J. Dairy Sci. 90:2141–2146
doi:10.3168/jds.2006-690

Use of Whey Permeate for Cultivating *Ganoderma lucidum* Mycelia

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ABSTRACT

A novel approach to utilizing whey permeate, the cultivation of mycelia of the edible mushroom *Ganoderma lucidum*, is introduced. The major objective of this research was to use whey permeate as an alternative growth medium for the cultivation of mycelia of edible mushroom *G. lucidum* and to find an optimum condition for solid-state cultivation. Response surface analysis was applied to determine the combination of substrate concentration (25 to 45 g of lactose/L), pH (3.5 to 5.5), and temperature (25 to 35°C) resulting in a maximal mycelial growth. The radial extension rates, estimated by measuring the diameters of growing colonies on the Petri dishes, were used as the growth of the mycelia at different conditions. In the model, pH and temperature significantly affected mycelial growth, but lactose concentration did not. The condition predicted to maximize the radial extension rate of 17.6 ± 0.4 mm/d was determined to be pH 4.4 and temperature 29.4°C. Therefore, the results suggest that whey permeate could be utilized as a growth substrate for the cultivation of mycelia from the edible mushroom *G. lucidum*, enhancing the use of this by-product by the cheese manufacturing industry.
Key words: *Ganoderma lucidum*, mycelial cultivation, whey permeate

INTRODUCTION

Cheese whey is a by-product of cheese manufacture that remains when CN and butter fat are separated as curd from milk. Whey permeate is further produced when proteins in whey are recovered as a whey protein concentrate by ultrafiltration (Haast et al., 1986; Lee et al., 2003). Whey permeate contains nutrients necessary for microbial growth and holds almost 100% of lactose in milk (Table 1).

However, because of its high lactose content (4 to 5%; González Siso, 1996), disposal of large volumes of whey permeate causes serious environmental problems because of its chemical oxygen demand (40 to 60 g/L; Martin, 1998). One possible solution to this problem is to use this potential pollutant as a growth substrate for economically valuable products.

Mushrooms have long been valued as foods and components of medicines by societies throughout the world (Smith et al., 2002). *Ganoderma lucidum* is popular as an ingredient in health foods and medicines because of its perceived health benefits (Eo et al., 1999b; Bao et al., 2001). In Northern Asia, this mushroom has been used for centuries as a health food and popular folk medicine for treating various human diseases such as hepatitis, hypertension, hypercholesterolemia, gastric cancer, arthritis, and bronchitis. *Ganoderma lucidum* produces the polysaccharide 1,3-β-D-glucan, which inhibits a variety of cancers by enhancing the host's immune functions (Wang et al., 1997; Fang and Zhong, 2002). *Ganoderma lucidum* may also have hypoglycemic activity, anti-inflammatory effects, and cytotoxicity toward hepatoma cells (Eo et al., 1999b; Bao et al., 2001).

Bioactive polysaccharides in mushrooms can often be extracted from mycelia of the species without waiting for a full fruiting body to develop (Song et al., 1998; Hatvani, 2001). Therefore, mycelial cultivation has received great interest as an efficient method for industrial production of valuable metabolites, and various agro-industrial by-products have been tried as inexpensive growth substrates (Hatvani, 2001; Fang and Zhong, 2002).

In recent years, solid-state cultivation (**SSC**) of mycelia has led to a wide range of applications at the laboratory scale because information from SSC can be applied to more commonly used liquid-state cultivation (Lekha and Lonsane, 1994; Maldonado and de Saad, 1998). Solid-state cultivation has also been frequently utilized in preliminary tests for cultivating microorganisms under experimental conditions because it requires less time and is less labor intensive than liquid-state cultivation.

Control of experimental conditions is vital to maximizing production efficiency (Eo et al., 1999b; Bao et al., 2001; Fang and Zhong, 2002). In particular, substrate concentration, pH, and temperature are key vari-

Received October 19, 2006.
Accepted January 23, 2007.
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Table 1. Characteristics of whey permeate used in this research

Organics	Concentration (mg/L)	Inorganics	Concentration (mg/L)
Chemical oxygen demand	60,781 ± 329	Calcium	200 ± 5
Total organic carbon	22,640 ± 125	Magnesium	84 ± 2
Carbohydrate	40,885 ± 240	Potassium	723 ± 21
Lactose	40,000 ± 119	Phosphate	587 ± 28
Lactic acid	1,051 ± 63	Sulfate	134 ± 5
Ammonia nitrogen	523 ± 12	Iron	3 ± 1
Crude fat	530 ± 25	Zinc	6 ± 1

ables to optimize mycelial growth. However, little information is available regarding the optimization of environmental factors affecting the growth of *G. lucidum* on various substrates (in particular, agro-industrial residues) in SSC. The objective of this research was to find an optimum condition with respect to the simultaneous effects of substrate concentration, pH, and temperature, which maximize growth of *G. lucidum* mycelia grown on whey permeate under SSC.

MATERIALS AND METHODS

Preparation of Media

Culture media containing different concentrations of lactose were prepared by dissolving dried whey permeate powder in distilled water, adding commercial agar (Becton Dickinson and Co., Sparks, MD) in the ratio of 1.5% (wt/vol), mixing, and autoclaving (120°C, 20 min). The pH was adjusted as required using 0.5 *M* NaOH or 0.5 *M* HCl. Media were poured into Petri dishes and allowed to solidify. Because the purpose of this research was to provide information about the treatment of raw cheese whey permeate as a growth substrate for mushroom mycelia, no other nutrients were added.

Mycelial Culture

Stock cultures of *G. lucidum* (KCTC 6283, Korean Collection for Type Culture) were obtained by transferring mycelial samples to Petri dishes (i.e., 90 mm in diameter) containing potato dextrose agar medium. The cultures were incubated at 25°C for 4 d in darkness. Inocula for the experiments were obtained from these cultures using a round cutter to excise 5-mm discs containing mycelium.

Radial extension rates [i.e., rate of change in radius (mm/d)] were calculated by measuring the size of each colony daily for 5 d after transfer to experimental conditions. Size was defined as the average of 4 diameter measurements along lines crossing at right angles.

Analysis of Mycelial Extension Rate

Response surface analysis (**RSA**) was used to determine the relationship of radial extension rate to environmental conditions (lactose concentration, pH, and temperature). Response surface analysis is a collection of mathematical and statistical techniques for building empirical models (Sen and Swaminathan, 1997; Hwang et al., 2001). It was applied to evaluate the relative significance of the environmental factors and to determine an optimum condition (Montgomery, 2001) under which mycelial growth is maximum, within the experimental range of the independent variables.

The experiment (Table 2) was based on the central composite in cube design (Montgomery, 2001) and consisted of a 3×2 orthogonal design (lactose concentration, pH, and temperature, each at 2 levels). The ranges of independent variables were 25 to 45 g of lactose/L, 3.5 to 5.5 pH, and 25 to 35°C. Each treatment with a center point (i.e., 35 g of lactose/L, pH 4.5, and 30°C) was replicated 5 times as previously described (Adinarayana et al., 2003; Lee et al., 2003). This type of design was used to minimize the number of trials needed to obtain statistically valid results.

A sequential procedure of collecting data, estimating polynomials, and checking the adequacy of the model was used. The method of least squares was used to estimate the parameters in the approximating polynomials. For the statistical analysis, EChip software (version 7.01, EChip Inc., Hockessin, DE) was used to establish the experimental design and to test complex polynomials to model the data.

RESULTS

The region of exploration for the RSA was decided as 3.5 to 5.5 pH and 25 to 35°C because conditions for culturing the mycelia on synthetic media were acidic and mesophilic (Hatvani, 2001; Kim et al., 2002). We performed preliminary experiments to estimate the maximum radial extension rates of *G. lucidum* in various concentrations of whey permeate (i.e., 2, 3, 10, 20, 30, 40, and 70 g of lactose/L). The radial extension rates of *G. lucidum* at different substrate concentrations were then fitted to an equation suggested by Shi et al. (1999; Figure 1). The substrate concentration that maximized the radial extension rate was assumed to

Table 2. Experimental design and observed radial extension rate of *Ganoderma lucidum* mycelia grown on reconstituted dry whey permeate

Trial	Independent variable			Radial extension rate[2] (mm/d)
	Lactose concentration (g/L)	Media pH	Incubation temperature (°C)	
Linear design				
1	25	3.5	25	11.8 ± 0.4
2	45	3.5	25	12.2 ± 0.9
3	25	5.5	25	10.6 ± 0.8
4	45	5.5	25	11.8 ± 1.1
5	25	3.5	35	9.9 ± 0.9
6	45	3.5	35	8.6 ± 0.9
7	25	5.5	35	6.9 ± 0.7
8	45	5.5	35	8.6 ± 0.3
9[1]	35	4.5	30	17.6 ± 3.4
Quadratic design				
10	25	4.5	30	17.3 ± 1.2
11	45	4.5	30	16.4 ± 0.1
12	35	3.5	30	14.9 ± 0.8
13	35	5.5	30	15.1 ± 1.4
14	35	4.5	25	12.4 ± 0.3
15	35	4.5	35	11.2 ± 0.6

[1]Center point.

[2]Average value of each trial replicated 5 times.

be 35 g of lactose/L, which was used as a center point for RSA model building.

A total of 19 trials, including a center point, were run to approximate the response surface for the mycelial growth of *G. lucidum*. To find the maximum radial extension rate, increasingly complex equations from linear to quadratic were sequentially tested to model the data obtained from the trials in Table 2. When the data were analyzed using the various models, the *P*-value of regression was significant at the 0.1% α-level, whereas lack of fit was not significant at the 5% α-level only for the quadratic model (equation [1]):

$$\eta = 206.6 + 7.2 \times 10^{-2}x_1 + 17.4x_2 + 12.6x_3 + 4.7 \times 10^{-2}x_1x_2 - 3.4x_1x_3 \times 10^{-3} + 3.5 \times 10^{-2}x_2x_3 - 2.4 \times 10^{-3}x_1^2 - 2.1x_2^2 - 2.1 \times 10^{-1}x_3^2, \quad [1]$$

where η = experimental value of the radial extension rate (mm/d), and x_i = independent variable i (i = 1 for lactose concentration, 2 for pH, 3 for temperature).

An ANOVA using equation [1] was initially performed to investigate the possible interaction between variables (Table 3). None of the 3 possible 2-way interactions among the variables (lactose concentration × temperature, pH × temperature, and lactose concentration × pH) was significant at the 5% α-level. This meant that the 2 independent variables (lactose concentration,

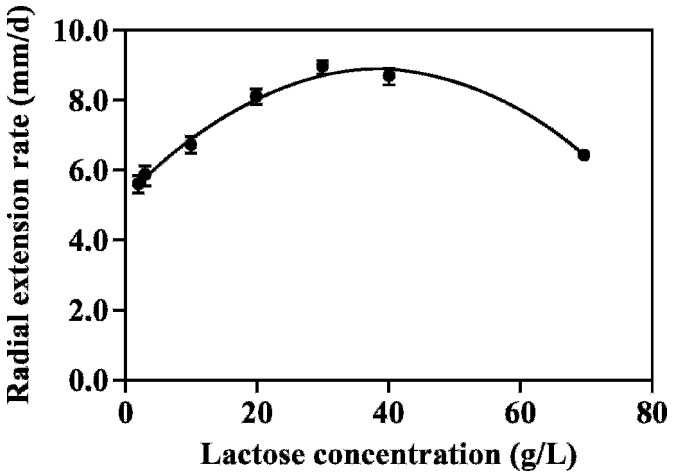

Figure 1. Observed and predicted mycelial growth rate of *Ganoderma lucidum* at different lactose concentrations at 25°C, pH 5.6. Bars represent the standard error of 5 replications: (●) observed mycelial growth rate; (—) model predictions. An equation suggested by Shi et al. (1999) was used to fit the radial extension rates of *G. lucidum* with different substrate concentrations.

Table 3. ANOVA for independent variables and their interactions

Source	Mean square	df	*P*-value
Lactose concentration	0.1447	2	0.8165
pH	46.6178	2	0.0049
Temperature	6.6968	2	0.0000
Lactose concentration vs. pH	0.2320	1	0.5661
Lactose concentration vs. temperature	0.2343	1	0.5690
pH vs. temperature	0.6570	1	0.5651

pH, and temperature at 2 levels each) were not interdependent, respectively. Further statistical inspection showed that pH and temperature affected the radial extension rate significantly at the 1% α-level, but the effect of lactose concentration was not significant at the 5% α-level. Therefore, another quadratic model excluding the lactose term was used to describe the response surface of the mycelial extension rate and was

$$\eta = -212.1 + 19.9x_1 + 12.7x_2 - 3.5 \times 10^{-2}x_1x_2 - 2.1x_1^2 - 2.1 \times 10^{-1}x_2^2, \quad [2]$$

where η = experimental value of the radial extension rate (mm/d), and x_i = independent variable i (i = 1 for pH, 2 for temperature).

The P-value of regression was significant at 0.1% α-level, whereas lack of fit was not significant at the 5% α-level only for the quadratic model (equation [2]). The regression coefficient and residual standard deviation of the quadratic model were 0.97 and 0.79, respectively. Therefore, this equation was used to determine the conditions that would maximize the radial extension rate by setting the partial derivatives of the equation to zero with respect to the independent variables. The RSA model estimated a maximal radial extension rate (17.6 ± 0.4 mm/d) at pH 4.4 and 29.4°C.

Two- and three-dimensional response surfaces of the quadratic model for the radial extension rate (Figure 2) showed a clear peak, which indicated that the optimum condition was well inside the design boundary. In the contour surfaces, the effects of independent variables on the response were evaluated using the grade of the contour lines along transects from the optimum condition toward the design boundary from equation [2]. As conditions depart from the optimum (i.e., at the peak) to the low and high limit, the grades were 2.61 and 1.78 per unit change in pH, 0.82 and 1.36 per unit change in temperature. These results indicate that the radial extension rate decreases sharply if media pH and incubation temperature move away from the optimum condition.

The residual plots for the model and the experimental data set showed no patterns or trends (Figure 3). Therefore, it was concluded that the model was able to accurately predict optimal growth conditions for *G. lucidum* mycelia using whey permeate as a growth substrate in SSC.

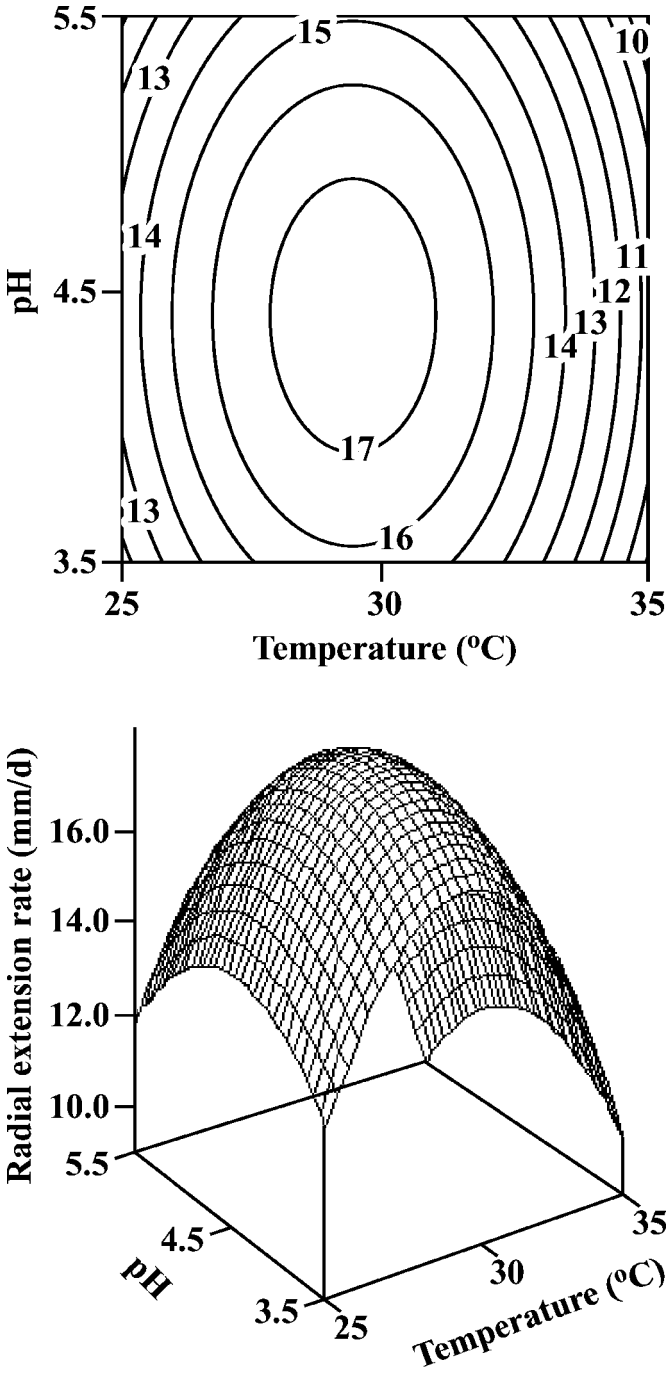

Figure 2. Two- and three-dimensional contour plots of the quadratic model for the mycelial growth of *Ganoderma lucidum* with respect to temperature and pH within the design boundaries.

DISCUSSION

Because lactose is the major carbohydrate in whey permeate, β-galactosidase is used to hydrolyze it (Madigan et al., 2003). *Ganoderma lucidum* produces β-galactosidase (Tang and Zhong, 2002), and this enzyme is presumably the basis of the mycelial growth observed in this research. The half-saturation coefficient of fungal β-galactosidase on lactose is 4.8 g/L at pH 4.5 and 55°C (Nikolaev et al., 1989; Samoshina and Samoshin, 2005). This reported value is much smaller than the

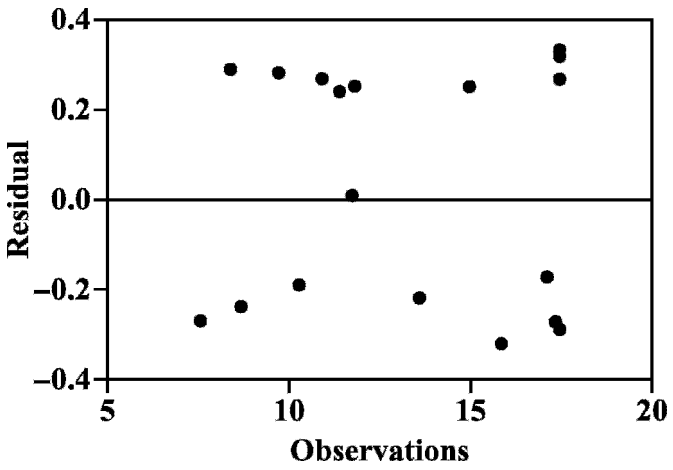

Figure 3. Residual plots of the quadratic model for radial extension rate. Each residual was calculated using equation [2].

range of lactose concentration [i.e., 25 ≤ lactose (g/L) ≤ 45] in this research. Therefore, the change of lactose concentration could have no measurable effect on the radial extension rate of *G. lucidum* within the design region. Changes in pH and temperature significantly affected the radial extension rate of *G. lucidum* mycelia grown on whey permeate (Figure 2). The optimum pH and temperature of purified fungal β-galactosidase lactose hydrolysis are pH 4.0 to 4.6 and 55 to 60°C (Cruz et al., 1999). This pH agrees well with that at which the maximum radial extension rate occurred in the present experiment, but the temperature does not. Clearly, cell growth and enzymatic activities are not necessarily affected in the same way by temperature. Generally, temperatures above the maximum at which growth can occur are lethal because high temperature may affect the breakdown of cell membranes or disruption of cuticular waxes (Madigan et al., 2003). *Ganoderma lucidum* is a mesophile fungus, which has a minimum temperature for growth above 0°C, a maximum below 50°C, and an optimum between 15 and 40°C (Griffin, 1994). The cell membranes of *G. lucidum* mycelia consist of 39.6% saturated fatty acids and 59.4% unsaturated fatty acids. Among the unsaturated fatty acids, linoleic acid (i.e., the melting point is –9°C) and oleic acid (i.e., the melting point is 13.4°C) are predominant components that compose 51.1% of the total fatty acids (Tseng et al., 1984).

Mycelial hyphae grow linearly under SSC, whereas mycelial mass in suspension culture increases exponentially in a fashion similar to a change in specific growth rate, because branching increases the number of apices (Pazouki and Panda, 2000). Optimal conditions for maximum mycelial production of *G. lucidum* in submerged culture using whey permeate were pH 4.2 at 28.3°C (Lee et al., 2003), which are very close to the optimal growth conditions estimated in this research. This suggests that SSC of mushroom mycelia with an adequate experimental design can be a cost-effective and convenient method to evaluate optimal growth conditions compared with suspension culture of mycelia.

CONCLUSIONS

Response surface analysis was successfully applied to determine the optimal conditions with respect to pH and temperature for growth of mycelia of *G. lucidum* used for bioconversion of whey permeate in SSC and to approximate the response surface describing the radial extension rate to changes in these variables. In the model, the effect of an interaction between the variables was not significant at the 5% α-level, but pH and temperature affected the radial extension rate significantly at the 1% α-level. The optimum condition for the maximum radial extension rate was pH 4.4 at 29.4°C. The random distribution of residuals as well as constant variance indicated adequacy of the quadratic model. The result shows that cultivation of *G. lucidum* mycelia at the optimum pH and temperature is a potential cost-effective solution for alternative treatment of cheese whey permeate.

ACKNOWLEDGMENTS

This research was supported in part by the Korean Institute of Environmental Science and Technology (EcoTechnopia-21), BK-21, and Advanced Environmental Biotechnology Research Center (AEBRC; Grant No: R11-2003-006-02002-0) programs.

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