *Trichoderma* spp. were 2.3%, 3.5%, and 4.3% from Kapyung, Kwangju city, and Yangpyung, respectively (Table 5). In all regions, *Penicillium*, *Trichoderma*, and *Aspergillus* spp. as green molds occurred similarly, suggesting that green mold disease of mushrooms might be prevalent in all three regions. In our previous study, the occurrence of green mold disease was similar in all three regions (An *et al*., 2009).

The microbial population sizes of bacteria and fungi depending on the years of cultivation ranged from 0.5×10^3 to 10.6×10^3 and from 0.9×10^3 to 13.5×10^3 CFU/m^3, respectively for 3 to 20 years of cultivation. There was no correlation between cultivation years and microbial densities by statistical analysis (data not shown), implying that higher microbial density might be related to sanitation condition or some other unknown factors rather than continuous yearly cultivation.

Our study observed that even though the facility is fairly new in the region of Yanpyung, Kyunggi province, if sanitation is not good, the microbial population density was high. Jhune (2002) reported that there was no correlation between cultivation years and microbial population densities although he did not present any data. He suggested that mushroom disease could be related to a combination of several factors such as the density of mushroom pathogen, pathogenicity, media condition and mushroom resistance to pathogen, etc, instead of a single factor

In conclusion, *Psuedomonas* spp., *Pedobacter* spp., and *Bacillus* spp. are the most frequent isolates of bacteria in all three regions. *Penicillium* species with the percent isolation of 62.5% was most commonly isolated from the air of cultivation facility of oyster mushrooms and outside of the mush-

room cultivation facility, followed by *Cladosporium* spp. with 23.65%, *Aspergillus* spp. with 7.09%, and *Trichoderma* spp. with 3.38% out of 296 isolates in total from all the air contents collected from mushroom cultivation facilities and outside of the mushroom cultivation facilities.

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