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ORIGINAL PAPER

Mycelial cultivation of *Phellinus linteus* using cheese-processing waste and optimization of bioconversion conditions

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Abstract A medicinal mushroom, *Phellinus linteus*, was successfully cultivated using a cheese-processing waste, whey, and the optimal bioconversion conditions for the maximum mycelial growth rate was also estimated through solid-state cultivation experiments. Response surface analysis with a face-centered design (center point replication = 5) was applied to statistically approximate the simultaneous effects of the three variables, i.e., substrate concentration (10–30 g lactose l^{-1}), temperature (20–30°C), and pH (4–6), on the mycelial growth rate of *P. linteus*. The following is a partial cubic model where η is the mycelial growth rate (K_r) and x_k is the corresponding variable term (k = substrate concentration, temperature, and pH in order): $\eta = -23.8 + 8.67 \times 10^{-2}x_1 + 1.48x_2 + 1.77x_3 + 8.00 \times 10^{-4}x_1x_2 + 7.25 \times 10^{-2}x_1x_3 + 5.13 \times 10^{-2}$ $x_2x_3 - 1.28 \times 10^{-2}x_1^2 - 3.18 \times 10^{-2}x_2^2. - 2.64 \times 10^{-1}$ $x_3^2 \quad -3.28 \times 10^{-3}x_1x_2 \quad x_3 + 4.68 \times 10^{-4}x_1^2x_2$. The produced response surface model proved to be significant ($r^2 > 0.99$, P-value <0.0001, coefficient of variation <5%) to describe the explored space. Temperature was found to be the most significant factor of dominant effects on the mycelial growth rate, and other variables such as temperature^2, pH, pH^2, and (substrate concentration^2 × temperature) also showed significant effects on the model output. The maximum mycelial growth rate was predicted to be 2.80 mm d^{-1} at 29.7 g lactose l^{-1}, 26.2°C, and pH 5. Our results proved a good potential of whey to serve as an alternative growth medium for cultivating *P. linteus* mycelia. This may provide another potential for managing this nutrient-rich waste in a cost-effective way.

Keywords Cheese-processing wastewater · Mushroom mycelia · Mycelial cultivation · *Phellinus linteus* · Response surface analysis

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Introduction

Mushrooms have been extensively used as a source of nutritional diet and folk medicine since ancient times. The consumption of mushrooms these days is growing fast due to the balanced nutritional composition and remedial effects (Seo et al. 2003). Especially, *Phellinus linteus* is among the most well known medicinal mushrooms for its extraordinary efficacy in

suppressing cancer and potentiating immunity (Wasser 2002; Zhu et al. 2008). Although the complex nature of pharmacological constituents in *P. linteus* is yet unidentified, recent studies have identified several bioactive substances of immunomodulating and/or anticancer effects (Zhu et al. 2008). In addition, contrary to conventional chemical medicines, these biologically synthesized natural compounds are free from toxic or side effects (Kim et al. 2003). This unique feature of *P. linteus* makes it a promising source for developing alternative anticancer agents or novel immunity boosters.

Phellinus linteus in the wild grows on hardwood trees such as mulberry and elm, but its natural fruiting body is very rare and as a result very expensive. Although the artificial cultivation of the fruiting body is possible, it requires complicated steps of initial mycelial cultivation, spawn body preparation, colonization on logs, and further cultivation on logs with temperature and humidity control (Hong et al. 2002; Hur 2008), which make it time-consuming and less-economic. When we move our focus from the mushroom to the bioactive substances it produces, there is no need to wait for a fruiting body to develop since the biocompounds should also be extracted from the mycelia (Hatvani 2001). This simple fact has triggered studies on the mycelial cultivation of medicinal mushrooms including *P. linteus* in solid-state and submerged growth settings (Hwang et al. 2003; Kim et al. 2002; Zou et al. 2009). However, previous studies mostly have focused on forming higher amount of target biocompounds and used sterile synthetic media containing pure sugars, suggesting a margin for cost reduction. Recent efforts to cultivate useful mushroom mycelia on organic wastes like food-processing byproducts, have demonstrated a high potential use of waste organics as economic alternative substrates (Hatvani 2001; Lee et al. 20032008a). However, little study has been so far conducted on waste-based cultivation of *P. linteus*, except a recent work which used a starch-processing waste (Lee et al. 2008b). On this, we tested another strong candidate for its use as a substrate supporting *P. linteus* cultivation.

Whey is a high-strength organic waste produced in bulk from cheese-processing industry. It is nutrient-rich and easily biodegradable, and thus has a wide use from food additives to health supplements (Pescuma et al. 2008). World whey production in 2006 was about 41 million tons equivalent to 2 million tons of lactose, the major organic compound in whey, but only 43% were used to process whey products (Ling 2008). This means an enormous amount of whey is still to be disposed as waste, causing high treatment costs and environmental problems. More options to utilize whey are desired to mitigate this matter. Given the high nutrient concentration, a promising way to use whey is as a substrate for microbial growth. Whey has been successfully used as substrate in diverse microbial systems such as acid/alcohol fermentation (Demirer et al. 2000; Kim et al. 2008; Lee et al. 2006; Pescuma et al. 2008; Tejayadi and Cheryan 1995), indicating its potential to support microbial growth. This study thus aimed to evaluate the applicability of whey as a substrate for *P. linteus* cultivation, achieving mycelia production and pollution reduction at once. We also statistically estimated the optimal bioconversion conditions for the maximum mycelial growth rate. For this purpose, temperature, pH, and substrate concentration were tested as affecting factors on growth. A solid-state cultivation (SSC) technique was employed due to the advantage of less time and labor requirements compared to a liquid-state method (Lee et al. 2008b).

Materials and methods

Wastewater and mycelial cultivation

Powdered whey was dissolved in distilled water to have different concentrations of lactose for subsequent experiments. Since the purpose of this study is to evaluate the potential use of whey as an alternative substrate for *P. linteus* cultivation, no supplementary nutrients were added. The rehydrated whey solutions were solidified by adding agar (1.5% w/v) followed by autoclave at 120°C for 20 min. Before pouring into petri dishes, pH was adjusted using 0.5 M HCl or 0.5 M NaOH solutions to meet the designed experimental conditions in Table 1. The solidified plates were used as the growth media for *P. linteus* mycelial cultivation.

Phellinus linteus (KCTC 6719) was obtained from Korean Collection for Type Cultures and inoculated to potato dextrose agar (PDA) plates. After 4 days of incubation at 25°C, a round-cut mycelial agar disk (diameter, 5 mm) was transferred onto the center of

Table 1 Experimental design and observed mycelial growth rates

Trials	Conditions			Observation
	Substrate concentration (g lactose l^{-1})	Temperature (°C)[a]	pH	Mycelial growth rate (K_r, mm d^{-1})
First order design				
1	10	20	4	1.00
2	30	20	4	0.79
3	10	20	6	1.46
4	30	20	6	1.53
5	10	30	4	1.19
6	30	30	4	2.26
7	10	30	6	2.02
8	30	30	6	2.06
9[b]	20	25	5	2.74 ± 0.05
Second order design				
10	10	25	5	2.29
11	30	25	5	2.75
12	20	25	4	2.14
13	20	25	6	2.60
14	20	20	5	1.73
15	20	30	5	1.95

[a] Temperature

[b] Center point was replicated five times (average value ± standard deviation)

each whey agar plate. The inoculated whey plates were then incubated at different temperatures as per the conditions in Table 1. The growth of *P. linteus* was monitored every 24 h during 10 day of incubation. Since the colonies grew in a circular fashion, the mycelial growth was measured in radial extension (Sang et al. 2001). The radius was measured using a laboratory calipers in four different positions and the average value was plotted over time (Lee et al. 2008b). The slope of the linear regression line of the resulting plot, i.e., the average radial extension rate (K_r), was assumed to be the mycelial growth rate under the given conditions (Sang et al. 2001).

Experimental design for response surface analysis

Response surface analysis (RSA) was employed to evaluate the simultaneous effects of substrate concentration, temperature, and pH on the growth rate of *P. linteus* utilizing whey. A 3×2 face-centered design (FCD), which consisted of an orthogonal 2^2 factorial design with a center point being replicated five times (for first-order model fitting, trials 1–9 in Table 1) further augmented by six face-centered points (for second-order model fitting; trials 10–15 in Table 1), was applied (Lee et al. 2008b; Montgomery 2001). This type of experimental design was chosen to obtain statistically sound results with minimum number of trials. Because whey has not previously been used for cultivating *P. linteus*, its growth was tested at different substrate concentrations (i.e., 1, 4, 7, 21, 35, 49, 63 g lactose l^{-1}) at pH 5.0 and 25°C (Kim et al. 2001). The radial extension rates at different substrate concentrations were plotted and the resulting plot was fitted to the Haldane equation (Andrews 1968) to find the point that shows the maximum growth rate at the given pH and temperature. Based on the calculated value, the exploring ranges (center point ± variance) of substrate concentration, temperature, and pH for RSA were determined to be 20 ± 10 g l^{-1}, 25 ± 5°C, and 5 ± 1 (Table 1). A sequential procedure of collecting experimental data from each trial, estimating polynomial equations, and evaluating model adequacy was performed. Increasingly complex polynomials from low to high orders (i.e., linear to partial cubic) were fitted to the experimental results to find the best-fit model describing the response surface. The model adequacy was checked based on the *p*-value of regression, lack-of-fit, and model coefficients.

Design-Expert 7 software (Stat-Ease, Minneapoils, MN) was used for experimental design and polynomial regression.

Analytical methods

Chemical oxygen demand (COD) was measured following the closed reflux colorimetric method (APHA-AWWA-WEF. 2005). Lactose was enzymatically quantified using a commercial kit (Lactose/d-Galactose test kit, Boehringer Mannheim, Germany). The concentration of lactate was determined using an ion chromatograph (Personal 790 IC, Metrohm, Switzerland) equipped with a PRP-×300 ion exclusion column (Hamilton, Reno, NV). Total Kjeldahl nitrogen (TKN) and crude protein concentrations were measured using the Kjeldahl method (APHA-AWWA-WEF. 2005) Crude fat was quantified using an automated extraction unit (Soxtec 2050, FOSS, Sweden). Two identical ion chromatographs (Personal 790 IC, Metrohm), equipped with a Metrosep A Supp 5–100 column (Metrohm) or a Metrosep Cation 1–2 column (Metrohm), were used to quantify anions or cations, respectively. A gas chromatograph (6890 PLUS, Agilent, Palo Alto, CA) equipped with a flame ionization detector (Agilent) and an Innowax capillary column (Agilent) was used to determine the concentrations of acetic, propionic, and butyric acids.

Results and discussion

Wastewater characteristics

Table 2 summarizes the biochemical characteristics of the whey used in this study. The total to soluble COD ratio of 87.8% indicated that the organic compounds in whey are mostly soluble. The COD contribution of lactose, the major organic compound in whey, was 38264 mg l^{-1}(COD conversion factor of lactose = 1.123 g g^{-1}) accounting for 79.0% of soluble COD and 69.3% of total COD. This means readily utilizable lactose is the main substrate for microbial growth in the whey we tested. All measured ions were at their non-inhibitory levels to microbial growth.

Response surface analysis modeling

Figure 1 shows the results of the preliminary test to decide the center point condition for substrate concentration. For each substrate concentration, the average mycelial growth rate (K_r) value ($n = 3$) was plotted. The K_r increased, with increasing lactose concentration, to its maximum and then decreased with further increasing lactose concentration, indicating a substrate inhibition pattern. The plotted data were well fitted to the Haldane equation (Andrews 1968) with a high r^2 (>0.95) and the curve-fitted model calculated the substrate concentration maximizing mycelial growth rate to be 18.7 g lactose l^{-1}, at the fixed pH and temperature of 5.0 and 25°C (Kim et al. 2001). Based on these values, the RSA exploring area was designed as shown in Table 1.

Thirteen trials including the center point (trials 1–9 in Table 1) were run first to test the adequacy of a first-order model describing the response surface of mycelial growth rate. The first-order regression was not statistically significant (P-value >0.68) with a poor r^2 of <0.2, indicating that the first-order model is not adequate to approximate the mycelial growth rate. This poor first-order fitting means the presence

Table 2 Composition and characteristics of whey wastewater

Parameter	Concentration (mg/l)	Parameter	Concentration (mg/l)
COD	55,182 (329)	TKN	665 (10)
Soluble COD	48,462 (698)	NH_4^+	81 (2)
Lactose	34,073 (805)	K^+	1,073 (21)
Crude protein	3,588 (63)	Na^+	573 (6)
Crude fat	360 (25)	Ca^{2+}	200 (5)
Lactic acid	895 (63)	Mg^{2+}	84 (5)
Acetic acid	482 (15)	PO_4^{2-}	987 (68)
Propionic acid	186 (17)	SO_4^{2-}	134 (5)
Butyric acid	25 (2)	Cl^-	1,125 (95)

Standard deviations are in parentheses

Raw wastewater (50 g whey powder l^{-1} solution)

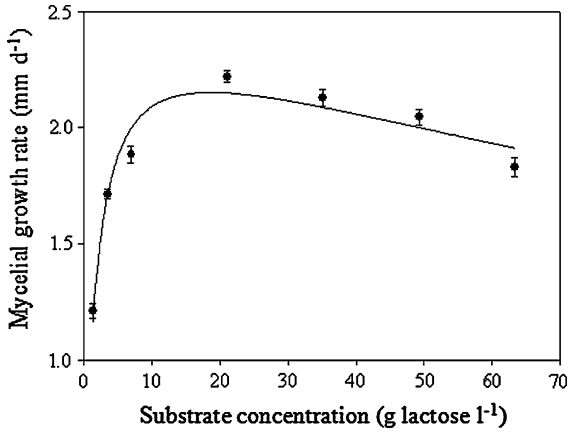

Fig. 1 Observation (closed circle) and model prediction (solid line) of *P. linteus* mycelial growth rate (K_r) at different substrate concentrations (25°C; pH 5.0; $n = 3$)

of a curvature in the response surface within the explored region and we can find the inflection point using a higher-order model. To test higher-order models, six augmentation points (trials 10–15 in Table 1) were run and analyzed. Increasingly complex polynomials from linear to partial cubic were tested to model the augmented data set. The adequacy of each model was assessed based on the r^2, regression *P*-value, and lack-of-fit (LOF) and the simplest model of best fit was selected. As a result, a modified partial cubic model (Eq. 1) was used to approximate the response surface of mycelial growth rate (K_r) to find the optimal cultivation conditions.

$$\begin{aligned}\eta = &-23.8+8.67\times10^{-2}x_1+1.48x_2+1.77x_3\\ &+8.00\times10^{-4}x_1x_2+7.25\times10^{-2}x_1x_3\\ &+5.13\times10^{-2}x_2x_3-1.28\times10^{-2}x_1^2-3.18\\ &\times10^{-2}x_2.^2-2.64\times10^{-1}x_3^2-3.28\\ &\times10^{-3}x_1x_2x_3+4.68\times10^{-4}x_1^2x_2 \qquad (1)\end{aligned}$$

where η: estimated mycelial growth rate (K_r, mm d^{-1}), x_k value of the independent variable k (k = substrate concentration, temperature, and pH in order).

The obtained response surface model showed a high r^2 of >0.99 and an excellent *P*-value of <0.0001. The model *LOF* was not significant at 5% α-level and the coefficient of variation of the model was 4.71%. The model adequacy was also proved with a high adequate precision (*AP*) value of 24.8. *AP* is a measure of the range in predicted response relative to its associated error, in other words, a signal to noise ratio. It compares the range of the predicted values at the design points to the average prediction error, and thus a high AP value (i.e., a large signal in comparison to the noise) means a statistically sound model. A ratio greater than 4 is generally desired for an adequate signal (Design Expert 7; Rodrigues et al. 2006). Consequently, the resulted mycelial growth rate response surface model proved adequate to navigate the design space. In addition, the residuals for all experimental points were examined for any weakness of the selected model. The plotted residuals showed least variances compared to the other models and scattered with no pattern or trend (data not shown), indicating random and homogeneous variances (Montgomery 2001). This again supports the adequacy of our model.

The optimal conditions for maximizing the mycelial growth rate were estimated using the produced model (Eq. 1) by setting the partial derivatives of the equation to '0' with respect to the independent variables. The calculated optimal conditions were 29.7 g lactose l^{-1}, 26.2°C, and pH 5.2 for substrate concentration, temperature, and pH, respectively. The predicted model output at the estimated optimal point was 2.80 mm d^{-1} (95% confidence interval, 2.65–2.96). Two- and three-dimensional response surfaces, with the optimal conditions marked, with respect to the test variables are shown in Figs. 2, and 3, 4.

Further inspection on the individual coefficients showed that x_2^2 (squared temperature) affected the

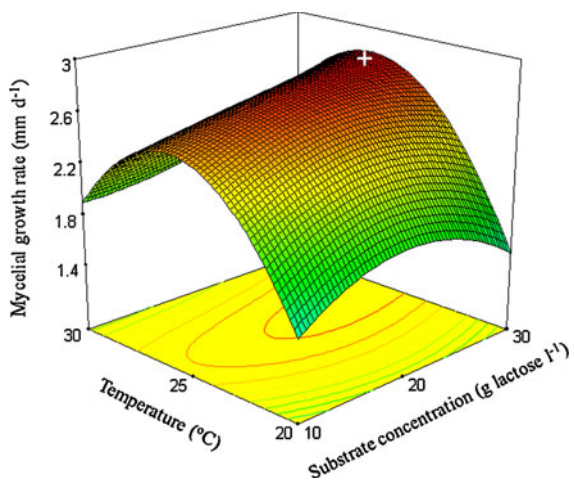

Fig. 2 The response surface plot for the mycelial growth of *P. linteus* with respect to substrate concentration and temperature within the explored region

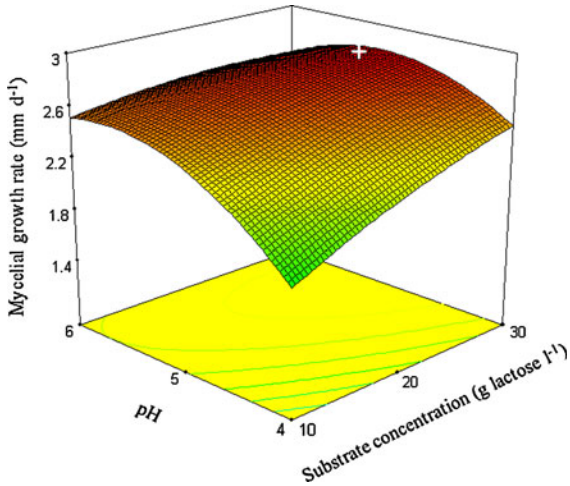

Fig. 3 The response surface plot for the mycelial growth of *P. linteus* with respect to substrate concentration and pH within the explored region

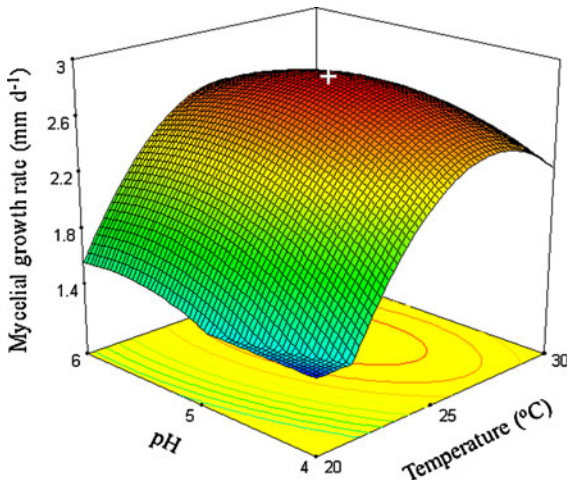

Fig. 4 The response surface plot for the mycelial growth of *P. linteus* with respect to temperature and pH within the explored region

model output (i.e., mycelial growth rate of *P. linteus*) most significantly (*P*-value <0.0001), whereas the linear effect of temperature (x_2) was not significant at 5% α-level. On the other hand, the squared substrate concentration term (x_1^2) was not significant at 5% % α-level but its first-order term was at 1% α-level. For pH, both first- and second-order terms (x_3 and x_3^2) were significant at 1% α-level. The strong and significant effect of temperature on the mycelial growth is clearly reflected in Fig. 2 and 4, where the mycelial growth rate response shows sizable changes along the temperature axis while the response contours are elongated along the substrate concentration or pH axes to form a long elliptic surface. This indicates the even lower sensitivity of the model to substrate concentration and pH than to temperature. This observation accords with the fact that temperature is generally the most critical factor in controlling microbial growth (Madigan et al. 2009). Among the two-way interaction terms, x_1x_2 (substrate concentration × temperature) and x_1x_3 (substrate concentration × pH) were significant at 1% and 5% α-levels, respectively. This means that the variables in each interaction term were interdependent to each other. On the other hand, the temperature and pH interaction term (x_2x_3) was not significant at 5% α-level, indicating that they are not interdependent. The other interaction terms $x_1x_2x_3$ (substrate concentration × temperature × pH) and $x_1^2x_2$ (substrate concentration^2 × temperature) were significant at 1% and 5% α-levels, respectively. These statistical inspection results are summarized in Table 3. Based on *P*-value and effect-on-response (*ER*), the most significant coefficients for the high-rate mycelial cultivation of *P. linteus* on whey were determined to be x_2^2 (temperature^2), x_3 (pH), x_3^2 (pH^2), and $x_1^2x_2$ (substrate concentration2 × temperature). The *ER*

Table 3 Statistical significance of estimated model coefficients

Term[a]	Coefficient	*P*-value	*ER*[b]
x_1	8.67×10^{-2}	0.0024	+0.28
x_2	1.48	0.1563	+0.22
x_3	1.77	0.0002	+0.46
x_1x_2	8.00×10^{-4}	0.0028	+0.32
x_1x_3	7.25×10^{-2}	0.0303	−0.19
x_2x_3	5.13×10^{-2}	0.0787	−0.14
x_1^2	c1.28×10^{-2}	0.0961	−0.22
x_2^2	-3.18×10^{-2}	< 0.0001	−1.48
x_3^2	-2.64×10^{-1}	0.0030	−0.52
$x_1x_2x_3$	-3.28×10^{-3}	0.0021	−0.32
$x_1^2x_2$	4.68×10^{-4}	0.0194	+0.46

[a] x_k, independent variable k (k = substrate concentration, temperature, and pH in order)

[b] *Effect on response* (*ER*) = cR, where c is the estimated coefficient and R is the explored range of the term. Both c and R were calculated based on coded variables

value is calculated by multiplying the estimated coefficient value (c) by the explored range of the term (i.e., independent variable, R), both of which are estimated based on coded variables. This value (cR) measures the direction ($\pm$) and magnitude of a term's effect on the model output (Do et al. 2008).

The necessity for reducing pollutant and for maximizing returns on raw material has been an issue in food or dairy industries producing massive amount of strong nutrient-rich byproducts every year, driving them to seek new options to use the wastes. This study presents the potential of whey as an alternative substrate for cultivating *P. linteus* mycelia and the optimal conditions for maximizing its growth rate on whey. This may provide an option to reduce the pollution load from surplus whey and at once make an additional return, value-added *P. linteus* mycelia, from it.

Conclusions

RSA was successfully applied to examine the effects of substrate (i.e., lactose) concentration, temperature, and pH on the mycelial growth rate of *P. linteus* on whey. A modified partial cubic model was constructed to describe the response surface, and the model adequacy was verified by statistical tests ($r^2 > 0.99$, P-value < 0.0001, coefficient of variation <5%) and residual plotting. The obtained model predicted that the maximum mycelial growth rate will be 2.80 mm d^{-1} (95% confidence interval, 2.65–2.96) at 29.7 g lactose l^{-1}, 26.2°C, and pH 5.2 condition. Statistical assessment showed that temperature was the most significant environmental factor of dominant effects on the model response, and that x_2^2 (temperature^2), x_3 (pH), x_3^2 (pH^2), and $x_1^2x_2$ (substrate concentration^2 × temperature) are significant and effective terms in the model. The overall results prove the potential use of whey as a growth medium, with no additives, for cultivating *P. linteus* mycelia.

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