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Production of Submerged Mycelium of *Laricifomes officinalis* (Vill) Kotl. et Pouzar

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Abstract: In recent years in Russia, interest has grown in the development of food and fodder additives and pharmaceutical drugs based on higher mushrooms and their metabolites. Most of these research works are concerned with basidiomycetes, which are widely investigated in many countries all over the world. The involvement of rarely encountered fungal organisms, with complex trophism and ecology, into the circle of investigations, not only would extend the range of practically valuable species but also provide new information on their biology, chemistry and genetics. Our attention was attracted by a representative of the unique group of xylotroph basidiomycetes, *Laricifomes officinalis*. Lately, this fungus has started to be studied as a producer of biologically active compounds. The following culture medium for the submerged cultivation of *L. officinalis* in laboratory was selected, g/l: glucose, 20.0; NH_4NO_3, 3.5; KCl, 0.5; K_2PO_4, 1.0; $MgSO_4$, 0.5; beer wort of 15 degrees Balling, 115.0. The optimal physical conditions for growing *L. officinalis* submerged culture either in flasks on a rotary shaker or in a fermenter were selected: temperature, 25-28°C; stirring at 180 rpm; duration, 14 days.

Key words: *Laricifomes officinalis* · Submerged mycelium · Cultivation parameters · Composition of the medium

INTRODUCTION

Currently, Russian and world science shows keen interest in investigating mushrooms. This is related, first of all, to crucial revision of the views on significance and uniqueness of the ecological functions that are controlled by mushrooms in natural ecosystems. Second, mushrooms have always been among the major and promising objects of biotechnology. The basidiomycetes have attracted attention of mycologists and biotechnologists as a source of several efficient pharmaceutical drugs: anticancer compounds (polysaccharides and proteins), antibiotics, psychotropic agents and anti-AIDS drugs [1-3].

Particular attention is attracted by a representative of the unique group of xylotroph basidiomycetes, *Laricifomes officinalis*, which grows on stems of larch trees. It has been actively used in folk and official medicine for several thousand years. This species has a clear-cut physiological activity; it shows sedative action and controls bleeding. This mushroom is best known in medicine of Asian-Pacific region, especially in Japan [4]. The principal active ingredient is agaric acid, which is an inducer of calcium ion ingress into mitochondria [5] and an adenine nucleotide translocase inhibitor [6]. Although this mushroom is abundant in the northern and far eastern regions of Russia [7-9] and is exported from there abroad, there are very few publications dealing with this mushroom in the Russian and world literature.

Lately, *Laricifomes officinalis* has been studied as a producer of biologically active compounds. Compounds isolated from the fruiting body of *L. officinalis* include carotenoids, sterols, unsaturated fatty acids, agaric acid, bioflavonoids and vitamins of B, P, E and A groups [10]. Antibiotic activities of the fruiting body [11] and the mycelial culture with respect to some pathogenic bacteria were established [12]. The antiviral activity of the fruiting body of *L. officinalis* was identified [13].

This brings about the need for artificial cultivation of *L. officinalis*. Submerged cultivation is considered to be an efficient method being promising for fast production of mycelium with specified characteristics [14-16]; moreover, mycelium may be found to contain compounds that are absent in the fruiting bodies [17]. For production of a

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submerged culture in the most active physiological state, it is necessary to study the effect of addition of various components to known liquid media.

The purpose of this work was to select the optimal nutrient medium for submerged cultivation of *L. officinalis* and to elucidate the optimal physical conditions that would ensure the most active accumulation of the biomass.

MATERIALS AND METHODS

The strain *Laricifomes officinalis* used in the study was isolated from the basidioma of *Laricifomes officinalis (Vill.) Kotl. et Pouzar* (= *Fomitopsis officinalis (Vill.) Bondartsev et Singer*) (herbarium VLA M20673). The mushroom parasitized a Dahurian larch (Larix dahurica (Rupr.) Rupr. "Bastak" national reserve at the Jewish Autonomous Region. The strain and the basidioma are deposited in the culture collection of the Institute of Biology and Soil Science, Far-East Branch, Russian Academy of Sciences.

The culture was stored at 4°C on wort agar (the sugar content in the wort was 4° Balling) with preliminary growth for 7-10 days at room temperature. Subculturing to a fresh nutrient medium was once a year.

For the preparation of submerged mycelium, the quinine fungus (*Fomitopsis officinalis*) was grown in 750 ml Erlenmeyer flasks with 100 ml of nutrient medium placed on a rotary shaker or in a 10-liter fermenter ("Applikon", the Netherlands).

The cultivation temperature and the rotation speed of the shaker were selected experimentally. To compare the growth of the culture on different media and under different conditions, a ten-day *L. officinalis* culture grown on wort agar was used as the inoculum. Agar blocks of the 14-day culture with 5 mm diameter were used as the inocula for the flasks. In the fermenter, a flask-grown 7-day culture was used (20% by volume). After completion of cultivation (every 2 days), mycelium was filtered off from the culture liquid, washed with distilled water and dried to a constant weight at a temperature not exceeding 45°C. The biomass formed was measured by the weighing method.

The following growth media were used [18, 19]:

- Beer wort (BW) 4° Balling (°B);
- Glucose medium (GM), g/l: glucose, 20.0; NH_4NO_3, 3.5; KCl, 0.5; K_2PO_4, 1.0; $MgSO_4$, 0.5; BW, 15 °B, 115.0;
- Starch medium (SM), g/l: starch, 10.0; soy flour, 2.0; NH_4Cl, 2.4; $MgSO_4*7Í_2Î$, 0.38;
- Flour medium with serum (FMS), g/l: wheat flour, prime quality, 14.0; milk serum (10 % by volume); K_2PO_4, 1.0; $MgSO_4*7Í_2Î$, 0.38;
- Corn soy flour (CSF), g/l: soy flour, 40.0; corn flour, 17.3; $KÍ_2PO_4$, 0.5;
- Flour medium (FM), g/l: wheat flour, prime quality, 57.3, $KÍ_2PO_4$, 0.5;
- Glucose peptone medium (GPM), g/l: $KÍ_2PO_4$, 1.0; $MgSO_4$, 1.0; KCl, 0.5, peptone, 5.0; glucose, 30.0;

The statistical treatment of the obtained data was performed using Microsoft Excel 2003 statistic functions and Statistica 6.0 program package.

RESULTS AND DISCUSSION

The mycelial growth of the mushroom on rich, natural nutrient media containing soy, wheat, or corn flour and milk serum was very weak if any. Meanwhile, on synthetic media with beer wort added, the biomass crop reached 7.1 g/l, which is a sort of record value for quinine fungi. In view of experimental observations and published data [19, 20], this species refers to a slowly growing type of fungi as compared with other basidiomycetes. Indeed, the average rate of growth of *Ganoderma lucidum* reaches 15.89 g/l over a period of 7 days [21].

Attention is attracted by the fact that for the identical composition of the medium, the addition of wort increases the yield of mycelium biomass. It is also noteworthy that upon cultivation in wort-containing media, the biomass and the culture liquid acquire the smell of stewed fruit with a slight honey tint. More acrid and sour odor appears upon the addition of serum. Thus, the composition of the nutrient medium influences the odor of the grown mycelium.

Different morphology of the colonies was noted. The colonies grown on semisynthetic media had a more or less regular round shape, cream color. They were of different size, fluffy and had star-shaped projections. The colonies grown on rich natural media had irregular shape and very small size (Fig.1).

The most important factors that control the growth and the metabolism of higher basidiomycetes in the culture include temperature, ph of the nutrient medium and aeration. These factors affect the solubility of salts, the ionic state of the substrates and the cell morphology and structure; they determine the physiological activity of the cultures, in particular, they influence the properties of

Fig. 1: Morphology of the submerged culture of L. officinalis.

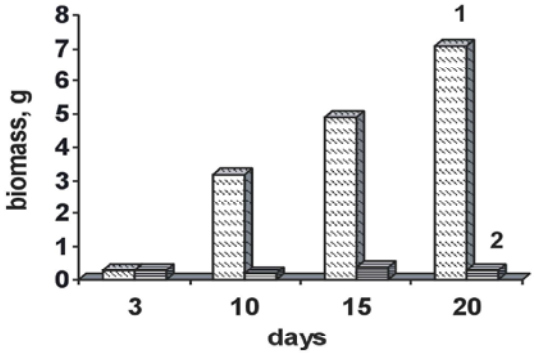

Fig. 2: Mycelial biomass accumulation of *L. officinalis* 2 on glucose medium at 25-28°C (1) and at 6-10°C (2).

cell walls, nutrient transport, membrane reactions, the growth rate and metabolism and the ability to assimilate particular nutrient sources [22, 23].

The minimum temperature needed for the development of higher mushrooms is known to be between 0 and +5°C. The maximum temperature varies from 27°C to 35°C. Wood-destroying fungi can be divided into three groups with optimal temperatures of: (i) 20-24°C, (ii) 24-32°C, (iii) above 32°C. This is apparently related to ecological f eatures of these groups. The particular nutrient requirements of fungi, the character of spore germination and the formation of antibiotics and other biologically active compounds may be temperature-dependent [24, 25].

Therefore, the quinine fungus culture was grown on the above-mentioned media at temperatures of 6-10°C and 25-28°C. According to experimental data, the highest yield of the biomass was observed at temperature of 25-28°C, all other factors being equal. Conversely, at 6-10°C, no increase in the culture biomass was observed (Fig. 2), which is confirmed by published data. Indeed, O.P. Nizovskaya and N.M. Milova [19] noted that 26°C is the temperature of choice for the growth of bracket fungi.

The acidity of the nutrient medium is an important factor controlling the culture growth and development of mushrooms. The ingress of particular nutrients into the cell, the activity of enzymes and the formation of pigments, vitamins, antibiotics and other biologically active compounds depend on the acidity. Most mushrooms can grow upon substantial pH variation, although the optimal values are often at ph 5.0 - 6.0. O.P. Nizovskaya and N.M. Milova [19] classify *L. officinalis* as a culture with high optimal acidity. However, the *L. officinalis* strain studied here demonstrated active growth in a fairly broad range of ph, from 3.7 to 7.6.

It is noteworthy that the culture considerably acidified the medium during the growth. For example, during the growth on beer wort, the ph decreased from 6.50 to 3.71, while on a starch medium, the decrease was from 7.52 to 5.24 (Table 1), which is also supported by published data. O.P. Nizovskaya and N.M. Milova [19] assigned *L. officinalis* to the type of cultures that strongly acidify the medium at any initial ph. This is apparently due to the fact that during growth, the quinine fungus forms up to 30-35% organic acids [25], which are responsible for the decrease in the pH. For example, O.N. Efimenko and L.V. Ageenkova [25] isolated oxalic, citric and agaric acids and two triterpenic acids from the fruiting body of *L. officinalis*.

However, in our opinion, apart from the listed characteristics, of no less importance for mushroom physical processes is the sugar content in the medium. When the amount of sugar was 1.0 to 2.8 °B, no culture growth took place. However, even 3.2 °B resulted in a slight increase in the biomass. The most active mycelium growth occurred at the sugar content of 3.8-4°B.

Also, the rotation speed of the shaker proved to be an important factor. The optimal rotation speed we established was 200 rpm.

Correlations were noted between pH of the nutrient medium, the sugar content in the medium and the submerged culture biomass yield; in particular, biomass growth results in higher amount of sugar in the medium and lower pH (Fig. 3). Each component of the nutrient medium was found to affect in one way or another the fungal mycelium. Study of stimulation and inhibition of its vital activity by nutrient medium components is important, in particular, for the development of cultivation processes for the production of biomass with specified properties and secondary metabolites.

Table 1: Accumulation of *F. officinalis* mycelium biomass in the submerged culture.

		ph			Sugar, degrees Balling			Biomass, g/l	
No	Nutrient medium	Primary	10th day	20th day	Primary	10th day	20th day	10th day	20th day
1	beer wort	6.50	5.42	3.71	4.0	4.5	4.7	2.5	6.1
2	glucose medium 1	6.60	5.36	4.50	4.0	4.6	4.9	2.8	6.3
3	glucose medium 2	6.60	5.36	4.70	4.0	4.6	4.9	3.2	7.1
4	starch medium	7.52	5.82	5.24	2.8	3.0	3.8	0.6	0.8
5	flour medium with serum	5.64	5.64	5.63	1.0	1.1	1.0	0	0
6	corn-soy flour	5.51	5.51	5.49	1.2	1.2	1.2	0	0
7	flour medium	5.59	5.59	5.57	1.5	1.6	1.4	0	0
8	glucose-peptone medium	7.79	7.51	7.25	2.1	1.8	1.6	0.9	0.3
9	glucose medium 3	6.52	5.31	4.41	3.7	4.1	4.5	2.6	6.0

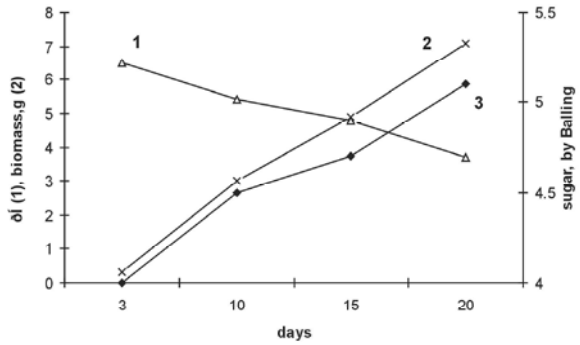

Fig. 3: Correlation between the acidity of the nutrient medium (1), the content of sugar in the medium (3) and the growth of the biomass (2) for submerged culture of *F. officinalis*. Fig. 4. Growth of biomass in the fermenter as a function of the stirrer speed

The most appropriate medium for submerged cultivation of the bracket fungus under laboratory conditions was selected: this is the glucose medium of the following composition (g/l): glucose, 20.0; NH_4NO_3, 3.5; KCl, 0.5; K_3PO_4, 1.; $MgSO_4$, 0.5; beer wort 15° Balling, 115.0.

The optimal physical conditions for cultivation of the submerged culture of *L. officinalis* on a flask shaker were determined to be as follows: temperature, 25-27°C; ph = 5.5-5.8; sugar content, 3.8-4.0 °B; rotation speed of the shaker, 200 rpm.

The submerged cultivation of basidiomycetes in a fermenter as a method for production of their mycelium for the subsequent isolation of biologically active metabolites has a number of advantages. A fermenter is suitable for submerged growth of the mushroom culture in the nutrient medium under sterile conditions, with vigorous stirring and with continuous bubbling of sterile air. Also, in a fermenter, all required parameters (temperature, acidity, etc.) can be monitored and maintained at specified levels.

For cultivation of *L. officinalis* in a fermenter, we selected a semisynthetic medium, which was optimal for submerged cultivation of this mushroom. The temperature and acidity conditions and the sugar content were selected in accordance with the results obtained in our flask experiments. Stirring of the culture in the fermenter was performed by a mechanical stirrer, the rotation speed of which was initially set to be equal to the rotation speed of the shaker, namely 200 rpm. However, it was found during the experiments that the highest yield of the mushroom biomass is attained at mechanical stirring at 180 rpm, all other factor being the same (Fig. 4).

CONCLUSION

Thus, submerged cultivation of *Laricifomes officinalis* in a fermenter should be performed in a glucose medium of the specified composition, a temperature of 25-28°C and rotation at 180 rpm for 14 days. Longer cultivation is inexpedient because the weight of the biomass remains constant and then the biomass sours. There are prospects for cultivating *Laricifomes officinalis* in larger fermenters, which would markedly reduce the required amount of the fruiting body of the mushroom and thus preserve its natural occurrence.

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