Scientia Horticulturae 161 (2013) 65–69

Contents lists available at ScienceDirect

Scientia Horticulturae

journal homepage: www.elsevier.com/locate/scihorti

Production of liquid spawn of an edible grey oyster mushroom, *Pleurotus pulmonarius* (Fr.) Quél by submerged fermentation and sporophore yield on rubber wood sawdust

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ARTICLE INFO

Article history:
Received 10 December 2012
Received in revised form 17 June 2013
Accepted 17 June 2013

Keywords:
Pleurotus sajor-caju
Bioreactor
Liquid inoculum
Mycelia
Mushroom cultivation

ABSTRACT

Production of quality spawn having good ability to colonise fruiting substrates at low risk of contamination is of utmost importance to the mushroom industry. The aim of this study was to investigate the potential of producing liquid spawn of an edible mushroom, *Pleurotus pulmonarius* (grey oyster) by submerged fermentation in a 2-L stirred-tank bioreactor under controlled conditions and to evaluate its ability to colonise rubber wood sawdust substrate for sporophore production. The liquid spawn cultivation medium contain 20 g L^{-1} of brown sugar, 4 g L^{-1} of rice bran, 4 g L^{-1} of malt extract, and 4 g L^{-1} of yeast extract (BRMY) with initial pH of 5.5 and incubated at 28 °C with agitation speed of 250 rpm and oxygen partial pressure of 30–40%. Maximum *P. pulmonarius* dry biomass production of 11.72 ± 5.26 g L^{-1} was achieved after 3 days of fermentation. The liquid spawn has the ability to colonise sterile rubber wood sawdust as fruiting substrates in a shortened time and produced higher yield of sporophores compared to the traditionally used grain spawn.

1. Introduction

Liquid culture technology for the production of mycelia of mushrooms can be extended to the mushroom spawn industry where the production process is currently based on the solid-state fermentation of cereal grain. In the edible mushroom cultivation industry, usually grain spawn is used. However, it has been reported that preparation of grain spawn has longer growth period and poses higher risk of contamination compared to liquid spawn (Confortin et al., 2008). Mycelia or liquid spawn produced by submerged fermentation is an alternative method for generating spawn as it produces higher yield and more uniform mycelial biomass in a shorter period (Stamets, 2000), favours mycelium dispersion, adaptation and enables inoculation to be carried out under a relatively more stringent aseptic condition (Bettin et al., 2009).

The cultivation of mushroom species in a bioreactor has been studied for the production of secondary metabolites, polysaccharides or the large quantity of biomass (Moreira et al., 2004). Many species of mushrooms such as *Agaricus bisporus* (Frieal and McLoughlin, 2000), *Agaricus blazei* (Lin and Yang, 2006), *Pleurotus ostreatus* (Papaspyridi et al., 2012), *Ganoderma lucidum* (Songulashvili et al., 2011) and *Lentinula edodes* (Zhong et al., 2012) have been grown in submerged culture. One of the pre-requisites for growing fungi in submerged cultures is the nutritional and environmental condition.

Nutrient sources affect the production of high-density mycelium in submerged fermentation. Hamachi et al. (2003) reported that brown sugar is generally recognised as the most readily utilisable carbon source for most mushrooms cultures because it contains approximately of 94–98.5% (w/w) sucrose and various types of non-sucrose components ranging from 1.5–6% (w/w). Further, it is acknowledged that the supplementation of rice bran produces mycelium vigour whilst soluble nitrogen sources produce dense mycelium (Bettin et al., 2009). The findings of our previous investigation using shake flasks to formulate an optimum growth medium of nine edible mushrooms, showed that a medium containing brown-sugar (2%), rice-bran (0.4%), malt (0.4%) and yeast (0.4%) extracts (BRMY) supported highest mycelia dry weights of *Schizophyllum commune* (14.64 ± 2.39 g L^{-1}) followed by *Pleurotus pulmonarius* (9.96 ± 1.14 g L^{-1}), and *Pleurotus sapidus* (7.56 ± 2.03 g L^{-1}) (unpublished data).

Hence, the objective of this study is to produce mycelial biomass of a popular edible mushroom, *P. pulmonarius* (liquid spawn) at a larger scale using BRMY formulation medium under controlled conditions in an automated bioreactor and to evaluate the performance of the liquid spawn for the production of sporophores compared to grain spawn.

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http://dx.doi.org/10.1016/j.scienta.2013.06.026

2. Materials and methods

2.1. Culture of P. pulmonarius and preparation of inoculum

Mushroom culture was received from a local mushroom grower as *Pleurotus sajor-caju* but was authenticated as *Pleurotus pulmonarius* (KUM61119) based on morphology and molecular sequencing by experts in Mushroom Research Centre (MRC), Faculty of Science, University of Malaya. The real *P. sajor-caju* (Fr.) Singer is in fact a separate species of mushroom, which was returned to the genus *Lentinus* by Pegler (1975), and is now called *Lentinus sajor-caju* (Fr.) Fries. *P. pulmonarius* culture investigated was deposited in MRC and maintained on malt extract agar (MEA) slants stored at 4 °C.

The seed culture of *P. pulmonarius* was prepared in 150-mL Erlenmeyer flasks that contained 45 mL of brown-sugar:rice-bran:malt:yeast extract medium (BRMY) previously optimised. BRMY consists of g L^{-1} of brown sugar (20), rice bran (4), malt extract (4), and yeast extract (4). The medium was then autoclaved at 121 °C and 15 psi for 20 min and was inoculated with 5 mL of *P. pulmonarius* mycelium suspended in sterile water. The mycelial suspension was prepared by scrapping the surface of five-day old mycelium of *P. pulmonarius* grown on BRMY slants. Seed culture of *P. pulmonarius* was incubated at 25 ± 1 °C shaken at 250 rpm for 5 days. Then 10% (v/v) of seed culture was inoculated into the bioreactor.

2.2. Cultivation of P. pulmonarius mycelium in a bioreactor

The fermentation was carried out using a 2-L stirred-tank bioreactor (Biostat B+ Sartorius, BBI). The previously optimised cultivation conditions used are as follows: cultivation temperature of 28 °C, agitation speed of 250 rpm, initial pH of 5.5, and oxygen partial pressure of 30–40%. The pH of the fermentation medium was controlled using automatic pH control, whereby either 0.5 M of NaOH or 0.5 M of H_2SO_4 was added when the pH deviated from a pre-set value of 5.5. The temperature was maintained by circulating cold water through a water jacket surrounding the bioreactor. A thermostated circulator bath was used to control the temperature of cooling water with dead band of ±0.5 °C. A variable speed motor powered the stirrer. The dissolved oxygen was monitored using an oxygen partial pressure probe, which was connected to a computer system for data acquisition. The pre-set oxygen partial pressure was maintained using O_2-enrichment cascade mode. The batch cultivation of *P. pulmonarius* was carried out for four days and the mycelial biomass, reducing sugar concentration and pellet morphology were observed everyday. Three batches of fermentation were done and data were represented as average.

2.3. Cultivation profiles of P. pulmonarius in an automated bioreactor

Sampling of the growth media (10 mL) in the bioreactor was done at regular time interval. Mycelium was separated from the broth by centrifugation at 6300 g for 10 min. After repeated washing with distilled water, mycelial pellets were dried at 70 °C until constant weight was achieved. Reducing sugar concentrations in the supernatant was determined by the dinitrosalicylic acid (DNS) method (Miller, 1959) and expressed as glucose equivalents. Samples of fresh pellets in growth medium were placed on glass slides and photographed for determination of pellet sizes and morphological observation under the microscope. The diameter sizes of 50 mycelial pellets were measured and averaged. Results from the three batches of fermentation were presented as average ± SEM.

2.4. Mycelium colonisation rate and sporophores yield of P. pulmonarius liquid spawn on rubber wood sawdust

Liquid spawn of *P. pulmonarius* (10 mL) harvested from the bioreactor was inoculated onto sterile fruiting substrate in plastic bags after autoclaving for 1 h at 121 °C. The fruiting substrate dry weight per bag was 270 g and the composition consists of sawdust 89% (w/w), rice bran 10% (w/w) and $CaCO_3$ 1% (w/w). The moisture content of the substrate was adjusted to 70% using distilled water. Twenty replicate bags of diameter 10 cm and 21 cm height were inoculated on top and incubated at room temperature (28–32 °C) in the mushroom house for mycelial growth. Mycelia growth extension was recorded at regular intervals for a period of one month. At the end of mycelial run, the bags were cut on the sides and subjected to an environmental temperature of 25 °C, relative humidity of 90–95% and good ventilation to initiate sporophore formation. Harvesting was done as sporophore matured and was done over a period of three weeks. The yield of sporophores was expressed as biological efficiency and was caclculated as below. The mycelial growth of liquid spawn and sporophore yield were compared with grain spawn. Grain spawn was prepared using 200 g of wheat grains pre-soaked overnight and then autoclaved for 1 h at 121 °C. Then mycelial discs of *P. pulmonarius* were inoculated and incubated for 7 days. Ten grams of mycelial colonised grain spawn was inoculated into sterile fruiting substrate in bags as above.

$$\text{Percentage biological efficiency (BE)} = \frac{\text{Grams of fresh sporophore produced}}{\text{Grams of dry substrate used}}$$

2.5. Statistical analysis

All statistical analysis were subjected to a one way analysis of variance (ANOVA) and the significant of difference between means was determined by Duncan's multiple range test and *t*-test at 95% least significant difference ($p < 0.05$).

3. Results

3.1. Production of P. pulmonarius liquid spawn in an automated bioreactor

Three batches of cultivation of *P. pulmonarius* were carried out for four days and at intervals of 24 h, samples were analysed for dry biomass production, reducing sugar concentrations, and the maximum pellet size (Table 1). The highest biomass production was obtained on the third day of fermentation yielding 11.72 ± 5.26 g L^{-1} dry weights followed by a decrease on the fourth day of fermentation. The reducing sugar concentration in the medium decreased sharply after 24 h of fermentation followed by a further gradual decrease until the fourth day with the reducing sugar concentration of 0.29 ± 0.01 g L^{-1}. The pellet size of *P. pulmonarius* after 24 h of fermentation was increased attaining a maximum pellet diameter of 2.50 ± 0.25 mm on day 3 of fermentation. This was followed by a decreased in pellet diameter on day 4 at 2.20 ± 0.17 mm. Hence, the time taken to achieve maximum pellet size coincides with the maximum dry mycelial biomass production (Table 1). Therefore, day 3 was selected as the optimum time to harvest the mycelium for inoculating the bags. The morphology of the pellets on day 3 was fluffy, hairy, and circular shape (Fig. 1).