

Submerged Cultivation of *Ganoderma lucidum* Biomass in Stirred Tank Reactor



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M. Berovič^{a,*} and M. Popovic^b

^aDepartment of Chemical, Biochemical & Ecology Engineering,
Faculty of Chemistry and Chemical Engineering, University of Ljubljana,
Večna pot 113, 1000 Ljubljana, Slovenia

doi: 10.15255/CABEQ.2018.1371

^bDepartment of Biotechnology, University of Applied Sciences,
Biotechnikum, Seestrassse 64, D-13347 Berlin, Germany

Original scientific paper

Received: May 1, 2018

Accepted: December 21, 2018

European isolated strain *Ganoderma lucidum*, its biomass formation, intracellular and extracellular polysaccharide production in batch and repeated fedbatch cultivation, were studied. The best fed batch results were obtained in the first feeding at 141 h at productivity of biomass $P_x = 0.016 \text{ g g}^{-1} \text{ h}^{-1}$, intracellular $P_i = 0.010 \text{ g g}^{-1} \text{ h}^{-1}$, extracellular polysaccharides $P_e = 0.007 \text{ g g}^{-1} \text{ h}^{-1}$, and percent value of polysaccharides per cell dry weight $P_i/x = 5.0 \%$ and $P_e/x = 3.0 \%$. Although four feedings increased the amount of fungal biomass by 42.66 %, productivities of fungal polysaccharides $P_i = 0.010 \text{ g g}^{-1} \text{ L}^{-1}$ and $P_e = 0.011 \text{ g g}^{-1} \text{ L}^{-1}$ showed that at the fourth feeding, the biomass was already exhausted and polysaccharide production was reduced. Therefore, 210 h batch cultivation with $P_x = 0.095 \text{ g g}^{-1} \text{ L}^{-1}$, $P_i = 0.078 \text{ g g}^{-1} \text{ L}^{-1}$ at $P_i/x = 33 \%$ and extracellular polysaccharides $P_e = 0.095 \text{ g g}^{-1} \text{ L}^{-1}$ at $P_e/x = 6.8 \%$, was found to be the best mode for *G. lucidum* fungal biomass and polysaccharides production. Non-Newtonian cultivation broth rheology was found to be influenced entirely by the concentration and morphology of the biomass.

Keywords:

Ganoderma lucidum, batch fermentation, repeated fed batch, stirred tank reactor, fungal polysaccharides, hydrodynamic behaviour

Introduction

In the last decades, basidiomycetes of various *Ganoderma* spp. and their wide range of pharmaceutically interesting products are one of the most attractive groups of natural products in Asia and North America. Most of the research has been related to *Ganoderma lucidum*, a recognised traditional remedy used in Chinese and Japanese traditional medicine. In Asian traditional medicine, the fruiting body of *Ganoderma lucidum*, called *Ling-Zhi* in Chinese and *Reishi* in Japanese, has been used for treatment of a series of diseases, including hepatitis, arthritis, nephritis, bronchitis, asthma, arteriosclerosis, hypertension, cancers and gastric ulcer^{1–3}. Several investigations report on its antiallergenic constituents⁴, immunomodulatory action⁵ and treatment of HIV infections, antitumor⁶ and cardiovascular effects⁷, liver protection and detoxification, and effects on nervous system⁸.

Pharmaceutically active compounds from *G. lucidum* include triterpenoids, proteins, steroids, alkaloids, nucleotides, lactones, and fatty acids. Polysaccharides (especially β -D-glucanes) have been

recognised as an effective anti-cancer drug⁹. In *Ganoderma* polysaccharide research, special attention was paid to their immunomodulatory effects^{10,11}.

For a large-scale production of medicinal fungi biomass and their active pharmaceutical compounds, antitumor, and anticancer acting polysaccharides, submerged cultivation is one of the most reliable technologies. The mechanism of fungal growth in nature is strongly connected with pasting the fungal tips on the solid matrix. For this purpose, large amounts of sticky fungal polysaccharides are produced and accumulated on the tip of young hyphae cells. This jelly-like material serves as the sticky plaster for anchoring the fungal tips onto the solid matrix. The polysaccharide gel serves also as the matrix for the transfer of cellulolytic and xylolytic enzymes on the surface of cellulose materials. On solid matrixes, fungal mycelia also use polysaccharide gels as a basis for moving on the surface.

For fungal biomass growth in submerged cultivations, accumulation of polysaccharides is not necessary. However, in high mixing shear fields, polysaccharides are secreted at the tips of young fungal hyphae cells. This gel-like material enters the liquid substrate where it increases the pseudoplasticity of the submerged cultivation broth. Increased pseudo-

*Corresponding author: Tel. + 386 40 636 943;
E-mail: Marin.Berovic@fkt.uni-lj.si

plasticity of the cultivation broth rapidly influences the reduction of mass and heat transfer. When the oxygen transfer is limited, higher agitation and more intensive aeration are required to increase the dissolved oxygen concentration in the media. Increased shear forces occur by increasing the oxygen transfer in the media, which strongly influences the fungal disintegration, resulting in growth damage.

Modern biotechnological cultivations in bioreactors, especially with submerged liquid substrate cultivation have been developed previously^{12–15}. The quality and content of physiologically active substances vary from strain to strain, and also depending on the location, culture conditions¹⁴, growth stage of the fungus¹⁷, processing procedures, and formulation preparation of β -1-3-D, β -1-4-D and β -1-6-D glucans¹⁸.

In general, for high amounts of *G. lucidum* biomass and polysaccharide production, stirred tank reactors (STR) are still the most used types on the industrial scale¹⁷. The main disadvantages of using STRs are the high energy-consumption and maintenance costs. In addition, changes in rheology from Newtonian to non-Newtonian conditions appear over the time of cultivation, due to the secretion of the fungal polysaccharides, which results in pseudoplastic behaviour of the culture broth. STRs are very suitable, especially in processes of cultivation broth that represent great reductions of hydrodynamic conditions, which have significant influence on the mass transfer²¹.

Submerged cultivation of large amounts of fungal biomass is the most widely used alternative to farming production^{19,12,24,25,28}. Besides optimization of process rheology and mass transport, an efficient cultivation process combines the production of fungal biomass and secretion of fungal polysaccharides in high shear fields. However, few investigations have considered the optimization of some other cultivation parameters, such as rate of agitation²⁰, pH²¹, inoculation density²⁶, temperature, and medium composition^{22,27}. According to Zhu *et al.*²⁸, roughly half of the studies on *G. lucidum* submerged cultivation were performed in Erlenmeyer flasks, but not in bioreactors.

Batch cultivation has been found to be the simplest and most often used and reported technique^{12,23,28}. A fed-batch process for simultaneous production of ganoderic acids and polysaccharides by *G. lucidum* has been described by Tang and Zhong²³. Ganoderic acids production remarkably improved by the pulse feeding of lactose, when its residual concentration was between 10 and 5 g L⁻¹. Effects of oxygen supply and lactose feeding on fed-batch submerged culture of *G. lucidum* have also been investigated^{23,28,29}. A higher volumetric oxygen transfer coefficient ($k_L a$) value led to a high-

er biomass density and a higher productivity of both intracellular polysaccharides and ganoderic acids. In a stirred bioreactor, at an initial $k_L a$ of 78.2 h⁻¹, obtained were a maximal cell concentration of 15.6 g L⁻¹ by dry weight, and a maximal intracellular polysaccharides production of 2.2 g L⁻¹. An increase in initial $k_L a$ from 16.4 h⁻¹ to 96.0 h⁻¹ led to a 1.8-fold higher production of ganoderic acids. Tang *et al.*^{22,23} achieved a significant synergistic enhancement of ganoderic acids accumulation, up to 754.6 mg L⁻¹, which was the highest reported in the submerged cultivation of *G. lucidum*.

Submerged fungal biomass cultivation is a process that produces significant amounts of extracellular fungal polysaccharides. Contrary to most of the publications using various Asiatic *G. lucidum* sp. strains, in this study, the engineering aspects of the original European strain and substrate were investigated.

The aim of present research was to find out which kind of mode of operation is more suitable as a platform for large scale production of *G. Lucidum* biomass and fungal pharmaceutical active polysaccharides.

Materials and methods

Microorganism

A Slovenian *G. lucidum* strain Ga. 1 4 (BFWS,1996) (Fig. 1), was used in all of the experiments. The strain was cryopreserved¹² and maintained on Petri dishes on potato dextrose agar (PDA) (Difco, USA) prepared by dissolving 39 g of dehydrated agar in 1 L distilled water at $T = 24$ °C. It was maintained at 24 °C, and re-inoculated every 3 weeks to maintain its viability and activity¹².

Cultivation of inoculum

The inoculum consisted of five 1-cm² cuts of a 7-day-old culture cultivated on PDA (Difco, USA) at 24°C¹². After the inoculation of 100 mL of liquid substrate, the biomass was cultivated in 250-mL Erlenmeyer flasks on a rotary shaker for 14 days ($N = 100$ rpm; $T = 30$ °C), and transferred to a bioreactor in its exponential growth phase¹².

Submerged cultivation

Substrate

Preliminary studies including substrate optimization using Plackett-Burman method have already been mentioned¹². For the preparation of the cultivation medium, 3.0 kg of peeled and cut potatoes were cooked in 2 L demineralised water, the mash was filtered, and the filtrate was added to 10 L de-

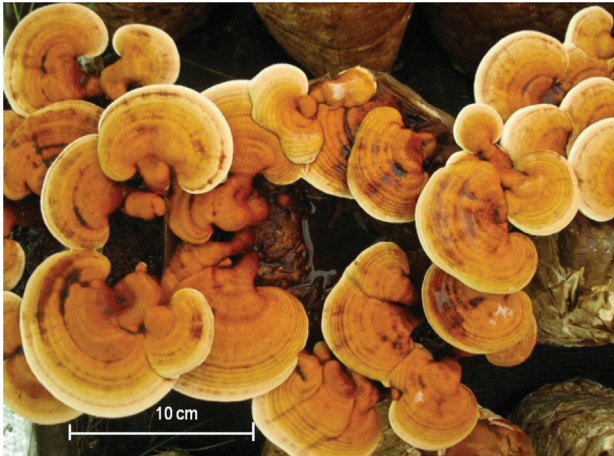


Fig. 1 – *Ganoderma lucidum* in nature (Photo A. Gregori)

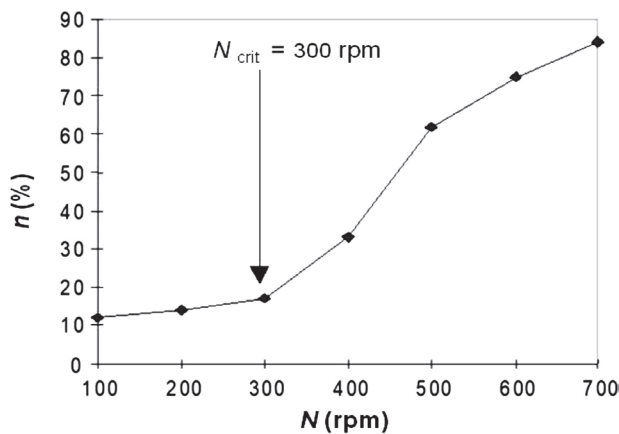


Fig. 2 – Critical agitation speed for hyphae damage in *Ganoderma lucidum* cultivation in stirred tank reactor; n (%) percentage of hyphae damage

mineralised water. Upon cooling and filtration, 4.5 L of the mash filtrate was diluted with demineralised water up to 10 L. Twenty grams of glucose per litre and 2 % (v/v) of olive oil were added, and pH was adjusted with 0.5 M sulphuric acid to 5.8. Substrate was sterilised *in-situ* in the bioreactor for 20 min ($T = 121$ °C at $p = 1.2 \cdot 10^5$ Pa) at a stirrer speed of $N = 100$ rpm.

Bioreactor

All the experiments were performed in a 10-L stirred tank reactor (Bioengineering AG, Switzerland) mixed by three Ruston turbines ($d = 60$ mm) with four baffles and standard tank configuration. Oxygen partial pressure was measured by polarographic sensor IL MGF 509, the redox potential by Ingold Pt 4865, and the pH by Ingold 465-35 k1 sensor. Inoculum in concentration of 17 % (wet weight) was used. All experiments were performed at the aeration rate of $\dot{Q}_g = 1.0$ L air/L substrate/min. A 20 % decrease in cultivation broth volume

caused by evaporation and sampling was taken into account in all experiments. Cultivation temperature was $T = 30$ °C.

Mixing

In previous experiments, the critical mixing rate was determined. Each cultivation experiment was performed at constant mixing rate in the range from 100 to 700 rpm. A ten-millilitre biomass sample was taken every 24 h, and the percentage of damaged hyphae, n (%) was determined¹² from optical microscope photos (magnification 100) (Fig. 2).

Cultivation of fungal biomass

Cultivation conditions were as follows: temperature of cultivation 30 °C; mixing rate 300 rpm; aeration 1 vvm; average measured values of pH 5.8 to 4.2; oxygen partial pressure 70–80 %, and redox potential 300 to 400 mV. Repeated fed batch cultivation started at the same conditions as the batch, first feeding started at 141 h, the second feeding started at 367 h, the third feeding at 573 h, and finally, the fourth feeding started at 902 h of fermentation. In each feeding, 1/3 of the cultivation broth was replaced with new fresh substrate.

Analytical methods

Biomass

Ten millilitres of the submerged cultivation broth was filtered, and the biomass on the filter was washed 3 times with distilled water, and after drying for 24 h at 105 °C, determined gravimetrically.

Extraction and fractionation of polysaccharides

Polysaccharides were determined by disintegration and extraction of 15 g of fungal pellet biomass sample with boiling water for 5 h. The suspension was filtered to remove the insoluble matter, and the polysaccharides were then precipitated by adding the 3-fold volume of 96 % ethanol. The precipitate was then freeze-dried.⁷

Extracellular polysaccharides

For determination of extracellular polysaccharides, 20 mL of the broth sample was filtered, filtrate was diluted with 100 mL of demineralized water, precipitated by adding 3-fold volume of 96 % ethanol, and left for 12 h at 0–4 °C. Polysaccharides were washed with 2 x 20 mL acetone and ether.

Intracellular polysaccharides

Filter cake from the previous procedure was used for determination of intracellular cell wall polysaccharides. Filter cake was cooked with 10

mL of distilled water for 3 h at 100 °C. Polysaccharides were then precipitated with 3-fold volume of 96 % ethanol, and solution was left for 12 h at 0–4 °C. The filter cake was washed with 2 x 20 mL acetone and ethyl ether, dried for 24 h in a drying chamber at 105 °C, and weighed.

Volumetric oxygen transport coefficient

Volumetric oxygen transport coefficient in cultivation broth was measured by dynamic method²⁸ using a polarographic electrode MFG 509 with IL Amplifier Type 531 (Industrial Lab, Switzerland). Measurement were corrected according to electrode response time ($\tau = 22$ s), gas and liquid dynamics³⁰.

Rheological measurements

Rheological measurements of flow behaviour and fluid consistency index were performed using a Rheotest 3 viscometer in a double cylinder configuration (2VEB MLW, Pruferte-Werk, Medingen, Germany). A rotating cylinder ($d = 37.7$ mm inner diameter) and measuring cell (D040.39 mm outer diameter) were used. The rheological properties were characterized by the Ostwald-de Waele power law model³¹.

Microscopy

Cell morphology was examined with light microscope Axioscope, Zeiss (Fig. 6b). The SEM images were taken using a field-emission electron-source SEM JEOL 7600F, Tokyo, Japan, operated at 15 kV (Fig. 6a). Macro-photos were taken by Nikon XF10 (Fig. 7).

Statistics

All of the cultivation experiments were performed at least in three runs. The results were analyzed for statistical significance by one-way analysis of variance (ANOVA) test using the Statistical Package of the Social Science (SPSS) version 11.0 (SPSS Inc., Chicago, IL, USA). Statistical significance of the test effects was evaluated at $p < 0.05$.

Results and discussion

Batch mode of fermentation

In the first part, the batch mode, i.e., the simplest and the most used process for submerged cultivation of *G. lucidum* cultivation, was studied. The cultivation proceeded for 210 h. Productivity of biomass was $P_x = 0.095$ g g⁻¹ L⁻¹, while the productivity of intracellular $P_i = 0.078$ g g⁻¹ L⁻¹ at $P_i/x = 33$ % of percent value of polysaccharides per cell dry weight and extracellular polysaccharides $P_e = 0.095$ g g⁻¹ L⁻¹ at $P_e/x = 6.8$ %.

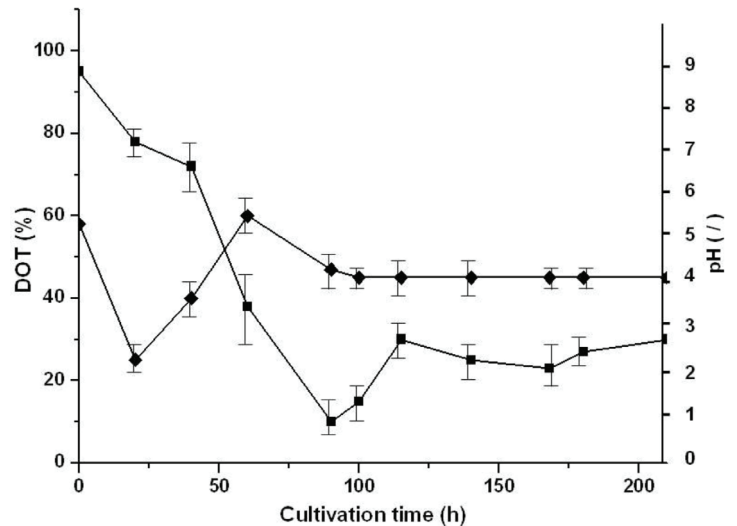


Fig. 3 – Batch cultivation of *Ganoderma lucidum* ♦ pH; ■ dissolved oxygen tension (DOT) expressed in percentage of saturation pressure

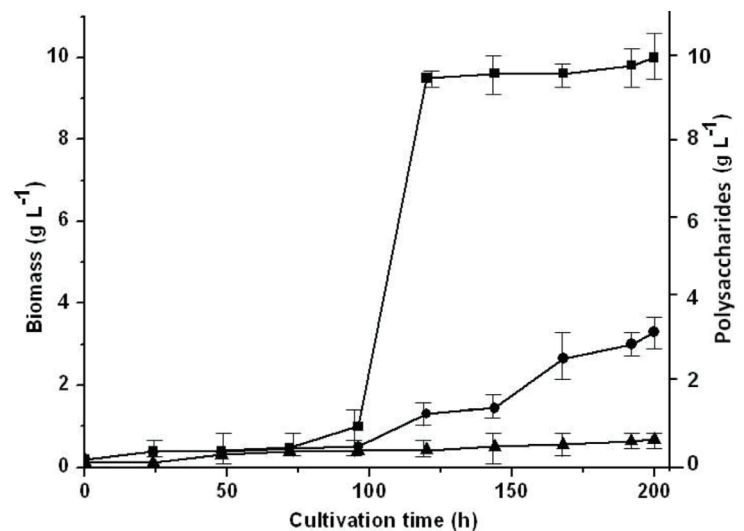


Fig. 4 – Batch cultivation of *Ganoderma lucidum* ■ biomass; ● intracellular; ▲ extracellular polysaccharides

Rheologically, *G. lucidum* cultivation broth was characterized by biomass concentration and its physiology state. The secretion of ganoderic acids that had already started from 12 to 24 h, influenced pH to decrease to 2.5. In further cultivation, the pH increased to 6 and then stabilized at 4.5. Microbial growth may cause marked changes in the pH of a substrate. Acid production, due to incomplete oxidation of the substrate or uptake of ammonium ions, could influence the decrease in pH, whereas the release of ammonia by deamination of urea or other amines will increase the pH (Fig. 3).

In this first part up to 18 h, microbial growth was characterized by hyphae forms mycelia and low biomass concentration of up to 2.5 ± 0.04 g L⁻¹, where typical Newtonian behaviour with flow behaviour index $n = 1.00 - 0.95 \pm 0.02$ was indicated.

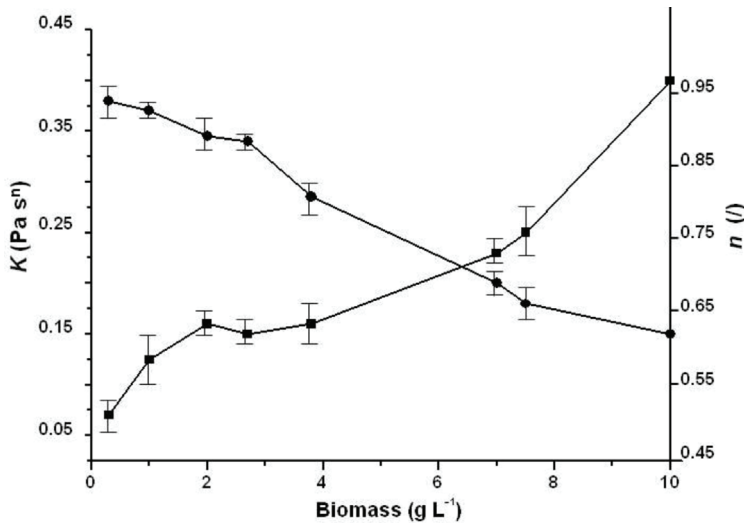


Fig. 5 – Changes in flow behaviour index n (●) and fluid consistency index K (■) influenced by the increase in biomass concentration, X

From 90 to 143 h, the growth turned from hyphae to spherical microbial pellets ($d_p = 0.3\text{--}0.8 \cdot 10^{-3}$ m) formed by sticking the tips of hyphae in the shear field, and biomass concentration from 3.25 g L^{-1} to $10.0 \pm 0.5 \text{ g L}^{-1}$ was indicated (Fig. 4).

In the exponential growth phase, high oxygen consumption was detected (Fig. 3), and at 90 h, the dissolved oxygen tension decreased to 10 % of saturation followed also by intensive intracellular (3.30 g L^{-1}) and extracellular polysaccharides (0.68 g L^{-1}) production (Fig. 4). In this period, significant non-Newtonian pseudoplastic behaviour of the cultivation broth was detected. The flow behaviour index was drastically reduced to $n = 0.68 \pm 0.05$, and

the fluid consistency index K increased up to $0.380 \pm 0.04 \text{ Pa s}^n$ (Fig. 5). Increasing biomass and pseudoplasticity of cultivation broth strongly influenced the $k_L a$ coefficient that was reduced from 250.0 h^{-1} to final 58.5 h^{-1} at the end of the cultivation at 210 h.

In the range of applicable shear rates, rheological behaviour of the cultivation broth can be characterised by Ostwald-de Waele model. The changes in the flow behaviour index n and consistency index K with biomass concentration can be observed in Fig. 5. Time change of the rheological properties during the presented biomass cultivations depended on morphology, biomass concentration, and production of sticky polysaccharides on the tip of the hyphae (Fig. 6a). The polysaccharides were turbine sucked and transferred into the cultivation broth through the created shear field effected by Rushton turbine, Fig. 6b.

Rheological behaviour of the cultivation media is strongly related to the growth stage of the fungal biomass. Although the cultivation broth from 18 to 210 h expressed a significant pseudoplastic non-Newtonian flow behaviour, the viscosity of the filtrate in all samples of this period was low with $(2.00 \pm 0.08) \cdot 10^{-3} \text{ Pa s}$, and did not change significantly over the cultivation time.

In the exponential growth phase, intensive foaming was detected. The foam was produced by the fungal biomass floating on the liquid surface in the reactor. It was controlled using the contact sensor through addition of an emulsion of polypropylene glycol in silicone oil. In the parts below the contact sensor and in the the head space parts, stuck pellets started growing.

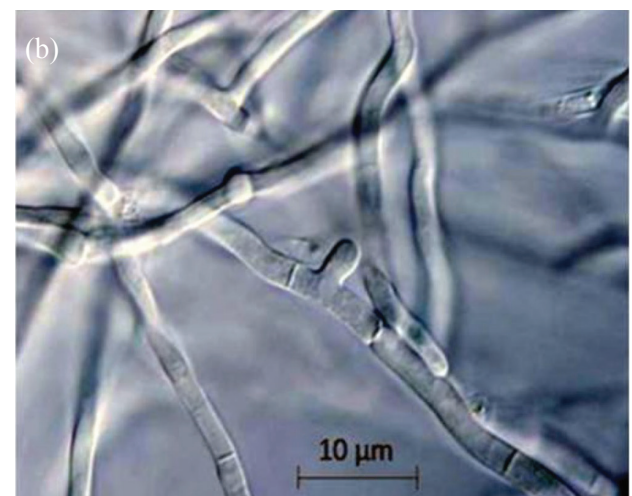
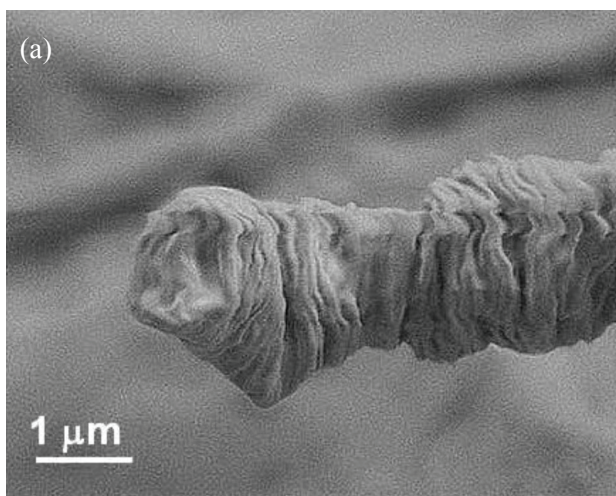


Fig. 6 – (a) Accumulation of external fungal polysaccharides on the tip of hyphae in nature (left); (b) naked and external polysaccharides free hyphae in submerged *Ganoderma lucidum* cultivation (right)

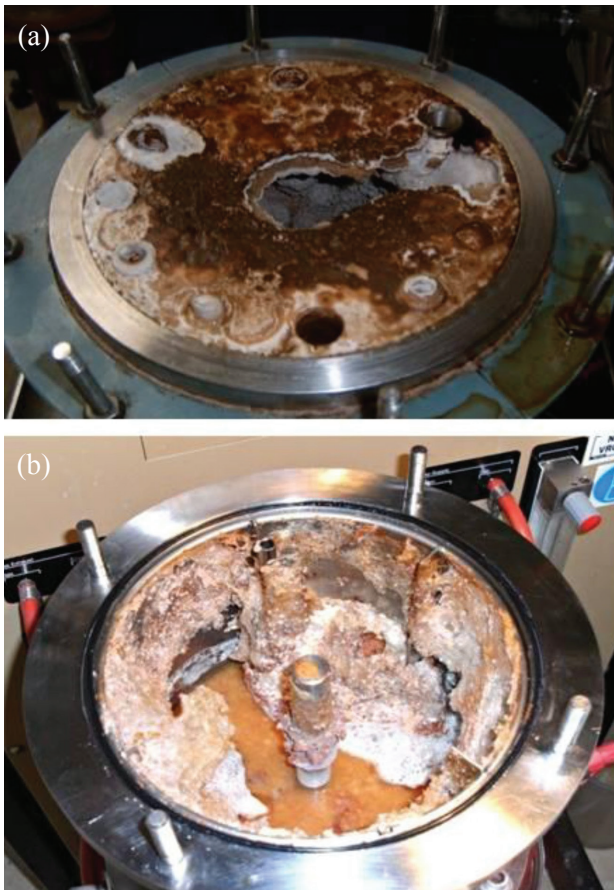


Fig. 7 – (a) Overgrown head space (left), (b) overgrown stirrer and sensors in stirred tank reactor (right)

In further cultivations, fungal biomass in the headspace intensively grew covering the tips of the sensors, including the foam detector (Fig. 7). Consequently, excess of antifoam agent was added and

the pseudoplasticity of the broth drastically increased. Dissolved oxygen tension dropped to 3.2 ± 0.2 % of saturation and higher mixing intensity was required. Concerning the preliminary experiments for the applied Rushton turbine as described previously, the stirring rate was not increased above the critical value of 300 rpm, in order to prevent hyphae damage (Fig. 7).

Repeated fed batch cultivation

In the second part of the experiment, fresh substrate feeding at various time intervals in a repeated fed batch process was introduced (Fig. 8). In the further cultivation, four feedings were applied. After exhausting the substrate, with each feeding, 1/3 of the total volume was replaced with a fresh substrate. The first feeding of the substrate took place at 141 h. In the first feeding, the biomass was diluted from 7.56 to 3.08 g L⁻¹, intracellular polysaccharide dropped from 3.00 to 1.30 g L⁻¹, and extracellular concentration changed from 0.73 to 0.32 g L⁻¹. Productivity of biomass in this part was $P_x = 0.019$ g g⁻¹ L⁻¹, while the productivity of intracellular $P_i = 0.015$ g g⁻¹ L⁻¹ at $P_i/x = 5.0$ % of percent value of polysaccharides per cell dry weight and extracellular polysaccharides $P_e = 0.014$ g g⁻¹ L⁻¹ at $P_e/x = 3.0$ %.

After the second feeding at 373 h, the biomass dropped from 10.16 to 6.44 g L⁻¹, intracellular polysaccharides from 2.45 to 1.60 g L⁻¹, and extracellular from 0.60 to 0.40 g L⁻¹ at $P_x = 0.014$ g g⁻¹ L⁻¹ at the productivity of intracellular $P_i = 0.010$ g g⁻¹ L⁻¹ and $P_e = 0.0087$ g g⁻¹ L⁻¹ $P_i/x = 11.32$ % and $P_e/x = 5.06$ %.

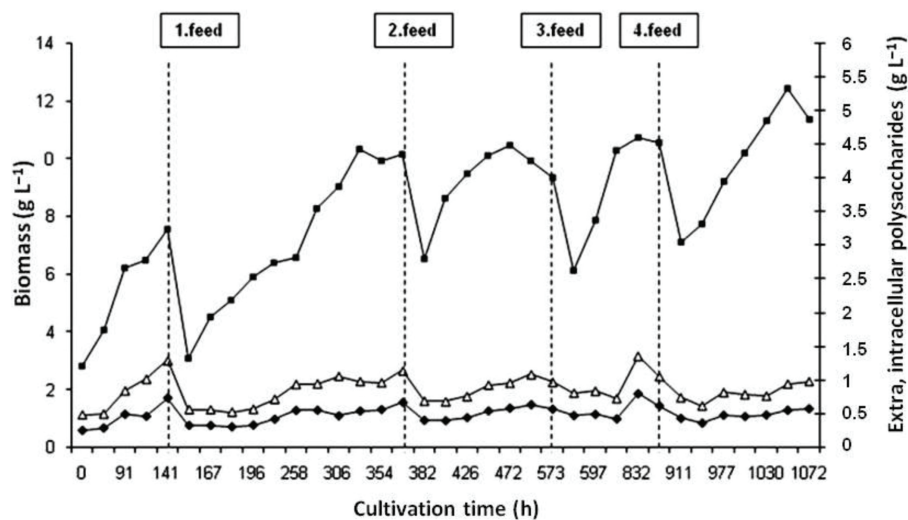


Fig. 8 – Production of biomass and fungal polysaccharides in fed-batch cultivation, feeding at 141, 367, 573, and 902 h; ■ biomass; Δ intracellular; ♦ extracellular polysaccharides

The third feeding was at 573 h. By the third feeding, the values of all three measured parameters declined, biomass to 6.22 g L^{-1} , intracellular polysaccharides to 0.82 g L^{-1} , and extracellular to 0.5 g L^{-1} , at $P_x = 0.0090 \text{ g g}^{-1} \text{ L}^{-1}$ at the productivity of intracellular $P_i = 0.010 \text{ g g}^{-1} \text{ L}^{-1}$ and $P_e = 0.010 \text{ g g}^{-1} \text{ L}^{-1}$, and at $P_i/x = 12.72 \%$ and $P_e/x = 7.27 \%$.

The last, fourth feeding was performed at 904 h. It lowered the biomass from 10.57 to 7.11 g L^{-1} as well as the level of polysaccharides from 1.07 to 0.80 g L^{-1} for intracellular and from 0.62 to 0.43 g L^{-1} for extracellular polysaccharides at $P_x = 0.010 \text{ g g}^{-1} \text{ L}^{-1}$ at the productivity of intracellular $P_i = 0.010 \text{ g g}^{-1} \text{ L}^{-1}$ and $P_e = 0.011 \text{ g g}^{-1} \text{ L}^{-1}$. After the fourth feeding, the cultivation proceeded to 1072 h, and finally 12.40 g L^{-1} of biomass, 1.0 g L^{-1} of intracellular polysaccharides and 0.58 g L^{-1} of extracellular polysaccharides were obtained. In the last 98 h, at $P_i/x = 8.06 \%$ and $P_e/x = 6.8 \%$.

Conclusions

Cultivation of *G. lucidum* submerged cultivation showed significant changes in rheological properties of the cultivation broth. Shear field of the radial stirrer slipped the external fungal polysaccharides from the tips of hyphae and sucked them into the cultivation broth, which influenced the change from Newtonian to the pseudoplasticity character of the broth. Non-Newtonian properties of the cultivation broth depended entirely on the concentration and morphology of the biomass, since the liquid phase was a low-viscosity Newtonian fluid.

Fed batch results lead to the conclusion that the best results in biomass and polysaccharide production were obtained in the first batch part ($P_x = 0.019 \text{ g g}^{-1} \text{ h}^{-1}$, $P_i = 0.012 \text{ g g}^{-1} \text{ h}^{-1}$, $P_e = 0.009 \text{ g g}^{-1} \text{ h}^{-1}$), while from the feeding procedure, the best results $P_x = 0.016 \text{ g g}^{-1} \text{ h}^{-1}$, $P_i = 0.010 \text{ g g}^{-1} \text{ h}^{-1}$, $P_e = 0.007 \text{ g g}^{-1} \text{ h}^{-1}$ were obtained at the first feeding. Although four feedings enabled the increasing in fungal biomass by 42.66 %, $P_i/x = 8.06 \%$ and $P_e/x = 6.8 \%$ were low, which indicates low productivity of fungal polysaccharides $P_i = 0.010 \text{ g g}^{-1} \text{ L}^{-1}$ and $P_e = 0.011 \text{ g g}^{-1} \text{ L}^{-1}$. After four feedings, the biomass was already exhausted and polysaccharide production was reduced.

Comparing fed batch results to the batch run, with $P_x = 0.095 \text{ g g}^{-1} \text{ L}^{-1}$, $P_i = 0.078 \text{ g g}^{-1} \text{ L}^{-1}$ at $P_i/x = 33 \%$ and extracellular polysaccharides $P_e = 0.095 \text{ g g}^{-1} \text{ L}^{-1}$ at $P_e/x = 6.8 \%$, for the best production of *G. lucidum* fungal biomass and polysaccharides, 210 h of batch cultivation is proposed. According to the process optimization, shorter cultivation time also means lower energy consumption.

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