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Production of *Ganoderma lucidum* mycelium using cheese whey as an alternative substrate: response surface analysis and biokinetics

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Abstract

A novel approach to utilize cheese whey, cultivating mycelium of an edible mushroom *Ganoderma lucidum* using cheese whey as a substrate, was introduced. Response surface analysis (RSA) with central composite in cube design was successfully applied to determine the optimal conditions where the maximum mycelial production occurred, which was at pH 4.2 and 28.3 °C. The high extract ratio as well as high content of polysaccharide (i.e., 1.2 g/l) indicated that the whey could be an alternative substrate for the mycelial production. Soluble chemical oxygen demand (SCOD) removal ranged from 80.7 to 93.1% within the design boundary. Therefore, cultivation of *G. lucidum* mycelia using cheese whey can provide a unique solution to solve the dual problems of an alternative utilization of the whey and waste management. The substrate inhibition biokinetics at the optimal conditions were also evaluated using a method of fourth-order Runge–Kutta approximation. The nonlinear least-squares (NLLS) method with 95% confidence interval was used. The maximum microbial growth rate, μ_{max}, and half saturation coefficient, K_s, for lactose and SCOD were determined to be 2.28 ± 0.11 and 2.27 ± 0.15 per day, and 95.5 ± 9.1 and 128.0 ± 12.1 g/l, respectively. The microbial yield coefficient, Y, and microbial decay rate coefficient, k_d, for lactose and SCOD were determined to be 0.49 ± 0.03 and 0.39 ± 0.03 g VSS/g of each substrate, and 0.05 ± 0.01 and 0.05 ± 0.01 per day, respectively. Inhibition coefficients were 37.6 ± 2.9 and 49.3 ± 3.3 g/l for lactose and SCOD, respectively.

Keywords: Bioconversion; Biokinetics; *Ganoderma lucidum*; Modeling; Optimization; Wastewater treatment

1. Introduction

Cheese whey is a by-product of cheese production that remains when casein and butter fat are separated as curd from milk. Depending on the type of cheese being made, up to 9 l of whey is generated for every kilogram of cheese produced. The organic matter in cheese whey causes a high chemical oxygen demand (COD) in the range of 40–70 g/l [1,2]. Cheese production and the resultant whey by-product worldwide, which was approximately 150 million tonnes in 2001 [3], has been one of the largest single visible sources of potential pollution. Continued growth of the cheese industry, necessity of reducing pollutants in the effluent, and the need to maximize returns on raw material encourage producers to seek new ways of using cheese whey.

Dairy wastes should be viewed as an inexpensive potential source of raw material from which valuable products can be produced. Cheese whey includes approximately half of the original nutrients of milk; 4% lactose, nitrogenous compounds, trace minerals and vitamins make it nutritionally valuable [4]. In this research, we hypothesized that a unique solution to solve the cheese whey management problem and to reduce high disposal cost would be to use cheese whey as an alternative substrate for cultivating mycelium of an edible mushroom, *Ganoderma lucidum*.

Mycelium of *G. lucidum* is widely used as an ingredient in many health foods and therapeutic medicines because of its perceived health benefits [5,6]. Recent increase in human consumption of *G. lucidum* is due to the fact that the mushroom is low in calories and rich in vegetable proteins, chitin, vitamins, and minerals [7,8]. Fruiting bodies of mushrooms including *G. lucidum* have traditionally been produced in solid cultures using substrates such as grain, sawdust or wood. However, it usually takes several months to complete a fruiting body culture, and it is often difficult to control the product quality. Therefore, submerged fermentation for mycelial culture has recently received great interest as a promising alternative for efficient production of cellular materials and valuable metabolites such as polysaccharide mainly due to its short period of mycelial cultivation (i.e., usually less than 2 weeks) [9–11].

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The control of environmental conditions as well as the modification of media composition has been vital in order to enhance the production efficiency in mycelial culture [7,9]. Despite this effort, little information is available regarding the optimization of environmental factors affecting the growth of *G. lucidum* for various substrates in submerged culture. Furthermore, biokinetics of *G. ludicum* using whey, essential to control and to predict the production efficiency, is lacking in the literatures. Therefore, the objectives of this research were to: (1) find the optimal conditions with respect to the simultaneous effects of pH and temperature where mycelial production of *G. lucidum* is maximized using cheese whey, and (2) evaluate the biokinetics of the mycelium at the optimal growth conditions.

2. Materials and methods

2.1. Cheese whey as a substrate

Dried whey powder from Samik Co., Korea, was dissolved in distilled water (57.1 g/l dry powder) to obtain the lactose concentration of typical cheese whey (i.e., 40 g/l lactose) because lactose is the major carbonaceous compound in cheese whey. The resulting whey was used as a substrate for cultivating mycelia of *G. lucimum*. Since cheese whey contains most of the essential nutrients for microbial growth, and in order to obtain information about treatment of raw cheese whey, no additional nutrients were added. However, the content of ammonium nitrogen and phosphorus in the form of orthophosphate was carefully monitored in every trial in order to ensure these two critical nutrients were not limiting.

2.2. Microbial strain and culture conditions

G. lucidum (KCTC 6283) was obtained from the Korean Collection for Type Cultures (KCTC) and was maintained in a potato dextrose agar (PDA) slant at 4 °C. The seed culture of *G. lucidum* was transferred to Petri dishes containing PDA media and incubated at 25 °C for 4 days. Mycelial agar discs (5 mm) were obtained by a round cutter and were used as inocula for subsequent experiments.

Three identical fermentors with working volumes of 4 l, equipped with temperature, pH, and dissolved oxygen (DO) controllers, were used to incubate the mycelia in batch mode. Each bioreactor was inoculated with 15 agar discs containing *G. lucidum* that was grown on the surface of PDA medium as described previously. Air, purified through filters, was supplied to the bioreactors at a rate of 1 (v/v)/min to maintain a minimum DO concentration of 2 mg/l. Buffers of 2 N sodium hydroxide and 2 N sulfuric acid were separately used to adjust the pH to the desired levels. Foam was controlled using 10% antifoaming agent (A5758, Sigma).

2.3. Optimization and evaluation of biokinetics

Because pH and temperature have been key variables to maximize the mycelial production of various edible mushrooms in submerged cultures, the mycelial growth of *G. lucidum* associated with simultaneous changes in these two parameters was examined. The initial starting points of pH and temperature were selected as close as possible to the literature values of similar conditions [9,12,13].

Response surface analysis (RSA) was applied to optimize the factors affecting the growth (i.e., temperature and pH). In this experiment, "optimum conditions" meant the operating conditions for maximizing the mycelial production within the investigated space of the independent variables. A sequential procedure of collecting data, estimating polynomials (Eq. (1)), and checking the adequacy of the model was used:

$$\eta = c_0 + \sum_{i=1}^{n} \alpha_i x_i + \sum_{i=1}^{n} \alpha_{ii} x_i^2 + \sum_{\substack{i \\ i<j}} \sum_{j} \alpha_{ij} x_i x_j \quad (1)$$

where η is the experimental value of mycelial concentration (g/l), x_i the independent variable i (i = pH and temperature in order), c_0 the regression constant, α_i the regression coefficients of the independent variable i (i = pH and temperature in order).

The method of least-squares was used to estimate the parameters in the approximating polynomials. The central composite in cube (CCC) design [14], which consists of an orthogonal 2^2 factorial design augmented by a center and 2×2 axial points (Table 1), was employed in this research.

Batch data during fermentation at the estimated optimum conditions were used to evaluate the system performance and growth kinetics of *G. lucidum*. We used the substrate inhibition biokinetic expressions for microbial growth and substrate utilization [15], and the numerical approximations are given as

$$X_{t+1} = \left\{ 1 + \left(\frac{\mu_m S_t}{K_s + S_t + (S_t^2 / K_{si})} - k_d \right) \Delta t \right\} X_t \quad (2)$$

Table 1
Experimental conditions and results of the central composite design

Trials	Conditions of variables		Responses[a]	
	pH	Temperature (°C)	Mycelial dry weight (g/l)	Residual SCOD concentration (g/l)
1	3.5	25	14.3	6.8
2	4.5	25	16.5	4.1
3	3.5	35	13.4	10.3
4	4.5	35	15.1	7.8
5[b]	4.0	30	17.0 (0.5)	4.5 (0.8)
6	4.0	37.1	14.0	8.5
7	4.0	22.9	15.8	6.2
8	4.7	30.0	16.3	4.6
9	3.3	30.0	14.3	8.9

[a] The values in parenthesis refer to standard deviation.
[b] Center point. Experiment was replicated five times and the response presented average values.

$$S_{t+1} = \left(1 - \frac{\mu_m}{Y} \frac{X_t}{K_s + S_t + (S_t^2/K_{si})} \Delta t\right) S_t \quad (3)$$

where X_t is the microbial concentration in the bioreactor at time t (microbial mass/volume), μ_m the maximum specific growth rate (time^{-1}), S_t the residual substrate concentration at time t (mass substrate/volume), K_s the half saturation coefficient numerically equal to the substrate concentration at which specific growth rate is half of its maximum (mass substrate/volume), K_{si} the substrate inhibition coefficient (mass substrate/volume), k_d the specific decay rate of the microorganism (time^{-1}), Δt the time increment (time), Y the microbial yield coefficient (microbial mass/mass substrate utilized).

A fourth-order Runge–Kutta approximation [16] along with a multiresponse, nonlinear least-squares (NLLS) method was employed to approximate kinetic coefficients with 95% confidence interval (CI).

2.4. Analytical methods

The COD of the whey and mixed liquor of the fermentors was measured by the closed reflux colorimetric method [17]. Two identical ion-exchange chromatographs (790, Metrohom) were used to quantify the cations and anions in the samples. A high performance liquid chromatograph (HPLC 1100 series, Agilent) equipped with a refractive index detector was used to quantify lactose.

Samples taken at each stationary growth phase were filtered using Whatman no. 2 filters and dried at 103 °C to measure concentration of mycelial dry mass in freely suspended culture. Total polysaccharide in the culture medium was determined by phenol-sulfuric acid assay [6]. The amount of protein was measure according to the Kjeldahl method [17]. An automated turbidity reader (Bioscreen C, Labsytems) was used to estimate mycelial growth rate at different substrate concentrations to validate the model accuracy.

3. Results and discussion

3.1. Optimizing mycelial production of G. lucidum using cheese whey

A total of 13 trials were run to approximate the response surface of the mycelial production of *G. lucidum*. Initial substrate concentration was 53.0 g/l soluble COD (SCOD). For all trials, the presence of nitrogen, with a minimum observed value of 40 mg/l NH_4^+, and phosphorus, which was 250 mg/l PO_4^{3-}, indicated that these essential nutrients were not limiting.

In order to find a maximum in the response, models from first to partial cubic were sequentially tested with the trials (Table 1). The *P*-values of regression, lack of fit, and corresponding coefficients were also tested. Residual variance and plots were simultaneously analyzed to discriminate models if multiple models were statistically significant to describe the response. Regression was significant at 1% α level and lack of fit was not significant at 5% α level for quadratic model, which was

$$\eta = -9.1 \times 10^4 + 3.3 \times 10^4 x_1 + 2.7 \times 10^3 x_2 - 52 x_1 x_2 - 3.7 \times 10^4 x_1^2 - 44 x_2^2 \quad (4)$$

The regression coefficient and residual standard deviation of the quadratic model were 0.95 and 0.4, respectively. This indicated that curvature existed in the response surface of mycelial dry weight within the experimental region.

The optimal conditions for mycelial production were calculated by setting the partial derivatives of the function to zero with respect to the corresponding independent variables of pH and temperature, which were pH 4.2 and 28.3 °C, respectively. The calculated model output at the optimal conditions was 18.1 ± 0.9 g/l dry weight.

Two- and three-dimensional response surfaces of the quadratic model for mycelial production, with estimated optimums, are shown in Fig. 1. The response surface of the mycelial production with respect to pH and temperature showed a clear peak with constant contour lines. This meant that the two independent variables were not interdependent and the combined effect on the mycelial production of *G. lucidum* using cheese whey was not significant.

The same protocol, applied in optimizing the mycelial production, was separately applied to approximate the response surfaces of the residual SCOD concentration at the stationary growth phase (Table 1). The partial cubic model

$$\text{SCOD} = -309752 + 169858 x_1 + 13652 x_2 - 7534 x_1 x_2 - 19837 x_1^2 - 7 x_2^2 + 16 x_1 x_2^2 + 821 x_1^2 x_2 \quad (5)$$

was selected to describe the response surface based on the statistical significance. The SCOD removal ranged from 80.7 to 93.1% within the design boundary, where the conditions for maximum SCOD removal were pH 4.6 and 27.1 °C. The model response at the estimated conditions was 3.6 ± 0.6 g/l SCOD, which was 93.1% SCOD reduction. Using Eq. (5), the residual SCOD concentration at the optimum conditions for mycelial production was 3.8 g/l, which was close to the minimum value. This indicates the grade of this elliptical region is not steep, thus the optimum conditions for mycelial production were likely to be used for the overall process parameter to maximize mycelial production of *G. lucidum* using whey with nearly maximum reduction of SCOD concentration.

The adequacy of the model prediction was verified by comparing the maximum model output with duplicated experimental values at optimal conditions; residual plots for all observed values were then examined for any weakness in the models [14]. The experimental value at the optimum conditions was 20.1 ± 0.8 g/l dry mycelia, which was close to the model output. The residual plots for the models and data set

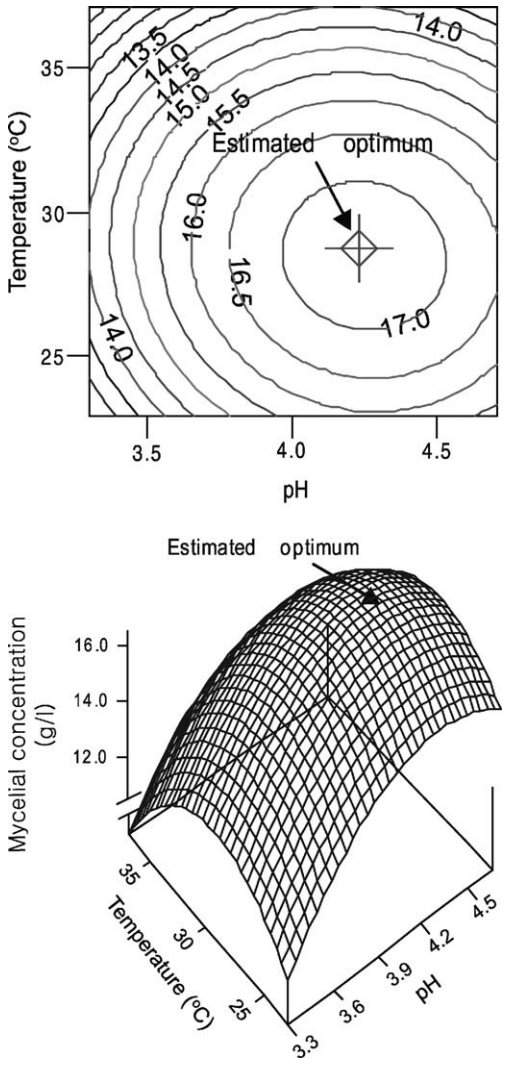

Fig. 1. Two- and three-dimensional contour plots of the quadratic model for the mycelial production with respect to pH and temperature.

showed no patterns or trends (Fig. 2). A check of the constant variance assumption could also be addressed because a random plot of residuals meant homogeneous error variances across the observed values. Excellent prediction of maximum response along with constant variance in residual plots indicated adequacy of the model, which meant the quadratic model allowed the prediction of response of mycelial production in this work, but within the studied range of temperature and pH. Therefore, it could be concluded that the RSA with the CCC design could be used to locate the conditions that maximized the mycelial production of *G. lucidum* using cheese whey within the investigated experimental region.

The polysaccharides in the extract of *G. lucidum* mycelia are glucans such as branched 1,3-β-D-glucans, which are used in a variety of commercial applications including health foods and medicines [9]. Because the polysaccharide content as well as productivity of the extract is an important parameter to indicate process efficiency in mycelial cultivation [11], we investigated extraction efficiency and characteristics of the extract from *G. lucidum* mycelia cultivated at the optimal conditions.

A total of 1.8 g/l extract was obtained from the submerged culture. Polysaccharide, protein, phosphorus, and potassium were the major components composing 66.9% of dry weight of the mycelial extract and corresponding concentrations were 1.210 ± 13, 32 ± 3, 40.2 ± 1.0, and 24.9 ± 3.8 mg/l. Although the production of *G. lucidum* polysaccharides is greatly dependent on culture conditions such as pH, temperature, and types of carbon sources, typical yield usually varies from 0.6 to 1.0 g/l polysaccharide using glucose medium [9,11]. The high yield of extract as well as high content of polysaccharide (i.e., 1.2 g/l) in this experiment indicated that the cheese whey could be an alternative substrate for mycelial production of *G. lucidum*. Therefore, cultivation of *G. lucidum* mycelia along with high SCOD stabilization can offer a potential cost-effective solution for an alternative utilization of cheese whey.

3.2. Growth kinetics of *G. lucidum* mycelium

Fig. 3 represents the changes of microbial and residual substrate concentrations in batch culture of the mycelium at the optimal conditions. The mass of *G. lucidum* gradually increased to 20.1±0.8 g/l dry weight for 172 h of incubation. Residual substrate concentration decreased to 3.5 ± 0.1 g/l SCOD for the same period, which was a 93.4% reduction of the initial wastewater strength. Nearly complete utilization of lactose, 99.1% of the initial concentration, was achieved.

The concentrations of mycelial cells, SCOD, and lactose shown in Fig. 3 were simultaneously used to estimate biokinetic coefficients in Eqs. (2)–(4). The substrate inhibition model was used to fit the data because the residual analysis of the model with the experimental data showed less and constant variance compared to the classical Monod expression. Table 2 summarizes the values of biokinetic coefficients, which were verified by comparing the simulated responses of SCOD, lactose, and cell concentrations with observed values. It should be noted that the rate of lactose degradation was relatively slower than that of SCOD (Fig. 3). This indicated that the rate of microbial utilization

Table 2
The values of biokinetic coefficients using data from batch culture of *G. lucidum*

Kinetic parameters	Biokinetic values (mean ± 95% CI)	
	Lactose[a]	SCOD[b]
μ_m (per day)	2.28 ± 0.11	2.27 ± 0.15
k_d (per day)	0.05 ± 0.01	0.05 ± 0.01
K_s (g/l substrate)	95.5 ± 9.1	128.0 ± 12.1
K_{si} (g/l substrate)	37.6 ± 2.9	49.3 ± 3.3
Y (g dry weight/g substrate)	0.49 ± 0.03	0.39 ± 0.03

[a] Biokinetic coefficients were evaluated based on lactose concentration.
[b] Biokinetic coefficients were evaluated based on SCOD concentration.

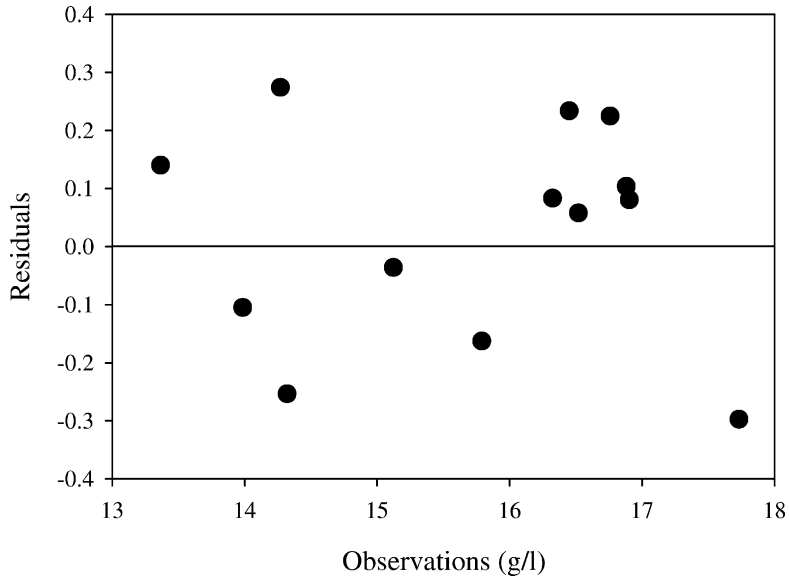

Fig. 2. Residual plots of the quadratic model for mycelial concentration at stationary growth phase.

of other organics in cheese whey, mostly milk protein, was higher than that of lactose, which is a major SCOD contributing organic in cheese whey. Therefore, it was likely that *G. lucidum* utilized the protein faster than lactose.

Fig. 4 shows that rapid mineralization of free ammonia (i.e., as low as 42.0 ± 2.0 mg/l) occurred in about 36 h of incubation and then the ammonia concentration gradually increased to 91.8 ± 5.3 mg/l toward the end of incubation. Since ammonia nitrogen exists mostly in the form of ammonium ion at acidic condition [18], the loss of nitrogen due to ammonia volatilization was negligible in the experiments. Therefore, the rate of ammonia formation resulting from protein degradation exceeded the rate of ammonia uptake by microbial activities, thus ammonia accumulated in the system. Phosphate, another essential nutrient, was not growth limiting. The change of phosphate concentration was similar to that of ammonia reduction for 48 h of incubation, which might be another indication of the rapid mycelial uptake of the nutrients at early growth stage. Concentration of phosphate in the medium, however, gradually decreased to 295.0±24.7 mg/l, which was 53.2% reduction of the influent concentration. Continuous increase in ammonia concentration along with gradual decrease in phosphate concentration after 84 h of incubation also indicated that the mycelia continuously utilized the protein as their carbon and ammonia sources.

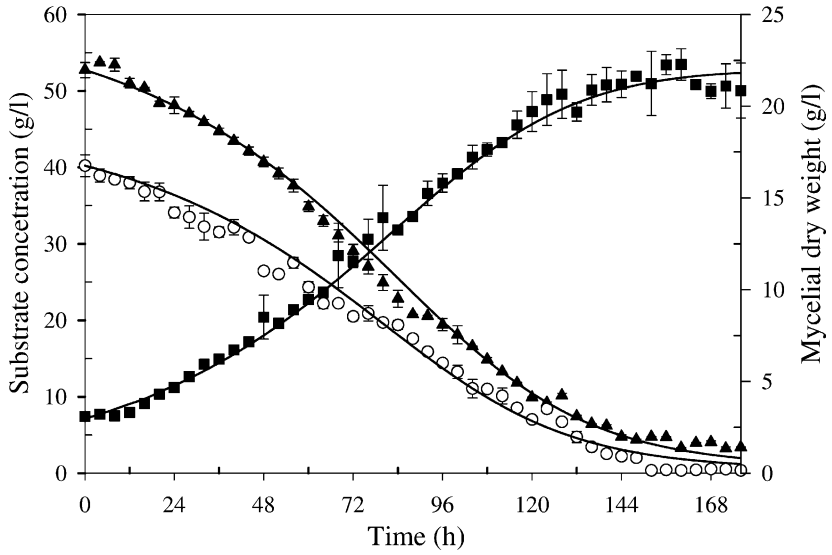

Fig. 3. Observed and predicted concentrations of substrates and *G. lucidum* in batch culture: (■) observed mycelial mass; (○) observed lactose; (▲) observed SCOD; (—) model predictions.

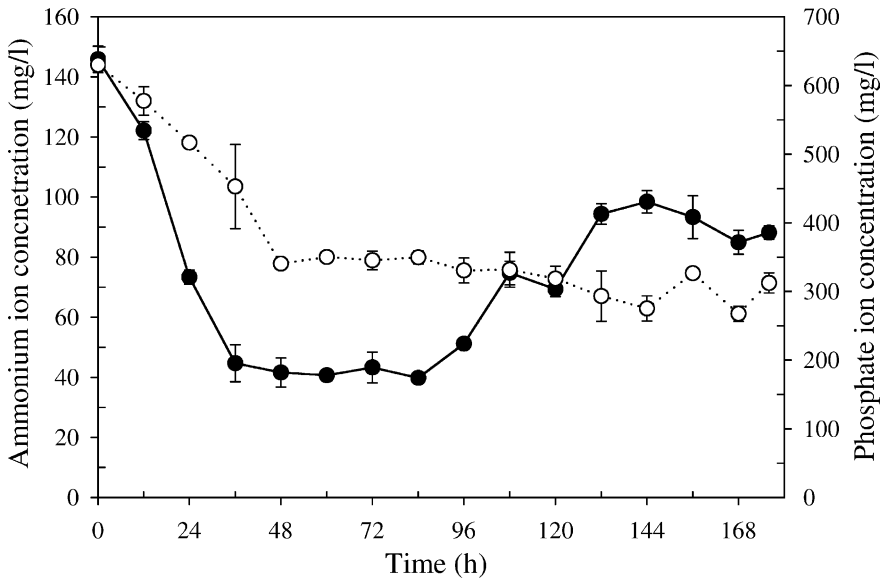

Fig. 4. The change of residual concentrations of ammonium and phosphate in batch culture of *G. lucidum*. Bars show standard errors of triplication: (●) ammonium; (—) phosphate.

An additional four trials of different whey powder concentrations, equivalent to the lactose concentrations of 20, 30, 50 and 90 g/l, were conducted to validate the model adequacy. The SCOD concentrations at corresponding lactose levels were 26.5, 39.7, 66.2 and 119.2 g/l. The experimental specific growth rates using new substrate concentrations were compared to the simulated responses using Eq. (6), and are shown in Fig. 5:

$$\mu = \frac{\mu_m S}{K_s + S + (S^2/K_{si})} \quad (6)$$

where μ is the specific growth rate of the microorganism (time^{-1}).

From Figs. 3 and 5, we could confirm the model outputs were in good agreement with the experimental values. Therefore, it could be concluded that the kinetic coefficients estimated in this study could be used to design and to control a system for cultivating mycelium of *G. lucidum* with the cheese whey as a substrate. This includes determination of the mycelial production period and prediction of process performance, such as a degree of pollution reduction as well as the amount of the mycelium produced per unit time period in a scaled-up process.

It must be recognized that the kinetic coefficients are variables as functions of process conditions like temperature or substrate characteristics. The kinetic values may

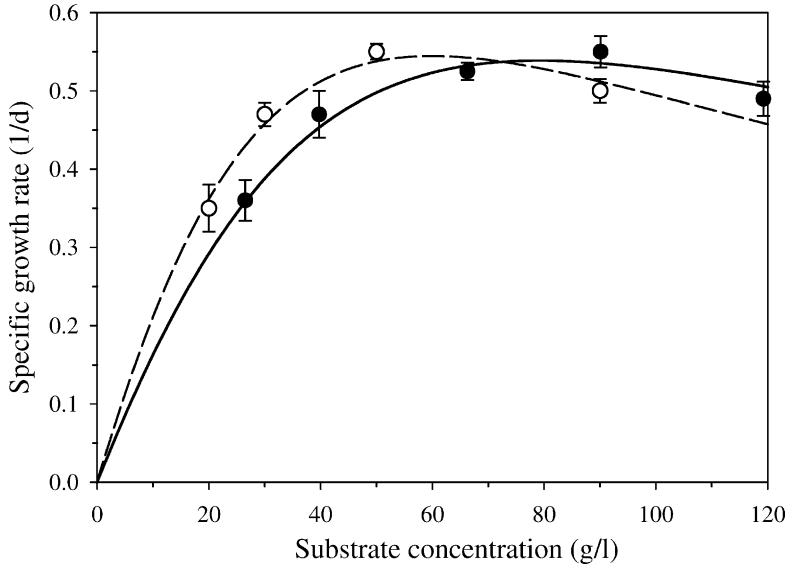

Fig. 5. Observed and predicted specific growth rate of *G. lucidum* at different substrate concentrations. Specific growth rate based on: (●) observed SCOD; (○) observed lactose; (—) calculated SCOD; (- - -) calculated lactose.

vary if some conditions differ from those used in this experiment.

4. Conclusions

For bioconversion of cheese whey, a set of experiments was carried out to cultivate mycelia of *G. lucidum*. The following conclusions are based on the results of the data from batch fermentations:

1. Response surface methodology was successfully applied to determine the optimal physiological conditions for the maximum production of the mycelia. These conditions were found to be at a pH of 4.2 at 28.3 °C. The predicted and experimental values for mycelial weight at the optimal conditions were 18.1 ± 0.9 and 20.1 ± 0.8 g/l dry mycelia, respectively. The SCOD removal ranged from 80.7 to 93.1% with a minimum of 3.5 ± 0.1 g/l residual SCOD.
2. The high extract ratio along with a high content of polysaccharide of 1.2 g/l at the optimal conditions indicated that cultivation of *G. lucidum* mycelia would offer a cost-effective solution as an alternative treatment of cheese whey wastewater.
3. A close association between experimental and model outputs indicated that the substrate inhibition biokinetics can be used in designing a system for producing mycelium of *G. lucidum* using cheese whey. These include a production schedule and amount of the mycelial production as well as reduction of waste strength.

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