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Full Length Research Paper

Optimization of mycelial biomass and protease production by *Laccocephalum mylittae* in submerged fermentation

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Laccocephalum mylittae **is a traditional Chinese medicinal fungus. Its wild sclerotium has been used in anthelmintic therapy for many years. Previous studies revealed that the main pharmaceutical component for the anthelmintic function of *L. mylittae* is an intracellular neutral protease, isolated from the wild sclerotium of this fungus. In this study, the mycelial biomass and protease production by *L. mylittae* in submerged fermentation was optimized using response surface methodology (RSM) and orthogonal matrix method. Under the optimum medium and conditions, the maximum activity of the anthelmintic protease from the mycelia of *L. mylittae* reached 4557.22 ± 147.36 U per gram mycelial dry weight (U/g), with the biomass of 11.65 ± 0.33 g/l after 3 d batch fermentation in a 15 l fermenter. This is a 2.71-fold and a 3.32-fold increase in protease productivity and biomass yield respectively, compared with that in the initial un-optimized fermentation process. The production time was also reduced from 4.5 d to 3 d. In addition, protease activity from the mycelia in optimized fermentation is 8.14-fold higher than that from the wild sclerotium of *L. mylittae*. These results provided valuable information for the further industrial production and applications of this fungus as well as its main pharmaceutical component.**

Key words: *Laccocephalum mylittae*, protease production, submerged fermentation, optimization, response surface methodology, orthogonal matrix method.

INTRODUCTION

In recent years, mushrooms have become an attractive source of various proteases, such as a fibrinolytic protease from *Flammulina velutipes* and a metallo-protease from *Perenniporia fraxinea* (Park et al., 2007; Kim et al., 2008). Proteases are the most important industrial enzymes used in detergents, food, agrochemicals and pharmaceuticals (Gupta et al., 2002).

Laccocephalum mylittae Núñez and Ryvarden (Chinese Lei-Wan; Synonym: *Polyporus mylittae* Cooke and Massee, *Omphalia lapidescens* Cohn and Schroet) is an economically medicinal fungus growing in New Zealand, South Australia and in vast regions of China (Cunningham, 1965; Núñez and Ryvarden, 1995; Buchanan and Ryvarden, 2000; Buchanan, 2001; Pan and Sun, 2005). Submerged sclerotium is the part of the fungus most likely to be met with and has been used throughout history as a vermifuge against many kinds of parasites, including roundworms, cestodes and ancylostomes (Miyazaki and Nishijima, 1980; Dedman, 2000; Pan and Sun, 2005). It is effective in anthelmintic therapy and results in little side-effects or environmental pollution, which are the main problems caused by chemotherapeutic agents (Wynn, 1996; Agaie and Onyeyili, 2007). Previous reports have indicated that the main anthelmintic component in *L. mylittae* is an intracellular neutral protease, isolated from the wild sclerotium (Guo et al., 1997; Zhao et al., 1998). This biologically active protease is able to destroy the parasite proteins and exhibits strong lethal effects on parasites. Our *in vitro* anthelmintic tests against the third stage larvae of *Ascaris*

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suum confirmed these findings, and the protease showed no toxicity or other side-effects in the acute toxicity test (unpublished data).

Although widely used in clinics and agriculture, the wild sclerotium of *L. mylittae* is difficult to collect in the natural environment because it grows underground and its development is limited by season, climate and other conditions (Dedman, 2000; Pan and Sun, 2005). Furthermore, collection of the wild sclerotium is getting harder and harder as the natural environment being destroyed. Consequently, producing this fungus and its main anthelmintic component under artificial control by submerged fermentation is much more efficient and important for environmental protection. Many fungi species have been obtained on a large scale by this method, and their products have been proved to have the same or stronger pharmacological activities compared to that of wild fungi (Parra et al., 2005; Revankar and Lele, 2006; Xu et al., 2006). To the best of our knowledge, little study has focused on the process control for the submerged culture of *L. mylittae* and the production of its main pharmaceutical component to date. The objective of the present work was to establish and optimize the entire fermentation process of mycelial biomass and the main pharmaceutical component of *L. mylittae*. The optimum culture media and conditions for biomass and protease production in submerged fermentation were obtained by response surface methodology (RSM) and orthogonal matrix method.

MATERIALS AND METHODS

Fungal strain

L. mylittae strain L66 was isolated from the wild sclerotium of *L. mylittae*, collected in Sichuan, China. The corresponding strain number in China General Microbiological Culture Collection Center (CGMCC) is AS 5.158.

Culture media

The medium of stock slants consisted of (g/l): potato, 200; dextrose, 20; agar, 15. The basal medium for seed culture and fermentation was determined as follows according to Lin (2000) and our preliminary experiments (g/l): dextrose, 10; yeast extract, 10; KH_2PO_4, 1.5; $MgSO_4 \cdot 7H_2O$, 0.5; Vitamin B_1, 0.01, at an initial pH of 6.0.

Culture conditions

Stock slants were incubated at 26°C for 10 d before storing at 4°C. For seed cultivation, mycelia from fresh slants was inoculated into 100 ml of fermentation medium in 500 ml Erlenmeyer flasks, incubated on a rotary shaker incubator at 180 rpm and 27°C for 3 d. For original culture in shake flasks, 10% (v/v) inoculum was inoculated into 100 ml of fermentation medium in 500 ml Erlenmeyer flasks, incubated on a rotary shaker incubator for 4.5 d at 180 rpm and 27°C. The original batch fermentation was carried out in a 15 l fermenter, equipped with two 6-bladed disc impellers and sensors measuring temperature, dissolved oxygen (DO) and pH (FUS-15, Shanghai Guoqiang Bioengineering Equipment CO. LTD., China). The working volume was 8 l. The biomass and protease production was conducted for 4.5 d at 27°C, with an initial pH of 6.0, an aeration rate of 1:0.8 vvm (volume of air per volume of medium per minute), an agitation rate of 180 rpm, and 10% (v/v) inoculum of 3 d seed cultivation. The variations of temperature DO and pH during the batch fermentation were monitored and recorded automatically by the fermentation system.

Mycelial biomass measurement

Mycelial biomass was measured as the mycelial dry weight after the mycelia were harvested by filtration through eight layers of gauze and subsequently freeze dried at -50°C.

Enzyme assay

After the cultured mycelia were filtered and freeze dried, the anthelmintic protease was purified from the mycelia by lixiviation, ion exchange chromatography and gel filtration chromatography according to Du and Li (1987). Protease activity was measured at its optimum reaction pH of 7.5 and temperature of 48°C (Du and Li, 1987), by hydrolysis of 2% (w/v) casein using a modified Folin-phenol method as previously described (Wu et al., 2006). One unit of protease activity is defined as the amount of protease capable of releasing 1 µg tyrosine of reducing casein per minute under the assay conditions.

Selection of carbon and nitrogen sources for fermentation in shake flasks

The effect of carbon sources on mycelial biomass and protease activity was investigated by a set of experiments using six kinds of carbon sources separately at the concentration of 10 g/l based on the basal medium, including dextrose, D-fructose, glycerol, maltose, soluble starch and sucrose. In addition, dextrose and sucrose were combined in a ratio of 1:1 as a single factor at the same concentration (10 g/l) to verify whether it had a superior effect as mentioned in previous research (Lin, 2000). The effect of nitrogen sources on biomass and protease activity was investigated using seven kinds of different nitrogen sources separately at the concentration of 10 g/l based on the basal medium, including beef extract, corn steep liquor, NH_4Cl, peptone, tryptone, urea and yeast extract. Concentrations of other components in the medium were fixed at original levels. Fermentation was carried out under original culture conditions.

Optimization by RSM on fermentation in shake flasks

Significant factors for biomass and protease production were screened using the two-level Plackett-Burman (PB) design. The factors investigated were separated into two groups, fermentation medium and fermentation conditions. Two screening experiments were conducted separately to avoid the effects of factors from one group being disguised by the factors from the other group; thus more accurate results could be obtained. Firstly, the screening experiment for identifying significant factors of fermentation medium was performed by PB design with four independent factors including yeast extract concentration (X_1), corn steep liquor concentration (X_3), dextrose/sucrose concentration (X_5) and initial pH (X_7), while X_2, X_4, X_6, X_8 were dummy variables used to evaluate the errors (Table 1). Secondly, the screening experiment for identifying significant factors of fermentation conditions were carried out by PB design with four new independent factors including ino-

Table 1. Plackett-Burman (PB) design of fermentation medium for biomass and protease production by *Laccocephalum mylittae*.

Run No.	Coded factor level[a]								Biomass (g/l) (Y_1)[b]	Protease activity (U/g) (Y_2)[b]
	YE (X_1)	Dum (X_2)	CSL (X_3)	Dum (X_4)	DS (X_5)	Dum (X_6)	pH (X_7)	Dum (X_8)		
1	1	-1	1	-1	-1	-1	1	1	5.31	2031.23
2	1	1	-1	1	-1	-1	-1	1	4.52	1951.84
3	-1	1	1	-1	1	-1	-1	-1	4.97	1995.56
4	1	-1	1	1	-1	1	-1	-1	4.69	1868.77
5	1	1	-1	1	1	-1	1	-1	6.30	2130.10
6	1	1	1	-1	1	1	-1	1	6.76	2176.58
7	-1	1	1	1	-1	1	1	-1	4.38	1938.39
8	-1	-1	1	1	1	-1	1	1	4.81	1980.94
9	-1	-1	-1	1	1	1	-1	1	6.17	2117.09
10	1	-1	-1	-1	1	1	1	-1	5.95	2095.71
11	-1	1	-1	-1	-1	1	1	1	4.72	1871.73
12	-1	-1	-1	-1	-1	-1	-1	-1	3.75	1775.32

YE: yeast extract; CSL: corn steep liquor; DS: dextrose/sucrose; Dum: dummy.
[a]The actual factor levels, coded as values of -1, 1 in the table, were as follows: yeast extract, 10 (-1) and 20 (1) g/l; corn steep liquor, 10 (-1) and 20 (1) g/l; dextrose/sucrose, 10 (-1) and 20 (1) g/l; initial pH, 6.0 (-1) and 7.0 (1).
[b]All factorial points are means of triplicate values.

culum size (X_9), liquid volume (X_{11}), inoculum age (X_{13}), temperature (X_{15}) and shaking speed (X_{16}), with three dummy variables X_{10}, X_{12}, X_{14} (Table 2). Finally, a three-factor-five-level central composite rotatable design (CCRD) was performed on the basis of the PB tests, (Table 3). Modeling and statistical analyses were performed by SAS (Version 8.0, SAS Inst., Inc., Cary, NC), while maps of response surfaces and contour plots were made and evaluated by Matlab (Version 7.1, the MathWorks, Inc., Natick, MA).

Optimization by orthogonal matrix method on fermentation in a 15 l fermenter

L_9 (3^4) orthogonal array was used for the optimization of biomass and protease production in the batch fermentation in a 15 l fermenter. Four factors on three different levels were involved in the design, including aeration rate, agitation rate, temperature and initial pH (Table 4). Medium composition, inoculum size and inoculum age were kept at optimum levels according to the results from optimized fermentation in shake flasks. Data were analyzed using MINITAB 13.30 (Minitab Inc., State College, PA., USA).
Protease activity was considered as the primary response for optimization, and biomass was taken into account as a secondary response. All experiments including fermentation and assay were performed in triplicate.

RESULTS AND DICUSSION

Selection of carbon and nitrogen sources

Selection of appropriate carbon and nitrogen sources is crucial in the development of an efficient and economical process. Hence, these two parameters were selected in the present work.

The effect of carbon source on biomass and protease production by *L. mylittae* was investigated using one-factor-at-a-time method (Figure 1). All the carbon sources tested could be utilized by *L. mylittae*, differing distinctly in degree. Dextrose and sucrose were the best two sources for both biomass and protease production, especially when these two were combined together in a ratio of 1:1 in the medium. This is in agreement with the previous literature (Lin, 2000) and probably because dextrose, as a monosaccharide, is favorable for fast mycelia growth while sucrose, as a disaccharide, is helpful for later metabolite accumulation. As a result, dextrose and sucrose were selected as a combined source in a ratio of 1:1 for both the seed cultivation and subsequent fermental optimization.

Among the various nitrogen sources tested by one-factor-at-a-time method (Figure 2), neither NH_4Cl nor urea was beneficial for biomass or protease production as inorganic nitrogen sources. Compared to organic nitrogen source, inorganic nitrogen source has been found less favorable for fungus growth and metabolism in some previous reports (Chung and Tzeng, 2004; Rayati et al., 2001). For mycelia growth, yeast extract was the best nitrogen source followed by peptone, both of which are simple organic nitrogen sources. The preferred utilization of yeast extract by higher fungi has been reported in the literature (Fasidi and Olorunmaiye, 1994; Gbolagade et al., 2006). The stimulatory action of yeast extract on biomass yield may be linked with its high carbohydrate, amino acids and vitamins composition (Gbolagade et al., 2006). Corn steep liquor gave less biomass than yeast extract or peptone. This is probably due to the usually slower speed of nitrogen utilization by

Table 2. Plackett-Burman (PB) design of fermentation conditions for biomass and protease production by *Laccocephalum mylittae*.

Run No.	Coded factor level[a]								Biomass (g/l) (Y_1)[b]	Protease activity (U/g) (Y_2)[b]
	IS (X_9)	Dum (X_{10})	LV (X_{11})	Dum (X_{12})	IA (X_{13})	Dum (X_{14})	T (X_{15})	SS (X_{16})		
1	1	-1	1	-1	-1	-1	1	1	2.17	1617.33
2	1	1	-1	1	-1	-1	-1	1	3.05	1704.74
3	-1	1	1	-1	1	-1	-1	-1	2.07	1606.92
4	1	-1	1	1	-1	1	-1	-1	1.50	1549.76
5	1	1	-1	1	1	-1	1	-1	2.85	1684.61
6	1	1	1	-1	1	1	-1	1	4.41	1840.84
7	-1	1	1	1	-1	1	1	-1	1.46	1546.16
8	-1	-1	1	1	1	-1	1	1	2.83	1682.75
9	-1	-1	-1	1	1	1	-1	1	2.46	1646.19
10	1	-1	-1	-1	1	1	1	-1	3.27	1726.88
11	-1	1	-1	-1	-1	1	1	1	1.30	1530.21
12	-1	-1	-1	-1	-1	-1	-1	-1	0.49	1349.18

IS: inoculum size; LV: liquid volume; IA: inoculum age; T: temperature; SS: shaking speed; Dum: dummy.
[a] The actual factor levels, coded as values of -1, 1 in the table, were as follows: inoculum size, 10% (-1) and 15% (1) (v/v); liquid volume, 100/500 (-1) and 150/500 (1) (ml/ml); inoculum age, 3 (-1) and 4 d (1); temperature, 25 (-1) and 30ºC (1); shaking speed, 160 (-1) and 220 rpm (1).
[b] All factorial points are means of triplicate values.

Table 3. Central composite rotatable design (CCRD) matrix for biomass and protease production by *Laccocephalum mylittae*.

Run no.	Coded factor level[a]			Biomass (g/l) (Y_1)[b]	Protease activity (U/g) (Y_2)[b]	
	DS (X_1)	IS (X_2)	IA (X_3)		Experimental	Predicted
1	-1	-1	-1	4.43	2742.84	2823.23
2	-1	-1	1	6.99	2998.55	2994.92
3	-1	1	-1	4.89	2788.92	2815.67
4	-1	1	1	6.48	2948.16	3012.08
5	1	-1	-1	5.64	2864.31	2847.63
6	1	-1	1	8.76	3176.43	3196.92
7	1	1	-1	6.29	2828.48	2879.67
8	1	1	1	8.86	3286.50	3253.68
9	-1.68179	0	0	4.12	2711.71	2637.94
10	1.68179	0	0	6.54	2853.89	2861.65
11	0	-1.68179	0	7.44	3044.53	3018.18
12	0	1.68179	0	7.98	3098.55	3059.18
13	0	0	-1.68179	7.53	3052.92	2992.73
14	0	0	1.68179	10.16	3456.16	3451.66
15	0	0	0	10.12	3415.35	3389.18
16	0	0	0	9.14	3453.42	3389.18
17	0	0	0	10.15	3450.63	3389.18
18	0	0	0	10.05	3347.74	3389.18
19	0	0	0	8.76	3275.68	3389.18
20	0	0	0	10.08	3376.15	3389.18

DS: dextrose/sucrose concentration; IS: inoculum size; IA: inoculum age.
[a] The actual factor levels, coded as values of -1, 1 in the table, were as follows: dextrose/sucrose concentration, 5.2 (-1.68179), 12 (-1), 22 (0), 32 (1) and 38.8 (1.68179) g/l; inoculum size, 10% (-1.68179), 12% (-1), 15% (0), 18% (1) and 20% (1.68179) (v/v); inoculum age, 2.66 (-1.68179), 3 (-1), 3.5 (0), 4 (1) and 4.34 (1.68179) d.
[b] All factorial points are means of triplicate values.

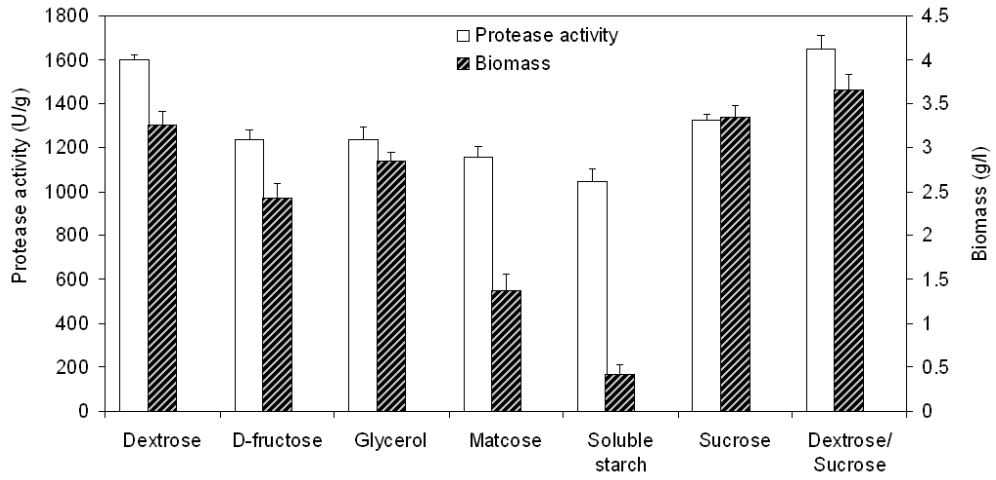

Figure 1. Effect of carbon sources on biomass and protease production by *Laccocephalum mylittae*. Concentrations of other components in fermentation medium were fixed at original levels. Fermentation was carried out under original culture conditions.

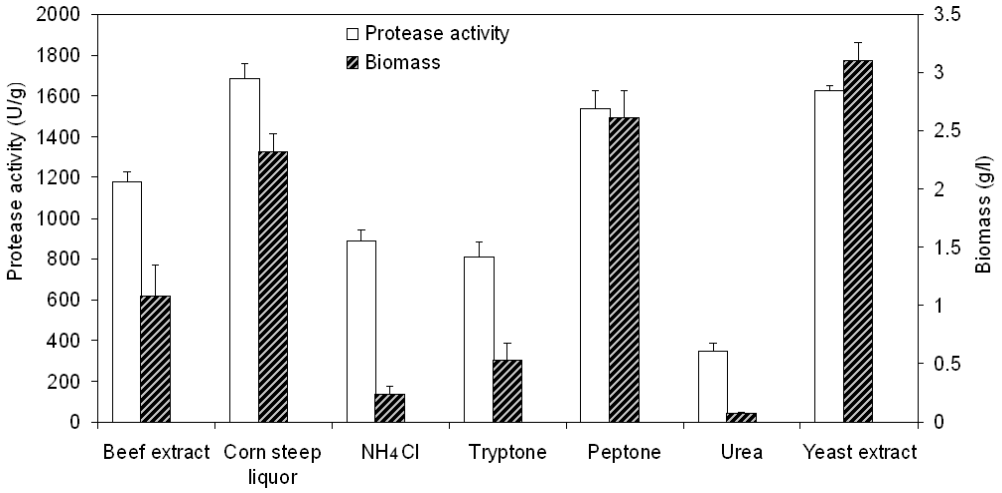

Figure 2. Effect of nitrogen sources on biomass and protease production by *Laccocephalum mylittae*. Concentrations of other components in fermentation medium were fixed at original levels. Fermentation was carried out under original culture conditions.

fungi on this complex organic nitrogen source. For protease production, however, corn steep liquor could enhance the production of protease better than the other nitrogen sources. Similar results have been observed by other researchers (Yang and Lee, 2001; Lima et al., 2005). This stimulating effect was probably caused by certain nutrients present in the rich composition of corn steep liquor (De Azeredo et al., 2006). Consequently, yeast extract and peptone were chosen in the improved medium of seed cultivation, to obtain more productive mycelia at a faster speed. Meanwhile, yeast extract and corn steep liquor were selected in the improved fermentation medium for both the mycelia growth and subsequent metabolite accumulation.

Optimization of biomass and protease production by RSM

Table 1 and 2 present the results of the two-level PB design for the screening of significant factors in fermentation medium and conditions. Statistical analysis of these data by SAS indicated that dextrose/sucrose concentration; inoculum size and inoculum age had significant influences ($P < 0.05$) and positive effects on biomass (data not shown) and protease production (Table 5), while the other factors were found to have insignificant influences. Thus, these three factors were selected for further optimization in the following CCRD experiment. It is worth noting that the effect of shaking speed was posi-

tive and close to the significant point (P = 0.05), indicating it was also an effective factor for positive regulation in biomass and protease production.

After the significant factors were determined according to the PB tests, both linear and quadratic effects on maximum protease activity were estimated by RSM combined with CCRD. The design matrix for CCRD was carried out with the fixed middle point at 22 g/l of dextrose/sucrose concentration, 15% (v/v) inoculum and 3.5 d of inoculum age (Table 3). Adequacy and fitness were evaluated by analysis of variance (ANOVA) and calculations of regression coefficients (Table 6). The effects of all the three parameters, namely dextrose/ sucrose concentration, inoculum size and inoculum age on protease production were found to be significant (P < 0.05), while their interactions with each other showed no significance. In addition, all the quadratic terms were found to be significant (P < 0.05) for protease production.

The best explanatory equation to fit the second-order model and subsequently produce the response surfaces was expressed as follows:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \quad (1)$$

Where Y is the dependent variable (response variable) to be modeled; β_0 is the intercept; β_1, β_2, and β_3 are linear coefficients; β_{11}, β_{22}, and β_{33} are squared coefficients; β_{12}, β_{13}, and β_{23} are interaction coefficients; X_1, X_2, and X_3 are the coded independent factors. In this study, the following second order polynomial equation giving the protease activity as a function (Y_2) of dextrose/sucrose concentration (X_1), inoculum size (X_2) and inoculum age (X_3) was obtained:

$$Y_2 = 70.06X_1 + 403.02X_2 + 1668.32X_3 - 2.26X_1^2 - 14.02X_2^2 - 236.09X_3^2 + 0.33X_1X_2 + 8.88X_1X_3 + 4.12X_2X_3 - 3905.08 \quad (2)$$

The model was reduced and simplified by deleting insignificant terms (P > 0.05) from Equation (2). A simpler model was obtained as follows:

$$Y_2 = 70.06X_1 + 403.02X_2 + 1668.32X_3 - 2.26X_1^2 - 14.02X_2^2 - 236.09X_3^2 - 3905.08 \quad (3)$$

The ANOVA results indicated that the quadratic regression to produce the second-order model was significant (P < 0.0001), and the lack-of-fit test showed no significance (P = 0.4366). The coefficient of determination (R^2) was 0.9608, which implied that 96.08% of the total variation could be explained by the model. This suggested that the model accurately represented the data in the experimental region.

The response surfaces and contour plots for the optimization are shown in Figures 3 - 5. Each figure presents the effect of two factors on protease activity, while the third factor was held at the middle level. These data revealed that the protease activity would increase as X_1 (dextrose/sucrose concentration) and X_2 (inoculum size) increase, but further increases in these two factors after the optimal point would reverse the trend (Figure 3). Similar effects on protease activity were observed for X_2 (inoculum size) vs. X_3 (inoculum age) in Figure 4 and X_1 (dextrose/sucrose concentration) vs. X_3 (inoculum age) in Figure 5. From equation (2), it could be deduced that the optimum point for maximum protease activity of X_1, X_2 and X_3 was 0.1634, 0.0530 and 0.7513, respectively. The corresponding levels of these three factors were approximately 25 g/l of dextrose/sucrose concentration, 15% (v/v) inoculum, and 4 d of inoculum age. Estimated maximum protease activity on these levels was 3484.51

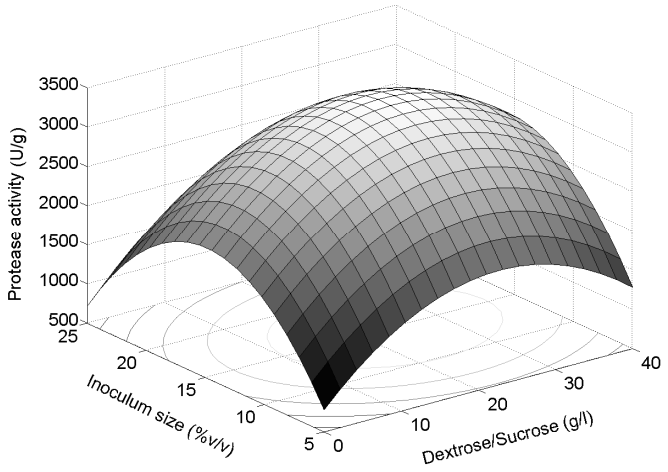

Figure 3. Response surface and contour plot of dextrose/sucrose concentration vs. inoculum size on protease production by *Laccocephalum mylittae*. Inoculum age was kept constant at 3.5 d.

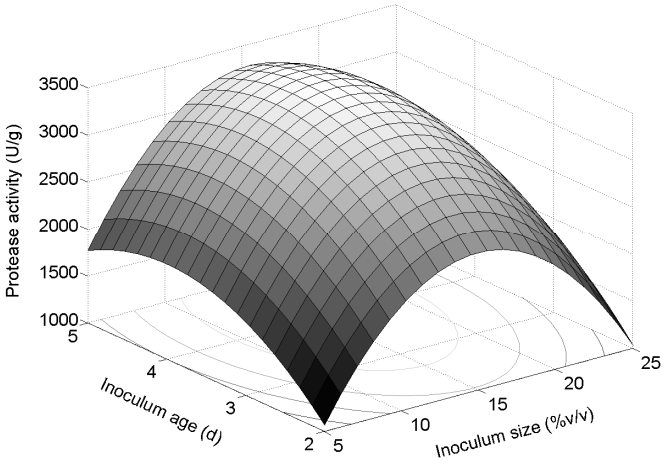

Figure 4. Response surface and contour plot of inoculum size vs. inoculum age on protease production by *Laccocephalum mylittae*. Dextrose/sucrose concentration was kept constant at 22 g/l.

Table 4. L_9 (3^4) orthogonal array for biomass and protease production by batch fermentation in a 15 l fermenter.

Run No.	Coded factor level[a]				Biomass (g/l)[b]	Protease activity (U/g)[b]
	Aeration rate	Agitation rate	Temperature	Initial pH		
1	1	1	1	1	9.24	1720.37
2	1	2	2	2	9.65	4127.08
3	1	3	3	3	9.96	3494.29
4	2	1	2	3	9.02	3320.87
5	2	2	3	1	9.72	4396.15
6	2	3	1	2	12.52	4078.63
7	3	1	3	2	7.87	2573.54
8	3	2	1	3	7.11	3841.37
9	3	3	2	1	10.98	3153.54

[a] The actual factor levels, coded as values of 1, 2, 3 in the table, were as follows: aeration rate, 1:0.8 (1), 1:1.0 (2) and 1:1.2 (3) vvm; agitation rate, 180 (1), 200 (2) and 220 (3) rpm; temperature, 24 (1), 27 (2) and 30 ºC (3); initial pH, 6.0 (1), 6.5 (2) and 7.0 (3).
[b] All factorial points are means of triplicate values.

Table 5. Identifying significant factors for protease production by *Laccocephalum mylittae* using Plackett–Burman (PB) design.

Factor	Effect	Standard error	*t*-value	*P*-value
Yeast extract	95.867	50.829	1.8861	0.1558
Corn steep liquor	8.28	50.829	0.1629	0.8810
Dextrose/Sucrose	176.45	50.829	3.4714	0.0403[a]
pH	27.157	50.829	0.53427	0.6302
Inoculum size	127.12	31.121	4.0849	0.0265[a]
Liquid volume	33.658	31.121	1.0815	0.3586
Inoculum age	148.47	31.121	4.7708	0.0175[a]
Temperature	15.052	31.121	0.48366	0.6617
Shaking speed	93.092	31.121	2.9913	0.0581

[a] Significant at 5% level ($P < 0.05$).

U per gram mycelial dry weight (U/g). All these predictions from the regression model were further ascertained by validation experiments, and the actual maximum protease activity was 3396.62 ± 136.13 U/g, within the 95% confidence interval predicted (from 3310.28 to 3658.74 U/g of protease activity).

Optimization by orthogonal matrix method in a 15 l fermenter

Processes of fermentation in shake flasks and in fermenters are not exactly the same. Compared with fermentation in shake flasks, control of biomass and protease production is much more complicated when submerged fermentation is carried out in the larger environment of an industrial fermenter. As a result, it is necessary to explore suitable fermentation conditions for biomass and protease production in fermenters based on the optimized results of fermentation in shake flasks.

Table 4 documents the experimental set of L_9 (3^4) orthogonal array with four widely studied factors on three different levels and the results. The means and delta values of biomass and protease activity are shown in Table 7. The larger the delta value for a factor, the larger the effect the variable has on the biomass and protease production. Analysis of the data indicated that agitation rate had the greatest effect on both biomass and protease production, followed by aeration rate, initial pH and temperature in order. Moreover, the agitation rate at level 3 (220 rpm) was found optimal for biomass increase while it was optimal for protease production at the middle level 2 (200 rpm). Optimal values of the other three factors were all at their middle levels for both biomass and protease production. Therefore, an agitation rate of 200 rpm, an aeration rate of 1:1.0 vvm, an initial pH of 6.5 and a temperature of 27°C were the optimum fermentation conditions for maximum protease production in a 15 l fermenter.

Figure 6 presents the time courses of biomass and pro-

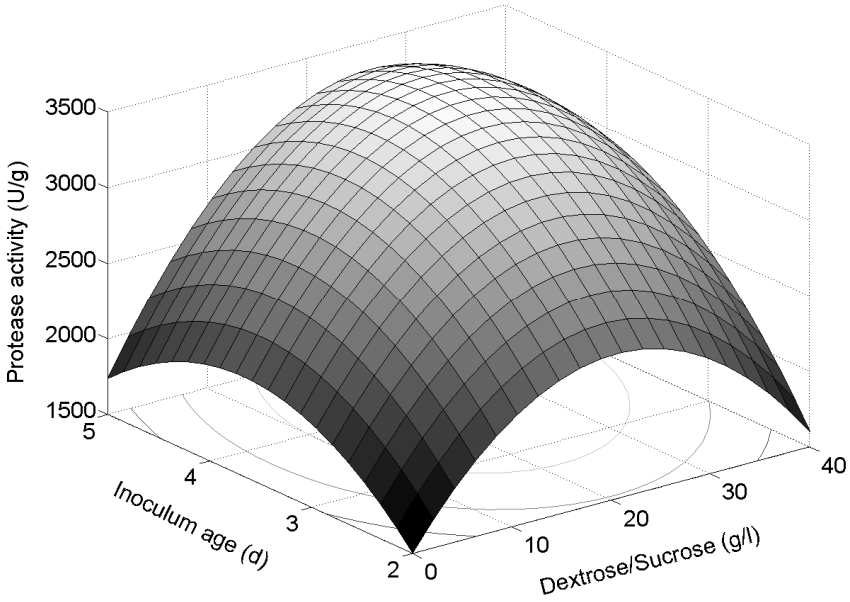

Figure 5. Response surface and contour plot of dextrose/sucrose concentration vs. inoculum age on protease production by *Laccocephalum mylittae*. Inoculum size was kept constant at 15%.

Table 6. Analysis of variance (ANOVA) for the regression model[a] of protease production obtained from the results of central composite rotatable design (CCRD).

Factor[b]	Degree of Freedom	Coefficient estimate	Standard error	*t*-value	*P*-value
Intercept	1	c = -3905.079102	1470.307651	-2.66	0.0241[c]
X_1	1	c_1 = 70.057943	23.185866	3.02	0.0129[c]
X_2	1	c_2 = 403.022249	88.367636	4.56	0.0010[c]
X_3	1	c_3 = 1668.315898	592.107025	2.82	0.0182[c]
X_1^2	1	c_{11} = -2.257658	0.186915	-12.08	<.0001[c]
X_2^2	1	c_{22} = -14.023541	2.107731	-6.65	<.0001[c]
X_3^2	1	c_{33} = -236.086567	74.766142	-3.16	0.0102[c]
X_1X_2	1	c_{12} = 0.327292	0.836710	0.39	0.7039
X_1X_3	1	c_{13} = 8.879750	5.020257	1.77	0.1074
X_2X_3	1	c_{23} = 4.119167	16.734191	0.25	0.8105

[a] Determination coefficient R^2 = 0.9608, $R^2_{adj.}$ = 0.9477.
[b] X_1, dextrose/sucrose concentration; X_2, inoculum size; X_3, inoculum age.
[c] Significant at 5% level (P < 0.05).

tease production in optimized and initial un-optimized fermentation. The profiles of DO and pH during the optimized batch fermentation in a 15 l fermenter are shown in Figure 7. Under the optimized fermentation medium and conditions, the mycelial biomass of *L. mylittae* increased exponentially and reached its maximum value after 60 h of incubation. The DO profile was in good agreement with the mycelial growth, exhibited a rapid decrease between 24 and 60 h. Maximum protease activity was observed around 72 h, during the stationary phase of mycelial growth. After the peak, however, the protease activity reduced gradually, probably due to the continuous increase of culture pH and associated with the decrease in mycelial productivity. Compared with the initial un-optimized fermentation process, the maximum protease activity in optimized fermentation reached 4557.22 ± 147.36 U/g after 72 h of incubation, with mycelial biomass of 11.65 ± 0.33 g/l at this point. This is a 2.71-fold and a 3.32-fold increase in protease productivity and biomass production, respectively. The production time was also reduced from 4.5 to 3 d. The anthelmintic protease derived from the cultured mycelia by optimized fermentation exhibited distinct lethal effects on the third stage larvae of *A. suum* in our anthelmintic tests *in vitro*,

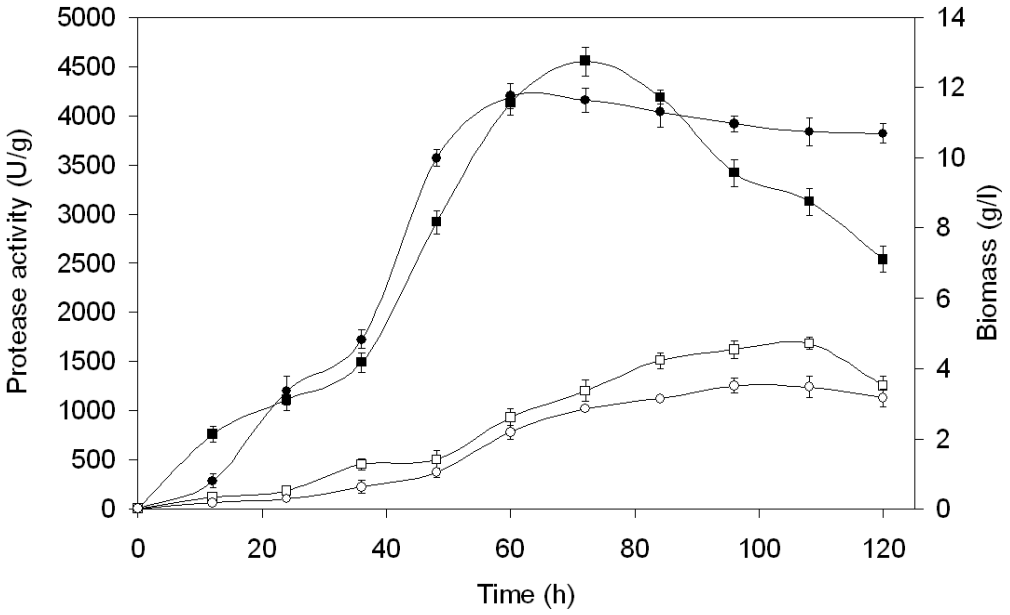

Figure 6. Time courses of growth (●, ○) and protease production (■, □) by *Laccocephalum mylittae* in optimized (black) and initial non-optimized (white) batch fermentation in a 15 l fermenter. Data are given as means ± SD, n = 3.

Table 7. Responses for means and delta values of biomass and protease production in the L_9 (3^4) orthogonal array.

Level	Aeration rate	Agitation rate	Temperature	Initial pH	Response
1	9.617	8.710	9.623	9.980	Biomass (g/l)
2	10.420	8.827	9.883	10.013	
3	8.653	11.153	9.183	8.697	
Delta	1.767	2.443	0.700	1.316	
1	3113.913	2538.260	3213.457	3090.020	Protease activity (U/g)
2	3931.883	4121.533	3533.830	3593.083	
3	3189.483	3575.487	3487.993	3552.177	
Delta	817.970	1583.273	320.373	503.063	

while little toxicity or other side-effects were observed in the acute toxicity test (unpublished data). In addition, the protease activity from the mycelia of *L. mylittae* by optimized fermentation was 8.14-fold higher than that from the wild sclerotium of this fungus.

Submerged fermentation of pharmaceutical fungi provides an effective method for industrial medicine production. This method could enhance the effective density of active ingredients and could regulate a variety of metabolites derived from the fungi. Simultaneously, artificial culture is of importance to environmental protection. The present study is the first to establish an optimum fermentation process for the production of mycelial biomass and the main pharmaceutical component of *L. mylittae*. It sets up a basis for further industrial production and applications of this fungus.

Conclusion

This research established an optimized fermentation process for the production of mycelial biomass and the main pharmaceutical component of *L. mylittae*, a medicinal fungus used in anthelmintic therapy. The optimum fermentation medium is composed of (g/l): dextrose/ sucrose, 25; yeast extract, 10; corn steep liquor, 10; KH_2PO_4, 1.5; $MgSO_4{\cdot}7H_2O$, 0.5; Vitamin B_1, 0.01, at an initial pH of 6.5. The optimum fermentation conditions are: aeration rate, 1:1.0 vvm; agitation rate, 200 rpm; inoculum age, 4 d; inoculum size, 15% (v/v); temperature, 27℃. Under the optimum medium and conditions above, the maximum activity of the anthelmintic protease was 4557.22 ± 147.36 U/g, with mycelial biomass of 11.65 ± 0.33 g/l after 3 d batch fermentation in a 15 l fermenter.

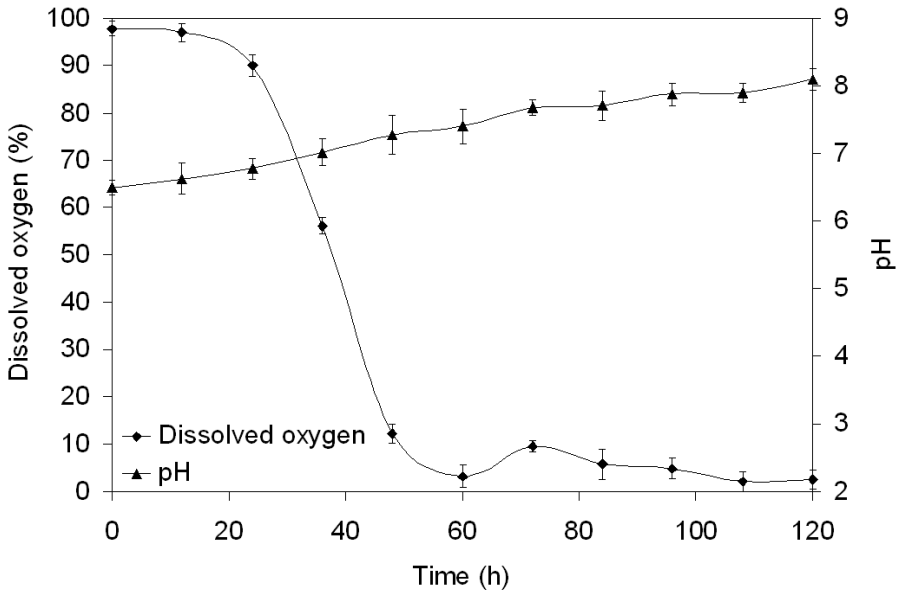

Figure 7. Time profiles of dissolved oxygen (DO) and pH for optimized biomass and protease production of *Laccocephalum mylittae* by batch fermentation in a 15 l fermenter. Data are given as means ± SD, n = 3.

This is a 2.71-fold and a 3.32-fold increase in protease productivity and biomass production respectively, when compared with that in the initial un-optimized fermentation process. The optimization also resulted in a reduction of the production time, from 4.5 to 3 d. In addition, protease activity from the mycelia by optimized fermentation is 8.14-fold greater than that from the wild sclerotium of *L. mylittae*. Based on these findings, the industrial production and applications of *L. mylittae* and its main pharmaceutical component can be regarded as possible and economically attractive.

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