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The Relevance of Solid-state Substrate Moisturing on *Ganoderma lucidum* Biomass Cultivation

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Summary

Solid-state cultivation of *Ganoderma lucidum* biomass based on originally isolated Slovenian strain and production of its polysaccharides in horizontal stirred tank reactor (HSTR) were developed. For solid state *G. lucidum* biomass cultivation and polysaccharide production, moisture fraction of solid substrate 70 % was found as a critical value. Moisture fractions higher than 70 % promote the growth and polysaccharide production, while below 70 % the growth of mycelium is slower and polysaccharide production is reduced. As the moisture of the substrate drops to 55 %, growth of mycelium stops, therefore, after 7 days of cultivation continuous moisturing of the substrate is necessary for high biomass and polysaccharide production.

Key words: solid-state cultivation, moisture content, *Ganoderma lucidum*

Introduction

Ganoderma lucidum (Fr.) Karst has been widely used as traditional medicine for promotion of health and longevity in China and other countries *(1)*.

It is a species of basidiomycetes which belongs to the order of *Aphyllophorales*, family *Polyporaceae* (or *Ganodermaceae*), class *Hymenomycetes* (2). Its fruiting body is large, dark reddish-brown with a glossy exterior *(1)*. It posesses numerous pharmaceutically active compounds, polysaccharides, triterpenoides, adenosine and derivatives, oleic acids, proteins and it is used to treat various human diseases, such as hepatitis, hypertension, hyperglycemia, chronic bronchitis, bronchial astma, cancer and other. Especially polysaccharides (β-D-glucan) are the most effective drug for cancer. A mushroom produces viscous extracellular polysaccharides for protection against low moisture conditions. Products of *G. lucidum* have recently undergone clinical trials and are available as a syrup, injection, tablet, tincture, bolus of powdered medicine and honey, both in solution and mixture *(3)*.

Ganoderma lucidum strain BFWS, Gal 4 of Slovenian origin has been isolated. It is rare in nature, especially in Europe where it is found exeptionally *(4)*.

Many workers have attempted to obtain the mycelium of *G. lucidum* using solid-state fermentation in plastic bags, on stumps and logs *(1)*, but very little information is available about the procedure and conditions of solid-state cultivation in bioreactors *(3)*.

Cultivation reported in this study was carried out in a horizontal stirred tank reactor (HSTR) under controlled conditions such as temperature, moisture fraction and airflow. This procedure is based on using cheap secondary raw materials and it represents the basis for a cheap and simple industrial production of high amounts of biomass and polysaccharides.

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Materials and Methods

Microorganism

Ganoderma lucidum, original Slovenian strain, BFWS, Gal 4 was used in this work. It was maintained on potatoe dextrose agar at 30 °C. Vegetative inoculum was obtained in three steps. The mycelium from agar plate was first transmitted to the liquid medium by cutting out 5 rolls (5 mm diameter) of mycelium with agar and transmitting it into a 500 mL baffled Erlenmayer flask containing 100 mL of a medium A. The flasks were incubated for 14 days as shake cultures at 30 °C and 100 rpm. The volume of 0.8 L of inoculum was used for inoculation of 8 L of medium A. At this stage the cultivating conditions in the submerged bioreactor were at 30 °C, pH=5.8, partial oxygen pressure=70–80 %, redox potential=300–400 mV, mixing=300 rpm, aeration=10 L/min *(4)*. To obtain higher concentrations of vegetative inoculum the culture was fed after 8 days with the same medium. At maximum biomass concentration, after few days, the culture was prepared to inoculate the solid medium for solid-state cultivation. The volume of liquid inoculum was 2 L.

Medium

The medium for mycelium propagation (medium A): water from cooked peeled potatoes 300 g/L, glucose 20 g/L, olive oil 2 %, distilled water. The medium for the solid-state cultivation (medium B): 800 g beech saw-dust, 20 mL olive oil, 50 mg $(NH_4)_2SO_4$, 200 mg KH_2PO_4, 50 mg $CaCl_2{\cdot}2H_2O$, 50 mg $MgSO_4{\cdot}7H_2O$, 150 mg $FeSO_4{\cdot}7H_2O$, trace elements diluted in 1.5 L distilled water that was used to moisten the saw-dust.

Bioreactor

Experiments were carried out in a horizontal stirred tank reactor (HSTR) of our own construction and design. The cultivation was carried out at 30 °C and airflow 2 L/min and periodical mixing of 80 rpm during 2 min (in the first 7 days every second day and 2 min every day in the last part of cultivation). After one week of cultivation a gas washing vessel with sterilized distilled water was connected to the air pump to moisturize the substrate (Fig. 1).

Analytical methods

Biomass concentration on particles was determined by measuring chitin contents with glucosamine assay with 3 methyl-2-benzothiazole hydrazone *(5)*. Polysacharides were determined by disintegration and extraction of 15 g sample (mycelium with saw-dust) with boiling water for 5 h and filtration of the suspension to remove the insoluble matter and then precipitation of polysaccharides by adding 3 volumes of (96 %) ethanol. The precipitate was then freeze-dried *(6)*.

Results and Discussion

Two different series of experiments were carried out. In the first one, solid substrate was left to dry through the cultivation by aeration and reaction heat emission, while in the second one it was moisturized using a gas washing vessel. Two representative experiments of each run are presented (Fig. 2).

In the first experiment a drying effect was studied. In the first 35 days, aeration reduced the moisture fraction of solid particles from 80 to 10 % (Fig. 2). In this period until the 30th day of cultivation biomas fraction of glucosamine increased up to 0.5 mg/g of solid substrate and the amount of polysaccharide up to 3.0 mg/g. While at the second experiment after decreasing the moisture to 35 % the gas moisturing device was connected and continuous moisturing of the substrate increased the solid substrate moisture fraction to 78 %. Higher moisture fraction rapidly increased the amounts of fungal biomass glucosamine to 0.68 mg/g and amount of polysaccharide up to 6.0 mg/g. The average rate of biomas glucosamine concentration was 0.016 mg/g/day, while the rate of polysaccharide production was also very high 0.16 mg/g/day (Figs. 3 and 4).

The conditions in the first week of cultivation, were highly favourable for mycelium growth and polysaccharide production. Biomass and polysaccharide production were in this period very comparable in the both series of experiments. Fungal polysaccharides are highly impor-

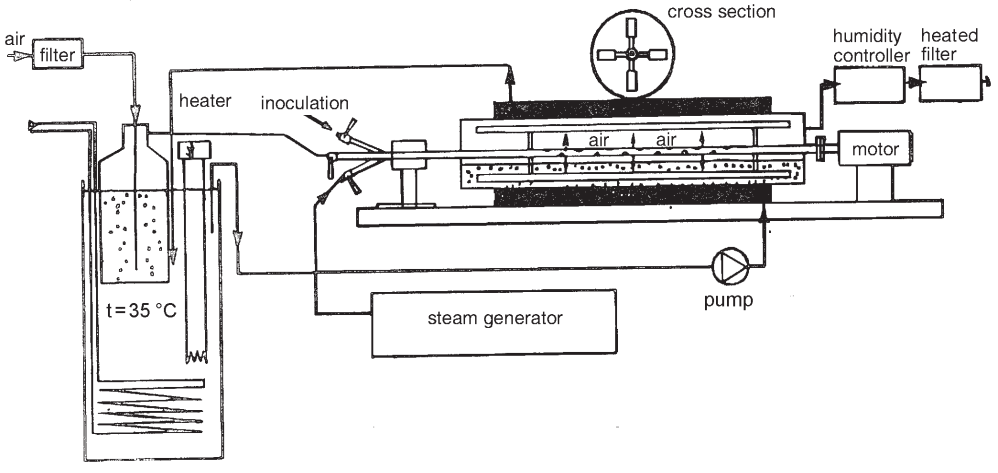

Fig. 1. Horizontal stirred tank reactor

tant for growth of mycelia especially at the beginning of cultivation. Their primary role is fastening of hyphae to the surface of the solid particles, but their great importance is also to stabilize the hyphae, protecting them against the evaporation and mechanical disturbances *(7)*.

As the moisture fraction dropped under the critical point of 70 %, the production of polysaccharides stopped and the polysaccharide fence around the cells has became thinner and thinner. When the moisture fraction dropped below 55 %, the growth of the mycelium has stopped and polysaccharide cover dried. In natural habitat polysaccharides, with their viscous structures, protect the hyphae against lower moisture conditions. They maintain and regulate the surroundings of the hyphae moisture for diffussion of different molecules (vitamins, minerals, oxygen, sugar components...) to the cell surface and into the cell, but when this protection is blocked, all of the cell activities stopped.

At 55 % the moisture fraction has been so low that diffusion of the soluble organic and mineral molecules from the substrate to the hyphae was finally blocked. At this conditions polysaccharide cover is dryed and hyphae began to dry and decline because of scarce supply of water and nutrients.

Due to rigid and impermeable cell walls the mycelia survived even at the moisture level of 40 %. If the substrate was moisturized again the mycelium revive very quickly and began to grow and to produce polysaccharides at a rate even higher than at the beginning of the cultivation (Figs. 3 and 4).

The first part of the second experiment proceeded almost identically as in the first experiment. But after moisturizing, when moisture content has reached values over 70 %, the growth rate has become 3 times higher then in the first part of the cultivation (Fig. 4).

The dried hyphae are able to absorbe large quantities of water and the mechanism of diffussion has been re-established again. The degradation process of solid matrix has started even at higher rate. While at the end of the cultivation biomass glucosamine concentration from the first experiment reached 0.2135 mg/g/day, at the end of the second experiment it was 0.6542 mg/g/day (Fig. 4).

The production of polysaccharides in the first part of the second experiment was also slowly increasing. Because of the low moisture content after 21 days of the cultivation, the production has fallen as in the first experiment. As the moisturizing began after 28 days of cultivation, the polysaccharide concentration has immediately increased to 5.7 mg/g at the end of the cultivation. The concentration of polysaccharides was 4.5 times higher in the second experiment than in the first one. This strong increase is probably due to strong enzyme activation because of the shock which mycelium has survived (Fig. 4).

The moisturizing of solid substrate has to start after 7 days of cultivation. In this way it keeps the moisture fraction over 70 %. It was found that in the first 7 days of the cultivation because of thin and delicate structure of the mycelium, it is enough to agitate 2 min per every second day. In the last part of cultivation mixing of 2

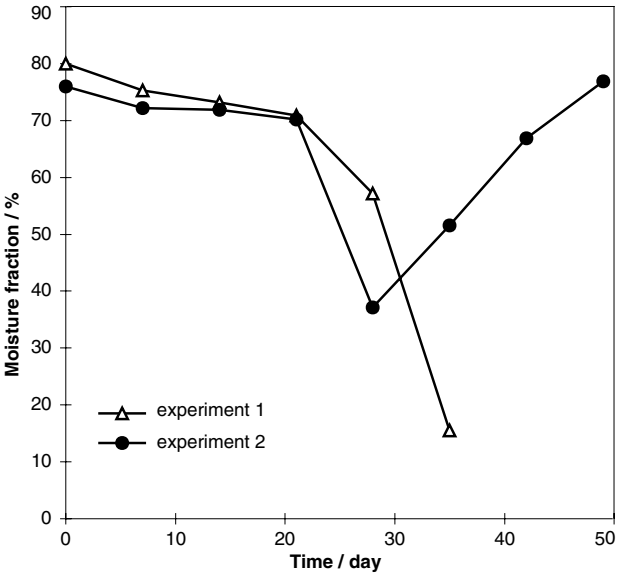

Fig. 2. Solid substrate moisture content in bioprocess time course, Δ without moisturizing, ● with moisturizing

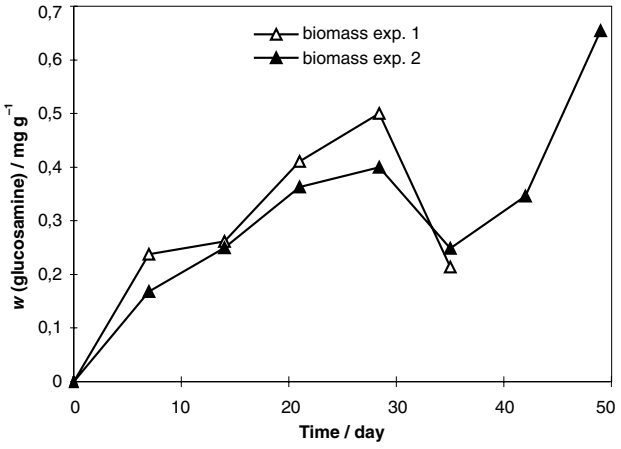

Fig. 3. Biomass content in bioprocess time course, Δ without moisturizing, ▲ with moisturizing

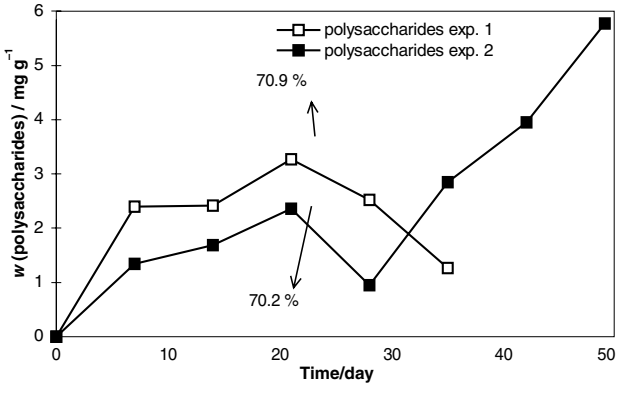

Fig. 4. Polysaccharide production in bioprocess time course, □ without moisturizing, ■ with moisturizing

min per every day is recommednable. It enables to reach equal moisture distribution and more uniform of the micelial growth.

Conclusions

For solid-state *G. lucidum* biomass cultivation and polysaccharide production, moisture fraction of solid substrate is of crucial importance. A moisture fraction of 70 % was found as a critical value. Moistures higher than 70 % promote the growth of *G. lucidum* mycelium and polysaccharide glucosamine production up to 0.68 mg/g and the amount of polysaccharide up to 6.0 mg/g. As the moisture fraction of the substrate drops to 55 %, growth of mycelium stops. However, mycelium survives moisture fraction drop even to 40 % and it begins to grow with maximal growth rate when the substrate is moistured again.

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Primjenjivost ovlaživanja supstrata u čvrstom stanju na uzgoj biomase *Ganoderma lucidum*

Sažetak

Razrađen je postupak za uzgoj biomase *Ganoderma lucidum*, izvornog izoliranog slovenskog soja, na čvrstom supstratu te proizvodnja polisaharida u horizontalnom reaktoru s miješalicom. Za uzgoj *G. lucidum* biomase na čvrstom supstratu i za proizvodnju polisaharida bilo je potrebno da čvrsti supstrat sadržava 70 % vlage. Vlažnost iznad 70 % potiče rast i proizvodnju polisaharida, dok je pri vrijednostima ispod 70 % usporen rast micelija i smanjena proizvodnja polisaharida. Kada vlažnost padne na 55 %, zaustavlja se rast micelija, pa je stoga potrebno tijekom 7 dana uzgoja stalno vlažiti supstrat kako bi se dobio veliki prinos biomase i polisaharida.