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Processing characteristics of submerged fermentation of *Antrodia cinnamomea* in airlift bioreactor

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ABSTRACT

Three 5-L airlift bioreactors including airlift reactor with solid draft tube (ALs), airlift reactor with net draft tube (ALn) and bubble column reactor (BC) were investigated for their suitability for cultivating *Antrodia cinnamomea*, and a stirred tank reactor (ST) was used for comparison. Results indicated that after 7 days fermentation, ALs yielded the highest mycelium content (313 mg/100 mL) and had the lowest dissolved oxygen in the broth. Among different aeration rates (0.025, 0.05, 0.1, 0.5, 1 vvm) used during cultivation of *A. cinnamomea* in ALs, the aeration rate 0.1 vvm resulted in a volumetric oxygen transfer coefficient of 10.8 h^{-1} and produced the highest mycelium content. When the optimal conditions were used for the fermentation of *A. cinnamomea* in an industrial 500-L ALs, the mycelium content in the broth reached 542 mg/100 mL in 28 days. The IC_{50} values of the ethanol extracts of *A. cinnamomea* mycelium cultivated in 5-L and 500-L ALs for 28 days were 23 and 17 μg/mL, respectively, for hepatocellular carcinoma cells HepG2. And after 42 days cultivation in 500-L ALs, the IC_{50} value of the mycelium ethanol extract was reduced to 10 μg/mL.

1. Introduction

The fungus *Antrodia cinnamomea*, a native species in Taiwan, forms fruit body naturally on *Cinnamomum kanehirai* Hay. The fruit body of this fungus is generally recognized as a precious traditional medicine and functional food materials, but it grows very slow and its market price is very high. In fact, its recent market value reaches approximately twenty thousand US dollar per kilogram. Therefore, the commercial practice nowadays for cultivating this fungus often adopts submerged fermentation to produce mycelium of *A. cinnamomea*. The mycelium of *A. cinnamomea* is also recognized as a health promoting product. A wide range of biological functions of the mycelium of *A. cinnamomea* are recognized, including anti-oxidation [1–3], anti-inflammatory [4–6], antitumor [7–13], hepatoprotective [14,15], vasorelaxative [16] and anti-hepatitis B virus [17]. A total of 78 compounds, including terpenoids, benzenoids, lignans and benzoquinone derivatives have been identified in the mycelium of *A. cinnamomea*. Among terpenoids group, the triterpenoids are considered as the most biologically active components [18].

For microbial cultivation, the fermentation environment and conditions provided by the bioreactor need to fit specific needs of the microorganisms to get high productivity of the desired products. The most critical function that a fermentor should provide is to satisfy the need of gas–liquid mass transfer. The common feature of airlift bioreactors is the gas supply at the bottom of the reactor, which creates a density difference in the liquid and causes circulation, and helps to promote mass transfer in the bioreactor. The advantages of the airlift bioreactor include no moving parts, low power consumption, high mass and heat transfer rates, good solids suspension, homogeneous shear and rapid mixing [19]. Airlift bioreactors could be used as fermentors in many biochemical processes, such as aerobic fermentation for the production of enzymes, antibiotics, proteins, biomass and other biotechnology products. For instance, an external-loop airlift bioreactor was used to produce α-amylase by *Bacillus subtilis*, and found that the α-amylase activity of the fermentation broth (432.3 U/mL) was higher than that obtained in a mechanically stirred tank bioreactor (397.2 U/mL) under optimized operating conditions [20]. An aeration strategy was proposed for cultivation of *Bacillus thuringiensis* for thuringiensin production in an airlift reactor with double wire mesh draft tubes. Based on the proposed strategy, the thuringiensin production was 70% higher than that of using the bubble column bioreactor with addition of antifoam agents for foam control [21].

In the previous study [22] we investigated the effect of elicitation on triterpenoids production of *A. cinnamomea* and found that airlift bioreactor with dual-net draft tube had higher k_La value than that with solid draft tube. But there was a technical problem associated with the net draft tube, i.e. the mycelium

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would be trapped by the net that made biomass recovery difficult. Therefore, we decided to carry out a thorough study for determining the best type of bioreactor which should be used for culturing *A. cinnamomea*. The 5-L bioreactors tested in this study were bubble column reactor (BC), airlift reactor with solid draft tube (ALs) and airlift reactor with net draft tube (ALn), and the stirred tank reactor (ST) was used for comparison. Once the optimal fermentation conditions and the type of airlift bioreactor for cultivating *A. cinnamomea* were determined, the process was tested on industrial 500-L ALs to investigate the feasibility of scaling-up the process. Finally, the inhibitory effect of the ethanol extract of *A. cinnamomea* mycelium on HepG2 cells was examined to confirm the bioactivity of the fermentation product. Therefore, the overall objective of this study was to determine the best type of airlift bioreactor for the fermentation of *A. cinnamomea* and to understand the processing characteristics during fermentation.

2. Materials and methods

2.1. Microorganism and reagents

Antrodia cinnamomea BCRC35716 was obtained from the Bioresoures Collection and Research Center (BCRC) in Food Industry Research and Development Institute (Hsinchu, Taiwan). The culture was maintained on 39 g/L potato dextrose agar at 25 °C and transferred to a fresh agar plate every month. To prepare the inoculum, the mycelium of *A. cinnamomea* was transferred from Petri dish to 250 mL flask containing 100 mL medium (component: glucose 2.0%, malt extract 2.0% and peptone 0.1%) and incubated at 25 °C for 7 days for mycelium growth.

Potato dextrose agar (PDA), malt extract and peptone were obtained from Difco (Sparks, MD, USA). Sodium hydrogen carbonate ($NaHCO_3$) was provided by Merck Chemical (USA). Trypsin, Dulbecco's Modified Eagle's Medium (DMEM) and fetal calf serum (FCS) were from Hyclone (Logan, UT, USA). Dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT), and other reagents used in the study were from Sigma Chemical (St. Louis, MO, USA).

2.2. Submerged fermentation in 5-L and 500-L airlift reactors

The 5-L airlift bioreactors (Firstek, Taiwan) including bubble column reactor (BC), airlift reactor with solid draft tube (ALs), airlift reactor with net draft tube (ALn) and stirred tank reactor (ST) were investigated. The ALs with 12.8 cm in diameter and 46 cm in height contained a solid or net draft tube with 8 cm in diameter and 25 cm in height. The mesh number of the net draft tube was 24. Without the draft tube, the reactor becomes the BC. The ST was 16 cm in diameter and 36 cm in height. The medium used for cultivating *A. cinnamomea* is composed of glucose 2.0%, malt extract 2.0% and peptone 0.1%. The pH of the medium was adjusted to 5 by adding 0.1 N NaOH or 0.1 N HCl, and then sterilized at 121 °C for 20 min. The fermentation was operated with 1% inoculum, and the fermentation temperature was at 25 °C based on the previous study [23]. The aeration rates including 0.025, 0.05, 0.1, 0.5 and 1.0 vvm were investigated to find out the optimal fermentation conditions for yielding the maximum amount of mycelium concentration.

The 500-L airlift reactor used in this study was 76 cm in diameter and 162 cm in height, and equipped with a solid draft tube which was 30 cm in diameter and 85 cm in height. The fermentor was charged with 400 L of medium consisting of glucose 2.0%, malt extract 2.0% and peptone 0.1%. The fermentation conditions were 1% inoculum, initial pH 5, 25 °C and aeration rate 0.1 vvm.

2.3. Experimental determination of k_La

Oxygen transfer from gas bubble to microorganism is usually limited by oxygen transfer through the liquid film surrounding the gas bubbles. The oxygen transfer rate (OTR, mg O_2/l h) from gas bubbles to liquid phase is given by

$$\mathrm{OTR} = \frac{dC_L}{dt} = k_La(C_L^* - C_L) \quad (1)$$

where k_L is the oxygen transfer coefficient (cm/h), a is the gas–liquid interfacial area (cm^2/cm^3), k_La is the volumetric oxygen mass transfer coefficient (h^{-1}), C_L^* is the saturated dissolved oxygen concentration in liquid phase at gas–liquid interface (mg/L), C_L is the actual dissolved oxygen concentration in the liquid phase (mg/L).

Eq. (1) can be integrated from time 0 to time t, and obtains:

$$\ln\left(\frac{C^* - C_L}{C^* - C_{L0}}\right) = -k_La \cdot t \quad (2)$$

where C_{L0} is the initial dissolved oxygen concentration in the liquid phase (mg/L). The k_La was determined by the gassing-out method as described by Shuler and Kargi [24]. Firstly, oxygen is removed from the system by sparging with nitrogen gas, air is then introduced and the change in dissolved oxygen (DO) is monitored until the solution is nearly saturated. Thus, k_La can be determined from the slope of $\ln(C^* - C_L/C^* - C_{L0})$ versus t. The k_La values were measured using the medium as liquid phase in various bioreactors.

2.4. Biomass determination and preparation of ethanol extract of mycelium

The concentration of mycelium in the fermentation broth was determined by filtering the broth through Whatman No.1 filter paper and washed twice with distilled water. Mycelium was vacuum dried at 50 °C until a constant weight obtained.

The ethanol extract of mycelium (EEM) was obtained by extracting vacuum-dry mycelium (0.5 g) with 95% ethanol (100 mL) at 25 °C for 48 h. The extract was filtered through Whatman No.1 filter paper, and then vacuum dried at 50 °C to obtain EEM.

2.5. Cell viability assay

The human hepatocellular carcinoma cell line (HepG2) was obtained from BCRC in Food Industry Research and Development Institute (Hsinchu, Taiwan). The cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1% penicillin–streptomycin in a humidified incubator (5% CO_2 in air at 37 °C). Cells were seeded in 96 well plates prior to EEM addition. The HepG2 cells were incubated with EEM at various concentrations for 72 h.

The tetrazolium dye colorimetric test (MTT test) was used to monitor cell growth as indicated by the conversion of the tetrazolium salt to formazan, a colored product. Briefly, the HepG2 cell numbers were counted by hemocytometer and seeded in 96-well microplates (1×10^4 cells/well in 200 μL of complete DMEM medium) for 24 h. Cells were then washed with PBS and incubated with EEM at various concentrations for 72 h. The medium was removed at the end of incubation, then, 100 μL of 2 mg/mL MTT was added to each well, and incubation was allowed to continue for 2 h. Finally, 100 μL of DMSO was added to each well and incubated for 15 min. The plate was read by a UV/vis Spectrophotometer (Jasco Model 7800, Japan) at a wavelength of 570 nm. The cell viability was calculated by dividing the absorbance of each experimental sample by the control sample, the medium. The sample concentration required to inhibit 50% cell growth (IC_{50}) was determined by interpolation from dose–response curves.