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Cultivation of Medicinal Mushrooms Ganoderma lucidum and Grifola frondosa Mycelia and Polysaccharides in Bioreactors

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Abstract **:** Original strains of *Ganoderma lucidum* (MZKI G97) and *Grifola frondosa* (GF3) isolated from Slovenian forests were cultivated using submerged and solid state cultivation. In 14 days submerged *Ganoderma lucidum* fed batch cultivation extracellular (1,7 g/L) and intracellular (0.45 g/L) polysaccharide fractions were isolated, up to 17.0 g/L dry fungal biomass was produced, while in 28 days *Grifola frondosa* fed batch cultivation 3.65 g/L of extracellular and 1.30 g/L intracellular polysaccharide and 15.2 g/L dry biomass was produced. In *Ganoderma lucidum* solid state cultivation in 18 days 5.77 mg /g extracellular and 1.45 mg /g intracellular polysaccharide was produced while in *Grifola frondosa* in 38 days cultivation of 0.70 extracellular and 3.80 mg/g of intracellular polysaccharide were produced. The isolated polysaccharides were mainly β-D-glucanes. Immunostimulatory effects of isolates were tested on induction of cytokine (TNF-α , IFN-γ and IL12) synthesis in primary cultures of human mononuclear cells (PBMC) isolated from a buffy coat.

Key words : *Ganoderma lucidum, Grifola frondosa, polysaccharides,* induction of cytokines, bioreactors, submerged, solid state cultivation

1. Introduction

Basidiomycets of various species and their wide range of pharmaceutically interesting products are in the last decades one of the most attractive groups of natural products in Asia and North America. Research in phisiology, basical and applied studies in fungal metabolism, process engineering aspects and clinical studies last two decades represent large cotribution to the development of this potentials that initiates the development of new drugs, specially those on the palette of over counter remedies [1-4].

Ganoderma lucidum, ancient immunostimulant (*Ling-Zhie* in China, *Reishi* in Japan) and *Grifola frondosa* (*Hui Shu Hua* in China, *Maitake* in Japan) and are lignin degrading basidiomycetes with highest potential medicinal active substances. Pharmaceutically active compounds from *Ganoderma lucidum* include triterpenoids, polysaccharides (1,6-β-D-glucans and 1,3-β-D-glucans), proteins, proteoglycans, steroids, alkaloids, nucleotides, lactones and fatty acids, amino acids, nucleotides, alkaloids, steroids, lactones and enzymes. Over 100 triterpenoids were found in *Ganoderma* spp., such as ganoderic (highly oxygenated C_{30} lanostane-type triterpenoids), lucidenic, ganodermic, ganoderenic and ganolucidic acids, lucidones, ganoderals and ganoderols *Grifola frondosa* active compounds primarilly belong to the group of polysaccharides (especially 1,6-β-D-glucans and 1,3-β-D-glucans), glycoproteins, and proteins. This products have been used for treatment of a series of diseases, including hepatitis, arthritis, nephritis, bronchitis, asthma, arteriosclerosis, hypertension, cancer and gastric ulcer. Newer investigations report on *Grifola frondosa* antiallergenic constituents, immunomodulatory action and treatment of HIV infections, antitumor and cardiovascular effects, liver protection and detoxification and effects on nervous system [2,3,5].

Ganoderma lucidum isolates effects on angiogenesis, reduction of benign prostatic hyperplasia, antibacterial and antiviral effects, effects on lipid metabolism and hypertension, antidiabetic activity, vitality and performance enhancement, antioxidant effects, and beneficial cosmetic effects on skin [6].

As *Grifola frondosa* and especially *Ganoderma lucidum* are very scarce in nature, the amount of wild mushroom is not sufficient for commercial exploitation. Cultivation on solid substrates, exploing various agricultural wastes from food and wood industy, stationary liquid medium or, in the last time, by submerged cultivation using various complex media, have become essential to meet the increasing demands on the international markets.

Ganoderma lucidum and *Grifola frondosa* polysaccharides (especially β-D-glucanes) have been recognised as effective anti-cancer drugs remarqably improving immunosystem in human, veterinary an fishery use. In human body they induce activity of cytokines IL-1, IL-6, TNF-α and IFN-γ production by human macrophages and T-lymphocytes.

A successful artificial cultivation of *Ganoderma lucidum* and *Grifola frondosa* has been reported on solid substrates, utilising e.g. sawdust and agricultural wastes as the main media components [7], as well as submerged cultivation in liquid media [8]. The quality and content of physiologically active substances vary from strain to strain and also depends on location, culture conditions [9] and growth of the mushroom [10].

The main goal of this research were to test the abbility of submerged as well as solid state production of *Ganoderma lucidum* biomass in bioreactors and to evaluate the potential immunostimulatory effects of polysaccharides tested on the induction of cytokine (TNF-α, IFN-γ) synthesis in primary cultures of human mononuclear cells. Present results were served as the basis for scale-up of large scale production

2. Materials and methods

2.1. Microorganism

Ganoderma lucidum strain Ga.l 4 (BFWS,1996) was originally isolated from the Slovenian forest was used in all experiments. The strain was cryopreserved 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. It was maintained at 24 ºC and was re-inoculated every 3 weeks to maintain its viability and activity [11,12].

Grifola frondosa strain Gf5, was originally isolated from the Slovenian forest was used in all experiments deposited in the fungal collection of the Institute for Natural Sciences, Slovenia, were maintained on Potato Dextrose Agar (Biolife, Italy) at 24 ^{o}C. The cultures were maintained active by regular transfers on fresh agar plates every 14 days [13].

2.2. Inoculum

The inoculum consisted of five 1 cm^2 cuts of a 7-day old culture cultivated on PDA. After inoculation of 100 ml of potato dextrose substrate in 250 ml Erlenmayer flasks at pH 5.8. The vegetative inoculum biomass was cultivated on rotary shaker for 14 days (100 rpm / 28 and 30 ºC) and transferred to bioreactors [11,12].

2.3. Substrates

Submerged cultivation - *Ganoderma lucidum*. For the preparation of cultivation medium, 3.0 kg of peeled and cut potatoes in 2 L demineralised water were cooked, the mash was filtered and the filtrate was added to 10 L with demineralised 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 was added, and pH was adjusted with 0.1N sulphuric acid to 5.8.

Grifola frondosa cultivation medium consisted of 45,2 g/L glucose; 6,58 g/L peptone, 2,97 g/L KH_2PO_4 and 2% (v/v) of olive oil at pH 5.8. Substrates were sterilised *in-situ* 20 min in bioreactor (T = 121 ºC at P = 1.2× 10^5 Pa) at a stirrer speed N =300 rpm.

Solid state cultivation. *Ganoderma lucidum* was performed on beech saw dust, wheat bran substrate. *Grifola frondosa* was cultivated on milled whole corn plant straw (*Zea mays*). As the additives mineral salts and olive oil used. In both cases 15 kg of substrates were used. Original technology of cultivation of fungal biomass has been developed recently for small and pilot-plant production of intra- and extracellular polysaccharides [11,12,14].

2.4. Bioreactors

Submerged cultivation. All the experiments were performed in 10-L stirred tank reactor (STR) (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.

Solid state cultivation was performed in 30 *l* horizontal stirred tank reactor (HSSR). Sterilisation was performed *in-situ.* For the process monitoring and control O_2/CO_2 analyser ECHOd.o.o, was used.

2.5. Cultivation

Submerged cultivation. Cultivation conditions were as following: temperature of cultivation /was at *Ganoderma lucidum* 30 ºC and 28 ºC and *Grifola frondosa* ; mixing 300 min^{-1}; aeration, 10 L min^{-1}; average measured values of pH 5.8 to 4.2; oxygen partial pressure 70- 80% and redox potential 300 to 400 mV. The first feeding in fed batch cultivation

started at 66 hours, at the second group at 198 and in the third group at 141 hours. In each period of feeding 2/3 of the fermentation broth was replaced with new fresh substrate.

Solid state cultivation. In *Ganoderma lucidum* cultivation temperature was 30°C, while at *Grifola frondosa* used temperature was 28°C. Aeration in solid state cultivation aeration was 5 *l* $min.^{-1}$ and only periodically mixing N = 8 rpm, 5min/day was used with both fungi in all of the experiments.

2.6. Analytical methods

Biomass

Submerged cultivation. Biomass in submerged cultivation was determined gravimetrically after filtration and drying for 24 h at 104 ºC.

Solid state cultivation. In solid state cultivation for biomass determination on solid particles determination of glucosamine (chitin) contents and glucosamine assay with 3 methyl-2-benzothiazole hydrazone were used [15].

Polysaccharides

Submerged cultivation. 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 hours at 0-4 ° C. Polysaccharides were washed with acetone and ether. Filter cake was used for determination of intracellular polysaccharides.

Solid state cultivation. Polysaccharides in solid state cultivation were determined by disintegration and extraction of 15 g taken sample with boiling water for 5 hours, filtration the suspension to remove the insoluble matter For determination of intracellular cell wall polysaccharides filter cake that was cooked with 10 ml of distilled water for 3 hours at 100 ° C. Polysaccharides were then precipitated with 3-fold volume of 96% ethanol and solution was left for 12 hours at 0-4 ° C. Filter cake was washed with acetone and ethyether, dried for 24 hours at 105 ° C and weighted. The precipitate was then freeze-dried [16].

Analysis of cytokine concentrations

Cytokine levels in PBMC culture supernatants were determined by commercially available ELISA kits and in accordance with the manufacturer's instructions. The TNF-α concentration was evaluated with TNF-α Assay Kit from Millenia Biotec (Germany), and IFN-γ and IL-12 with respective Assay Kits from Pierce Biotechnology (USA). The detection limits for TNF-α were 15 pg/mL, INF-γ 1 pg/mL and IL-12 1 pg/mL.

3. Results and discussion

Fungal mycelium was separated from the submerged cultivation broth by vacuum filtration. Cultivation medium was concentrated at T = 50 ^{o}C and a reduced pressure.
Extracellular polysaccharides were precipitated from the concentrate by 96 % ethanol and filtered. Intracellular polysaccharides were extracted with hot water (T = 100 ^{o}C, 3 hours), filtered, concentrated and precipitated by 96 % ethanol. Polysaccharides were further purified by ion-exchange chromatography on DEAE-cellulose (column 20 x 3.0 cm, elution with water, 0.1M $NaHCO_3$, 0.3M $NaHCO_3$, 0.5M $NaHCO_3$ and 0.1M NaOH), gel filtration on Sepharose 4B (column 70 x 1.2 cm, elution with water), and affinity chromatography on Concavalin A-Sepharose 4B (column 20 x 1.2 cm). For β□-polysaccharides, the column was eluted with 0.1 M phosphate buffer (pH=7,0) in 1M NaCl, and for α-polysaccharides with 0.1 M glucose in 1M NaCl. In all cases, the absorbance of chromatographic fractions was measured at 480-490 nm by Dubois method.

Human peripheral blood mononuclear cells (PBMC) from buffy coat of healthy blood donors were isolated by density gradient centrifugation with Ficoll-Paque (Pharmacia Biotech, Sweden) and washed with phosphate buffered saline. The cells were cultured in RPMI 1640 tissue culture medium supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin and heat-inactivated AB normal human serum. After that, $1x10^6$ cells (final culture volume: 1.5 mL) were plated in 24 well plates(Nunc,Denmark)with different concentrations of extracts. Final concentration of extracts was 12.5, 100 and 200 µg/mL at 37 ^{o}C in a 95% humidified (relative humidity) atmosphere with 5% CO_2. Polymixin B (10 µg/mL) was added to samples to rule out a possible contamination with lipopolysaccharide (LPS). Cultures of untreated cells in RPMI 1640 with and without Polymixin B, and without the mushrooms extracts, were used as negative controls. A control experiment was performed with10 ng/mL LPS with and without Polymixin B addition on untreated cells to check the neutralizing effect of Polymixin B. The incubation period for TNF-α determination was 4 hours. The incubation period for INF-γ and IL-12 determination was 72 hours. Before ELISA analysis the cell suspensions were centrifuged at 3000 RPM for 5 minutes, and the supernatants were frozen at -70 ^{o}C. All chemicals used were obtained from Sigma (USA).

Human peripheral blood mononuclear cells (PBMC) from the buffy coat of a healthy blood donor were isolated by a density gradient centrifugation with Ficoll-Paque (Pharmacia,

Sweden). The cells were cultured in a tissue culture medium RPMI 1640 (Sigma, USA) supplemented with 100 U/mL penicillin (Sigma, USA), 100 μg/mL streptomycin (Sigma, USA), 20 mM Hepes (Sigma, USA) and 10 % heat-inactivated AB normal human serum (Sigma, USA). The 1x 10^6 cells (final culture volume 1,5 mL) were plated in 24-well culture plates (Nunc, Denmark) with each of five fractions alone in different concentrations (3.25, 12.5, 50, 100, 400 μg/mL), at T=37°C in a humidified atmosphere of 5% CO_2 in air. Cultures of untreated cells in RPMI 1640 without active substances were considered as a negative control. To rule out a possible contamination by the endotoxin - a lipopolysaccharide from the Gram negative bacterial cell wall (LPS) of our polysaccharide samples, the samples with polysaccharide concentrations of 12.5, 100, 400 μg/mL with added polymyxin B (Sigma, USA) in concentration 10 μg/mL, were tested parallel.
The concentration of cytokines (pg/mL) in PBMC culture supernatant was measured by commercially available ELISA kits, TNF-α from DPC (USA) and IFN-γ from Endogen (USA), according to the manufacturer instructions. The detection limit for TNF- α was 15.0 pg/mL and for IFN-γ 1.0 pg/mL, respectively .

In-vitro testing of immunomodulatory effects from *Ganoderma lucidum* mycelium isolates from β glucanes the highest induction of moderate amounts of 2450 pg/mL, TNF-α shown fraction B and from polysaccharide fractions after gel filtration 2250 pg/mL fraction C. In induction of IFγ the highest extent of 10.8 pg/ mL was indicated at fraction B, while at induction of IL-12 the highest activity 4300 pg/mL indincated fraction B and from polysaccharide fractions after gel filtration 3759 fraction C. At TNF-α induction at *Grifola frondosa* was up 475 pg/mL, in induction of IFγ the highest extent of 180 pg/ mL was indicated at fraction B, while at induction of IL-12 the highest activity 1100 pg/mL indincated fraction B at a polysaccharide concentration of 200 μg/mL.

4. Conclusions

The reported results represent valuable information on active fungal polysaccharides produced and isolated from the European *Ganoderma and Grifola spp.* Cultivation of fungal biomass of *Ganoderma lucidum* and *Grifola frondosa* isolated from the Slovenian forest by submerged and solid state cultivation enables high production of pharmaceutically active fungal biomas. The extra and intra cellular polysaccharides represents mostly 1-3 and 1 – 6 β-D-glucanes, which are main immunomodulatory substances of both fungi. Present results shown that the polysaccharide isolates of both fungi shown remarkable induction of cytokines. Polysaccharide fractions from both fungal mycelium proved to be inducers of production of cytokines TNF-α, IFN-γ and at *Grifola frondosa* also IL-12, that are comparable to the those ammounts of cytokines that are inducing activity of romurtide, which has been used as a supporting therapy in cancer patients treated with radiotherapy and/or chemotherapy.

In *Ganoderma lucidum* in 18 days of solid state cultivation to 5.77 mg /g of extracellular and 1.45 mg /g intracellular polysaccharide was produced at the end of the cultivation. In *Grifola frondosa* in solid state cultivation in 38 days of cultivation 3.80 mg/g of extracellular and 0.70 mg/g of intracellular polysaccharide was produced.

Comparing to traditional farming production of both fungi fruit boddies submerged cultivation of *Ganoderma lucidum* and *Grifola frondosa* biomass in bioreactors enable much shorter cultivation of large ammounts of fungal biomass.

In 14 days submerged *Ganoderma lucidum* fed batch cultivation extracellular (1,7 g/L) and intracellular (0.45 g/L) polysaccharide fractions were isolated, up to 17.0 g/L dry fungal biomass was produced, while in 28 days *Grifola frondosa* fed batch cultivation 3.65 g/L of extracellular and 1.30 g/L intracellular polysaccharide and 15.2 g/L dry biomass was produced.

In *Ganoderma lucidum* solid state cultivation in 18 days 5.77 mg /g extracellular and 1.45 mg /g intracellular polysaccharide was produced while in *Grifola frondosa* in 38 days cultivation of 0.70 extracellular and 3.80 mg/g of intracellular polysaccharide were produced.

Submerged cultivation in large scale bioreactors up to 200 m^3 is more suitable for production of dietary supplements in human use, while solid state cultivation up to 10 m^3 scale enable large scale production of feed grade imunostimulatory compounds suitable for veterinary use.

Both fungi are already long term cultivated in China and Japan, as well as in the other Asiatic countries, in large ammounts by farming, Consequently, Slovenian strains of *Ganoderma lucidum* and *Grifola frondosa* prooved a potential and promising source for production of the fungal biomass and polysaccharides - the natural immunomodulatory substances that could be efficiently and economically produced by submerged and solid state cultivation. Comparing to the traditional production of both fungi biomass, the results of present research shown that cultivation of large ammounts of fungal biomass produced in bioreactors could remarkably shortern the cultivation time in farming from 4 - 6 months

to 14-18 days in submerged and to 18-38 days by solid state cultivation.

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