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## Suitability of Oil Palm Empty Fruit Bunch and Sago Waste for *Auricularia polytricha* Cultivation

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**Abstract:** Environmental issues caused by oil palm empty fruit bunch and sago waste in addition to their availability have encouraged studies and researches in utilizing these lignocellulosic wastes. Four indigenous *Auricularia polytricha* were isolated and identified based on molecular and morphological characteristics. Cultivation potentials were evaluated on different substrate formulations consist of empty fruit bunch, sago waste, rubber wood sawdust and rice bran. Substrates for mushroom cultivation were formulated according to the C/N ratios. Growth and yield performances were determined using bag cultivation method in completely randomized design. Results showed that identification based on Internal Transcribed Spacer (ITS) sequences were consistent with morphological characteristics while 18S rDNA sequences showed rather inconclusive identification. *Auricularia polytricha* failed to colonize substrate consisted of empty fruit bunch alone whereas the use of empty fruit bunch as supplementary substrate significantly enhances colonization rate and fruiting bodies production. Sago waste was less suitable as main substrate due to reduced colonization rate, delayed primordia formation and poorer yield as compared to rubber wood sawdust. Among isolates, BF07 recorded fastest primordia formation and highest biological efficiency (34.3-37.4%) on substrate A (Rubber wood sawdust with rice bran, 80:20) and C (Rubber wood sawdust with empty fruit bunch, 75:25). The selection of substrates and isolates to be used can be crucial in *Auricularia polytricha* cultivation. Isolate BF07 and empty fruit bunch as supplementary substrate can be recommended to mushroom growers for improving growth and yield of this mushroom.

**Key words:** Black jelly mushroom, oil palm empty fruit bunch (EFB), sago hampas, C/N ratio, substrate formulations, mushroom cultivation

### INTRODUCTION

*Auricularia polytricha* or commonly known as Black Jelly mushroom is an edible mushroom which has been widely used as food and tonic agent especially among the Asians (Kim, 1993). Locally known as 'Kulat telinga nera', it is highly demanded by the local communities due to its unique texture, nutritional properties and numerous medicinal benefits (Razak *et al.*, 2013). Its unique gelatinous texture absorbs the flavor of soup, making it a popular ingredient in traditional Chinese cuisine. The mushroom not only contains carbohydrate (88%), protein (7%), lipid (2%) but also various vitamins such as Vitamin C, Thiamine and Niacin (Hung and Nhi, 2012; Afiukwa *et al.*, 2013). Hassan and Ghada (2012) also reported that the nutritional values of *A. polytricha* are higher than of many vegetables and fruits.

Apart from its unique texture, flavour and nutritional properties, *A. polytricha* is also well known for their pharmaceutical effects in Chinese herbal medicine. It is popular among the Chinese community due to its ability to improve fluidity of blood and blood circulation (Razak *et al.*, 2013). Studies showed that this mushroom has many other biological activities including antioxidant, anticancer, antitumor, anti-inflammatory and immunomodulatory (Ukai *et al.*, 1983; Sheu *et al.*, 2004; Yu *et al.*, 2009; Song and Du, 2010, 2012; Sun *et al.*, 2010). Koyama *et al.* (2002) also reported that the methanol extract contains antinociceptive compounds.

*Auricularia polytricha* has been reported to be the first cultivated species around AD 600 in China (Chang and Miles, 1987). It is cultivated mainly on sawdust of various tree species (Irawati *et al.*, 2012). However, shortage of suitable sawdust due to

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competition from other industries (fibre and wood-based boards) has urged researchers to look for alternative substrates for cultivation (Razak *et al.*, 2013). The use of alternative substrates such as wheat straw, paddy straw, rapeseed straw, maize stalk and wheat bran have been reported (Kushwaha *et al.*, 2006).

In Malaysia, the oil palm industry contributed export earnings of RM 65.2 billions, covering 41% of the world's supply of palm oil. Recognized as one of the largest global producers and exporters in oil palm, this industry also produce huge amount of agricultural waste materials. It was estimated that 66.3 mt of oil palm waste were generated from palm oil mills while Empty Fruit Bunch (EFB) account for 6.76 mt in terms of dry weight (Hon, 2010). Difficulties in disposal and improper management of these waste materials has led to serious environmental issues including emission of bad odours, habitat for pest and creating unproductive dumping sites (Sulaiman *et al.*, 2008). Another lignocellulosic waste which contributes to the pollution of water bodies in Malaysia is the sago waste. Locally known as sago 'hampas', these are fibrous residues left behind after most of the starch has been extracted out of the sago palm (*Metroxylon sagu*). Sago production is one of the important economical industries in Malaysia, especially the state of Sarawak. In Sarawak alone, 59-110 t of sago 'hampas' are produced daily from sago processing mill (Awg-Adeni *et al.*, 2010). Large quantities of these lignocellulosic wastes produced in localized areas making it a potential raw material for recycling.

Mushroom cultivation represents one of the most efficient ways of utilizing agricultural wastes while producing superior food sources with high nutritional and medicinal values. The EFB and sago 'hampas' have been used as substrates for cultivation of various mushrooms such as *Volvariella* sp., *Pleurotus* sp., *Pleurotus ostreatus* and *Ganoderma boninense* (Tabi *et al.*, 2008; Awg-Adeni *et al.*, 2010; Abbas *et al.*, 2011; Sudirman *et al.*, 2011). However, studies on optimization and domestication of *A. polytricha* on local condition and substrates are still insufficient (Razak *et al.*, 2011). In this study, the possible use of EFB and sago 'hampas' for cultivation of indigenous *A. polytricha* isolates were evaluated.

## MATERIALS AND METHODS

*Auricularia polytricha* were collected from the local markets in Bintulu and various habitats in Universiti Putra Malaysia Bintulu Sarawak Campus (UPMKB). Basidiocarps were washed in sterile distilled water and surface sterilized with 75% ethanol for 10 sec. Surface sterilization was repeated 2-3 times to reduce microbial contamination. The basidiocarps were then rinsed again

in sterile distilled water then dried by pressing between sterile paper towels. A small piece of inner tissue was taken using a sterile surgical blade and placed on Potato Dextrose Agar (PDA) supplemented with 0.01% chloramphenicol to prevent bacterial contamination.

Morphological identification of the fungi was done as described by Musngi *et al.* (2005). The texture and color of the pileus and hymenia were observed. Cross sections of ~0.1 mm thick were observed under compound microscope. Measurement of each zone from the hymenia to the pileus was used to identify the fungi accurately (Kobayashi, 1981).

Molecular identification of fungi was done by DNA sequencing. DNA extraction was done by using a simplified Sodium Dodecyl Sulphate (SDS) extraction method without boiling (Edkona *et al.*, 2013). Polymerase Chain Reaction (PCR) amplification of fungal DNA was achieved using ITS (ITS1/ITS4) (White *et al.*, 1990) and 18S rDNA primers (EF4/EF3) (Smit *et al.*, 1999) with minor modification. The annealing temperature for EF4/EF3 primers was increased to 50°C and total amplification was reduced to 35 cycles. Amplified DNA was precipitated by mixing 50 µL of PCR product with 0.1 volumes of 3 M potassium acetate (pH 4.8) followed by 2.5 volumes of absolute ethanol. The mixture was incubated on ice for 30 min prior to centrifugation at 12,000 g for 15 min at 4°C. The DNA pellet was washed in 0.5 mL of 75% ethanol and repelleted at 12,000 g for 5 min. The DNA was air dried and resuspended in 15 µL sterile distilled water. DNA was visualized using AlphaView SA version 3.3.1 and quantification was done based on band intensity using GeneRuler™ 1 kb DNA ladder (Fermentas) as the standard. Purified PCR products were subjected to automated DNA sequencing using forward primer. A BLAST search was carried out with gene bank database. Spawn was prepared from crushed corn as describe by Razak *et al.* (2011). Four raw materials namely sago 'hampas', rubberwood sawdust, EFB and rice bran were used to evaluate yield of *A. polytricha* fruiting bodies and production time. Substrate formulations were based on the C/N ratios of raw materials measured by combustion (Chefetz *et al.*, 1996) and Kjeldahl (Tan, 2005) methods. Dolomite (2%) and gypsum (1%) were used to increase the pH of substrates as well as to provide supplementary minerals for mycelia growth. Table sugar (1%) was also added to provide easily available energy source for initial growth. The moisture content was adjusted to ~65% and 1 kg of formulated substrates were packed tightly in PP bags. Sterilized substrate bags were inoculated with 10 g of fully grown spawn. The bags were then incubated at 25±2°C in dark condition. After full mycelia run, the bags were transferred to a shelf in humidified chamber at 28±2°C. The bags were slit horizontally below the PVC pipe to allow formation of fruiting bodies. The first flush

of matured fruiting bodies were harvested 6-10 days after primordia formation. The second and third flushes were harvested in the same manner. Biological efficiency was calculated based on the equation:

$$\text{Biological efficiency (\%)} = \frac{\text{Grams of fresh fruiting bodies produced}}{\text{Grams of dry substrate used}} \times 100$$

Time for complete colonization, primordia formation and yield were recorded.

**Statistical analysis:** The experiment was arranged in completely randomized design and statistical analysis of data was done using SAS version 9.0. Analysis of variance (ANOVA) and Tukey's mean homogeneity test were used to compare mean values at  $p \leq 0.05$ .

### RESULTS

Four *Auricularia* isolates designated as BF05, BF07, BF12 and BF13 were collected and grown into pure mycelia cultures. The BF05, BF12 and BF13 were collected from the local market while BF07 was found growing on fallen trunk of rubber tree (*Hevea brasiliensis*) in rubber estate of UPMKB. All isolates were identified as *A. polytricha* based on morphological characteristics described by Musngi *et al.* (2005) and Kobayashi (1981) (Fig. 1).

PCR products of the 18S rDNA and ITS were observed as single bands in agarose gel at sizes of approximately 1.5 kb and 500 bp respectively (Fig. 2). Sequences of ITS (490-559 bp) and 18S rDNA (600-980 bp) revealed that the two DNA markers had different

capacities to identify the mushroom isolates. ITS sequences of all *Auricularia* isolates had high similarities (99-100%) with *A. polytricha* reference sequences and were clustered together in the phylogenetic tree (Fig. 3). The *A. polytricha* clade was separated from other closely related species by a strongly supported branch (81% bootstrap support) while isolate BF07 was found to be weakly separated from other members with 54% bootstrap support. The 18S rDNA sequences of the isolates were indistinguishable from *A. auricular judae* and *Exidia glandulosa* in a single clade (Fig. 4). In the clade, isolate BF07 was again slightly differentiated from the other isolates with 46% bootstrap support.

The C/N ratios of sago 'hampas' (SH), rubber wood sawdust (RW), EFB and rice bran (RB) was 189.9, 177.62, 77.96 and 56.67, respectively (Table 1). EFB and RB were used as the supplementary substrates due to their high nitrogen content (0.88 and 0.69%) while SH and RW were used as main substrates. The C/N ratios of formulated substrates were in the range of 127.5-140.3.

From results shown in Table 2 the fastest colonization rate was found in substrate C, whereby all isolates achieved full colonization within 18-25 days followed by

Table 1: C/N ratios of raw materials and formulated substrates

Raw material/ substrates	Composition (%)	Carbon (%)	Nitrogen (%)	C:N ratio
RB	Rice bran	49.76±0.03	0.88±0.12	56.67
EFB	Empty fruit bunch	53.88±0.04	0.69±0.04	77.96
RW	Rubberwood sawdust	56.41±0.05	0.32±0.03	177.62
SH	Sago 'hampas'	54.98±0.02	0.29±0.02	189.90
Substrate A	RW 80+RB 20	55.08	0.43	127.50
Substrate B	SH 80+RB 20	53.94	0.41	132.20
Substrate C	RW 75+EFB 25	55.78	0.41	135.22
Substrate D	SH 75+EFB 25	54.71	0.39	140.27

Carbon and nitrogen content of substrates were calculated base on their raw material composition

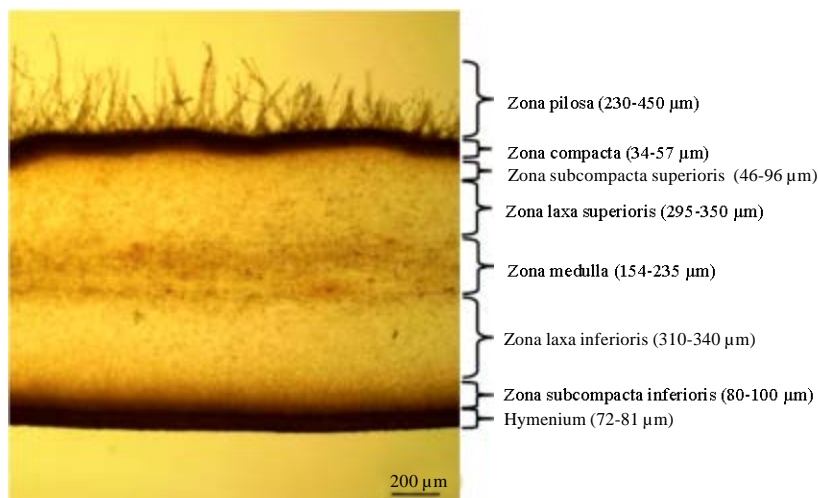


Fig. 1: Cross section of *Auricularia polytricha* (40x) showing hyphal zonation for identification

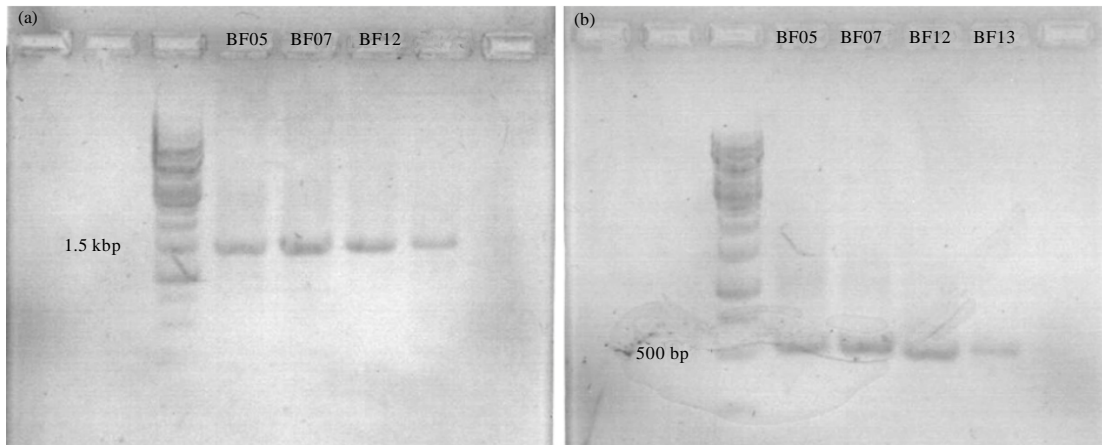


Fig. 2(a-b): Gel image of PCR product using (a) EF4/EF3 and (b) ITS1/ITS4 primers

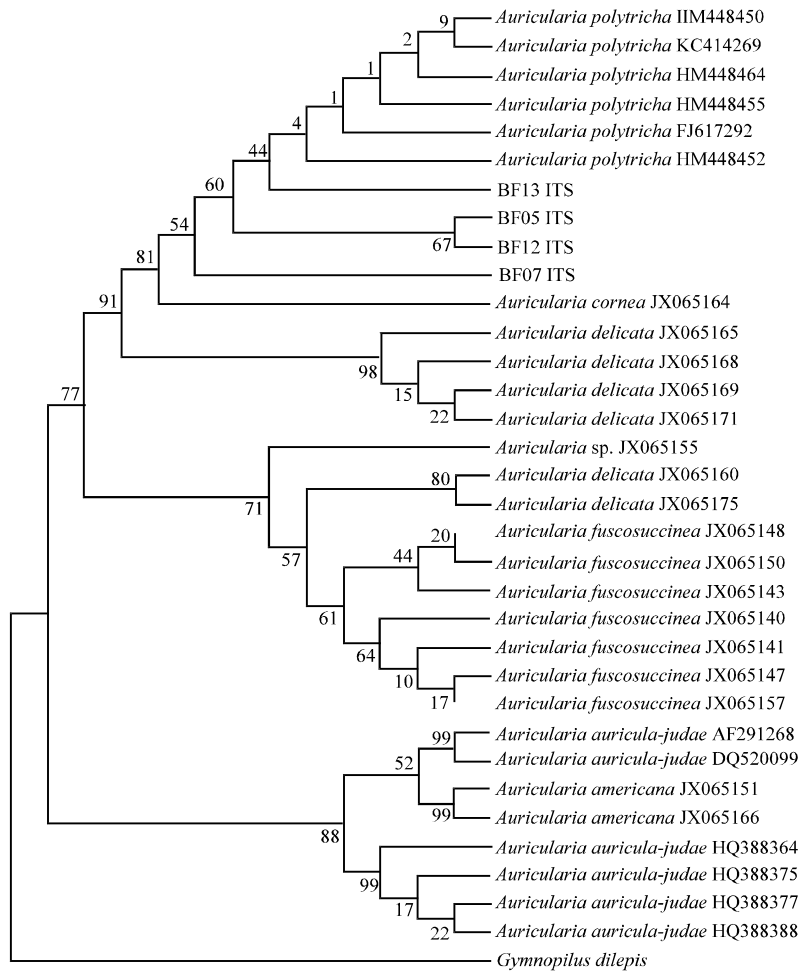


Fig. 3: Neighbour-joining tree showing relationship between 29 ITS sequence (430 bp aligned) of closely related species and the *A. polytricha* isolates (BF05, BF07, BF12, BF13). Bootstrap values are shown in each branches in a bootstrap analysis of 1000 replicates

Table 2: Time for complete colonization, primordia formation, total yield and biological efficiency (BE) of *A. polytricha* isolates on different substrate

Isolate and substrate	Colonization (day)	Primordia formation (day)	Total yield (g)	BE (%)
<b>BF05</b>				
A	34.3±1.5 <sup>de</sup>	5.0±1.7 <sup>def</sup>	94.9±3.10 <sup>cd</sup>	27.1±0.9
B	43.7±3.5 <sup>ab</sup>	9.7±1.5 <sup>abc</sup>	60.7±10.2 <sup>gh</sup>	17.4±2.9
C	24.7±1.2 <sup>fg</sup>	7.3±1.2 <sup>bcd</sup>	118.2±4.20 <sup>ab</sup>	33.8±1.2
D	41.0±2.7 <sup>bc</sup>	8.7±0.6 <sup>bde</sup>	78.6±7.00 <sup>def</sup>	22.4±2.0
<b>BF07</b>				
A	31.0±1.0 <sup>e</sup>	4.0±1.0 <sup>f</sup>	130.7±6.00 <sup>a</sup>	37.4±1.7
B	38.3±2.1 <sup>cd</sup>	10.0±2.0 <sup>abc</sup>	76.5±3.10 <sup>def</sup>	21.8±0.9
C	22.0±1.0 <sup>gh</sup>	4.3±1.5 <sup>ef</sup>	120.2±16.8 <sup>ab</sup>	34.3±4.8
D	36.7±2.5 <sup>cd</sup>	8.3±1.5 <sup>bcd</sup>	67.3±8.40 <sup>efg</sup>	19.2±2.4
<b>BF12</b>				
A	29.3±0.6 <sup>f</sup>	7.0±1.0 <sup>def</sup>	92.8±2.30 <sup>cd</sup>	26.5±0.7
B	48.7±2.5 <sup>a</sup>	9.3±0.6 <sup>abcd</sup>	39.9±7.30 <sup>f</sup>	11.4±2.1
C	21.7±0.6 <sup>h</sup>	6.3±1.5 <sup>def</sup>	89.0±2.70 <sup>d</sup>	25.4±0.8
D	41.7±1.5 <sup>bc</sup>	11.7±2.1 <sup>ab</sup>	55.5±4.00 <sup>gh</sup>	15.9±1.2
<b>BF13</b>				
A	24.7±0.6 <sup>g</sup>	6.3±1.5 <sup>def</sup>	82.9±3.20 <sup>de</sup>	23.7±0.9
B	36.7±0.6 <sup>d</sup>	13.7±1.5 <sup>a</sup>	46.9±4.80 <sup>hi</sup>	13.4±1.4
C	18.0±1.0 <sup>h</sup>	5.7±0.6 <sup>def</sup>	109.5±1.80 <sup>bc</sup>	31.3±0.5
D	33.7±2.1 <sup>de</sup>	10.0±2.0 <sup>abc</sup>	62.4±4.40 <sup>gh</sup>	17.8±1.3
LSD (5%)	5.4	4.4	20.2	-
CV (%)	5.4	18.2	8.1	-

In a column, means with different letters are significantly different at  $p \leq 0.05$

substrate A (25-34 days). Substrate B and D had significantly longer colonization durations with 37-49 days and 34-42 days, respectively. Primordia formation occurred within 4-7 days for substrate A and C, whereas substrate B and D resulted in slower primordia emergence (8-14 days). Yield of fruiting bodies were higher in substrate A (82.9-130.7 g) and C (89.0-120.2 g) as compared to B (39.9-76.5 g) and D (55.5-78.6 g).

Fruiting bodies were harvested for 3 flushes (Fig. 5). The RB supplemented substrates (A and B) recorded highest yield in the first harvest followed by second and third for all isolates. However, the production patterns for EFB supplemented substrates (C and D) were rather inconsistent. In substrate C, isolate BF05 showed highest yield during the 2nd harvest (38.7% of total yield) while yield of isolate BF13 increase with each harvest. Isolate BF07 and BF13 also showed highest yield during the 2nd harvest in substrate D with 39.5 and 38.6% of total yield, respectively.

Isolates used had variable effect on colonization rate, primordia formation and yield. Isolate BF13 recorded numerically fastest colonization rate (18-37 days) while comparing the same substrate among isolates. However, the fastest primordia formation (~4 days) and highest yield (120.2-130.7 g) was observed in isolate BF07 on substrate A and C. Stages of fruiting bodies development including primordia emergence, fructification and matured fruiting bodies of *A. polytricha* were shown in Fig. 6.

## DISCUSSION

Molecular study showed that the results of ITS sequences were more consistent with morphological

identification. The 18S rDNA sequences were unable to provide conclusive result as the sequences of *A. polytricha*, *A. auricular judae* and *Exidia glandulosa* were highly similar. White *et al.* (1990) claimed that ITS evolves faster and is suitable for interspecies identification. On the other hand, 18S rDNA evolves relatively slower and it is often used to study distantly related taxa. This was supported by Anderson *et al.* (2003) stating that 18S rDNA sequences are highly conserved and generally only able to resolve taxonomic groups to the genus level.

The use of EFB as substrate for mushroom cultivation showed variable results. Tabi *et al.* (2008) reported that *Pleurotus ostreatus* was unable to grow on EFB alone. This was in agreement with the present results whereby all isolates failed to colonize substrate consists solely of EFB. This might be due to the complex structure of EFB lignin which was relatively resistant to degradation. The presence of hydroxyphenylpropane units together with syringyl and guaiacylpropane units making it structurally more complicated than wood lignin (Sun *et al.*, 2000). However, Sudirman *et al.* (2011) demonstrated that *Pleurotus* sp. and *Ganoderma boninense* were able to grow on EFB supplemented with 15% rice bran. On the other hand, Rizki and Tamai (2011) had cultivated *Pleurotus ostreatus* using un-supplemented EFB. These contradictory results might be due to the different composting stage of the EFB. The C/N ratios of EFB used by Sudirman *et al.* (2011) and Rizki and Tamai (2011) were 57.8 and 20.39, respectively while the C/N ratio of EFB used in this study was 77.96. From these C/N ratios, the EFB used by those studies were partially decomposed while this study utilizes relatively under decomposed EFB.

Although, the present results showed that under decomposed EFB was not suitable to be used as sole substrate but it can be potentially used as supplementary substrate for *A. polytricha* cultivation. Substrates with EFB supplementation showed superior performances in growth and yield as compared to RB. Higher mycelia densities were observed on EFB supplemented substrates. This was in agreement with Ayodele and Akpaja (2007), whereby the supplementation of sawdust with 20% oil palm fruit fibre enhanced mycelia growth and yield of *Lentinus squarrosulus*. Razak *et al.* (2013) also revealed that the use of EFB and sawdust at equal portion (50:50) greatly enhanced growth of *A. polytricha* as compared to sawdust alone. In addition, the availability of EFB all year round making it a better choice of supplementary substrate as compared to RB which is only available at certain period.

According to Onyango *et al.* (2011), *Auricularia* sp. benefits from substrates with high proportion of lipids. It was also reported that the growth rate of mushroom



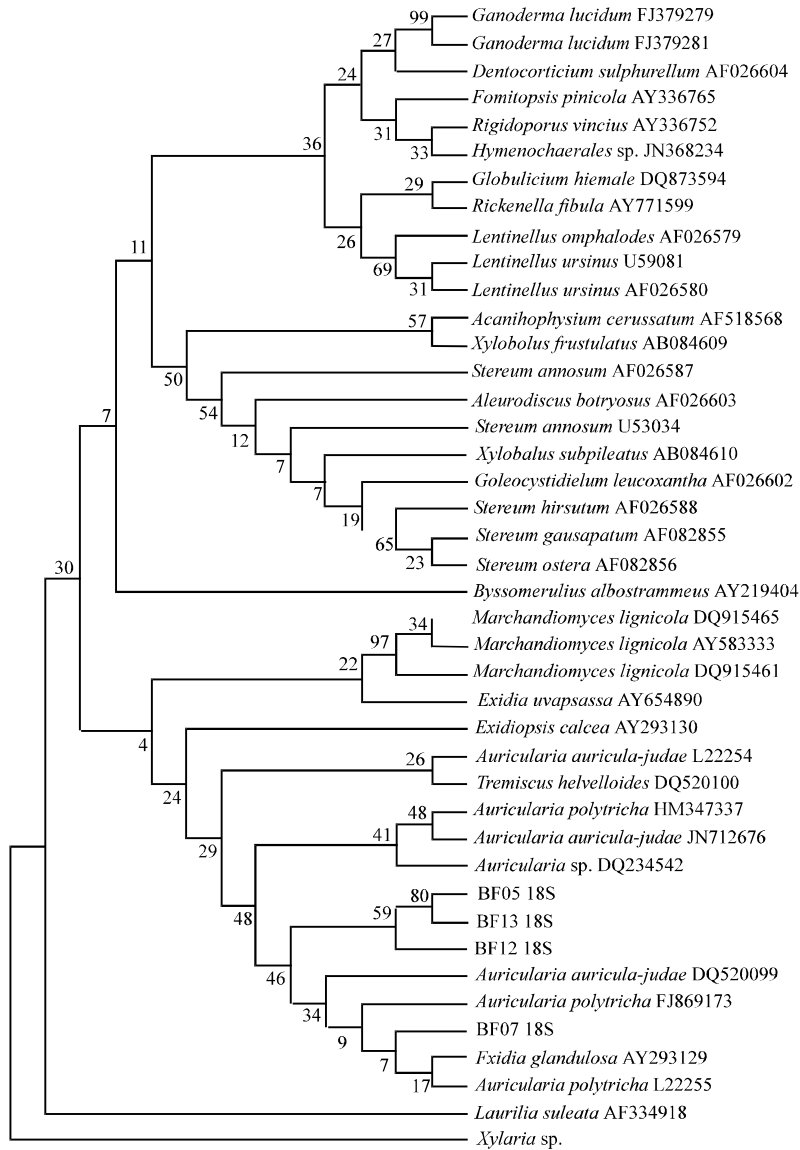


Fig. 4: Neighbour-joining tree showing relationship between 37 18S rDNA sequence (530 bp aligned) of closely related species and the *Auricularia* isolates (BF05, BF07, BF12, BF13). Bootstrap values are shown in each branches in a bootstrap analysis of 1000 replicates

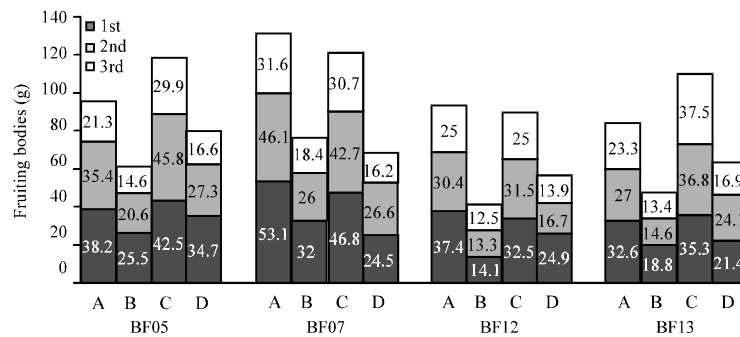


Fig. 5: Yield of fruiting bodies at 1st, 2nd and 3rd harvest



Fig. 6(a-c): Image of the (a) Primordia (b) Fruitification and (c) Matured fruiting bodies of *A. polytricha*

mycelia were enhanced by addition of lipids to the basal media (Wardle and Schisler, 1969). Schisler (1967) speculated that the linoleic acid in vegetable oil stimulated the yield and production of mushroom fruiting bodies. From these studies, it is likely that the oil residues in EFB (1.6%) (Baharuddin *et al.*, 2011) could have certain role in promoting growth and yield performances of *A. polytricha*. Complex mosaic of substrate components are found to be beneficial for mushroom formation (Razak *et al.*, 2013). Hence, a mixture of fine RW and course EFB fibre was considered ideal for mushroom cultivation. However, thorough mixing was required to ensure homogeneity during substrates preparation. It was also noted that the sharp spikelets of EFB fibre can be problematic during packing of substrate bags. Protruding spikelets could cause injuries and contamination during handling and incubation period. Thicker PP bag ( $\geq 0.05$  mm) should be recommended for packing substrates with EFB.

The abnormal yield pattern observed in EFB supplemented substrates can be explained by looking at its degradability. EFB was relatively resistant to degradation compare to other substrates. The nutrients contained inside EFB requires a longer period of time to be released and absorbed by the fungi which resulted in delayed maximum yield. This was shown by several isolates (BF05, BF07 and BF13) producing highest yield during the second or third harvest in substrate C and D (Fig. 5).

SH was found to be less favourable for cultivation of *A. polytricha*. It has been reported that SH was suitable to be used as compost for mushroom cultures (Awg-Adeni *et al.*, 2010) and laccase production through solid substrate fermentation of *Pleurotus sajor-caju* (Kumaran *et al.*, 1997). However, the present results demonstrated that substrates with 70-75% SH generated poor colonization rate, delayed primordial formation and lower yield in *A. polytricha* cultivation.

Apart from cultivation substrates, different isolates or strains of mushroom culture played an important role in mushroom cultivation. Onyango *et al.* (2011) showed that

different strains of *Auricularia auricula* responded differently to various substrates. The diverse characteristics and biological properties of strains or isolates were also shown in other mushrooms such as *Pleurotus ostreatus* (Sastre-Ahuatzi *et al.*, 2007), *Agaricus bisporus* (De Andrade *et al.*, 2008), *Agaricus blazei* (Colauto *et al.*, 2011) and *Lentinula edodes* (Gaitan-Hernandez *et al.*, 2011). In this study, isolate BF07 was observed with the fastest primordia formation (~4 days) and highest biological efficiency (34.3-37.4%) on substrate A and C. The exceptional performance of isolate BF07 may be associated with its position in phylogenetic tree which differ from other isolates (Fig. 3 and 4). The variation in ITS and 18S rDNA sequences may contributed to the significant performances in isolate BF07. Morphologically, it was also observed that isolate BF07 had darker and thicker fruiting bodies as compared to other isolates. However, more studies have to be carried out for a better understanding of genetic variations in mushroom cultivation performances.

## CONCLUSION

This study clearly indicated that EFB have high potential to be utilized for *A. polytricha* cultivation. EFB expressed superiority over RB as supplementary substrate which resulted in faster colonization and increased yield. However, SH was not suitable for cultivation of *A. polytricha* as it resulted in slower colonization rate, delayed primordial formation and lower yield. Isolate BF07 can be recommended for used for further research or commercial scale production in Malaysia.

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