



## Resource recovery using whey permeate to cultivate *Phellinus linteus* mycelium: Solid-state and submerged liquid fermentation

Kyungjin Cho,\* Joonyeob Lee,† Gyuseong Han,† Na Kyung Kim,‡ Hyokwan Bae,\* and Seokhwan Hwang†<sup>1</sup>

\*Center for Water Resource Cycle Research, Korea Institute of Science and Technology, 39-1 Hawolgok-Dong, Seongbuk-Gu, Seoul 136-791, Republic of Korea

†School of Environmental Science and Engineering, Pohang University of Science and Technology (POSTECH), Pohang, Gyeongbuk 790-784, Republic of Korea

‡Department of Civil and Environmental Engineering, University of Illinois at Urbana-Champaign, 205 North Mathews Ave., Urbana 61801

### ABSTRACT

The growth characteristics of *Phellinus linteus* mycelium were assessed and compared under solid-state fermentation (SSF) and submerged liquid fermentation (SLF) systems on whey permeate medium. Response surface methodology was used to investigate the growth rates of mycelia under various conditions of operating temperature ( $T_0$ ), initial pH, and substrate concentration ( $[S]$ ). The optimal growth conditions of *P. linteus* mycelium were determined to be 26.1°C, pH 4.6, and 60.3 g of lactose/L in the SSF system, and 29.0°C, pH 5.0, and 65.3 g of lactose/L in the SLF system. The maximum growth rates were predicted to be  $1.92 \pm 0.01$  mm/d in SSF and  $192.1 \pm 0.0$  mg/L per day in SLF. Random trials were conducted to experimentally validate the evaluated optimal conditions. The differences between the modeled and observed values were only 5.3% in the SSF system and 6.1% in the SLF system. Significant engineering factors differed between the fermentation techniques;  $T_0$  was significant in both cultivation systems, whereas initial pH was significant in SSF but  $[S]$  was significant in SLF. Our findings can be used to guide the operation of the bioconversion process for cultivating *P. linteus* mycelium using whey permeate wastewater.

**Key words:** whey permeate wastewater, *Phellinus linteus*, response surface methodology, bioconversion

### INTRODUCTION

Whey is a liquid by-product of cheese production, and world production of cheese whey is currently more than 145,000,000 tonnes per year and is increasing (Pais et al., 2014). The whey is filtered to recover whey proteins,

and the final waste of this process is whey permeate, which consists of small molecules such as lactose and mineral salts (Dallas et al., 2014). Disposal of this dairy wastewater causes serious environmental problems due to its high chemical oxygen demand (COD) and large volume (Song et al., 2007). However, whey permeate wastewater is regarded as a nutritionally valuable resource for microbial growth because it contains nutrients such as nitrogen and trace elements. In fact, the potential of using whey wastewater as a substrate for diverse bioconversion processes such as anaerobic digestion and bio-ethanol production has been demonstrated (Sansonetti et al., 2009; Carvalho et al., 2013). However, whey wastewater is not an ideal substrate for anaerobic digestion because the acidic pH and highly biodegradable content of the wastewater result in rapid acidification (Diamantis et al., 2014). In addition, use of whey permeate for bio-ethanol production is not currently feasible because microorganisms that efficiently ferment lactose to bio-ethanol are not available (Akbas et al., 2014). Therefore, efficient utilization of the nutrient resources in whey permeate wastewater requires development of a more appropriate bioprocess that can stably produce economically valuable commodities; we suggest using it to cultivate *Phellinus linteus* mycelia.

*Phellinus linteus* is a medicinal basidiomycete (mushroom) that has been used as a traditional treatment for gastroenteric dysfunction, diarrhea, and hemorrhage, and that may have anticancer properties (Mei et al., 2015). Fruiting bodies of *P. linteus* are very expensive because their production is complicated and takes 5 to 6 mo (Lee et al., 2011). However, the beneficial bioactive compounds of mushrooms can be extracted directly from the mycelia, so cultivation time can be reduced (Hatvani and Meés, 2001). Therefore, if *P. linteus* mycelia can be successfully grown in whey permeate wastewater, economic benefits could be achieved by producing valuable products while reducing the amount of organic waste. In addition, bioconversion of *P. linteus* from the wastewater can be a suitable bio-

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<sup>1</sup>Corresponding author: shwang@postech.ac.kr

process strategy because fungi tolerate acidic pH better than do bacterial communities (Carvalho et al., 2013). However, use of *P. linteus* mycelia for this process requires information on how various parameters affect its growth characteristics.

Two fermentation techniques, solid-state fermentation (SSF) and submerged liquid fermentation (SLF), have been used to cultivate mycelia. The 2 systems have unique advantages and disadvantages. Solid-state fermentation refers to any fermentation process in which microorganisms grow on a water-insoluble solid substrate in the absence of water flow (Liang et al., 2009); SSF has been used to produce high-value products such as enzymes from mycelia (Singhania et al., 2009). It uses a small fermentation volume and allows easy separation of byproducts, so it costs little to install and operate (Shuler and Kargi, 2001). However, scale-up of SSF system operations is complicated by the difficulty in monitoring and controlling factors such as operating temperature ( $T_0$ ) and pH.

Submerged liquid fermentation refers to fermentation in an aqueous medium; SLF has been applied to mass production of mycelia (Singhania et al., 2009). A crucial advantage of SLF is that it provides good mixing conditions during bioprocess operation. The mixing ensures homogeneity of various factors such as dissolved oxygen,  $T_0$ , and substrate concentration ( $[S]$ ; Raghavarao et al., 2003). Moreover, the homogeneity enables precise monitoring and efficient control of these factors, so scale-up for mycelial cultivation is easier for SLF than for SSF (Singhania et al., 2009). However, the SLF system is more vulnerable to contamination by bacteria or yeasts than the SSF system because of the high water content in SLF (Shuler and Kargi, 2001). Therefore, a suitable cultivation strategy should be selected according to the cultivation purpose.

Because of the complementary advantages and disadvantages of SSF and SLF, we investigated the growth rate of *P. linteus* grown in SSF and SLF systems on whey permeate wastewater medium, and compared the growth characteristics of the mycelia in the 2 systems. Three engineering factors:  $T_0$ , initial pH, and  $[S]$  were considered the independent variables for the statistical test. The significant engineering factors were evaluated by ANOVA.

## MATERIALS AND METHODS

### Microorganism

*Phellinus linteus* (KCTC 6719) was obtained from the Korean Collection for Type Cultures (KCTC; Daejeon, Korea) and maintained in a potato dextrose agar slant

at 4°C. This seed culture of *P. linteus* was transferred into petri dishes filled with potato dextrose agar medium and incubated at 25°C for 4 d. After incubation, as an inoculum for subsequent experiments, an active site of the mycelial agar disk with 5-mm diameter was excised using a sterilized round cutter.

### Whey Permeate Wastewater

Dried whey permeate powder was obtained from Gossner's cheese plant (Logan, UT). To provide practical operating information for the application in a commercial plant, the whey permeate was used as the sole substrate (Table 1). The whey permeate powder was dissolved in distilled water to obtain different  $[S]$  for subsequent experiments (Table 2);  $[S]$  was adjusted based on lactose content because lactose was the primary carbon source of whey permeate wastewater.

For SSF, the whey permeate powder was dissolved in a 250-mL flask with several target  $[S]$  (Table 2). At that point, commercial agar (Becton Dickinson and Co., Sparks, MD) was added (1.5% wt/vol) to the flask to solidify the whey permeate wastewater. The pH was adjusted by using 2 N HCl or 2 N NaOH to meet the experimental conditions, and the medium was sterilized by autoclaving at 121°C for 20 min (Table 2). The sterilized solution was poured into petri dishes, allowed to solidify, and then used as the medium for cultivation of *P. linteus* mycelium. The whey permeate wastewater was prepared in the same for SLF as for SSF except for the solidification step.

### SSF and SLF

Solid-state fermentation was conducted in petri dishes. A mycelial agar disk was transferred to the center of each dish and the dishes were incubated at different  $T_0$  according to the experimental design (Table 2). The radial extension of the mycelium was monitored every 24 h for 30 d to evaluate the growth rate, because the colony grows in a circular fashion (Lee et al., 2008b). The radius was measured using a laboratory calipers at 4 different positions and averaged. The slope of the linear regression of radius over time (i.e., the average radial extension rate) was regarded as the mycelial growth rate under the given condition.

Submerged liquid fermentation was conducted in 250-mL Erlenmeyer flasks with 100-mL working volume. Five mycelial discs were inoculated into each flask, and the flasks were incubated at different  $T_0$  with 100-rpm agitation (Table 2). The mycelial growth rate in the SLF system was evaluated by measuring the concentration of volatile suspended solids (VSS). The VSS

**Table 1.** Characteristics of whey permeate wastewater (1 g of whey permeate powder/L of water)

Classification	Measurement parameter <sup>1</sup>	Concentration (mg/L)		
		Mean	SD	
Solids	TS	1,074	39	
	TSS	41	3	
	TDS	1,032	41	
	VS	929	29	
	VSS	35	7	
	VDS	894	35	
Organic nutrients	Total COD	957	11	
	Soluble COD	945	15	
	Total carbohydrate	883	77	
	Lactose	691	16	
Ions	Cation	Na <sup>+</sup>	12.1	0.5
		NH <sub>4</sub> <sup>+</sup>	1.5	0.4
		K <sup>+</sup>	42.4	0.0
		Mg <sup>2+</sup>	1.9	0.0
		Ca <sup>2+</sup>	5.4	1.0
	Anion	Cl <sup>-</sup>	27.2	0.0
		NO <sub>2</sub> <sup>-</sup>	0.2	1.0
		NO <sub>3</sub> <sup>-</sup>	1.6	0.0
		PO <sub>4</sub> <sup>3-</sup>	20.5	0.0
		SO <sub>4</sub> <sup>2-</sup>	3.1	0.4

<sup>1</sup>TSS = total suspension solid; TDS = total dissolved solid; VS = volatile solid; VSS = volatile suspension solid; VDS = volatile dissolved solid; COD = chemical oxygen demand.

concentration was a reasonable indicator of the growth of *P. linteus* because 96.1% of total compounds existed in soluble form in the whey permeate wastewater [i.e., the ratio of total dissolved solid (TDS) to total solid (TS); Table 1]. The increase of mycelial biomass per unit time was considered the mycelial growth rate.

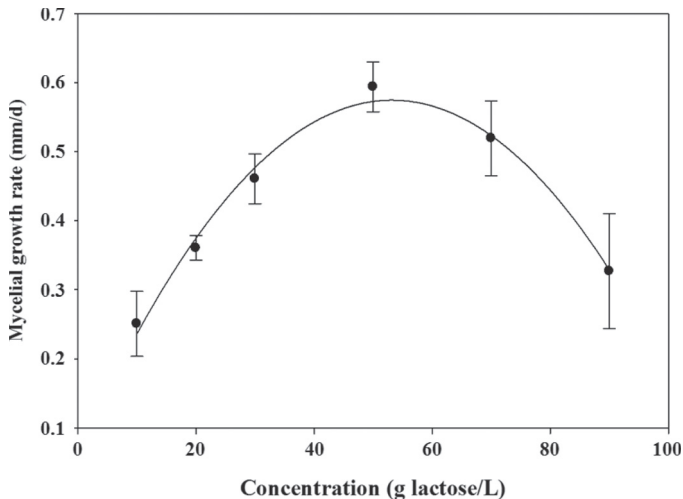
**Table 2.** Experimental conditions by central composite design and observed growth rates

Item	Trial	Conditions of variables			Response <sup>1</sup>	
		Temperature (°C)	Initial pH	Substrate concentration (g of lactose/L)	Mycelial growth rate in SSF system (mm/d)	Mycelial growth rate in SLF system (mg/L per day)
Linear design	1	20	4.0	45	0.76	148
	2	20	4.0	65	0.85	133
	3	20	6.0	45	0.86	131
	4	20	6.0	65	0.68	103
	5	30	4.0	45	0.85	96
	6	30	4.0	65	1.75	195
	7	30	6.0	45	0.47	56
	8	30	6.0	65	0.55	195
	9 <sup>2</sup>	25	5.0	55	1.86 ± 0.18	175 ± 16
Quadratic design	10	25	5.0	45	1.72	108
	11	25	5.0	65	1.70	193
	12	25	4.0	55	1.62	169
	13	25	6.0	55	1.01	140
	14	20	5.0	55	1.34	143
	15	30	5.0	55	1.43	188
Augmented design <sup>3</sup>	16	35	5.0	72.5	—	176
	17	35	4.0	72.5	—	149
	18	35	5.0	55	—	95
	19	35	4.0	55	—	101
Validation experiment	20-1	26	4.6	64	2.01	—
	20-2	30	5.2	67	—	204

<sup>1</sup>SSF = solid-state fermentation; SLF = submerged liquid fermentation.

<sup>2</sup>Center point (mean ± SD, n = 5).

<sup>3</sup>Augmented trials were only performed for the SLF system to find optimum growth conditions.



**Figure 1.** Observed and predicted mycelial growth rates of *Phellinus linteus* at different whey permeate concentrations based on the lactose content. ● = observed mycelial growth rates; solid line = model predictions; error bars indicate standard deviation;  $n = 8$  at each condition.

### Experimental Design for Response Surface Methodology

Response surface methodology (RSM) was used to evaluate the simultaneous effects of  $T_0$ , initial pH, and  $[S]$  on the growth rate of *P. linteus* utilizing whey permeate wastewater. The experimental design was conducted based on a central composite in cube design with a center point replicated 5 times (Lee et al., 2008a). This type of experimental design is useful to minimize the number of experimental trials required to obtain statistically significant results under given conditions. The experimental conditions for RSM were set at  $20 \leq T_0 \leq 30^\circ\text{C}$  and  $4.0 \leq \text{initial pH} \leq 6.0$  based on the growth characteristics of *P. linteus* in previous studies (Hwang et al., 2003; Lee et al., 2008b, 2011). Preliminary experiments were conducted at different  $[S]$  (i.e., 10, 20, 30, 50, 70, and 90 g of lactose/L) at pH 5.5 and  $25^\circ\text{C}$  to determine the experimental range because information about the effects of  $[S]$  on growth of *P. linteus* in whey permeate wastewater medium is very limited. After using the least squares method to estimate polynomials, the adequacy of the models was checked by inspecting residual plots and by considering statistical values. Additional trials were conducted under random conditions to verify the statistical model for each cultivation system experimentally.

### Analytical Methods

Lactose concentration was analyzed using an HPLC [Agilent Technologies, Palo Alto, CA; RI detector; Supel-

cosil LC-NH<sub>2</sub> chromatographic column (Supelco, Bellefonte, PA); 5  $\mu\text{m}$ , 250 mm  $\times$  4.6 mm; acetonitrile:H<sub>2</sub>O (83:17) as a mobile phase]. Chemical oxygen demand and soluble COD (SCOD) concentrations were measured using colorimetry. Total carbohydrate (TC) concentration was measured using a phenol-sulfuric assay (DuBois et al., 1956). Solid concentrations such as TS, TDS, total suspended solids (TSS), volatile solids (VS), volatile dissolved solids (VDS), and VSS were determined according to the procedures in standard methods (APHA-AWWA-WEF, 2005). Two identical ion chromatographs (790 Personal IC, Metrohm, Herisau, Appenzell Ausserrhoden, Switzerland) were used to measure the concentrations of cations and anions in the samples.

## RESULTS

### Characteristics of Whey Permeate Wastewater and Preliminary Test

Physico-chemical analyses of the whey permeate wastewater were performed (Table 1). The ratio of soluble to total COD was 98.7%; that is, the organic compounds contained in the whey permeate wastewater were mostly soluble. The ratio of lactose to total carbohydrate was 78.3%, confirming that lactose was the primary carbon source. In addition to the high organic contents, the whey permeate wastewater contained various inorganic ingredients, which could support the growth of mycelia (Carvalho et al., 2013).

In the preliminary experiments, the growth rate of *P. linteus* was greatest at  $[S] \approx 55.0$  g of lactose/L; the growth pattern was successfully fit by the Shi model ( $R^2 = 0.98$ ; Shi et al., 1999; Figure 1). Therefore, the center point in the RSM experiment was set at  $[S] = 55$  g of lactose/L.

### Modeling and Analyzing Mycelial Growth Rate in SSF

Thirteen trials including the center point (trials 1 to 9; Table 2) were run on solid medium. Initially, the data were fit using least squares to a first-order model to access the location of the optimum condition. The  $P$ -values, correlation coefficient  $R^2$ , and lack of fit (LOF) were calculated to validate the model adequacy. The linear model had  $P = 0.79$  and  $R^2 = 0.11$ ; the LOF was significant at the 1%  $\alpha$ -level, suggesting that this model was not statistically significant to describe the mycelial growth rate of *P. linteus*. The interaction model was also statistically insignificant ( $P = 0.91$ ,  $R^2 = 0.24$ ) with LOF that was also significant at 1%  $\alpha$ -level. A second- or higher-order model could not be fit using the data from trials 1 to 9 because of the lack



of axial data points, so 6 additional trials (trials 10 to 15; Table 2) were conducted to collect sufficient data to estimate polynomials that describe the mycelial growth rate. From the results, a quadratic model (Eq. [1]) was adequate ( $P < 0.01$ ,  $R^2 = 0.951$ , LOF not significant at the 1%  $\alpha$ -level) to describe the growth rate ( $\eta_1$ ) of the mycelial *P. linteus* in the SSF system (Figure 2):

$$\begin{aligned} \eta_1 = & 1.84 + 1.1 \times 10^{-2}X_1 - 2.4 \times 10^{-1}X_2 + 8.1 \\ & \times 10^{-3}X_3 - 3.8 \times 10^{-2}X_1X_2 + 2.7 \times 10^{-3}X_1X_3 - 1.4 \\ & \times 10^{-2}X_2X_3 - 1.7 \times 10^{-2}X_1^2 - 4.4 \times 10^{-1}X_2^2 \\ & - 1.4 \times 10^{-3}X_3^2, \end{aligned} \quad [1]$$

where  $\eta_1$  = mycelial growth rate in the SSF culture (mm/d) and  $X_i$  = independent variable  $i$  [ $i = T_0$  ( $^{\circ}\text{C}$ ), pH, and  $[S]$  (g of lactose/L) in order].

The residual plot (Figure 3) demonstrated no patterns or trends, so no weakness of the model was detected (Lee et al., 2011). Using Eq. [1], the optimum growth condition was determined to be  $T_0 = 26.1^{\circ}\text{C}$ , pH = 4.6, and  $[S] = 60.3$  g of lactose/L, at which the predicted  $\eta_1 = 1.92 \pm 0.01$  mm/d. The additional trial (trial 20–1 in Table 2) was conducted under random conditions (i.e.,  $T_0 = 26.0^{\circ}\text{C}$ , pH = 4.6,  $[S] = 63.7$  g of lactose/L) to assess the accuracy of the model predictions. The growth rate at this random condition was 2.01 mm/d, which differed by only 5.3% from the model response of 1.90 mm/d, indicating that the model adequately represented the SSF experimental sets. The significance of independent variables was also evaluated through ANOVA. The growth rate of *P. linteus* was significantly affected by  $T_0$  and pH at 1%  $\alpha$ -level but was not affected for the values of  $[S]$  tested (Table 3). The results indicate that  $T_0$  and pH are important engineering factors when SSF is used to cultivate *P. linteus* mycelia in whey permeate wastewater.

### Modeling and Analyzing Mycelial Growth Rate in SLF

Model evaluation for the SLF system was conducted in the same way as for SSF. In SLF, the first-order model was not statistically significant to describe the growth rate of *P. linteus* ( $P > 0.05$ , for linear and interaction model; LOF significant at 5%  $\alpha$ -level). Therefore, 6 additional trials (trials 10 to 15; Table 2) were conducted to find the optimum growth conditions. A quadratic equation was adequate ( $P < 0.001$ ,  $R^2 = 0.91$ ; LOF insignificant at 5%  $\alpha$ -level) to describe the relationship. However, the optimal conditions of  $T_0$  and  $[S]$  (i.e.,  $T_0 = 29.0^{\circ}\text{C}$ ,  $[S] = 65$  g of lactose/L) were located near the boundary of the experimental design. Therefore, the design boundary was expanded to find the optimum

growth condition by performing 4 additional trials (trials 16 to 19; Table 2).

Finally, a quadratic model (Eq. [2]) was adequate ( $P < 0.01$ ,  $R^2 = 0.874$ ; LOF not significant at the  $\alpha = 0.05$  level) to describe mycelial growth rate in the SLF system (Figure 4):

$$\begin{aligned} \eta_2 = & 185.4 - 1.2X_1 - 6.3X_2 + 2.3X_3 + 7.2 \times 10^{-1}X_1X_2 \\ & + 5.9 \times 10^{-1}X_1X_3 + 6.3 \times 10^{-1}X_2X_3 - 8.4 \\ & \times 10^{-1}X_1^2 - 5.4X_2^2 - 2.4 \times 10^{-1}X_3^2, \end{aligned} \quad [2]$$

where  $\eta_2$  = mycelial growth rate in the SLF culture (mg/L per day) and  $X_i$  = independent variable  $i$  [ $i = T_0$  ( $^{\circ}\text{C}$ ), pH, and  $[S]$  (g of lactose/L) in order].

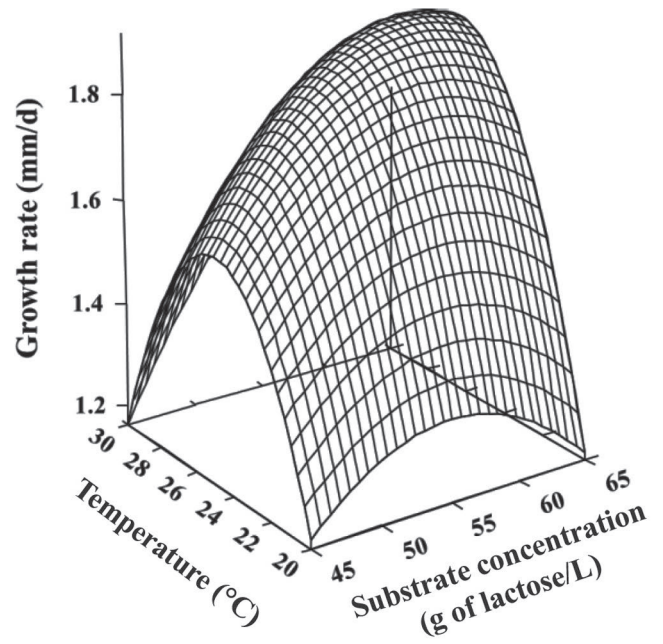
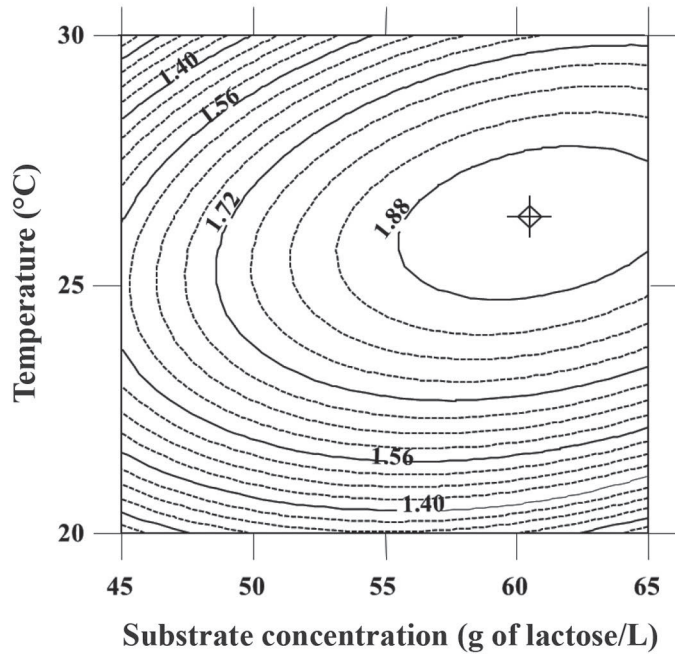
The residual plots demonstrated no patterns or trends, so no weakness of the model was detected (Figure 3). Using Eq. [2], the optimal growth condition was determined to be  $T_0 = 29.0^{\circ}\text{C}$ , pH = 5.0, and  $[S] = 65.3$  g of lactose/L, at which the predicted  $\eta_2 = 192.1 \pm 0.0$  mg/L per day. An additional trial was conducted at a random growth condition (i.e.,  $T_0 = 30.0^{\circ}\text{C}$ , pH = 5.2,  $[S] = 67.0$  g of lactose/L) to validate model accuracy (trial 20–2; Table 2). The observed growth rate was 204.0 mg/L per day, differing by only 6.1% from the model response of 191.5 mg/L per day. In addition,  $T_0$  and  $[S]$  were statistically significant at 1%  $\alpha$ -level for the SLF system, whereas pH were not. The results indicate that  $T_0$  and  $[S]$  are the crucial engineering factors in the SLF system (Table 3).

## DISCUSSION

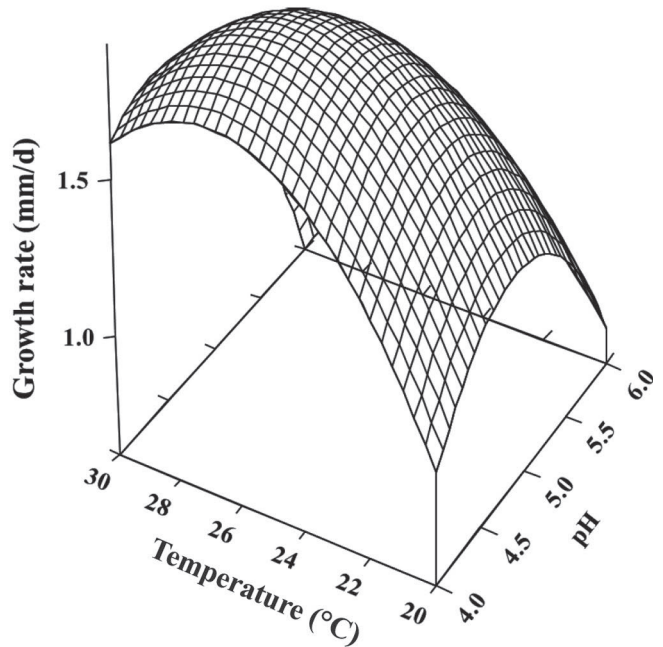
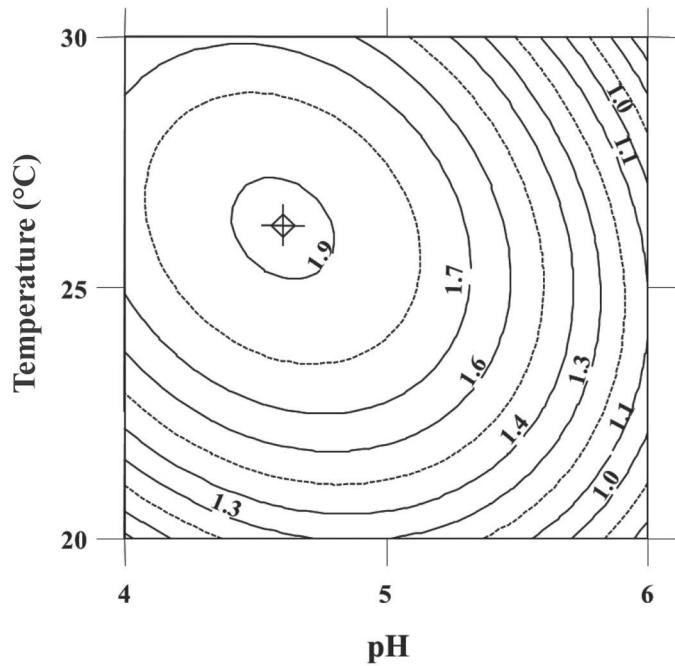
Effects of  $T_0$ , pH, and  $[S]$  on mycelial growth of *P. linteus* were determined in SSF and SLF systems operating with whey permeate wastewater as the sole nutrient source (Figures 2 and 4). The results indicate that whey permeate wastewater can be used as the sole substrate for mycelial growth of *P. linteus*. However, the optimal growth conditions and the significant factors differed between the SSF and SLF systems.

In both systems,  $T_0$  significantly affected mycelial growth rate. Growth rate of the mycelial *P. linteus* was greatest at  $T_0 = 26.1^{\circ}\text{C}$  in the SSF system and at  $T_0 = 29.0^{\circ}\text{C}$  in the SLF system. These  $T_0$  values are reasonable because *P. linteus* grows optimally in the range of 25.0 to 30.0 $^{\circ}\text{C}$  (Lee et al., 2003). The optimum  $T_0$  of *P. linteus* was 2.9 $^{\circ}\text{C}$  lower in the SSF system than in the SLF system due to the different cultivation characteristics. In general, metabolic heat generated by microbial activity is difficult to dissipate in SSF systems (Raghavarao et al., 2003). In fact, active fungal growth can generate a rapid temperature gradient (i.e., 3.0 $^{\circ}\text{C}/\text{cm}$ )

(a)



(b)



**Figure 2.** Two- and 3-dimensional contour plots of the models for the growth rates of *Phellinus linteus* mycelia in the solid-state fermentation (SSF) system with respect to (a) temperature and substrate concentration and (b) temperature and pH within the design boundaries.

**Table 3.** Results of ANOVA for independent variables

Term	Solid-state fermentation system			Submerged liquid fermentation system		
	Mean square	df	P-value	Mean square	df	P-value
Temperature	0.254	2	0.006	3,599.1	2	0.002
pH	0.541	2	<0.001	220.3	2	0.531
Substrate concentration	0.061	2	0.164	8,034.6	2	<0.001
Temperature × pH	0.285	1	0.010	144.5	1	0.521
Temperature × substrate concentration	0.143	1	0.048	9,773.4	1	<0.001
Substrate concentration × pH	0.149	1	0.045	373.7	1	0.308

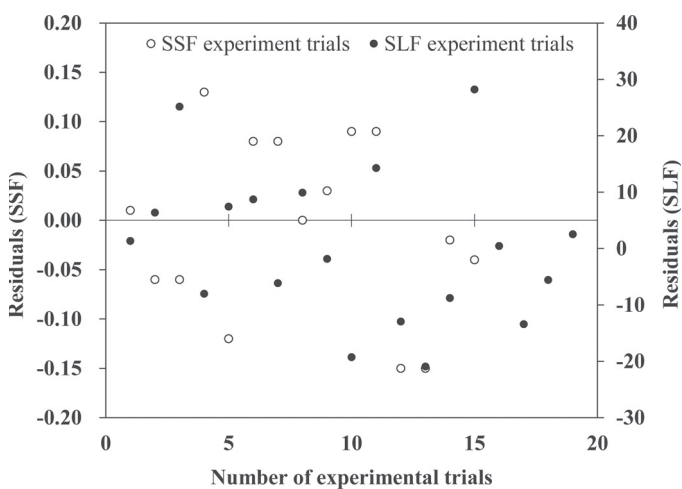
in solid-type cultures (Lonsane et al., 1985). Therefore, the retained metabolic heat in the SSF system may have reduced the optimum  $T_0$  for growth of mycelial *P. linteus*. The results suggest that the SSF system should be operated at lower  $T_0$  than the SLF system during bioconversion operation using *P. linteus*.

In addition,  $T_0$  significantly affected the growth of *P. linteus* in both fermentation systems (Figures 2 and 4) because temperature is a critical factor that directly affects microbial activity (Madigan et al., 2008). The significant effect of temperature on the growth is clearly reflected in Figures 2 and 4, where the responses demonstrate considerable change along with the temperature axis. For example, when  $T_0$  decreased from the optimum condition to 20.0°C, mycelial growth rates decreased by 37% in SSF and by 42% in SLF. Therefore,  $T_0$  must be strictly controlled when cultivating mycelial *P. linteus* in whey permeate wastewater, regardless of fermentation method.

The effect of initial pH on mycelial growth rate was statistically significant at the 1%  $\alpha$ -level for the SSF system but insignificant at the 5%  $\alpha$ -level for the SLF

system (Figure 2, Table 3). In fact, the growth rate of *P. linteus* mycelium varied considerably with the change of initial pH in the SSF system only (Figure 2). The optimal initial pH values in both systems were similar to that reported in a previous study—pH 5.0 (Kim et al., 2001). However, the optimum growth pH of *P. linteus* is controversial. In fact, Lee et al. (2008b) reported that the mycelial *P. linteus* grew optimally at pH 6.0 in starch wastewater medium (Lee et al., 2008b). The difference might be related to the difference of extracellular enzymes and their activity in ambient pH conditions. In the present study, *P. linteus* might secrete an extracellular enzyme,  $\beta$ -D-galactosidase, which degrades the lactose to soluble substrate that can pass through the cell wall. In fact, the optimum pH range of  $\beta$ -D-galactosidase is pH 3.8 to 5.2, which covers the optimal growth pH of *P. linteus* in this study (Macris and Markakis, 1981). In contrast, the optimal growth pH of the previous study, pH 6.0, corresponded to the optimum pH range (i.e., pH 5.2 to 6.9) of  $\alpha$ -amylase, which degrades starch to soluble sugars (Ishikawa et al., 1990; Lee et al., 2008b). Therefore, the optimum growth pH at the SSF and the SLF systems may be determined by the unique substrate characteristic of the whey permeate wastewater; that is, the lactose richness.

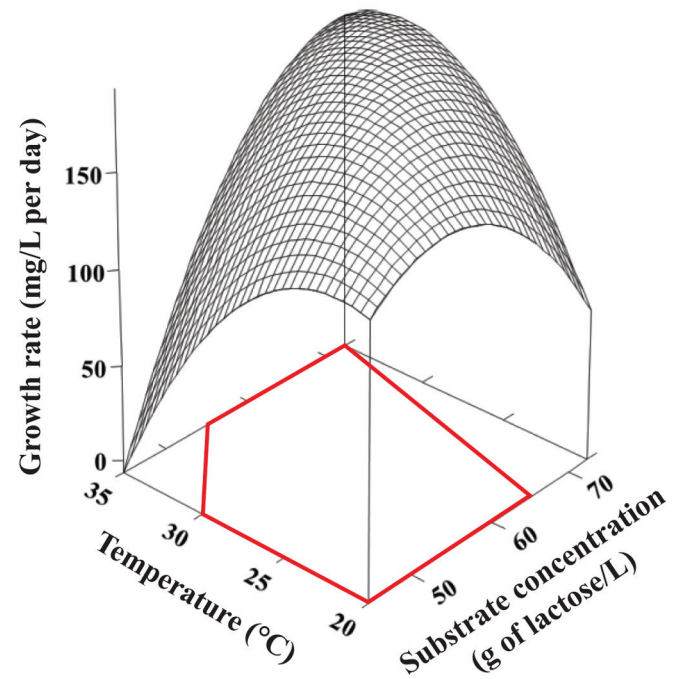
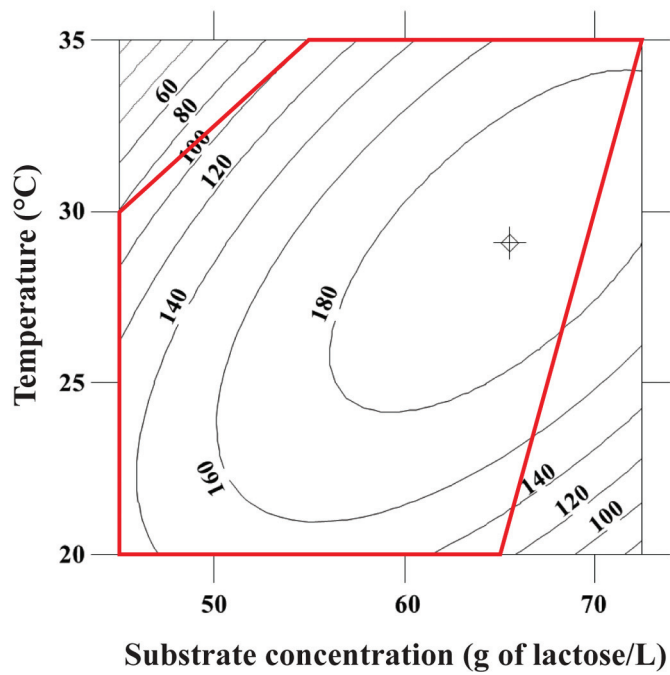
The difference between the SSF and the SLF systems of the optimal growth pH and its significance could have 3 causes: (1) pH decrease in the cultivation medium due to the actions of extracellular enzymes, (2) the hyphal growth characteristic of mycelia, and (3) the different mixing properties of the fermentation systems. During fermentation, fungi secrete extracellular enzymes to hydrolyze particular substrates; this process decreases the pH in the growth medium, and ultimately generates a pH gradient in the cultivation system (Mitchell et al., 2000). Interestingly, the actively growing tip of a mycelial hypha can overcome severe ambient conditions such as local pH decrease and substrate depletion in SSF systems, because the active tip of the hypha extends to intake the substrate (Riquelme, 2013; Sanati Nezhad and Geitmann, 2013). This ability implies that



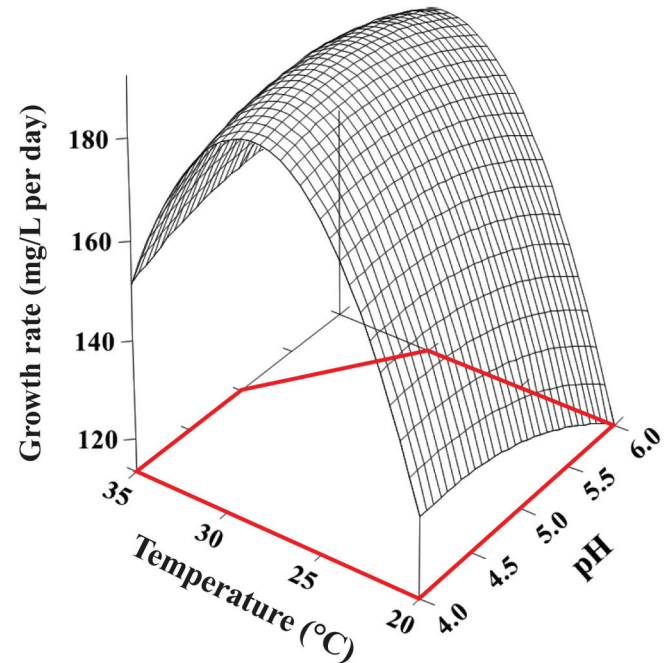
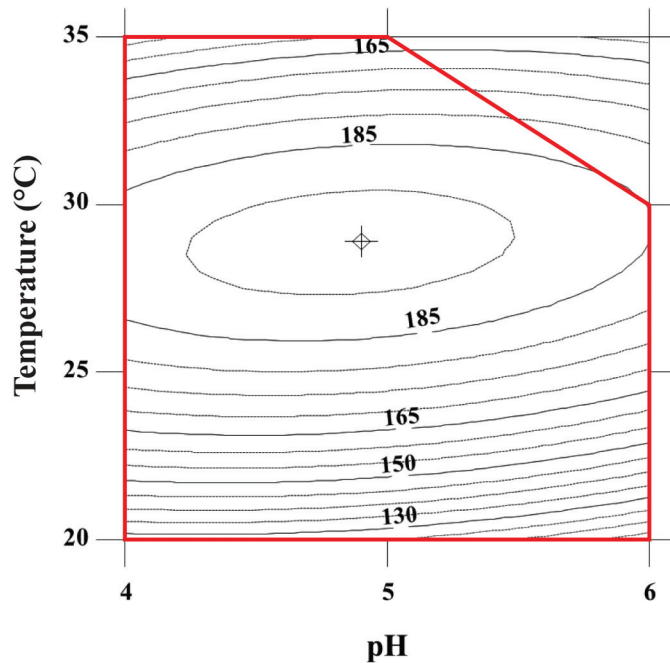
**Figure 3.** Residual plots of the evaluated models for the solid-state fermentation (SSF; ○) and submerged liquid fermentation (SLF; ●) systems.



(a)



(b)



**Figure 4.** Two- and 3-dimensional contour plots of the models for the growth rates of *Phellinus linteus* mycelia in the submerged liquid fermentation system with respect to (a) temperature and substrate concentration, and (b) temperature and pH within the design boundaries. The dashed linear (red) lines on the contour plots were based on the ranges of the independent variables described in Table 2 and represent the boundary conditions to calculate the growth rate using the evaluated models. Color version available online.



the hyphal tip of *P. linteus* may have contacted fresh substrate that was at constant pH; that is, initial pH directly affected the growth pH of the mycelia. However, in SLF systems, the pH decreases in the entire medium due to mixing, so that *P. linteus* mycelia can be exposed to varied pH. This implies that the initial pH would have relatively little effect on the growth of *P. linteus* mycelia in SLF. This argument may explain why the effect of initial pH on growth of mycelial *P. linteus* was more significant in the SSF system than in the SLF system (Table 3, Figure 2). These observations suggest that control of initial pH enhances the process stability of SSF for mycelial bioconversion utilizing whey permeate wastewater. Moreover, in the same context, the optimal initial pH may have been higher in the SLF system than in the SSF system because extracellular enzymes might reduce the pH over time. The results indicate that initial pH has different effects on the growth of *P. linteus* mycelia despite the use of the same substrate.

The optimal  $[S]$  was 60.3 g of lactose/L for the SSF system and 65.3 g of lactose/L for the SLF system, but it had a significant effect on mycelial growth only in the SSF system (Table 3, Figure 2). These values are about twice as high as the optimal substrate concentration of 29.7 g of lactose/L when using cheese whey as a sole substrate for cultivation of the mycelial *P. linteus* in an SSF system (Lee et al., 2011). In general, whey permeate is generated by ultrafiltration of raw cheese whey; this process removes whey proteins that enhance microbial growth (i.e.,  $\beta$ -LG, BSA,  $\alpha$ -LA, and so on; Singh et al., 2014), so whey permeate wastewater contains fewer nutrients than does cheese whey; this deficiency explains why higher  $[S]$  was required for optimal growth of mycelial *P. linteus* in our study than when whey was used. This hypothesis could be tested by reducing permeate loading or adding whey proteins in further research.

The differences in the optimum  $[S]$  and in the significance of  $[S]$  between the systems could be caused by the different substrate delivery characteristics—diffusion for SSF and mixing for SLF. In principle, diffusivity is significantly affected by the distance  $D$  between the microorganism and the source of the substrate that determines its growth rate (Harms and Bosma, 1997; Gonzo et al., 2014), so growth rate is inversely related to  $D$ . Several previous studies reported that the microbial activity depends on  $D$  (Harms and Bosma, 1997; Knudsen et al., 2013). In addition, during growth of filamentous fungi in SSF systems, hyphae penetrate the solid substrate matrix, so extracellular enzymes can reach deeply into it (Barrios-Gonzalez, 2012); this hyphal penetration enhances the substrate diffusivity by reducing  $D$  (Mitchell et al., 2000). However, in SLF sys-

tems, substrate is delivered mainly by mixing, which is a different mechanism than occurs in the SSF systems. This unique property can decrease substrate uptake by fungi in SLF systems. For example, the turbulent current generated by mixing can hinder substrate uptake by disturbing relevant contact between the substrate and fungi (Raghavarao et al., 2003). In addition, the shear force created by agitation can damage the hyphae, and thereby cause variation in growth rate (Tang et al., 2007). Therefore, these comparisons suggest that *P. linteus* mycelia can efficiently utilize the substrate despite the low  $[S]$  in the SSF system, so the optimal growth rate can be achieved at lower  $[S]$  in the SSF system than in the SLF system. Moreover,  $[S]$  would not have a significant effect on mycelial growth the SSF system because substrate utilization efficiency can be high regardless of  $[S]$  due to hyphal penetration into the substrate. However,  $[S]$  would significantly affect the growth rate in the SLF system due to mixing (Figure 4). Therefore, the results suggest that  $[S]$  should be carefully controlled in SLF systems for cultivating *P. linteus*.

## CONCLUSIONS

This study evaluated the use of whey permeate wastewater medium as a sole nutrient source for cultivation of *P. linteus* mycelia in SSF and SLF systems. Optimal growth conditions were  $T_0 = 26.1^\circ\text{C}$ ,  $\text{pH} = 4.6$ , and  $[S] = 60.3$  g of lactose/L in the SSF system and  $T_0 = 29.0^\circ\text{C}$ ,  $\text{pH} = 5.0$ , and  $[S] = 65.3$  g of lactose/L in the SLF system. Operating temperature had a statistically significant effect on growth rates in both fermentation systems; initial pH (but not  $[S]$ ) was significant in the SSF system, whereas  $[S]$  (but not initial pH) was significant in the SLF system. These findings can be used to guide operation of processes to cultivate *P. linteus* mycelia using whey permeate wastewater.

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