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ORIGINAL ARTICLE

MEDICINAL PROPERTIES AND GROWTH OF MUSHROOM LIGNOSUS RHINOCEROTIS RYVARDEN

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Abstract: Lignosus rhinocerotis, a wild medicinal mushroom that has been "generally gained in Malaysia recently. Due to the interest in its medicinal properties and the fact that it has not been successfully domesticated (commercial cultivation), Mushroom cultivation is an efficient and economical way to recycle agricultural and industrial wastes. optimization of fruiting substrate using readily available lignocellulosic agroresidues (sawdust, paddy straw and oil palm empty fruit bunch) with supplementation of spent brewery yeast as the nitrogen source was investigated. Further optimization using MINITAB analysis showed that only sawdust had significant effect on the mycelial growth rate for substrate formulation consisting of sawdust and paddy straw.
Keywords: mushroom,Polyporaceae, Sclerotia,Paddy straw.

INTRODUCTION

Lignosus rhinocerotis (as “rhinocerus”) (Cooke) Ryvarden, previously known as Polyporus rhinocerus (Cooke) or Fomes rhinocerus(Cooke), belongs to Basidiomycota, Agaromycetes, Polyporales and Polyporaceae (Kirk et al., 2008). Polyporaceae is a very diverse family having a complex macrostructure, whereby their flesh is composed of several kinds of hyphae. The strength and the long life of these fungi are mainly due to the binding of their hyphae during their fruiting. The genus Lignosus consists of six species, including Lignosus dimiticus Ryvarden (1975), Lignosus ekombitii DouanlaMeli and Langer (2003), Lignosus goetzii (Henn.) Ryvarden (1972), L.rhinocerotis (Cooke) Ryvarden (1972), Lignosus sacer (Afzel. ex Fr.)Ryvarden (1972) and Lignosus hainanensis Cui et al. (2010).

Morphology of Lignosus spp. is unusual for polypores because the sporophore (fruiting body) consists of a cap on a central stem (which occurs in a few polypore genera) and grows from a sclerotium in the ground (which is even rarer), rather than from wood,as is the case with most polypores.

Sclerotia are specialized vegetative structures containing reserve materials important in the survival of the vegetative stage of the fungus during unsuitable conditions.They are round or of variable shape and size with cream cortex made up of intertwined hyphae embedding innumerable large starch grains, and produce from the upper surface many rhizomorphs which penetrate the humus.

L. rhinocerotis can be found in Australia, Papua New Guinea,Borneo, Philippines, Indonesia, Malaysia, Sri Lanka and Vanuatu.The sclerotium of L. rhinocerotis is subterranean with a spherical,oval, or even irregular shape (about 4–5 cm in diameter). The rough and wrinkly surface (rind) of the sclerotia (which is white to pale brown in color), on which 3–7 orbicular pilei (that are tea brown in color, ciliated, and depressed in the center) are produced.Pileus is up to 2 mm thick, and the internal structure is white andpowdery (Ryvarden and Johansen, 1980).

L. rhinocerotis is one of the most economically important sclerotium-forming fungi in China besides Pleurotus tuber-regium.The sclerotium is regarded as an expensive folk medicine for the treatment of chronic hepatitis, gastric ulcers and liver cancer (Wong and Cheung, 2008). The indigenous communities in Malaysia claimed that L. rhinocerotis can be used as a medicine totreat cough, asthma, fever, cancer and food poisoning. The medicineis usually prepared by boiling sliced sclerotium of L. rhinocerotis and the resulting decoction is then drunk (Lee et al., 2009).

The main source for medicinal use at present is still wild sclerotia in their natural habitats which limits its availability. Hence, it ishighly priced ranging from US$15–25 per sporophore including the sclerotium. For exploration of mushroom sclerotia for nutraceuticals and as a functional food, research on the cultivation of mushroom sclerotia needs to be comprehensively conducted, so that their scale and efficiency of production can be improved and their commercialization

facilitated. In contrast to P. tuber-regium,information concerning the cultivation of L. rhinocerotis sclerotium is very limited (Wong and Cheung, 2008).

Nutrient status and nature of lignocellulose material used as fruiting substrate influences mycelial growth, sclerotial yield and biological efficiency. Rubberwood sawdust (SD) is predominantly used as substrate for mushroom cultivation as it is similar to decaying logs in the natural habitat of basidiomycetes. Agroresidues such as paddy straw and oil palm empty fruit bunches which are found in abundance in Malaysia can also be exploited. At present, Malaysiais the largest exporter of palm oil in the international market. In the process of extraction of palm oil from oil palm fruit, empty fruit bunches (EFBs) are generated as waste products. Paddy is one of Malaysian major crop and producing huge amount of paddy straw(PS) as solid biomass waste seasonally. The paddy straw came after the stripping process of rice using machine at the field, where the paddy straw was removed and left to dry. It has no further use apart from being used as fodder. Both EFB and PS have been shown to be suitable substrates for cultivation of oyster mushroom(Nageswaran et al., 2003; Mohd Tabi et al., 2008).

Hence, the objective of this study was to optimize the formulation of selected agroresidues to support mycelial growth as well as sporophore and sclerotia formation for the efficient cultivation of L. rhinocerotis to overcome the limited supply of sclerotia used as medicinals and for conservation of this species.

MATERIALS AND METHODS

PREPARATION OF L. RHINOCEROTIS MYCELIAL CULTURE

L. rhinocerotis (KUM61075) culture was authenticated and deposited in Mushroom Research Centre, Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia. The culture was maintained on BSSYM agar slants consisting of brown sugar (2%),spent yeast (1%) and malt extract (1%) (w/v). Inoculum was pre-pared by periodic transfer of 9-mm diameter mycelia plugs cut from the periphery of an 8-day old colony growing on BSSYM agar media in Petri plates.

SELECTION OF SUBSTRATE FORMULATION BASED ON MYCELIAL GROWTH

Rate Agroresidues investigated as fruiting substrate consisted of rubberwood sawdust (SD), paddy straw (PD), and oil palm empty fruit bunch (EFB). Brewery spent yeast (SY) obtained from Carlsberg factory (Petaling Jaya, Selangor, Malaysia) was also incorporated as a source of nitrogen. Fifteen different substrate formulations consisting of single or mixed formulations were investigated according to percentage ratios as shown in Table 1. The moisture content of each formulation was fixed to 60% and the pH adjusted to 6.0 using cooking vinegar. The agroresidues mixture was then packed in a glass Petri dish, containing approximately 20 g of substrate.Three replicate plates were prepared for each formulation. The agroresidues mixture was then autoclaved at 121 ◦C for 1 h and upon cooling inoculated with a single agar plug placed on top of the substrate in the center of the dish and incubated at 28 ◦C. Radial growth of the fungus was measured at four equidistant points from the center of the growing colony, and the rate of mycelia growth(mm/day) determined over 29 days. The data obtained were then tabulated and a linear graph was plotted whereby the gradient of the linear line was determined as the mycelial growth rate.

Table 1 Formulations and percentage ratios of agroresidues and nitrogen source to determine the optimum fruiting substrate of L. rhinocerotis based on mycelial growth rate.

Sawdust (SD)	Paddy straw (PS)	Empty fruit bunch (EFB)	Brewery spent yeast (SY)
99	–	–	1
–	99	–	1
–	–	99	1
89	10	–	1
82	10	–	8
10	89	–	1
10	82	–	8
–	10	89	1
–	10	82	8
89	–	10	1
82	–	10	8
–	89	10	1
–	82	10	8
–	10	89	1
–	10	82	8

Table 2 Levels of sawdust, paddy straw and spent yeast applied for the optimization studies.

Fruiting substrate components	Level (wt)	
	High	Low
SD	7.9	1
PS	7.9	1
SY	2	1

Optimization of substrate consisting of sawdust, paddy straw and spent yeast based on mycelial growth rate

Further optimization of the formulation consisting of sawdust,paddy straw and spent yeast that exhibited profound effect on mycelial growth (as determined above) was carried out. Each variable was studied at two concentration levels representing high and low set points as shown in Table 2. The experimental combinations were designed using MINITAB®14 software. From the selected range, eight different substrate formulations/ratios were determined as follows and the preparation was carried out as above.

1. SD + PS + SY (1:1:1)
2. SD + PS + SY (1:1:2)
3. SD + PS + SY (1:7.9:1)
4. SD + PS +SY (1:7.9:2)
5. SD + PS + SY (7.9:1:1)
6. SD + PS + SY (7.9:1:2)
7. SD + PS +SY (7.9:7.9:1)
8. SD + PS + SY (7.9:7.9:2)

The mycelial growth rate obtained from each substrate formulation was analyzed using the software to obtain the optimum substrate formulation and levels for the cultivation of L. rhinocerotis.

2.4. Statistical analysis

One-way ANOVA was used to analyze the data to establish significance differences between the means ($p = 0.05$). Calculations were performed using Statgraphics Plus v. 3.0 software (StatisticalGraphics Corp., Princeton, NJ, USA).

2.5. Cultivation of L. rhinocerotis on optimized substrate formulae

SPAWN PREPARATION

Eight days old mycelia culture of L. rhinocerotis was grown on BSSYM agar media. Optimized substrate, as determined above, wasprepared and the moisture was adjusted to approximately 60% (v/w). The pH was adjusted to pH 6.0 using cooking vinegar. Substrate was filled in polypropylene bags (diameter: 90 mm) to a height of approximately 80 mm and weighing around 200 g. The bags were covered with plastic caps without holes and then, autoclaved at 121 ◦C for 1 h. The bags were left to cool overnight before inoculated with three 9 mm-diameter mycelia plugs and the plastic caps were replaced with cotton-plugged plastic caps with holes.The bags were incubated at 28 ◦C for 3 weeks for full spawn development.

MYCELIUM RUNNING, SCLEROTIA AND SPOROPHORE DEVELOPMENT ON OPTIMIZED FRUITING SUBSTRATE

The optimized substrate formulation was packed into polypropylene bags to a height of 100 mm and sealed with plastic cap without holes. Fifty replicate bags were prepared and sterilized by autoclaving at 121 ◦C for 1 h. Upon cooling they were inoculated with 3 weeks old L. rhinocerotis spawn, covered with cotton-plugged plastic caps with holes and incubated at room temperature in the dark. Mycelium running (linear growth rate)was recorded by measuring the penetration of mycelium into the substrate along 4 lines drawn at four sides of the bag for a period of 1 month The data obtained was then tabulated and a linear graph was plotted whereby the gradient determined represents the rate of mycelial run. After 3 months of incubation, the plastic bags were removed and the mycelia-colonized substrate blocks were buried in loam soil at 15 cm depth for 6–12 months for the development of sclerotia and sporophores. The size of the beds was 1.00 m (length) and 0.15 m (width). The burial site was roofed but exposed to external environmental condition, with the average temperature ranging from 28 to 32 ◦C. Throughout the cultivation period, the soil was watered every 2–3 days.

RESULTS AND DISCUSSION

SELECTION OF SUBSTRATE FORMULATION BASED ON MYCELIAL GROWTH RATE

Many agricultural by-products and waste materials have been used to produce edible-medicinal mushrooms such as oyster(Salmones et al., 2005), shiitake (Martinez-Guerrero et al., 2011),jelly mushroom (Abdul Razak et al., 2012) and Lingzhi mushroom(Veena and Pandey, 2011). According to Wong and Cheung (2008),there is only one report on cultivation of P. rhinocerus (synonym for L. rhinocerotis) on sawdust as substrate by Huang (1999); however,no data on yield were reported. No work has been reported on the use of other agroresidues for the cultivation of this medicinal mushroom. The aim of this study was to select agroresidues used singly or in combinations to optimize mycelial growth for cultivation of L. rhinocerotis.

The growth rate of L. rhinocerotis on BSSYM agar media was 4.5 ± 0.5 mm which is slow compared to other edible-medicinal mushrooms. The colony color was white to beige or light yellow upon maturation. The mycelium texture appeared fluffy or velvety as shown in Fig. 1. Fig. 2 shows the mycelial growth rate of L. rhinocerotis on agroresidues formulations consisting of sawdust (SD), paddy straw (PS), empty fruit bunch (EFB) as carbon sources and spent yeast (SY) as nitrogen source. The formulation consisting of SD + PS + SY at percentage ratio of 82:10:8 gave the highest mycelial growth rate of 3.0 ± 0.1 mm/day. This is followed by SD + PS + SY (89:10:1) and PS + SD + SY (89:10:1) formulations with mycelia growth rate of 2.8 mm/day.

Fig. 1. Colony of L. rhinocerotis on BSSYM agar media.

Formulations consisting of SD or PS as the dominant substrate in combination with other agroresidues were observed to havehigher growth rates compared to using EFB as the dominant substrate. In addition, SD or PS used in combinations exhibited higher mycelial growth rate than using SD or PS singly however, the effect was non-significant. The lowest growth rate was shown by EFB (100%) with mycelial growth rate of 1.7 ± 0.3 mm/day. This is in accordance with Mohd Tabi et al. (2008) who reported lowest mycelial colonization of P. ostreatus on EFB compared to SD while Nageswaran et al. (2003) reported good mycelia colonization and fruiting bodies yield on paddy straw.

OPTIMIZATION OF SAWDUST AND PADDY STRAW FORMULATIONS

Sawdust and paddy straw were selected for further formulation optimization using MINITAB®14 software. A maximum range of 7.9 and the lowest range of 1.0 for sawdust and paddy straw and a maximum range of 2.0 and lowest range of 1.0 for spent yeast were set as the limit for optimization. Fig. 3 shows the average mycelial growth rate for the optimization of sawdust, paddy straw and spent yeast levels based on the statistical experimental design.

From ANOVA (data not shown), it was found that only sawdust as the main effect was significant ($p = 0.001$) in the formulation;other main effects viz. paddy straw and spent yeast

were found to be not significant at p = 0.05. The analysis of the regression coefficients showed that an increase in sawdust level would result in a minimum of four- and eight times increase in average mycelial growth rate as compared to paddy straw and spent yeast, respectively.Based on the main effect plot (not shown), the increase in sawdust level from 1.0 to 7.9% resulted in a linear increase in the average mycelial growth rate i.e. from 2.7 to 3.2 mm/day.

Selection of the substrate components is very critical in obtaining good mycelium run. According to Elliot (1994), mushroom substrate, the main source of nutrients, is one of the crucial factors that greatly affect the growth and fructification of mushrooms.

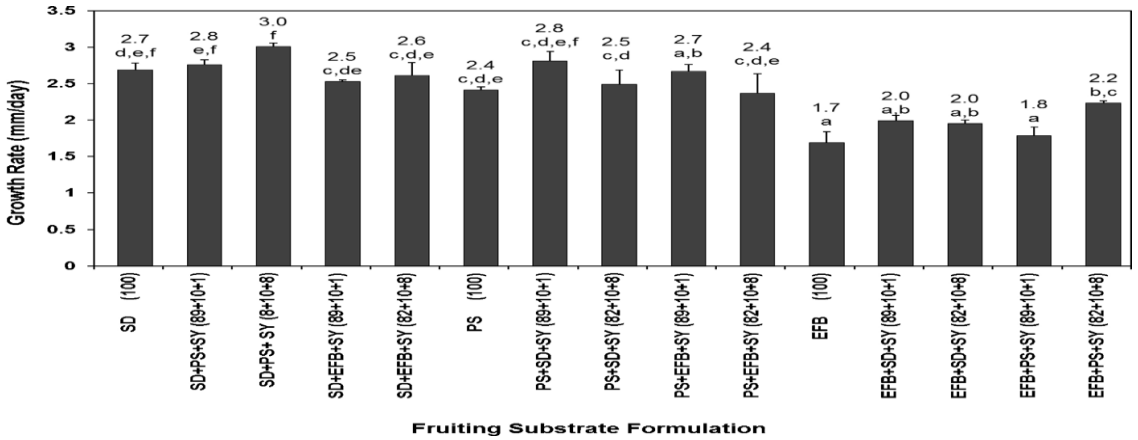

Fig. 2. Mycelial growth rate on substrate formulations consisting of sawdust, paddy straw, empty fruit bunch and spent yeast. Results were expressed as mean ± standard deviation of three determinations. Different letters (a–f) denote the means were significantly different at p = 0.05.

Furthermore, it is found that different species of cultivated mushroom have different substrate requirements ranging from hardwood to waste residues (Philippoussis et al., 2003).

The subsequent validation experiments of optimized substrate formulation consisting of sawdust, paddy straw and spent yeast (7.9:1:1) as predicted by MINITAB®14 analysis was carried out in Petri plates and mycelial growth rate obtained was 3.3 ± 0.1 mm/day, while the average mycelium run from the spawn in bags using this formulation was 3.8 ± 0.8 mm/day. These observed experimental mycelial growth rate values were in good agreement to the MINITAB®14 predicted value of 3.4 mm/day.

CONCLUSION

Mushroom cultivation is an efficient and economical way to recycle agricultural and industrial wastes. Rubberwood sawdust,paddy straw and oil palm empty fruit bunches, which are normally burnt or dumped, can be formulated into artificial media for cultivation of L. rhinocerotis. We showed that rubberwood sawdust mixed with paddy straw and supplemented with spent yeast as nitrogen source at a ratio of 7.9:1:1 supported the best mycelial growth and the pilot cultivation technology which, successfully produced the sclerotia and sporophores, can be easily adopted by commercial mushroom growers in tropical countries. This is the first report of successful artificial cultivation of L. rhinocerotis with descriptions of the major developmental stages leading to formation of sclerotia and sporophores.

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