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ORIGINAL RESEARCH

Recycling spent *Pleurotus eryngii* substrate supplemented with *Tenebrio molitor* feces for cultivation of *Agrocybe chaxingu*

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Abstract
Purpose In the industrialized production of mushrooms usually only one flush of fruitbody is harvested, so that nutrients and energy in the substrate is not fully exploited. In this study, the spent *Pleurotus eryngii* substrate was recycled for the cultivation of *Agrocybe chaxingu* under ambient temperature.
Method Six formulae were tested: (1) Control: 98% spent substrate, 1% sucrose, 1% lime; (2) Control + 10% wheat bran; (3) Control + 20% wheat bran; (4) Control + 10% *T. molitor* feces; (5) Control + 20% *T. molitor* feces; (6) Control + 10% wheat bran + 10% *T. molitor* feces.
Results Two flushes of fruitbody were harvested, the control substrate resulted in a biological efficiency of 40.42%; the formulae with supplementation of 10% wheat bran, 20% wheat bran and 10% *T. molitor* feces significantly increased biological efficiency to 52.50, 54.61 and 51.56%, respectively, and supplementation of 20% *T. molitor* feces, or 10% wheat bran plus 10% feces further significantly increased biological efficiency to 62.95 and 61.10%, respectively. All supplemented substrates had significantly higher cellulose and laccase activity than the Control (cellulase 0.10 U/g; laccase 41.00 U/g), which were 10% wheat bran (0.15 U/g; 72.67 U/g), 10% *T. molitor* feces (0.17 U/g; 98.33 U/g), 20% wheat bran (0.22 U/g; 76.00 U/g), 20% *T. molitor* feces (0.27 U/g; 87.00 U/g), 10% wheat bran plus 10% *T. molitor* feces (0.25 U/g; 97.67 U/g), respectively.
Conclusion Spent *Pleurotus eryngii* substrate was promising for cultivation of *Agrocybe chaxingu*, especially when supplemented with 20% *T. molitor* feces, or with 10% *T. molitor* feces plus 10% wheat bran.

Keywords Spent mushroom substrate · Fruitbody · Biological efficiency · Cellulase · Laccase

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Introduction

In the industrialized production of low temperature fruiting type mushrooms like *Pleurotus eryngi* and *Flammulina velutipes*, usually only one flush of fruitbodies is harvested (biological efficiency 60–65%) because the biological efficiency of the successive flushes are not high enough (approximately 30%) to make a profit where facilities and air cooling systems are expensive. Currently, most of the spent substrate is burnt to generate steam for substrate sterilization and heating of mushroom farms, some spent substrate is used as organic fertilizers in orchards. Nutrients and energy in the substrate is not fully exploited, as evidenced by over 100% total biological efficiency in non-industrialized mushroom production under natural environmental conditions where 3–4 flushes were harvested (Philippoussis et al. 2001; Mandeel et al. 2005). In recent years, many experiments have been conducted to recycle the spent substrate for cultivation of other mushrooms (usually high temperature fruiting types) (Royse 1992; Li 2013).

Agrocybe chaxingu (in some previous cases mistakenly termed *A. cylindracea* or *A. aegerita*) (Callac et al. 2011) is a popular mushroom with a sweet aroma and many

medicinal benefits. It is an antioxidant (Choi et al. 2009), possessing properties that aid the curing of cancers (Hyun et al. 1996), diabetes (Lee et al. 2010), etc. With a view to recycle spent *P. eryngii* substrate for cultivation of other mushrooms in a low cost way, in the present study, *A. chaxingu* was chosen because it is a moderate temperature fruiting type mushroom suitable for cultivation at a broad range of ambient temperatures for a period from late spring to autumn in south China.

To formulate recycled substrate for *A. chaxingu* cultivation, the nutrient composition of spent *P. erygnii* substrate was analyzed and compared with the unused substrate. *Tenebrio molitor* rearing has expanded rapidly in China, mainly as animal and pet feed, and to a lesser degree for human consumption. The sand-like feces of *T. molitor* larvae contain digested fiber, crude protein (14–18%), crude lipid (15–18%) and minerals (Wang et al. 2012; Lee and Rho 2014), therefore, *T. molitor* feces could be an ideal ingredient used for mushroom cultivation, but very few such studies were reported (Gan et al. 2008). Currently *T. molitor* feces is used mainly as garden fertilizer or livestock feed.

The purpose of the present study was to test if the spent substrate of *P. eryngii* in the industrialized production setting was a good substrate for production of *A. chaxingu*; and the effect of supplementing 10–20% *T. molitor* feces or/and wheat bran in improving fruiting body yield.

Material and methods

A. chaxingu and spent *P. eryngii* substrate

The experimental *A. chaxingu* strain was purchased from Xue Shan Er Precious Edible Mushroom Institute, Gutian county, Fujian province. Spent *P. eryngii* substrate was provided by Guangdong Lantian Agricultural Co., Ltd., Fengshun county, Guangdong province. The formula of the unused substrate for *P. eryngii* production was: 50% sugarcane bagasse, 20% cottonseed hulls, 20% wheat bran, 5% cornmeal, 3% soymeal, 1% lime, 1% gypsum. *T. molitor* feces was purchased via Taobao.com from Hong Chang Feed Rearing Farm in Binzhou city, Shandong province. Wheat bran and other ingredients and materials were purchased from a local market.

Determination of composition of *P. eryngii* substrate

Total carbon content of the spent and unused *P. eryngii* substrate was determined by potassium dichromate method (ISO 14235 1998). Total nitrogen content was determined by the Kjeldahl method (Bremner and Breitenbeck 1983). Soluble sugar content was determined by the anthrone–sulfuric acid method (Spiro 1966). Starch content was determined with the method described by Holm et al. (1986). Cellulose, hemicellulose, lignin and ash content were determined according to Goering and Van Soest (1970).

Agrocybe chaxingu cultivation experiment

Preparation of substrate

Mushroom cultivation was carried out in a chamber in our laboratory. The following 6 formulae were adopted, representing without or with supplementation of 10–20% *T. molitor* feces or/and wheat bran. For each formula 30 bags were inoculated.

1. 98% Spent substrate, 1% sucrose, 1% lime.
2. 88% Spent substrate, 1% sucrose, 1% lime,10% wheat bran.
3. 78% spent substrate, 1% sucrose, 1% lime, 20% wheat bran.
4. 88% Spent substrate, 1% sucrose, 1% lime, 10% *T. molitor* feces.
5. 78% Spent substrate, 1% sucrose, 1% lime, 20% *T. molitor* feces.
6. 78% Spent substrate, 1% sucrose, 1% lime, 10% wheat bran,10% *T. molitor* feces.

The spent *P. eryngii* substrate was fragmented by hand and sun dried for use, and after mixing thoroughly with other ingredients, the 1% sucrose was solved in required water to make sure the wet substrate contain 65% water. Then the wet substrate was filled into 33 × 17 cm HDPE plastic bags with each bag containing 857 g (equals to 300 g dry substrate). The substrate was pressed to a compactness so that the bag side was slightly tensioned to leave no free space for primordial occurrence during cropping stage. The bags were not fully filled so that an empty space was left in the bag to maintain moisture during cropping. After sealing with neck rings and cotton-free (sponge) caps, the bags of substrate were autoclaved (Tomy SS325) at 121 °C for 2 h.

Inoculation and mycelial culture

As the temperature in the autoclave dropped to 60–70 °C, the bags of substrate were moved to a biosafety cabinet for further cooling and UV light sterilization of surface microorganisms (2–3 h). When the substrate was cooled thoroughly each bag was inoculated with about 20 g solid spawn previously cultured in bags with spent *P. eryngii* substrate. Then the mycelium bags were cultured in the dark (with a closed curtain) at room temperature (18–23 °C), with air relative humidity maintained at 65–70% by occasionally adding moisture with a spray humidifier.

Cropping management

As most of the bags of all six formulae were fully colonized (upon spawn run completion), after 10 additional days of mycelial growth, the neck rings and caps were removed from the bags, and natural light was supplied to induce primordial formation. At the same time the air relative humidity was maintained at 80–90% with a humidifier.

Determination of mycelial cellulase and laccase activity during spawn run

Five samples were taken of each formula for enzyme determination upon completion of substrate colonization. Crude enzyme was extracted by placing 2.0 g fresh mycelial culture into a 250 mL flask added with 20 mL 0.1 mol/L citrate buffer (pH 5.0) which was shaken in a rotary shaker at 28 °C, 200 rpm for 2 h. The extracted solution together with substrate was centrifuged at 4000 rpm for 10 min and the supernatant was used as crude enzyme for activity assay.

The carboxymethyl cellulase (CMCase) activity assay followed the method of Ghose (1987). 1 unit CMCase activity was defined as the enzyme amount required to transfer substrate into 1 μmol glucose and expressed in U/g fresh mycelial culture. Laccase activity was measured by following the method described by Heinzkill et al. (1998), with modification of doubling both the sample volume and reagent volume to suit a 1 cm cuvette. Laccase activity was expressed as U/g substrate (fresh weight), where 1 U was defined as 1 μmol of substrate oxidized per min.

Spawn run period, fruiting body yield and biological efficiency

The spawn run period (the number of days from inoculation to colonization completion of the substrate by the mycelium) was recorded. Two flushes of fruiting bodies were harvested. The fresh weights of fruiting body were recorded and biological efficiency (BE %) was calculated by dividing fresh weight of fruiting body by dry substrate weight per bag.

Data statistical analysis

Original data were processed using EXCEL (Microsoft, WA, USA) and Scheffe's tests were performed using SPSS 17.0 (SPSS Inc., Chicago, IL, USA).

Results and discussion

Composition of unused and spent *P. eryngii* substrate

As indicated in Table 1, the content of total carbon, total nitrogen and C/N ratio in the spent *P. eryngii* substrate was merely slightly reduced as compared with unused substrate. The more easily digestible ingredients (soluble sugar, starch and hemicellulose) are significantly reduced to 40.14, 11.14 and 54.28% of the unused substrate values. The cellulose content in the spent substrate was 91.55% that of the unused substrate, which was slightly reduced, and lignin in the spent substrate was 81.88% that of the unused substrate, reducing to a larger degree than cellulose. The ash in the spent substrate was more than twice that of the unused substrate, reflecting the dry matter loss through respiration by *P. eryngii*. The data indicated that to obtain good fruitbody yield the spent substrate should be replenished with the easily digestible ingredients like soluble sugar, starch and hemicellulose, but extra nitrogen source (e.g., soymeal), lignocellulosic ingredients were not required to be supplemented, hence wheat bran, cornmeal and *T. molitor* feces could satisfy this purpose. In fact, too much nitrogen in the substrate can lead to ammonia accumulation during storage or preparation which inhibited mycelial growth (Choi 2004; Mohamed et al. 2016).

Mycelial cellulase and laccase activity and spawn run period

During vegetative growth edible fungi produce a wide range of extracellular enzymes to degrade the lignocellulosic substrates, including cellulase, laccases, peroxidases, xylanase, protease, etc. (Magnelli and Forchiassin 1999). In the present study, the mycelial cellulase and laccase activity at 40 day of spawn run (upon colonization of substrate) were determined to reveal possible associations of substrate degradation rate with substrate formulae (Table 2). Mn peroxidase was not determined in this study because it displayed a similar pattern to laccase (Zeng et al. 2013).

As shown in Table 2, the cellulase activity in mycelia of Formulae 2 and 4 were not significantly different from that in Formula 1, but Formulae 3, 5 and 6 had significantly higher cellulase activity than Formula 1, which indicated that both the inclusion of wheat bran and *T. molitor* feces significantly enhanced cellulase activity as the inclusion rate reached 20% (either separately or combined), and supplementation of 20% feces (Formula 5) demonstrated the highest value.

Table 1 Comparison of main composition of unused and spent *P. eryngii* substrate (%)

Component	Unused substrate	Spent substrate	Relative remaining value
Total carbon	44.77 ± 3.25	42.66 ± 2.58	95.29
Total nitrogen	1.21 ± 0.14	1.18 ± 0.09	97.52
C/N ratio	37:1	36:1	97.30
Soluble sugar	1.47 ± 0.11	0.59 ± 0.06	40.14
Starch	7.81 ± 0.23	0.87 ± 0.15	11.37
Cellulose	33.26 ± 4.36	30.45 ± 2.37	91.55
Hemicellulose	15.42 ± 1.58	8.37 ± 1.74	54.28
Lignin	20.81 ± 2.92	17.04 ± 1.98	81.88
Ash	2.80 ± 0.20	6.03 ± 0.25	215.36

Data are presented in mean ± SD ($n = 3$)

Table 2 CMCase and Lac activity, spawn run period of *A. chaxingu* on different formulae of spent *P. eryngii* substrate at 40 days of spawn run

Formula	CMCase activity (U/g)	Lac activity (U/g)	Spawn run period (day)
1 CK	0.10 ± 0.02[c]	41.00 ± 8.89[c]	40.17 ± 1.55[c]
2 CK + 10% bran	0.15 ± 0.04[bc]	72.67 ± 8.79[b]	43.70 ± 0.47[ab]
3 CK + 20% bran	0.22 ± 0.05[ab]	76.00 ± 9.00[b]	43.30 ± 1.2[b]
4 CK + 10% feces	0.17 ± 0.06[bc]	98.33 ± 10.26[a]	40.70 ± 0.80[c]
5 CK + 20% feces	0.27 ± 0.08[a]	87.00 ± 9.27[ab]	44.75 ± 0.60[a]
6 CK + 10% bran +10% feces	0.25 ± 0.09[ab]	97.67 ± 10.02[a]	44.50 ± 0.95[a]

Data are presented in mean ± SD ($n = 5$ for enzyme activity; $n = 30$ for spawn run period)

Means within each column bearing no common superscripts are significantly different ($P < 0.05$)

All supplemented formulae demonstrated significantly higher laccase activity than the Control, indicating that either supplementation of wheat bran or *T. molitor* feces could stimulate laccase excretion. And Formulae 4 (10% feces) and 6 (10% feces + 10% bran) had significantly higher laccase activity than Formulae 2 and 3 (10 and 20% bran), but not higher than Formula 5 (20% feces), indicating *T. molitor* feces was superior to wheat bran in stimulating laccase activity in *Ganoderma*. According to substrate inducing theory, such enzyme activity enhancing effect of *T. molitor* feces might be due to the presence of digested products of cellulose, chitin and other polymeric compounds.

The spawn run period reflects how the substrate suits the mycelial growth, in the present study the spawn run periods with all the 6 formulae of substrate were over 40 days as the mycelia were cultured under natural temperatures (18–23 °C). It can be seen from Table 3 that the spawn run period of *Chaxingu* was the shortest (40 days) in the substrate of Formulae 1 and 4, supplementation of 10–20% (Formulae 2, 3) wheat bran significantly extended the spawn run period by about 3 days as compared with Formula 1, and supplementation of 20% *T. molitor* feces or 10% wheat bran plus 10% feces (Formulae 5, 6) significantly extended the spawn run period by about 4 days; The extended spawn run period by supplementation of wheat bran or/and feces might be due to two reasons: (1) both wheat bran and *T. molitor* feces were not elastic as was sugarcane bagasse and cottonseed hulls, thus reducing the porosity of supplemented substrates with poorer oxygen transmission within the substrate; (2) supplementation of the ingredients led the mycelia to allocate more resources to excrete lignocellulosic enzymes (as evidenced by data in Table 2) and other enzymes such as protease, which facilitated final degradation of substrate but slowed vegetative mycelial growth in the first stage.

Fruitbody yield and biological efficiency

Primordia emerged successively in bags of all formulae of substrate one week after applying cropping management (as previously described measures, including cap removing, curtain opening and addition of air humidity) except seemingly slightly later on Formula 6. Due to the limited number ($n = 30$) of bags for each formula it was difficult to collect synchronical fruitbodies at a time, the separately collected representative fruiting bodies of each formula were shown in Fig. 1. The fruitbody yield and biological efficiency of each formula are shown in Table 3.

It can be seen from Table 3 that in the first flush, all the supplemented formulae had significant higher fruitbody yields than Formula 1, and no significant differences

Table 3 Fruitbody yield and biological efficiency of *A. chaxingu* on different spent *P. eryngii* substrates

Substrate formula	1st flush fruitbody yield(g/kg)	2nd flush fruitbody yield(g/kg)	Total fruitbody yield(g/kg)	Biological efficiency (%)
1. CK	262.60 ± 40.80^{b}	141.57 ± 45.80^{b}	404.17 ± 43.30^{c}	40.42^{c}
2. CK + 10% bran	323.73 ± 50.30^{a}	201.27 ± 45.40^{b}	525.00 ± 47.87^{b}	52.50^{b}
3. CK + 20% bran	311.37 ± 52.27^{a}	234.76 ± 51.83^{a}	546.13 ± 52.06^{b}	54.61^{b}
4. CK + 10% feces	308.00 ± 42.93^{a}	207.57 ± 56.40^{b}	515.57 ± 49.67^{b}	51.56^{b}
5. CK + 20% feces	337.87 ± 53.33^{a}	291.63 ± 61.23^{a}	629.50 ± 57.30^{a}	62.95^{a}
6. CK + 10% bran +10% feces	334.87 ± 48.13^{a}	276.17 ± 57.97^{a}	611.03 ± 53.07^{a}	61.10^{a}

Data are presented in mean ± SD ($n = 30$)

Means within each column bearing no common superscripts are significantly different ($P < 0.05$)

Fig. 1 Representative fruitbodies of Formulae *1–6* from left to right. Photos were taken with bags separately since fruitbodies of all formulae were not uniformly matured

existed among the supplemented formulae. In the second flush the fruitbody yield of Formulae 3, 5 and 6 were significantly higher than Formulae 2 and 4 as well as Formula 1, indicating that as compared with the 10% supplementation level, supplementing 20% wheat bran or/and *T. molitor* feces could increase yield of successive flushes, and therefore, the total yield.

By looking at the total fruitbody yield of the six Formulae, it can be seen that the spent *P. eryngii* substrate supplemented with only 1% sucrose and 1% lime (Formula 1) could yield 404.17 g/kg fruitbody for 2 flushes, namely achieving a biological efficiency of 40.42%, which was satisfactorily obtained from a farm waste (Jeznabadi et al. 2016). Supplementation of 10–20% wheat bran (Formulae 2, 3) or 10% feces (Formula 4) significantly increased total fruitbody yield, so that the biological efficiency was raised to over 51.56%. Supplementation of 20% (Formula 5) or 10% feces plus 10% wheat bran (Formula 6) resulted in the highest biological efficiency of over 61.10% which was not only significantly higher than Formula 1 but also higher than the 10–20% wheat bran supplementation formula and the 10% feces supplementation formula. Therefore, *T. molitor* feces was superior to wheat bran to supplement the spent *P. eryngii* substrate for cultivation of *A. chaxingu*. It was shown previously that the nutritive value of *T. molitor* was superior to livestock meat (Rumpold and Schlüter 2013) and insects including *T. molitor* are far more efficient in transforming plant biomass into animal biomass than conventional livestock (Nakagaki and DeFoliart 1991).

Conclusion

Spent *Pleurotus eryngii* substrate was promising for cultivation of *Agrocybe chaxingu* under ambient temperatures. On the recycled substrate supplemented with only 1% sugar and 1% lime a biological efficiency of 40.42% was achieved. The formulae with an additional supplementation of 10% wheat bran or 10% *Tenebrio molitor* raised the biological efficiency to 52.50 and 51.56%, respectively. The formulae with an additional supplementation of 20% *T. molitor* feces or 10% *T. molitor* feces plus 10% wheat bran demonstrated the highest biological efficiency of 62.95 and 61.10%, respectively, significantly higher than that of the formula with supplementation of 20% wheat bran, therefore, *T. molitor* feces was an excellent

supplement that was superior to wheat bran for *Agrocybe chaxingu* cultivation.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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