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ORIGINAL PAPER

Bioconversion of starch processing waste to *Phellinus linteus* mycelium in solid-state cultivation

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Abstract The objective of the experiment was to use starch processing waste as an alternative growth medium for cultivation of mycelia of the mushroom *Phellinus linteus* and to find an optimum condition under solid-state cultivation. Response surface analysis along with a central composite design was successfully applied to approximate the simultaneous effects of the substrate concentration (16–36 g l^{-1}), pH (4.5–6.5), and temperature (25–35 °C) on the mycelial growth rate. In the model, pH and temperature significantly affected the mycelial growth but substrate concentration did not. The optimal substrate concentration, pH, and temperature for maximizing growth rate of *P. linteus* mycelia were found to be 16.5 g l^{-1}, pH 6.0, and 29.7 °C, respectively. Subsequent verification of these levels agreed with model predictions and the maximum mycelial growth rate at these conditions was 6.1 ± 0.8 mm day^{-1}. Therefore, the results of the experiments suggest that starch processing waste could be utilized as a growth substrate for the cultivation of the mushroom mycelia of *P. linteus*, enhancing the usefulness of this byproduct of the starch manufacturing industry. This approach is likely to be useful for establishing similar parameters for the cultivation of other fungi.

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Introduction

The commercial starch processing industry generally employs the corn wet-milling process and generates large quantities of slurry waste. Approximately, 44% of raw materials are generated as wastes such as bran residues [6]. This starch processing waste (SPW) disposal causes serious environmental problems mainly because of its large volume, high chemical oxygen demand (COD), organic suspended solids, and presence of potential foaming substances such as corn protein [3, 11]. However, SPW should be considered as a byproduct that can be used as a growth medium for economically valuable products such as fungal biomass rich in bioactive compounds [10].

Mushrooms have long been a source of nutritional foods and folk medicine. Especially, *Phellinus linteus* has been well known as a medicinal mushroom due to pharmacological properties and antitumor activity [19]. *P. linteus* has been used as a traditional medicine to cure gastroenteric disorder and lymphatic disease, and to lower total cholesterol level [12, 13]. Recent research into this mushroom has also revealed the presence of various biocompounds that activates immunomodulator, induces NAD(P)H:quinone oxidoreductase, and represses gap junctional intercellular communication by hydrogen peroxide (H_2O_2) [5, 13, 22].

Because mushrooms can excrete various enzymes to make use of almost any substrate as a growth medium, various agro-industrial byproducts have been tried as inexpensive substrates by different mushroom species [1, 7,

8, 20]. The beneficial bioactive compounds contained in mushrooms can be extracted from the mycelia of the species without waiting for a full fruiting body to develop [8]. Thus, mycelial cultivation has received great interest as an efficient method for industrial production of valuable metabolites [14, 16]. In this experiment, therefore, we hypothesized that a unique solution to the waste treatment problem and to reduce disposal cost would be to use SPW as a substrate for value-added *P. linteus* mycelial cultivation.

Although the efficacy of the bioactive compounds has been shown to be directly affected by the culture conditions in which the mycelia were grown [15–17], information about the effect of environmental factors for maximum growth of the *P. linteus* mycelia on SPW is lacking in literature. Among the many culture conditions affecting fungal growth, substrate concentration, pH, and temperature are considered as key variables for the enhanced growth of mycelia. Therefore, the objective of this research was to optimize growth condition of *P. linteus* using SPW with respect to the simultaneous effects of substrate concentration, pH, and temperature. A solid-state cultivation technique (SSC) was employed due to the advantages of less time and work requirements than that of liquid-state cultivation [23]. In this research, "optimum conditions" meant the operating conditions for maximizing the mycelial growth rate with respect to the independent variable.

Materials and methods

Starch processing waste and mycelia strain

One batch of SPW (i.e. 0.2 m^3) was obtained from Corn Product Korea (Icheon, Korea). The raw SPW, in slurry form, was initially dried at 60 °C to remove moisture, pulverized, and stored at 4 °C until used for the experiments.

Dried SPW powder was suspended in distilled water to obtain differing solids concentrations for subsequent experiments. Since the purpose of the research was to provide information about the use of raw SPW as an alternative substrate for mycelial cultivation, no additional nutrients were added. Commercial agar (Becton Dickinson and Co., Sparks, MD, USA) used for bacterial plate counts was added to the rehydrated SPW solution in the ratio of 1.5% (w/v). The pH was adjusted by addition of 0.5 M HCl or 0.5 M NaOH as needed to meet the experimental conditions in Table 1, followed by autoclaving at 120 °C for 20 min. The solution was poured into Petri dishes and allowed to solidify. These plates then became the growth media for the mycelial cultivation.

Phellinus linteus (KCTC 6719) was obtained from the Korean Collection for Type Cultures (KCTC) and maintained in a potato dextrose agar (PDA) slant at 4 °C. The seed culture of *P. linteus* was transferred to Petri dishes containing PDA media and incubated at 25 °C for 4 days.

Table 1 Experimental design and observed mycelial growth rate of *Phellinus linteus* mycelium grown on reconstituted starch processing waste

	Trials	Independent variables			Mycelial growth rate (mm day^{-1})
		Substrate concentration (g l^{-1})	Media pH	Incubation temperature (°C)	
Linear design	1	16	4.5	25	4.3
	2	36	4.5	25	4.3
	3	16	6.5	25	4.9
	4	36	6.5	25	4.4
	5	16	4.5	35	3.0
	6	36	4.5	35	3.0
	7	16	6.5	35	4.7
	8	36	6.5	35	4.4
	9[a]	26	5.5	30	5.9 ± 0.1
Quadratic design	10	16	5.5	30	5.9
	11	36	5.5	30	5.4
	12	26	4.5	30	4.2
	13	26	6.5	30	5.9
	14	26	5.5	25	4.7
	15	26	5.5	35	4.2
Validation	16	26	5.7	30	5.6 ± 0.1

[a] The experiment was repeated three times and the response represented average values

Mycelial agar disks (5 mm) were cut using a round cutter and used as inocula. After inoculation, the SPW-containing Petri dishes were placed in two identical incubators at different temperatures according to the experimental design listed in Table 1.

Experimental design and analysis

Petri dishes inoculated with actively growing mycelia were removed from the incubator every 24 h for 10 days to collect growth rate data. Since the colonies grow in a circular fashion, the data was collected by using standard laboratory calipers to measure the diameter of each mycelial colony in millimeter as it grew on the Petri dish. The diameter was measured daily in four different places along lines crossing at right angles. The average size of the colony was plotted against the cultivation periods, and the slope of the regression line was estimated by a least squares method. The slope represented the mycelial extension rate and was assumed to be the growth rate of the mycelia under the given conditions [23]. These rates along with their corresponding culture environments were used to obtain the set of conditions that would maximize the mycelial growth rate by response surface analysis (RSA).

RSA is an iterative statistical technique to approximate multivariate responses and is represented by the following equation:

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

where η = experimental value of the mycelial growth rate (mm day^{-1}), C_0 = regression constant, and x_i = independent variable i (i = substrate concentration, pH, temperature in order).

A sequential procedure of collecting data, estimating polynomials with the least squares method, and checking the adequacy of the model was used. The experimental design was based on a 3 × 2 central composite in cube (CCC) design with a center point being replicated three times as previously described [4, 23]. This type of design was used to minimize the number of trials needed to obtain statistically significant results. Experimental conditions at the central point were selected to be as close as possible to the optimal environment of *P. linteus* mycelia cultivation found in the literature [9, 19].

The COD of the dried SPW powder was measured by the closed reflux colorimetric method, and the amount of protein was measured according to the Kjeldahl method [2]. Total organic carbon (TOC) was quantified using a TOC analyzer (TOC-V_{CHP}, Shimadzu). Two identical ion-exchange chromatographs (790 Personal IC, Metrohom) were used to quantify the cations and anions in the sample. Carbohydrate in the sample was determined by phenol–sulfuric acid assay. Solids concentrations were determined according to the procedures in standard methods [2].

Results and discussion

Waste characteristics

Table 2 summarizes the physical and biochemical characteristics of the SPW used in this research. Most of the solids were in suspended form [i.e., 96.7% (w/w)]. A high ratio of volatile suspended solids (VSS) (i.e., organic residues remaining after ignition at 550 °C) to total suspended solids (TSS), 99.0%, indicated that most of the solids were potentially biodegradable organics. Carbohydrates and proteins, 70.9% (wt/wt), were likely to be major COD contributing organics in the waste.

Response surface analysis and optimal culture conditions

A total of 17 trials were run to approximate the response of the mycelial growth rate. Because SPW has not previously been used for the mycelial cultivation of *P. linteus*, we performed preliminary experiments testing the growth rate of *P. linteus* in various concentrations of SPW (i.e., 3, 10, 30, 50, 70, 90 g SPW powder l^{-1}) at 5.5 pH and at 30 °C. The radial extension rates of *P. linteus* at different substrate concentrations were then fitted to an equation suggested by Shi et al. [21]. The substrate concentration that maximized

Table 2 Composition and characteristics of the starch processing waste (mg g^{-1} dry weight)

Parameter	Concentration	Parameter	Concentration
Moisture content (%)	90 (2)	Crude protein[a]	207 (19)
COD	1,822 (81)	Ammonium	6.4 (0.2)
SCOD[b]	93 (2)	Calcium	7.9 (1.1)
TOC	491 (0.1)	Magnesium	2.4 (0.3)
Carbohydrate	502 (56)	Potassium	11.4 (0.4)
Total nitrogen	45 (3)	Sodium	6.8 (0.2)
TKN[c]	36 (3)	Phosphate	42 (0.4)
TSS	967 (83)	Sulfate	31 (0.6)
VSS	957 (86)	Chloride	32 (0.1)

Standard deviations are in parentheses

[a] Protein content = (TKN − ammonia nitrogen) × 6.25

[b] Soluble COD

[c] Total Kjeldahl nitrogen

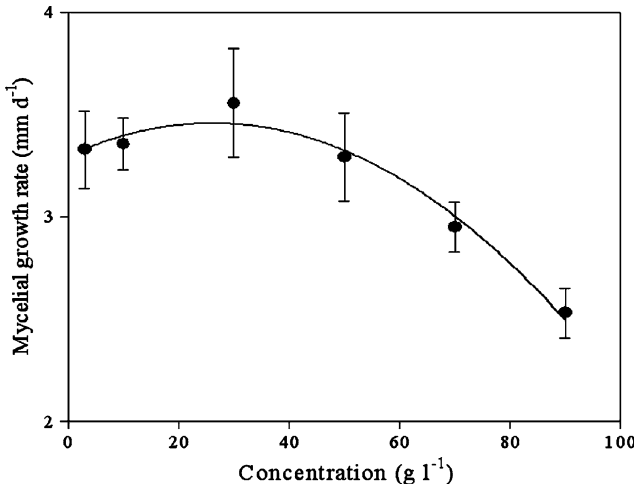

Fig. 1 Observed and predicted mycelial growth rate of *P. linteus* at different substrate concentrations: (*filled circle*) observed mycelial growth rate; (*solid line*) model predictions. An equation suggested by Shi et al. (1999) was used to fit the mycelial growth rate of *P. linteus* with different substrate concentrations

the mycelial growth rate was determined to be 26 g l^{-1} (Fig. 1). This value was used as a center point for RSA modeling.

The region of exploration for the *P. linteus* mycelia cultivation was decided to be 16–36 g l^{-1}, 4.5–6.5 pH, and 25–35 °C. Eleven trials including a center point (from trials 1 to 9 in Table 1) were run first. Initially, these data were fit using a first-order model with least squares to investigate the location of the optimum condition. The regression coefficient, the lack of fit (LOF) of the first-order model, and *P* values of the parameter estimations were used to validate the model. The *P* value for the LOF was significant, while the regression coefficient for the mycelial growth rate was not significant at the 5% α-level. Therefore, it was concluded that the first-order model was not an adequate approximation for the mycelial growth rate of *P. linteus*. The curvature in the response of the mycelial growth rate indicated that the conditions were likely to be near the optimum.

A second or higher order model could not be fit using the data from trials 1 to 9 in Table 1 due to lack of axial data points. An additional six trials (from 10 to 15 in Table 1) were augmented with previous trials to make a CCC design. To find more precise polynomials for a maximum mycelial growth rate in the response, increasingly complex equations from linear to quadratic were tried to model the data in Table 1.

The *P* value of regression was significant at 0.1% α-level and LOF was not significant at 5% α-level only for the quadratic model (Eq. 2). The regression coefficient and residual standard deviation of the quadratic model were 0.97 and 0.28, respectively.

$$\begin{aligned}\eta = {} & -41.4 + 7.9 \times 10^{-4}x_1 + 4.6x_2 + 2.3x_3 \\ & - 9.5 \times 10^{-3}x_1x_2 + 5.9 \times 10^{-4}x_1x_3 \\ & + 5.8 \times 10^{-2}x_2x_3 + 3.9 \times 10^{-4}x_1^2 - 0.5x_2^2 \\ & - 4.5 \times 10^{-2}x_3^2\end{aligned} \quad (2)$$

Therefore, the second-order model was selected to describe the response surface of the mycelial growth rate within this region. Residual plots represent the difference between the experimental and calculated values using models. Residual plots for all experimental values were examined for any weakness of the selected model and showed no patterns or trends (Fig. 2). If the model is adequate, the residual should be structureless [18]. Therefore, the scattered data points indicated that the model (Eq. 1) was adequate approximation for *P. linteus* mycelial growth rate with respect to the independent variables. The plots also showed least variance compared to other models and a random plot of residuals indicated homogeneous error variances across the observed values.

An additional trial was run to verify the accuracy of the model prediction at randomly selected experimental conditions (26 g l^{-1}, pH 5.7, 30 °C). The experiment was replicated five times. The observed value along with standard error was 5.6 ± 0.1 mm day^{-1}, which was close to the model response of 5.5 mm day^{-1}. Therefore, it was concluded that the model was able to predict accurately the response surfaces of growth conditions for *P. linteus* mycelia using SPW. The equation was then used to determine the optimal conditions maximizing the mycelial growth rate by setting the partial derivatives of the equation at zero with respect to the corresponding variables. The optimum condition for the maximal mycelial growth rate was 16.5 g l^{-1}, 6.0 pH, and 29.7 °C. The calculated model output at the optimal condition was 6.1 ± 0.8 mm day^{-1}.

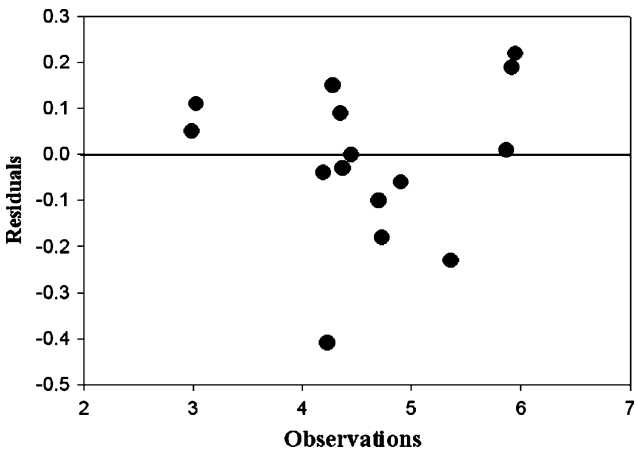

Fig. 2 Residual plots of the quadratic model for mycelial growth rate. Each residual was calculated using Eq. 2

Analysis of variance using Eq. 2 was initially performed to investigate the three possible two-way interactions among the independent variables (substrate concentration × temperature, pH × temperature, and substrate concentration × pH). The effect on the response was significant at 5% confidence level only for the interaction term of pH and temperature, which indicated that pH and temperature were interdependent. This interaction can be seen as a region of an elongated ellipse in the response surface of the mycelial growth rate (Fig. 3). The rounded ridge, inside the design boundary, runs diagonally on the plot from lower left to the upper right. In the contour surface, the effect of independent variables on the response was evaluated using the grade of the contour lines along transects from the optimum condition toward the design boundary. As shown in Fig. 3, movement away from the optimum conditions along the path of steepest ascent was 31 °C increase in temperature for every unit decrease in pH. Therefore, the mycelial growth rate is likely to drop sharply if the operational conditions with respect to the two variables are simultaneously increased or decreased as described, such as a 31 °C decrease in temperature per unit of pH increases. Contrary to the steepest ascent, 1 °C decrease in temperature for every unit decrease in pH was the gentlest slope of the response.

Thus, it could be calculated that variation in the mycelial growth rate would be less than 5.0%, compared to the maximum value, if the two independent variables were changed in the range 29.5 ± 2.5 °C and 5.9 ± 0.6 pH. The limits of the conditional boundary within the contour plots are shown as straight lines forming a tetragon on the plots (Figs. 3, 4).

Further statistical inspection showed that temperature and pH affected the mycelial growth rate significantly at 1% α-level, but the effect of substrate concentration was not significant at 5% α-level. Figure 4 represents 2D and 3D plots of the effect of temperature and substrate concentration on the mycelial growth rate at pH 5.5. It can clearly be seen that the response contours were concentric ellipses that were elongated along the substrate concentration axis, forming a stationary maximum system or a line maximum [18]. Insensitivity of the response to movement along the substrate concentration axis rendered almost no change in the response off the value at the stationary point. However, sizable changes in the response surfaces were shown along the temperature axis, which indicated that temperature had a much bigger effect than substrate concentration.

Factorial design, used most often in biological research, yields discrete results according to the values that the investigator assigns to the variables. It is hard to visualize the effect of the variables that were not assigned (for example, middle point between two variable values). With

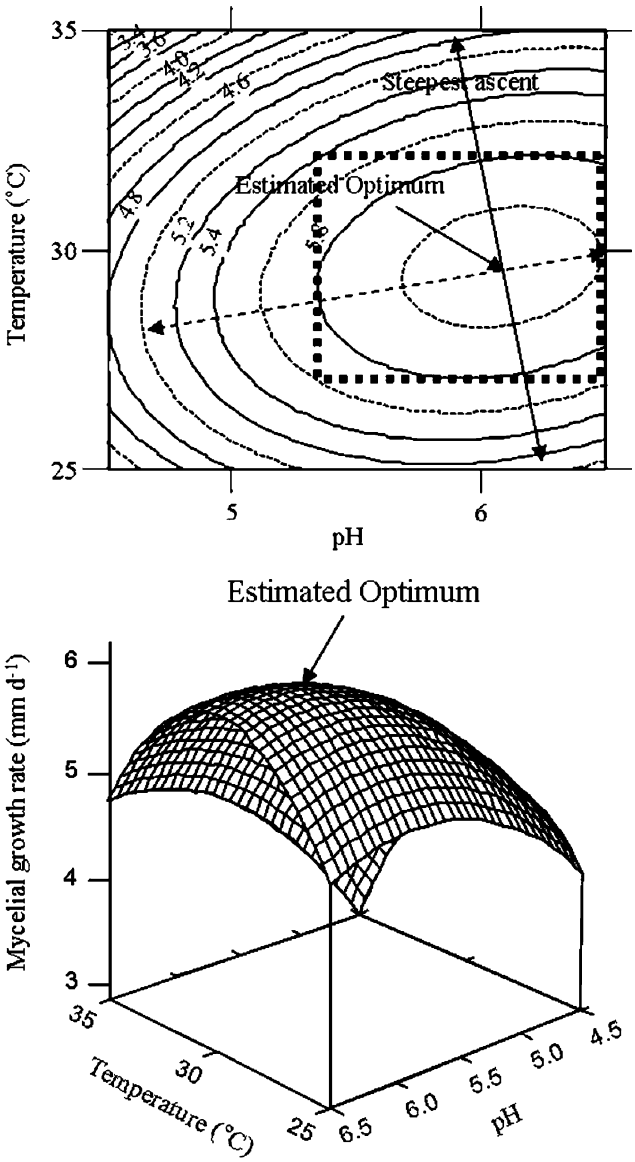

Fig. 3 Two-dimensional and three-dimensional contour plots of the quadratic model for the mycelial growth of *P. linteus* with respect to substrate concentration and temperature within the orthogonal design boundaries. The designed intervals were $4.5 \leq \text{pH} \leq 6.5$ and $25 \leq \text{temperature (°C)} \leq 35$, respectively

the proper selection of variable interval, RSA continuously approximates the response surface, which allows the researcher to investigate the entire region of variables. The unbounded polynomial models that defined the response surface might predict the location of the optimum response outside the design boundary. Since the polynomial models extend to infinity, however, the models used to predict the response surface here should be restricted within specified boundaries. It should also be noted that the response contours would appear slightly different if a higher order polynomial had been chosen because of greater flexibility of the polynomial, but so long as there was no LOF for the

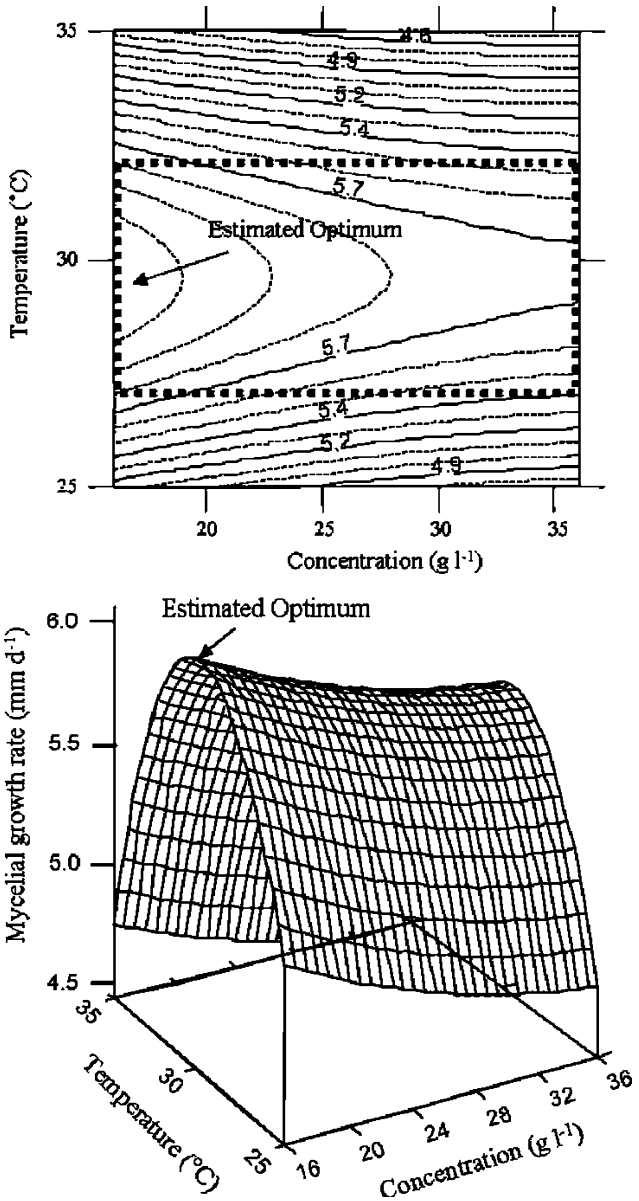

Fig. 4 Two-dimensional and three-dimensional contour plots of the quadratic model for the mycelial growth of *P. linteus* with respect to substrate concentration and temperature within the orthogonal design boundaries. The designed intervals were $16 \leq$ concentration (g l^{-1}) ≤ 36 and $25 \leq$ temperature (°C) ≤ 35, respectively

partial cubic model, the differences would not have been significant.

The necessity for reduction for pollutant and the need to maximize returns on raw material have encouraged the starch producers to seek new way of utilizing the wastes, which have widely relied on using it as cheap ingredient of animal feed. Based on the results in this experiment, a unique solution to solve the waste disposal problem and to provide additional benefit to starch processing industries would be to use SPW as a substrate for value-added *P. linteus* mycelia cultivation.

Conclusions

RSA was successfully applied to evaluate the effects of substrate (i.e. SPW) concentration, pH, and temperature on growth rate of *P. linteus* mycelia. A quadratic model was selected to approximate the response, and the adequacy of the model was verified by the results of validation data and residual plots. In the predictive model for mycelial growth rate of *P. linteus*, the effects of pH and temperature were significant at the 0.1 and 1% α-level, respectively, but the substrate concentration did not affect the mycelial growth rate significantly at the 5% α-level. The optimum condition for the maximum mycelial growth rate was 16.5 g l^{-1}, 6.0 pH, and 29.7 °C. Maximum growth rate of the mycelia at the optimal condition was calculated and to be 6.1 ± 0.8 mm day^{-1}. It can be concluded that SPW could be used as an alternative growth substrate for the cultivation of *P. linteus* mycelia.

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