

Beneficial bacterial strains on *Agaricus blazei* cultivation

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Abstract – The objective of this work was to evaluate the effects of six bacterial strains isolated from *Agaricus blazei* (ABM) on its cultivation. The six strains were characterized as to their effects on the productivity, polysaccharide-protein complex (PSPC), and polysaccharide contents of ABM cultured on sterilized casing soils. Three isolates enhanced ABM mycelium growth. Inoculation of *Arthrobacter* sp. or *Exiguobacterium* sp. on sterile peat casing soil resulted in 64% increase in ABM mushroom total fresh matter yield compared to the uninoculated control. Inoculation of *Exiguobacterium* sp., *Microbacterium esteraromaticum* or *Pseudomonas resinovorans* on sterilized loamy casing soil resulted in 62, 95, and 59% increase in ABM mushroom total fresh matter yield, respectively. The PSPC content in ABM increased 7 to 10% in casing soil inoculated with five of the six isolates compared to the uninoculated control. *Exiguobacterium* sp. inoculated on casing soil resulted in a mushroom-polysaccharide content 15% higher than the control. Moreover, inoculation of five of the six isolates on the casing soil reduced the harvesting time from 10 to 27 days. The evaluated beneficial microbes improve the yield, PSPC, and polysaccharide contents, besides reducing the harvesting time in ABM culture.

Index terms: *Agaricus blazei*, beneficial microbe, casing soil, mushroom productivity, polysaccharide-protein complex, probiotics.

Estirpes bacterianas benéficas ao cultivo de *Agaricus blazei*

Resumo – O objetivo deste trabalho foi avaliar os efeitos de seis estirpes bacterianas isoladas de *Agaricus blazei* (ABM) sobre o seu cultivo. As seis estirpes foram caracterizadas quanto a seus efeitos sobre a produtividade, o conteúdo do complexo polissacarídeo-proteína (CPPP) e o conteúdo de polissacarídeos, em ABM cultivado em solos esterilizados. Três isolados aumentaram o crescimento micelial de ABM. A inoculação de *Arthrobacter* sp. ou *Exiguobacterium* sp. em cobertura de turfa estéril resultou em aumento de 64% na produção total de cogumelos, em comparação ao controle sem inoculação. A inoculação de *Exiguobacterium* sp., *Microbacterium esteraromaticum* ou *Pseudomonas resinovorans* em solo argiloso esterilizado resultou em 62, 95, e 59% de aumento na produção total de cogumelos, respectivamente. O conteúdo de CPPP em ABM aumentou de 7 a 10% em solo com inoculação de cinco dos seis isolados, em comparação ao controle sem inoculação. A inoculação de *Exiguobacterium* sp. em terra de cobertura resultou em conteúdo de polissacarídeo nos cogumelos 15% superior ao do controle. Além disso, a inoculação de cinco dos seis isolados na terra de cobertura reduziu o tempo de colheita de 10 a 27 dias. Os microrganismos benéficos avaliados melhoram a produção e os conteúdos de CPPP e de polissacarídeos, além de reduzir o tempo de colheita no cultivo de ABM.

Termos para indexação: *Agaricus blazei*, microrganismo benéfico, terra de cobertura, produtividade de cogumelos, complexo polissacarídeo-proteína, probiótico.

Introduction

Agaricus blazei Murrill is a medicinal mushroom that contains bioactive polysaccharides and polysaccharide-protein complexes (PSPC) which have been shown to function as potent antioxidants, antitumorogenic, and anticancer agents (Endo et al., 2010; Ishii et al., 2011). Due to its pharmacological activities, ABM has drawn the attention of food scientists, and biotechnologists, as well as industrial growers.

Conventional culturing of ABM requires the initial spawning of the mycelium on a solid culture medium (15 to 21 days), and subsequent covering with suitable casing soil to stimulate fruiting-body formation, which normally takes around 45 to 60 days (Colauto et al., 2010; Chu et al., 2012). The time required to culture ABM from spawning to a harvestable mushroom size is long, compared to related *Agaricus* sp., such as *A. bisporus* (25 to 35 days, Pardo-Gimenez et al., 2010). Furthermore, the biological efficiency (BE) – defined as the ratio of the productivity of an organism to its

energy supply – for ABM cultures are often in the range of 40–70% (Mendonça et al., 2005; Donini et al., 2006; Andrade et al., 2007; Pokhrel & Ohga, 2007; Colauto et al., 2010; Chu et al., 2012). As such, it would be beneficial to identify techniques to decrease the time and energy required to culture ABM.

A previous report showed that a loamy soil can substitute peat soil (PS) as a casing material and significantly improve the total fresh yield, production time and BE of ABM (Chu et al., 2012). In addition, chemical constituents as Cu and Zn, in the casing soil, have shown significant negative correlation with ABM total yield (Chu et al., 2012).

Biological approaches to improve crop growth have been practiced worldwide. In animal systems, beneficial microbes (BM) or probiotics are essential for maintaining the health of an individual (Malo et al., 2010). Similarly, endophytes and BM that dwell in the rhizosphere contribute to plant growth and development (Chen et al., 2006; Young & Shen, 2007; Hardoim et al., 2008). Reports on the application of BM for improving the productivity of edible mushrooms are scarce. The thermophilic fungus *Scytalidium thermophilum*, identified in the early 1990s, was shown to enhance mycelial growth and improve the productivity of *A. bisporus* (Straatsma et al., 1994). The bacterium *Pseudomonas* sp. P7014 was shown to enhance the mycelial growth and reduce the production time of the edible mushroom *Pleurotus eryngii* (Kim et al., 2008). In addition, the effects of *Trichoderma* sp. or *Chaetomium olivacearum* co-cultures with spawned ABM mycelium on the productivity of ABM was assessed, and the results showed co-culturing of these two fungi did not affect ABM productivity (Andrade et al., 2007). Further reports on the effects of BM in ABM cultivation are limited.

Several novel bacterial species were recently identified from the base of ABM stipes, including *Agaricicola taiwanensis*, *Microbacterium agarici* sp. nov., *Microbacterium humi* sp. nov., and *Microbacterium pseudoresistens* sp. nov. (Chu et al., 2010; Young et al., 2010), and the evaluation of the possible beneficial effects of these and other species on ABM productivity are still needed.

The objective of this work was to evaluate the effects of six bacterial strains isolated from ABM on its cultivation, specifically their effects on ABM

productivity, harvesting time, and polysaccharide and PSPC contents.

Materials and Methods

Farm-grown ABM was collected from Nantou, Taiwan (23°57'26" N, 120°58'29" E). The base of the mushroom stipe was excised, minced, soaked and incubated in distilled water by shaking for 30 min at 25°C. Aliquots of the stipe-soil-solution were plated on nutrient agar (Himedia, Mumbai, India) by serial dilution, and incubated at 25°C for 3 days. Bacterial colonies were subcultured into single colonies, which were individually cultured on nutrient broth for DNA extraction, using MO BIO Ultraclean Microbial DNA Isolation Kit (MO BIO, Carlsbad, CA, USA).

16S rRNA gene was amplified using 1F and 9R universal primers (5'-GAG TTT GAT CAT GGC TCAG-3' and 5'-AAG GAG GTG ATC CAA CCG CA-3', according to Edward et al., 1989), with a standard reaction kit (GeneMark Technology Co., Taiwan) and a thermal cycler (TechneFlexigene, Cambridge, UK). DNA fragments of ~1,500 bp were purified using QIAquick Gel Extraction Kit, (QIAGEN, Hilden Germany) and sequenced as described in Young et al. (2005). Cycle sequencing primers used were:

- 3F, 5'-CCT ACG GGA GGC AGC AG-3';
- 4R, 5'-TTA CCG CGG CTG CTG GCAC-3'; and
- 5F, 5'-AAA CTC AAA TGA ATT GAC GGGG-3'

(Edward et al., 1989). The 16S rRNA gene sequence (~1,500 continuous nucleotides) was analyzed using Blast (National Center for Biotechnology Information, 2012). Only the identity that showed the highest level of similarity between 16S rRNA gene and the submitted sequence was mentioned.

Amongst the identified bacteria, six isolates (Table 1) were selected for this study, due to their similar fast-growing characteristics. All six microbes belong to the risk group 1 microbia, based on the German Collection of Microorganisms and Cell Cultures (2011) and, thus, are potential BM for improving ABM production. Individual microbes were co-cultured with ABM on potato dextrose agar, in order to determine their effects on the mycelium growth.

The ABM strain BCRC36814 was purchased from The Food Industry Research and Development Institute, Hsin-Chu, Taiwan. To assess the effect of

these six bacteria on ABM mycelium growth, a 0.5 cm diameter block of ABM mycelium was spotted on a potato dextrose agar (Difco, Detroit, MT, USA) plate along with individual microbium. The microbium was drawn in a line approximately 2 cm away from the spotted ABM mycelium block with a sterilized inoculation loop. After 7 days at 28°C, the ABM mycelium diameter was measured and compared to the control without microbial inoculation. The above ABM mycelium growth tests for each bacteria treatment were performed with three replicates.

Sawdust medium, purchased from Q-YO (Bio Technology Farm, Ta-Chun, Chang-Hua, Taiwan), was used as the culture medium for the spawning of ABM mycelium (Chu et al., 2012). In addition to the commonly used peat soil (PS), local loamy soil was also used as the casing soil for culturing ABM (Pokhrel & Ohga, 2007; Colauto et al., 2010; Chu et al., 2012). The physicochemical properties of the culture medium and casing soils are summarized in Table 2. The contents of soil ash, organic matter, and organic carbon were analyzed according to Nelson & Sommers (1982), and total N was analyzed using the Kjeldahl method (Bremner & Mulvaney, 1982). The culture medium

and casing soils were soaked separately in water at a 1:5 ratio (w/v), and the pH and electrical conductivity were determined using a pH-electroconductivity meter. Bulk density (BD) was determined by measuring the ratio (DW/TV) between soil dry matter weight (DW) and its fixed volume (TV) (Blake & Hartge, 1986). The particle density (PD) of the soil samples was averaged from two measurements (Blake & Hartge, 1986). Porosity (f) was calculated by the equation: $f = [(1-BD/PD) \times 100\%]$. Water-holding capacity (WHC) was calculated using the weight of water-saturated casing soil divided by the weight of oven-dried casing soil.

The ABM stock was activated and subcultured on potato dextrose agar medium to produce fresh ABM and, subsequently, cultured on sterilized wheat to produce ABM grain spawn (Chu et al., 2012). Sterilized sawdust medium was evenly packed in bag logs in a 250 mL pot with a total weight of 100 g. The moisture content of the sawdust medium was 57%. Two grams of ABM grain spawn were inoculated on sterilized sawdust medium and placed in an incubator at 28°C for spawning. The light intensity in the growth chamber was 20 ± 2 lux, measured at 45 ± 5 cm (Digital Light Meter, TES-1335, Taiwan). After complete spawning (1 month), two separate casing treatments of imported PS – peat soil (Saprists, Histosols; FAO, 1988) – or loamy soil (loamy, mixed, nonacid, hyperthermic, Typic Udorthent; FAO, 1988) were covered on top of the medium with 2 to 2.5-cm thickness (Chu et al., 2012). The casing soils and sawdust medium were sterilized before use (121°C , 1.033 kg cm^{-2} , 1 hour for 3 times), in order to avoid possible microbial contaminants. We also included an uninoculated nonsterile casing soil as control.

To assess the effects of the bacterial isolates on the productivity of ABM, individual bacterial strains were inoculated on sterilized casing soil instead of the culture medium because the bacteria strains were isolated from the mushroom stipe along with the casing

Table 1. Microbes with most similar 16S rRNA gene sequences to those isolated from the base of *Agaricus blazei* stipe, and the effects of each isolate on mycelium growth⁽¹⁾.

| Isolate code | Scientific name ⁽²⁾ | 16S rRNA (% identity) | Mycelium diameter (cm) |
|--------------|---------------------------------------|-----------------------|------------------------|
| JN03 | <i>Exiguobacterium</i> sp. | 99.4 | 3.47a |
| JN05 | <i>Bacillus psychrodurans</i> | 99.1 | 3.67a |
| JN10 | <i>Microbacterium esteraromaticum</i> | 99.2 | 3.67a |
| JN12 | <i>Arthrobacter</i> sp. | 99.2 | 4.74b |
| JN16 | <i>Pseudomonas resinovorans</i> | 98.6 | 4.97b |
| JN17 | <i>Pseudomonas alcaliphila</i> | 99.6 | 4.97b |
| Control | - | - | 3.51a |

⁽¹⁾Means followed by equal letters do not significantly differ by Duncan's multiple range test, at 5% probability. ⁽²⁾16S rRNA gene sequence (~1,500 continuous nucleotides) was analyzed using Blast (National Center for Biotechnology Information, 2012).

Table 2. Physicochemical properties of the casing soils and of the sawdust medium used for culturing *Agaricus blazei*.

| Material ⁽¹⁾ | pH | EC ($\mu\text{S cm}^{-1}$) | BD ---- (g cm^{-3}) ---- | PD ----- | f ----- | WHC ----- | Ash ----- | OM, OC, N, C/N ----- (%) ----- | | | |
|-------------------------|------|---------------------------------|--|-------------|------------|--------------|--------------|-----------------------------------|------|------|------|
| | | | | | | | | OM | OC | N | C/N |
| LS | 5.82 | 177 | 1.32 | 2.55 | 48.2 | 147 | 94.5 | 4.35 | 2.29 | 0.10 | 22.9 |
| PS | 4.09 | 3340 | 0.55 | 1.44 | 61.8 | 263 | 10.8 | 85.5 | 45.0 | 2.01 | 22.4 |
| Sawdust | 7.47 | 2.72 | nd | nd | nd | nd | 15.0 | 79.2 | 46.0 | 2.58 | 17.8 |

⁽¹⁾LS, loamy soil/loamy, mixed, nonacid, hyperthermic, Typic Udorthent; PS, peat soil/Saprists, Histosols. EC, electric conductivity; BD, bulk density; PD, particle density; f, porosity; WHC, water holding capacity; OM, organic matter; OC, organic carbon; N, total nitrogen; C/N, total nitrogen carbon ratio; nd, not determined.

soil. Five milliliters of nutrient broth-cultured microbe (10^9 cfu mL⁻¹; calculated using the serial dilution method) were evenly applied directly on top of the casing soil weekly until harvesting. The same volume of bacterial-free nutrient broth was applied on the uninoculated control. Each experiment was conducted in a randomized block design with three replicates.

Harvesting of the fruiting body takes place when the mushrooms reach their highest biomass, which occurs during the immature stage, when the veil membrane is enclosed and the gills are intact (Mendonça et al., 2005; Pokhrel & Ohga, 2007; Chu et al., 2012). Total fresh matter yield (FW, g per pot) was measured, and the BE was determined after harvesting by the equation $BE = FW/DWC$ (Andrade et al., 2007), where DWC is the dry weight of the culture medium.

Polysaccharide-protein complex content was determined according to Sarangi et al. (2006). Lyophilized PSPC was used to determine the polysaccharide content, using the phenol-sulfuric acid method (Sarangi et al., 2006). All standards were purchased from Sigma Chemical Co., St. Louis, MO, USA.

Statistical analyses were performed using the CoStat's statistical procedures (CoHort Software, 2002), and means were compared by Duncan's test, at 5% probability.

Results and Discussion

JN12, JN16 and JN17 bacterial isolates were able to positively affect ABM mycelium growth, when compared to the control without bacteria co-culturing. However, JN03, JN05 and JN10 isolates have not affected ABM mycelium growth. Several reports have indicated that co-cultivation with certain microbial strains results in positive effects on mushroom mycelial growth and subsequent mushroom production (Straatsma et al., 1994; Kim et al., 2008). Naturally occurring thermophilic fungi such as *S. thermophilum* have been reported to be important for the preparation of matured compost for mushroom culture. Furthermore, inoculation of sterilized culture medium with *S. thermophilum* increased the productivity of *A. bisporus* (Straatsma et al., 1994).

After 90 days in lab conditions, nonsterilized uninoculated PS casing (BK) produced many primordia, but very few of them developed into harvestable mushrooms. This resulted in a very low total fresh matter

yield and BE (Table 3). On the contrary, sterilized PS casing without microbe inoculation (CK) resulted in higher total fresh matter yield and BE. Compared to the CK treatment, inoculation of both *Exiguobacterium* sp. (JN03) and *Arthrobacter* sp. (JN12) on the sterile PS casing resulted in a significant 64% increase in ABM total fresh matter yield. The increase in the total fresh matter yield of the fruiting body also reflected a significant increase in the BE. However, inoculations with *B. psychrodurans* (JN05) and *P. resinovorans* (JN16) on the sterile PS casing did not show significant effects on ABM total fresh matter yield and BE, in comparison to CK treatment. Furthermore, inoculation of *M. esteraromaticum* (JN10) and *P. alcaliphila* (JN17) resulted in a significant reduction in the total fresh matter yield and BE, in comparison to CK treatment. It is interestingly to note that the enhancement of ABM mycelial growth by individual isolates (JN12, JN16, JN17) does not correlate with the total fresh matter yield. Similar observations were reported for *A. bisporus* (Wiegant et al., 1992). Results warrant further development of some of the microbes identified here as BM for application in commercial ABM production.

Previous reports have shown that loamy soil can substitute PS as a casing soil for the cultivation of ABM (Pokhrel & Ohga, 2007; Colauto et al., 2010; Chu et al., 2012). Individual isolates were inoculated on loamy casing soil to assess their effects on ABM production. Inoculation of all isolates, except for *B. psychrodurans* (JN05), on the sterilized loamy casing soil, during the cultivation period, resulted in a 10 to 27-day reduction of the harvesting time (Table 4). *Exiguobacterium* sp. (JN03) and

Table 3. Effect of microbe inoculation in peat casing soil on the productivity of *Agaricus blazei*⁽¹⁾.

| Inoculation treatment | Mushrooms total fresh yield | | Biological efficiency ⁽³⁾ (%) |
|--|-----------------------------|--------------------|--|
| | (g per pot) | (%) ⁽²⁾ | |
| <i>Exiguobacterium</i> sp. (JN03) | 19.3a | 164 | 44.5a |
| <i>Bacillus psychrodurans</i> (JN05) | 13.1bc | 111 | 30.3bc |
| <i>Microbacterium esteraromaticum</i> (JN10) | 9.3d | 78.8 | 21.6d |
| <i>Arthrobacter</i> sp. (JN12) | 19.3a | 164 | 44.6a |
| <i>Pseudomonas resinovorans</i> (JN16) | 12.2bc | 103 | 28.2bc |
| <i>Pseudomonas alcaliphila</i> (JN17) | 8.7d | 73.7 | 20.1d |
| Sterile soil without inoculation (CK) | 11.8c | 100 | 27.1bc |
| Nonsterile soil without inoculation (BK) | 3.3e | - | 7.6e |

⁽¹⁾Means followed by equal letters do not significantly differ by Duncan's multiple range test, at 5% probability. ⁽²⁾Increase or reduction of variables in relation to uninoculated control (CK). ⁽³⁾Biological efficiency = (fresh weight of mushroom/dry weight of cultural compost) × 100.

Table 4. Effect of microbe inoculation in loamy casing soil on the harvesting time, productivity, polysaccharide protein complex (PSPC) and polysaccharide contents of *Agaricus blazei*⁽¹⁾.

| Inoculation treatment | Days to the first harvest after casing | Mushroom total fresh yield in two harvestings | | PSPC contents ⁽²⁾ | | Polysaccharide contents | |
|--|--|---|--------------------|------------------------------|--------------------|-------------------------|--------------------|
| | | (g per pot) | (%) ⁽³⁾ | (g kg ⁻¹) | (%) ⁽³⁾ | (g kg ⁻¹) | (%) ⁽³⁾ |
| <i>Exiguobacterium</i> sp. (JN03) | 40d | 35.2b | 162 | 5.36ab | 110 | 1.84a | 115 |
| <i>Bacillus psychrodurans</i> (JN05) | 76a | 24.5cd | 113 | 5.38ab | 110 | 1.60b | 100 |
| <i>Microbacterium esteraromaticum</i> (JN10) | 57c | 42.3a | 195 | 4.87b | 100 | 1.64b | 103 |
| <i>Arthrobacter</i> sp. (JN12) | 46cd | 29.5c | 136 | 5.24ab | 107 | 1.34c | 84 |
| <i>Pseudomonas resinovorans</i> (JN16) | 56c | 34.6b | 159 | 5.27ab | 108 | 1.57b | 98 |
| <i>Pseudomonas alcaliphila</i> (JN17) | 41d | 27.9c | 129 | 5.25ab | 107 | 1.45c | 91 |
| Sterile soil without inoculation (CK) | 67b | 21.7d | 100 | 4.89b | 100 | 1.60b | 100 |

⁽¹⁾Means followed by equal letters do not significantly differ by Duncan's multiple range test, at 5% probability. ⁽²⁾PSPC and polysaccharide contents were analyzed using mushroom harvested from first flush. ⁽³⁾Increase or reduction of variables in relation to uninoculated control (CK).

P. alcaliphila (JN17) reduced harvesting times to 40 and 41 days, respectively, compared with the control (67 days). Conversely, inoculation of *B. psychrodurans* (JN05) on the loamy casing soil prolonged harvesting time by 9 days. These results suggest that several of the tested BM promote ABM fruiting and effectively reduce the required time for harvesting, by as many as 27 days. It is worth noting that both *Exiguobacterium* sp. (JN03) and *P. alcaliphila* (JN17), which showed the best fruiting-body promotion, are alkaliphilic bacteria (Yumoto et al., 2001; Vishnivetskaya et al., 2009). Studies on the relationship between the alkaliphilic properties of these bacteria and ABM fruiting-body production will be a topic of future research.

Inoculation of all individual isolates, except for *B. psychrodurans* (JN05), on the loamy casing soil increased the total fresh matter yield from 29 to 95% of the control without inoculation (Table 4). In particular, *M. esteraromaticum* (JN10), *Exiguobacterium* sp. (JN03) and *P. resinovorans* (JN16) inoculation resulted in total fresh yield increases of 95, 62 and 59% over the CK, respectively. Interestingly, the effect of *M. esteraromaticum* (JN10) and *P. alcaliphila* (JN17) in PS was opposite to their effect in local loamy casing soil. The most extreme difference was for *M. esteraromaticum* (JN10) inoculation; 78.8 vs 195% of the control (100%) in PS and loamy soil casing, respectively. This disparity may be due to differences in microbe count after application to the soils, despite weekly application of individual microbes. More likely, the difference in the physicochemical characteristics between the two casing soils – for instance, the relatively acidic pH of PS – may have affected the

alkaliphilic microbe *M. esteraromaticum* (Habe et al., 2004). In any case, further characterization of microbial viability and microbial exudation in the two different environments could be insightful.

Pharmacologically active substances, including PSPC and polysaccharides, were measured in the dried fruiting bodies harvested from flush 1. None of the microbe inoculation treatments showed significant differences in PSPC content from the uninoculated control. Only *Exiguobacterium* sp. (JN03) inoculation resulted in a significant increase in polysaccharide content (Table 4).

Beneficial microbes are important components of plant ecosystems, where they function to improve plant growth, increase yield, and enhance resistance to diseases (Young & Shen, 2007). Cultivation of ABM to a harvestable size takes a long time, often with low BE. Thus, approaches that could improve ABM productivity are of crucial importance, considering its commercial value.

Conclusions

1. Beneficial microbes can be identified and applied in *Agaricus blazei* (ABM) cultivation.
2. Application of *Exiguobacterium* sp. on sterilized casing soil can increase biomass, shorten harvesting time and increase polysaccharide production in ABM.

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