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EXPERIMENTAL ARTICLES

Optimization of Nutrient Medium for Submerged Cultivation of *Ganoderma lucidum* (Curt.: Fr.) P. Karst

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Abstract—The dependence of the amount of the grown vegetative mycelium of *Ganoderma lucidum* on the composition of the nutrient medium has been studied under conditions of submerged cultivation. The medium was optimized using full factorial and steepest ascent experimental designs. The addition of two carbon sources to the medium considerably improved the submerged growth of the mushroom. An optimized medium provided for a high yield (20–20.95 g/l) of the morphologically homogeneous mycelium and shortened the cultivation period to 3–4 days.

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Key words: optimization, submerged cultivation, biomass, *Ganoderma lucidum*.

The basidial xylotrophic Reishi mushroom *Ganoderma lucidum* (Curt.: Fr.) P. Karst has been used in traditional medicine for as many as two thousand years. The metabolites isolated from this mushroom possess anticancer, immunomodulatory, antimicrobial, hypolipidemic, hepatoprotective, and sedative activities [1–3].

The solid-state cultivation of *G. lucidum* on lignocellulose substrates is commonly used to obtain the fruiting bodies of this mushroom. Submerged cultivation is preferable to solid-state cultivation when the nutritive requirements of a mushroom are to be studied, since it provides for better growth of the mushroom and allows the addition of many substrates and the wide variation of their concentrations without worsening the mass transfer conditions in the culture. Recently, the submerged growth of *G. lucidum* was studied as a function of the qualitative composition of the nutrient medium, which contained glucose, sucrose, lactose, maltose, peptone, yeast extract, corn extract, mineral salts, and other ingredients [4]. The yield of the *G. lucidum* mycelium in submerged culture typically comprises 12 g dry wt./l over a cultivation period of 10 days [4, 5]. The maximal known yields of the *G. lucidum* mycelium amount to 21.9–22.1 g/l after 12 days of growth in bioreactors [4] and 20.9 g/l after 16 h of growth in flasks [5]. The significance of the results of these studies is depreciated because of the absence of sufficient information on the composition of the nutrient media used.

The qualitative and quantitative composition of nutrient media can easily be optimized using mathematically designed experiments. The aim of this work was to study the effect of the composition of the nutrient medium on the amount of the grown vegetative mycelium of *G. lucidum* and the cultivation length under conditions of submerged cultivation.

MATERIALS AND METHODS

The *Ganoderma lucidum* strain 5 used in this study was selected from the culture collection at the Laboratory of Biosynthesis of Biologically Active Substances, Gauze Institute of New Antibiotics [6, 7]. The mushroom was cultivated for 3–7 days as described elsewhere [6]. Following cultivation, the mycelium was

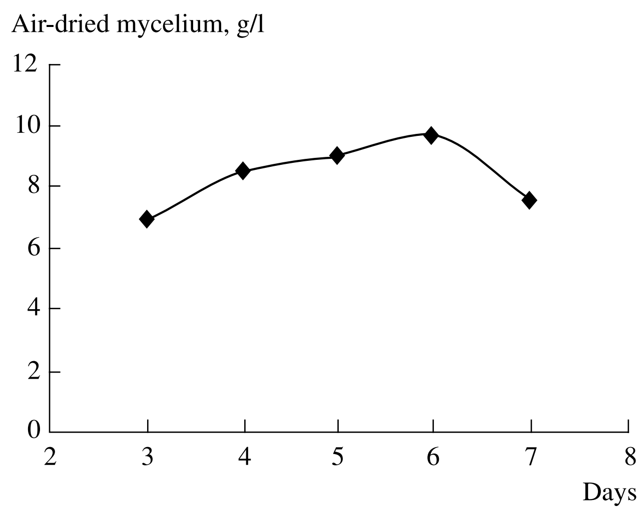

Fig. 1. Growth of *G. lucidum* strain 5 in the basal cultivation medium.

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Table 1. Levels of variable factors in FFD Experiment 1

Factor notation	Factor substance	X_{max}, g/l	X_{min}, g/l	λ, variation step length
X1	Soybean meal	15	5	5
X2	Glucose	25	15	5
X3	KH_2PO_4	4	2	1
X4	$MgSO_4 \cdot 7H_2O$	0.3	0.1	0.1

separated from the culture liquid by filtration, washed with water, and dried to a constant weight at a temperature not exceeding 45°C. The resultant dry mycelium is referred to hereafter as an air-dried mycelium.

The composition of the nutrient medium for *G. lucidum* was optimized using mathematically designed experiments [8]. The data obtained were statistically processed using the Statistica 6.0 program.

RESULTS AND DISCUSSION

The nutrient medium for *G. lucidum* was optimized in experiments that were designed according to full factorial and steepest ascent strategies [8]. Our earlier studies showed that soybean meal is one of the most important nutrients for *G. lucidum* [6]. The media that were prepared with this nutrient provided for a biomass yield of 9–10 g/l after 5–6 days of cultivation (Fig. 1).

The basal medium for optimization contained the following ingredients (in g/l): glucose, 20; soybean meal, 10; potassium phosphate, 3; and $MgSO_4$, 0.2. These concentrations of the ingredients were taken as centerpoint levels in the full factorial design (FFD) matrix 1 (Table 1). The total number of factor combinations in this matrix is 2^4 (Table 2). The cultivation period was 6 days. The response variable (Y_u) was the air-dried mycelium yield.

The data obtained in these experiments (Table 2) made it possible to derive the regression coefficients b_i. Comparing them with the confidence limit ε showed that the response of the biomass yield to three factors (the concentrations of soybean meal, glucose, and potassium phosphate) was statistically significant, the

Table 2. FFD Matrix 1 and experimental results

u Run number	X1 Soybean meal	X2 Glucose	X3 KH_2PO_4	X4 $MgSO_4 \cdot 7H_2O$	Y_u Air-dried mycelium, g/l	Factor and their interactions	Regression coefficients b_i
1	–	–	–	–	5.4	"1"	7.63
2	+	–	–	–	11.8	x1	1.54
3	–	+	–	–	5.3	x2	–0.93
4	+	+	–	–	9.7	x1x2	–0.59
5	–	–	+	–	6.9	x3	–0.49
6	+	–	+	–	7.7	x1x3	–0.56
7	–	+	+	–	5.5	x2x3	0.14
8	+	+	+	–	7.2	x1x2x3	0.25
9	–	–	–	+	7.1	x4	0.19
10	+	–	–	+	12.5	x1x4	–0.12
11	–	+	–	+	6.3	x2x4	–0.42
12	+	+	–	+	6.9	x1x2x4	–0.45
13	–	–	+	+	6.35	x3x4	0.12
14	+	–	+	+	10.8	x1x3x4	0.48
15	–	+	+	+	5.9	x2x3x4	0.10
16	+	+	+	+	6.8	x1x2x3x4	–0.10

Note: Confidence limit ε = 0.33 at a significance level of 5%.

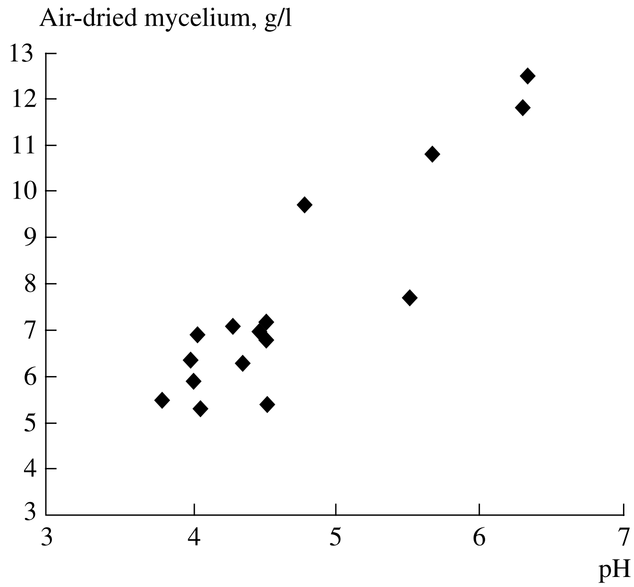

Fig. 2. The yield of the *G. lucidum* strain 5 mycelium versus the final pH of the cultivation medium.

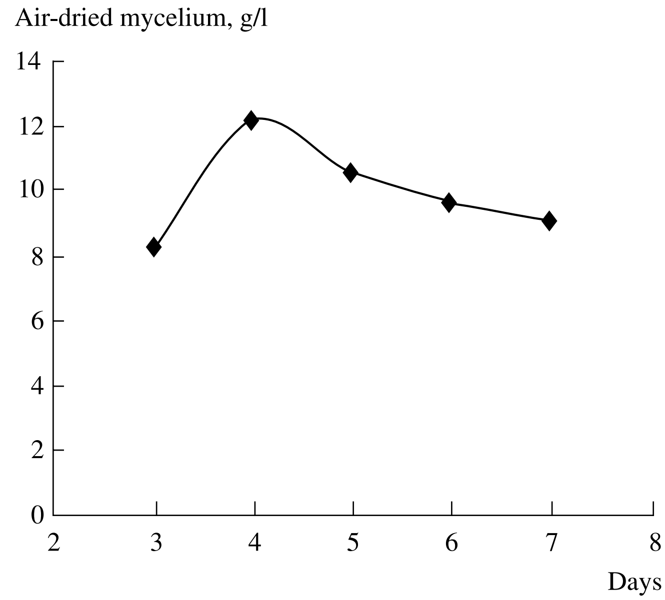

Fig. 3. Growth of *G. lucidum* strain 5 in medium 2.4.

concentration of soybean meal being limiting and those of glucose and potassium phosphate being inhibitory. Such results suggested that, in order to optimize the nutrient medium, the concentration of soybean meal should be increased and those of glucose and potassium phosphate should be lowered.

The observed negative relationship between the limiting nitrogen source (soybean meal) and the inhibitory sources of carbon (glucose) and phosphorus (potassium phosphate) indicated that the beneficial effect of soybean meal can be diminished by increasing the concentrations of glucose and potassium phosphate. On the other hand, the negative relationship between the inhibitory factor glucose and the insignificant factor magnesium sulfate suggested that the detrimental effect of glucose can be enhanced by raising the concentrations of magnesium sulfate and that the beneficial effect of the insignificant factor magnesium sulfate can become inferior at high concentrations of glucose. The observed two-way interaction effects between the factors did not determine a further optimization strategy. The three-way interaction effect of all three factors (soybean meal, glucose, and potassium phosphate), in fact, was the sum of the three-way interaction effects.

Table 3. The results of Steepest Ascent Experiment 1

Media	Biomass, g/l	Final pH of the culture liquid
Basal medium	8.80	3.2
1.1	8.05	3.4
1.2	9.10	3.7
1.3	10.25	4.9
1.4	9.85	5.2
1.5	**10.35**	**5.6**
1.6	9.60	6.0

It is known that pH can greatly influence the submerged growth of basidiomycetes. In our experiments, the decrease in the pH of the culture liquid of *G. lucidum* below 4.5 considerably diminished the mycelium yield in all but one case. On the other hand, the high final values of pH in the culture liquid correlated with the high mycelium yields (Fig. 2). According to the available data [9], the observed acidification of the cultivation medium of the wood-decaying mushroom *G. lucidum* may be due to accumulation of organic acids, including oxalic acid, in the medium.

The next experiment was designed according to the steepest ascent strategy and taking into account the regression coefficients [8]. The experiment (designated "Steepest Ascent Experiment 1"), which proceeded from the original composition of the nutrient medium, made it possible to improve the mycelium yield by 17% compared to the original medium (Table 3).

Steepest Ascent Design Matrix 2 (Table 4) was constructed using a shorter step in the soybean meal concentration, whereas the concentrations of glucose and potassium phosphate were the same as in Steepest Ascent Experiment 1. The maximum mycelium yield

Table 4. Steepest Ascent Matrix 2 and experimental results

Media	Soybean meal, g/l	Glucose, g/l	KH_2PO_4, g/l	Biomass, g/l	Final pH of the culture liquid
Basal medium	10	20	3	9.62	3.4
2.1	11	18.1	2.8	10.21	4.5
2.2	12	16.2	2.6	10.26	4.5
2.3	13	14.3	2.4	11.05	5.0
2.4	14	12.4	2.2	**11.55**	**6.2**
2.5	15	10.5	2.0	10.92	6.5

Table 5. Levels of variable factors in FFD Matrix 2

Factor notation	Factor substance	X_{max}, g/l	X_{min}, g/l	λ, variation step length
X1	Carbon source 1	8	4	2
X2	Carbon source 2	7	3	2

was observed in the case of medium 2.4 (Table 4), which was chosen for further studies.

One of the important technological parameters is the period of growth. The study of the growth dynamics of *G. lucidum* 5 showed that the biomass of this strain reached a maximum on the fourth day of growth (Fig. 3) compared to the sixth day in the case of the original medium (Fig. 1).

On the whole, one full factorial and two steepest ascent designed experiments made it possible to improve the mycelium yield by 20% and to shorten the cultivation period by two days. Moreover, these experiments showed that final pH values below 4.0 are stressful to submerged *G. lucidum* cultures. As might be expected, low final values of pH were observed when the concentration of glucose in the cultivation medium was high.

The detrimental effect of low pH values can be mitigated either by raising the initial pH of the nutrient medium, by adding a buffer salt to the medium, or by lowering the glucose concentration. Each of these methods has its own drawbacks: high initial pH values will unfavorably affect fungal growth at the early stages and the increase in the concentration of phosphates (as buffer salts) or the decrease in the concentration of glucose (as the carbon source) will inevitably diminish the mycelium yield, as is evident from the above results.

To avoid these limitations, we attempted to supplement the cultivation medium of *G. lucidum* with an additional carbon source, a mixture of saturated and unsaturated fatty acids of a natural origin (Carbon Source 2), leaving unchanged the total concentration of carbon in the medium and the concentrations of the other ingredients. The basal medium in these experi-

Table 6. FFD Matrix 2 and experimental results

u Run number	X1 Carbon source 1	X2 Carbon source 2	Y_u Air-dried mycelium, g/l	Factors and their interactions	Regression coefficients b_i
1	–	–	12	"1"	13.475
2	+	–	13.4	X1	0.675
3	–	+	13.6	X2	0.775
4	+	+	14.9	X1X2	–0.025

Note: Confidence limit ε = 0.65 at a significance level of 10%.

Table 7. Results of Steepest Ascent Experiment 2

Media	Biomass, g/l	Final pH of the culture liquid
3.1	14.5	6.3
3.2	16.1	6.2
3.3	16.5	5.7
3.4	16.0	5.3
3.5	**18.4**	5.9
3.6	17.6	5.5

Table 8. Levels of variable factors in FFD Experiment 3

Factor notation	Factor substance	X_{max}, g/l	X_{min}, g/l	λ, variation step length
X1	Carbon source 2	11	5	3
X2	Soybean meal	18	10	4
X3	Glucose	12	5	3.5

ments was 2.4. The proportion between glucose and Carbon Source 2 was 1 : 1. The factor levels used in FFD Matrix 2 are presented in Table 5. The total number of factor interactions was equal to 2^2.

FFD Experiment 2 (Table 6) showed an increase in the mycelium yield. The final pH of the cultivation medium did not fall below 4.9. The concentrations of both carbon sources were found to be limiting.

Based on these results, the second steepest ascent experiment was designed, in which the concentrations of the carbon sources were varied according to the regression coefficients derived in FFD Experiment 2. The maximum mycelium yield in this experiment (18.4 g/l after 4 days of growth) was observed on medium 3.5 (Table 7).

Two more experiments were aimed at further optimizing medium 3.5. In FFD Experiment 3 (Table 8), the variable factors were the concentrations of the two carbon sources and one nitrogen source, whereas the concentrations of mineral salts were constant and corresponded to those in medium 2.4.

A comparison of the regression coefficients b_i with the confidence limit ε showed that the mycelium yield response to soybean meal and Carbon Source 2 was statistically significant, the concentration of both these factors being limiting. This finding showed that, in order to optimize the nutrient medium, the concentrations of soybean meal and Carbon Source 2 should be further increased. The concentration of glucose was found to be an insignificant factor.

Based on these data, the final steepest ascent experiment was designed (Table 10), which allowed us to obtain a mycelium yield of 20.2 g/l.

Thus, the optimization strategies described in this paper made it possible to improve the biomass yield of *G. lucidum* strain 5 about twofold and to significantly shorten the cultivation period. On optimized cultivation media, biomass yield typically reached 19–21 g/l on the

Table 9. FFD Matrix 3 and experimental results

u Run number	X1 Carbon source 2	X2 Soybean meal	X3 Glucose	Y_u Air-dried mycelium, g/l	Factors and their interactions	Regression coefficients b_i
1	–	–	–	10.2	1	13.61
2	+	–	–	13.5	x1	2.09
3	–	+	–	11.2	x2	1.72
4	+	+	–	17.0	x1x2	0.85
5	–	–	+	11.1	x3	0.64
6	+	–	+	12.7	x1x3	–0.21
7	–	+	+	13.6	x2x3	0.61
8	+	+	+	19.5	x1x2x3	0.22

Note: Confidence limit ε = 0.73 at a significance level of 5%.

Table 10. Results of Steepest Ascent Experiment 3

Media	Air-dried mycelium, g/l	Final pH of the culture liquid
Basal medium (medium 3.5)	16.7	3.9
4.1	11.1	4.55
4.2	12.0	4.1
4.3	13.9	4.8
4.4	15.9	4.9
4.5	16.9	4.5
4.6	16.5	4.9
4.7	**20.2**	**5.0**
4.8	17.1	4.6
4.9	19.4	4.75

fourth day (in some experiments, it was possible to achieve 20 g/l on the third day). A considerable increase in the biomass yield was observed when the cultivation medium was supplemented with an additional carbon source. The mathematically designed sequential optimization of the cultivation medium of the mushroom *G. lucidum* 5 not only increased the mycelium yield, but also shortened the cultivation period and made the cultivation process more reproducible in terms of biomass yield, cultivation length, final pH of the culture liquid, and morphological homogeneity of the mycelium.

REFERENCES

1. Hobbs, C., *Medicinal Mushrooms: An Exploration of Tradition, Healing and Cultures*, Interweave Press, 1995.
2. Wasser, S. and Weis, A., *Medicinal Mushrooms: Reishi Mushroom* (*Ganoderma lucidum* (Curtis: Fr.) P. Karst), Haifa, 1997.
3. Denisova, N.P., *Lechebnye svoistva gribov: Etnomikologicheskii ocherk* (Curative Properties of Fungi: An Ethnomycological Essay), St. Petersburg: SPGMU, 1998.
4. Wagner, R., Mitchell, D.A., Sassaki, G.L., Lopes de Almeida Amazonas, M.A., and Berovic, M., Current Techniques for the Cultivation of *Ganoderma lucidum* for the Production of Biomass, Ganoderic Acid, and Polysaccharides, *Food Technol. Biotechnol.*, 2003, vol. 41, no. 4, pp. 371–382.
5. Fang, Q.H. and Zhong, J.J., Two-Stage Culture Process for Improved Production of Ganoderic Acid by Liquid Fermentation of Higher mushroom *Ganoderma lucidum, Biotechnol. Prog.*, 2002, vol. 18, no. 1, pp. 51–54.
6. Garibova, L.V., Antimonova, A.V., Zav'yalova, L.A., and Krasnopol'skaya, L.M., Growth and Morphological Characteristics of the Reishi mushroom *Ganoderma lucidum* as Functions of Cultivation Conditions, *Mikol. Fitopatol.*, 2003, vol. 37, no. 3, pp. 14–19.
7. Krasnopol'skaya, L.M., Antimonova, A.V., Belitskii, I.V., Bukhman, V.M., Isakova, E.B., Libenzon, A.V., Zav'yalova, L.A., and Garibova, L.V., Medicinal Reishi mushroom *Ganoderma lucidum* (Curt.: Fr.) P. Karst: Submerged Cultivation and Anticancer Properties, in *Netraditsionnye prirodnye resursy, innovatsionnye tekhnologii i produkty* (Natural Substances, Products, and Innovation Technologies in Traditional Medicine), Moscow, 2003, issue 9, pp. 46– 55.
8. Maksimov, V.N., *Mnogofaktornyi eksperiment v biologii* (Multifactorial Experiments in Biology), Moscow: Mosk. Gos. Univ., 1980.
9. Shivrina, A.N., Nizkovskaya, O.P., Falina, N.N., *et al.*, *Biosinteticheskaya deyatel'nost' vysshikh gribov* (The Biosynthetic Activity of Higher Fungi), Leningrad: Nauka, 1969.