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Optimization of Parameters for Mycelia Growth by *Schizophyllum commune* and a Kinetic Model Study of its Growth Morphology

Yi Peng Teoh and Mashitah Mat Don
School of Chemical Engineering, Universiti Sains Malaysia, Seri Ampangan,
14300, Nibong Tebal, Seberang Perai Selatan, Penang, Malaysia

Abstract: The ever expanding trend of rubberwood-based industry in Malaysia brings concerns about biodegradation problem on rubberwood for sustainable production. Conventional chemical control has been a successful method of preventing staining fungal growth. But the effects of these chemicals are of concern because they create problems for the environmental and public health. *Schizophyllum commune* is a well known edible white-rot fungus that is capable of producing secondary metabolites. Statistical optimization was employed to optimize the culture parameters for maximum mycelia growth of this fungus in shake flask culture. A Response Surface Methodology (RSM) coupled with Box-Behnken Design (BBD) was applied and three experimental parameters (pH, incubation temperature and agitation) were selected. The optimum condition for the maximal growth (33.1404 g L^{-1}) was found to be at pH 6.78, 30.23°C and 174.14 rpm, respectively. Under such condition, the predicted maximum growth was in good agreement with the experimental data with 0.213-0.602% error. The growth kinetics of the *Schizophyllum commune* was investigated using an unstructured kinetic model, the Logistic model. The model provide reasonable result with R^2>0.9.

Key words: *Schizophyllum commune*, biomass, Box-Behnken design, kinetics, logistic model

INTRODUCTION

Schizophyllum commune is very common species of mushroom in the family Schizophyllaceae of Agaricales (Hao *et al*., 2010), which having more than 28,000 distinct sexes (Douglas, 2006). It was probably the most widespread fungus in existence, being found on every continent except Antarctica, where there is no wood to be used as a substrate (Teoh *et al*., 2011; Ohm *et al*., 2010). Thus, this fungus was reported as the common invader of rotten wood, which was able to colonize and cause severe infections in humans. On the other hand, this fungus exhibit other properties, such as tolerance to the fungicide benomyl, susceptibility to high concentrations of cycloheximide, a dolipore-type septum and mating tests (Guarro *et al*., 1999). Recently, this fungus has demonstrated that it can produce a variety of schizophyllan and hydrolytic enzymes, such as xylanases. This exopolysaccharide contained a homoglucan that possesses a β-1,3-linked backbone with single β-1.6-linked glucose side chain at approximately every third residue (Teoh *et al*., 2011; Jayakumar *et al*., 2010). As reported by Hao *et al*. (2010), schizophyllan has numerous potential applications, such as thickener for cosmetic lotions, act as oxygen-impermeable films for food preservation, biological response modifier and a non-specific stimulator of immune system and enhance the effect of vaccines and anti-cancer therapies and also anti-tumor agent.

There is a growing interesting for the *S. commune* fermentation in order to accelerate the mycelia growth In fact, the mycelia growth rate has been shown to vary with the environmental conditions, such as solution pH, incubation temperature and agitation speed. The pH of the growth medium played an important role by inducing morphological changes in the organism. The pH change was observed during growth of the organism which affected product in the medium (Teoh and Mashitah, 2010). In fact, the unfavorable reactions within too alkaline or acidic condition could be attributed to the loss of nutritive value of protein and formation of potentially toxic substances such as lysinoalanine and thus influencing the growth of fungal (Liu *et al*., 2008). Besides, temperature is probably one of the important environmental factor that determining the fungal growth (Rousk and Baath, 2011). Most of the well-characterized microorganism live best at temperature form 25-40°C, many were thrive at high temperature and others might growth best (although slowly) at 0-15°C. In fact, every organism had an optimum temperature for growth, as

Corresponding Author: Mashitah Mat Don, School of Chemical Engineering, Universiti Sains Malaysia, Seri Ampangan, 14300, Nibong Tebal, Seberang Perai Selatan, Penang, Malaysia Tel: 604-5996468 Fax: 604-5941013

increasing the generation time, the temperature declines from that optimum (Teresa, 1999). Agitation intensity is another notable variable that influenced the mixing and oxygen transfer rates in much fungal fermentation and thus influencing the mycelia growth morphology (Teoh and Mashitah, 2010). For example, Rahman *et al.* (2005) reported that the small increment of enzyme activities as increasing the agitation speed from 150 to 350 rpm showed that aeration is an important factor for the growth of aerobic strains. In fact, a higher agitation speed is sometimes detrimental to mycelia growth (Teoh and Mashitah, 2010).

The classical method of optimization involves changing one variable at a time by keeping the others at fixed levels. It was time consuming and does not guarantee the determination of optimal conditions. Hence, experimental design techniques offered a considerable advantage over the one-factor-at-a-time approach for fermentation improvement (Aravindan *et al.*, 2007). Box-Behnken design proposed three level designs for fitting response surface, in which the resulting designs were usually efficient in terms of required runs, either in rotatable or nearly rotatable (Montgomery, 2001).

Filamentous fungi commonly exhibit different morphological forms in submerged culture, either in the form of mycelia filamentous or as densely interwoven mycelia masses (pellet). These morphological forms influence the mycelia growth (Feng *et al.*, 2010). In fact, the rationale design and optimization of the fermentation requires the understanding of production kinetics (Aravindan *et al.*, 2007). A kinetic model could be used to control a system for fungal fermentation process including determination of the mycelia production period and prediction of process (Feng *et al.*, 2010). It was found that an unstructured model, the Logistic model, provide a good approximation of the fermentation profile of fungal growth even though the complete mechanism is not considered (Feng *et al.*, 2010; Aravindan *et al.*, 2007).

In this present study, the optimization study of physical condition (pH, incubation temperature and agitation speed) for mycelia growth by *S. commune* was investigated using Response Surface Methodology (RSM) coupled with Box-Behnken Design (BBD). Also, to the authors' knowledge, there has been little investigation into the fungal growth behavior which is considered as one of the important parameter in industrial application. Thus, the growth kinetics of *S. commune* mycelia under optimized condition in shake flask culture was investigated.

MATERIALS AND METHODS

Fungal strain: The fungal strain, *S. commune* was obtained from the Biocomposite and Protection of Timber Forest Products Laboratory, Forest Research Institute Malaysia (FRIM), Kepong, Malaysia. The stock culture was grown on Malt Extract Agar (MEA) at 30°C and maintained on agar slants prior for subsequent studies.

Mycelia suspension preparation: Mycelia suspension was prepared by suspending mycelia discs from 7 days old culture plate in a sampling bottle containing sterilized distilled water and 0.1% (v/v) Tween 80. The disc of 5 mm diameter was punched on the mycelia mats of the agar plate using sterilized cork borer. A total of 10 discs for every 100 mL sterilized water were vortexed for 5 min in order to make the mycelia suspensions became homogeneous.

Fermentation condition: Ten milliliter (10% v/v) of the mycelia suspension was added to 90 mL medium in 250 mL Erlenmeyer flask containing following composition: Yeast extract 18 g L^{-1}, malt extract 10 g L^{-1}, glucose 38 g L^{-1}, KH_2PO_4 1 g L^{-1}, K_2HPO_4 1 g L^{-1}, $MgSO_4.7H_2O$ 0.6 g L^{-1} and $(NH_4)_2$ SO_4 2 g L^{-1}. Before transferring the mycelia into culture media, the media need to be autoclaved at 121°C for 15 min. The culture was incubated at 30±1°C, pH 6.5 in an incubator shaker at 150 rpm for 5 days. The culture broth was then harvested and centrifuged at 4000 rpm for 15 min. The mycelia produced were filtered prior placing it in an oven at 60°C for 24 h before weighing.

Experimental design using response surface methodology (RSM): RSM is a collection of mathematical and statistical techniques that are useful for the modeling and analysis of problem in which a response of interest is influenced by several variables and the objective is to optimize this response (Teoh *et al.*, 2011; Teoh and Mashitah, 2010). The behavior of the system is explained by the following quadratic equation (Feng *et al.*, 2010):

$$Y = \beta_0+\Sigma\beta_i x_i+\Sigma\beta_{ij} x_i x_j+\Sigma\beta_{ii} x_i^2 \quad (1)$$

where, Y is the predicted response, β_0 is the offset term, β_i is the linear offset, β_{ii} is the squared offset, β_{ij} is the interaction effect and x_i is the dimensionless coded value of X_i.

The RSM used in this present study was a Box-Behnken design involving three different factors, pH, incubation temperature and agitation, as shown in Table 1. The results were analyzed using Analysis of

Variance (ANOVA) by Design Expert 6.0.6 software. The simultaneous interactions of the three factors can be studied based on the three-dimensional plots. The optimum region was also identified based on the main parameters in the overlay plot. The experiment was then repeated for five times and each result obtained was compared with the predicted values in order to determine the validity of the model.

Growth kinetic of *Schizophyllum commune* mycelia: Pellet form mycelia were grown in 250 mL Erlenmeyer flask with a 10 mL of a suspension of pellets. Multiple flasks were run simultaneously and three flasks were taken each time for sampling.

As reported by Papagianni (2004), the mycelia growth kinetic of filamentous fungi in submerged culture was mainly based on the study with unicellular organisms. The Logistic equation was used to fit the biomass curve in this study. This equation is a substrate independent model and is widely used to describe the inhibition of biomass on mycelia growth (Gong and Lun, 1996). The Logistic model can be described as follows (Yazid, 2010):

$$\frac{dC}{dt}=\mu_m\left(1-\frac{C}{C_m}\right)C \quad (2)$$

where, C is the biomass (g L^{-1}), C_m is the maximum attainable biomass concentration (g L^{-1}) and μ_m is the maximum specific growth rate (h^{-1}). Then, the integrated form of Eq. 2 using $C = C_o$ (t = 0) gives a sigmoidal variation of C as a function of t which may represent both an exponential and a stationary phase, Eq. (3):

$$C(t)=\frac{C_oC_me^{\mu_m t}}{C_m-C_o+C_oe^{\mu_m t}} \quad (3)$$

The kinetic parameter, μ_m can be determined by rearranging Eq. 3 as:

$$\ln\left(\frac{C_i}{1-C_i}\right)=\mu_m t-\ln\left(\frac{C_m}{C_o}\right) \quad (4)$$

where, C_i indicated as C/C_m. If the logistic model describes the data suitably, then plot of $\ln[C_i/(1-C_i)]$ versus time (t) should give a straight line of slope as μ_m and intercept as $-\ln(C_m/C_o)$.

Table 1: Variables and levels used for Box-Behnken design

	Symbol		Level		
Independent variable	Actual	Coded	-1	0	+1
pH	X1	A	5.5	6.5	7.5
Incubation temperature (°C)	X2	B	25	30	35
Agitation (rpm)	X3	C	50	150	250

RESULTS AND DISCUSSION

Optimization of biomass production by *S. commune* using response surface methodology: A Box-Behnken Design (BBD) was used to develop a correlation between pH, incubation temperature and agitation rate in order to improve the mycelia growth by *S. commune*. There was a considerable variation in the biomass depending upon the fermentation conditions, as shown in Table 2. It can be seen that the replication at the centre point conditions resulted in higher biomass than at other levels.

Based on Table 2, the predicted response Y for the biomass is as follows:

$$Y = 32.54+1.39A+0.67B+2.39C-2.30A^2-5.97B^2-4.48C^2+1.11AB+0.51AC+0.21BC \quad (5)$$

Based on the above equation, the coefficients with one factor represented the effect of that particular factor, while the coefficients with two factors and those with second-order terms represented the interaction between the two factors and quadratic effect, respectively. In addition, the positive sign in front of the terms indicated synergistic effect, while the negative sign indicated antagonistic effect (Teoh and Mashitah, 2010).

Table 3 summarized the Analysis of Variance (ANOVA) of response surface quadratic model of mycelia growth. The p-values were used as a tool to check the significance of each coefficient and the model predicted, the smaller the value of p, the more significant was the model and corresponding coefficient (Teoh *et al*., 2011; Feng *et al*., 2010). In this study, the ANOVA of the regression model demonstrated that the model was significant, with an f-test of a very low possibility value ($p>f$) <0.0001). Also, three linear coefficients (A-C) and all

Table 2: Box-Behnken design matrix with biomass as response

	Independent variables			Response
Run	A	B	C	Biomass(g L^{-1})
1	1	0	-1	24.13±0.08
2	0	0	0	32.92±0.03
3	-1	0	0	26.38±0.07
4	0	1	-1	20.20±0.08
5	0	-1	-1	19.29±0.09
6	1	0	1	30.02±0.08
7	-1	0	-1	22.53±0.07
8	-1	1	0	22.37±0.06
9	0	0	0	31.94±0.03
10	0	1	1	25.33±0.08
11	0	0	0	32.74±0.08
12	1	1	0	27.53±0.04
13	0	-1	1	23.58±0.05
14	0	0	0	32.05±0.06
15	1	-1	0	23.96±0.08
16	0	0	0	33.07±0.05
17	-1	-1	0	23.23±0.02

quadratic coefficients were significant, as were two of the interaction coefficients (AB, AC). The insignificant coefficients were not omitted from Eq. 5, since it was a hierarchical model.

Table 3: Analysis of variance (ANOVA) of response surface quadratic model of mycelia growth

Source	Sum of squares	Mean square	f-value	(p)>F
Model	353.160	39.240	243.39	<0.0001
A	15.510	15.510	96.160	<0.0001
B	3.600	3.600	22.330	0.0021
C	45.850	45.850	284.30	<0.0001
A^2	22.330	22.330	138.45	<0.0001
B^2	149.950	149.950	929.69	<0.0001
C^2	84.370	84.370	523.11	<0.0001
AB	4.920	4.920	30.470	0.0009
AC	1.050	1.050	6.4800	0.0383
BC	0.180	0.180	1.1000	0.3282
Residual	1.130	0.160		
Lack of fit	0.065	0.022	0.081	0.9670
Pure error	1.060	0.270		
Cor total	354.290			
SD	0.400		Adj. R^2	0.993
R^2	0.997		Pred. R^2	0.992

Adj.: Adjusted; Pred.: Predicted

On the other hand, the value of determination coefficient, R^2 (0.997) for Eq. 5 suggested that the sample variation of 99.7% for biomass was attributed to the independent variables and only about 0.3% of the total variation could not be explained by the model. The Pred. R^2 of 0.992 was in reasonable agreement with the Adj. R^2 of 0.993.

The 3D-surface plot could visually show the response over a region of interesting factor levels, the relationship between the response and experimental levels of each variable and the type of interaction between test variables in order to deduce the optimum condition (Teoh *et al*., 2011; Feng *et al*., 2010). Figure 1(a) represented the effect of pH and incubation temperature on the biomass, while the agitation rate was fixed at 150 rpm. This plot showed that the biomass production

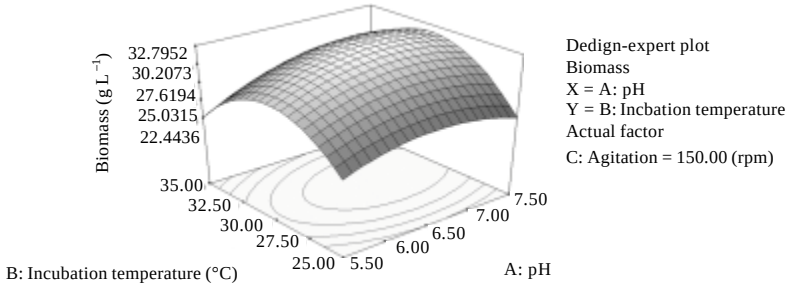

Fig. 1(a): The 3D plot showing the interaction between pH and incubation temperature at 150 rpm on biomass

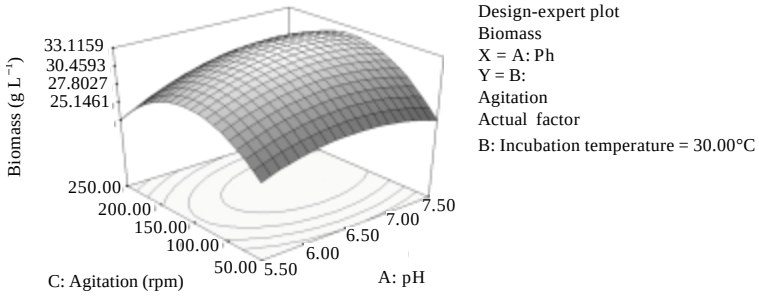

Fig. 1(b): The 3D plot showing the interaction between pH and agitation at 30°C on biomass

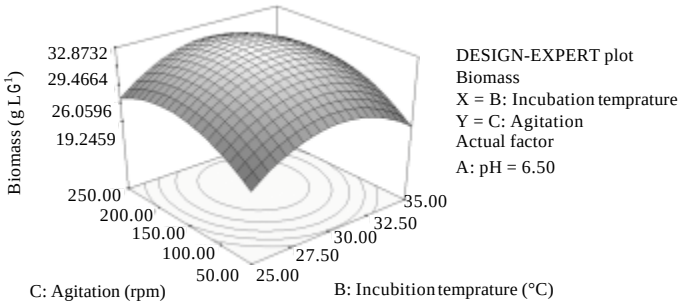

Fig. 1(c): The 3D plot showing the interaction between incubation temperature and agitation at pH 6.5 on biomass

significantly increased upon increasing temperature up to about 30°C. Beyond this point, a sharp decreased trend was observed. This phenomenon proved that the effect of incubation temperature on the growth was sensitive within the tested range, in which a maximal yield could be obtained. With this, the same trend is also indicated in Fig. 1b and c.

Optimum range of parameters: Design-expert plot illustrates the interaction between pH, incubation temperature and agitation speed on biomass production. The optimal conditions to obtain the maximal growth by *S. commune* were initial pH 6.78, incubation temperature at 30.23°C and agitation speed of 174.14 rpm (Fig. 2). In this present study, the desirability value was 1.000, which is evidence for the application of this model.

Verification experiments: In order to confirm the model adequacy, five sets of experiments were repeated randomly at optimum condition to obtain a maximum growth by *S. commune* experimentally. As shown in Table 4, the percentage error difference between the experimental and predicted value were in the range of 0.213-0.602%. Since, the differences between actual and predicted response were always less than 1%, thus providing its validity.

Table 4: Validation of the data and model constructed

Run	Experimental result (g L^{-1})	Predicted result (g L^{-1})	Error (%)
1	32.9500	33.1404	0.575
2	33.3110	33.1404	-0.515
3	33.0020	33.1404	0.418
4	33.2110	33.1404	-0.213
5	32.9410	33.1404	0.602

Table 5: Kinetic model parameters of mycelia biomass production by *Schizophyllum commune*

Kinetic parameters	Kinetic values
C_o (g L^{-1})	0.1922
C_m (g L^{-1})	32.8515
μ_m (h^{-1})	0.0514
Correlation coefficient (R^2)	0.9649

Growth kinetics of *S. commune* under optimized condition: Figure 3 shows the batch culture mycelia growth with time during fermentation. This growth curve was a typical S-figure curve, where after the lag phase (approximately 1 d); the mycelia growth suddenly entered the exponential phase. This phenomenon could be due to the fact that fungus had adapted to the environment and had sufficient source to catabolism the carbon sources in the production medium (Yazid, 2010).

The values from regression are presented in Table 5. The coefficient of determination (R^2), is a measure of the strength of the linear relationship between the experimental and predicted values, in which greater the proportion of the explained variation, the stronger was the degree of linear relationship (Aravindan *et al*., 2007). The kinetic profile of mycelia growth was well described by the Logistic model with R^2 value (0.9649) closer to 1.0. Based on the fitted model, the simulated value of C_m was lower than the experimental value (32.9361 g L^{-1}) by 0.26%. This could perhaps be attributed to the arrival of the decline phase (Feng *et al*., 2010). In this present study, the value of μ_m for the pellet form mycelia was at 0.0514 h^{-1}. This result was in good agreement with the study done by Feng *et al*. (2010), who's reported that the pellet form had a lower production rate and longer growth cycle as compared to the filamentous form of mycelia.

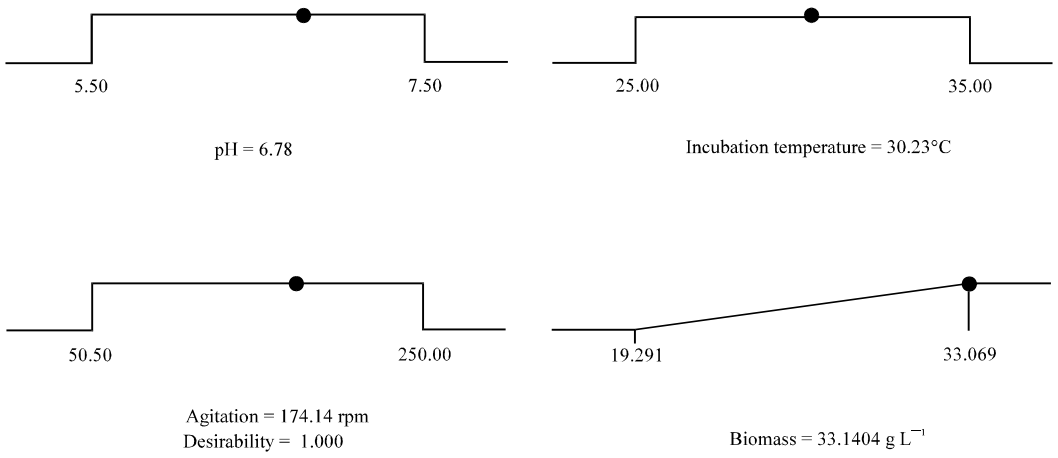

Fig. 2: Optimal condition and the desirability for growth by *Schizophyllum commune*

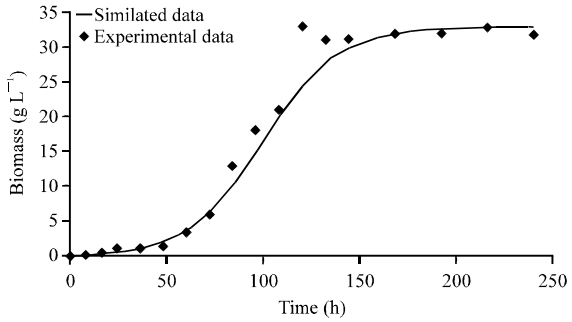

Fig. 3: The experimental data and regression curve of mycelia biomass produced by *Schizophyllum commune*

CONCLUSION

A statistical tool, RSM was used to optimize the mycelia growth by *S. commune* with pH, incubation temperature and agitation speed as the coded variables. A result showed that the highest biomass produced (33.1404 g L^{-1}) was detected at pH = 6.78, temperature 30.23°C and agitation speed 174.14 rpm in shake flask culture. Meanwhile, the experimental data fitted well with the model predicted values within 0.213-0.602% error. In addition, the Logistic model was used to predict the mycelia growth behavior in shake flask culture. It was found that the experimental data was well fitted into the model with $R^2 = 0.9649$.

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