



Cultivation of Pharmaceutically Active Fungal Biomass in Bioreactors

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WOOD-DEGRADING MUSHROOMS



White rot



Brown rot



Photo A. Gregori

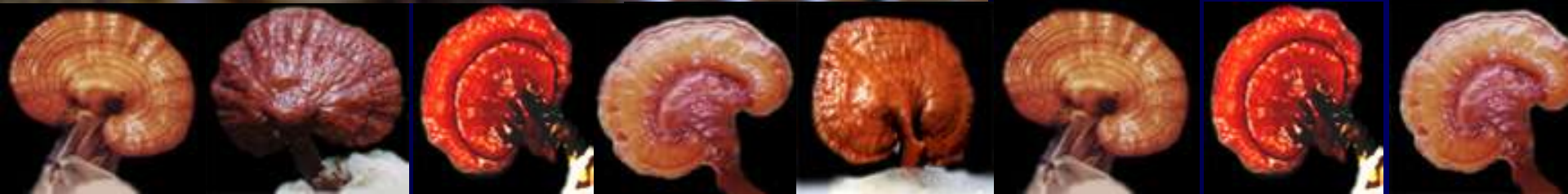


Ganoderma lucidum





©earthstongue.com



Ganoderma lucidum

Regnum: FUNGI

Phyllum: EUMYCOTA

Subphyllum: BASIDIOMYCOTINA

Clasis: HYMENOMYCETES

Ordo: APHYLLOPHORALES

Familia: POLYPORACEAE

Genus: GANODERMA

Species: LUCIDUM

**Slovenian habitate fruiting body of
Ganoderma lucidum
used in all our experiments**



F. Pohleven

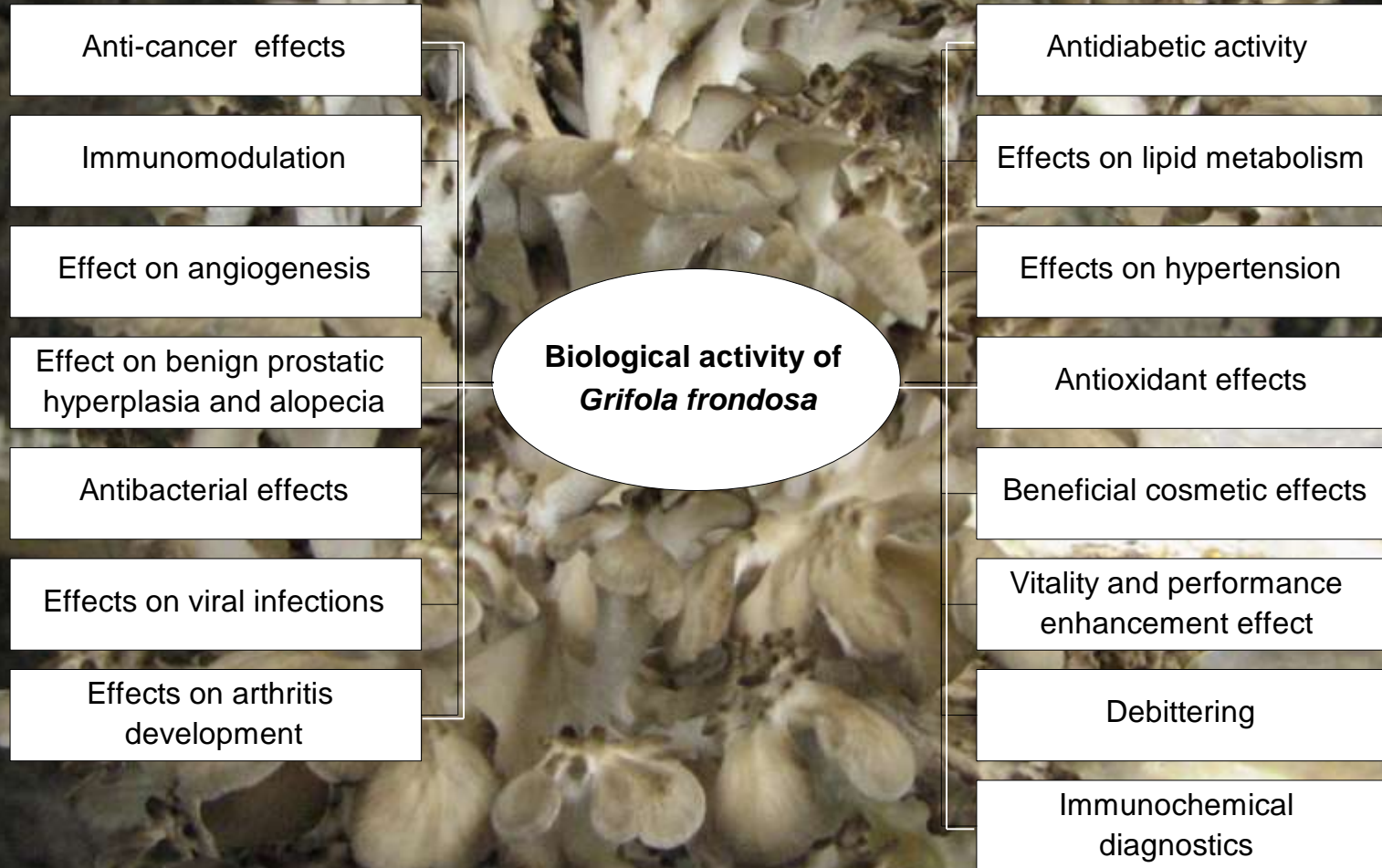
Ganoderma lucidum



Grifola frondosa



Biological activity of *G. frondosa*



Tametes versicolor



UGA0488101

A large, multi-colored bracket fungus (Trametes versicolor) growing on a dark, textured surface. The fungus has concentric rings of colors including brown, tan, and grey. The background is dark and appears to be a forest floor with some green ferns visible on the right side.

Trametes versicolor

Immunomodulation

Antibacterial

Anti-cancer activity

- **Gastric**

- **Lung**

- **Breast**

- **Colorectal**

- **Esophageal**

Polysaccharides

Beta glucans

- **Polysaccharide-peptide (PSP)**

Polysaccharide krestin (PSK)

Triterpenoides

UGA0488101

Hericium erinaceus



Hericium erinaceus

Anti-cancer activity

- Gastric ulcers
- Esophageal carcinoma

Anti-dementia activity

- Stimulate myelination
- Stimulate nerve cells
- Stimulate nerve growth factor

Beta glucans

Polysaccharides

Diterpenoides (Erinacines)

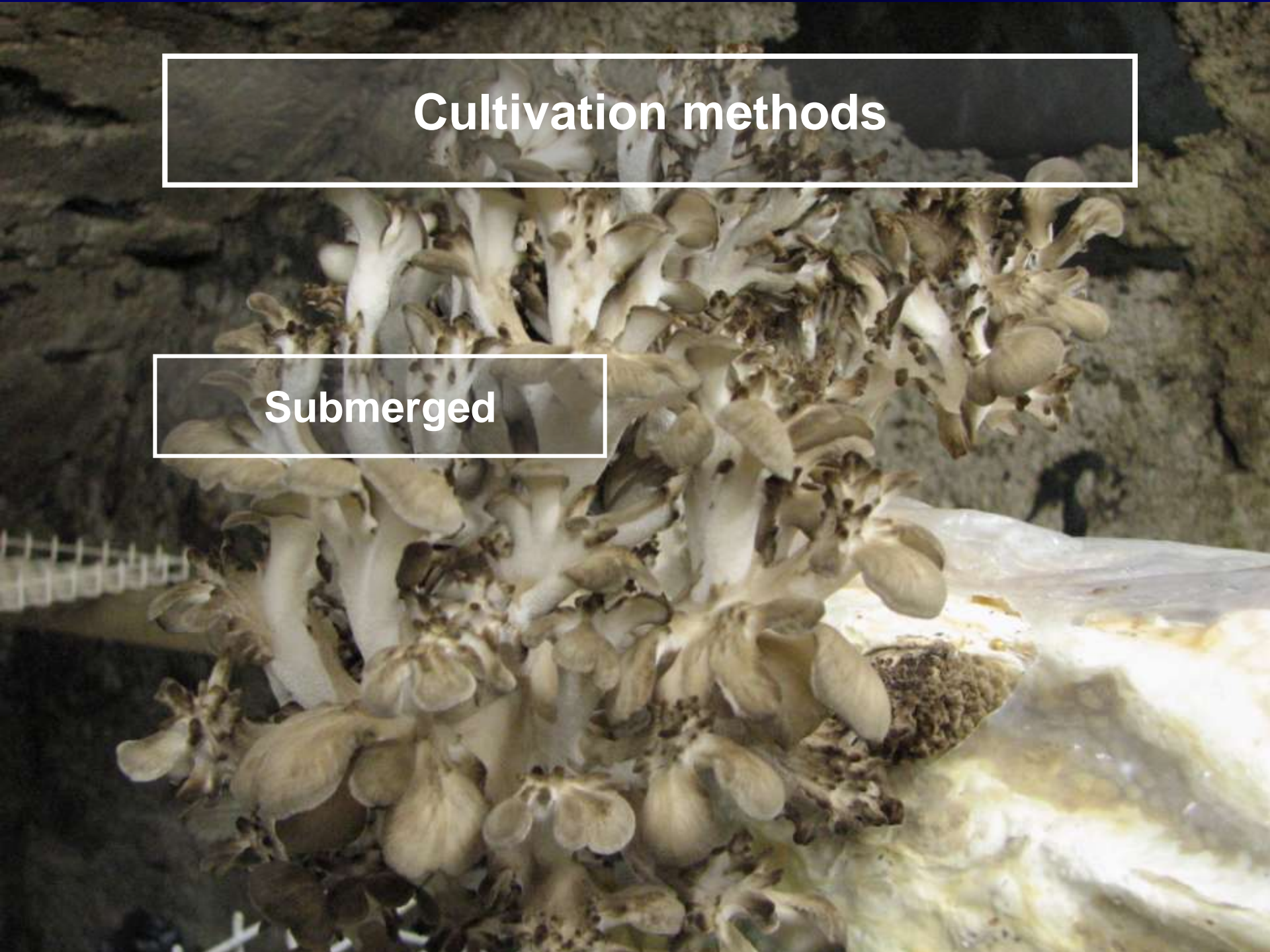
Triterpenoides

Cultivation methods



Cultivation methods

Submerged





Cultivation methods

Submerged

Solid state



Cultivation methods

Submerged

Solid state

Farming



BIOREACTOROMICS



BIOREACTOROMICS





Submerged cultivation



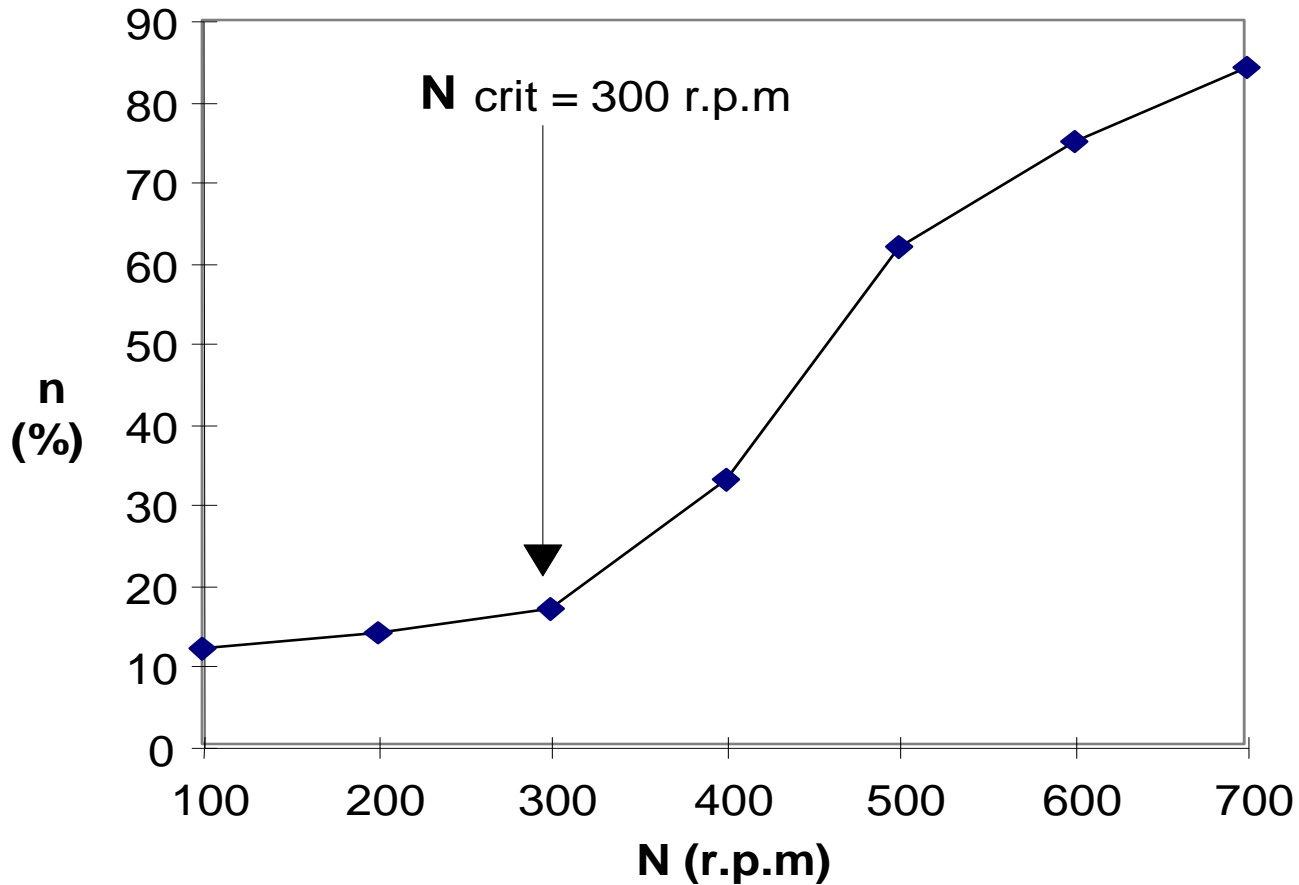
Submerged cultivation



Ganoderma lucidum
innoculum used in
submerged cultivation



Mixing limits in submerged cultivation



Influence of the impeller speed N on the number of cut filaments n



Ganoderma lucidum submerged cultivation

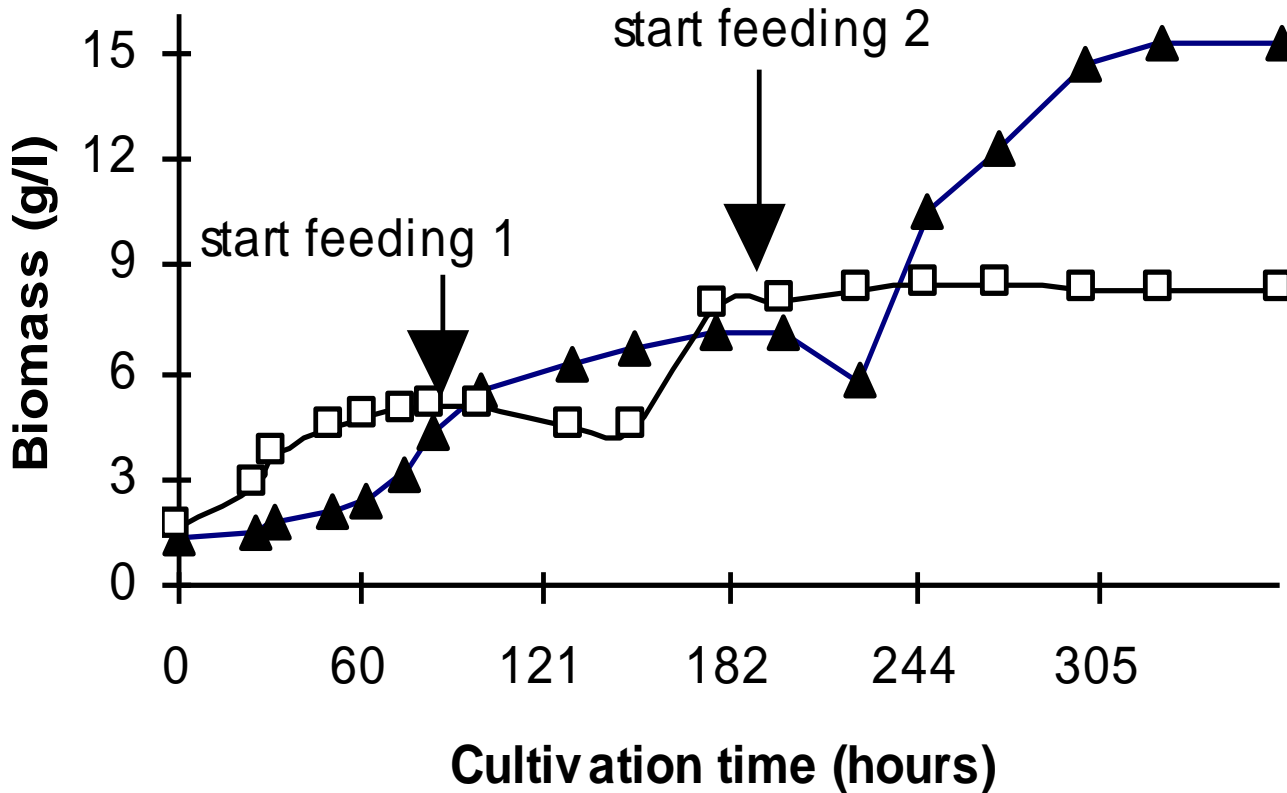


Influence of inoculum concentration on *Ganoderma lucidum* biomass production in batch cultivation, ($T = 30^{\circ}\text{C}$, $N = 300 \text{ min}^{-1}$, $Q_g = 10 \text{ L min}^{-1}$)

▲ 14 % inoculum ■ 17 % inoculum ● 20 % inoculum



Ganoderma lucidum submerged cultivation



Ganoderma lucidum fed batch cultivation. Time course of biomass accumulation
□ feeding at 90 hours □ feeding at 200 hours
($T = 30^{\circ}\text{C}$, $N = 300 \text{ min}^{-1}$, $Q_g = 10 \text{ L min}^{-1}$)

Grifola frondosa submerged cultivation

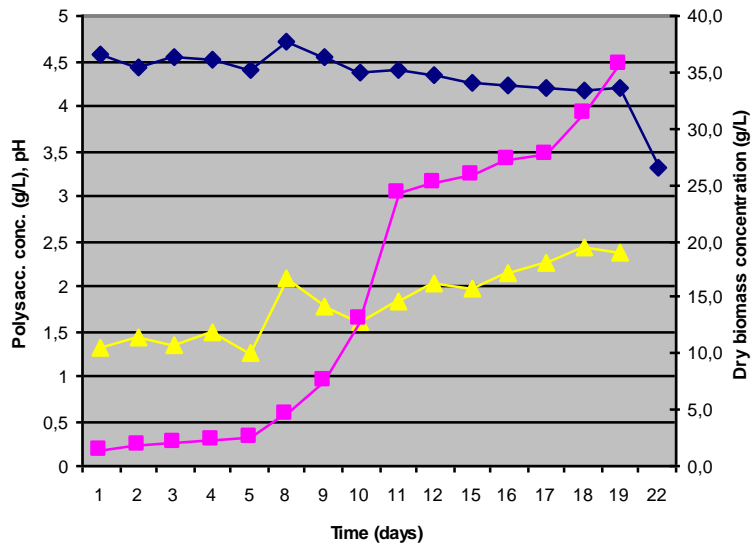


Grifola frondosa submerged cultivation

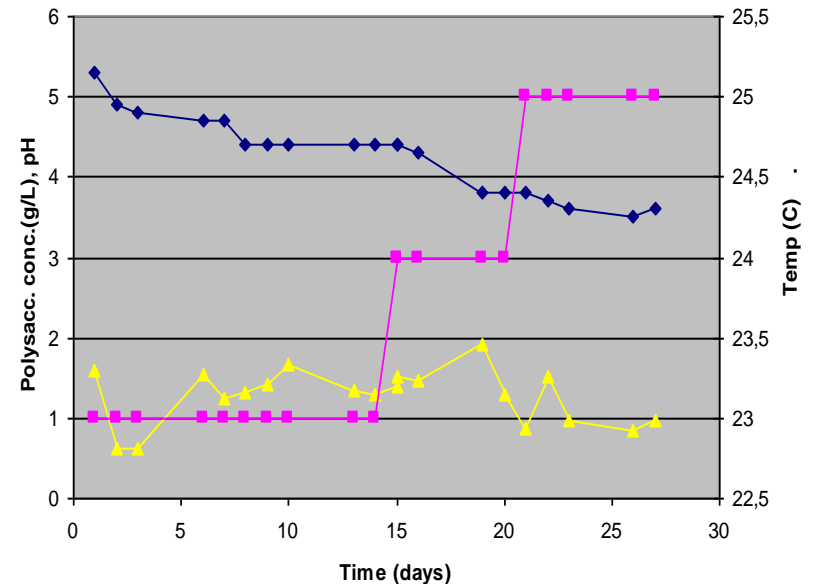


Tandem Connected Stirred tank reactors
5 and 25 l

Grifola frondosa submerged cultivation



Buble Column



Fixed Bed Column

Production of intra- and extracellular polysaccharides of *Grifola frondosa* by submerged cultivation



G. frondosa biomass and polysaccharides were produced by submerged cultivation in a liquid medium a 10-L bioreactor.

Slovenian isolate of *G. frondosa* (GF3), Fungal bank of the Biotechnical faculty, Department of Wood Science and Technology, University of Ljubljana, Slovenia) was used in all experiments. In all of the experiments, *G. frondosa* mycelium was cultivated in a liquid medium - in erlenmayer flasks and in a 10-L mixing bioreactor.

The liquid substrate consisted of glucose as a source of carbon, yeast extract and polypeptone as sources of nitrogen. Optimal growth conditions in a bioreactor were : temperature 28 °C, air flow 5 L/min, mixing speed 220 rpm. Mycelium was growing in the form of pellets from which intracellular polysaccharides were isolated.

After 30 days of cultivation, in shaken cultures 26 g/L of fresh biomass or 12 g/L dry biomass was obtained. The amount of total polysaccharides was 0.5 g/L of liquid medium.

After 60 days of fed batch cultivation in the stirred tank reactor, the amount of fresh biomass was 32.8 g/L and dry biomass 18 g/L. 0.36 g/L of dry extracellular polysaccharides were isolated from the mycelium and 0.13 g/L of dry intracellular polysaccharides.

Isolated polysaccharides were separated by ion-exchange chromatography, gel filtration and affinity chromatography. 5 fractions of extracellular β -D-glucanes were obtained, with total mass of 6.7 mg (40.1 μ g/g dry biomass or 4.0 μ g/L liquid substrate) and 2 fractions of intracellular β -D-glucanes with total mass of of 4.9 mg (29.3 μ g/g dry biomass or 0.5 μ g/L liquid substrate).

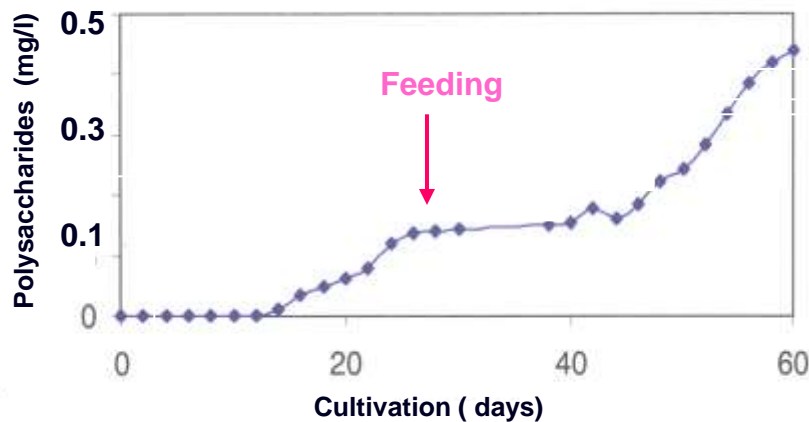


Fig. 1 Production of total fungal polysaccharides

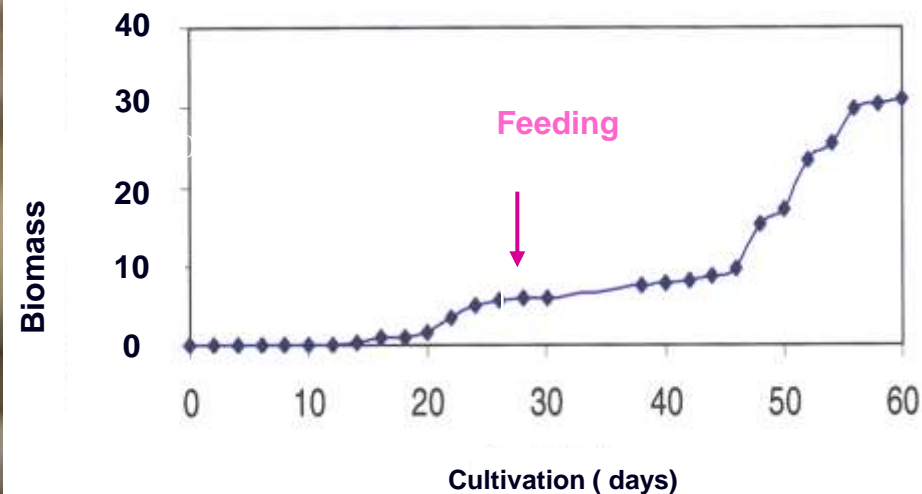


Fig. 2 Production of fungal biomass in stirred tank reactor



Bubble Colum Reactor



Yields (%)

Oil (%)	Biomass Biomass	Extracellular polysaccharides	Intracellular polysaccharides
0,0	9,798	0,826	1,883
0,25	12,571	0,902	2,305
0,5	11,242	1,124	1,707
1,0	9,995	0,804	2,151

Trametes versicolor

Polysaccharides

Potato dextrose	30 g/L
Yeast extract	6 g/L
Polypeptone	2 g/L
$\text{KH}_2\text{PO}_4 \cdot 3\text{H}_2\text{O}$	0,6 g/L
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0,5 g/L
$\text{MnSO}_4 \cdot 1\text{H}_2\text{O}$	0,2 g/L

Triterpenoides



Stirred Tank Reactor



Bubble Column



Stirred Tank Reactor

Hericium erinaceus cultivation



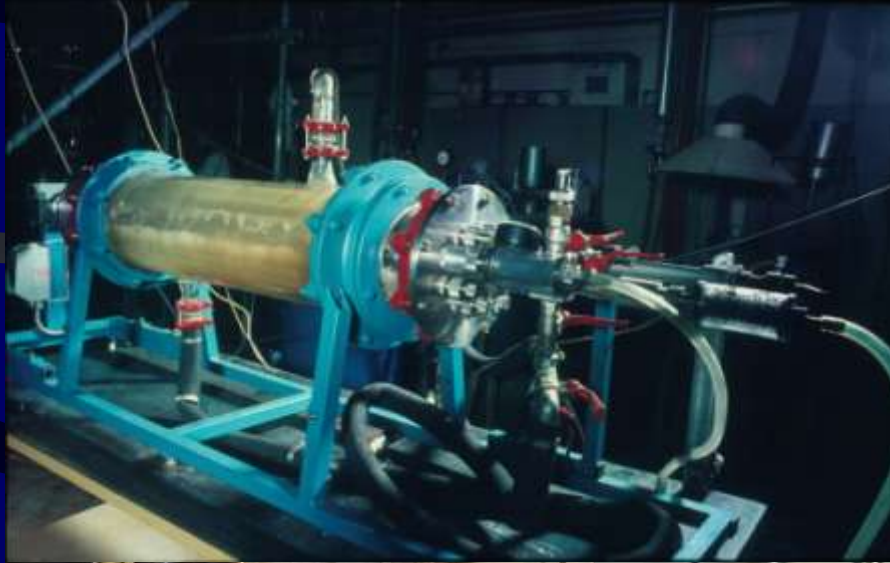
Stirred Tank Reactor

Solid state cultivation



Horizontal Stirred Tank Reactor

BIOREACTORS





Ganoderma lucidum



Mycelial growth after 6 weeks

Janic & M. Berovic

Ganoderma lucidum



I. Habijanac & M. Berovic

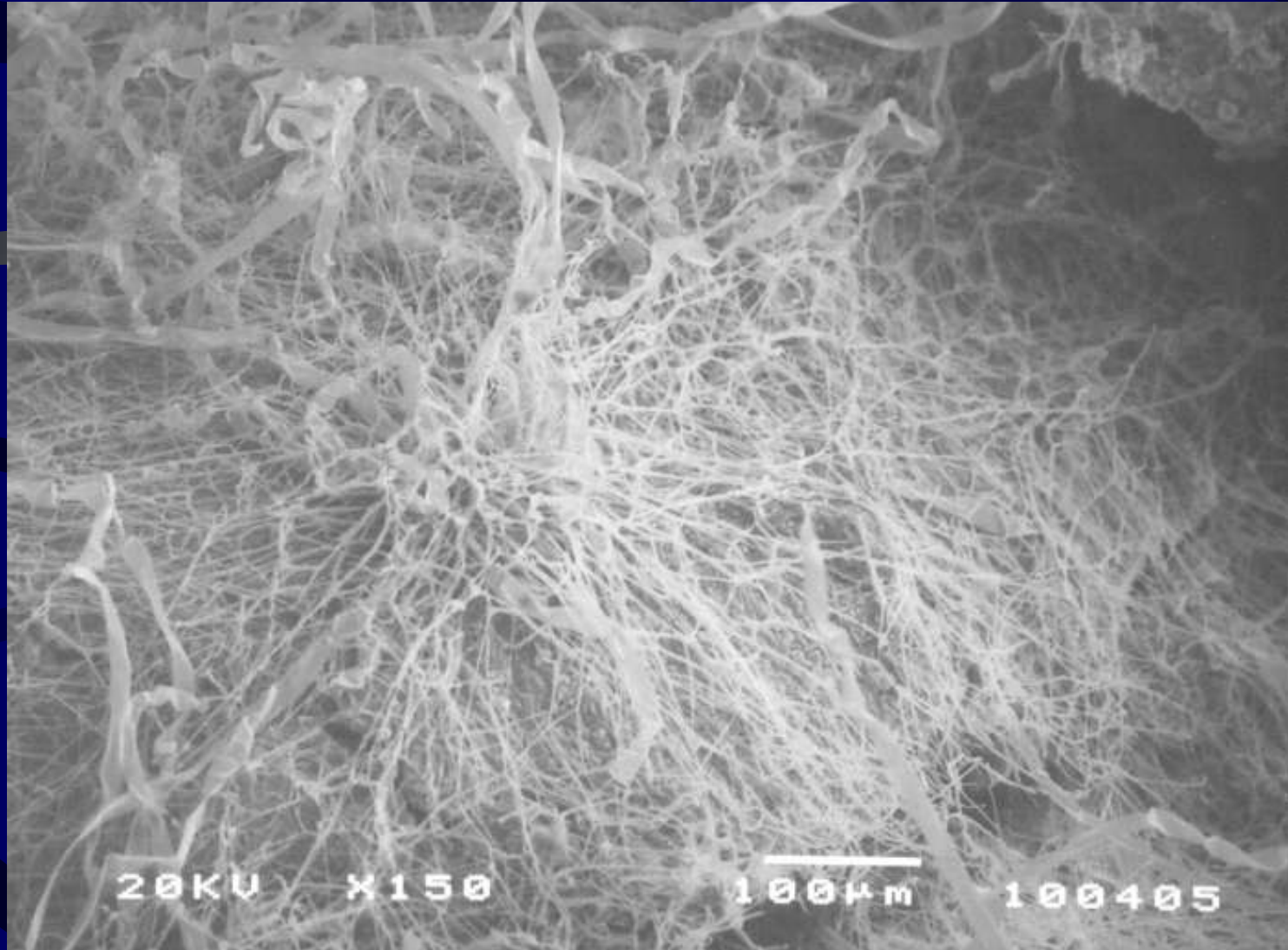
Mycelial growth after 6 weeks

Ganoderma lucidum



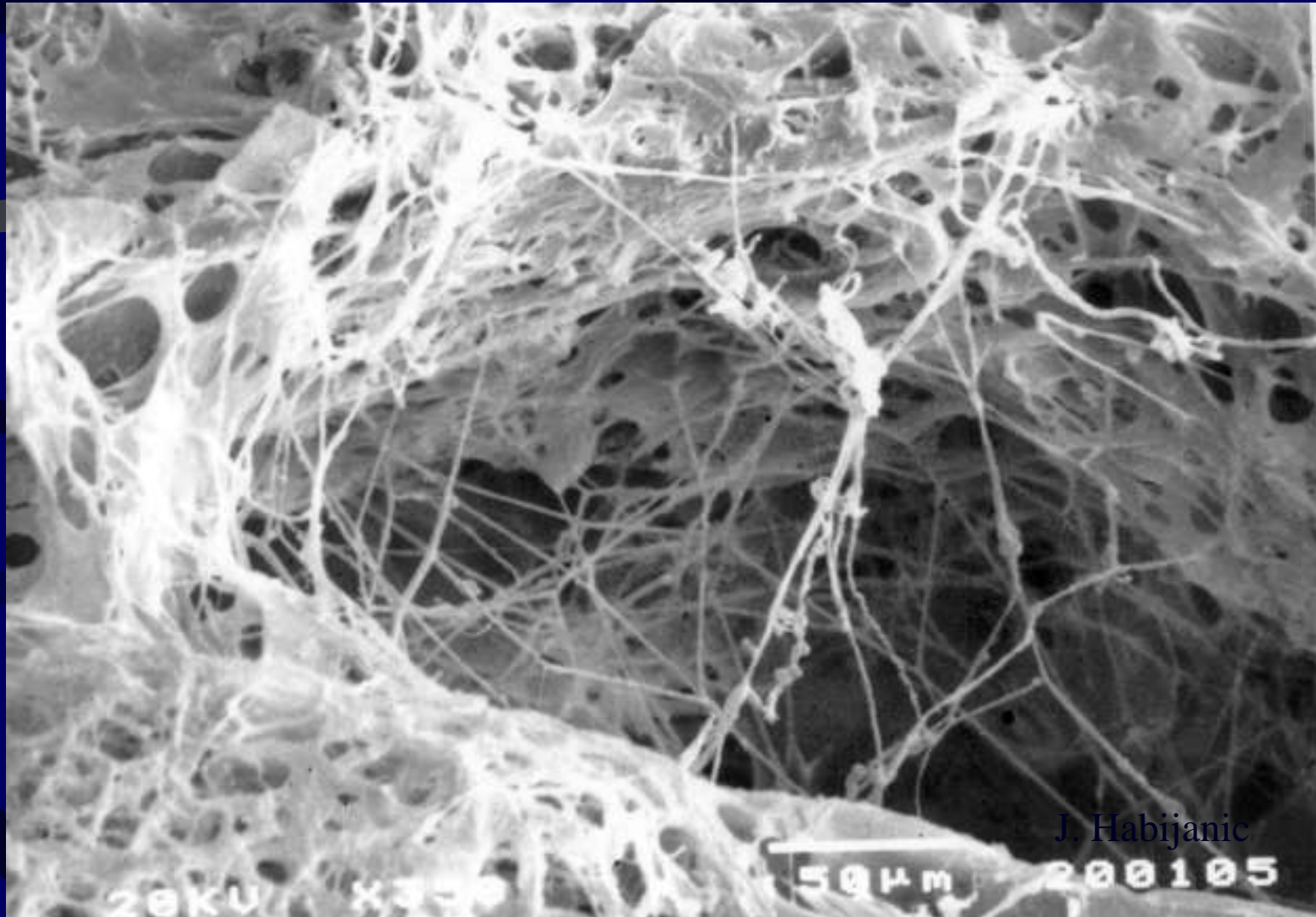
Mycelial growth after 6 weeks

GROWTH IN SSF



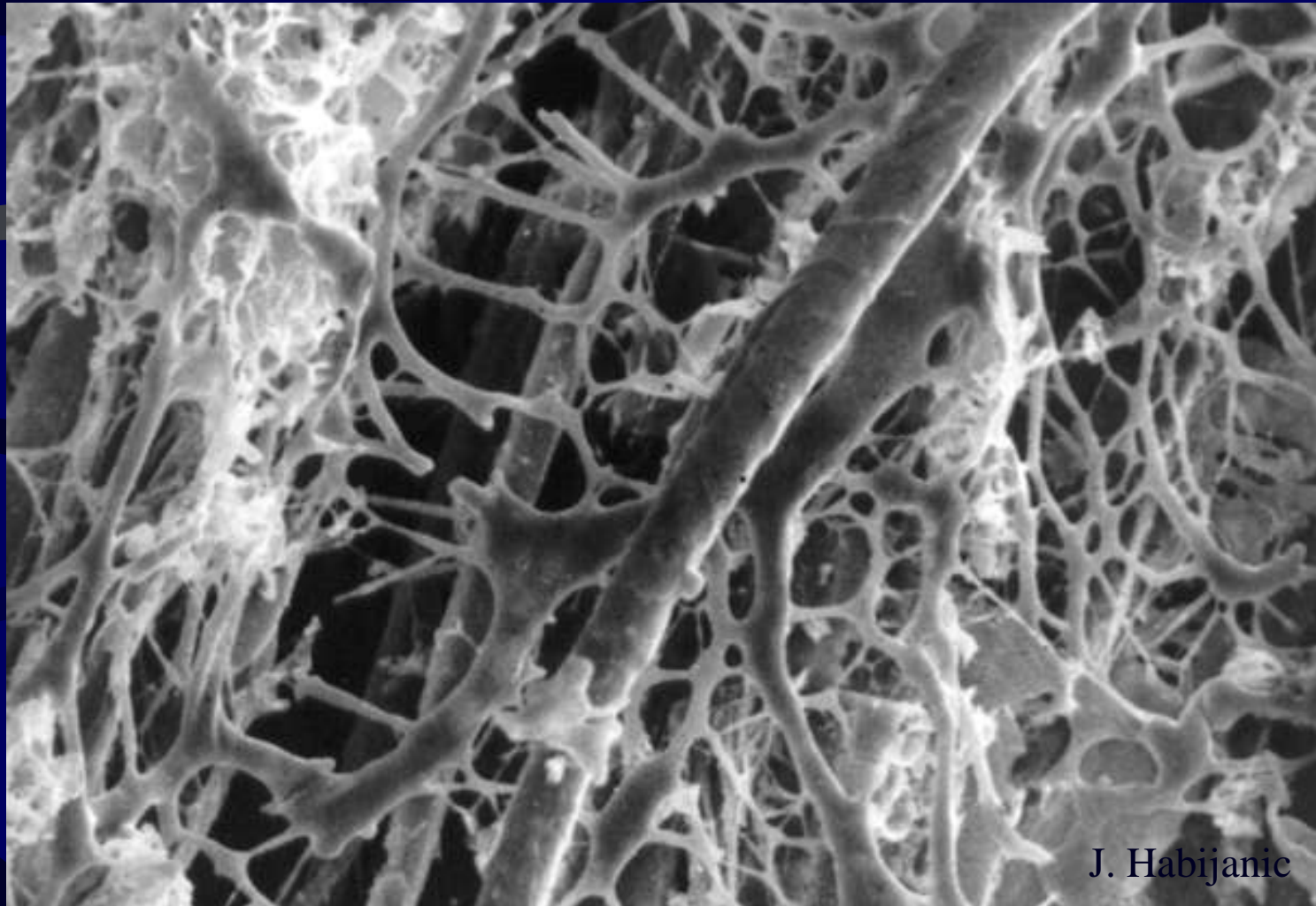
Mycelial growth on solid substrate after 7 days of cultivation (350x)

GROWTH IN SSF



Mycelial growth on solid substrate after 12 days of cultivation (350x)

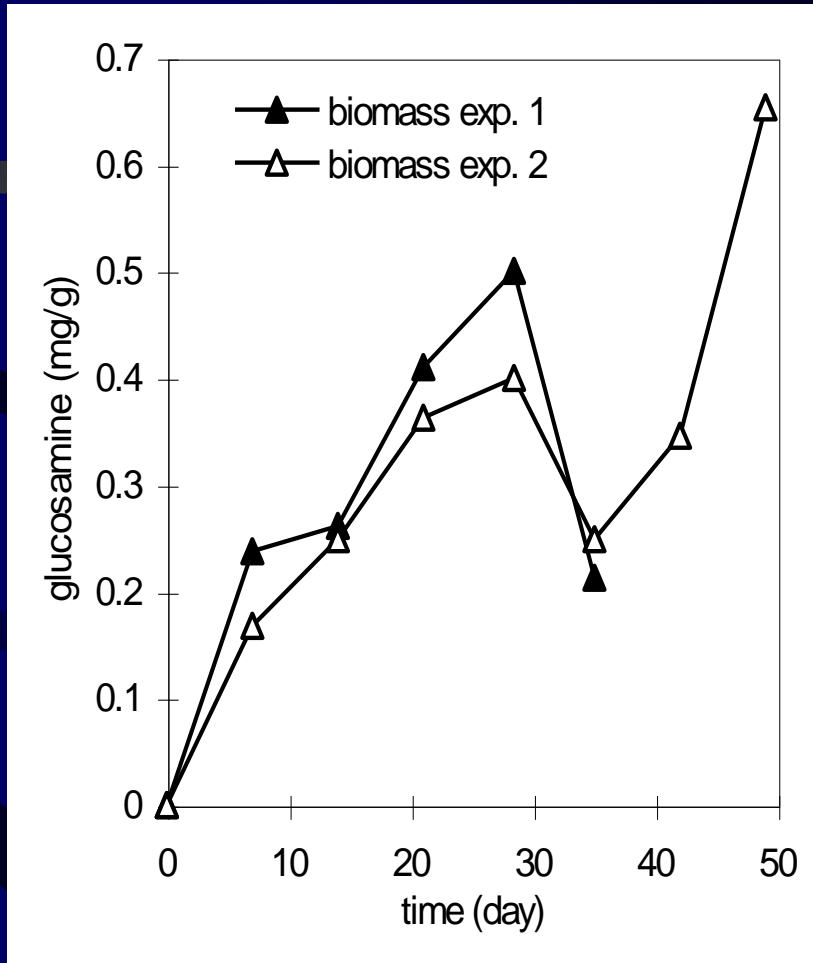
GROWTH IN SSF



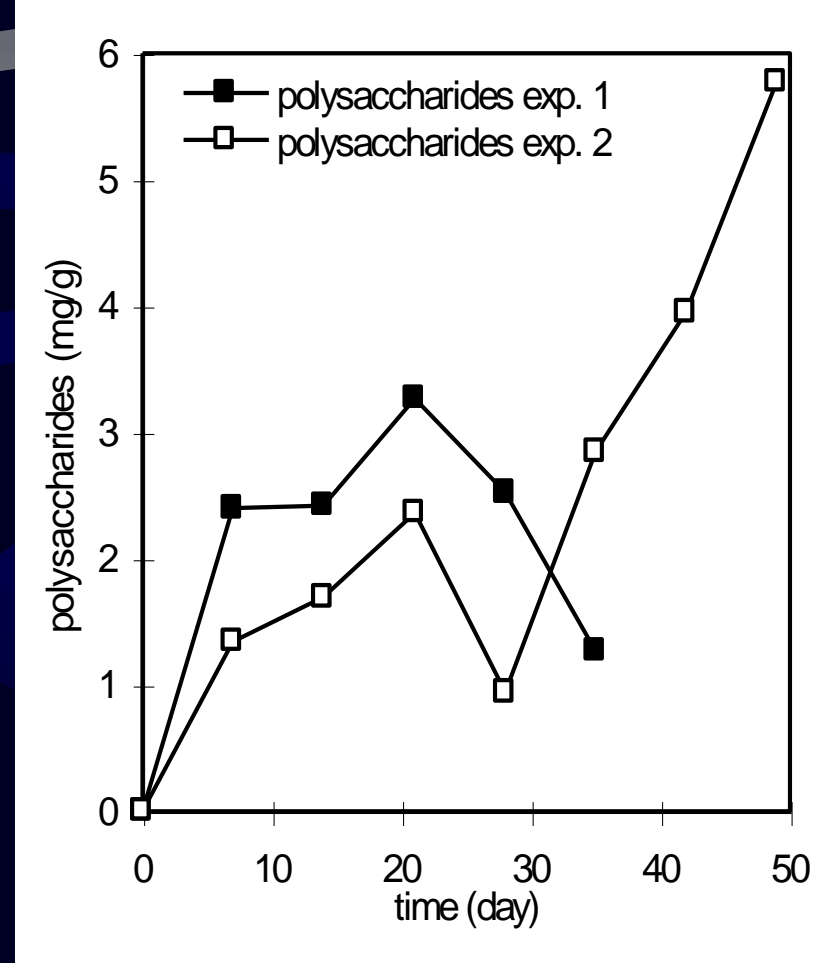
Mycelial growth on solid substrate after 14 days of cultivation (350x)



Ganoderma lucidum solid state experiments



Biomass growth during both experiments



Polysaccharide production during both experiments

Grifola frondosa solid state cultivation



Corn straw substrate

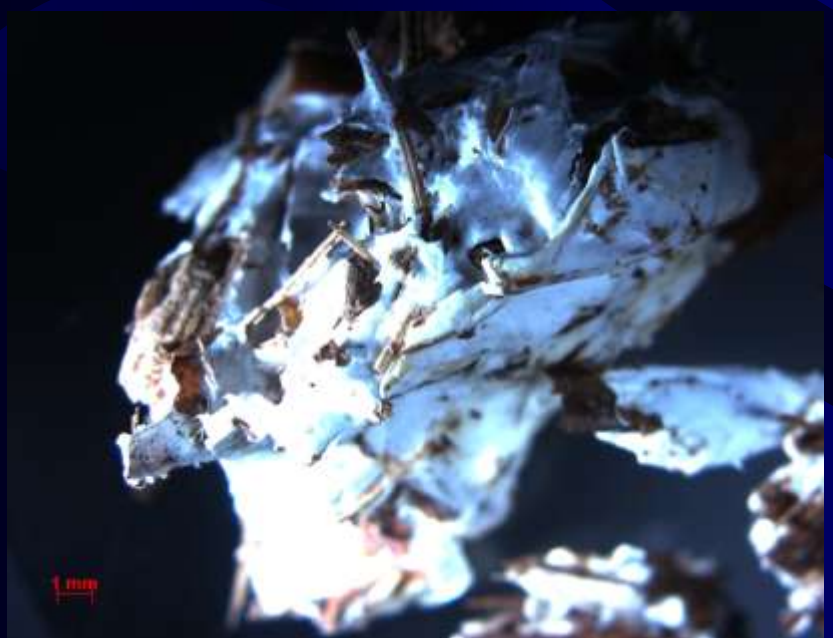
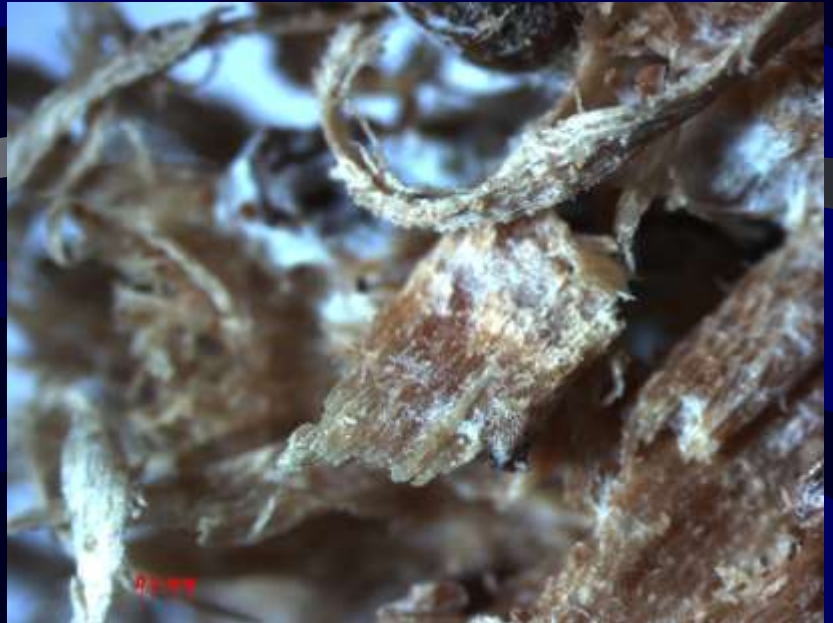
Grifola frondosa

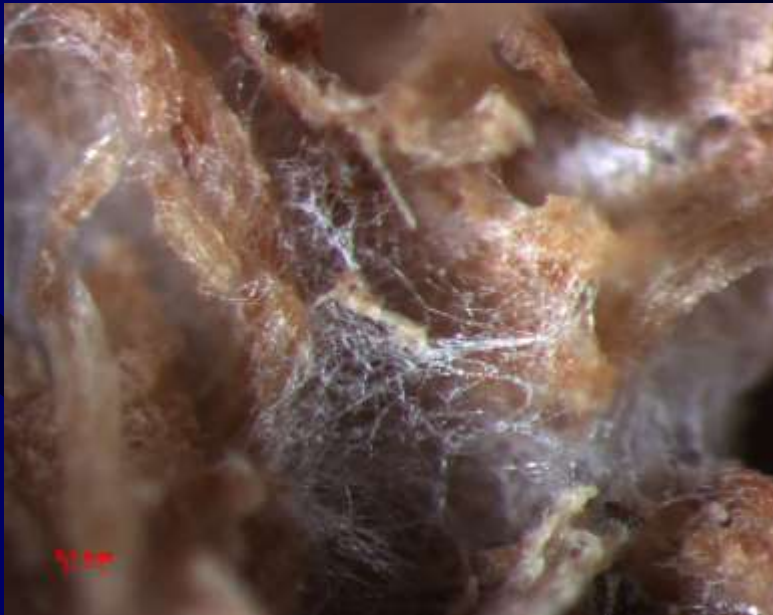
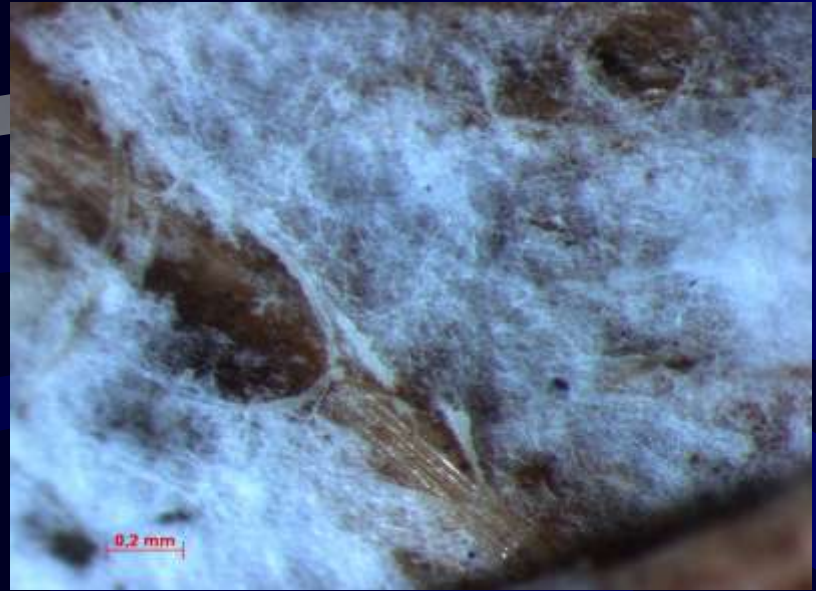


Horizontal Stirred Tank Reactor

Solid state cultivation of *Grifola frondosa* (Dicks: Fr) S.F. Gray biomass and production of intra- and extracellular polysaccharides







Production of intra- and extracellular polysaccharides of *Grifola frondosa* by solid state cultivation

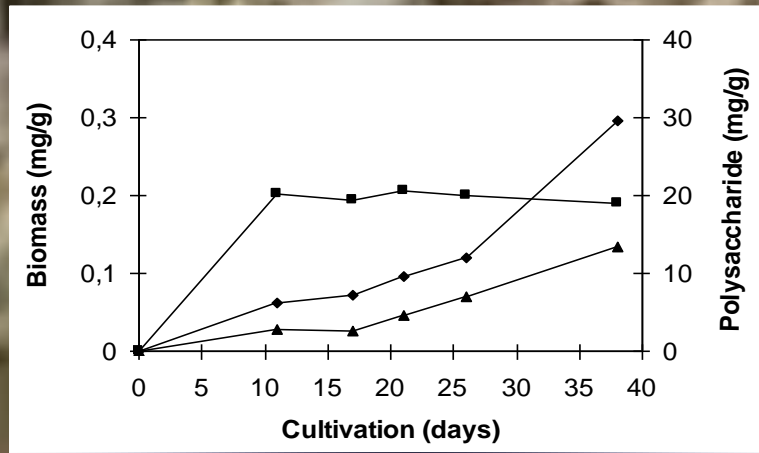
MATERIAL AND METHODS

Solid substrate mixture consisted of 1000 g of milled whole corn plant (*Zea mays*) 50 mg $(\text{NH}_4)_2\text{SO}_4$, 200 mg KH_2PO_4 , 50 mg $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 50 mg $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 150 mg $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 2 g CaSO_4 , 20 ml olive oil, 0,5 L distilled water, and (optionally) 500 g of olive press cake.

Experiments were carried out in a horizontal stirred tank bioreactor. Optimal cultivation conditions were: temperature 28°C, airflow 5 L per minute, periodical mixing after 14 days of cultivation, 1 minute, 80 rpm. Optimal moisture content for biomass growth and polysaccharide production was 60 %, the critical lowest moisture content point was 45 %, where the production of biomass and polysaccharides stopped.

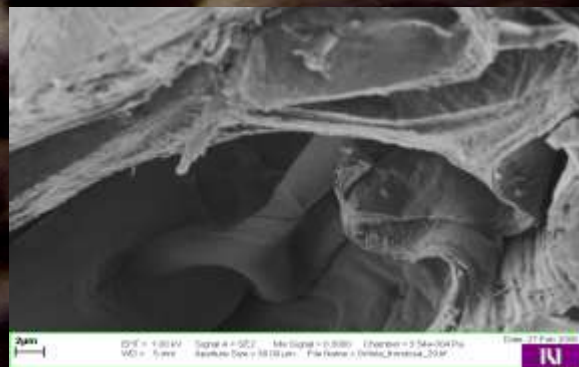
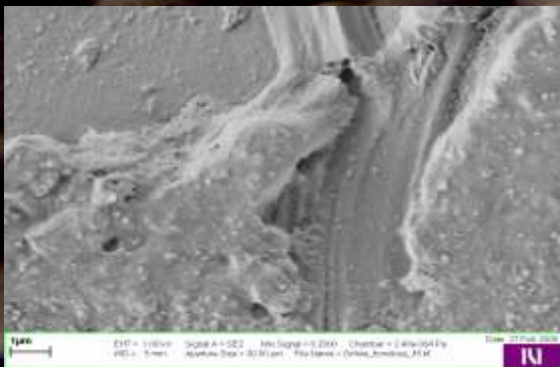
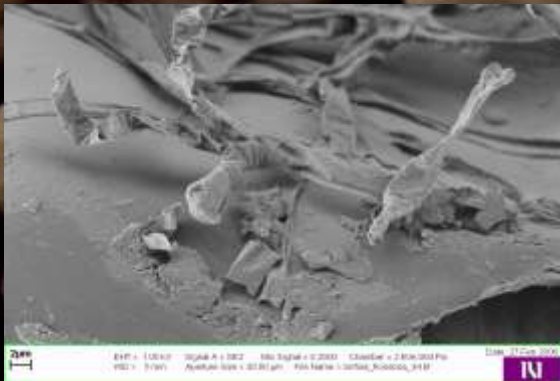
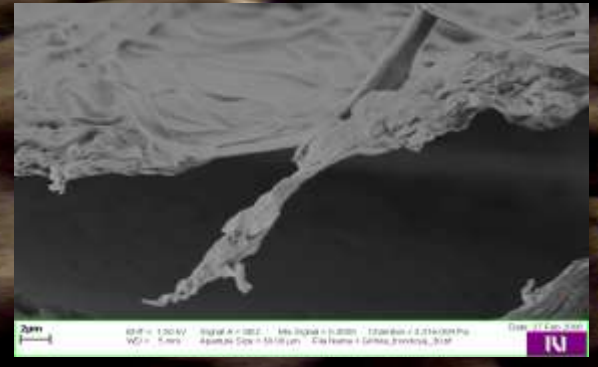
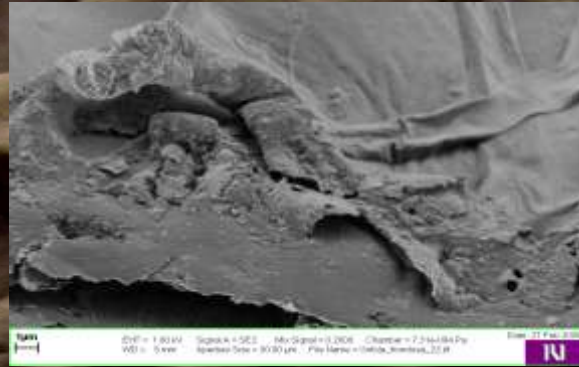
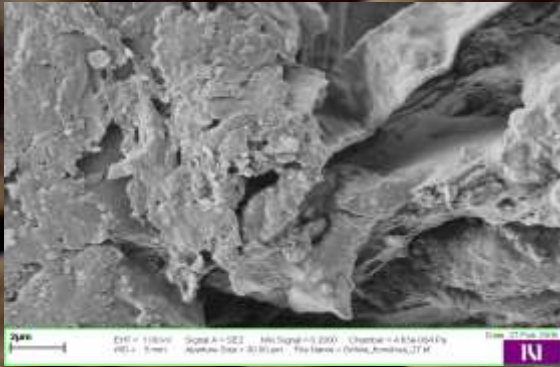
0.1 g of dry intracellular polysaccharides and 0.31 g of dry extracellular polysaccharides were isolated from 1g of dry mycelium. Crude polysaccharides were dialyzed and further separated by ion-exchange chromatography on DEAE cellulose, gel chromatography on Sepharose 4B, and affinity chromatography on Concanavalin A - Sepharose 4B. Four fractions of pure intracellular β -D-glucans were isolated (total mass 47.2 mg; 89.6 $\mu\text{g/g}$ of fresh biomass) and four fractions of pure extracellular β -D-glucans were isolated (total mass 127.2 mg; 241.1 $\mu\text{g/g}$ of fresh biomass).

70.0 g of fresh biomass was produced on 1000.0 g of substrate (yield 0.07 g of fresh biomass per 1 g of substrate). 38.0 mg/g of dry intracellular polysaccharides and 78.0 mg/g of dry extracellular polysaccharides were isolated from the mycelium.



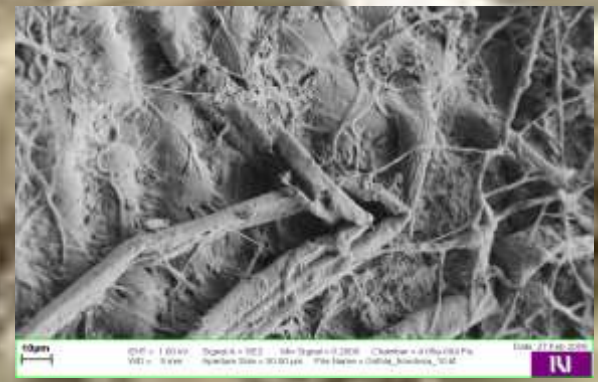
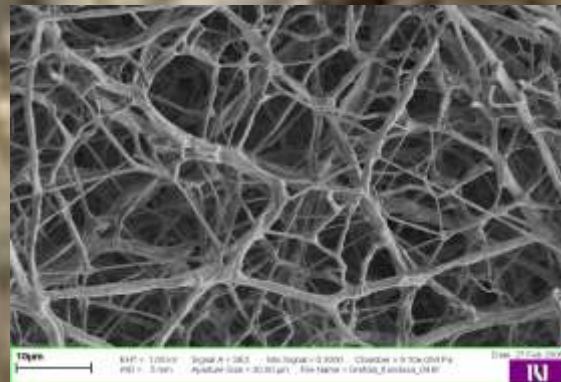
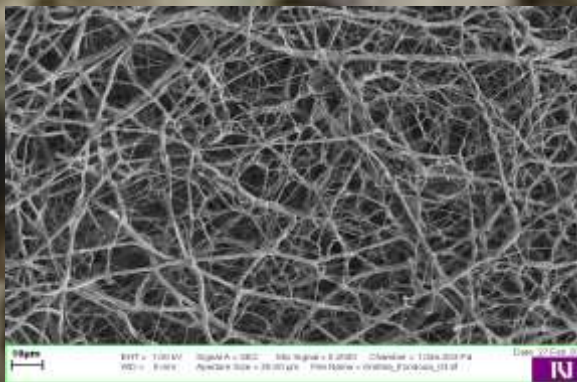
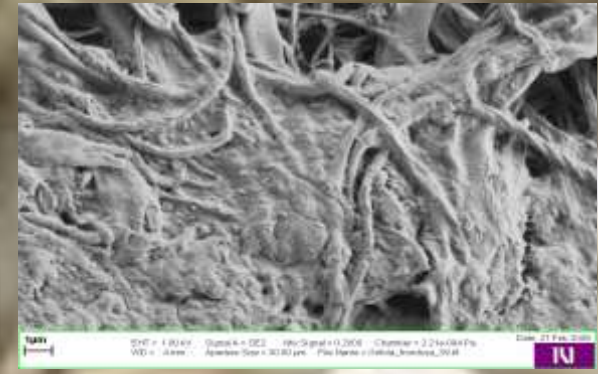
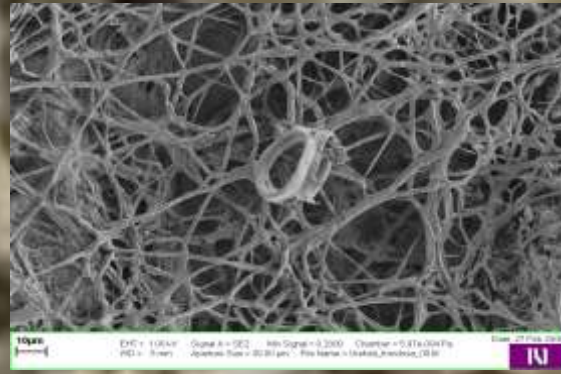
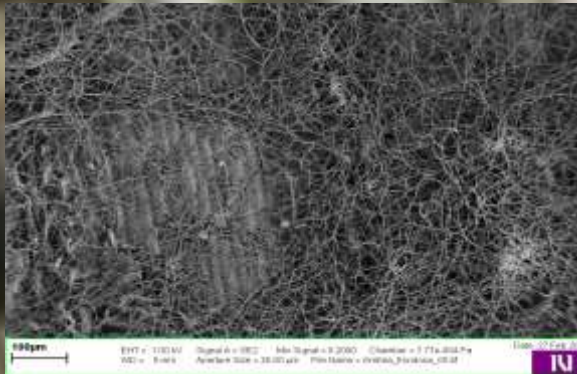
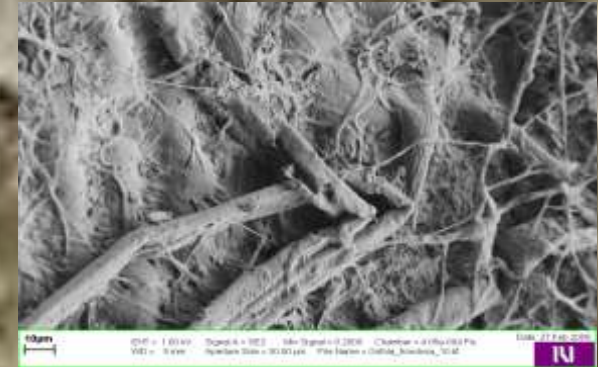
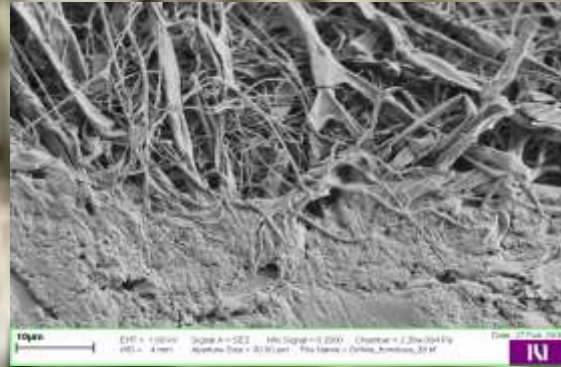
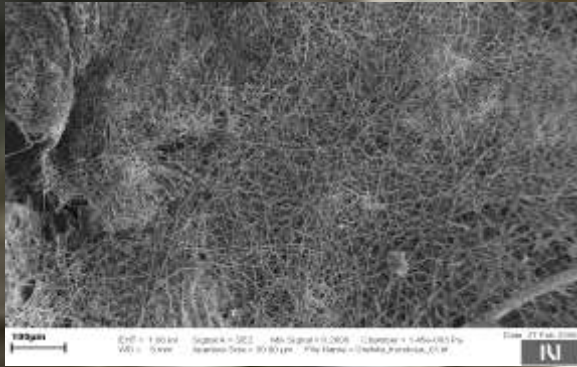
Grifola frondosa mycelium in horizontal stirred tank reactor

Grifola frondosa



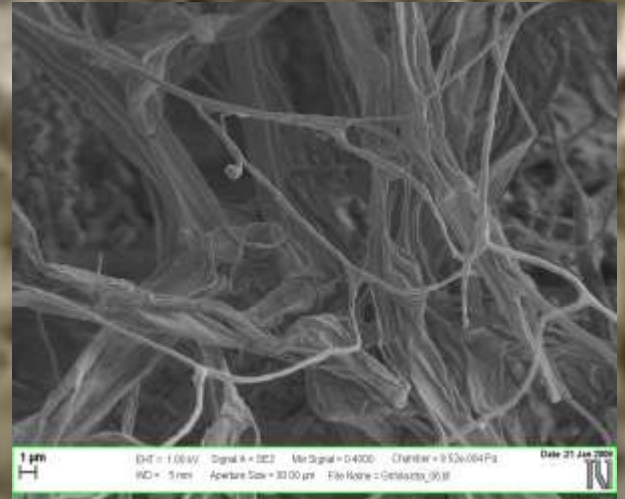
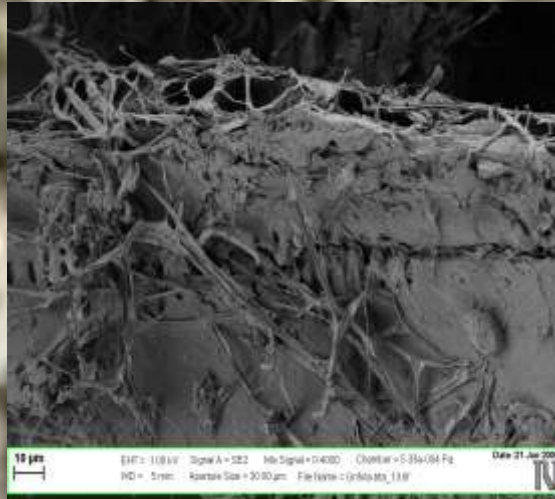
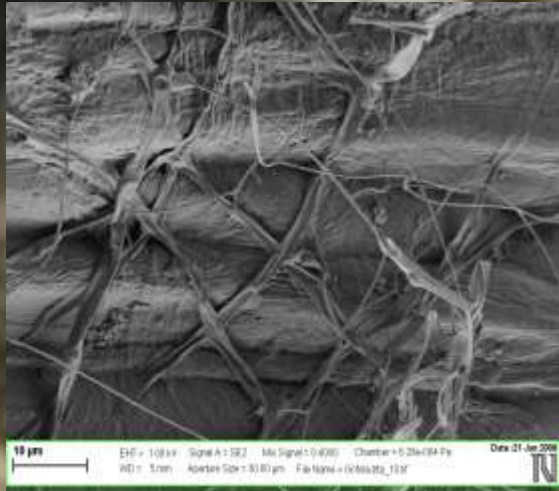
Corn cellulose

Grifola frondosa

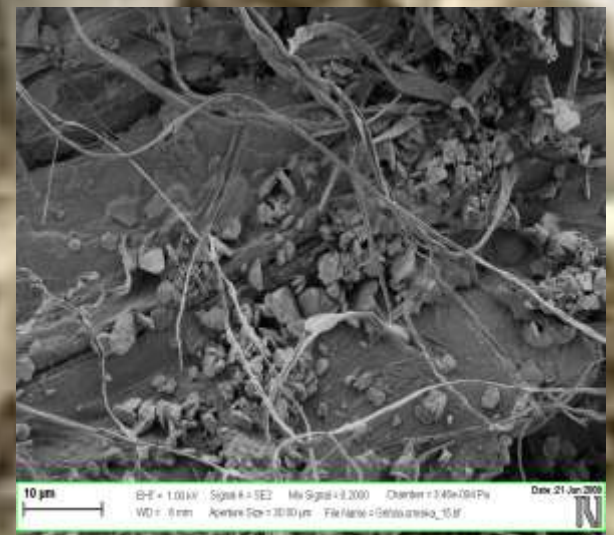
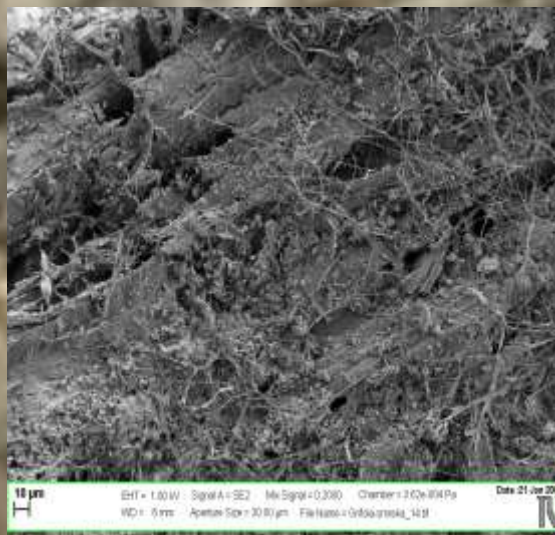


Corn cellulose

Grifola frondosa



Vine branch cellulose



Pine wood cellulose

Trametes versicolor
Solid state cultivation

Beech saw-dust

Corn straw



Medical Mushrooms Farming



Grifola frondosa farming



Submerged Cultivation *Trametes versicolor*

Potato dextrose	30 g/L
Yeast extract	6 g/L
Polypeptone	2 g/L
$\text{KH}_2\text{PO}_4 \cdot 3\text{H}_2\text{O}$	0,6 g/L
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0,5 g/L
$\text{MnSO}_4 \cdot 1\text{H}_2\text{O}$	0,2 g/L



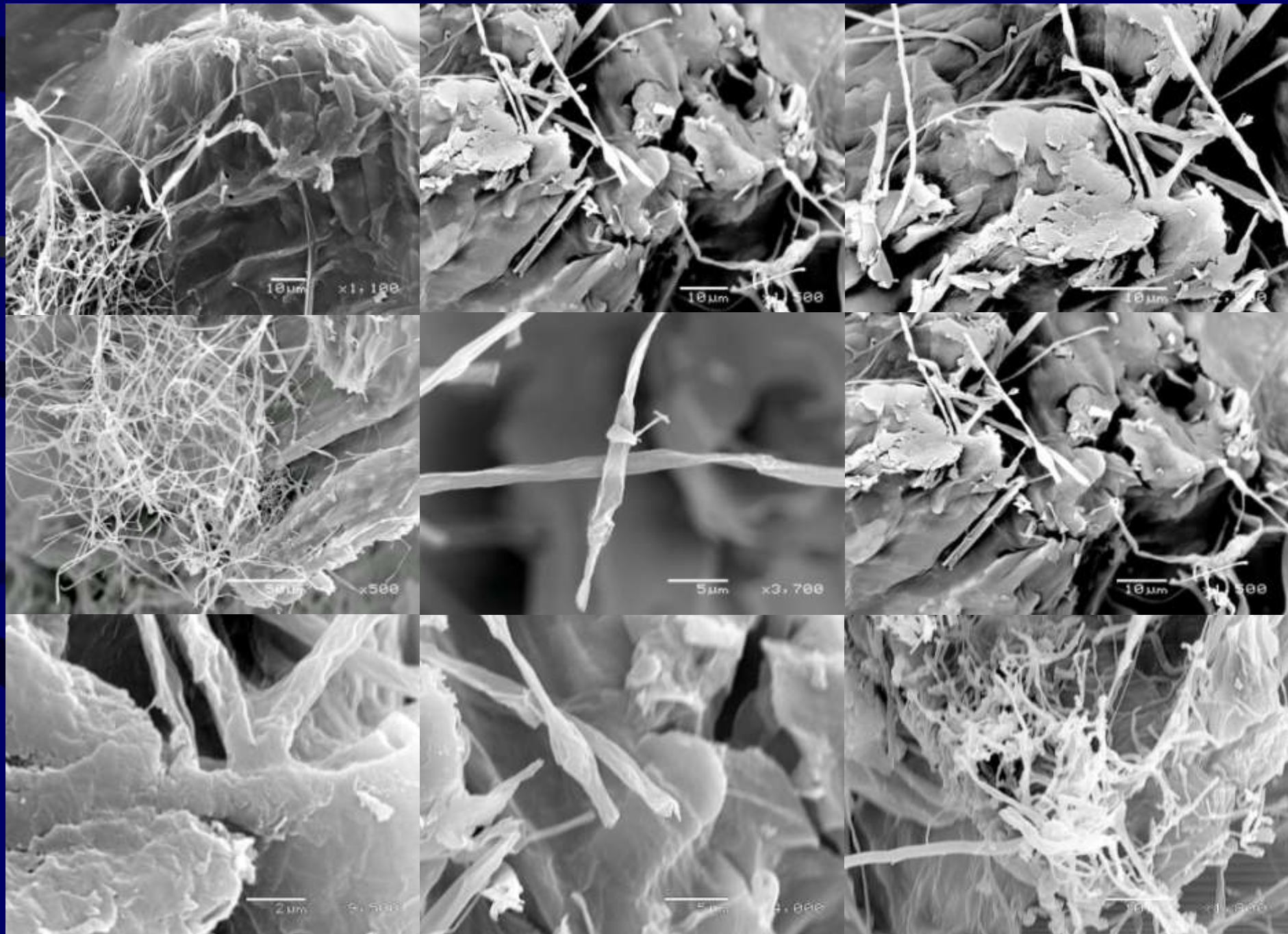
Stirred Tank Reactor

Submerged cultivation – *Hericium versicolor*

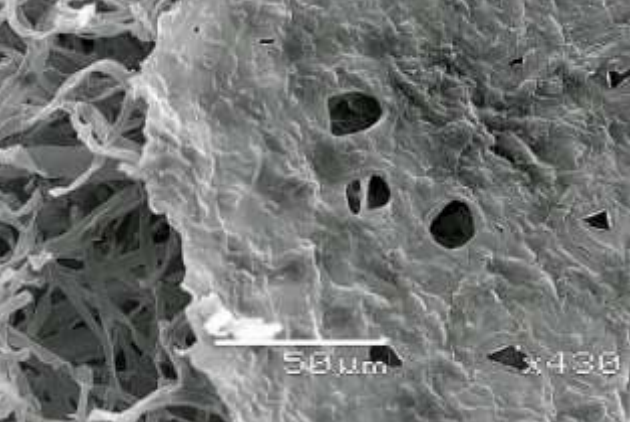
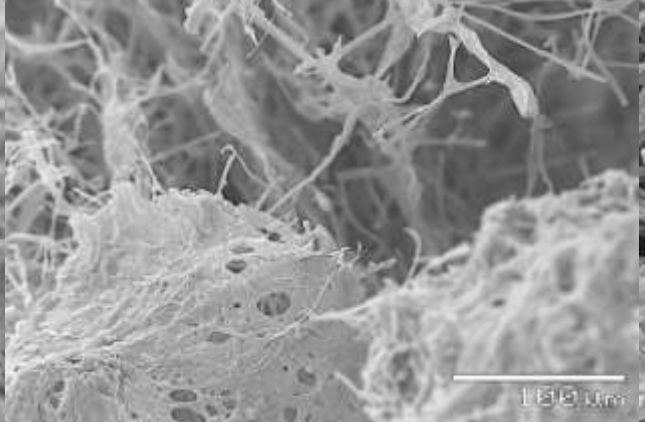
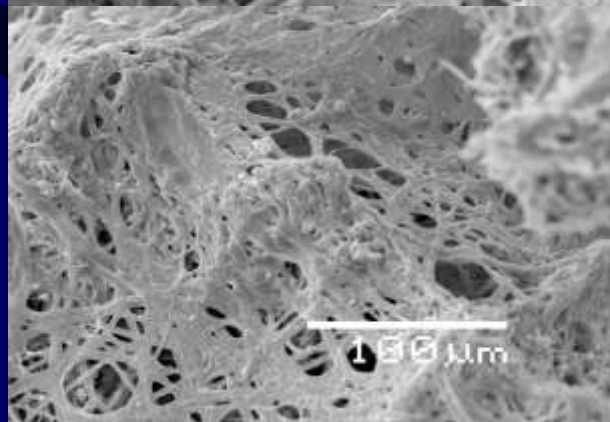
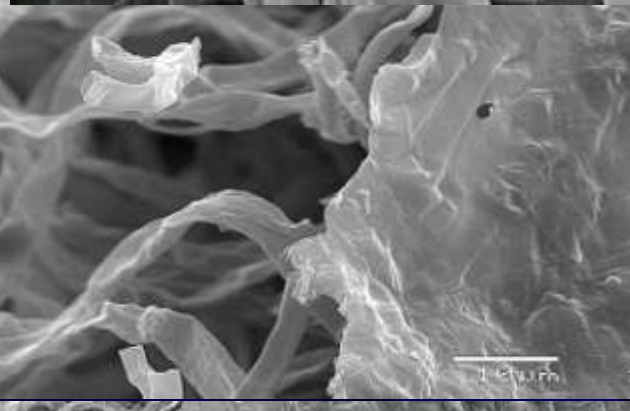
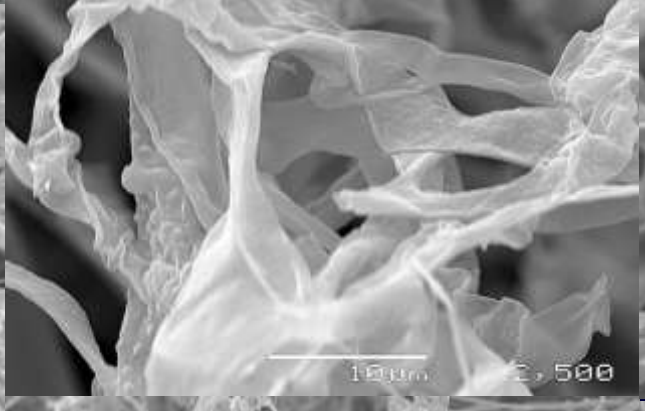
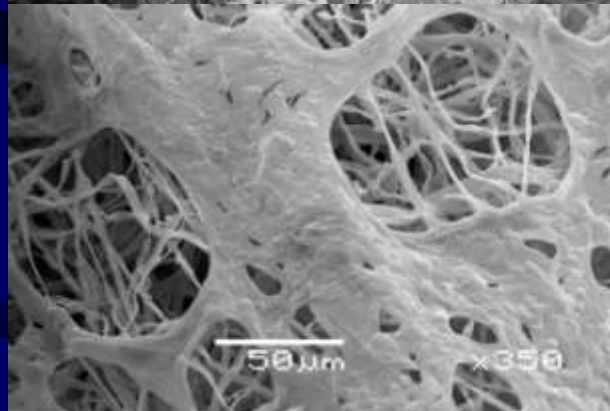
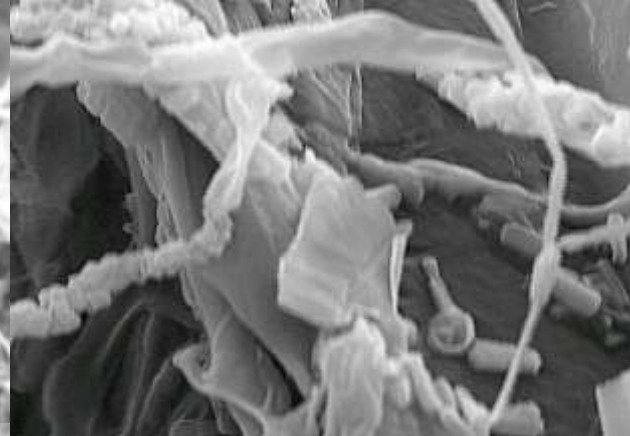
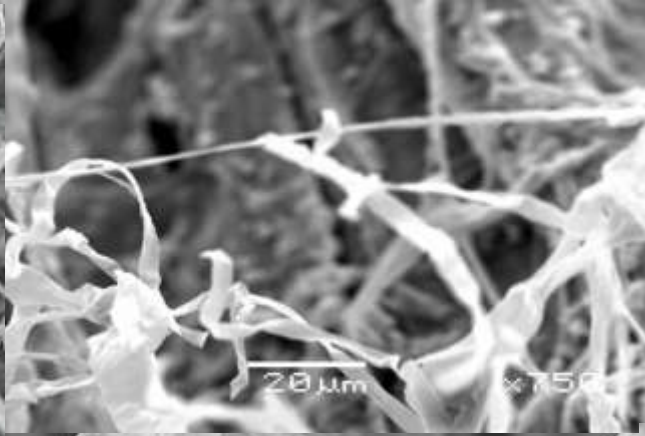
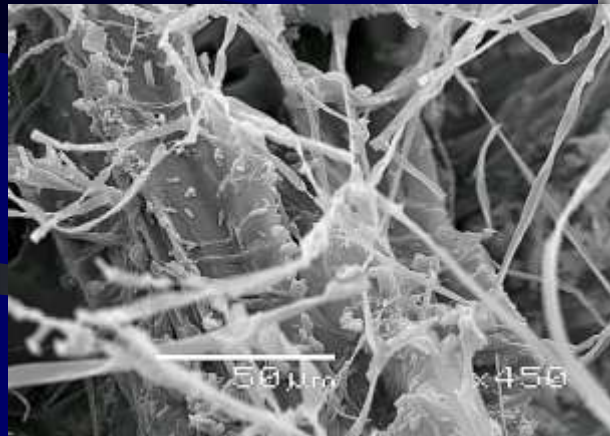
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$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0,5 g/L
$\text{MnSO}_4 \cdot 1\text{H}_2\text{O}$	0,2 g/L



Solid state cultivation *Trametes versicolor*



Solid state cultivation *Hericium erinaceum*



Grifola frondosa farming

Substrate compositions

Substrate	Strain	Beech sawdust (%)	Crushed hemp seeds (%)	Olive oil press cake (%)	Crushed corn seeds (%)	CaCO ₃ (%)	CaSO ₄ (%)	Water (%)
1	Gf1	0	0	52.5	14.8	1.6	0	31.1
2	Gf1	53.5	0	0	10.7	0	0.1	35.5
3	Gf1	31.6	31.6	0	0	0	1.3	35.5
4	Gf5	0	0	52.5	14.8	1.6	0	31.1
5	Gf5	53.5	0	0	10.7	0	0.1	35.5
6	Gf10	53.5	0	0	10.7	0	0	35.5

Grifola frondosa farming

Yields

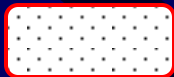
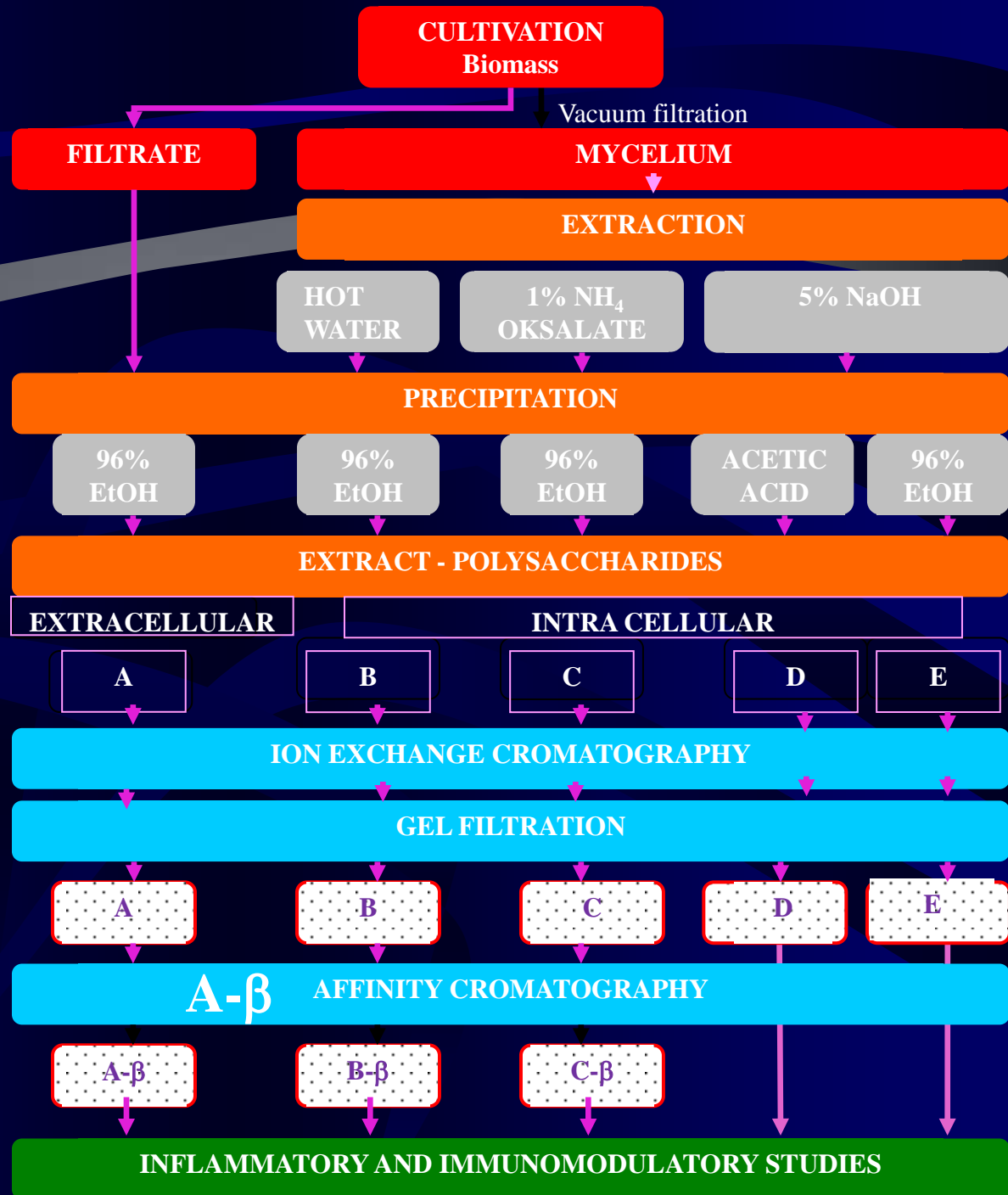
Fruiting body*	Mass of extract (g)	Yield (mg/g of dry fruiting body)	Sugar content (%)	Protein content (%)
1	0.69	50.4	70.3	13.2
2	10.9	104.8	42.3	9.5
3	1.25	63.5	39.5	7.2
4	0.71	65.7	75.9	12.4
5	0.87	47	41.9	9.1
6	0.57	44.5	41.8	9.4
7**	0.55	43.8	40.1	8.8

Grifola frondosa farming

β - glucan content

Fruiting body	Total glucan (%)	Alpha glucan (%)	β- glucan (%)
1	65.4	1.1	64.3
2	33.6	1.0	32,6
3	17.2	0.8	16.4
4	69.3	1.2	68.1
5	19.2	0.9	18.3
6	35.6	0.9	34.7
7	27.8	1.1	26.7

Study course



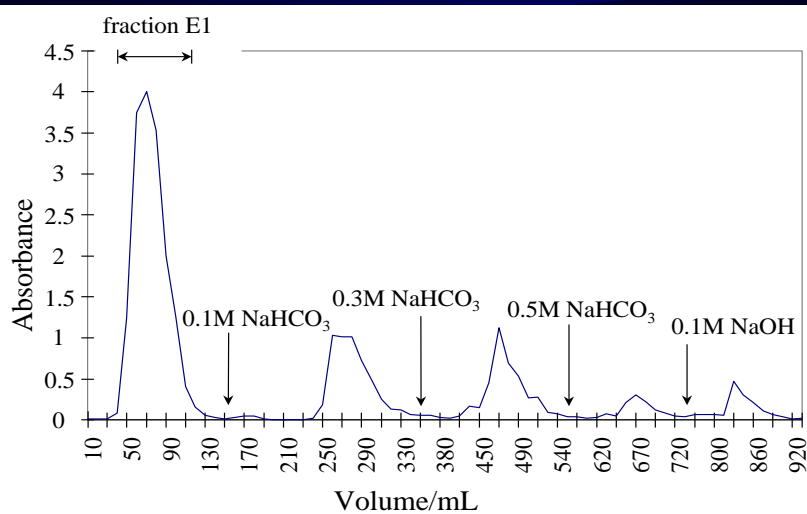
Tested in immuno-
studies



Ethanol-precipitated amorphous polysaccharides (37x)

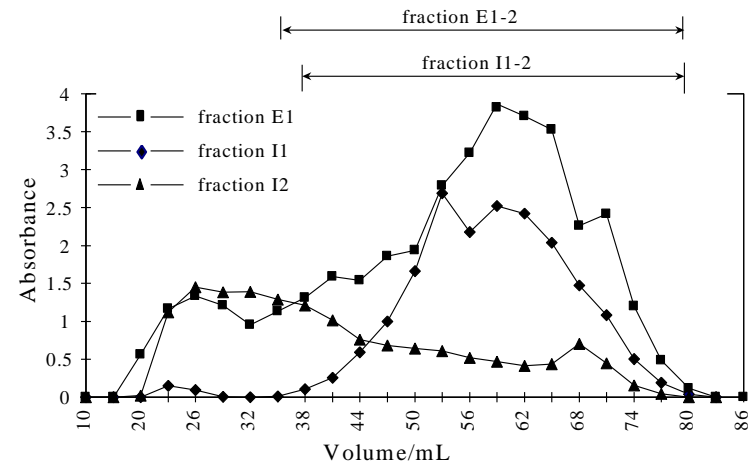
Ion-exchange chromatography

Elution chromatogram of a water soluble fraction of extracellular polysaccharides



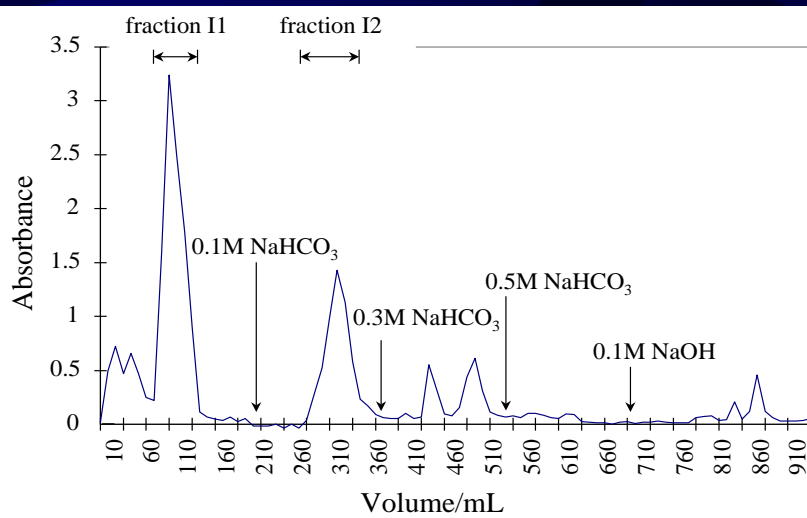
Gel filtration chromatography

elution chromatograms of extracellular polysaccharide



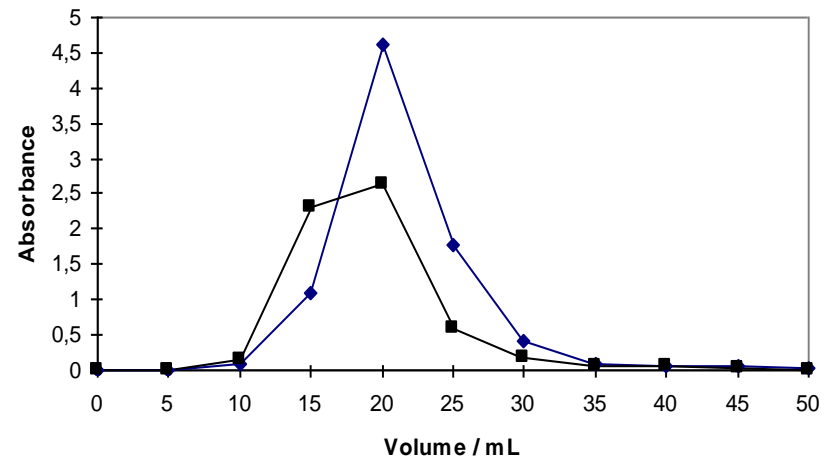
Ion-exchange chromatography

Elution chromatogram of a water soluble fraction of intracellular polysaccharides



Affinity chromatography

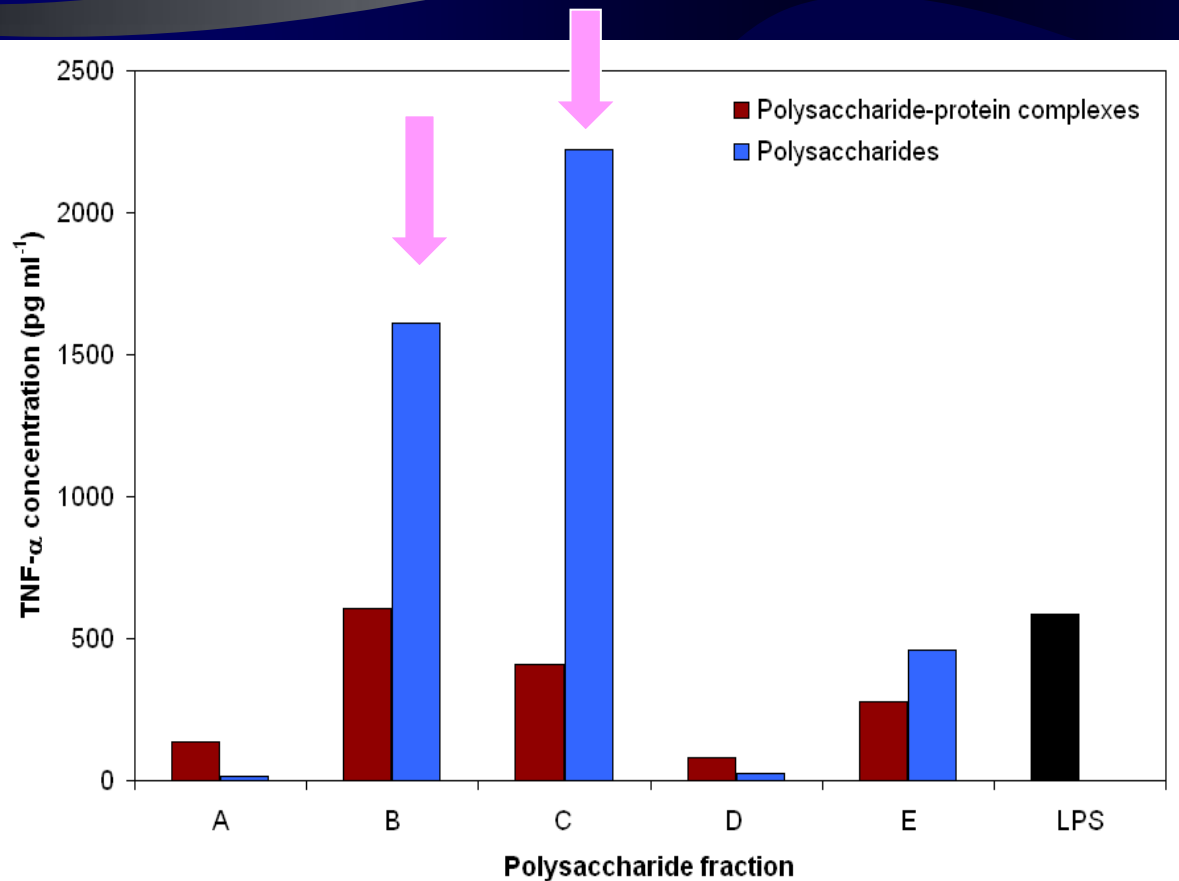
on Concanavalin A - Sepharose 4B:
chromatograms of β -polysaccharides



Inflammatory response on Polysaccharides vs. Polysaccharide-protein complexes

- The induced production of inflammatory cytokine TNF- α was evaluated and compared both for polysaccharide-protein complexes and polysaccharides.
- The comparison of the TNF- α inducing capacity of crude fractions fractionated and purified by ion-exchange chromatography and gel-filtration
- During our studies we concluded that concentration 100 $\mu\text{g/ml}$ is the most appropriate to study immune responses.
- Following the stimulation of PBMC with concentration 100 $\mu\text{g/ml}$, the supernatants were screened for the content of TNF- α after a 4h incubation.

Comparison of TNF- α inducing capacity of 5 polysaccharide and polysaccharide-protein complex fractions



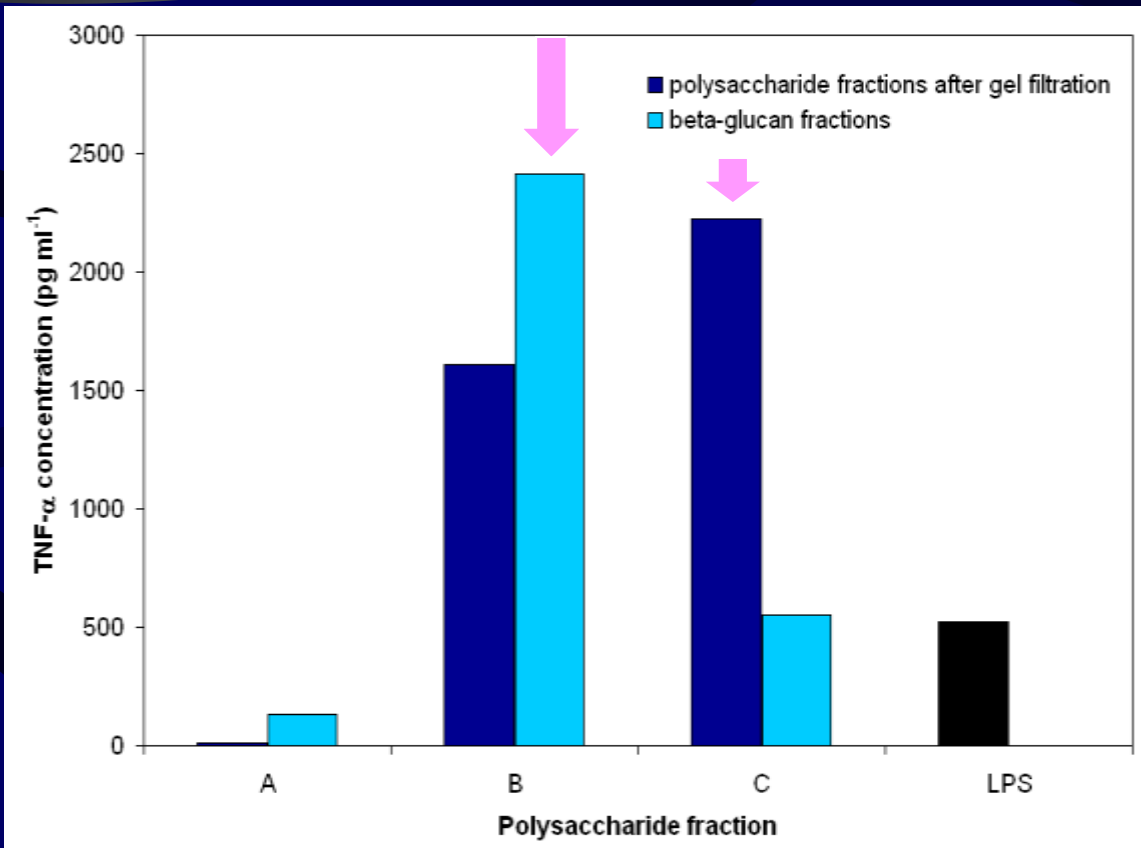
Polysaccharide fractions (without proteins) induce higher amounts of TNF- α (up to 2224 pg ml⁻¹) than polysaccharide-protein fractions.

The highest difference observed at cellular fractions C and B.

The effect of polysaccharides from *G. lucidum* on innate inflammatory response

- Since polysaccharides (without proteins) have demonstrated higher TNF- α inducing capacity versus polysaccharide-protein complexes (demonstrated in previous study) further studies have been focused on those.
- The aim of the study was:
 - to elucidate the effect of polysaccharides on primary inflammatory immune response including cytokine responses: TNF- α , IFN- γ and IL-12
 - compare the inducing capacity between polysaccharides obtained after gel filtration and pure β -glucans to study the further effect of purity.
- Extracellular (Fraction A) and two cellular polysaccharide fractions (Fractions B and C) with highest TNF- α inducing capacity (from previous study) have been studied.
- Polysaccharides have been further separated by affinity chromatography on Concanavalin A-Sepharose 4B column to obtain purified polysaccharides of β -configuration (β -glucans) and α -configuration (α -glucans). Our study was focused on β -glucan fractions.

TNF- α response



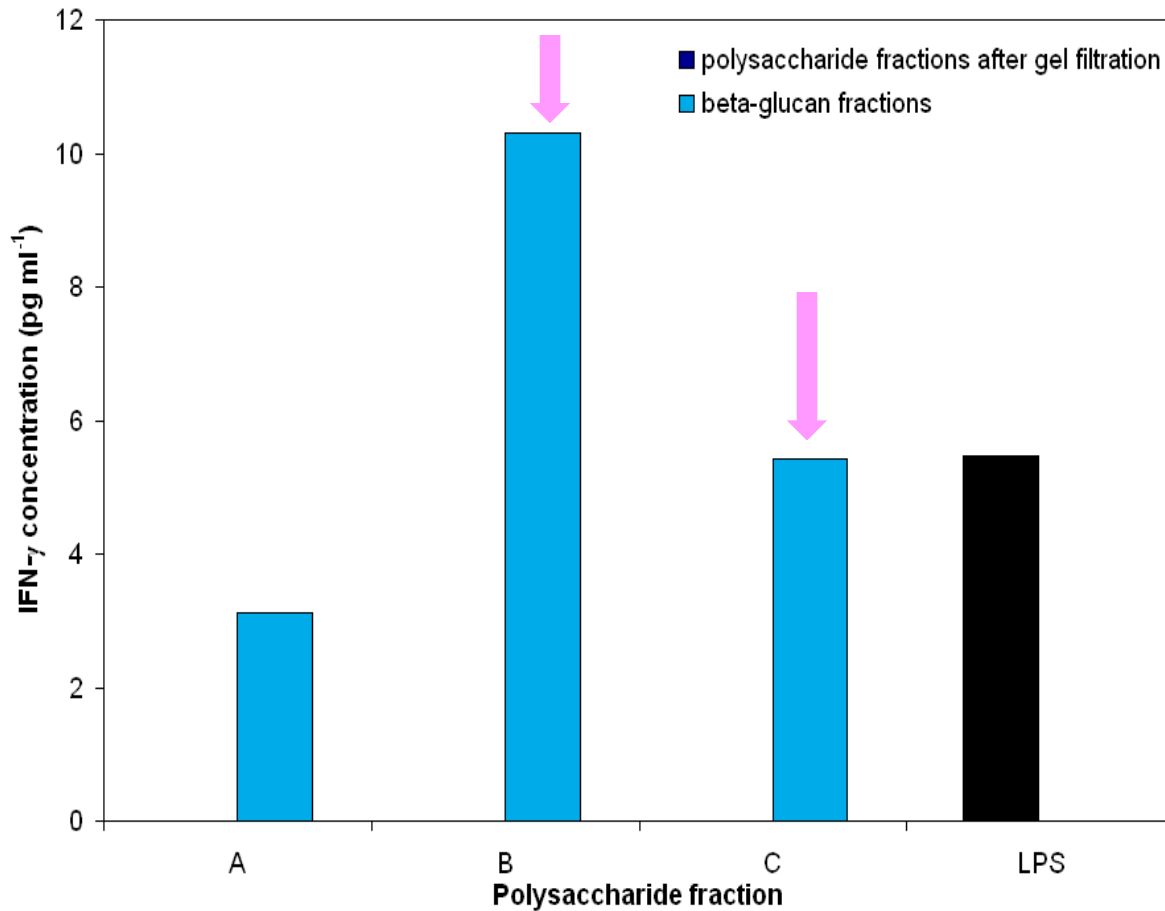
Intracellular polysaccharides

(Fraction B and C)

have significantly higher TNF- α inducing capacity than extracellular polysaccharides (Fraction A).

From all fractions the highest TNF- α inducing capacity (2413 pg ml⁻¹) is observed at Fraction B β -glucans.

IFN- γ response



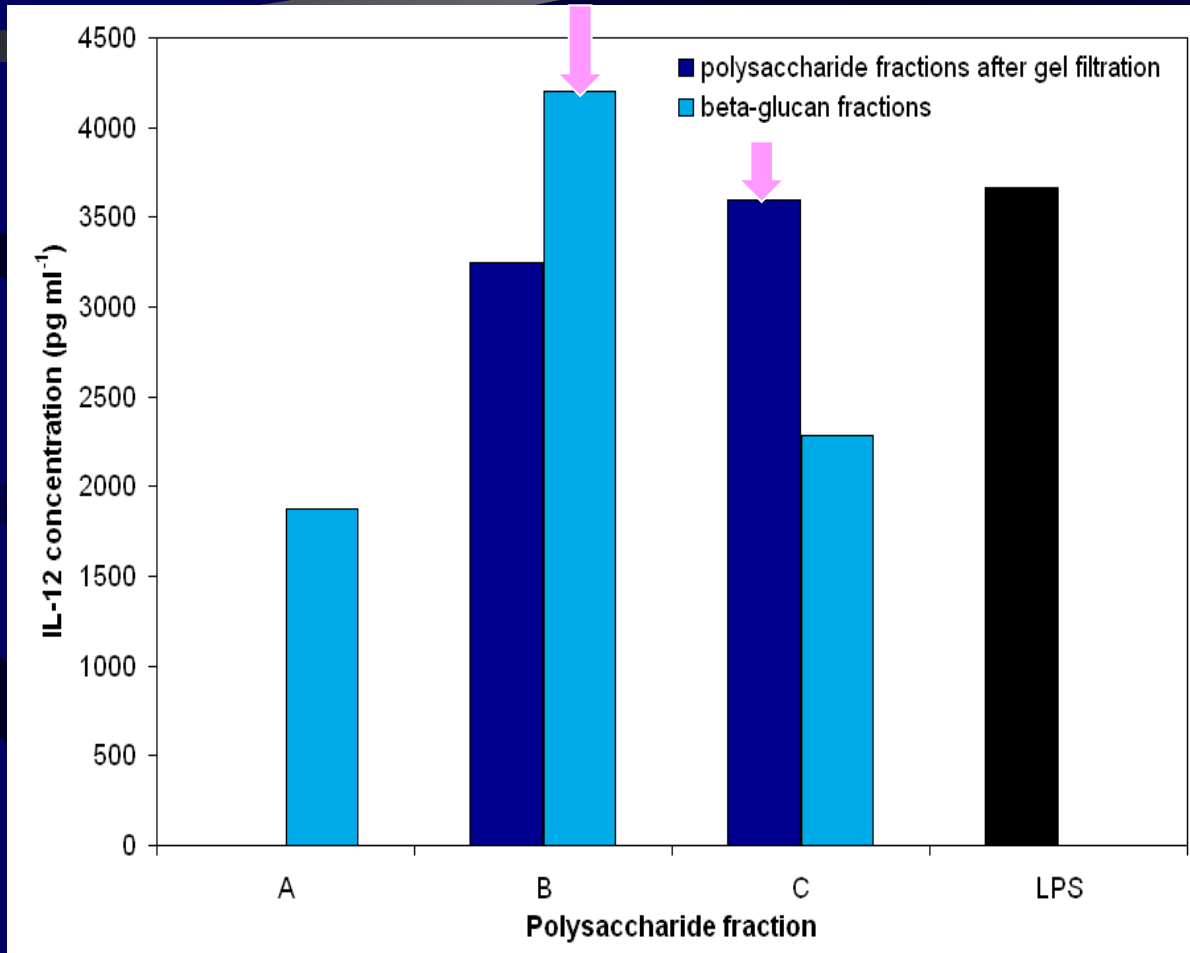
All β -glucan fractions induce very low levels of IFN- γ .

All three fractions obtained after gel filtration demonstrate capacity below the detection limit.

B fraction demonstrates the highest capacity among all fractions.

The lowest capacity is observed at extracellular polysaccharides.

IL-12 response



Intracellular polysaccharides (Fraction B and C) induce higher amounts of cytokine IL-12 than extracellular polysaccharides (Fraction A).

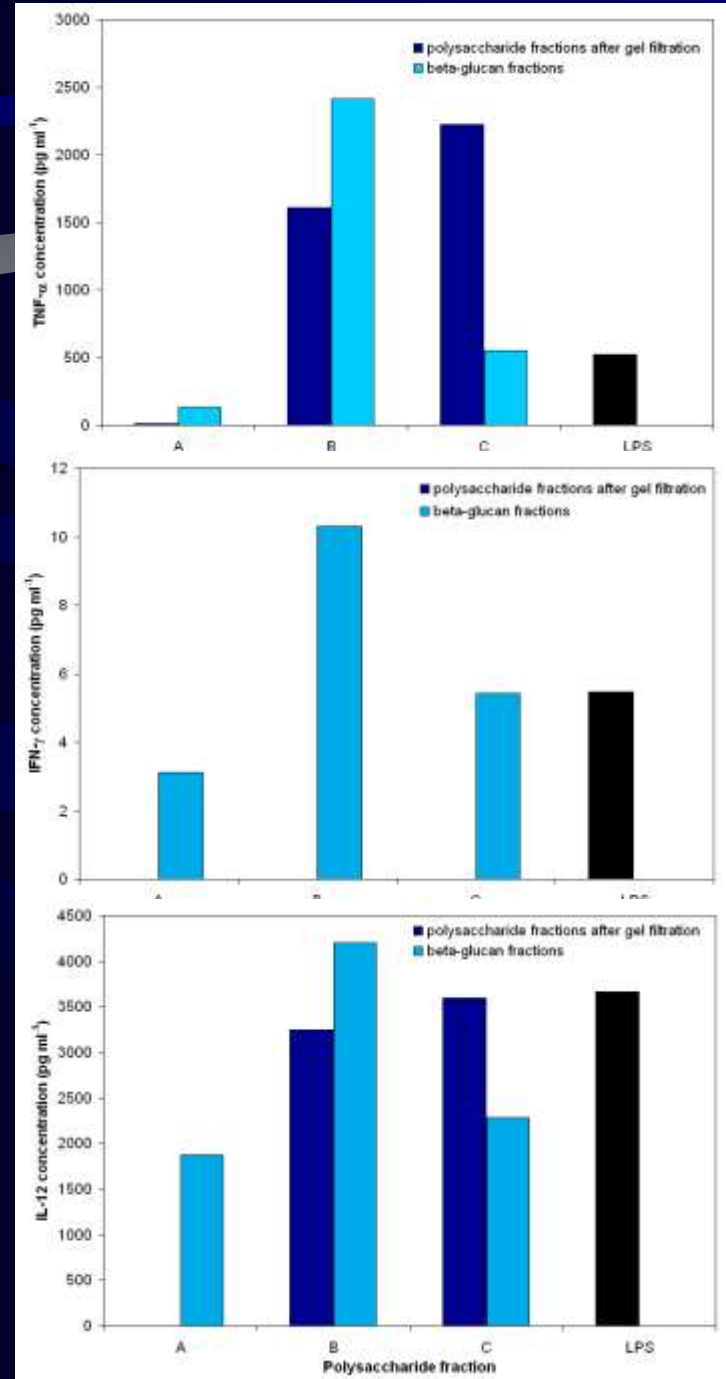
The highest IL-12 inducing capacity (3664 pg ml⁻¹) is observed at Fraction B β -glucan, followed by Fraction C obtained after gel filtration, which compared to inducing capacity of LPS.

Effect of polysaccharides on primary inflammatory immune response - summary

Cellular polysaccharides (Fractions B and C) induce higher inflammatory response (at all three cytokines) than extracellular polysaccharides (Fraction A). The graphical pattern of response is similar in all three Figures.

Water soluble β -glucans extracted by hot water (Fraction B) demonstrate highest ability to induce inflammatory response followed by water soluble polysaccharides extracted by 1 % ammonia oxalate solution and purified by ion-exchange chromatography and gel-filtration (Fraction C).

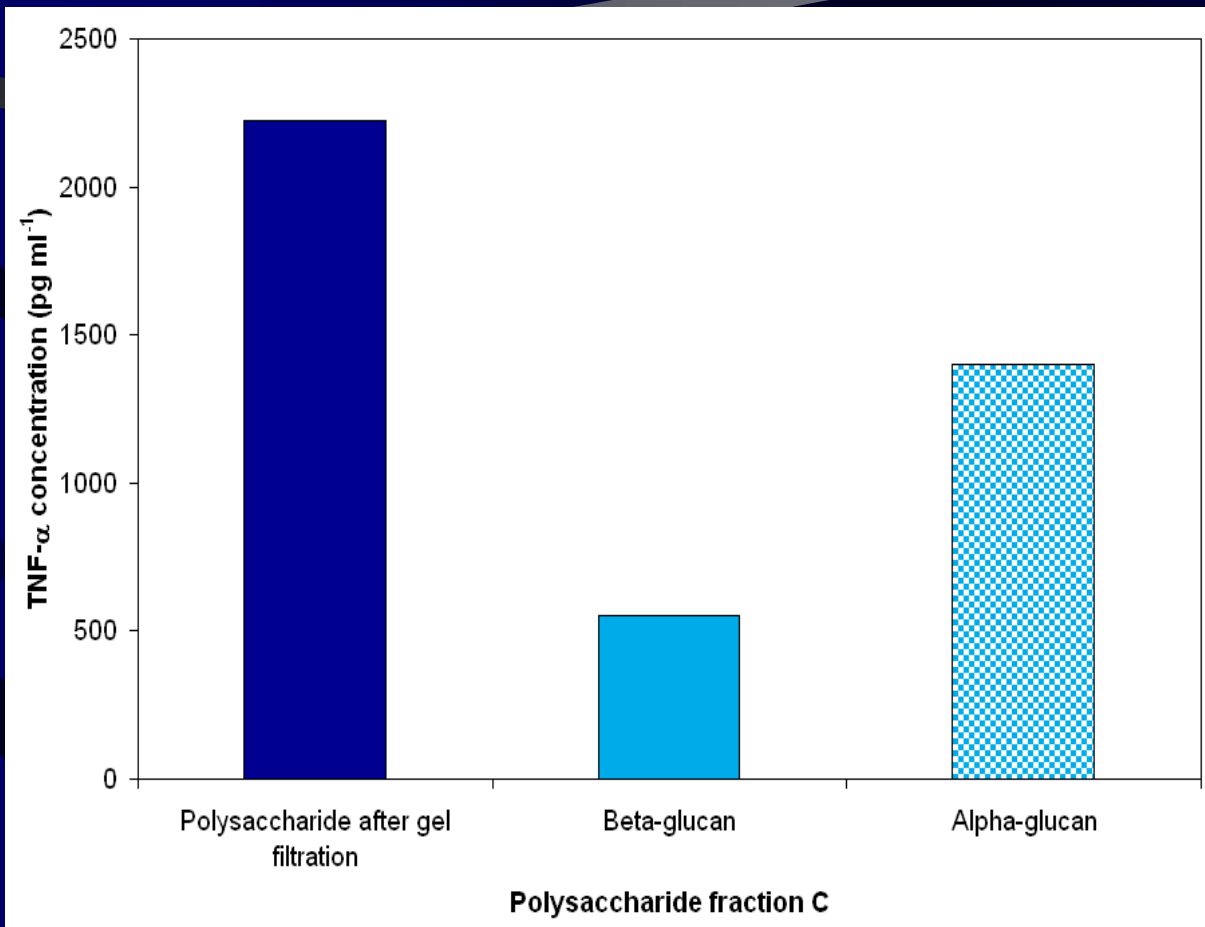
In general β -glucan fractions induce stronger inflammatory response than fractions obtained after gel filtration. Exceptions are observed at fractions C in case of TNF- α and IL-12 response.



Further study on fraction C on TNF- α inducing capacity

- Based on previous study Fraction C demonstrated higher inflammatory responses on polysaccharides obtained after gel filtration than pure β -glucans.
- The fraction obtained after gel filtration is a complex containing polysaccharides with β -configuration (β -glucan) and α -configuration (α -glucan). During affinity chromatography step those two are being separated.
- In our study the ability of Fraction C α -glucan has been tested on TNF- α inducing capacity and therefore potential contribution of α -glucan to overall inducing capacity in the complex.
- Following the stimulation of PBMC with Fraction C α -glucan in concentration 100 μ g/ml, the supernatants were screened for the content of TNF- α after a 4 h incubation.

TNF- α inducing capacity of Fraction C α -glucan



α -glucan demonstrates 2.5-higher capacity to induce TNF- α response than β -glucan.

α -glucan's higher capacity might contribute to TNF- α inducing capacity of the Fraction C complex obtained after gel filtration.

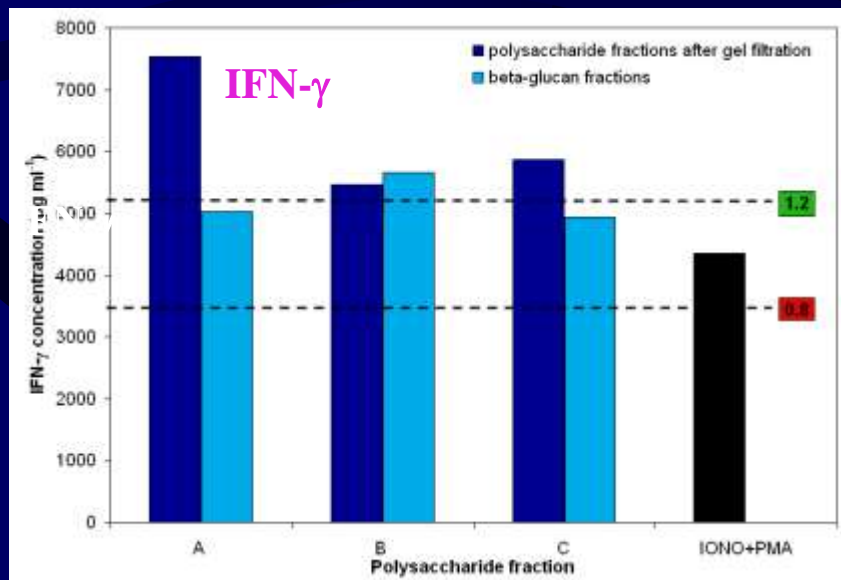
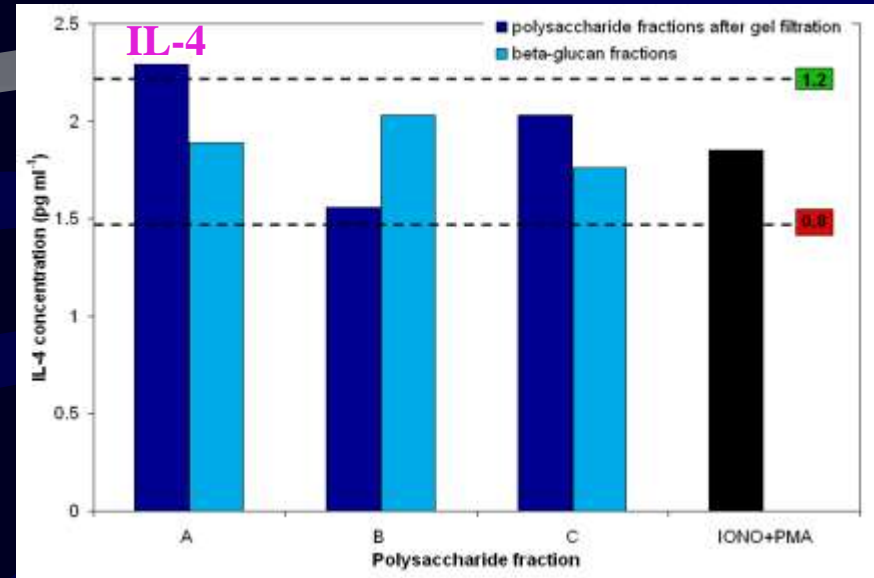
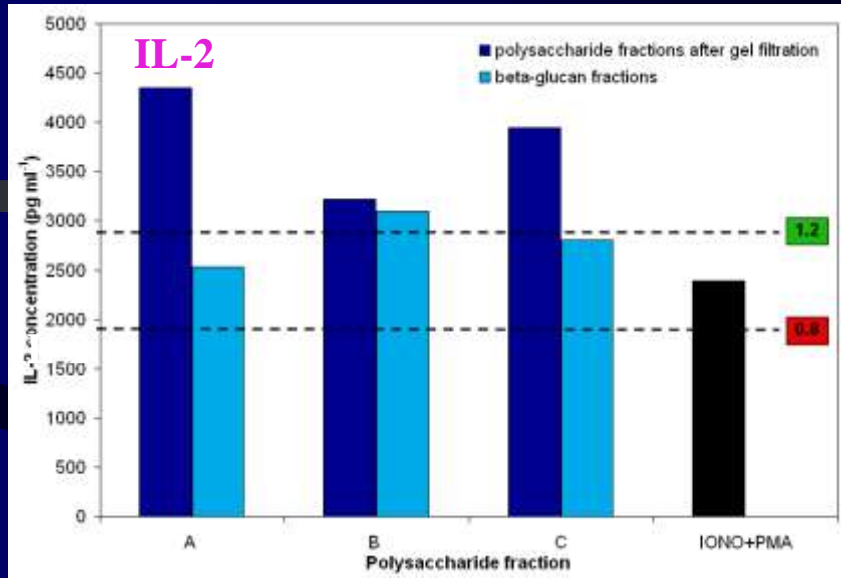
Immunomodulation studies

- **Studies on Th-1 (IL-2 and IFN- γ) vs. Th-2 response (IL-4)**
- Following the stimulation of PBMC with IONO + PMA and different polysaccharide fractions in concentration 100 $\mu\text{g/ml}$, the supernatants were screened for the content of **IL-2, IFN- γ and IL-4** after a 40-h incubation.

Positive Control: IONO+PMA

- Representation of results:
 - All results above 1.2 index line represent stimulatory/promotion effect on response
 - All results below 0.8 index line represent inhibitory effect on response
 - All results between the lines represent neutral response
- Calculation of index: $\text{ONO \& PMA + Fraction } x / \text{IONO \& PMA}$

Th1 vs. Th2 response



Polysaccharides direct lymphocyte response into Th1

- IL-2 and IFN- γ positive response and
- IL-4 negative to neutral response

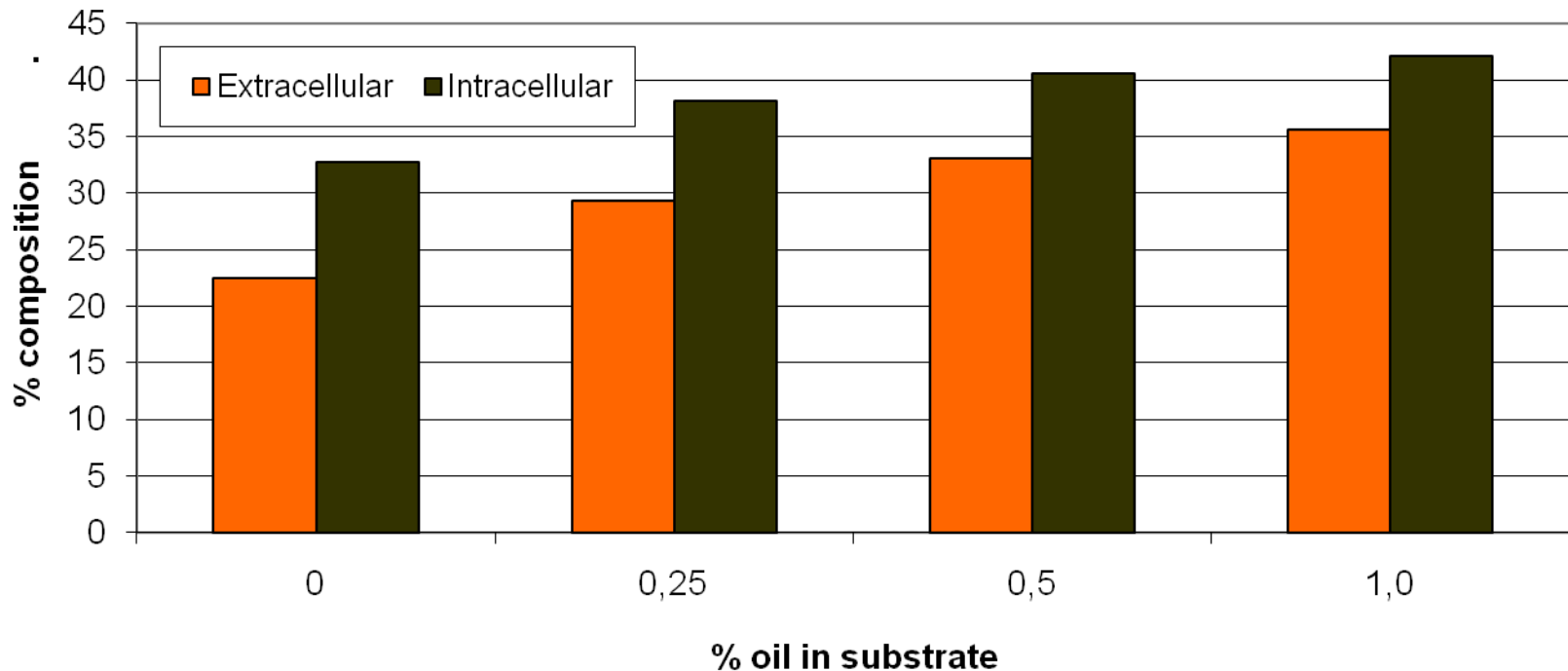
Grifola frondosa



Chemical composition of polysaccharides

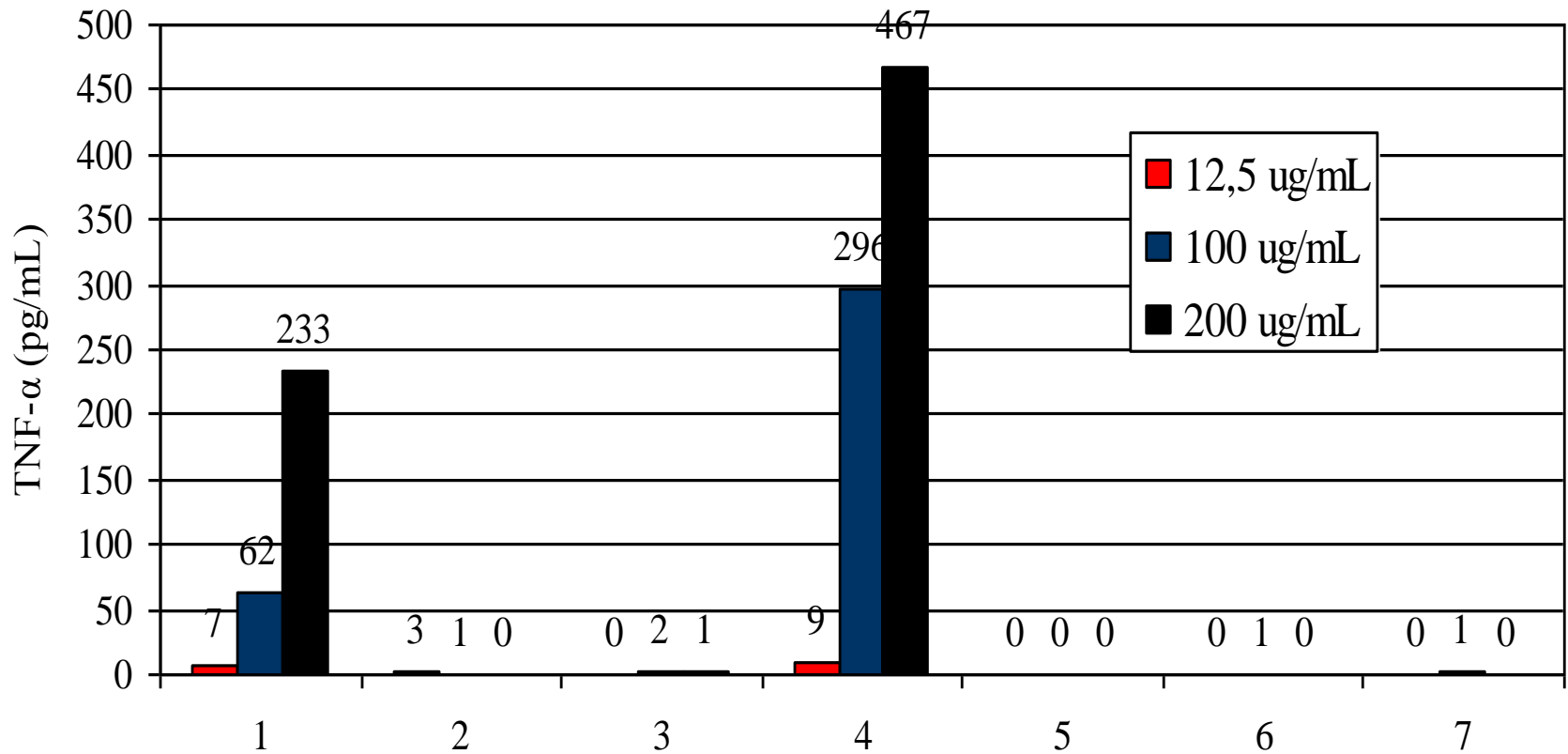
β -glucan trend – intracellular and extracellular

β -glucan content in extra- and intra-cellular polysaccharides



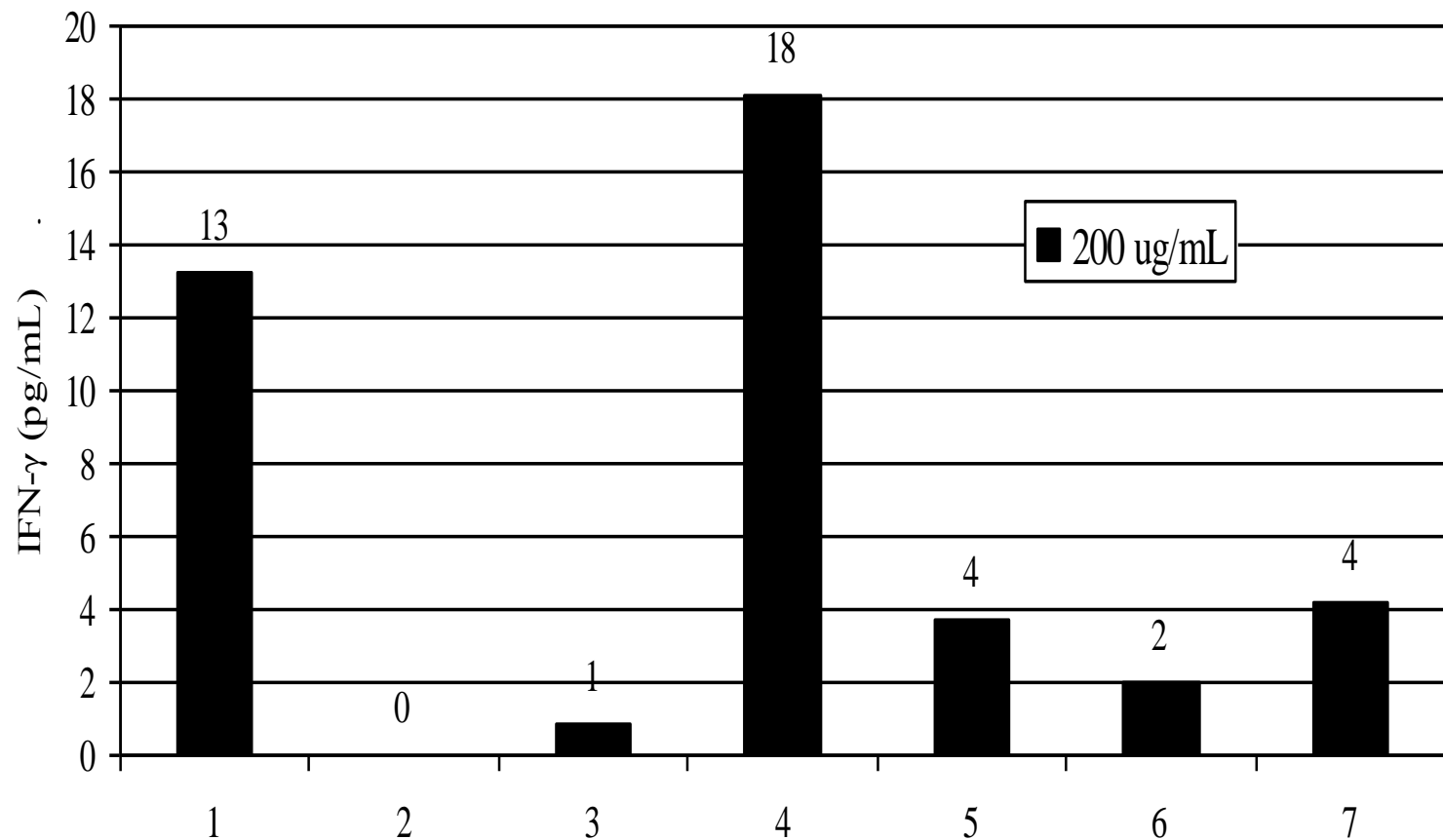
Grifola frondosa farming

TNF- α induction in PBMC



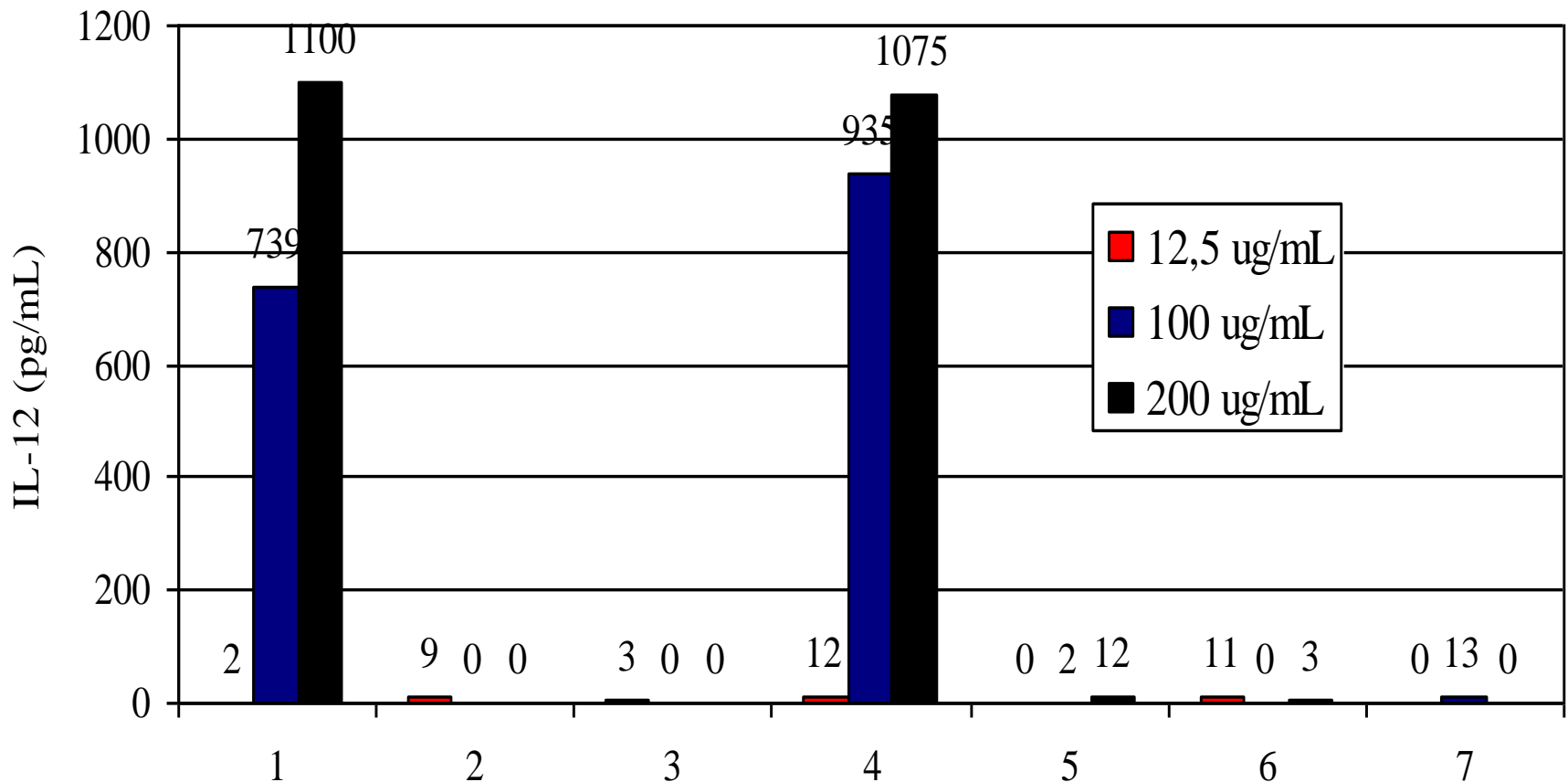
Grifola frondosa farming

IFN - γ induction in PBMC



Grifola frondosa farming

IL-12 induction in PBMC

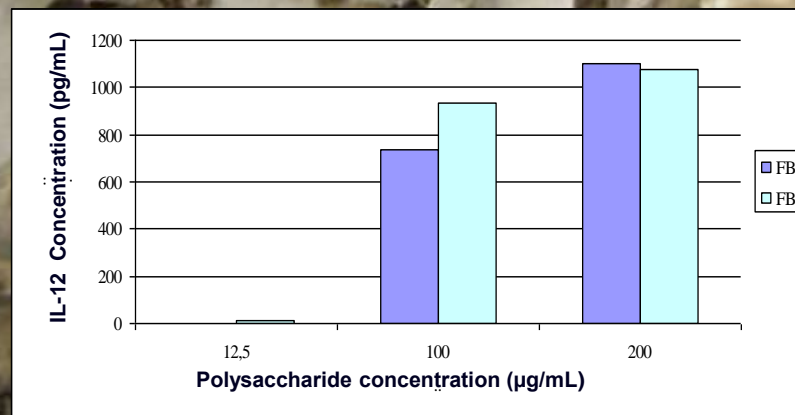
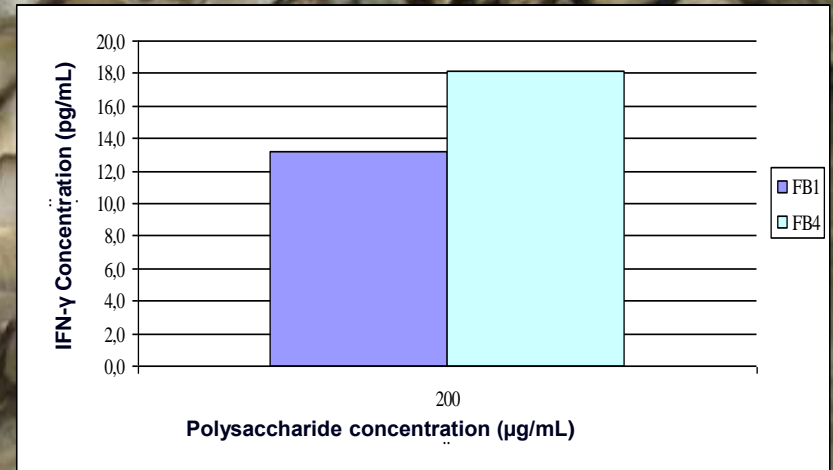
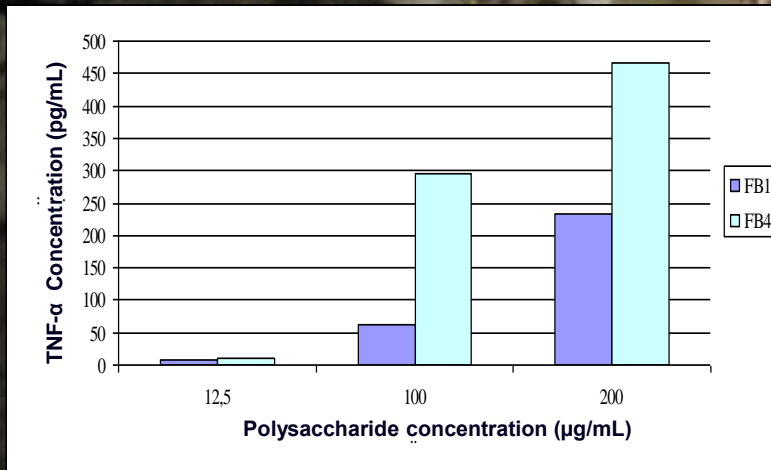


Immunostimulatory effects of *G.frondosa* polysaccharides

Submerged cultivation

Extracellular ■

Intracellular ■



The comparison of different isolates on induction of cytokines
TNF α , IF γ and IL-12

Summary of findings

Inflammatory response:

- Intracellular polysaccharides induce higher inflammatory response than extracellular polysaccharides. The highest response is observed with hot water extracted β -glucans.
- Polysaccharides have higher TNF- α inducing capacity than polysaccharide-protein complexes.
- Pure β -glucans induce higher inflammatory response (observed with all cytokines: TNF- α , IFN- γ and IL-12) than polysaccharides obtained after gel filtration. α -glucan could contribute to response of fraction C, which demonstrates opposite results.
- The graphical pattern of response is similar among all three cytokines, however IFN- γ response is very low or below detection limit.

Immunomodulation:

- Extracellular polysaccharides have higher response on lymphocyte immunomodulation than cellular polysaccharides. The highest effect is observed with fractions obtained after gel filtration.
- Polysaccharides direct lymphocyte response into Th1 response.

Conclusions

Original strains of *Ganoderma lucidum* (MZKI G97) and *Grifola frondosa* (GF3) were isolated from Slovenian forests.

Submerged and solid state cultivations were applied.

Ganoderma lucidum

In 14 days Submerged Cultivation fed batch cultivation extracellular (1,7 gl-1) and intracellular (0.45 gl-1) polysaccharide fractions were isolated, up to 17.0 gl-1dry fungal biomass was produced.

In in 18 days solid state cultivation extracellular (5.77 mg /g) of and intracellular (1.45 mg /g) polysaccharide was produced at the end of the cultivation.

Grifola frondosa

In 28 days fed batch cultivation submerged cultivation 3.65 mg/l of extracellular and 1.30 mg/l intracellular polysaccharide were produced.

In 38 days solid state cultivation in of cultivation 3.80 mg/g of extracellular and 0.70 mg/g of intracellular polysaccharide were produced.

Isolation of fungal polysaccharides

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.

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The latest News !!

Boletus Sexual Life

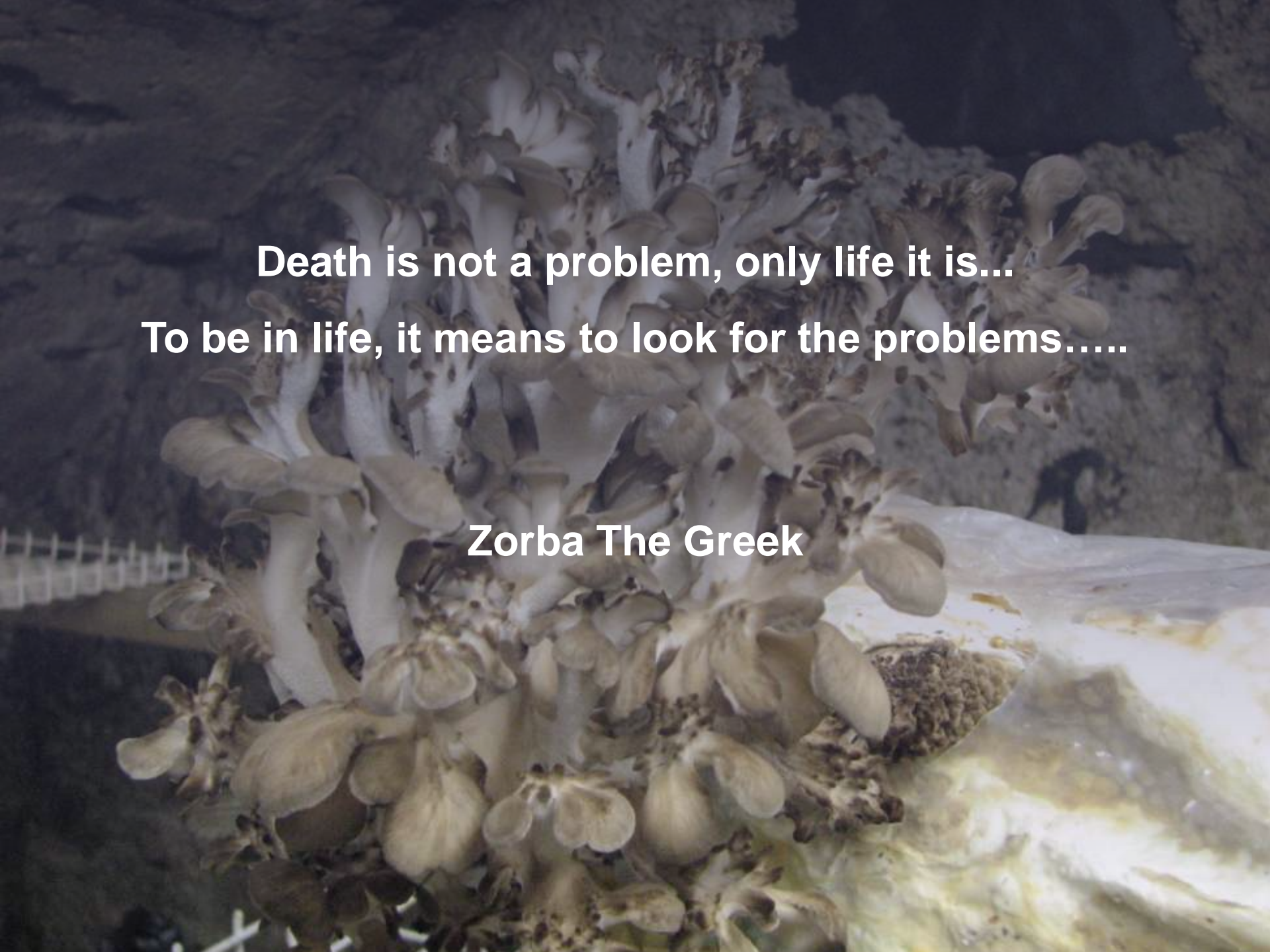
Boletus Sexual Life



Boletus Sexual Life



Source : Vatican State University



Death is not a problem, only life it is...
To be in life, it means to look for the problems.....

Zorba The Greek

Summary of findings (SLO)

Vnetje:

- Vnetje mocneje inducirajo polisaharidi celicne stene. Najvisji odziv opazen pri beta-glukanih ekstrahiranih z vroco vodo.
- Polisaharidi (brez proteinov) mocneje inducirajo sintezo TNF- α kot polisaharid-proteinski kompleksi.
- Cisti polisaharidi (beta-glukani) mocneje inducirajo vnetje kot polisaharidi po ciscenju z gelsko filtracijo. C frakcija je izjema, kjer je mozno da alfa del frakcije prispeva k odzivu kompleksa (iz gelse filtracije).
- Odzivi na TNF- α in IL-12 so zelo podobni, na IFN- γ so odzivi zelo nizki oziroma pod mejo detekcije.

Imunomodulacija:

- Limfocitne odzive mocneje modulirajo ekstracelularni polisaharidi. Navisji odziv opazen pri frakcijah ociscenih z gelsko filtracijo.
- Polisaharidi ojacijo Th1 odziv.