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OPTIMIZATION OF CULTURE CONDITIONS FOR LACCASE PRODUCTION FROM *ABORTIPORUS BIENNIS* IN A PILOT-SCALE BIOREACTOR

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Abstract - The optimal culture conditions of laccase productions in submerged culture by Abortiporus biennis in a pilot-scale bioreactor (300 L) were determined using the rotating simplex method. The optimal culture conditions were C/N ratio 25, agitation speed 200 rpm, aeration rate 1.5. In the optimal culture medium, the maximum laccase production was 1361 U/L in the pilot-scale bioreactor. Further investigation of the mycelial pellets and culture broth during the fermentation period revealed that the mean diameter and compactness of the pellet and viscosity of the broth were significantly positively correlated with laccase production. The results demonstrated the feasibility of A. biennis fermentation for large-scale production of laccase.

Key words: Mycelia; *Abortiporus biennis*; fermentation; morphology; rotating simplex method

INTRODUCTION

In recent years, there has been growing interest in the production of ligninolytic enzymes, especially laccase (Dhouib et al., 2005; Sarnthima and Khammuang, 2013). Laccase (EC 1.10.3.2) is an extracellular, copper-containing oxidase enzyme that is capable of oxidizing both phenolic and non-phenolic lignin-related compounds by a radical-catalyzed reaction mechanism with molecular oxygen (Martinez et al., 2005) Laccase plays an important role in lignin degradation and has wide application in the textile and food industries (Couto and Herrera, 2006).

Abortiporus biennis belongs to white rot Basidiomycetes, a group of fungi that possess the unique ability to degrade all wood components (Jaszek et al., 2006). It has been reported that a novel laccase from *A. biennis* was purified and demonstrated antiproliferative activity against Hep G2 and MCF-7 cells, and inhibitory activity against HIV-1 reverse transcriptase (Zhang et al., 2011).

The rotating simplex method is a simple and non-statistical optimization technique for obtaining a suitable combination of parameters for cultivation when the experiments have to be carried out sequentially due to the operating feasibility or limitation of equipment (Panda and Naidu, 2000). This method has been successfully used for many biosynthesis processes (Xu et al., 2006; Hendrix, 1980). This method is guided by the movement of the simplex in a response plane. It is a self-correcting, and an error causes the simplex to move in a

direction away from the optimum. Each time the simplex moves, a new combination trial is determined based on the outcome of the latest trial, and the process continues until the best combination of variables is obtained.

The optimum cultivation conditions in 250-ml Erlenmeyer flasks for high yields of laccase production from *A. biennis* have been investigated (Erden et al., 2009). However, no information is available in the literature on the laccase production and mycelial characteristics of *A. biennis* in pilot-scale fermentation. The present study explores the suitability of a newly isolated fungus (*A. biennis*) for the production of laccase. The strain was first isolated from soil and identified by an analysis of the ITS rDNA region sequence. The rotating simplex method was applied to optimize the culture conditions in a pilot-scale (300-L) stirred-tank fermenter for enhancing laccase production. Three variables, including C/N ratio, agitation speed and aeration rate, were selected as process (independent) variables while laccase production was the response (dependent variable). The mycelial morphology and broth rheology were analyzed during the fermentation and their relationships with laccase production discussed.

MATERIALS AND METHODS

Microorganism and growth conditions

Abortiporus biennis was isolated from the root of a camphor tree in Mianyang, Sichuan Province, and kept in the Forest Microbiological Research Center of Northeast Forestry University (no. SWUST2009111001). Stock cultures were maintained on potato dextrose agar (PDA) slants. The liquid culture of mycelia was initiated by transferring the fungal mycelia from the stock culture on a Petri dish into the 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 liquid medium was composed of 30 g glucose and 3 g peptone/L with an initial pH 7.0. The inoculums for the 15-L seed fermentor were prepared in the shake-flasks, and that for the industrial fermentor was prepared in the seed fermentor.

Isolation and identification of strain Abortiporus biennis

The isolated strain was phylogenetically identified by ITS-5.8S rDNA sequencing analysis. The chromosomal DNA of the strain was isolated from the fresh mycelium using a Fungal Genomic DNA Extraction Kit (Sangon Biotech Co., Ltd; Shanghai, China). The resulting genomic DNA was amplified using a DNA PCR Kit (Sangon Biotech Co., Ltd; Shanghai, China) and primers ITS1(5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') on an Applied Biosystems Veriti 96-well thermal cycler (Applied Biosystems, Foster City, CA) under the following conditions: 94°C for 5 min (1 cycle); 94°C for 45 s, 55°C for 50 s; 72°C for 60 s, (33cycles); 72°C for 5 min (1 cycle). The PCR products were sequenced in both directions by Sangon Biotech Co. Ltd (Shanghai, China). The obtained nucleotide sequence of the ribosomal sequence was compared with those of GeneBank using the NCBI Blast program, and sequence homology was comparatively analyzed using the Clustal X program (Glass and Donaldson, 1995).

Cultivation in bioreactors for mycelial and laccase production

Four percent (v/v) of mycelial broth was inoculated into a 300-L stirred-tank bioreactor (Bailun, Shanghai, China). Unless otherwise specified, medium and culture conditions were performed under the following conditions: 40 g/L maltose, 1.6-8 g/L peptone depending on requirement of C/N ratio, 0.68 g/L KH_2PO_4, 26°C, working volume 180 L, cultivation time 8 d. The C/N ratio, agitation rate and aeration rate were varied according to the experimental design. Samples were taken aseptically at intervals for analyses (Bae et al., 2000). The mycelial fermentation broth was sampled every two days and centrifuged at 8 000*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 (Park et al., 2002). The supernatant was filtered through Whatman No. 1 filter paper and used for the enzymatic assay. All experiments were performed in triplicate to ensure the trends observed were reproducible.

Optimization procedure

The method began with a set of four experiments considering the fact that the simplex takes the shape of a tetrahedron composed of three key variables viz. C/N ratio, aeration rate and agitation intensity. The levels of the variables for these four experiments were set up from first four earlier experience of the process. After these four experiments had been conducted, the experiment that gave the most unsatisfactory response was identified and replaced by a new combination of variables that should be a reflection of the worst point on the response plane. Science determining the reflection of a point of a tetrahedron in the response plane is a complex mathematical procedure and the following rule for the new experimental point was applied: twice the average of the best points minus the worst point. This new experiment was then carried out with the newly determined set of variables, and the worst response from the four remaining experiments was again identified and replaced. This iterative procedure was continued until no further improvement in activity was obtained.

Laccase assay

The laccase activity was determined using 2,2'-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) (Sigma) as the substrate. The laccase reaction mixture contained 0.5 ml of 0.45 mM ABTS, 1.2 mL of 0.1 M phosphate buffer (pH 6.0) and 0.5 mL of filtrate to give a final reaction volume of 2.2 mL. The oxidation of the substrate (ABTS) was monitored by the increase in the absorbance at 420 nm using Shimadzu UV-1800 spectrophotometer (Tokyo, Japan) over 90 s at 28°C±1. Enzymatic activity was expressed as 1 U = 1 μmoL of ABTS oxidized per min at 28°C±1 (Erden et al., 2009; Rasera et al., 2009).

Measurements of the viscosity and morphology

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, 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 the pellet was characterized by measuring the area and perimeter of the pellet core and the maximum diameter of the pellet. Forty-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 = (pellet/aggregate perimeter)2/(4π×pellet area). Viscosity measurements were performed on samples collected from the bioreactor at regular intervals using a Broolfield programmable LVD-VII + digital viscometer fitted with a small sample adapter (Park et al., 2002).

Statistical analysis

Data were expressed as mean ± S.D. The results were analyzed for statistical significance by one-way analysis of variance (ANOVA) using the Statistical Package of the Social Science (SPSS) version 11.0 (SPSS Inc., Chicago, IL, USA). The statistical differences were considered significant at $p<0.05$. Pearson's coefficient analysis was used to analyze the coefficient of determination (R^2) and evaluate the relationship among laccase production, mycelial morphology and broth viscosity.

RESULTS

Identification of fungus

The rDNA-ITS gene sequences (600-700 bp) were identified by polymerase chain reaction (PCR), sequencing and comparison to all sequences in GenBank. Its GenBank accession number was HQ833313. By analysis of the ITS rDNA gene sequence, this strain was found to be similar to *Abortiporus biennis* (99% based on ITS rDNA). Therefore, based on combined analysis of the fungal physicochemical characters (data not shown), the strain PG23 could be identified as *Abortiporus biennis* (Fig. 1).

Rotating simplex method for A. biennis culture

According to the rotating simplex method, a total of six fermentation experiments were necessary to obtain the best combination of physical parameters (e.g. C/N radio, agitation intensity and aeration rate) for laccase production in *A. biennis* (Table 1). During the initial four experiments, the levels of the variables were C/N ratio 5-15, aeration rate 1.0-2.0 vvm, agitation rate 100-250 rpm. No prior knowledge was available regarding the conditions of the laccase production. The above levels were set based on the previous experience in shake-flask cultures and general levels for fungal fermentation reported in literature. It was observed from the initial experiments that the laccase production was low (Run# 2 and 4) when the C/N ratio was low and agitation speed was high (Table 1). In Run# 5, the highest laccase activity of 1 309 U/L was obtained at a C/N ratio of 18.33 with an aeration of 0.67 vvm and agitation speed of 50 rpm compared to the first four runs (Table 1). This clearly indicates that the tetrahedron simplex is moving towards the optimum. In Run# 6, the laccase activity has decreased, which indicated that the simplex had moved away from the optimum. Hence, the condition of Run# 5 is considered as optimum. An almost 3-fold improvement in laccase production was obtained in Run# 5 compared with the laccase production obtained in unoptimized culture (Run# 2).

Characterization of fermentation kinetics, broth viscosity and mycelial morphology

In Table 1, the lowest laccase activity was observed in Run# 2, while the highest lowest laccase activity was observed in Run# 5. Thus, the different characterization of mycelial growth and laccase produc-

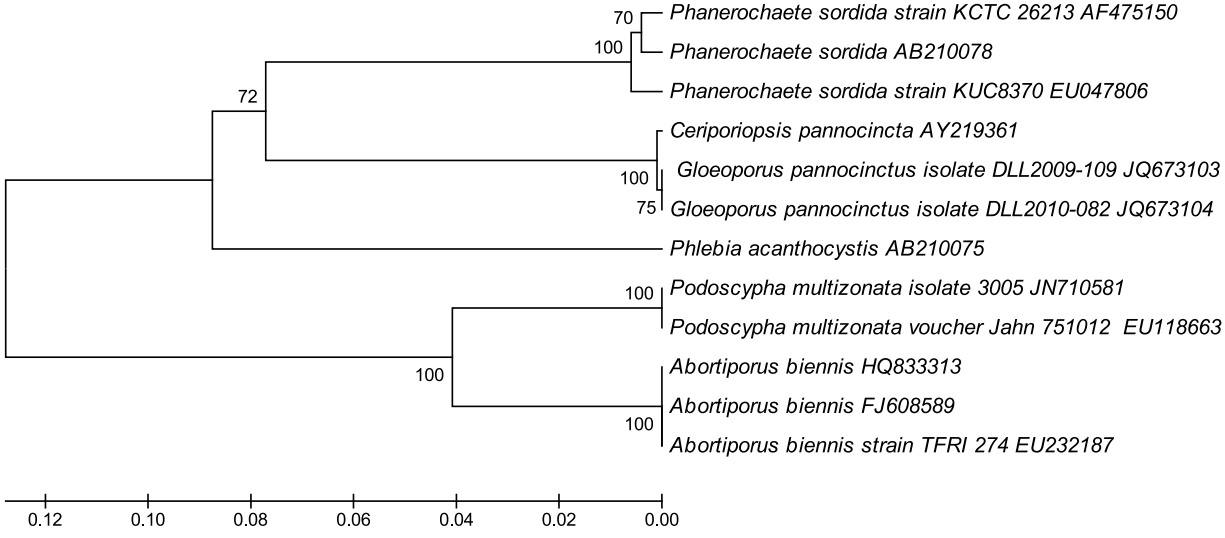

Fig. 1. The phylogenetic dendrogram for *A. biennis* and related strains based on the ITS rDNA sequence. Numbers in parentheses are accession numbers of published sequences. Bootstrap values were based on 1000 replicates.

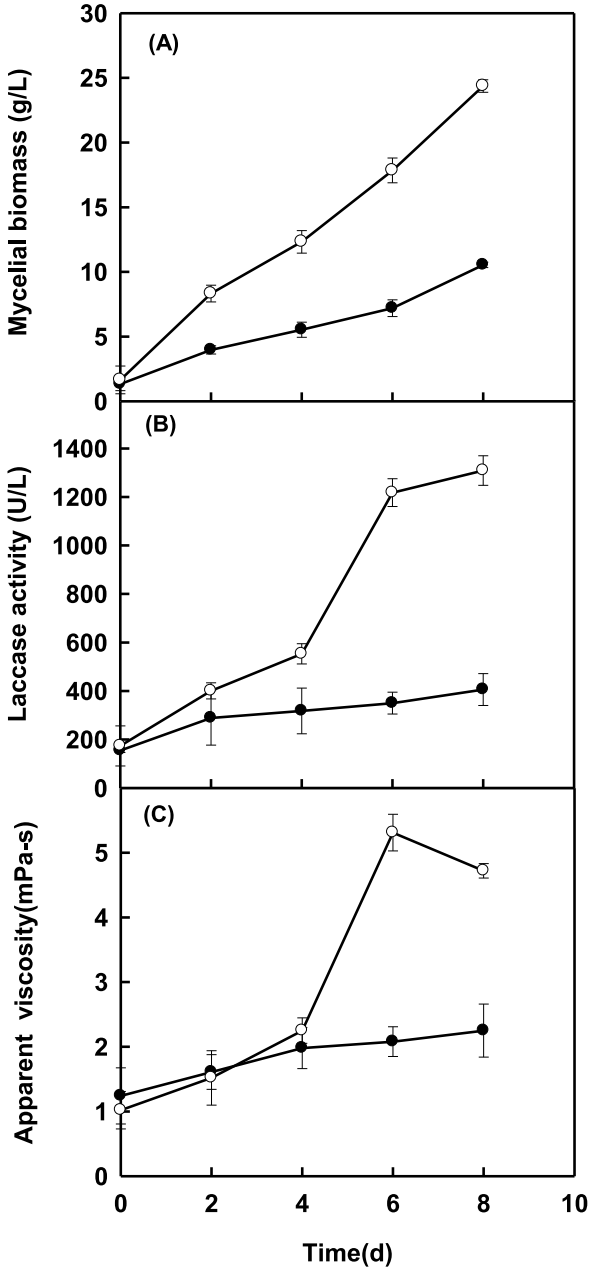

Fig. 2. Time profiles of (A) mycelial biomass, (B) laccase activity and (C) apparent viscosity of fermentation broth in submerged culture of *A. biennis* in a stirred-tank reactor. (●) Run# 2, (○) Run# 5.

tion, morphology and broth viscosity between Run# 2 and 5 was investigated. Fig. 2A shows the typical time courses of biomass growth (Run# 2 and 5) by *A. biennis* in a 300-L stirred-tank bioreactor. The mycelial biomass of Run# 5 grew more rapidly than that of Run# 2 during the fermentation period. The trends of biomass products were similar with those of laccase productions observed in Run# 2 and 5 (Fig. 2B). The apparent viscosity of the whole broth according to the fermentation period is depicted in Fig. 2C. As for both Run# 2 and 5, the viscosity of the fermentation broth increased rapidly as the cells entered their exponential growth. In Run#5, this continued up to day 6 (5.5 mPa·S), when cells entered their stationary phase and the viscosity of the broth, which was proportional to the cell concentration, declined accordingly. It was found that the broth viscosities of Run# 2 ($R^2 = 0.92$, $p<0.01$) and 5 ($R^2 = 0.97$, $p<0.01$) were positively correlated with laccase production during the course of fermentation.

Fig. 3 shows the typical morphological changes (2-d interval) during the entire fermentation period (0-8 d). The cells were observed to form mainly pellets throughout the culture period. The pellet diameter increased rapidly and the outer hairy regions of the pellets became fluffier. Fig. 4 shows the mean diameter (A), circularity (B), compactness (C), and roughness (D) of the pellets (Run# 2 and 5) during cultivation of *A. biennis*. During the fermentation periods of both Run# 2 and 5, the compactness of the pellets increased all the time, but no drastic changes in circularity and roughness were revealed. The mean diameter and compactness of the pellets during the fermentation of Run# 5 were larger than those of Run# 2, but there were no significant differences in circularity and roughness between Run# 2 and 5 during the fermentation except on day 8. Further investigation made clear that the changes in mean diameter ($R^2 = 0.86$, $p<0.05$) and compactness ($R^2 = 0.80$, $p<0.05$) in Run# 5 was coincidental with laccase production.

DISCUSSION

The C/N ratio of the fermentation medium is a key factor for optimum laccase production in *A. biennis*. This is evident from the observation that laccase production steadily increased with the increase of C/N

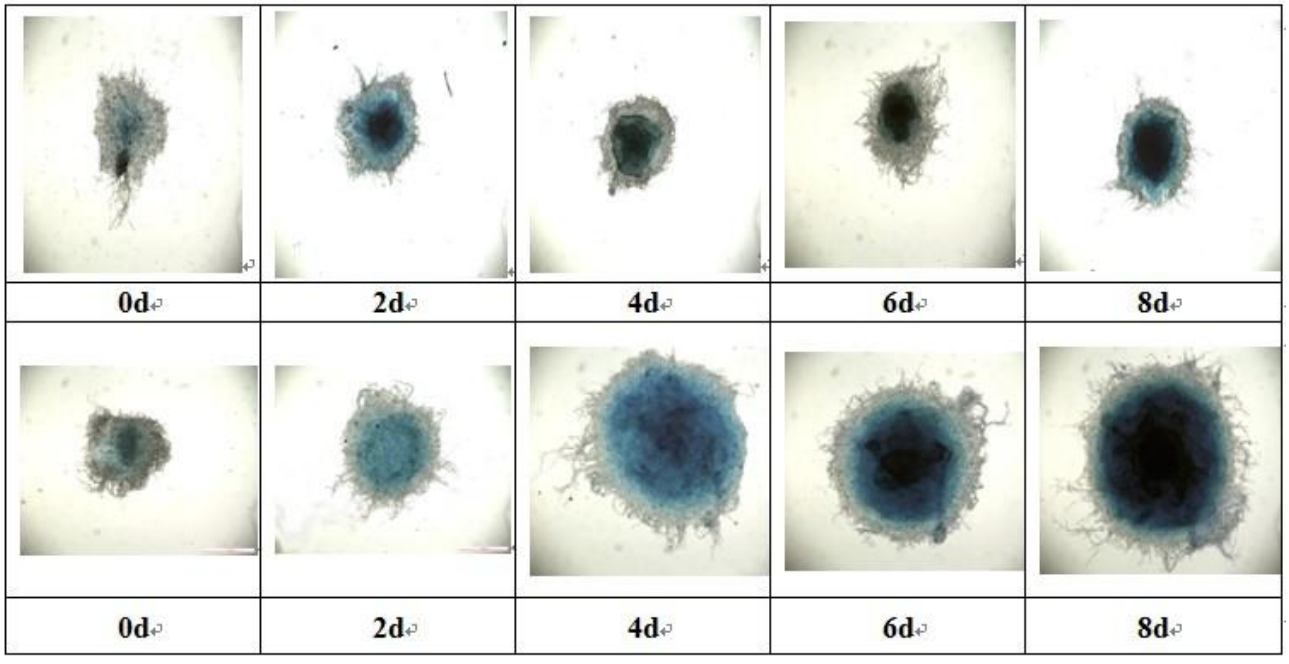

Fig. 3. The morphological changes in *A. biennis* in a stirred-tank fermenter. Representative images were taken at 40-fold magnification; 1-9 d – fermentation period in days. Upper Fig.: Run# 2, Lower figure: Run# 5.

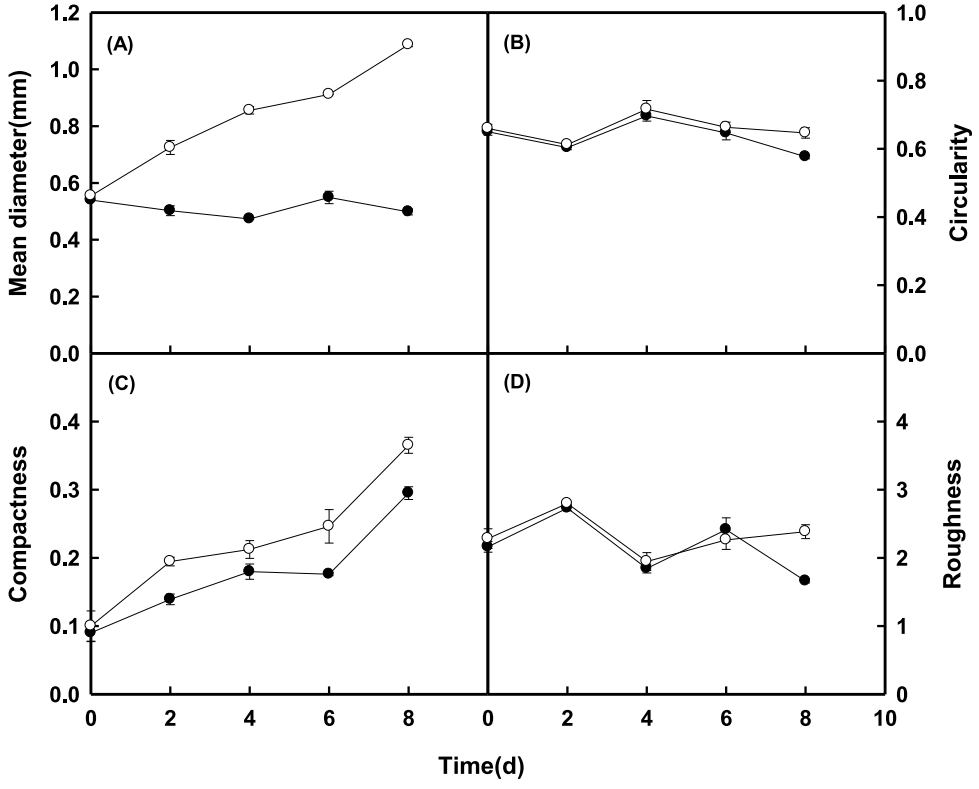

Fig. 4. Mean diameter (A), circularity (B), compactness (C) and roughness (D) of *A. biennis* pellets growing in a stirred-tank fermenter. (●) Run# 2, (○) Run# 5.

Table 1. Results of the rotating simplex method for optimization of culture conditions for laccase production by *A. biennis* in a batch bioreactor

Run no	C/N ratio*	Aeration rate (vvm)	Agitation speed (rpm)	Laccase activity (U/L)
1	15	1.0	100	1241 ±20
2	5	2.0	250	406 ±15
3	15	2.0	100	736 ±13
4	5	1.0	250	510 ±17
5	18.33	0.67	50	1309 ±37
6	27.22	1.45	0	685 ±14

*C: 40 g/L maltose, N: peptone concentration depends on requirement of C/N ratio

ratio up to 18.33 and decreased in regions of higher C/N ratio values (Table 1). It is apparent that the relatively lower aeration rate and agitation speed in the fermentor would contribute to laccase production (Table 1). This is in agreement with the results reported by Erden et al. (2009). They suggested that agitation speed significantly affects laccase production in 250-ml Erlenmeyer flasks and relatively lower agitation speed is more desirable for laccase production. This may be due to the shear inactivation of key enzymes necessary for synthesizing the laccase or the break-up of mycelial pellets, or both.

In submerged cultures of higher fungi such as entomopathogenic fungi and mushrooms, it was reported that pellet morphology and broth viscosity were two key factors directly affecting fermentation productivity (Park et al., 2002; Xu et al., 2006). In this study, the mean diameter and compactness of the pellet and viscosity of the broth were significantly positively correlated with laccase production. This is similar to the finding by Tepwong et al. (2011), which indicated that mycelium pellets' morphology, particularly mean diameter, was highly positively correlated with ergothioneine accumulation throughout the fermentation period of *Lentinula edodes*. Zhai et al. (2013) also reported the viscosity of *Stropharia rugosoannulata* was related to exopolysaccharides production.

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