

seed culture medium. The seed culture was propagated in a 250 ml Erlenmeyer flask containing 50 ml of liquid medium at 26°C on a shaking incubator at 150 rpm for 4 days. The fermentation liquid medium was composed of 30 g glucose and 3 g peptone (per liter) with an initial pH about 7.0 (not adjusted).

Fermentations in bioreactors

Four percent (v/v) of mycelial suspensions was carried out in a 5-L stirred-tank reactor with sixblade Rushton turbine impeller (Infors, Switzerland). Unless otherwise specified, fermentations were performed under the following conditions: 28°C, agitation speed 160 rpm, aeration rate 2 vvm, working volume 3 L, cultivation time 12 days. Samples were taken aseptically from the bioreactor at 2-day intervals for analyses.^[9] All experiments were performed in triplicate to ensure the trends observed were reproducible.

Measurements of the viscosity and morphology

The viscosity measurements were performed on samples collected from bioreactor at regular intervals using a Brookfield programmable LVDVII + digital viscometer (Brookfield Engineering Laboratories Inc., Stoughton, MA, USA) fitted with a small sample adapter. The morphological properties of the samples collected were evaluated using an image analyzer (DT2000 System, China) with software linked to a light microscope (Nikon, Japan) through a CCD camera. Samples were fixed with an equal volume of fixative (13 ml of 40% formaldehyde, 5 ml glacial acetic acid, and 200 mL of 50% ethanol). An aliquot (0.1 mL) of each fixed sample was transferred to a slide, air dried, and then stained with methylene blue (0.3 g of methylene blue, 30 ml of 95% ethanol in 100 mL water). For each sample, the morphology of pellet was characterized by measuring the area and perimeter of the pellet core and the maximum diameter of the pellet. Normally, a 40-fold magnification was used. The morphology of the pellets was characterized by their mean diameter, circularity, roughness, and compactness. The circularity was estimated as the ratio of the Fieret's minimum diameter to the Fieret's maximum diameter of the pellets or aggregates. The compactness was estimated as the ratio of the projected area of the hyphae in a clump to the projected convex area of that clump, the latter being the area after filling internal voids and concavities in the clump's external perimeter. In addition, the roughness (R) was measured using the following equation:

$$R = (\text{pellet}/\text{aggregateperimeter})^2 / (4\pi \times \text{pellet area})^{[10]}$$

Processing of mycelial fermentation broth for antiproliferation measurements

The mycelial fermentation broth was centrifuged at 8000 g for 15 min to obtain the mycelium biomass.

The mycelial pellet was washed thoroughly with distilled water and dried at 60°C in an oven till constant weight for measurement of the dry weight.^[11] The liquid broth containing mycelial biomass and extracellular metabolite products from the fermentors was processed in a batch by spray-drying into dried powder with an inlet air temperature 185-190°C. The power was divided into four equal portions and extracted with reflux for 3 times (1 h and each) by the following solvents (solid/liquid ratio 1:3) and methods: Boiling water (WE), WE with ultrasonication (30 min, 100 W) (WUE), butanol (BE) and ethyl acetate extract (EAE), respectively. The liquid extract was concentrated by evaporation and then freeze-dried. The WE, WUE, BE, and EAE were applied to the antiproliferation activity tests.

Evaluation of antiproliferation activity

For the antiproliferation effect study, LoVo cells of colon cancer were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum in a humidified 5% CO₂ atmosphere at 37°C.^[12] The proliferation of LoVo cells was determined using the colorimetric 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay described by Mosmann.^[13] Briefly, cells were seeded at a density of 3×10^4 cells/well in a 100 µL volume of the medium in 96-well plates and allowed to attach for 24 h. The dosages of extract were 1, 10, 100, and 1000 µg/mL while distilled water instead of the extract was used for the negative controls. Ten microliter MTT (0.4%) was added after 48 h. After incubated at 37°C for 4 h, the supernatant was aspirated and then 150 µL dimethyl sulfoxide was added to each well. Absorbance was measured at 490 nm by a 96 well microplate reader (Tecan, GENios ELIASA Co., Austria). All results *in vitro* were expressed as the inhibition rate of tumor cell proliferation as follows:

$$\text{Inhibition rate (\%)} = (1 - A_{\text{sample}} / A_{\text{control}}) \times 100\% \quad (1)$$

Where A_{sample} and A_{control} were defined as absorbances of the sample, control (without extract). And IC₅₀ was determined by nonlinear regression analysis using the GraphPad PRISMe statistics software package (Version. 2.0; San Diego, CA, USA).

Statistical analysis

Data were expressed as mean ± standard deviation. The results were analyzed for statistical significance by one-way analysis of variance test using the Statistical Package of the Social Science (SPSS) version 11.0 (SPSS Inc., Chicago, IL, USA). The statistical differences were considered significant at $P < 0.05$. Using the Pearson coefficient analysis was used to analyze the coefficient of determination (R^2) and evaluate the relationship among

mycelial production, mycelial morphology, and broth viscosity.

RESULTS AND DISCUSSION

Fermentation result in a stirred-tank reactor

Figure 1 shows the typical time courses of mycelial culture in a 5-L stirred-tank bioreactor, including biomass growth, nutrient consumption and medium pH change. The mycelial biomass grew rapidly (exponentially) from day 2 to 6 and reached a maximum biomass (12.5 g/L) in day 8 [Figure 1a]. As expected, the concentration of residual sugar dropped to below 5 g/l at day 8 and to nearly zero at day 12 [Figure 1b]. However, no drastic change in pH was recorded during the course of fermentation [Figure 1c].

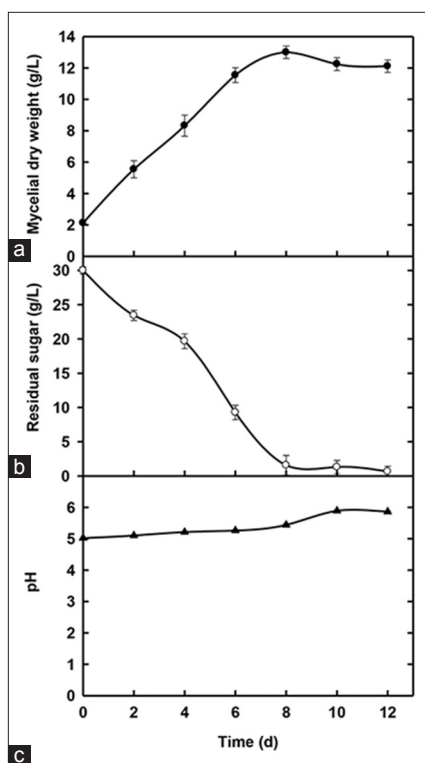


Figure 1: Time profiles of (a) mycelial biomass, (b) residual sugar, and (c) pH in submerged culture of *Phellinus vaninii* Ljup in a stirred-tank fermenter

Characterization of broth viscosity and mycelial morphology

The apparent viscosity of the whole broth according to the fermentation period is depicted in Figure 2. The viscosity of the fermentation broth at 300 rpm increased rapidly as the cells entered their exponential growth. This continued up to day 8 (64.5 mPa·S), which microorganisms entered their stationary phase and the viscosity of the broth, which was proportional to the cell concentration, declined accordingly. Between two typical fungal morphologies (e.g. filamentous and pelleted), pellet morphology was cited as one of the key factors directly affecting fermentation productivity.^[14] Figure 3 shows the typical morphological changes (2 days interval) during the entire fermentation period (0-12 days). The cells were observed to form mainly pellets during the entire culture period. The pellet diameter increased rapidly, and the outer hairy regions of the pellets became fluffier. Figure 4 shows the mean diameter (a), circularity (b), compactness (c), and roughness (d) of the pellets during cultivation of *P. vaninii* Ljup. The compactness, mean diameter and roughness of the pellets increased during the 4, 10, and 8 days of fermentation, respectively, and then decreased, but no drastic changes in circularity were revealed during the course of fermentation. Further investigation in the

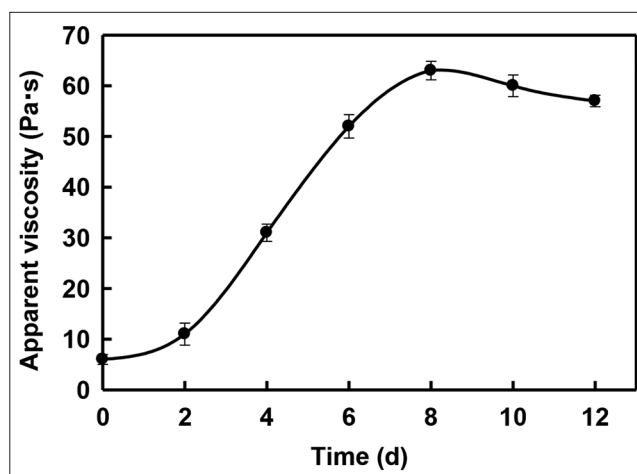


Figure 2: Time profile of apparent viscosity of fermentation broth of *Phellinus vaninii* Ljup in a stirred-tank fermenter

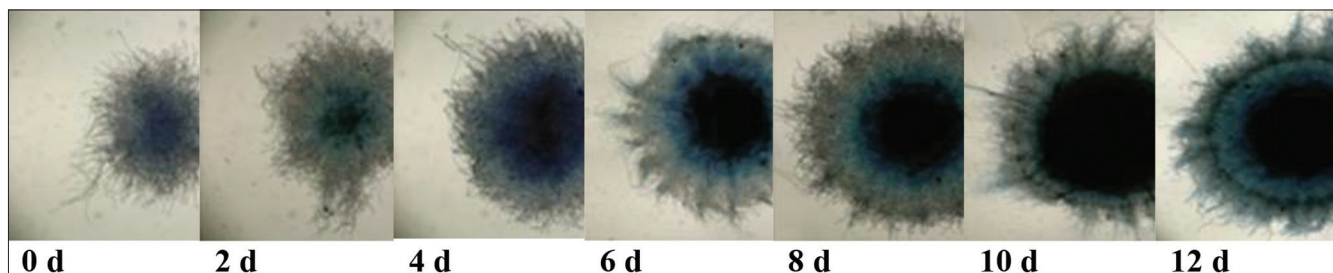


Figure 3: The morphological changes in *Phellinus vaninii* Ljup in a stirred-tank fermenter. Representative images were taken at 40-fold magnification. One to 9 day means fermentation period in day

mycelial pellets during the fermentation period revealed that mean diameter of the pellet was positively correlated with mycelial biomass ($R^2 = 0.82$, $P < 0.05$) and broth viscosity ($R^2 = 0.90$, $P < 0.01$), significantly. These results clearly show that the mycelial morphological change was coincidental with fungal biomass production and viscosity change. Our findings were consistent with Hwang *et al.*, who reported *Pbellinus baumii* showed the largest pellet size, which accordingly resulted in extremely high apparent viscosity.^[15] It is also found that the mycelial biomass was positively correlated with broth viscosity ($R^2 = 0.96$, $P < 0.01$) during the course of fermentation. It is similar to the finding by Hwang *et al.*, which indicated that the morphological variation of the three *Pbellinus* species was closely linked to the apparent viscosity of the whole broth.^[15]

Antiproliferative effects of mycelial fermentation products

Colon cancer is the third most commonly diagnosed cancer in the world; but, it is more common in developed

countries.^[16] In this work, to evaluate the effect of growth inhibition, various concentration of extracts from *P. vaninii* Ljup (i.e. WE, WUE, BE and EAE) ($1 \mu\text{g/L}$ - $1000 \mu\text{g/L}$) was added to the culture medium of colon cancer LoVo cells. It shows that all extracts have the inhibition effect on LoVo cell proliferation [Table 1]. The inhibition effects of BE and EAE are better than that of WE and WUE, and the effects were significantly dose-dependent. At $1000 \mu\text{g/ml}$ and 48 h after medicating, the inhibition rate of BE on colon cancer LoVo cell was 86.8%, the inhibition rate of EAE was 87.5%. Though the inhibition effects of BE and EAE are similar, taking into account that the evaporating temperature of ethyl acetate is lower than that of BE under vacuum, ethyl acetate is regarded as a desirable solvate. And the mean IC_{50} of EAE against LoVo cells was $53.459 \mu\text{g/mL}$. Similar to our results, Ajith and Janardhanan investigated the antitumor activities of ethyl acetate, methanol and aqueous extracts from the sporocarp of *Pbellinus rimosus* (Berk) Pilat and it showed that extract of ethyl acetate was the most effective than others to inhibit of solid tumor induced by Dalton's lymphoma ascites cell line.^[17] The incubation time of EAE ($100 \mu\text{g/mL}$) on inhibition effects of colon cancer LoVo cells was investigated and showed in Figure 5. The inhibition rate is enhanced corresponding to increase with the incubation time, and under the incubation time of 120 h, the inhibition rate on cancer cells reaches almost 100%.

Table 1: Antiproliferative activity of WE, WUE, BE, and EAE on tumor LoVo cells

Concentration ($\mu\text{g/mL}$)	Inhibition rate (%)			
	WE	WUE	BE	EAE
0	0.00 \pm 0.00	0.00 \pm 0.00	0.00 \pm 0.00	0.00 \pm 0.00
1	5.90 \pm 0.26	6.20 \pm 1.22	8.20 \pm 0.18	4.10 \pm 0.28
10	6.90 \pm 0.42	9.10 \pm 0.42	10.30 \pm 0.66	9.90 \pm 0.42
100	50.90 \pm 0.42	54.90 \pm 0.40	65.20 \pm 0.56	76.10 \pm 1.04
1000	57.80 \pm 0.46	58.30 \pm 0.62	86.80 \pm 0.06	87.50 \pm 0.04

*The results represent mean \pm SD (n=5). LoVo cells grown in 96-well plates and allowed to attach for 24 h. Cell viability was determined by MTT assay as described in the text. Concentration of 0 $\mu\text{g/mL}$ means negative control. SD: Standard deviation; MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide; WE: Boiling water; WUE: Water with ultrasonication; BE: Butanol; EAE: Ethyl acetate extract

CONCLUSIONS

In this work, mycelial fermentation characteristics of *P. vaninii* Ljup were evaluated in the fermentor for the first time. Our results indicated that the pellet formation and viscosity of *P. vaninii* Ljup were closely correlated during the fermentation. The EAE of *P. vaninii* Ljup

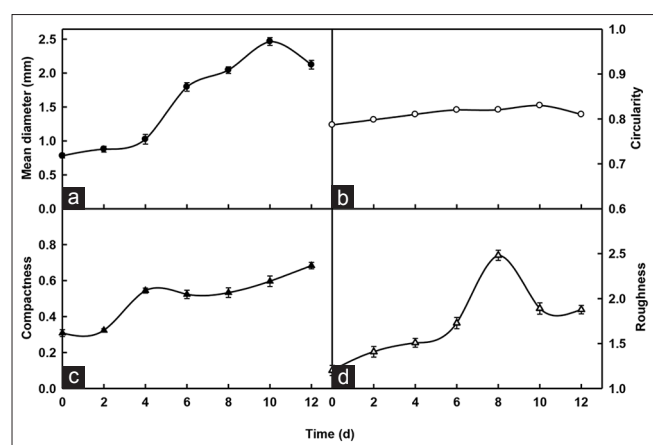


Figure 4: Mean diameter (a), circularity (b), compactness (c), and roughness (d) of *Phellinus vaninii* Ljup pellets growing in a stirred-tank fermenter

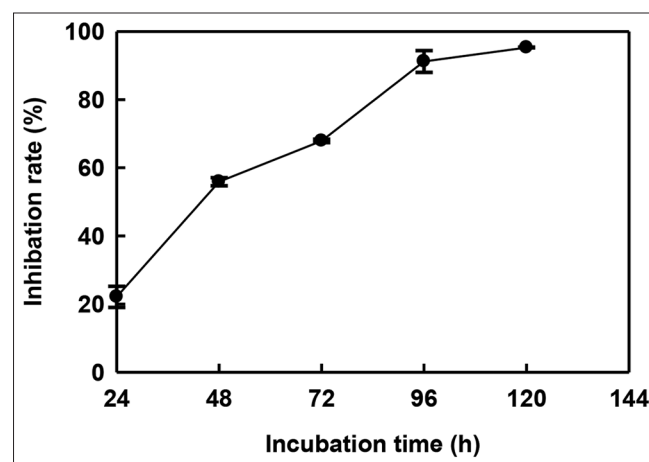


Figure 5: Effect of incubation time of ethyl acetate extract on tumor LoVo cells

exhibited the highest activity in the antiproliferative effect *in vitro*. In the future, pharmacological and biochemical investigations, especially acute toxicity, are needed to analyze the active compounds (could be polysaccharides) from *P. vaninii* Ljup.

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