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Contributed Paper

Fluid Flow Induced Shear Stress Affects Cell Growth and Total Flavone Production by *Phellinus igniarius* in Stirred-Tank Bioreactor

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ABSTRACT

The effect of fluid flow induced shear stress on dry cell mass and total flavone production in the cultivation of *Phellinus igniarius* has been studied. The results showed that at impeller tip speeds (ITS) of 0.628, 1.256 and 1.884 $m \cdot s^{-1}$, the maximal production of total flavone was 2.80, 3.17 and 2.19 $g \cdot L^{-1}$ respectively. A relatively high cell concentration of 7.8 $g \cdot L^{-1}$ by dry mycelial biomass was obtained in the bioreactor at an ITS of 1.256 $m \cdot s^{-1}$. At these ITSs, the maximal apparent viscosity was 1.123, 1.814 and 1.465 mPa·s and the exopolysaccharide production reached to 7.2, 9.4 and 8.1 $g \cdot L^{-1}$ respectively. The flow behavior of fermented broths was recorded photographically during submerged culture. The information obtained should assist progress towards the large scale production of flavone by submerged cultivation of *Phellinus igniarius*.

Keywords: Bioreactor, cell biomass, *Phellinus igniarius*, shear stress, flavone production

1. INTRODUCTION

Phellinus igniarius (L.:Fr.) Quél, a basidiomycete fungus belonging to the family *Polyporaceae*, is a medicinal mushroom containing many bioactive compounds including many flavones that have been reported to possess antibacterial, antiviral, antioxidative, antitumor and antimutagenic activities [2, 7, 11-14, 16, 21]. The fruiting bodies of *P. igniarius* are used in Chinese medicine for a variety of human diseases. Currently, commercial products from medicinal mushrooms are mostly obtained by field cultivation of the fruiting body. However, this does not guarantee a standardized product, with product composition varying from batch to batch [19, 21, 22]. Mushroom submerged fermentation is viewed as a promising alternative for efficient production [20]. Hydrodynamic shear stress is well known as an important factor for shear sensitive bioprocesses such as cell cultures of animal, plant and higher fungi [1, 3-5, 8-10, 15, 17, 19, 22]. Up to now there are only a few reports regarding shear

effects on submerged cultures of mushrooms [19]. In this study, the shear effect on cell growth and total flavone production by medicinal mushroom *P. igniarius* in bioreactor has been investigated.

2. MATERIALS AND METHODS

The strain of *P. igniarius* ATCC36121 was purchased from The American Type Culture Collection (USA). The stock culture was inoculated on a potato dextrose agar (PDA) slant (2.4 % potato dextrose broth and 2 % agar), incubated at 28°C for 6 days and then stored at 4°C. It was subcultured monthly. The preculture medium and preculture conditions have been described in detail elsewhere [6, 13]. Rutin was obtained from Winherb Medical S&T Development Co.Ltd (Shanghai, China). The reagent I used in the experiments was made up of 0.8 g boracic acid and 1.0 g sodium acetate dissolved in 100 ml 70% v/v ethano1 [20, 21].

For inoculum preparation, *P. igniarius* was initially grown on a PDA medium in a Petri dish, and then transferred to the seed culture medium by punching out a portion (5 mm diameter) of the agar plate with a sterilized homemade cutter. The seed culture was grown in a 500-mL flask containing 100 mL of PMP medium (2.4 % potato dextrose broth, 1 % malt extract, 0.1 % tryptone, pH normal) at 28°C with shaking at 130 rpm for 7 days.

A 5-L stirred-tank bioreactor (Biotech, Shanghai, China) equipped with pH probe, dissolved oxygen electrode and an impeller diameter of 8.0 cm was used to evaluate the hydrodynamic shear effect on the mushroom cell cultures. The hydrodynamic shear stress was evaluated by impeller tip speed (ITS = 3.14 × agitation speed × impeller diameter) as reported in other cases [18, 19]. An impeller tip speed (ITS) was set at 0.628, 1.256 and 1.884 $m \cdot s^{-1}$ by changing agitation speed to be 150, 300 and 450 r/min, respectively. Unless otherwise specified, submerged cultures of *P. igniarius* for the production of total flavone were performed in the stirred-tank fermentor under the following culture conditions: fructose 20 $g \cdot L^{-1}$, yeast extract 20 $g \cdot L^{-1}$, $CaCl_2$ 0.55 $g \cdot L^{-1}$, temperature 28°C, aeration rate 4 vvm, initial pH = 8.0, and working volume 3.5 L. All experiments were performed at least in duplicate.

The value for the shear stress can be interpreted as the maximal shear stress ($\gamma_{\max} \propto \pi DN$) or the average shear stress ($\gamma_{ave} \propto N$). Here the impeller tip speed (u_t) has been calculated in a form involving π, the impeller ratio (N), and the diameter of the impeller (D),

$$u_t = \pi ND \qquad (1)$$

and is close to the maximal shear stress, γ_{max}. The Reynolds number (R_e) has been calculated in a form involving the agitation rate (N), the diameter of impeller (D), the density (ρ), and the apparent viscosity (μ).

$$\mathrm{Re} = \frac{ND^2\rho}{\mu} \qquad (2)$$

The flow pattern in a bioreactor with baffles can be classified into two groups, depending on the value of R_e one of which is laminar flow (R_e)10) and the other is onflow (R_e)10^4) The agitation power is related to the flow pattern and the structure of the reactor and it increases with shear stress. It can be calculated as

$$P_0 = \Phi \rho N^3 D^5 F_r^y \qquad (3)$$

where

$$F_r = \frac{N^2 D}{g}$$

$$y = \frac{\alpha - \lg \mathrm{Re}}{\beta}$$

For the present system $\Phi \approx 6.0, \alpha \approx 1.0, \beta \approx 40.0$, g is the gravitational constant.

For the colorimetric assay, rutin was dissolved in 70% (v/v) ethanol to obtain a concentration of 400 μg mL^{-1} and filtered through a membrane filter (0.45 μm pore size) prior to the experiments. The rutin solution was used as the standard. The absorbance values (λ = 385 nm) of different volumes (0, 20, 50, 100, 200, 300 μl) of the standard rutin solution were then determined with 70% (v/v) ethano1 as control. Reaction mixtures consisted of 0.2 ml rutin standard, 0.8 ml 70% (v/v) ethanol, and 1 ml Reagent I. A standard curve was prepared according to the data obtained from the experiments [6].

Samples were taken at 24 hr intervals. Dry cell mass (DCW) was measured by the gravimetric method and total flavone content was assayed using the UV colorimetric method. Reaction mixtures consisted of 100 μl fermentation broth sample, 1ml 70% ethanol and 1ml stain reagent I. Spectra were determined at 385nm by comparison with controls in which the sample was replaced by 70% (v/v) ethano1. Sampleconcentrations were calculated from a standard curve made using diluted rutin. Several dilutions of sample were used to make sure the readings were within the standard curve range. The standard curve showing absorbance vs concentration was fitted with a linear equation for which y = 0.0361, x = -0.0034, and $R^2 = 0.999$, where x is the concentration of rutin and y is the absorbance value at 385 nm. This equation held over the concentration range 2.65 ~ 36.36 μg/ml.

The apparent viscosity was measured in the fermentation broth using a Brookfield viscometer LVT (Massachusetts, USA) with spindle number 4 at 30 rpm and 28°C. The apparent viscosity was calculated by

$$\mu = \gamma \cdot \rho \qquad (4)$$

where μ is the apparent viscosity of the fermented broth in mPa·s, γ is the kinematic viscosity of the fermented broth in mm^2/s, and ρ is the density of the fermented broth in kg/m^3.

3. RESULTS AND DISCUSSION

Table 1 shows that the flow pattern in the bioreactor was characteristic of onflow ($Re > 10^4$). The agitation power constant (Pd) and impeller tip speed (ITS = u_v) increase with the agitation, which reflects a higher shear stress. The mycelium growth kinetics at an ITS of 0.628, 1.256 and 1.884 $m \cdot s^{-1}$ in a 5-L stirred bioreactor are shown in Figure 1. After 8 days cultivation, a maximal cell density of 7.8 $g \cdot L^{-1}$ by DCW was obtained at an ITS of 1.256 $m \cdot s^{-1}$, while it was only 1.8 $g \cdot L^{-1}$ at 1.884 $m \cdot s^{-1}$. The results show that mycelium growth is inhibited by a relatively higher agitation. Figure 2 presents the time profiles of total flavone production. This was 2.80, 3.17 and 2.19 g/L, on day 7, 7 and 8 at an ITS of

Table 1. The operate parameters in bioreactor

Agitation (rpm)	Agitation N (r/s)	Re	Pd	ITS ($m \cdot s^{-1}$)
150	2.5	14801.2	19960.7	0.628
300	5.0	23544.5	144510.0	1.257
450	7.5	35534.0	443079.0	1.885

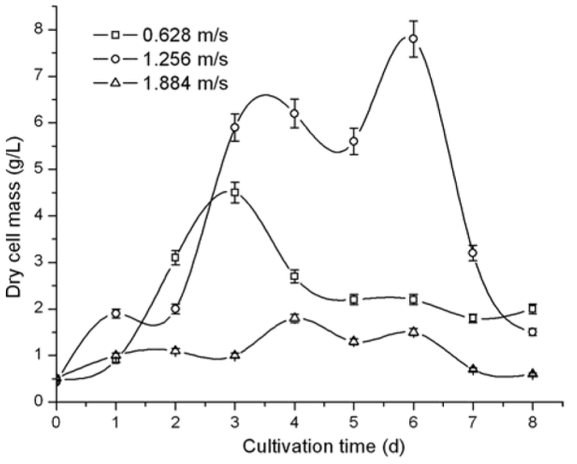

Figure 1. Time profiles of DCW in submerged cultures of *P. igniarius* in a 5-L stirred-tank bioreactor at different impeller tip speeds: % 0.628; % 1.256; % 1.884 $m \cdot s^{-1}$.

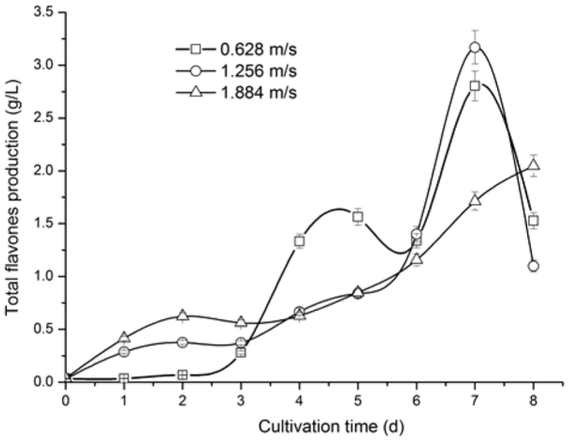

Figure 2. Time profiles of total flavone production in submerged cultures of *P. igniarius* in a 5-L stirred-tank bioreactor at impeller tip speeds: % 0.628; % 1.256; % 1.884 $m \cdot s^{-1}$.

0.628, 1.256 and 1.884 $m \cdot s^{-1}$, respectively, and the corresponding productivity was 0.40, 0.45 and 0.27 $g \cdot L^{-1}$ per day. An ITS of 1.256 $m \cdot s^{1}$ seemed to be optimal for total flavone productivity. The data imply that a moderate shear environment is favorable for the total flavone accumulation on day 7 at an ITS of 1.256 $m \cdot s^{-1}$. For comparison, Figure 3 shows apparent viscosity curves vs cultivation time at different ITS. The maximal apparent viscosity was 1.123, 1.814 and 1.465 mPa·s, on day 6, 5 and 4 at an ITS of 0.628, 1.256 and 1.884 $m \cdot s^{-1}$, respectively. At this time, the maximum response for exopolysaccharide production was 7.2, 9.4 and 8.1 $g \cdot L^{-1}$, respectively. The results indicate that the apparent viscosity of the fermentation broth is closely related to a secretion of exopolysaccharide. Hydrodynamic shear stress is well known as an important factor for shear sensitive bioprocesses. Much has been discovered about the effects of various types

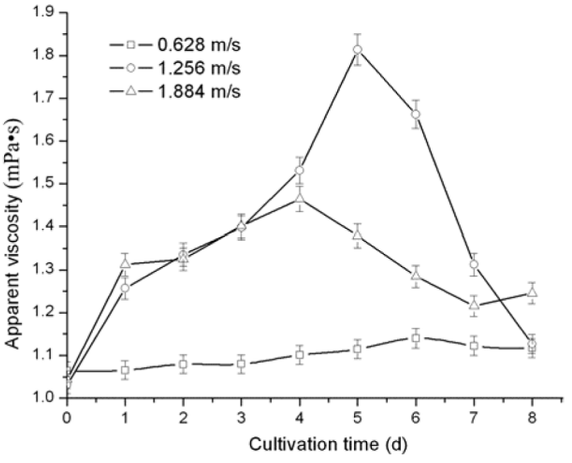

Figure 3. Time profiles of the apparent viscosity of fermentation broths in submerged cultures of *P. igniarius* in a 5-L stirred-tank bioreactor at impeller tip speeds: % 0.628; % 1.256; % 1.884 $m \cdot s^{-1}$.

of stress on microbiological processes in recent years. Taking dry mycelium mass, exopolysaccharide and total flavone as assessment indices, we have focused on the effect of hydrodynamic shear stress on cell growth and metabolite production of *P. igniarius* in a bioreactor where the agitation rate was set at 150, 300 and 450 r/min. It was demonstrated that application of moderate shear stress (1.256 $m \cdot s^{-1}$) is necessary for cell growth and metabolite production of *P. igniarius*. At the same time, adequate oxygen is conducive to mycelium growth under conditions of moderate stirring. If the agitation is too low, oxygen deficiency will result in the formation of large pellets and less biomass. The mycelium growth and metabolite secretion were also found to be inhibited by higher agitation. This is probably because of damage to the dispersed hyphae and pellets. This is supported by the observation that high agitation also increased the pH and pH is known to be increased by mycelium autolysis.

4. CONCLUSIONS

In conclusion, multiple effects of fluid flow induced shear stress on the growth, exopolysaccharide and total flavone production in *P. igniarius* cell cultures are demonstrated for the first time. The information obtained in this study should be valuable in optimizing scale-up of mushroom submerged cultivation in bioreactors to produce novel and potentially useful bioactive secondary metabolites on a large scale.

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