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Enhancement of Triterpenoids Production of *Antrodia cinnamomea* by Co-Culture with *Saccharomyces cerevisiae*

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Abstract

Antrodia cinnamomea (AC) has attracted much attention recently due to its pharmacological benefits, especially for anticancer, and immunomodulation effects. Triterpenoids are the major bioactive compounds present in AC, and responsible for cancer curing ability. Oxygen supply to fermentation is a crucial environmental parameter affecting triterpenoids production. Thus, 29.6 mg/g biomass dry weight (DW) of triterpenoids was obtained by static operation (limited oxygen supply) in the second stage of monoculture of AC. In this paper, we propose a novel approach to enhancing triterpenoids production of AC through two-stage fermentation by co-culture with *Saccharomyces cerevisiae* in the second stage under limited oxygen supply. As a result, 38.0 mg/g DW of triterpenoids was obtained in 250 ml shake flask. Triterpenoid content was further enhanced as 46.5 mg/g DW by increasing the inoculation level of *S. cerevisiae* to 25%. While a scale-up study of the novel fermentation strategy was executed in a 2 L airlift bioreactor, 62.2 mg/g DW of triterpenoids was achieved, which was 110% and 64% enhancement as compared to those of monoculture of AC in the flask and airlift bioreactor, respectively. This role of *S. cerevisiae* and its elicitors on triterpenoids production of AC were also investigated in this study.

Keywords: *Antrodia cinnamomea; Saccharomyces cerevisae;* Co-culture; Triterpenoids; Two-stage fermentation; Elicitors

Introduction

Antrodia cinnamomea [AC] is a medicinal mushroom endemic to Taiwan, locally popular with the name "Niu-Chang-ku", and is widely used as a traditional medicine. AC grows on the inner cavity wall of *Cinnamomum kanehirai* as its natural habitat [1-3]. AC is commonly used as remedy for food, alcohol, and drug intoxication, diarrhea, abdominal pain, hypertension, skin itching, and cancer [2,4,5]. In several Asian and European countries, AC is consumed as a dietary supplement for cancer prevention, and hepatoprotection from ancient period [6]. The phytochemical investigation of the fruiting bodies, spores, and mycelia of AC has led to isolation, and characterization of a series of sesquisterone lactones, polysaccharides, steroids, and terpenoids. Many of these compounds have been evaluated for biological activity [7-10]. The extracts of AC have different biological functions including anti-oxidation [11-13], anti-inflammotary [14-16], anti-tumor [17-23], hepatoprotective [24,25], vasorelaxative [26], and anti-hepatitis B virus surface antigen [27].

Among the terpenoids, the triterpenoids are considered as the most biologically active components in *A. cinnamomea*, responsible for its pharmacological and therapeutic efficacy ingniting main research focus on this group of compounds isolation, identification, application, and enhanced production [28]. Five new ergostane-type triterpenoids, antcin A, antcin B, antcin C, zhankuic acid B, and zhankuic acid C from the fruting bodies of AC were identified in 1995 [4,10]. Preliminary pharamacological studies found that antcin A, and antcin C exhibited anti-inflammatory activity, and cytotoxicity against various cancer cell lines like P388 murine leukaemia. Zhankuic acid B showed anticholinergic and antiserotonergic activities on a guinea pig ileum preparation of 10 pg/mL. Zhankuic acids A (antcin B), and C showed cytotoxicity against P388 murine leukemia cells with an IC50 value of 1.8, and 5.4 μg/mL, respectively [9,10]. Cherng et al. and Dai et al. reported four new ergostane-type triterpenoids isolated from the fruiting body of AC [8,29]. It was reported that both the mycelium and sporocarp of AC provide protection against acute liver damage induced by ethanol. Owing to its perceived health benefits, AC has gained wide popularity as a health food, and became the most valuable mushroom in Taiwan [30]. In fact, the current market price has skyrocketed to 15000 USD/kg [1].

Increase in consumers' demand for AC has triggered the aggressive harvesting of the wild AC fruiting body by cutting *C. kanehirae* trunk (an endangered species in Taiwan), and alarmed grave concern for the conservation of the tree [31]. Host specificity, slow growth rate, and rarity in nature are considered the main reasons behind the failure of meeting the consumer's demand, increased price, and illegal cutting of the host tree [32]. Therefore, scientists from academia and pharmaceutical industry have initiated intensive research on development of AC products in lab, and scaling up to industrial scale. Efforts are made to uplift the cultivation techniques, including solid state fermentation for the formation of the fruiting body or submerged state fermentation for harvesting mycelium in commercially available medium [2]. Also, quality control, efficacy approbation, and safety are important requirements for bringing the medicinal values of AC to mainstream international pharmaceutics, and have to be duly addressed [33].

Submerged culture is a commercial way for mass generation of microorganisms capable of producing secondary metabolites. Submerged culture gives rise to potential advantages of higher mycelial production in a compact space, and shorter time with lesser chances of contamination [34]. *A. cinnamomea* is a fungus which is sensitive to shear stress. Thus, airlift bioreactor can produce more mycelium

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than stirred tank reactor since airlift reactor includes no moving parts, low power consumption, high mass, and heat transfer rates, good solids suspension, homogenous shear, and rapid mixing [35,36]. Researchers investigated a two-stage submerged fermentation could lead to the maximum triterpenoids production 42 mg/g DW by using static culture to create oxygen limitation with temperature fluctuation between 25°C for 10 hr and 10°C for 14 hr [35].

Researchers found mixed cultivation of microorganisms could achieve satisfying results over pure culture. Co-culture of *Spirulina platensis* and *Rhodotorula glutinis* increased the accumulation of total biomass and total lipid yield [37]. Co-culture of *Aspergillus niger*, and *Trichoderma reesei* in solid state fermentation helped in the production of desired components of cellulase enzyme [38]. The concentrated and detoxified hydrolysate was fermented with mixed culture of *Saccharomyces cerevisiae*, and *Pichia stipitis* leading to conversion of both hexoses, and pentoses in the hydrolysate with high ethanol yields than the ethanol produced with monoculture of *S. cerevisiae* [39]. The oil accumulation in a mixed culture of *Chlorella* sp. and *Saccharomyces cerevisiae* was higher than that in the monoculture under the different light sources used [40].

However, so far, few studies in literature are focused on co-culture of AC. Therefore, the aim of this study was to investigate co-culture of AC and *S. cerevisiae* with two-stage operation, and varying environmental parameters. It was believed that the co-culture would help to mimic the natural growing condition of AC where different organisms might be playing role in triterpenoids production in wild AC. Oxygen supply is an important physical factor as it affects growth, morphology, nutrients uptake, and metabolite biosynthesis of a fungus. Rau et al. reported that oxygen limitation enhanced glucan formation of *Schizophyllum commune*, and *Sclerotium glucanieum*. The fungus reduced cell growth, and increased glucan accumulation when oxygen partial pressure in the culture broth was decreased to almost zero [41]. Oxygen limitation strategy was also successful in enhancing cordycepin production. Therefore, in this study we tested the effect of oxygen limitation by subjecting AC to grow in static, and cycling process in shake flask. Then, we discussed the interaction between *A. cinnamomea* and *S. cerevisiae* for triterpenoids production. Also, based on previous research about the role of elicitors in enhancing triterpenoids production in submerged production [36], we tested the effect of dead mycelia (chitosan present in the cell wall of fungus), and alcohol as elicitors. Finally, successful scaling-up the co-culture two-stage fermentation strategy was demonstrated from 250 ml shake flask to 2 L airlift bioreactor.

Materials and Methods

Microorganism and maintenance

Antrodia cinnamomea BCRC35396 and *Saccharomyces cerevisiae* BCRC 21812 were obtained from the Bioresources Collection and Research Center at the Food Industry Research and Development Institute (Hsinchu, Taiwan). *A. cinnamomea* was maintained on potato dextrose agar and transferred to a fresh agar plate every month, grown at 28°C, then stored at 4°C [35,42]. Since *S. cerevisiae* does not produce any triterpenoids as confirmed in our study, thus it is used as a model for co-culture with *A. cinnamomea*. The yeast was reactivated on yeast extract peptone dextrose (YPD) agar slants at 28°C, then maintained at 4°C, and subcultured every 3 months [43].

Inoculum preparation

The PMP medium for seed culture was made up of the following components (w/v): glucose 2.0%, malt extract 2.0% and peptone 0.1%. The pH value was initially adjusted to 5 by adding 1 N NaOH or 1 N HCl, followed by autoclaving at 15 psi, 121°C for 15 min. A suspension spore of AC was obtained by washing the 7 days old PDA slant with 10 ml sterile distilled water. Then, 10% (v/v) of the spore suspension was grown in a 250 mL Erlenmeyer flask containing 100 mL of the seed culture medium. The seed culture was incubated at 28°C for 7 days on a rotary shaker at 120 rpm [44]. A seed culture of *S. cerevisiae* was grown in an Erlenmeyer flask containing 100 mL PMP medium. The seed culture was incubated at 28°C for 2 days on a rotary shaker at 120 rpm.

Oxygen supply experiment

In order to understand the oxygen supply for the fermentation of *A. cinnamomea*, we used rotary shaker to control shaking, which was able to provide oxygen to the fermentation broth. For the first stage of AC monoculture, the flask culture experiments were performed in 250 ml flasks containing 100 ml of PMP medium after inoculating with 10% (v/v) of the seed culture of AC. The culture was incubated at 28°C on a rotary shaker incubator at 120 rpm for 12 days.

Then, the fungus was also cultivated in aerated, static or cycling (24 hr, 48 hr) conditions to 20th day in second stage. Cycling operation (24 hr) refers that the fungus was first cultivated in static condition to create oxygen limitation for 24 hr and then under shaking condition for 24 hr on rotary shaker at 120 rpm for oxygen supply. The samples were collected at various intervals from the shake flasks for analyzing cell density by UV-visible spectrophotometer, biomass and triterpenoids production. Values of cell density and corresponding biomass were indicators for the oxygen limitation tolerance ability of AC.

Co-culture experiment

In nature, AC grow with different organisms, therefore to imitate nature habitat, we designed co-culture experiments with S. *cerevisiae*. The first stage, 10% (v/v) of the seed culture of AC was grown in 250 ml flasks containing 100 ml of PMP medium, and the culture was incubated at 28°C on a rotary shaker incubator at 120 rpm for 12 days. Then, the 10% (v/v) of seed culture of *S. cerevisiae* was added. At the same time, the fungus was also cultivated in either static or cycling (24 hr, 48 hr, 23:1 hr) conditions to 20th day in second stage. Cycling operation (23:1 hr) means the fungus was cultivated in static condition for 23 hr followed by shaking condition for 1 hr.

Two-stage cultivation with elicitors

To the fermentation broth, live/dead *S. cerevisiae* and various concentration of alcohol were added in second stage. The effects of live and dead mycelium of *S. cerevisiae* treatment were studied by adding 10% (v/v) of seed culture of *S. cerevisiae*, which were either sterilized (dead) or live in second stage. Then the fungi were cultivated in either static or aerated conditions to 20th day. Effects of alcohol treatment were tested by adding various concentration of alcohol (0.1, 0.2, 0.5, 1 g/L) with static operation to 20th day in second stage. To reduce the influence of fermentation broth volume, 25% (v/v) of sterile distilled water was added.

Airlift bioreactor

A 2 L airlift bioreactor was used PMP medium for submerged cultivation of the fungus. The pH of the medium was adjusted to 5.0 by adding 1 N NaOH or 1 N HCl, and followed by autoclaving at 15 psi, 121°C for 15 min. For pure culture of *A. cinnamomea*, the fermentation medium was inoculated with 10% (v/v) of inoculants of AC, operated at 30°C and 1 vvm for 24 days.

For co-culture system, we combined airlift bioreactor with oxygen supply experiment. The fermentation medium was first inoculated with 10% (v/v) of inoculants of *A. cinnamomea*, operated at 30°C and 1 vvm for 16 days. Then, 25% (v/v) of inoculants *S. cerevisiae* was fed to the submerged culture. The aeration was shifted 1 vvm to 0.1 vvm, and 1 vvm to static condition, respectively.

Determination of mycelia dry weight

For biomass determination, the mycelia collected from fermentation broth were filtered through Advantec No.1 filter paper and washed with distilled water. The retained mycelia were vacuum dried at 50°C for sufficient time and weighed to obtain the biomass during fermentation [36].

Crude triterpenoids determination

The dried mycelium (0.1 g) was extracted with 95% ethanol (6 mL) for 24 hr. The extract was filtered through Advantec No.1 filter paper, and then dried by rotary evaporator. The dried filtrate was partitioned by H_2O and chloroform (1:1) first. The chloroform layer was further partitioned by 5% $NaHCO_3$ solution. The pH value of the $NaHCO_3$ layer was then adjusted to 3.0 by adding 1 N HCl, and partitioned again with chloroform. The chloroform layer was dried at 50°C under vacuum to obtain crude triterpenoids [36].

Determination of alcohol

To analyze the concentration of alcohol in fermentation broth, the sample was filtered through a 0.22 μm membrane. The filtrate was analyzed by Gas chromatographic (GC). The GC system was equipped with a capillary column DB-WAX (30 m × 0.25 mm i.d. × 0.25 μm thickness), a Flame Injector Detector (FID) with an operating temperature of 150°C an injector with a temperature of 120°C and an oven with a temperature of 72°C [43]. The carrier gas was hydrogen and air with pressure of 0.45 bar and 1.2 bar, respectively. The injection volume of sample was 1 μL.

Results and Discussion

Effect of oxygen supply on pure culture of AC in flasks for triterpenoids production

Zhang and Zhong reported that oxygen limitation caused poor cell growth and low ganoderic acid production in for *G. lucidum*, an aerobic fungus. However, a certain degree of oxygen limitation was beneficial to the ganoderic acid biosynthesis, while cell growth was fairly good [45]. Higher oxygen supply in the *A. cinnamomea* culture was favorable for cell growth and polysaccharide production, but was inhibitory on the triterpenoids production [1].

Triterpenoids are secondary metabolites of AC. In previous research, triterpenoids could be effectively increased by giving an anoxic environment which forces mycelium of AC to enter the death phase [36]. The main purpose of the cycling operation was creating oxygen limitation to stimulate fungus for production of triterpenoids during static operation, and reviving the survival of mycelia during shaking conditions i.e., oxygen was timely provided to avoid fungus rapid death. Results at three different two-stage operations of biomass and triterpenoids content in monoculture in 250 ml flask are shown in Table 1. Continuous shaking operation yielded highest amount of biomass 2.55 g/L which is fairly higher than the biomass obtained at static condition in 2nd stage amounting to 1.92 g/L. Sufficient oxygen supply resulted better mycelia growth thereby increasing biomass. However, even in the oxygen limitation condition during static operation, cell growth was fairly good which indicates that AC can tolerate the oxygen limitation condition to a certain extent. The oxygen limitation tolerance shown by AC in our cultures agrees with the findings by Zhang and Zhong [45]. The triterpenoids production obtained in the monoculture with static and cycling (24 hr and 48 hr) operation were 29.6, 24.1 and 20.5 mg/g DW, respectively. The highest triterpenoids content of the monoculture was observed during static operation. The results indicated the limitation of oxygen supply to the culture was responsible for increased triterpenoids production.

Effect of oxygen supply of co-culture of *A. cinnamomea* and *S. cerevisiae* in flasks for triterpenoids production

In this study, we added *S. cerevisiae,* and varied static or cycling operation in second stage. Results at three different two-stage operations of biomass and triterpenoids content in monoculture, and co-culture in 250 ml flask are shown in Figure 1a and 1b, respectively. Regardless of the different two-stage operation, biomass obtained in the monoculture was higher than those of co-culture, as seen in Figure 1a. AC and *S. cerevisiae* compete with each other for the available nutrients resulting in lesser cell growth. Biomass obtained in the monoculture with static and cycling (24 hr and 48 hr) operations were 1.96, 2.02 and 1.87 g/l, respectively.

In contrast, regardless of two-stage operation used, the triterpenoids production obtained in co-culture culture was higher than that in the monoculture, as shown in Figure 1b. The triterpenoids production obtained in the co-culture culture with static and cycling (24 hr and 48 hr) operation was 38, 32.9 and 23.6 mg/g DW, respectively. The highest triterpenoids content of the co-culture was observed with static operation. The effect of cycling operation was not satisfactory because the abundant availability of oxygen hindered triterpenoids production in mycelia.

Because static operation gave higher contents of triterpenoids, good oxygen suppply will increase biomass, a special strategy to reach maximal total triterpenoids per flask was designed to supply oxygen by shaking flask for 1 hr followed by static operation for 23 hr. The biomass and triterpenoids content for this condition was 1.94 g/l and 32.9 mg/g DW, and the maximal total triterpenoid was achieved as 63.83 mg/ flask as indicated in Table 2. The main reason for short duration of oxygen supply (1 hr) was to slow the death of mycelium caused by limitation of oxygen. However, the results indicated that manipulation of oxygen supply is essential to enhance production of triterpenoids of co-culture system.

The biomass ratio of *A. cinnamomea,* and *S. cerevisiae* in the broth would be an important factor to ensure sufficient availability of oxygen, nutrients etc. for both the organisms. In this study, for elevating triterpenoids production during submerged cultivation of *A. cinnamomea*, various inoculation level of *S. cerevisiae (*10%, 25% and 50% v/v*)* were added to the 12th day's fermentation broth, as shown in Figure 2. Among the tested inoculum levels, the inoculation level of 25% v/v resulted in the highest amounts of triterpenoids contents 46.5 mg/g DW.

Addition of *S. cerevisiae* is a successful strategy to increase production of triterpenoids. It is also based on our assumption of mimicking the natural environment of growth of wild AC in the forest. However, the mechanism of the mixed cultivation was still vague. Therefore, the study focused next on the investigation of the mechanism of co-culture cultivation.

Monoculture	Biomass (g/L)	Triterpenoids (mg/g DW)
Shaking condition	2.55 ± 0.21	18.0 ± 1.43
Static in 2nd stage	1.92 ± 0.012	29.6 ± 1.23
Cycling (24 hr)	2.02 ± 0.23	24.1 ± 2.20
Cycling (48 hr)	1.87 ± 0.16	20.5 ± 1.85

Table 1: Result of biomass and triterpenoids content in monoculture of *Antrodia cinnamomea* under different oxygen supply in the second stage in 250 mL shake flasks.

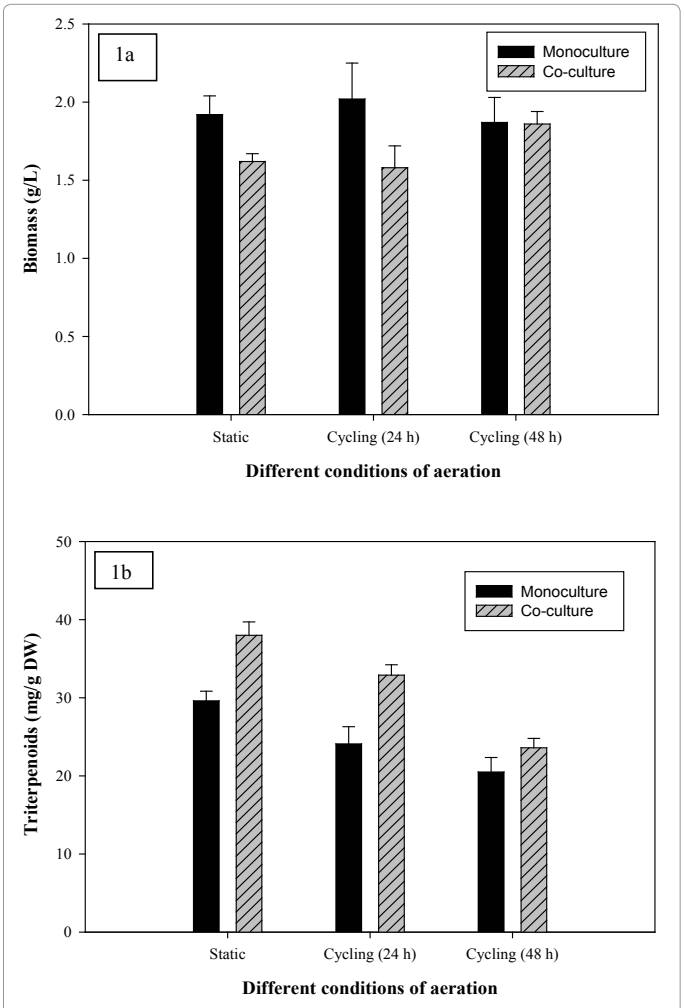

Figure 1: Biomass (a) and triterpenoid content (b) of different two-stage submerged cultivation in 250 mL shake flasks.

Co-culture	Biomass (g/L)	Triterpenoids (mg/g DW)	Triterpenoids (mg/ flask)
Static in 2nd stage	1.62 ± 0.05	38.0 ± 1.71	61.56
Cycling (1:23 hr)	1.94 ± 0.015	32.9 ± 0.21	63.83
Cycling (24 hr)	1.58 ± 0.14	32.9 ± 1.32	51.98
Cycling (48 hr)	1.86 ± 0.08	23.6 ± 1.20	43.9

Table 2: Result of biomass and triterpenoids content in the co-culture of *Antrodia cinnamomea* and *Saccharomyces cerevisiae* under different oxygen supply in the second stage (after 12 days of cultivation) in 250 mL shake flasks.

Role of elicitors in two staged submerged fermentation

The effect of addition of *S. cerevisiae* on triterpenoids production has two perspectives. The first one is fungal biomass; chitosan in cell wall of biomass (elicitor) may cause enhancement of triterpenoids production. The second one is the metabolites of *S. cerevisiae*; for example alcohol may affect AC growth. Thus, sterilized inoculant of *S. cerevisiae* was added to the 12th day's fermentation broth under both aerated and static conditions. Regardless of aeration or static operation in second stage, the production of triterpenoids by dead cells of *S. cerevisiae* (sterilized) dropped compared to live cells as indicated in Table 3. The production of triterpenoids obtained by adding live *S. cerevisiae* under static operation was 38.0 mg/g DW and highest among the given conditions. Based on this fact, it was assumed that alcohol produced by *S. cerevisiae* might have enhanced the triterpenoids production in co-culture.

Various concentrations of commercially available alcohol (1, 0.5, 0.2 and 0.1 g/l) were added to the 12th day's fermentation broth, as listed in Table 4 to test our assumption of role of alcohol produced by *S. cerevisiae* in enhancing triterpenoids production. It was found that alcohol could enhance the production of triterpenoids of *A. cinnamomea*. Among the tested dosages, the dosage level of 0.1 g/l alcohol resulted in the highest amounts of triterpenoids contents 36.7 mg/g DW. Higher dosage, such as 1 g/l alcohol might have inhibited AC growth thereby resulting in decreased triterpenoids production. Deionized water was added to the 12th day's fermentation broth. It was observed that addition of deionized water yielded 31.5 mg/g DW of triterpenoids which was not significant improvement in triterpenoids production.

These result indicated that alcohol produced by *S. cerevisiae* plays a vital role for increasing triterpenoids production during the submerged cultivation. Dilution effect is not the main reason to enhance production of triterpenoids during submerged cultivation of AC.

Scale up study in 2 L Airlift bioreactor

The proposed co-culture two-stage fermentation process was scaled up from 250 ml shake flask to 2 L airlift bioreactor with the purpose of studying the feasibility of the process in commercial production. Extensive literature studies revealed that AC was sensitive to shear stress resulting in decline of triterpenoids production [35]. Airlift bioreactor was a suitable choice than stirred tank reactor.

During monoculture of AC in a 2 L airlift bioreactor at 1 vvm aeration, the biomass reached the highest content 6.66 g/L on 16th day, as shown in Figure 3. Better oxygen supply in airlift bioreactor as compared to that of flask resulted in higher biomass as expected.

The maximum biomass reached at 16th day, this suggested that two-stage experiments culture with different oxygen supply would start from this day. By 16th day, AC yielded acidic metabolites causing pH value of the fermentation broth drop to 3.0. Reducing sugar was almost exhausted on 24th day. In the first four days, no triterpenoids production was observed. Then, triterpenoids increased with time, and the amount of triterpenoids content reached 38.2 mg/g DW on 24th day. The results indicated airlift bioreactor favored both biomass formation and triterpenoids production as compared to those of flask.

The next step was testing two-stage fermentation in co-culture with varied aeration. *S. cervisiae* was added on 16th day. Late addition of *S. cervisiae* was done with the purpose of ensuring sufficient nutrition and increased biomass of AC. In the second stage (from 16th day), the aeration level was varied from 1 vvm to 0.1 vvm for aerated co-culture and from 1 vvm to 0.0 vvm for static co-culture. The biomass obtained in the monoculture was higher than co-culture, as shown in Figure 4a. The triterpenoids content in the co-culture was higher than monoculture, as shown in Figure 4b. Among the tested two-stage operation, the static operation resulted in the highest amounts of

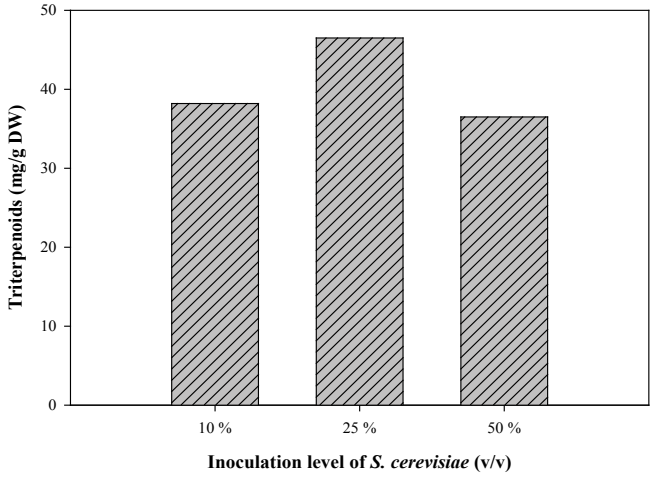

Figure 2: Result of different inoculation level of *S. cerevisiae* on triterpennoids content of *A. cinnamomea* in 250 mL shake flasks.

Co-culture	Biomass (g/L)	Triterpenoids (mg/g DW)	Alcohol (g/L)
Aerated w/ live yeast	2.55 ± 0.21	18.0 ± 1.43	0.0148
Aerated w/ sterilized yeast	2.59 ± 0.16	17.5 ± 1.21	0
Static in w/ live yeast	1.62 ± 0.11	38.0 ± 0.61	0.3677
Static w/ sterilized yeast	1.75 ± 0.23	20.4 ± 1.08	0

Table 3: Effect of addition of live and sterilized *Saccharomyce cerevisiae* in the second stage on the biomass and triterpenoids content of *Antrodia cinnamomea* in 250 mL shake flasks.

Monoculture	Biomass (g/L)	Triterpenoids (mg/g DW)
Static	1.81 ± 0.11	29.6 ± 0.061
Static + Alcohol (1 g/L)	1.92 ± 0.12	24.1 ± 0.38
Static + Alcohol (0.5 g/L)	1.91 ± 0.23	31.8 ± 1.72
Static + Alcohol (0.2 g/L)	2.13 ± 0.08	34.2 ± 1.12
Static + Alcohol (0.1 g/L)	1.94 ± 0.15	36.7 ± 0.58
Static + Water (25% v/v)	2.23 ± 0.06	31.5 ± 0.44

Table 4: Effect of alcohol treatment on the biomass and triterpenoids content of AC in 250 mL shake flasks.

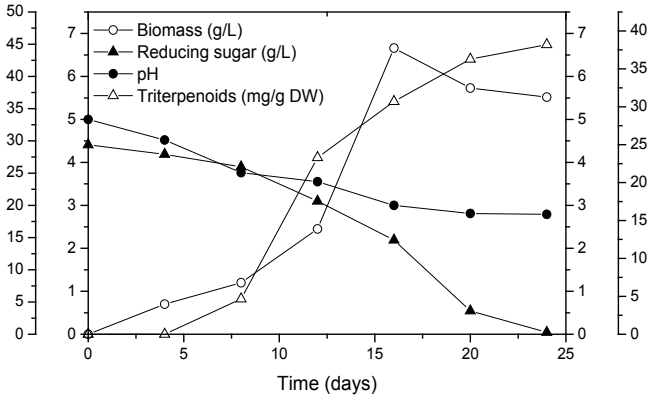

Figure 3: Time course of the mycelium growth of *A. cinnamomea*, final pH, reducing sugar and triterpenoids content in a 2 L airlift bioreactor at 1 vvm.

triterpenoids contents 62.2 mg/g DW. These results agrees with the flask conditions and indicates that scaling-up the process from 250 ml shake flask to 2 L airlift bioreactor is feasible. Hence, the strategy is applicable for commercial production of AC with increased triterpenoids content.

S. cerevisiae (also known as baker's yeast) converts reducing sugar into ethanol and carbon dioxide. Alcohol produced during co-culture in a 2 L airlift bioreactor under different aeration conditions is contributed by *S.* cerevisiae, and shown in Figure 5. Two-stage operation (static and 0.1 v.vm) produced alcohol concentrations of 0.69 and 0.065 g/l on 24th day, respectively. These results showed that triterpenoids contents are affected with alcohol concentrations. Compared to the addition of commercially available alcohol (0.1 g/L resulting highest triterpenoids content), the alcohol (concentration of 0.69 g/L) produced by *S. cerevisiae* during co-culture in airlift bioreactor at static operation contributed better results.

Conclusions

The effect of two-stage operation and inoculation level of *S. cerevisiae* on the production of triterpenoids in the mycelium of AC was demonstrated in this study. Based on our findings, co-culture of AC and *S. cerevisiae* in a 2 L airlift bioreactor with 25% v/v inoculation level of *S. cerevisiae* in second stage, and under static condition is optimal condition for enhanced triterpenoids production. Oxygen limitation plays a vital role for higher production of triterpenoids in AC cultures. Since, scaling up from 250 ml flask to a 2 L air-lift bioreactor has improved results; it

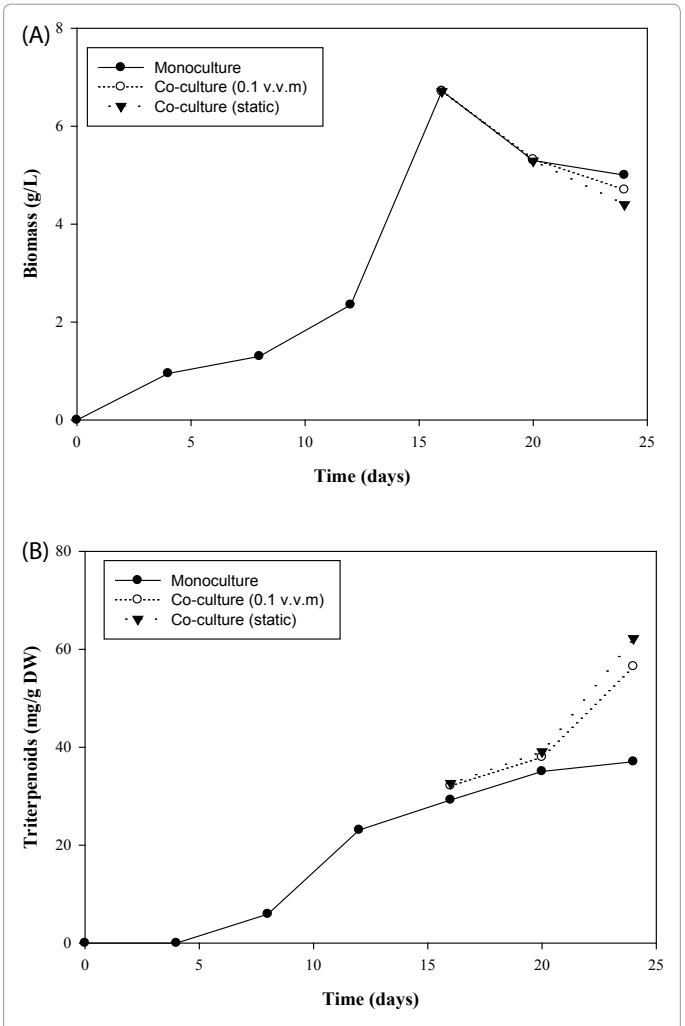

Figure 4: Biomass (a) and triterpenoid content (b) of different oxygen supply two-stage submerged cultivation in a 2 L airlift bioreactor.

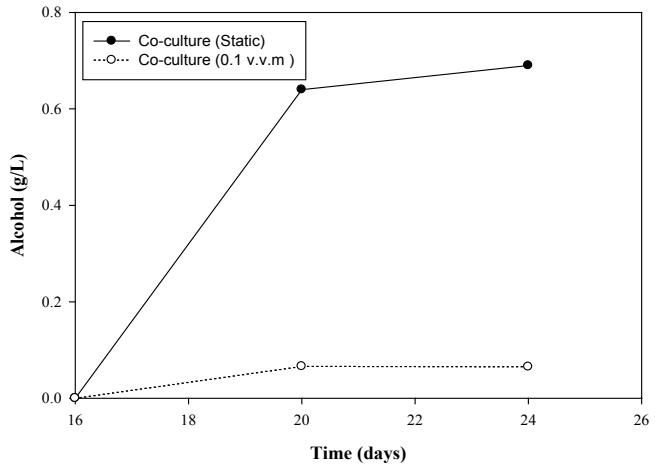

Figure 5: Alcohol concentrations of two-stage submerged cultivation under two different oxygen supply in a 2 L airlift bioreactor.

assures the application of this strategy for commercial production of AC with enhanced triterpenoids content.

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