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Optimization of Submerged Culture Conditions for the Production of Mycelial Biomass and Exopolysaccharides from *Lignosus rhinocerus*

(Pengoptimuman Kultur Tenggelam untuk Penghasilan Biojisim Miselium dan Eksopolisakarida *Lignosus rhinocerus*)

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ABSTRACT

*Tiger's Milk mushroom (*Lignosus rhinocerus*) is a highly priced medicinal mushroom utilized in traditional medicine to treat various diseases. However, due to insufficient wild* L. rhinocerus*, submerged culture conditions and nutritional requirements for the production of mycelial biomass and exopolysaccharide (EPS) from* L. rhinocerus *were studied using one-factor-at-a-time and orthogonal matrix method in shake flask culture. The optimal pH and temperature for ideal production of mycelial biomass and EPS were found to be at pH6 and 25°C, respectively. The optimal compositions for mycelial biomass production were 80 g/L of glucose, 4 g/L of potassium nitrate, 0.4 g/L of $FeSO_4.7H_2O$ and 0.1 g/L of $CaCI_2$. Subsequently, the optimal compositions for EPS production were 80 g/L of glucose, 4 g/L of potassium nitrate, 1.4 g/L of $FeSO_4.7H_2O$ and 1.1 g/L of $CaCI_2$. The maximum mycelial biomass and EPS concentrations achieved in a 1.5 L stirred-tank bioreactor were 6.3788 g/L and 1.2 g/L, respectively. Mycelial biomass production was about 3 times higher than that at the basal medium. However, EPS production indicated no significant difference at the basal medium. In addition, the concentrations for α-amylase, β-amylase, cellulase and invertase in optimal medium were 2.87, 1.07, 3.0 and 3.0 mg/mL, respectively. Current findings suggest that the production of mycelial biomass and EPS of* L. rhinocerus *can be enhanced dramatically by controlling the culture conditions and modifying the medium's composition.*

Keywords: Exopolysaccharides; Lignosus rhinoceros; *mycelial biomass; orthogonal matrix method; submerged culture*

ABSTRAK

*Cendawan Susu Harimau (*Lignosus rhinocerus*) adalah cendawan bernilai yang sering digunakan dalam perubatan tradisi untuk merawat pelbagai penyakit. Namun, disebabkan kekurangan bekalan* L. rhinocerus *liar, maka kajian penghasilan biojisim miselium dan eksopolisakarida (EPS) daripada cendawan ini dijalankan menggunakan kaedah matriks ortogon satu faktor pada satu masa untuk memperoleh keadaan pengkulturan tenggelam yang optimum. Nilai pH dan suhu optimum untuk penghasilan biojisim miselium dan EPS dalam medium lengkap cendawan (MLC) didapati adalah pada pH6 dan 25°C. Kompisisi optimum untuk penghasilan biojisim miselium ialah 80 g/L glukosa, 4 g/L kalium nitrat, 0.4 g/L $FeSO_4.7H_2O$ dan 0.1 g/L $CaCI_2$. Manakala, komposisi optimum untuk penghasilan EPS ialah 80 g/L glukosa, 4 g/L kalium nitrat, 1.4 g/L $FeSO_4.7H_2O$ dan 1.1 g/L $CaCI_2$. Penghasilan biojisim miselium dan EPS telah dipertingkatkan dengan menggunakan 1.5 L tangki bioreaktor dan penghasilan biojisim miselium dan EPS yang maksimum ialah 6.3788 g/L dan 1.2 g/L. Di samping itu, kepekatan α-amilase, β-amilase, selulase dan invertase dalam medium yang optimum masing-masing adalah 2.87, 1.07, 3.0 dan 3.0 mg/mL. Penemuan semasa mencadangkan bahawa penghasilan biojisim miselium dan EPS* L. rhinocerus *boleh dipertingkatkan secara mendadak melalui pengawalan keadaan pengkulturan dan komposisi medium.*

Kata kunci: Biojisim miselium; eksopolisakarida; kaedah matriks ortogon; kultur tenggelam; Lignosus rhinoceros

INTRODUCTION

Tiger's Milk mushroom (*Lignosus* spp.) are native to tropical parts of the world where it has been used in folk medicine to treat diseases (Lee et al. 2009) and is a potential candidate as adjuvant therapy to treat breast cancer (Edwards et al. 2005). Tiger's Milk mushroom comprises of six species - *L. dimiticus, L. ekombitii, L. goetzii, L. rhinocerus, L. sacer* (Douanla-Meli & Langer 2003; Núñez & Ryvarden 2001; Ryvarden & Johansen 1980) and *L. hainanensis* which was discovered in the tropical forests of Hainan Province, southern China recently (Cui et al. 2010). In Malaysia, Tiger's Milk mushroom (*Lignosus rhinocerus*) is collected occasionally from remote regions in the states of Pahang and Perak. The matured fruit body of a Tiger's Milk mushroom has three distinct parts: A cap (pileus), a stipe or stem and a tuber (sclerotium).

Previously, we reported the optimal solid-state culture condition for the mycelial growth of *L. rhinocerus* (Lai et al. 2011). Due to sluggish mycelial growth in solid culture and longer development period of fruit body and tuber from *L. rhinocerus*, this study was initiated to explore the potential of propagating mycelial cells of *L. rhinocerus*

in submerged culture. Propagating mycelial cells with submerged fermentation technology is a favorable alternative to overcome this setback due to its low cost, and higher mycelial biomass and exopolysaccharides (EPS) production in a compact space and shorter time with smaller odds of contamination (Chiu et al. 2008; Guo et al. 2009).

Exopolysaccharides (EPS) are produced by numerous microorganisms especially mushrooms due to their various biological and pharmacological activities (Bae et al. 2000). EPS are high-molecular-weight polymers that are composed of sugar residues and are found to have multifarious applications in various food and pharmaceutical industries. In mushroom, the roles of EPS have been described as a means of fungus adhesion to its substrate, immobilization of exocellular enzyme, prevention of hyphal dehydration and storage of excess nutrients (Elisashvili et al. 2009).

When fungi are grown in liquid media, a biosynthesis of EPS depends on several factors because EPS are partially dissolved in the culture medium (Manzoni & Rollini 2001). Hence, this study was initiated to design the optimal nutrients and culture conditions in submerged fermentation for the production of mycelial biomass and EPS from *L. rhinocerus* using an orthogonal matrix method.

Orthogonal design is one of the important statistical methods that use the Taguchi parameter design method (Montgomery 1999). This method is feasible for the investigation of the influence of controlled factors in a multivariable system; in addition, the method yields effective responses in the course of system optimization (Kim et al. 2005). In addition to determining the optimal setting for mycelial growth, orthogonal design has been successfully applied to the improvement of culture media or the production of primary and secondary metabolites in the fermentation process (Escamilla et al. 2000; Lee et al. 1997; Li et al. 2001; Xu et al. 2003).

Subsequently, concentrations of α-amylase, β-amylase, cellulase and invertase in optimal medium were analyzed. Amylases are commonly used to hydrolyze starch molecules into polymers composed of glucose units and the most popular and important forms of industrial amylases are α-amylase and β-amylase. In addition to amylases, cellulase plays a main role in breaking down cellulose in the cell walls of plants while invertase is an excellent enzyme to catalyze the hydrolysis of sucrose into glucose and fructose. These enzymes have potential application in a wide number of industrial processes such as food, fermentation and pharmaceutical industries (Paula & Pérola 2010).

Materials and Methods

Microorganism and medium

L. rhinocerus were collected from the state of Pahang, Malaysia in June, 2009. The stock cultures were grown on potato dextrose agar (PDA) medium. After that, the medium was incubated at 25°C for 15 days under the dark condition. In this study, the basal medium was MCM (Mushroom Complete Medium) which contained 20 g/L of glucose, 2 g/L of peptone, 2 g/L of yeast extract, 0.46 g/L of KH_2PO_4, 1 g/L of K_2HPO_4 and 0.5 g/L of $MgSO_4.7H_2O$.

Inoculum preparation and flask culture

The seed was grown in 250 mL flasks containing 50 mL of MCM at 25°C and incubated on a rotary shaker incubator at 150 rpm for 8 days. *L. rhinocerus* that was previously grown on PDA medium in a petri dish was transferred to the seed culture medium by punching out 5 mm of the agar plate culture with a cork borer. Culture conditions were conducted with the following factors: Temperature: The inoculated flasks were incubated at 15, 20, 25, 30 and 35°C; pH: The medium was adjusted to different pHs (4, 5, 6, 7, 8 and 9). In addition, the following factors were studied for suitable nutrient sources: Six sources of carbon - fructose, glucose, sucrose, xylose, maltose and lactose - were tested and supplemented to the basal medium containing 20 g/L carbon source; 2 g/L peptone, 2 g/L yeast extract, 0.46 g/L KH_2PO_4, 1 g/L K_2HPO_4 and 0.5 g/L $MgSO_4.7H_2O$; The basal medium for the nitrogen sources testing contained 20 g/L glucose, 0.46g/L KH_2PO_4, 1 g/L K_2HPO_4 and 0.5 g/L $MgSO_4.7H_2O$. The two sources of nitrogen were removed and twelve nitrogen sources - ammonium nitrate, calcium nitrate, ammonium acetate, potassium nitrate, sodium nitrate, glycine, asparagine, glutamine, alanine, phenylalanine, urea and glutamic acid - were separately provided in the basal medium; and Five mineral sources, $CaCI_2$, $FeSO_4.7H_2O$, KH_2PO_4, K_2HPO_4 and $MgSO_4.7H_2O$ were examined and added separately at concentrations of 7 mM, where two mineral ions used in the basal medium were removed.

Batch fermentation in 1.5 L fermentor

The fermentation medium was inoculated with 10% (v/v) of the seed culture and then cultivated at 25°C in a 1.5 L stirred-tank bioreactor (Biostat B, B.Braun Biotech International). Unless otherwise specified, fermentation was performed under the following conditions: temperature, 25°C; aeration rate, 2 vvm; and 100 rpm agitation speed. The seed culture was transferred to the fermentation medium and was cultivated for 11 days.

Analytical methods

The liquid fermentation of *L. rhinocerus* was filtered through Whatman No. 2 filter paper to separate the mycelium. The mycelial biomass was measured after the mycelial biomass was left drying at 70°C overnight at constant weight. The remaining liquid fermentation was studied to determine the production of EPS. Exopolysaccharides were extracted as follows: 2.0 mL sample was transferred into the falcon tube and 8 mL of absolute ethanol was mixed together. The mixture was stirred vigorously and left overnight at 4°C. The precipitated EPS was centrifuged at 9000 g for 15

min and 8.5 mL supernatant was discarded. The remaining supernatant and pellet in the tube were mixed together and transferred into a 1.5 mL eppendorf tube. Then, it was centrifuged again at 9000 g for 15 min. The supernatant was discarded and the weight of EPS was measured after drying at 70°C overnight at a constant weight.

ORTHOGONAL MATRIX METHOD

In this research, there were four design variables (A, B, C, D) and three levels of concentrations (1, 2 & 3). The orthogonal $L_9(3^4)$ was used as a suitable minimum orthogonal matrix method to obtain the optimal medium in submerged cultures. Glucose, potassium nitrate, $FeSO_4.7H_2O$ and $CaCI_2$ were presented as the carbon, nitrogen and mineral sources for the production of mycelial biomass and EPS successively after the test by the one-factor-at-a-time method. The levels' components of the culture medium are shown in Table 1.

ENZYME CONCENTRATION

The concentrations of enzymes were investigated using different methods depending on the different types of enzymes. Invertase concentration was determined by incubating 0.1 mL of enzyme solution with 0.9 mL of sucrose in 0.03 M acetate buffer (pH5.0) at 55°C for 20 min (Escamilla et al. 2000). α-Amylase was assayed with the addition of 50 µL enzyme solution to 0.5 mL of 1% (w/v) soluble starch made in 0.1 M acetate buffer (pH5.6) and incubated at 60°C for 30 min (Kammoun et al. 2008). Cellulase concentration was investigated by incubating 0.5 mL of enzyme solution with 0.5 mL of 1% carboxymethyl cellulose (CMC) in phosphate buffer (50 mM, pH5.0) for 30 min at 37°C (Coral et al. 2002). β-Amylase activity was measured with the mixture of 2.0 mL phosphate buffer (0.1 M, pH6.9) and 0.5 mL of 1% soluble starch before the addition of 0.5 mL enzyme solution (Adeniran et al. 2010). Then, the mixture was incubated at 60°C for 5 min in water bath. After incubation, dinitrosalicylic (DNS) reagent was added to stop the reaction. The amount of reducing sugar released was measured using dinitrosalicylic (DNS) acid method (Miller 1959). Then, the mixture was heated for 5 min in a boiling water bath. For invertase, α-amylase and cellulase, glucose was used to prepare the standard curve and the absorbance was read at 575 nm in spectrophotometer while for β-amylase, maltose was used as the standard curve and the absorbance was read at 540 nm in spectrophotometer.

STATISTICAL ANALYSIS

All results were expressed as mean±S.D. Statistical analysis was performed using one way analysis of variance (ANOVA) using Minitab® 15.1.1.0. A p-value < 0.05 was considered significant.

RESULTS AND DISCUSSION

OPTIMAL CULTURE CONDITIONS

The initial medium pH is a critical factor associated with the growth of fungi because it will affect the cell membrane function, cell morphology and structure, the solubility of salts, the ionic state of substrates, the uptake of various nutrients and product biosynthesis (Qing & Jian 2002). In this study, *L. rhinocerus* was cultivated in the basal medium with different initial pHs (4, 5, 6, 7, 8 & 9) in shake culture conditions to investigate the effects of pH on mycelial biomass and EPS production.

The optimal mycelial production was observed at pH6 with significant differences ($p< 0.05$) compared with pH4, 8, 9 (Figure 1). Conversely, no significant differences were found between pH6 and pH5 and 7 in the production of mycelial. In addition, pH6 yielded the highest EPS production followed by pH7 and 5; and none of these differences were significantly different ($p> 0.05$). These results are consistent with that of other studies and suggest that the mycelial of various species of mushrooms will grow over a wide range of pH values. A possible explanation for this might be that fungi have the ability to alter the pH of their surroundings during growth and this ability is related to the maintenance of a suitable internal pH and ionic balance (Cooke & Whipps 1993). The current finding corroborates with Yang and Liau (1998) that the most favorable pH range for most organism growth is from 5 to 7 and lower values of initial pH will be beneficial to inhibit the growth of bacterial contaminants (Yang & Liau 1998).

Temperature plays a crucial role in the growth and metabolites formation of filamentous fungi because it controls the rates of metabolic reactions (Lv et al. 2010). Hence, mycelial cells of *L. rhinocerus* were cultivated at various temperatures (15, 20, 25, 30 & 35°C) to determine the optimal production of mycelial biomass and EPS. Current results suggested that the favorable temperature for optimal production of mycelial biomass and EPS are at 25°C (Figure 2) and statistical analysis suggest that there are no significant differences ($p > 0.05$) between 25 and 30°C for both mycelial biomass and EPS production. Present results

TABLE 1. Experimental factors and their levels for orthogonal projects

Level	A (%)	B (%)	C (%)	D (%)
1	4	0.20	0.04	0.01
2	6	0.40	0.09	0.06
3	8	0.60	0.14	0.11

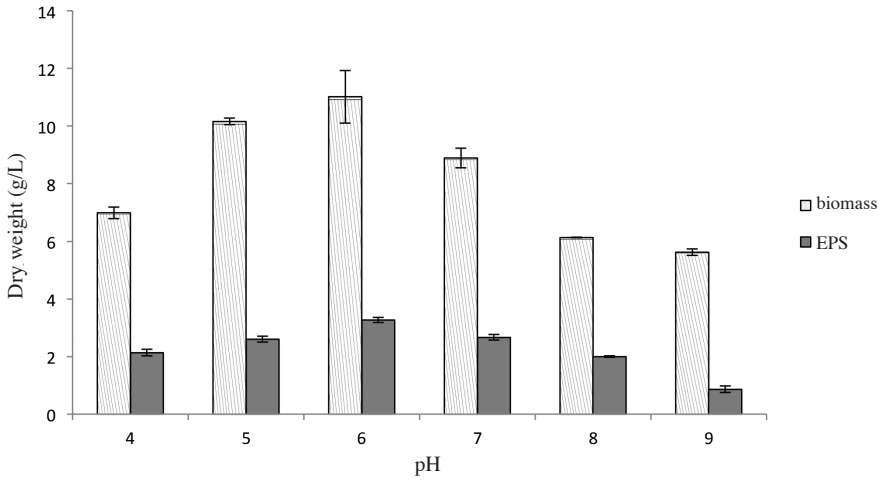

FIGURE 1. Effect of pH on the mycelial biomass and exopolysaccharides production by *L. rhinocerus* in shake flask cultures

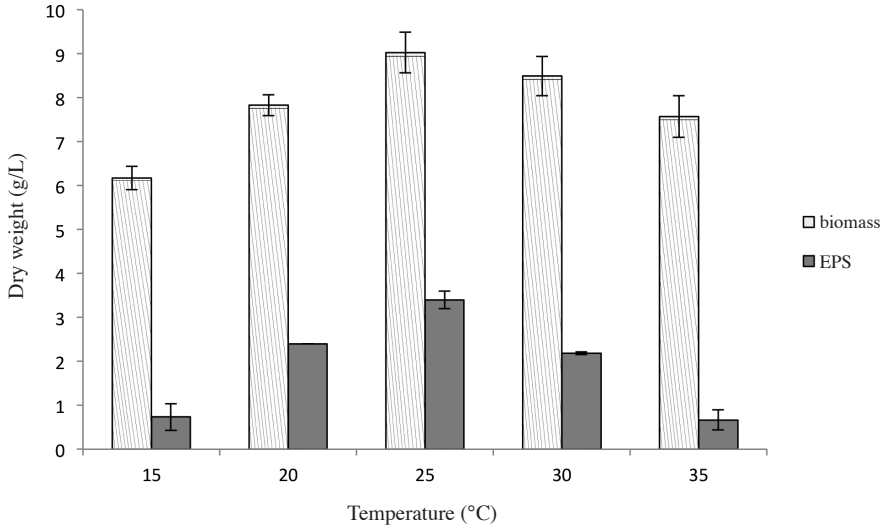

FIGURE 2. Effect of temperature on the mycelial biomass and exopolysaccharides production by *L. rhinocerus* in shake flask cultures

seem to be consistent with other researches which found the optimal temperature for basidiomycetes growth to be between 20 and 30°C (Boddy 1980; Cartwright & Findlay 1934).

OPTIMAL NUTRIENT SOURCES

In general, mycelial cells of mushrooms grow over a wide range of carbon source (Yang et al. 2003). To determine the suitable carbon source for the EPS production and mycelial growth in *L. rhinocerus*, seven carbon sources were separately provided at 20 g/L instead of glucose employed in the basal medium. Among the carbon sources tested, glucose yielded the highest mycelia growth and maximal EPS production at 6.84 and 2.67 g/L, respectively (Figure 3).

Medium containing glucose was statistically significant ($p< 0.05$) in yielding the highest mycelia growth and producing maximum EPS compared to the other carbon sources. This finding is consistent with previous study that glucose was the preferred carbon source for mycelial production of *L. rhinocerus* and EPS production of *Pleurotus florida* (Burns et al. 1994; Lai et al. 2011). In addition, glucose was also the most suitable carbon source for the production of mycelia biomass and EPS from *Phellinuss gilvus* (Hwang et al. 2003). This corroborates with the general concept expressed in early studies on fungi; stating that among the hexoses commonly studied, glucose is biologically the most effective energy source (Cochrane 1958). Furthermore, glucose is the best candidate for the carbon sources because of its easy-to-use and low cost compared to other carbon sources (Wei et al. 2008).

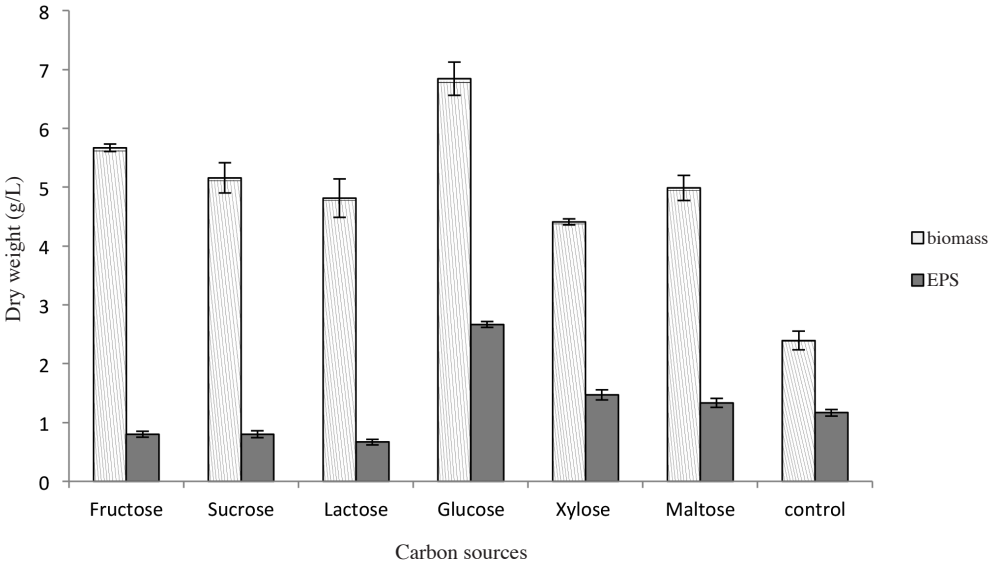

FIGURE 3. Effect of carbon sources on the mycelial biomass and exopolysaccharides production by *L. rhinocerus* in shake flask cultures

Nitrogen plays an important role in fungal growth and metabolite production (Kim et al. 2005). Thus, the effect of nitrogen on mycelial biomass and EPS production of *L. rhinocerus* in the form of ammonia, nitrate or in organic compounds, such as amino acids and proteins were studied. Among the twelve nitrogen sources that have been tested in this study, calcium nitrate was the most suitable for the growth of mycelial biomass (2.72 g/L) while potassium nitrate was the most favorable nitrogen source for the maximal production of EPS (1.17 g/L) (Figure 4). Statistical analysis suggested that potassium nitrate yielded maximum EPS production with significant differences ($p< 0.05$) compared to other nitrogen sources. However, mycelial growth in medium containing calcium nitrate was not statistically different ($p> 0.05$) compared to other nitrogen sources.

Current findings indicate that the mycelial growth and EPS production of *L. rhinocerus* prefer inorganic nitrogen sources. However, current findings are contrary to those of Shih et al. (2006) that suggested most basidiomycetes prefer complex organic nitrogen sources for their favorable submerged cultures because certain essential amino acids could scarcely be synthesized from inorganic nitrogen sources in the cultivation of higher fungi (Shih et al. 2006).

The effects of mineral sources on mycelia growth and EPS production were examined by supplementing various mineral sources at a concentration of 7 mM. Among the five minerals analyzed, medium with $CaCl_2$ yielded the highest mycelial biomass growth (3.47 g/L) while $FeSO_4.7H_2O$ was the most favorable mineral source for optimal EPS production (1.02 g/L) (Figure 5). Statistical analysis suggested that $CaCl_2$ yielded optimal mycelial

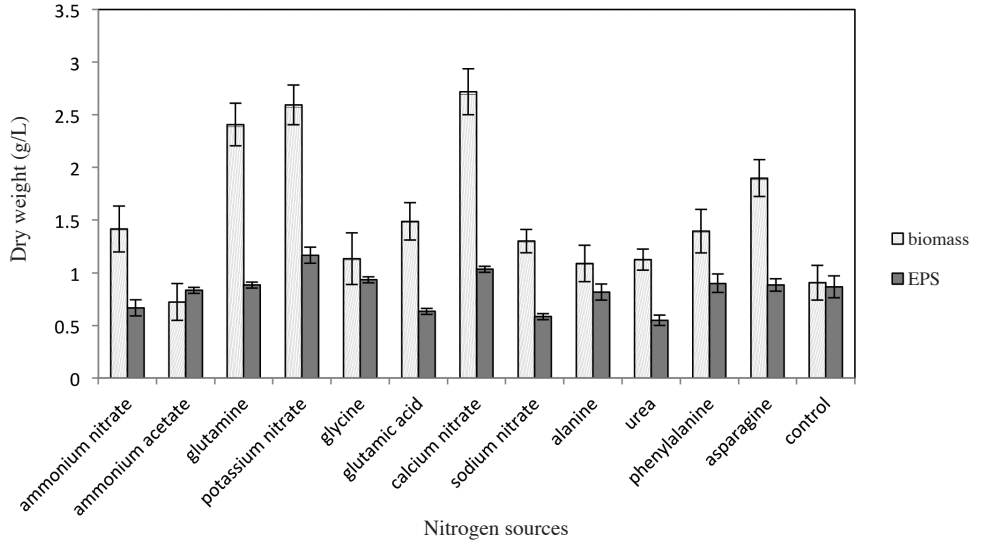

FIGURE 4. Effect of nitrogen sources on the mycelial biomass and exopolysaccharides production by *L. rhinocerus* in shake flask cultures

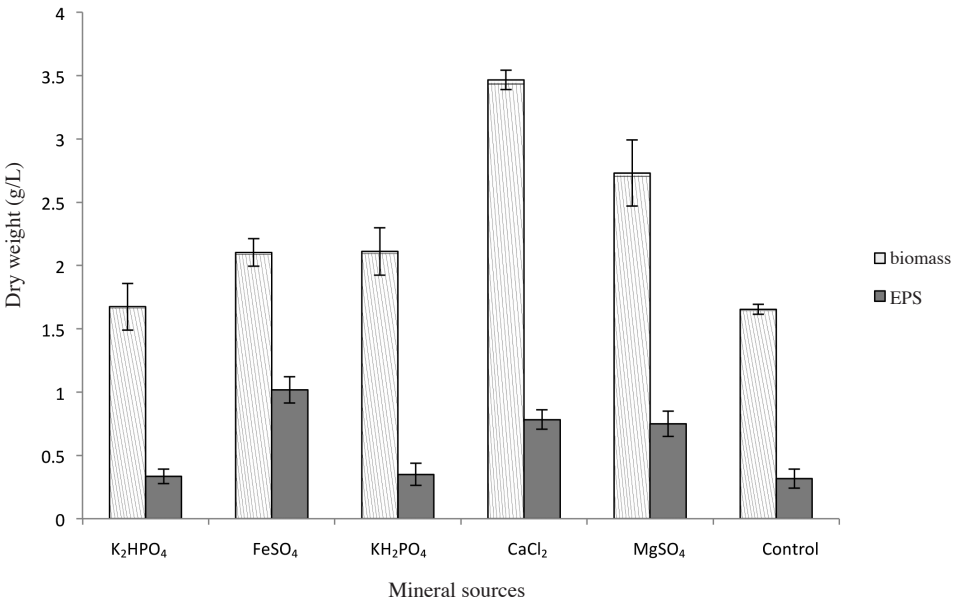

FIGURE 5. Effect of mineral sources on the mycelial biomass and exopolysaccharides production by *L. rhinocerus* in shake flask cultures

growth with significant differences ($p< 0.05$) compared to other mineral sources. In addition, one-way ANOVA showed $FeSO_4.7H_2O$ yielded maximum EPS production compared to other mineral sources and these differences were statistically significant ($p< 0.05$).

Among the various mineral sources examined, both Mg^{2+} and Ca^{2+} had a beneficial effect on mycelial growth, whereas the maximum EPS production was achieved in the media containing ferum ion. Current findings seem to be consistent with Chardonnet et al. (1999) that found $CaCl_2$ to be one of the favorable mineral sources for optimal mycelial growth (Chardonnet et al. 1999). Supplementation of calcium ion to the medium has been frequently regarded as one of the effective ways to accelerate mycelia growth and to induce some metabolites from many microorganisms in submerged cultures (Chardonnet et al. 1999). In addition, the importance of calcium ion was also recorded in submerged culture of *Cordyceps sinensis*, *Lentinus subnudus* and *Schizophyllum commune* (Dong & Yao 2005; Jonathan & Fasidi 2001).

MEDIA OPTIMIZATION BASED ON ORTHOGONAL MATRIX METHOD

The orthogonal matrix method was used to investigate the relationships between variables of medium components and optimize their concentrations for production of mycelial biomass and EPS. Based on the design of four factors and three levels (Table 1); A, B, C and D represent glucose, potassium nitrate, $FeSO_4.7H_2O$ and $CaCl_2$, respectively. Experimental conditions for each experimental group are listed in Table 2 with experimental results for mycelial biomass and EPS production.

The optimization levels of each factor (Table 3) were obtained using the intuitive analysis based on statistical calculation using the data in Table 2. In summary, the optimum compositions to produce mycelial biomass were 80 g/L of glucose, 6 g/L of potassium nitrate, 0.4 g/L of $FeSO_4.7H_2O$ and 0.1 g/L of $CaCl_2$ while the optimal medium compositions for EPS production were 80 g/L of glucose, 4 g/L of potassium nitrate, 1.4 g/L of $FeSO_4.7H_2O$ and 1.1 g/L of $CaCl_2$.

TABLE 2. Application of $L_9(3^4)$ orthogonal projects to mycelial biomass and exopolysaccharides production by *L.rhinocerus*

Run	A	B	C	D	Biomass (g/L)	Exopolysaccharides (g/L)
1	1	1	1	1	3.83 + 0.17	0.20 ± 0.01
2	1	2	2	2	2.75 + 0.03	0.20 ± 0.01
3	1	3	3	3	2.69 + 0.22	0.47 ± 0.12
4	2	1	2	3	2.71 + 0.11	0.47 ± 0.12
5	2	2	3	2	3.36 + 0.08	0.53 ± 0.23
6	2	3	1	1	5.72 + 0.78	0.40 ± 0.01
7	3	1	3	2	5.08 + 0.13	1.40 ± 0.20
8	3	2	1	3	3.85 + 0.59	1.53 ± 0.31
9	3	3	2	1	7.76 + 0.53	1.20 ± 0.40

The arrangements of column A, B, C and D were decided by orthogonal design for 4 (factor) X 9 (run number); every row of run number represents one experimental replicate; every run was carried out twice. Values are mean ± SD of double determinations

TABLE 3. Analysis of media on mycelia biomass and exopolysacchride production in shake flask cultures of *L.rhinocerus* with orthogonal projects

	Mycelia biomass (g/L)				Exopolysaccharides (g/L)			
	A	B	C	D	A	B	C	D
K_1	9.27 + 0.42	11.62+0.41	13.4+ 1.54	17.31+1.48	0.87+0.12	2.07+0.32	2.13+0.31	1.80+0.40
K_2	11.79+0.97	9.96+0.70	13.22+0.67	11.19+0.24	1.4+0.35	2.26+0.54	1.87+0.52	2.13+0.43
K_3	16.69+1.25	16.17+1.53	11.13+0.43	9.25+0.92	4.13+0.91	2.07+0.52	2.40+0.50	2.47+0.55
k_1	3.09+0.14	3.87+0.14	4.47+0.51	5.77+0.49	0.29+0.04	0.69+0.11	0.71+0.10	0.60+0.13
k_2	3.93+0.32	3.32+0.23	4.41+0.22	3.73+0.08	0.47+0.12	0.75+0.18	0.62+0.17	0.71+0.14
k_3	5.56+0.42	5.39+0.51	3.71+0.14	3.08+0.31	1.38+0.31	0.69+0.17	0.80+0.17	0.82+0.18
R	2.47+0.28	2.07+0.28	0.76+0.37	2.69+0.19	1.09+0.26	0.06+0.07	0.18+0.07	0.22+0.05
Optimal level	3	3	1	1	3	2	3	3

a) $K_i = \sum$ mycelial yield at K_i. Values are mean ± SD of double determinations; b) $k_i = \sum K_i / 3$. Values are mean ± SD of double determinations; c) R = max $\{k_i\}$- min $\{k_i\}$. Values are mean ± SD of double determinations

BATCH FERMENTATION

In the present study, EPS and mycelial biomass production in basal medium (glucose 20 g/L, KH_2PO_4 0.46 g/L, K_2HPO_4 1 g/L, $MgSO_4.7H_2O$ 0.5 g/L, peptone 2 g/L and yeast extract 2 g/L) were 1.1833 g/L and 2.178 g/L, respectively. Meanwhile, EPS and mycelial biomass production in optimized medium (glucose 80 g/L, potassium nitrate 4 g/L, $FeSO_4.7H_2O$ 1.4 g/L and $CaCI_2$ 1.1 g/L) in 1.5 L stirred-tank bioreactor were 1.2 g/L and 6.3788 g/L, respectively. Mycelial growth in optimized medium was about 3 times higher than that at basal medium but EPS production indicated the same values compared to that at the basal medium.

In addition, the presence of commercially important fungal extra-cellular enzymes were analyzed in optimized fermentation medium. Enzyme concentrations recorded for α-amylase, β-amylase, cellulase and invertase were 2.87, 1.07, 3.0 and 3.0 mg/mL, respectively. Current results suggested that among four enzymes analyzed, the expression of both cellulase and invertase concentration were similar and higher compared to the expression of α-amylase and β-amylase by mycelial cells of *L. rhinocerus*.

CONCLUSION

Current findings suggested that the production of mycelial biomass and EPS of *L. rhinocerus* can be enhanced dramatically by controlling the culture conditions and modifying the medium's composition. In addition, mycelial cells production of *L. rhinocerus* was successfully optimized in shake flask cultures using the orthogonal matrix method. However, more studies should be carried out to increase the production of polysaccharides with various biological activities that have the potential to be developed into functional foods. In addition, the optimization strategy established in this study may be worth attempting with other *Lignosus* species.

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