

ADVANCES IN CULTIVATION OF MEDICINAL FUNGI BIOMASS AND PHARMACEUTICAL COMPOUNDS 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 solid state cultivation. In *G. 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 *G. frondosa* solid state cultivation in 38 days of cultivation 3.80 mg/g of extracellular and 0.70 mg/g of intracellular polysaccharide was produced. Polysaccharides were further separated by ion-exchange, gel and affinity chromatography. 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.

Keywords: *Ganoderma lucidum*, *Grifola frondosa*, polysaccharides, immunomodulatory effects

INTRODUCTION

Basidiomycetes 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 physiology, basical and applied studies in fungal metabolism, process engineering aspects and clinical studies last decade represent large contribution to the development of this potentials that initiates the development of new drugs, specially those on the palette of over counter remedies.

G. frondosa also known as Maitake and *G. lucidum*, are lignin degrading basidiomycete with excellent nutritional and medicinal properties. Pharmaceutically active compounds from *G. 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 C₃₀ lanostane-type triterpenoids), lucidenic, ganodermic, ganoderenic and ganolucidic acids, lucidones, ganoderals and ganoderols. *G. frondosa* active compounds primarily belong to the group of polysaccharides (especially 1,6- β -D-glucans and 1,3- β -D-glucans), glycoproteins, and proteins. These 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 *G. frondosa* anti-allergenic constituents, immunomodulatory action and treatment of HIV infections, antitumor and cardiovascular effects, liver protection and detoxification and effects on nervous system [1].

A large and diverse spectrum of chemical compounds with a pharmacological activity has been isolated from the mycelium, fruiting bodies and sclerotia of *Ganoderma* mushrooms: triterpenoids, polysaccharides, proteins, amino acids, nucleotides, alkaloids, steroids, lactones, fatty acids and enzymes

G. 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.

As *G. frondosa* and especially *G. lucidum* are very rare in nature, the amount of wild mushroom is not sufficient for commercial exploitation. Cultivation on solid substrates, exploiting various agricultural wastes from food and wood industry,

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.

G. lucidum and *G. frondosa* polysaccharides (especially β -D-glucanes) have been recognised as effective anti-cancer drugs remarkably improving immunosystem in human, veterinary and 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 *G. lucidum* and *G. frondosa* has been reported on solid substrates, utilising e.g. sawdust and agricultural wastes as the main media components [2], as well as submerged cultivation in liquid media [3]. The quality and content of physiologically active substances vary from strain to strain and also depends on location, culture conditions [4] and growth of the mushroom [5].

The main goals of this research were to test the ability of submerged cultivation for the production of *G. lucidum* biomass, 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.

MATERIALS AND METHODS

Strains

Original strains of *G. lucidum* (MZKI G97) and *G. frondosa* (GF3) isolated from Slovenian forests were cultivated in solid state cultivation where beech saw dust, wheat bran and mineral salts as well as milled whole corn plant (*Zea mays*), olive oil and mineral salts substrates were used. Production of extracellular and intracellular polysaccharides in both cultivation was studied.

Substrates

Solid state cultivation of *G. lucidum* and *G. frondosa* biomass producing 1,6- β -D-glucans and 1,3- β -D-glucans in solid substrates from food and wood industry wastes were applied. Original technology of cultivation of fungal biomass has been developed recently for small and pilot-plant production of intra- and extracellular polysaccharides.

Bioreactor

In solid state cultivation in 30 l horizontal stirred tank reactor (HSSR) was used. (Fig. 1). Sterilisation was performed *in-situ*.

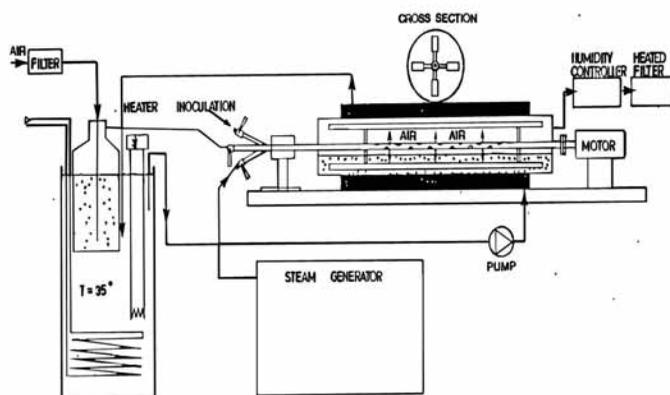


Figure 1. Horizontal stirred tank reactor and equipment

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

Biomass

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

Microscopy

Fungal growth in solid state cultivation was monitored by electron microscopy using Field-Emission Scanning Electron Microscope Karl Zeiss Supra 35 VP (Fig. 2).

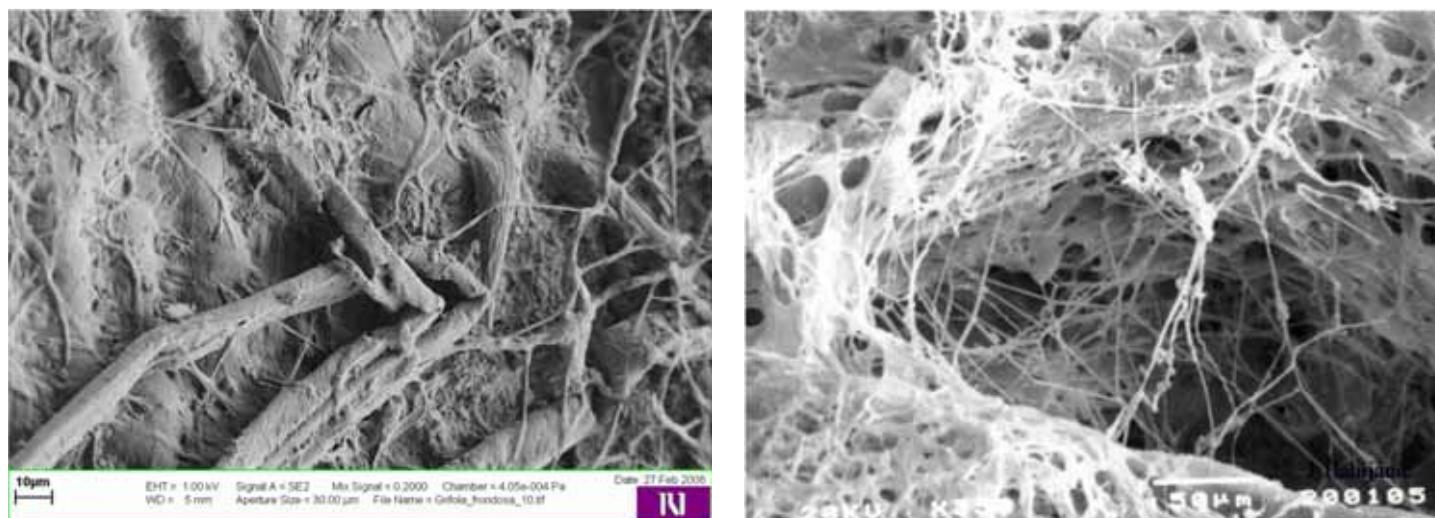


Figure 2. Fungal growth in solid state cultivation a) *G.rifola frondosa*, b) *Ganoderma lucidum* Field-Emission Scanning Electron Microscope Karl Zeiss Supra 35 VP

RESULTS AND DISCUSSION

Cultivation

G. lucidum in 18 days of solid state cultivation produced 5.77 mg/g of extracellular and 1.45 mg/g intracellular polysaccharide at the end of the cultivation

In *G. frondosa* in solid state cultivation in 38 days of cultivation produced 3.80 mg/g of extracellular and 0.70 mg/g of intracellular polysaccharide was produced. Polysaccharides were further separated by ion-exchange, gel and affinity chromatography.

Extraction and fractionation of polysaccharides

Fungal mycelium was separated from the submerged cultivation broth by vacuum filtration. Cultivation medium was concentrated at T = 50 °C and a reduced pressure. Extracellular polysaccharides were precipitated from the concentrate by 96% ethanol, filtered, washed with acetone and dried (fraction A). The mycelium was extracted with 85% ethanol to eliminate low molecular components. Then, the first fraction of intra-cellular polysaccharides was extracted with hot water (T = 100 °C, 3 hours), filtered, concentrated and precipitated by 96% ethanol (fraction B). The mycelium was further extracted with 1% ammonium oxalate solution (T=98 °C, 3 hours) (fraction C), and with 5% sodium hydroxide solution (T= 25 °C, 12 hours), from which polysaccharides were precipitated by acetic acid (fraction D), and from the remaining solution by ethanol (fraction E). Samples of fractions A - E were used in cytokine assays (Table 1).

Table 1. Extraction and fractionation of polysaccharides

Fraction Properties		Mass (mg)	Yield (%)
A	extracellular polysaccharides, water soluble, precipitated with 96% ethanol	1616	1,37
B	intracellular polysaccharides, hot water extract, precipitated with 96% ethanol	2414	2,04
C	intracellular polysaccharides, 1% ammonium oxalate solution extract, precipitated with 96% ethanol	1183	1,002
D	intracellular polysaccharides, 5% sodium hydroxide solution extract, precipitated with acetic acid	2068	1,75
E	intracellular polysaccharides, 5% sodium hydroxide solution extract, precipitated with 96% ethanol	650	0,55

Polysaccharide fractions A and B were further fractionated and purified by ion-exchange chromatography on DEAE-cellulose (column 20 x 3.0 cm, elution with water, 0.1M NaHCO₃, 0.3M NaHCO₃, 0.5M NaHCO₃ and 0.1M NaOH), gel filtration on Sepharose 4B (column 70 x 1.2 cm, elution with water), and affinity chromatography on Concanavalin 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.

Evaluation of cytokine inducing capacity

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⁻¹ penicillin (Sigma, USA), 100 mg ml⁻¹ streptomycin (Sigma, USA), 20 mM Hepes (Sigma, USA) and 10 % heat-inactivated AB normal human serum (Sigma, USA). The 1x 10⁶ 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 mg ml⁻¹), at T=37 °C in a humidified atmosphere of 5% CO₂ 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 mg ml⁻¹ with added polymyxin B (Sigma, USA) in concentration 10 mg ml⁻¹, were tested parallelly.

The concentration of cytokines (pg ml⁻¹) 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⁻¹ and for IFN-γ 1.0 pg ml⁻¹, respectively

Table 2. The concentration of IFN-γ in culture supernatants of human PBMC incubated for 72 hours with two polysaccharide fractions showing the strongest TNF-α inducing capacity (B and C), with polymyxin B

Concentration of polysaccharide fraction [mg/ml]	Concentration of IFN-γ pg/ml]	
	Polysaccharide fraction B	Polysaccharide fraction C
12.5	<1	<1
100	1.23	2.15
400	1.39	2.18

Immunomodulatory activity

In vitro testing of immunomodulatory effects of polysaccharide fractions from *G. lucidum* mycelium proved the induction of moderate amounts of TNF-α in the extent of < 3.0 pg ml⁻¹ to 630 pg ml⁻¹, while TNF-α induction at *G. frondosa* was

up 322 pg ml⁻¹ at a polysaccharide concentration of 200 µg ml⁻¹ for the intracellular fraction. of a culture supernatant (Figs.3 and 4 a,b,c).

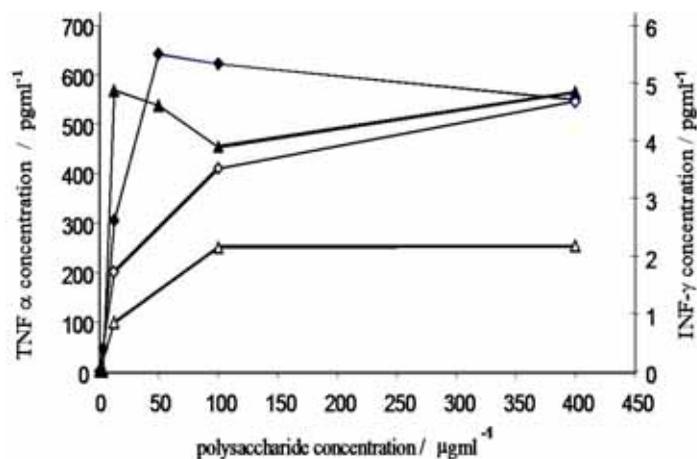


Figure 3. Induction of TNF-α and IFN-γ production by the most active *Ganoderma lucidum* polysaccharides (sample C) from ammonium-oxalate extract, in the presence of polymyxin and without polymyxin

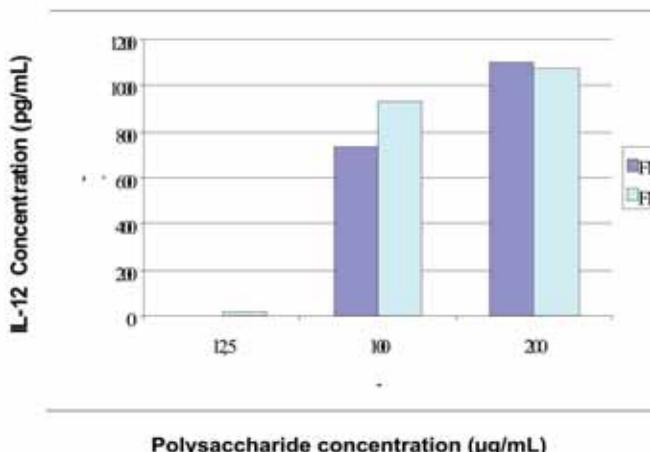
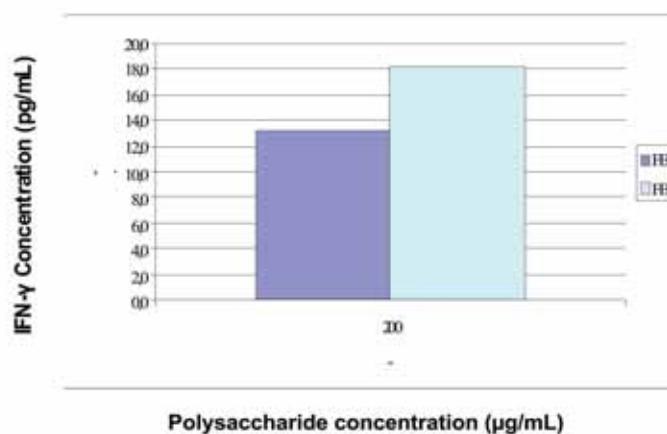
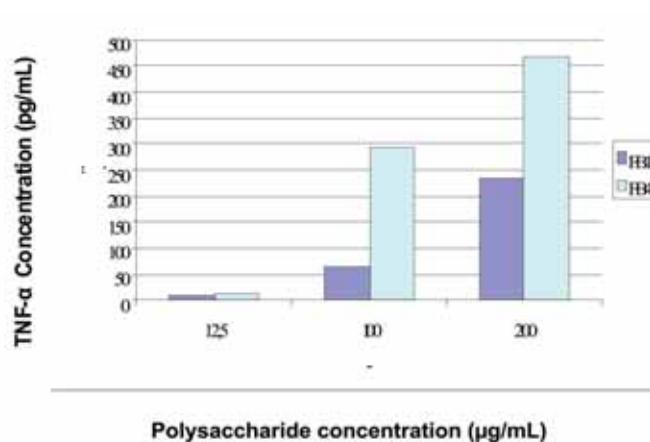
◆ TNF- ; ◇ TNF-α + polymyxin ; ▲ IFN-γ ; △ IFN-γ + polymyxin

CONCLUSION

Cultivation of fungal biomass of *G. lucidum* and *G. frondosa* isolated from the Slovenian forest by solid state cultivation enables high production of pharmaceutically active fungal biomass that enables the production of fungal polysaccharides mostly 1-3 and 1 – 6 β-D-glucanes, which are known from literature as the main immunomodulatory substances of both fungi.

Polysaccharide fractions from both fungal mycelium proved to be inducers of production of cytokines TNF-α, IFN-γ and at *G. frondosa* also IL-12, that are comparable to the those amounts 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.

Consequently, the polysaccharides isolated from the Slovenian *G. lucidum* strain represent a potential and promising natural immunomodulatory substance, which could be efficiently and economically produced by solid state cultivation and production of *G. lucidum* and *G. frondosa* biomass. The reported results represent valuable information on active fungal polysaccharides produced and isolated from the European *Ganoderma* and *Grifola* spp. Solid state



Figures 4 a,b,c. Immunostimulatory effects of *Grifola frondosa* polysaccharides. The comparison of different isolates on induction of cytokines TNF α , IFN- γ and IL-12

cultivation are enabling large scale production of fungal polysaccharides - particularly suitable as feed grade immunostimulatory compounds for veterinary use.

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