Large-scale submerged fermentation of *Antrodia cinnamomea* for anti-hepatoma activity

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Abstract

BACKGROUND: Submerged cultivation of *Antrodia cinnamomea* was carried out for manufacturing the fermentation product with anti-hepatoma activity. The fermentation process was optimized for different parameters at shake flask level to obtain products with high inhibition potency against Hep G2 hepatoma cells. Scale-up of the fermentation process was then achieved from 250 mL shake flask to 5 L, 500 L and 5-ton fermenters.

RESULTS: Glucose and malt extract were found to be the best carbon and nitrogen sources, respectively. The initial pH of 5.0 and an operating temperature of 22 °C were the best for a product with lowest IC_{50} value. A shorter cultivation time was required when the scale of fermentation increased from 5 L to 5 tons. The reducing sugar and solids contents of the broth filtrate were correlated exponentially with the IC_{50} of the ethanolic extract of mycelium for hepatoma cells, and the level of ergosterol in the mycelium extract follows the same profile as the increase in Hep G2 cells inhibition.

CONCLUSION: When *Antrodia cinnamomea* is cultured in a 5-ton fermenter, 4 weeks are required for the fermentation product to reach the highest anti-hepatoma activity. The solid and reducing sugar contents of the broth filtrate as well as the ergosterol content in the ethanol extract of mycelium can serve as the marker during fermentation for manufacturing product with anti-hepatoma activity.

Keywords: scale-up; submerged cultivation; *Antrodia cinnamomea*; anti-hepatoma activity; Hep G2 cells

INTRODUCTION

Antrodia cinnamomea, renamed from *Antrodia* camphorata,[1] is a fungus species native to Taiwan. Many studies have shown that *A. cinnamomea* possess various health-promoting activities including anti-inflammatory, antioxidant, anti-cancer and hepato-protective functions.[1–5] It is generally believed that steroids and triterpenoids may be the major components for the bioactivities of *A. cinnamomea.* Recent evidence, however, shows that *A. cinnamomea* also contains other active compounds such as polysaccharide in the mycelium. Polysaccharide from this fungus was found to exhibit anti-hepatitis B, anti-oxidative and immunomodulatory functions.[6]

Hepatocarcinoma is one of the most fatal cancers in Taiwan. This may be attributed to the fact that Taiwan is a hyperendemic area for hepatitis B virus infection, with approximately 16% of the general population infected with this virus.[7] In addition, chronic infection of Hepatitis B virus is a known risk factor of liver cancer. With such a high prevalence of liver cancer, people frequently use *A. cinnamomea* as an alternative medical treatment. Nowadays many *A. cinnamomea* products are being sold commercially.

A. cinnamomea is a parasitic fungus that lives on the wall of the inner cavity of *Cinnamonum kanehira*. The fruiting body of *A. cinnamomea* is very expensive because it has a high medicinal value and also because of its slow growth rate. In view of this, submerged cultivation has been adopted to meet the consumption demands of this medicinal fungus.[8] Yang *et al*. (2003) cultured *A. cinnamomea* using submerged fermentation with different carbon sources (glucose, sucrose, potato starch, corn powder) and nitrogen sources (yeast extract, YM broth, malt extract).[9] They found that fermentation with corn powder as carbon source and YM broth as nitrogen source yielded the maximum amount of mycelium. Song *et al*. cultured *A. cinnamomea* in a 500 L fermenter at 27–30 °C for 7 days, and found that the filtrate of the fermentation broth had no inhibitory effect on human hepatoma cells, but the methanolic extract of mycelium had an anti-hepatomatic effect.[3,10] Shu (2004) studied the effect of pH on the production and molecular weight distribution of exopolysaccharide produced by

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A. cinnamomea during fermentation, and found that the exopolysaccharide yielded at pH 3.0 had a higher molecular weight (800 kDa) and pH 5.0 gave a higher exopolysaccharide yield (5.05 m g^{-1}).[11] Huang (2002) cultivated a new strain of *A. cinnamomea* and evaluated the effect of the broth filtrate on different cancer cells.[12] Through 2 weeks of cultivation, they claimed that the broth displayed a reddish color and exhibited inhibition of eight cell lines, including Hela, ES2, Hep 3B, MCF7, AGS, COLO 205, COLO 320HSR and Caco-2 to various extents.

Although the health-promoting and disease preventive functions of this medicinal fungus are widely recognized, the biochemical process and components responsible for its bioactivity are still under investigation. Particularly, the relationship between fermentation process and the bioactivity of the fermentation products is still not clear. Thus the objectives of this study were to investigate the effect of fermentation conditions on the anti-hepatomatic activity of the products, and to identify the marker which can be used to monitor the process during fermentation of the *A. cinnamomea* on a commercial scale.

MATERIALS AND METHODS

Materials

Malt extract and peptone were purchased from Difico (Sparks, MD, USA). Ethyl acetate, dimethyl sulfoxide (DMSO), glucose, galactose, fructose, sucrose, lactose, 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT), and other reagents used in the study were purchased from Sigma Chemical (St Louis, MO, USA). Trpysin, Dulbecco's Modified Eagle's Medium (DMEM) and fetal calf serum (FCS) were purchased from Hyclone (Logan, UT, USA).

Organism and inoculum

Antrodia cinnamomea (BCRC35716) was obtained from the Bioresources Collection and Research Center (BCRC) in Food Industry Research and Development Institute (Hsinchu, Taiwan). The culture was maintained on potato dextrose agar (PDA) slopes. Slopes were inoculated and incubated at 25 °C for 4 weeks and stored at 4 °C. To prepare the inoculum, the mycelium of *A. cinnamomea* was transferred to a Petri dish containing PDA medium and incubated at 25 °C for 4 weeks. Mycelia agar discs (0.5 cm) were obtained using a self-designed cutter and used as inocula to cultivate the fungus in a shake flask.

Preparation of liquid cultures of *A. cinnamomea*

Colonies of *A. cinnamomea* were observed under a light microscope to ensure that no contamination occurred. The agar covered with fungal mycelia was cut into small pieces and then homogenized aseptically with liquid medium (2% glucose, 2% malt extract and 0.1% peptone) for 30 s. The suspension was used as inoculum for submerged cultivation of *A. cinnamomea*.

Submerged shake flask culture

In order to discover the optimal cultivation conditions the partial medium replacement method was used. The initial cultivation medium, composed of 2% malt extract and 2% glucose with pH adjusted to 5, was prepared in 250 mL flasks. For studying the influence of the carbon source, glucose was replaced with different carbon sources, including glucose, lactose, galactose, fructose and sucrose. Different nitrogen sources were also checked for optimal fermentation conditions, including malt extract, soy peptone, peptone, yeast extract and yeast malt extract. To investigate the effect of initial pH on fermentation, the culture medium was adjusted to various pH values from 2 to 9 using HCl and NaOH. The optimum temperature for the fermentation process was investigated by incubating the shake flasks at 22, 25 and 28 °C. Then, 5 mL homogenized *A. cinnamomea* inocula were added to each flask. The submerged cultures were incubated for 4 weeks with constant agitation at 100 rpm on an orbital rotary shaker.

Scale-up of submerged fermentation

The 5 L fermenter was inoculated with 50 mL of *A. cinnamomea* cultivated in a 250 mL flask for 2 weeks. The 500 L fermenter was inoculated with 1 L of *A. cinnamomea* cultivated in a 2 L flask for 2 weeks. Finally, the 5-ton fermenter was inoculated with 400 L of inoculum cultivated in 500 L fermenter for 2 weeks. The fermentation conditions were glucose 2%, malt extract 2%, initial pH 5, and cultured at 22 °C, 50 rpm agitation, and an aeration of 0.5 vvm.

The dimensions of the 5 L, 500 L and 5-ton fermenters were 16 cm × 36 cm (inside diameter × height), 75 cm × 150 cm and 130 cm × 366 cm, respectively. During fermentation, the working volume was approximately 80% of the total fermenter volume.

Fermentation broth processing after cultivation

At the end of the fermentation process, the fermentation broth was passed through Whatman No. 1 filter paper (for shake flask and 5 L fermenter) or centrifuged using a decanter to separate the mycelium from the broth filtrate. The pellets of mycelium were then washed twice with 50 mL distilled water to remove the broth adsorbed on the pellets. Both the mycelium and broth filtrate were freeze dried and stored at 4 °C for further use.

To prepare the ethanolic extract of the mycelium, 1 g of dry mycelium and 50 mL of 95% ethanol were homogenized using a Polytron homogenizer (Kinematica, AG, Lucerne, Switzerland), and then agitated for 24 h to extract the ethanol-soluble components from the mycelium. The extract thus obtained was concentrated using a rotary evaporator and then stored in a refrigerator at −80 °C for component analyses and cancer cell viability test.

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Influence of *A. cinnamomea* products on viability of cancer cell lines

Tumor cell lines were purchased from the BCRC, including AGS from human stomach adenocarcinoma (BCRC 60 102), HeLa from human cervical epithelioid carcinoma (BCRC 60 005), MCF7 from human breast adenocarcinoma (BCRC 60 436), Colo 320 HSR from human colon adenocarcinoma (BCRC 60 109), Hep 3B from human hepatocellular carcinoma (BCRC 60 434) and Hep G2 from human hepatoblastoma (BCRC 60 177). These cell lines were maintained as stocks in DMEM supplemented with 10% fetal bovine serum. The cells were maintained in a humidified atmosphere containing 5% CO_2 at 37 °C and passaged twice each week using trypsin–EDTA to detach the cells from cell culture flasks.

Cell viability was studied by MTT assay. Tumor cells were harvested, counted and inoculated into a 96-well microtiter plate at the designated concentrations. The start-up concentration of the cells was 10 000 cells per well. The total volume of the cell culture medium in each well was 100 μL, and cells were maintained in a humidified atmosphere containing 5% CO_2 at 37 °C. On the second day the fermentation products, including the dry matter of filtrate and the ethanolic extract of the mycelium, were diluted 20–20 000 times with serum-free DMEM and 0.1% ethanol. The diluted solutions were filtered through a 0.22 μm filter; 200 μL of the diluted samples was then applied to the culture wells, and the resultant cultures were incubated for 3 days under the same conditions as mentioned above. Subsequently, the supernatant was removed from each well, and 100 μL MTT (2 mg mL^{-1} in phosphate-buffered saline) was added to each well. After an additional 2 h incubation at 37 °C, the supernatant was removed from each well and 100 μL of 100% DMSO was added to solubilize the MTT–formazan product. After thorough mixing with a mechanical plate mixer, the absorbance at 570 nm was measured with an ELSA reader (Tecan Spectra, Wetzlar, Germany). The tumor cell viability was calculated by dividing the absorbance of each experimental sample by the corresponding control sample (the medium). The IC_{50} value (the concentration of the sample which inhibits 50% of the cancer cells to proliferate or the concentration of the sample which results in 50% of cell viability) was calculated by linear regression between the sample concentration in DMEM and cell viability.

High-performance liquid chromatographic (HPLC) analysis

HPLC analysis was performed using an ICI 1100 system, composed of LC1100 pump (ICI Australia), ICI LC 1200 UV-visible detector, SFD S5200 autosampler (set at 20 μL) (Bad Honnef, Germany) and online degasser ERC 3415 (Tokyo, Japan). SISC (Scientific Information Service Corporation Inc., Taipei, Taiwan) chromatography software was used for data acquisition and integration. Separations were carried out with a Lichrospher 100RP (Darmspadt, Frankfurt, Germany) column and detected at UV 280 nm. The mobile phase was methanol. All samples were filtered through a 0.45 μm filter before analysis. To identify the ergosterol in the fermentation product, pure compound of ergosterol purchased from Sigma Co. (St Louis, MO, USA) was used.

Statistical analysis

Statistical analysis was performed using two-way ANOVA and Tukey's multiple comparison test (SAS Institute Inc., Cary, NC, USA) to determine significant differences among means ($P < 0.05$).

RESULTS AND DISCUSSION

Influence of *A. cinnamomea* products on various cancer cell lines

Different cancer cell lines including MCF7, Hela, AGS, COLO, Hep 3B and Hep G2 maintained in DMEM were tested for anti-tumor activity of the ethanolic extract of *A. cinnamomea* mycelium cultured in a shake flask for 8 weeks. The ethanolic extract was found to be most inhibitory to the hepatoma cell lines Hep 3B and Hep G2, showing the lowest IC_{50} of 30 μg mL^{-1} for both of them (Fig. 1). The IC_{50} for the other cell lines were 150, 250, 90 and 90 μg mL^{-1} for AGS, Hela, MCF7 and COLO 320 HSR, respectively. This result validated that the ethanolic extract of *A. cinnamomea* was more effective in inhibiting the liver cancer cells than other cancer cells, and inhibition of cell growth might be brought about by upregulation of Fas/FasL to activate the caspase-3 and caspase-8 cascades, thus inducing cell apoptosis.[13]

Effects of carbon and nitrogen sources

A. cinnamomea cultured in a shake flask was optimized for various growth conditions. Various carbon sources were used to check their influence on growth

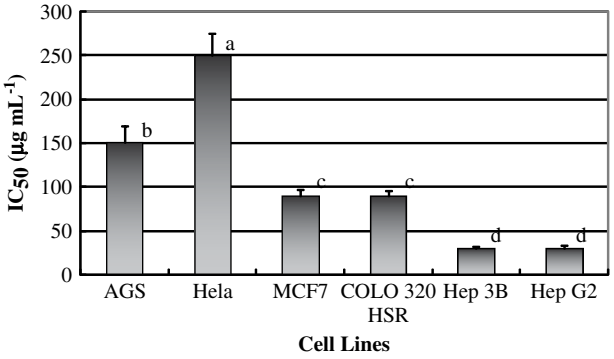

Figure 1. Effect of ethanolic extracts of *A. cinnamomea* mycelium cultured in a shake flask for 8 weeks on different cancer cells. AGS, human stomach adenocarcinoma (BCRC 60 102); HeLa, human cervical epithelioid carcinoma (BCRC 60 005); MCF7, human breast adenocarcinoma (BCRC 60 436); Colo 320 HSR, human colon adenocarcinoma (BCRC 60 109); Hep 3B, human hepatocellular carcinoma (BCRC 60 434); Hep G2, human hepatoblastoma (BCRC 60 177).

conditions of the fungus and IC_{50} values of the ethanolic extract of mycelium, as well as the broth filtrate obtained after submerged fermentation. It was found that glucose was the best carbon source for production of mycelium, with high inhibition potency towards Hep G2 cells (Table 1). In the case of broth filtrate, glucose, galactose and lactose were found to be the better carbon sources, giving a higher anti-Hep G2 activity (Table 2). The inhibitory activity of the broth filtrate might be attributed to secondary metabolites released from the mycelium such as triterpenoids and exopolysaccharide produced by *A. cinnamomea*.[8,14] Considering the cost of the medium, glucose was chosen for the subsequent studies.

Fermentation was performed with various nitrogen sources including malt extract, soy peptone, peptone, yeast extract and yeast malt extract. It was observed that with malt extract the highest Hep G2 cell inhibition was achieved both in case of mycelium extract and broth filtrate. Although the yield of mycelium for malt extract was lower than that for yeast extract and yeast malt extract, malt extract yielded the highest amount of dried product from the broth filtrate (Table 3). In view of the bioactivity as well as the yield of fermentation product, malt extract was chosen as the nitrogen source for submerged cultivation of *A. cinnamomea*.

Optimization of initial pH and temperature

Cultured medium adjusted to different pH was used for submerged cultivation of *A. cinnamomea* in a shake flask to optimize the initial pH for culture conditions. Maximum mycelium yield was obtained with an initial pH of 5.0, while the maximum broth filtrate dry weight was achieved with a pH of 2.0 (Table 4). These results were similar to those obtained by Shu *et al.*, who found that the initial pH of 5.0 gave the highest mycelium yield, though no biological activity of the mycelium was reported in this study.[11] The ethanolic extract of the mycelium grown at an initial pH value of 2.0 exhibited the maximum anti-hepatoma potency against Hep G2 cells. In the case of

Table 1. Effect of ethanolic extracts of mycelium of *A. cinnamomea* cultured with different carbon sources on Hep G2 cells

	Cell viability (%)				
Dosage ($\mu g\,mL^{-1}$)	Glucose	Galactose	Lactose	Sucrose	Fructose
1500	16.2 ± 1.8^{k}	21.0 ± 3.0^{jk}	37.1 ± 5.6^{ghijk}	42.4 ± 6.5^{ghij}	44.2 ± 5.0^{ghi}
1200	20.7 ± 2.3^{jk}	29.2 ± 4.1^{ijkh}	38.7 ± 5.8^{ghij}	49.6 ± 3.5^{fgh}	51.7 ± 5.3^{efgh}
900	21.0 ± 2.0^{jk}	37.6 ± 5.3^{ghijk}	71.8 ± 10.8^{cde}	87.5 ± 8.2^{abc}	81.5 ± 9.8^{bc}
600	25.5 ± 2.7^{ijk}	56.3 ± 7.9^{defg}	98.4 ± 14.8^{ab}	103.8 ± 12.5^{ab}	99.6 ± 11.7^{ab}
300	40.2 ± 4.1^{ghij}	70.2 ± 9.8^{cdef}	101.6 ± 15.2^{ab}	106.2 ± 11.6^{a}	101.3 ± 12.5^{ab}
150	74.2 ± 9.2^{dc}	106.9 ± 15.0^{a}	105.7 ± 15.9^{a}	107.4 ± 13.1^{a}	106.2 ± 10.4^{a}

Values followed by different letters are significantly different ($P < 0.05$) by Tukey's multiple comparison test ($n = 3$).

Table 2. Effect of broth filtrate of *A. cinnamomea* cultured with different carbon sources on Hep G2 cells

	Cell viability (%)				
Dosage ($\mu g\,mL^{-1}$)	Glucose	Galactose	Lactose	Sucrose	Fructose
1500	13.2 ± 1.7^{fg}	11.0 ± 2.4^{g}	12.0 ± 1.7^{g}	12.2 ± 1.6^{g}	15.0 ± 1.7^{fg}
1200	17.0 ± 1.8^{fg}	12.2 ± 3.1^{g}	13.3 ± 1.9^{fg}	11.3 ± 1.0^{g}	17.3 ± 1.0^{fg}
900	14.1 ± 2.1^{fg}	13.7 ± 3.2^{fg}	13.0 ± 1.8^{fg}	22.4 ± 2.8^{f}	35.6 ± 4.8^{e}
600	18.9 ± 2.2^{fg}	14.8 ± 3.8^{fg}	17.4 ± 2.4^{fg}	43.5 ± 5.8^{cde}	46.4 ± 8.4^{cde}
300	49.0 ± 3.2^{cd}	41.3 ± 6.0^{de}	42.5 ± 3.1^{cde}	75.2 ± 5.1^{b}	72.7 ± 9.3^{b}
150	65.6 ± 2.8^{b}	52.9 ± 11.1^{c}	45.0 ± 3.4^{cde}	109.0 ± 8.4^{a}	104.0 ± 7.4^{a}

Values followed by different letters are significantly different ($P < 0.05$) by Tukey's multiple comparison test ($n = 3$).

Table 3. Influence of nitrogen source on bioactivity and yield of *A. cinnamomea* fermentation products

Nitrogen source	Cell viability of mycelium ethanolic extract (%)[a]	Cell viability of broth filtrate (%)[a]	Dry weight of mycelium ($g\,100\,mL^{-1}$)	Dry weight of broth filtrate ($g\,100\,mL^{-1}$)
Malt extract	30.5 ± 2.8^{c}	31.1 ± 0.5^{e}	0.70 ± 0.01^{dc}	2.60 ± 0.03^{b}
Soy peptone	72.5 ± 5.6^{b}	62.7 ± 0.4^{d}	0.85 ± 0.02^{c}	0.82 ± 0.02^{c}
Peptone	68.5 ± 6.2^{b}	72.0 ± 1.0^{c}	0.65 ± 0.02^{d}	2.72 ± 0.04^{a}
Ye	86.4 ± 7.3^{a}	96.2 ± 1.3^{a}	1.26 ± 0.10^{a}	0.52 ± 0.01^{e}
YM	65.4 ± 6.2^{b}	76.3 ± 1.3^{b}	1.05 ± 0.08^{b}	0.67 ± 0.04^{d}

Values followed by different letters in the same column are significantly different ($P < 0.05$) by Tukey's multiple comparison test ($n = 3$).
[a] Hep G2 cells were treated with $400\,\mu g\,mL^{-1}$ *A. cinnamomea* fermentation product.

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Table 4. Influence of initial pH on bioactivity and yield of *A. cinnamomea* fermentation products

pH	Cell viability of mycelium ethanolic extract (%)[a]	Cell viability of broth filtrate (%)[a]	Dry weight of mycelium (g 100 mL^{-1})	Dry weight of broth filtrate (g 100 mL^{-1})
2	16.3 ± 2.6^{e}	43.2 ± 2.1^{c}	0.08 ± 0.01^{d}	3.21 ± 0.05^{a}
3	46.3 ± 2.5^{c}	42.9 ± 1.8^{c}	0.45 ± 0.02^{c}	2.87 ± 0.06^{b}
4	47.8 ± 2.8^{c}	39.6 ± 12.2^{cd}	0.53 ± 0.04^{b}	2.61 ± 0.08^{c}
5	28.6 ± 2.8^{d}	29.6 ± 2.5^{d}	0.69 ± 0.02^{a}	2.55 ± 0.04^{cd}
6	32.1 ± 3.2^{d}	30.6 ± 10.3^{d}	0.65 ± 0.03^{a}	2.42 ± 0.05^{de}
7	45.6 ± 1.5^{c}	36.5 ± 1.3^{cd}	0.55 ± 0.04^{b}	2.31 ± 0.08^{e}
8	62.4 ± 1.9^{b}	56.7 ± 2.5^{b}	0.41 ± 0.01^{c}	2.84 ± 0.05^{b}
9	68.7 ± 2.1^{a}	92.3 ± 8.2^{a}	0.42 ± 0.02^{c}	2.96 ± 0.07^{b}

Values followed by different letters in the same column are significantly different ($P < 0.05$) by Tukey's multiple comparison test ($n = 3$).
[a] Hep G2 cells were treated with 400 μg mL^{-1} *A. cinnamomea* fermentation product.

broth filtrate the initial pH of 5.0 gave the highest activity against Hep G2 cells (Table 4). Although the ethanolic extract of mycelium cultured at pH 2 had the highest anti-tumor activity, its mycelium content was also the lowest one. This result indicates that the active compounds are produced in extreme culture conditions – the lowest pH. However, since the mycelium yield was considerably reduced at such a low pH, pH 5.0 was used in subsequent studies.

A. cinnamomea has been submerge cultured at temperatures ranging from 25 to 30 °C.[9,14] However, the fruiting body of *A. cinnamomea* usually grows on the inner cavity of *Cinnamomum kanehirai* Hay (Lauraceae) on mountains at 450–1200 m above sea level at temperatures normally between 22 °C (evening) and 28 °C (daytime) in the summer. Therefore, the fermentation temperatures investigated in this study were 22, 25, and 28 °C. It was found that fermentation at 22 °C yields a product with a high inhibition value both in the case of filtrate broth as well as ethanolic extract of mycelium. Conversely, maximum yield was obtained at 25 °C for both mycelium ethanolic extract and filtrate broth (Table 5). We decided to use 22 °C as the fermentation temperature for the subsequent study because bioactivity was the primary concern.

Scale-up of submerged fermentation of *A. cinnamomea*

The fermentation process was scaled up in a stepwise manner. First the process was scaled up to 5 L fermentation with 50 mL inoculum broth, and the process was monitored by taking a small aliquot from the fermenter on a weekly basis. The pH, reducing sugar, mycelium content, solid content of the broth filtrate, and IC_{50} of the broth filtrate as well as the ethanolic extract of mycelium for Hep G2 cells were analyzed. In a similar manner, the fermentation process was scaled up to 500 L inoculated with 2 L inoculum. Finally, the process was scaled up to 5 tons using 400 L inoculum. It was observed that the fermentation carried out in the 5 L fermenter took 4 weeks to reach stationary phase, as indicated by the mycelium content. The reducing sugar and solid content of the broth filtrate decreased continuously with decreases in IC_{50} for both broth filtrate and ethanolic extract of mycelium (Table 6). When the IC_{50} of the ethanolic extract of mycelium is correlated with the contents of reducing sugar and total solids, a significant exponential relationship is found, with coefficients of determination (R^2) of approximately 0.9 (Fig. 2). It appears that both the solid content and reducing sugar content of the broth filtrate can serve as the marker during fermentation of *A. cinnamomea* for manufacturing product with anti-hepatoma activity. Since the cultivation of *A. cinnamomea* in the 5 L fermenter reached stationary phase in 4 weeks and the IC_{50} of the mycelium extract also reached its lowest point at almost the same time, we decided to limit the cultivation time to 4 weeks when scaling up the process using commercial scale fermenters. However, it is worth noting that while the IC_{50} of the mycelium extract remained the same after 4 weeks of fermentation, the IC_{50} of the broth filtrate decreased continuously as the fermentation time was prolonged. It was suspected that the bioactive metabolites started to be released from the mycelium to the broth after the fungal culture reached stationary phase.

When the process was scaled up to 500 L and then to the 5-ton fermenter, it was found that the culture

Table 5. Influence of temperature on bioactivity and productivity of *A. cinnamomea* fermentation products

Temperature	Cell viability of mycelium ethanolic extract (%)[a]	Cell viability of broth filtrate (%)[a]	Dry weight of mycelium (g 100 mL^{-1})	Dry weight of broth filtrate (g 100 mL^{-1})
22	26.8 ± 2.7^{a}	30.2 ± 2.5^{b}	0.71 ± 0.01^{b}	2.70 ± 0.04^{c}
25	28.2 ± 3.5^{a}	35.6 ± 2.3^{a}	0.80 ± 0.03^{a}	3.50 ± 0.03^{a}
28	29.4 ± 2.8^{a}	36.8 ± 3.2^{a}	0.82 ± 0.02^{a}	2.80 ± 0.02^{b}

Values followed by different letters in the same column are significantly different ($P < 0.05$) by Tukey's multiple comparison test ($n = 3$).
[a] Hep G2 cells were treated with 400 μg mL^{-1} *A. cinnamomea* fermentation product.

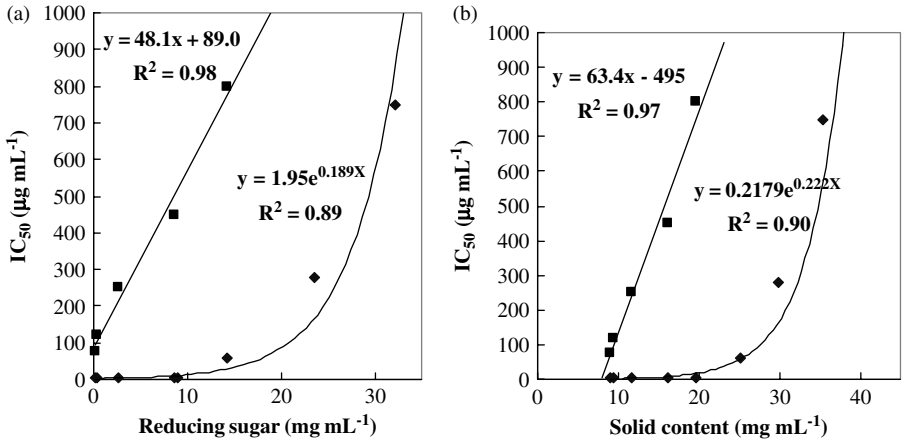

Figure 2. Relationships between IC_{50} of the ethanolic extracts of mycelium and broth filtrate *versus* the reducing sugar content (a) as well as the solid content (b) of the filtrate of the fermentation broth of *A. cinnamomea*. ■, IC_{50} of broth filtrate; ♦, IC_{50} of ethanolic extracts of mycelium.

Table 6. Influence of cultivation time on mycelium content, pH, reducing sugar, IC_{50} of broth and mycelium ethanolic extract in 5 L fermenter

Weeks	PH	Reducing sugar (mg mL^{-1})	Mycelium content (mg 100 mL^{-1})	Solid content of broth filtrate (g 100 mL^{-1})	IC_{50} of broth filtrate (μg mL^{-1})	IC_{50} of mycelium ethanolic extract (μg mL^{-1})
0	4.86^{a}	34.1^{a}	0	4.06^{a}	>1000	–
1	4.50^{b}	33.6^{a}	0.07^{e}	3.53^{b}	>1000	750^{a}
2	3.19^{c}	32.1^{b}	0.18^{d}	2.98^{c}	>1000	280^{b}
3	3.05^{cd}	23.5^{c}	0.24^{c}	2.52^{d}	>1000	60^{c}
4	2.95^{de}	14.2^{d}	0.35^{ab}	1.96^{e}	800^{a}	3.8^{c}
5	2.90^{de}	8.7^{e}	0.35^{ab}	1.62^{f}	450^{b}	4.2^{c}
6	2.85^{e}	2.6^{f}	0.36^{a}	1.16^{g}	250^{c}	3.5^{c}
7	2.85^{e}	0.4^{g}	0.35^{ab}	0.95^{h}	120^{d}	2.8^{c}
8	2.85^{e}	0.1^{g}	0.32^{b}	0.89^{h}	75^{d}	2.9^{c}

Values followed by different letters in the same column are significantly different ($P < 0.05$) by Tukey's multiple comparison test ($n = 3$).

Table 7. Performance of fermentation of *A. cinnamomea* in a 500 L fermenter

Weeks	pH	Reducing sugar (mg mL^{-1})	Mycelium content (mg 100 mL^{-1})	Solid content of broth filtrate (g 100 mL^{-1})	IC_{50} of broth filtrate (μg mL^{-1})	IC_{50} of mycelium ethanolic extract (μg mL^{-1})
0	4.33	34.0	–	4.05	>1000	>1000
1	3.20	17.6	0.14	2.09	950	235
2	3.13	0.26	0.41	1.37	555	85.0
3	3.04	0.09	0.54	0.76	390	8.75
4	2.97	0.06	0.57	0.67	250	3.25

also reached its stationary phase in 3–4 weeks, but the mycelium content in the larger-scale fermenters appeared to be higher than that in the 5 L fermenter (Tables 7 and 8). In addition, although the IC_{50} of the ethanolic extract of the mycelium from large-scale fermenters was basically the same as that from the laboratory fermenter after 4 weeks of fermentation, the broth filtrate from the large-scale fermenter appeared to have higher anti-hepatoma activity (lower IC_{50}) than that from the 5 L laboratory fermenter. It is very difficult to predict performance when the scale of fermentation changes. In this study, the surface:diameter ratio of the fermenters decreased dramatically during scale-up, which would reduce the surface aeration and might have an adverse effect on the growth of fungus. However, the oxygen supply by surface aeration in the large-scale fermenter might not be as important as the contribution by sparging. It was suspected that effective sparging and gas dispersion (by impeller) might have compensated for the insufficient surface aeration in large-scale fermenters. Nonetheless, this study has demonstrated that the fermentation of *A. cinnamomea* can be carried out in commercial fermenters with similar or even better performance than the laboratory fermenter.

HPLC analysis

HPLC analyses were performed on mycelium extracts and broth filtrates every week from the 5-ton fermenter, and the resulting profiles are given in Figs 3 and 4. It was observed that in the mycelium extract a peak at retention time 7.8 min – ergosterol – appeared

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Table 8. Performance of fermentation of *A. cinnamomea* in a 5-ton fermenter

Weeks	pH	Reducing sugar (mg mL^{-1})	Mycelium content (mg 100 mL^{-1})	Solid content of broth filtrate (g 100 mL^{-1})	IC_{50} of broth filtrate (μg mL^{-1})	IC_{50} of mycelium ethanolic extract (μg mL^{-1})
0	4.88	34.1	–	4.02	>1000	>1000
1	3.14	29.3	0.11	2.98	515	185
2	2.99	18.8	0.50	1.58	375	72.5
3	2.94	5.70	0.63	0.77	335	27.5
4	2.88	0.85	0.68	0.70	290	4.25

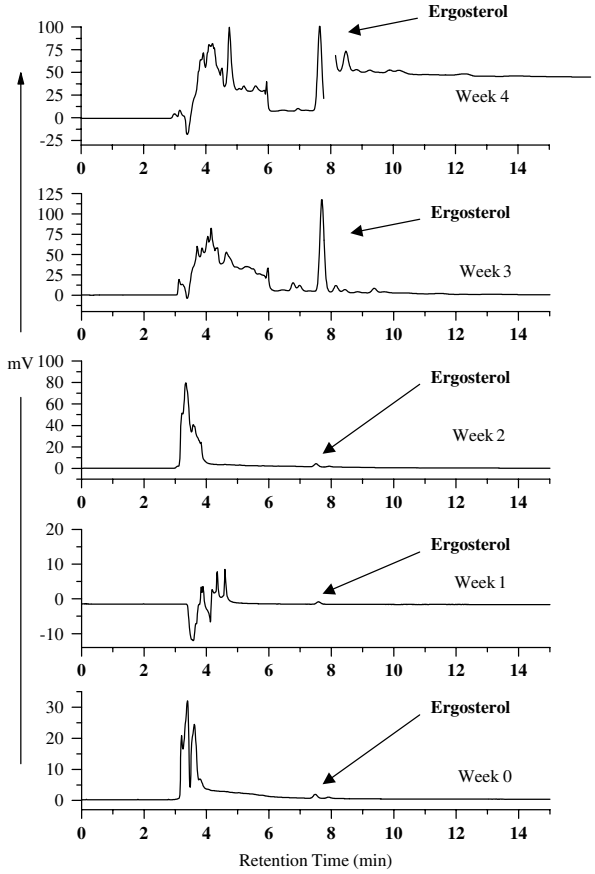

Figure 3. HPLC chromatograms of *A. cinnamomea* mycelium extract from 5-ton fermenter at different weeks.

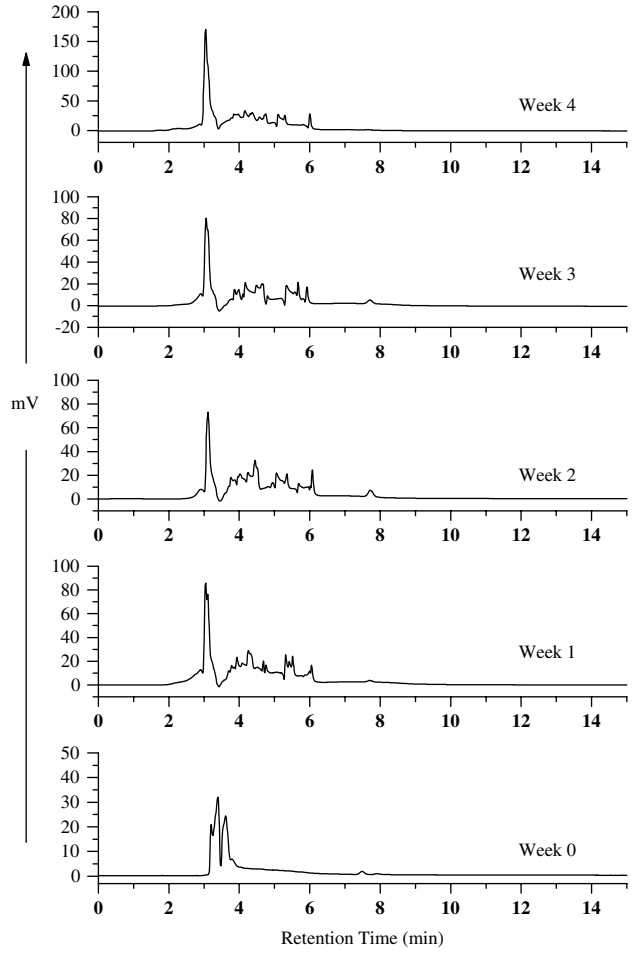

Figure 4. HPLC chromatograms of *A. cinnamomea* broth filtrate from 5-ton fermenter at different weeks.

after 3 weeks' fermentation (Fig. 3), but this characteristic peak was not found in the broth filtrate (Fig. 4). Ergosterol is the predominant sterol found in the membrane of most fungi.[15] Since the level of ergosterol in the mycelium extract follows the same profile as the increase in Hep G2 cell inhibition, it may also serve as a marker for fermentation product with high anti-hepatoma potency.

CONCLUSION

The process parameters for submerged cultivation of *A. cinnamomea*, including carbon source, nitrogen source, initial pH and temperature, were optimized to obtain mycelium and broth filtrate having the lowest IC_{50} values for anti-hepatoma activity. The fermentation process was then scaled up to the 5-ton fermenter. It was found that the fungal culture reached stationary phase in 3 weeks, while the lowest IC_{50} value both in the case of broth filtrate and mycelium extract was achieved in 4 weeks. Both the reducing sugar and solid content of the broth filtrate decreased continuously with the decreases in IC_{50} for both broth filtrate as well as ethanolic extract of mycelium, and significant exponential relationships were found between the IC_{50} of the ethanolic extract of mycelium and the contents of reducing sugar and total solids. Therefore, both the solid content and reducing sugar content of the broth filtrate can serve as the marker during fermentation of *A. cinnamomea* for manufacturing products with anti-hepatoma activity. In addition, HPLC analysis of the mycelium extract indicates that ergosterol can also serve as a marker for the fermentation of *A. cinnamomea* with high

anti-hepatoma potency. However, further studies are needed to fractionate and identify the bioactive metabolites responsible for anti-hepatoma activity of the fermentation products.

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