

Immunostimulatory and Imunomodulant Activities of Extra and Intracellular Fungal Polysaccharides of *Ganoderma lucidum* by Submerged and Solid State Cultivation

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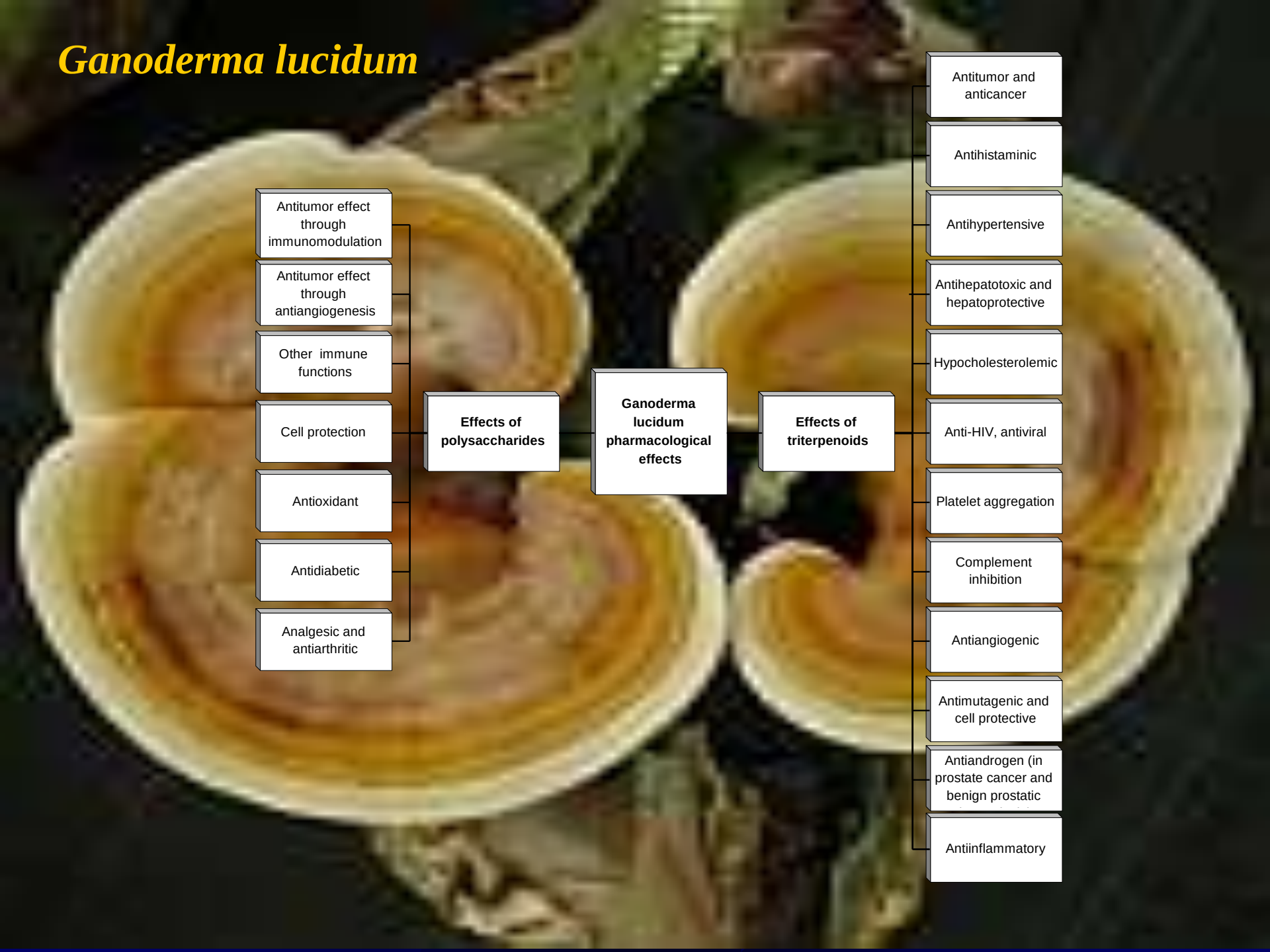
WOOD-DEGRADING MUSHROOMS

White rot

Brown rot


Ganoderma lucidum

Ganoderma lucidum









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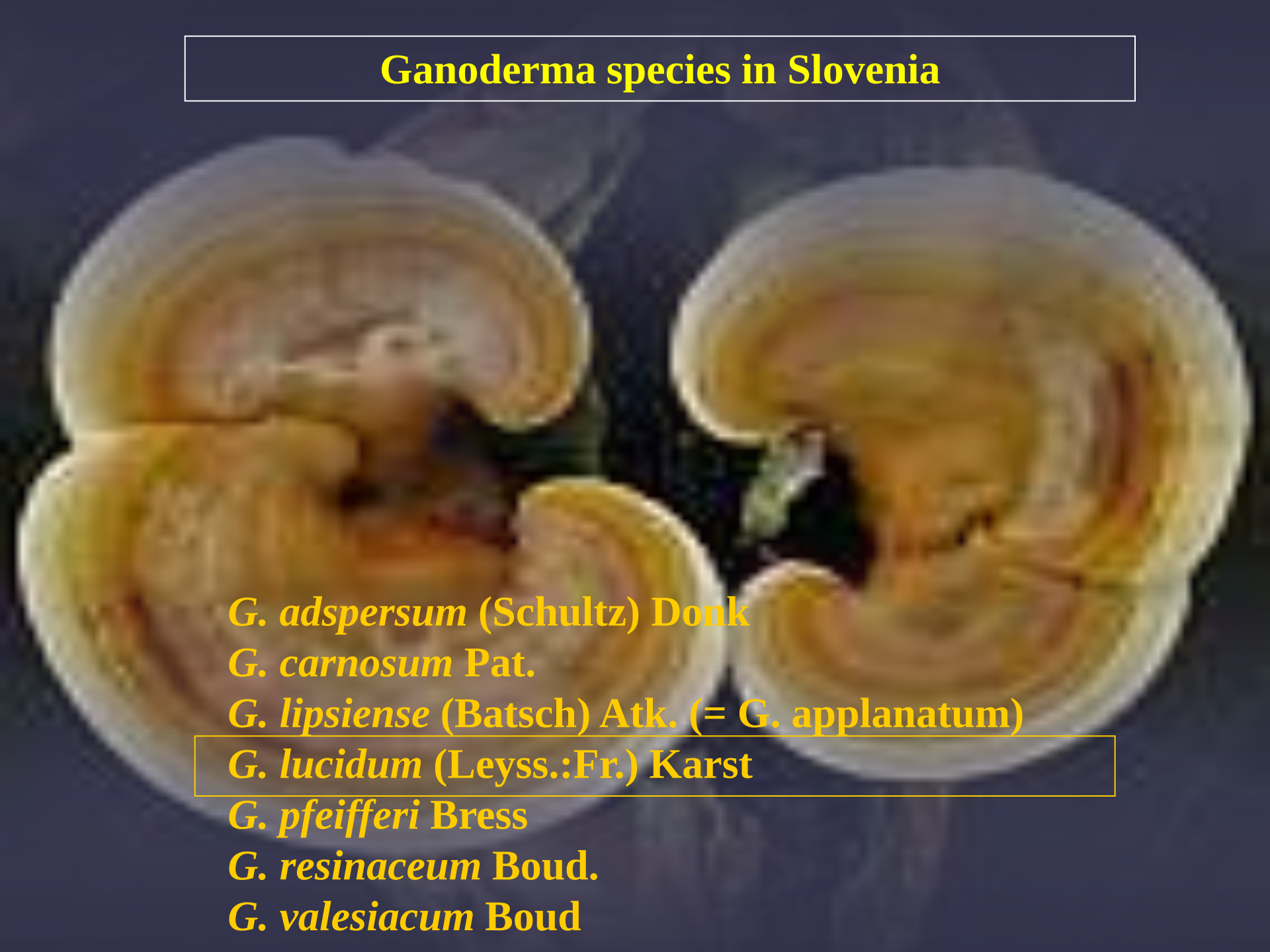
Ganoderma lucidum

Regnum: FUNGI
Phyllum: EUMYCOTA
Subphyllum: BASIDIOMYCOTINA
Clasis: HYMENOMYCETES
Ordo: APHYLLOPHORALES
Familia: POLYPORACEAE
Genus: GANODERMA
Species: LUCIDUM

Ganoderma species in Slovenia



G. adspersum (Schultz) Donk
G. carnosum Pat.
G. lipsiense (Batsch) Atk. (= G. applanatum)
G. lucidum (Leyss.:Fr.) Karst
G. pfeifferi Bress
G. resinaceum Boud.
G. valesiacum Boud

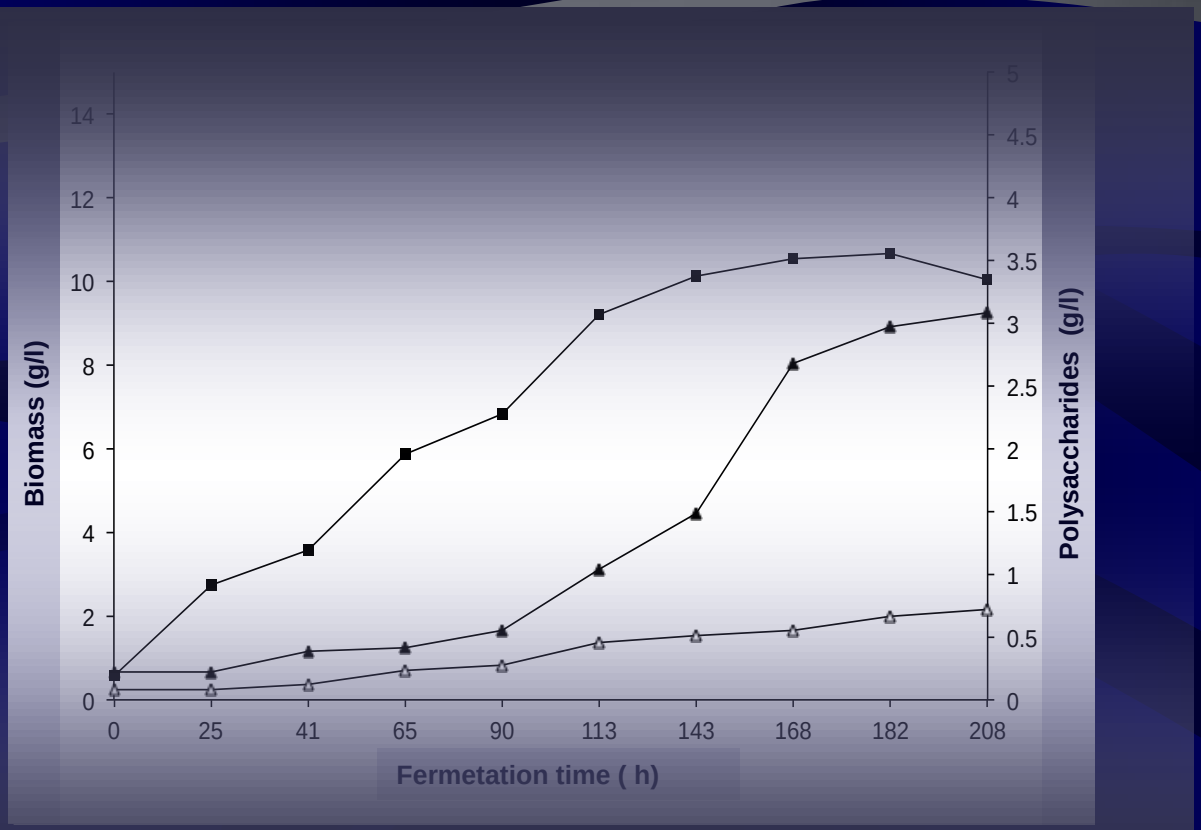
BIOREACTOROMICS

Submerged cultivation

Submerged cultivation

Ganoderma lucidum
innoculum used in
submerged cultivation

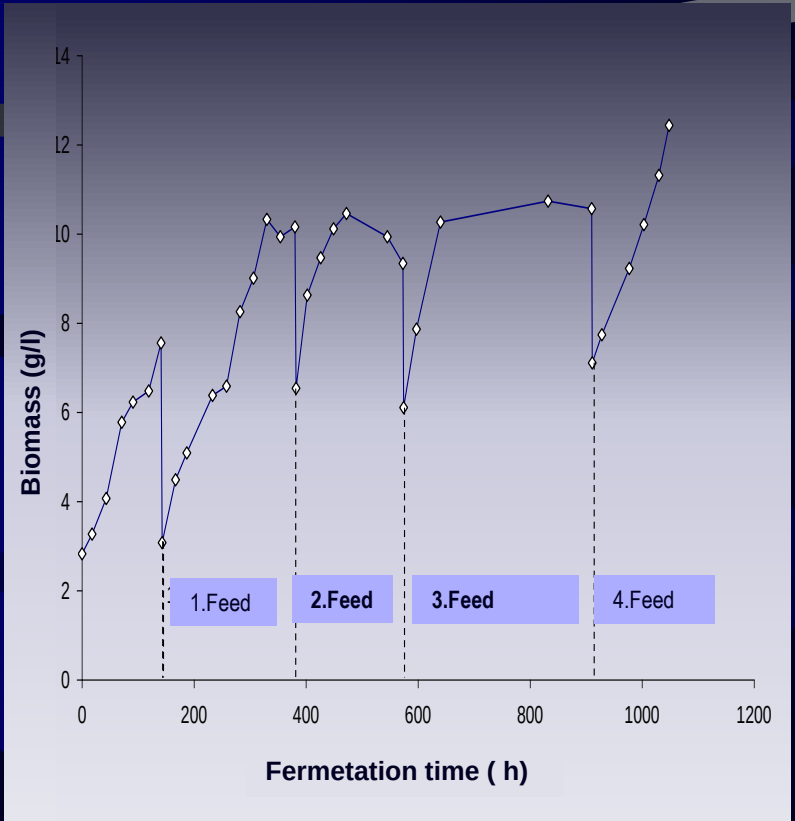
Ganoderma lucidum submerged cultivation

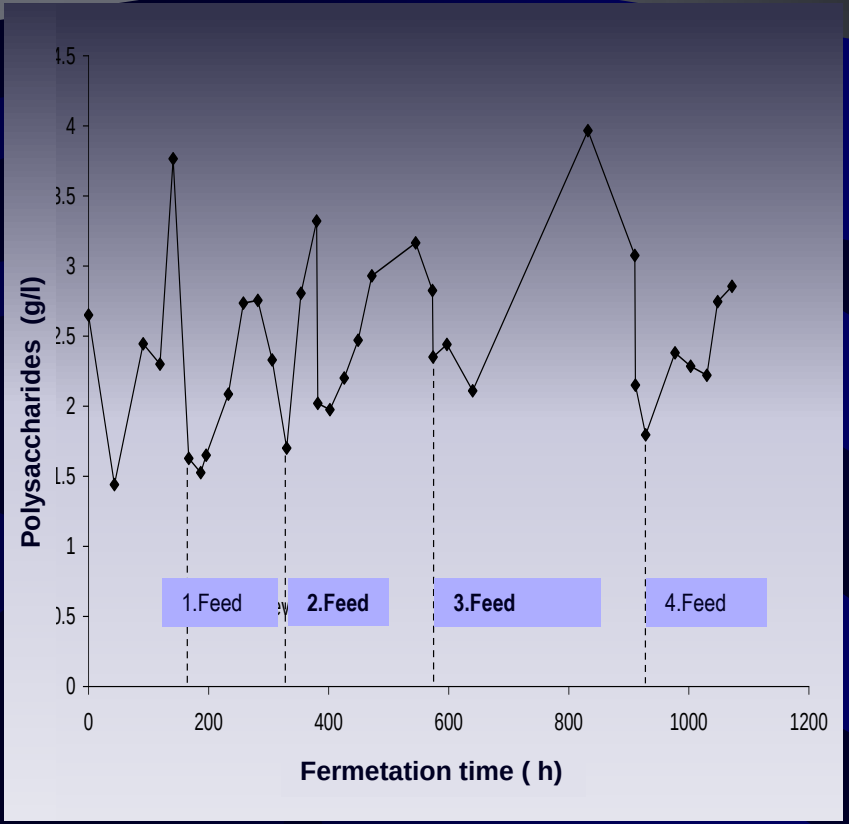


Batch cultivation of *Ganoderma lucidum* biomass and polysaccharide production ($T = 30°C$, $N = 300\ min^{-1}$, $Q_g = 10\ L\ min^{-1}$)

■ **Biomass ; Δ Extracellular ; ▲ Intracellular polysaccharides**

Ganoderma lucidum submerged cultivation





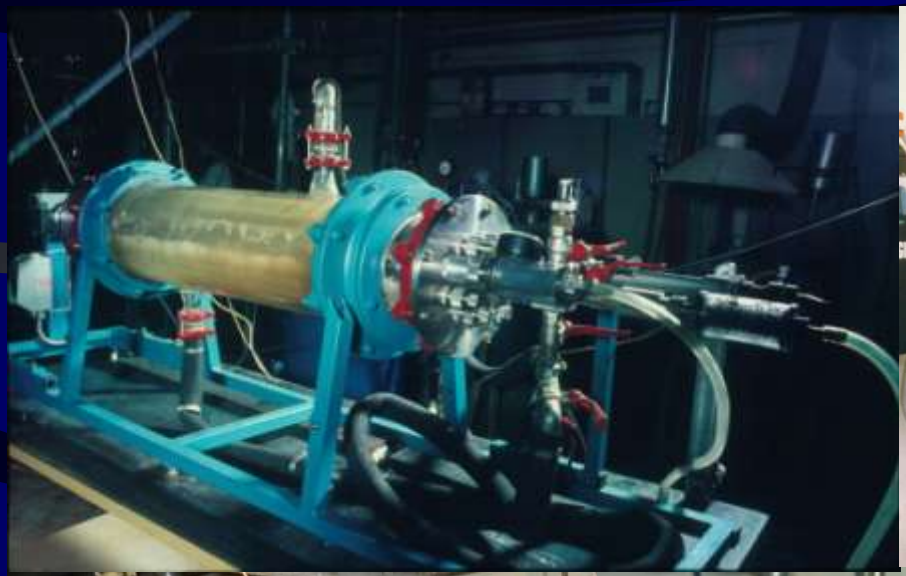
Influence of feding on *Ganoderma lucidum* biomass and polysaccharide production in fed batch cultivation, (T = 30°C, N = 300 min^{-1}, Q_g = 10 L min^{-1})

- **17 % inoculum**

Solid state cultivation

Horizontal Stirred Tank Reactor

BIOREACTORS



Ganoderma lucidum

Mycelial growth after 6 weeks

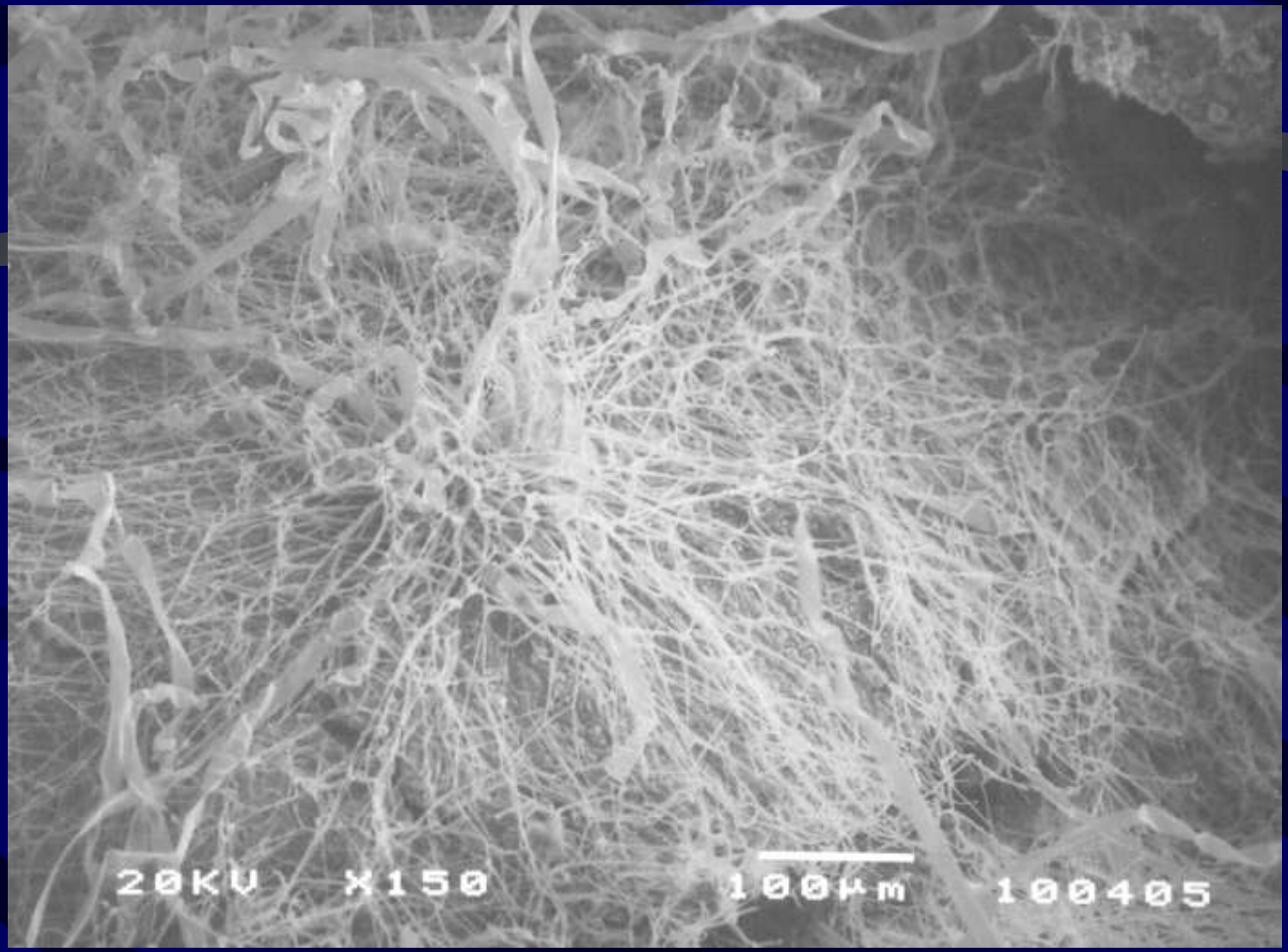
Ganoderma lucidum

Mycelial growth after 6 weeks

Ganoderma lucidum

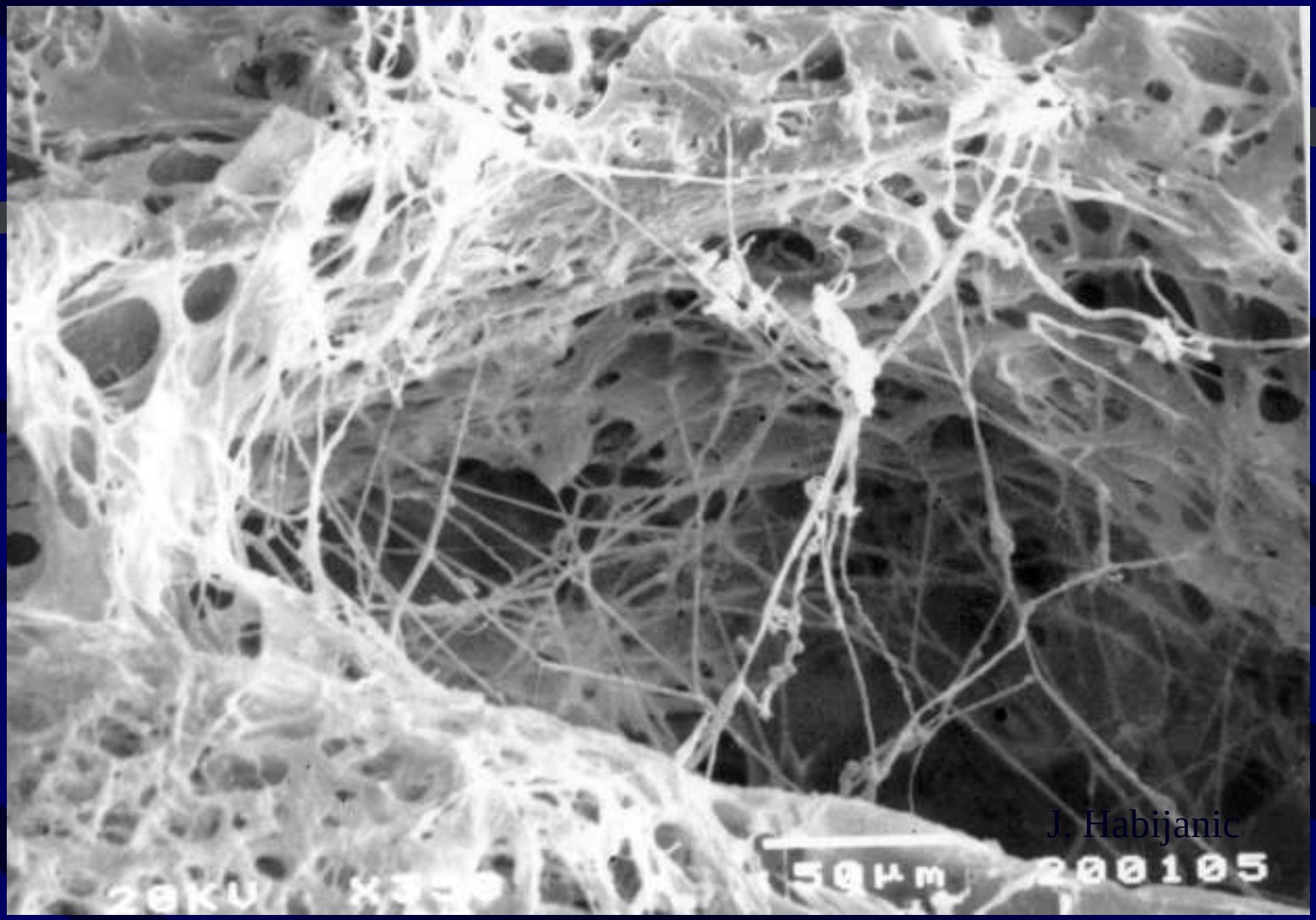
Mycelial growth after 6 weeks

GROWTH IN SSF



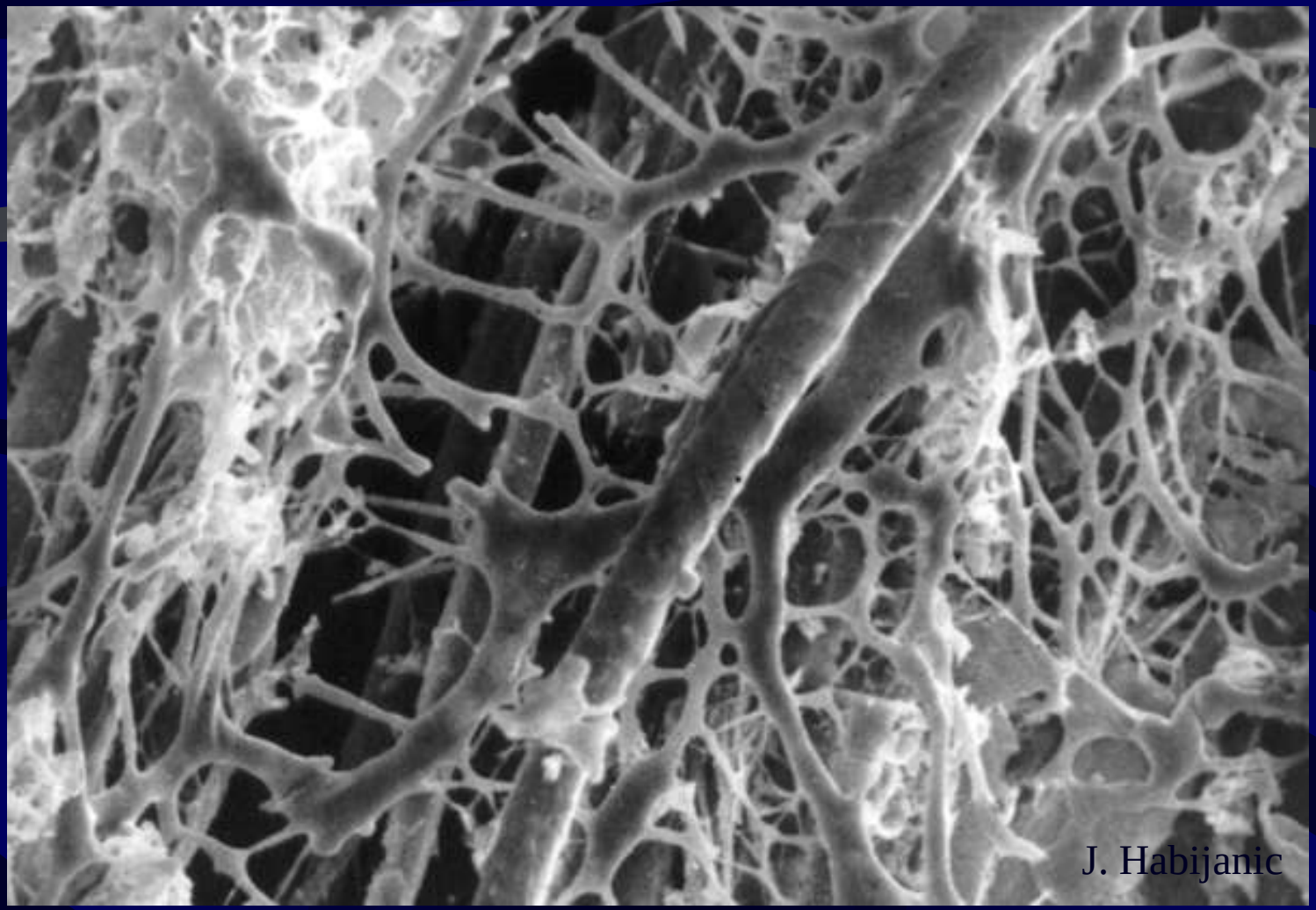
Mycelial growth on solid substrate after 7 days of cultivation (350x)

GROWTH IN SSF



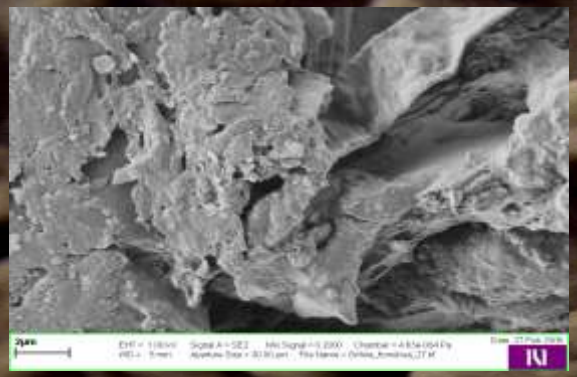
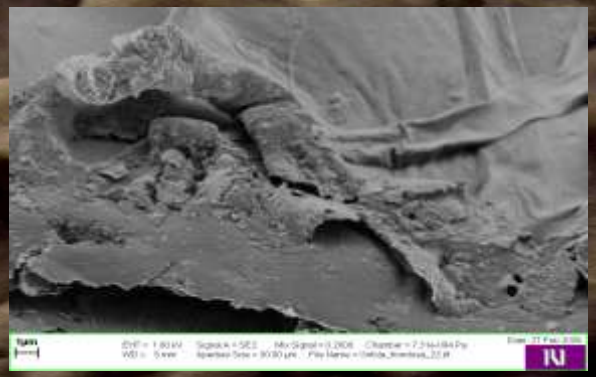
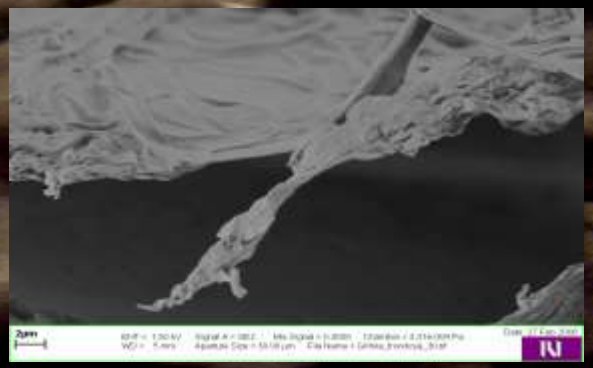
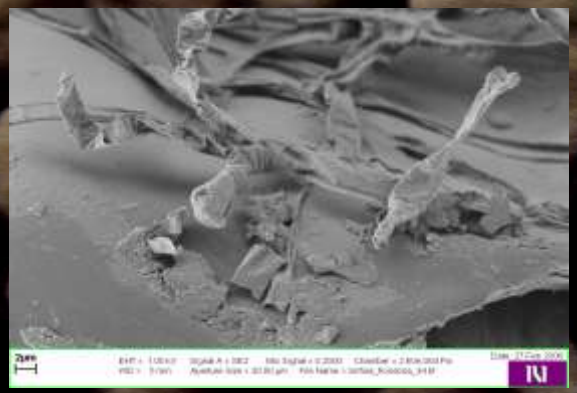
Mycelial growth on solid substrate after 12 days of cultivation (350x)

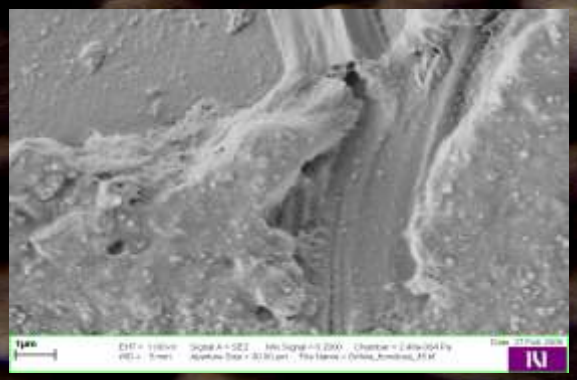
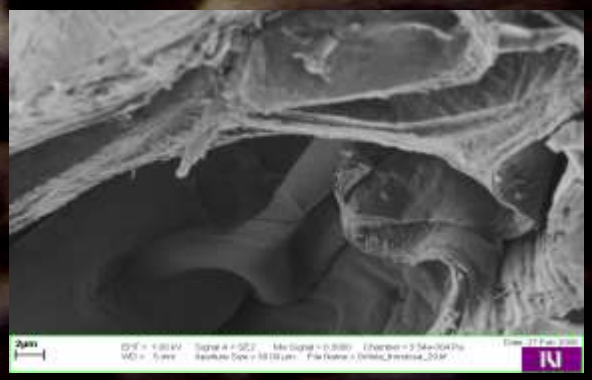
GROWTH IN SSF



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

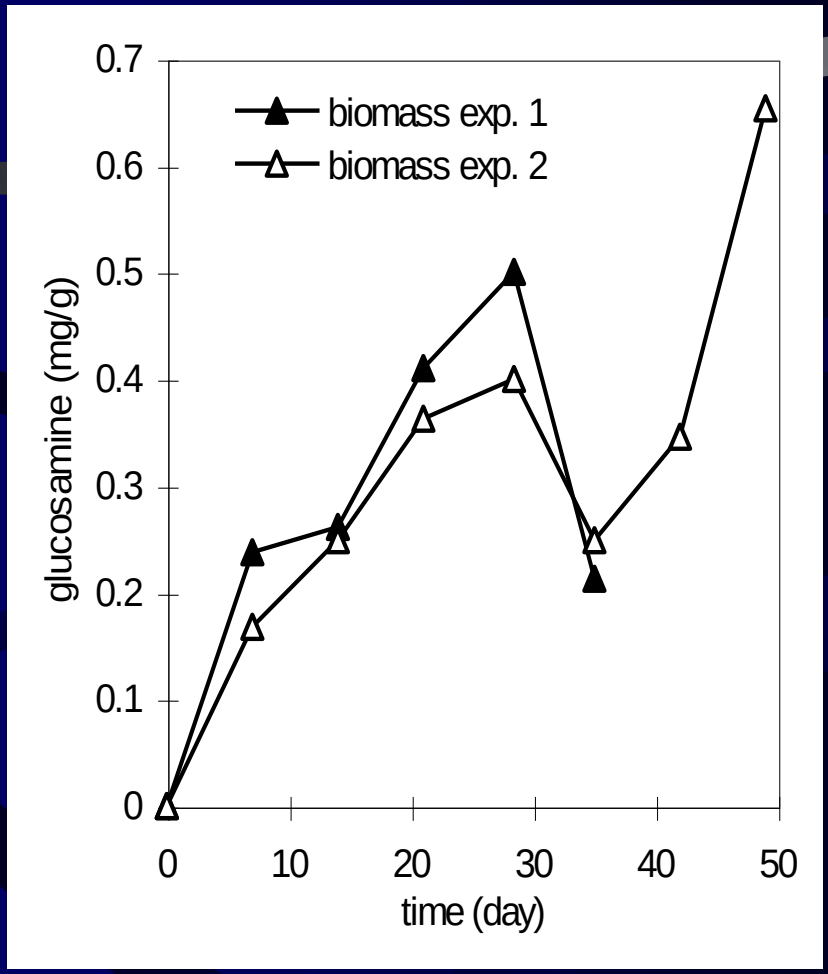
Grifola frondosa

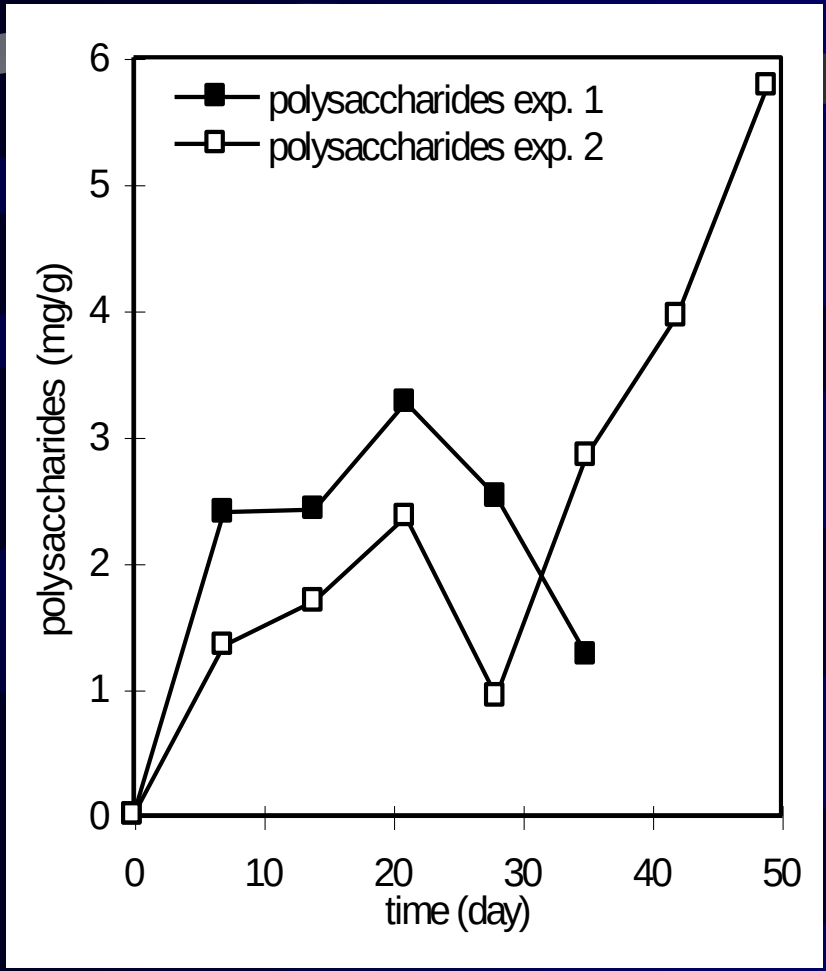





Corn cellulose

Ganoderma lucidum solid state experiments

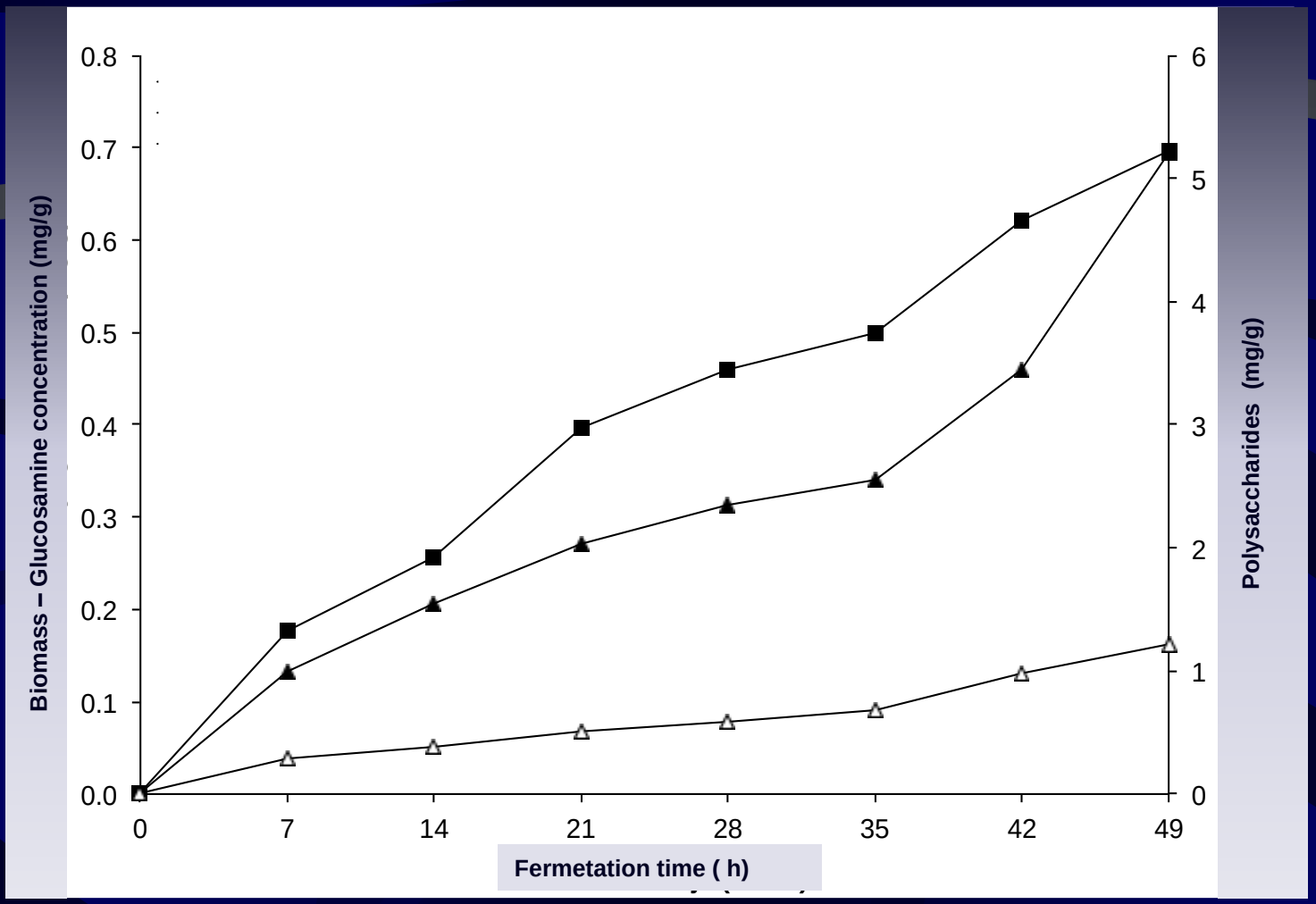


Biomass growth during both experiments



Polysaccharide production during both experiments

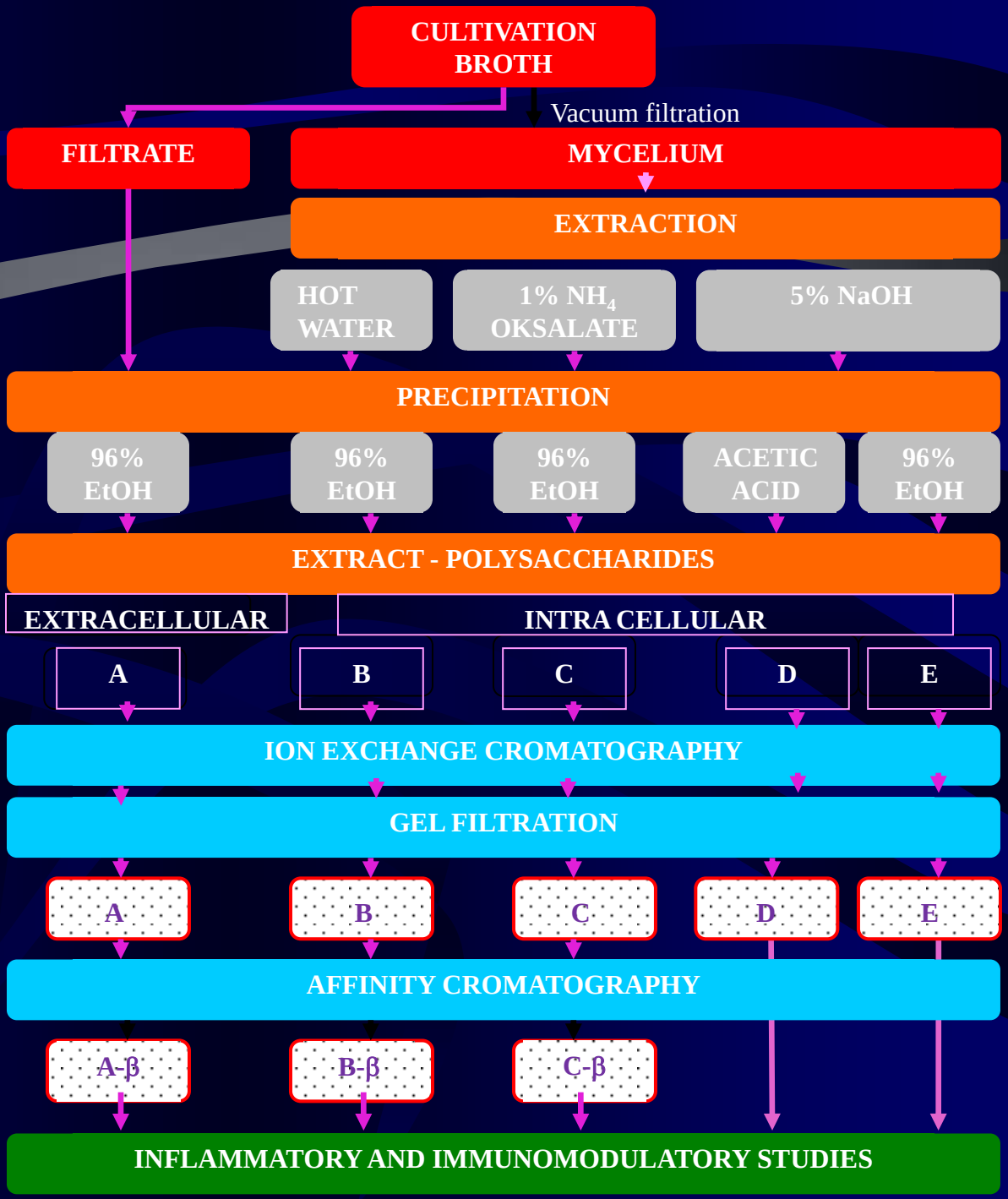
Ganoderma lucidum solid state experiments



Ganoderma lucidum biomass and polysaccharide production
($T = 30°C$, $N = 300\ min^{-1}$, $Q_g = 10\ L\ min^{-1}$)

■ Biomass ; Δ Extracellular ; ▲ Intracellular polysaccharides

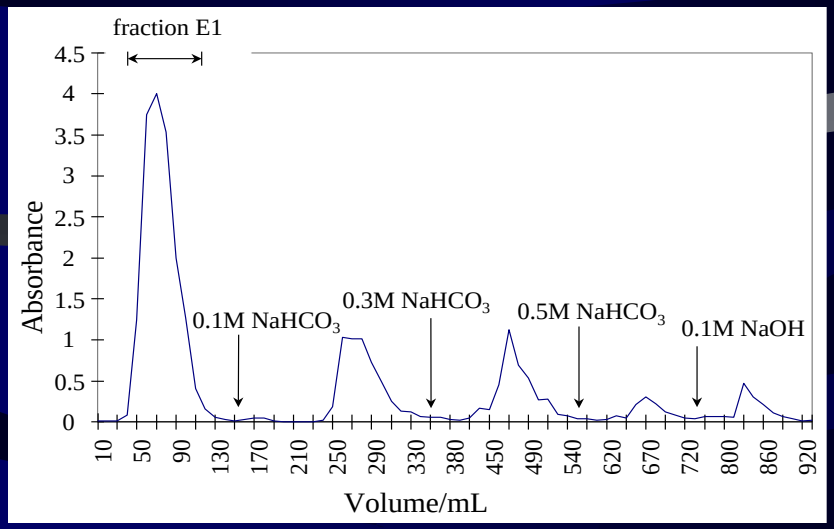
Study course



Tested in immuno-studies

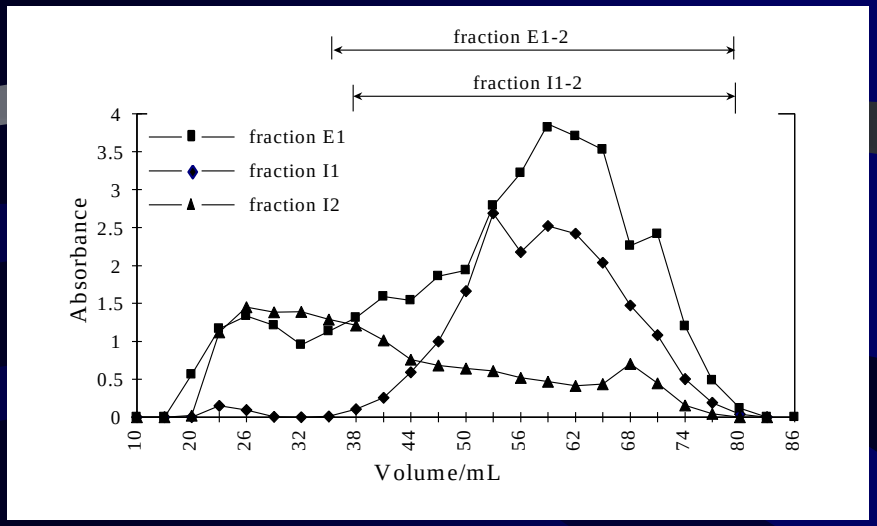
Ion-exchange chromatography

Elution chromatogram of a water soluble fraction of **extracellular polysaccharides**



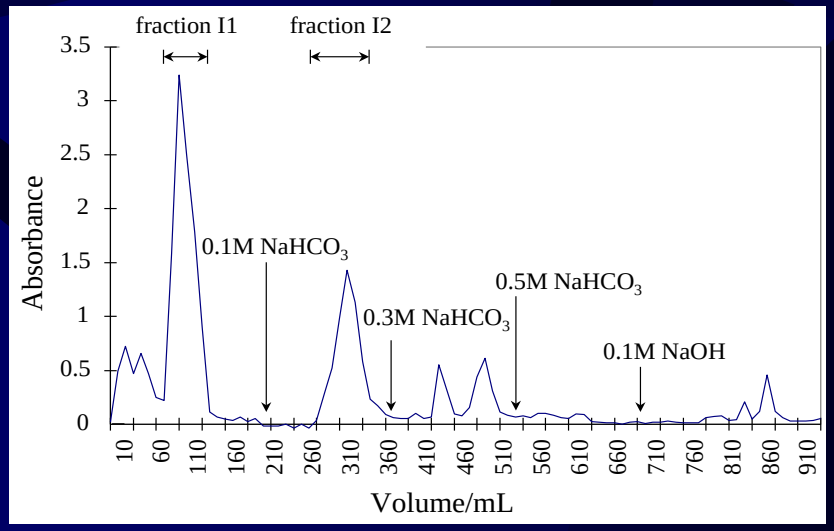
Gel filtration chromatography

elution chromatograms of extracellular polysaccharide



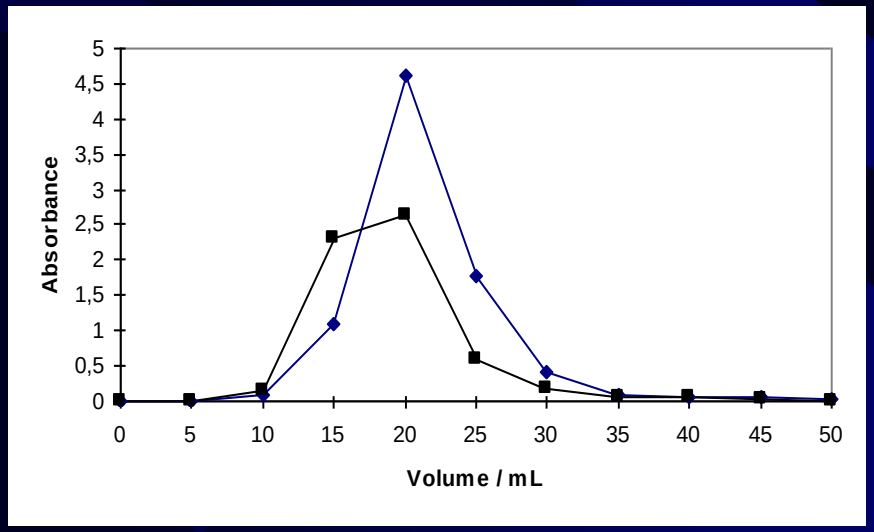
Ion-exchange chromatography

Elution chromatogram of a water soluble fraction of **intracellular polysaccharides**



Affinity chromatography

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



Extraction and fractionation of polysaccharides

- Cultivation broth vacuum filtered
- **Extracellular polysaccharides**: liquid medium concentrated, precipitated with 96 % ethanol, filtered, washed with acetone, dried - **Fraction A**.

Extraction and fractionation of polysaccharides

- **Intracellular polysaccharides** mycelium pre-extracted with 85 % ethanol, extraction with hot water, precipitation by 96 % ethanol **Fraction B**
- Mycelium further extracted with 1% ammonium oxalate solution - **Fraction C**
- and with 5 % sodium hydroxide solution; polysaccharides precipitated by acetic acid **Fraction D**
- from the remaining solution precipitated by ethanol- **Fraction E**.

Ethanol-precipitated amorphous polysaccharides (37x)

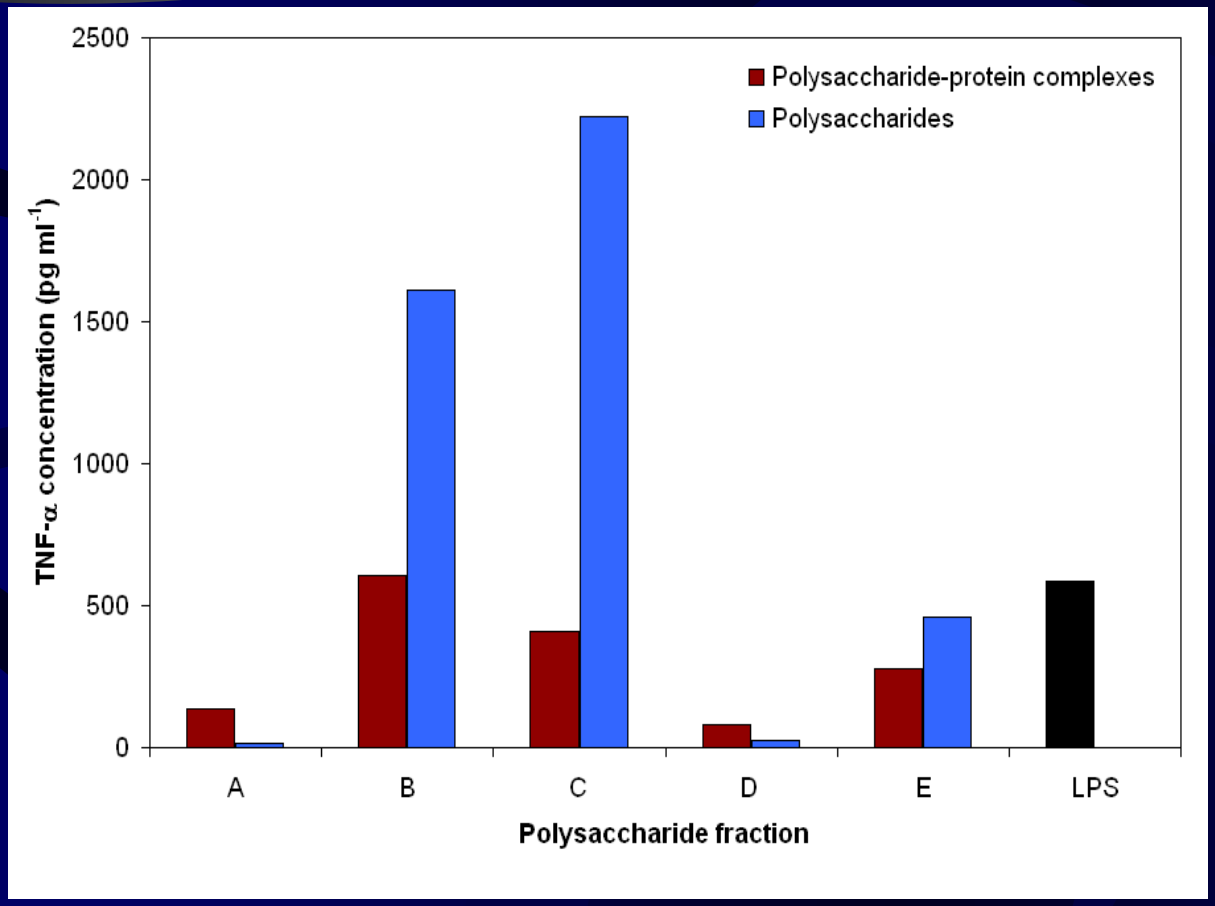
Characteristics and yields of polysaccharide fractions from *G. lucidum* mycelium

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

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 it was concluded that concentration 100 μg ml-1 is the most appropriate to study immune responses.
- Following the stimulation of PBMC with concentration 100 μg ml-1, the supernatants were screened for the content of TNF-α after a 4-h incubation.

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



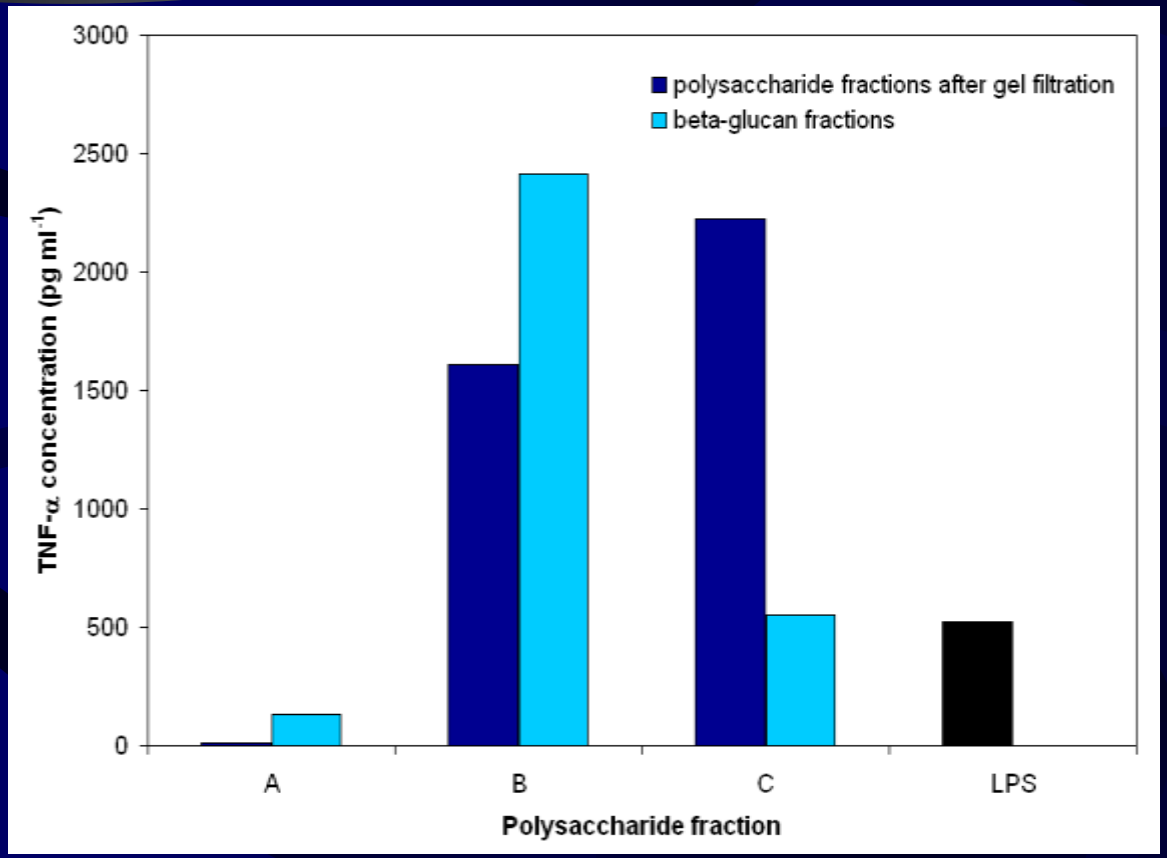
Polysaccharide fractions (without proteins) induce higher amounts of TNF-α (up to 2224 pg ml^{-1}) than polysaccharide-protein fractions.

The highest difference observed at cellular fractions C and B.

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

- Polysaccharides (without proteins) have demonstrated higher TNF-α inducing capacity versus polysaccharide-protein complexes (demonstrated in previous study) further studies have been focused on :
  - 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 Intracellular 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 Concavalin 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.

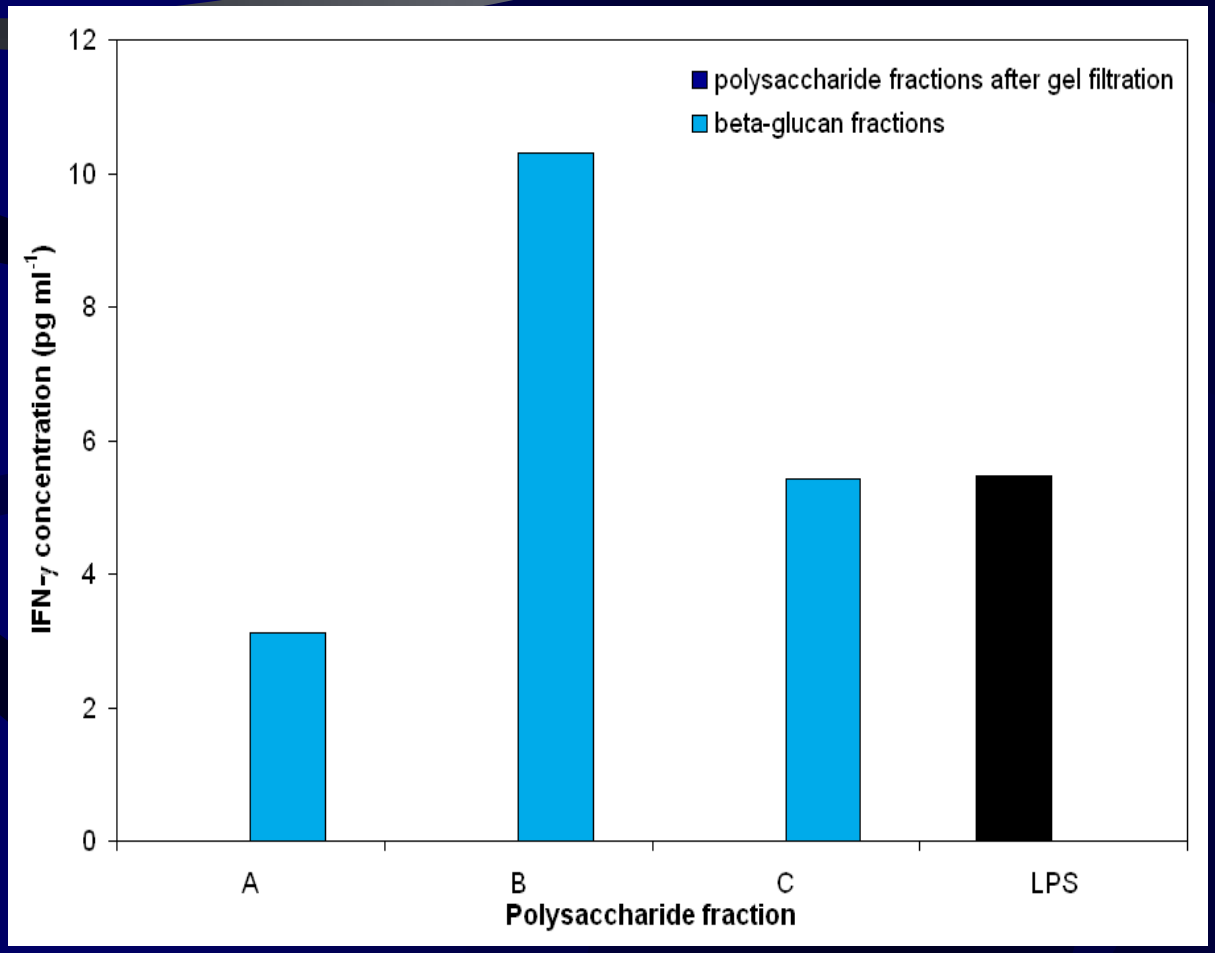
TNF- α response



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.

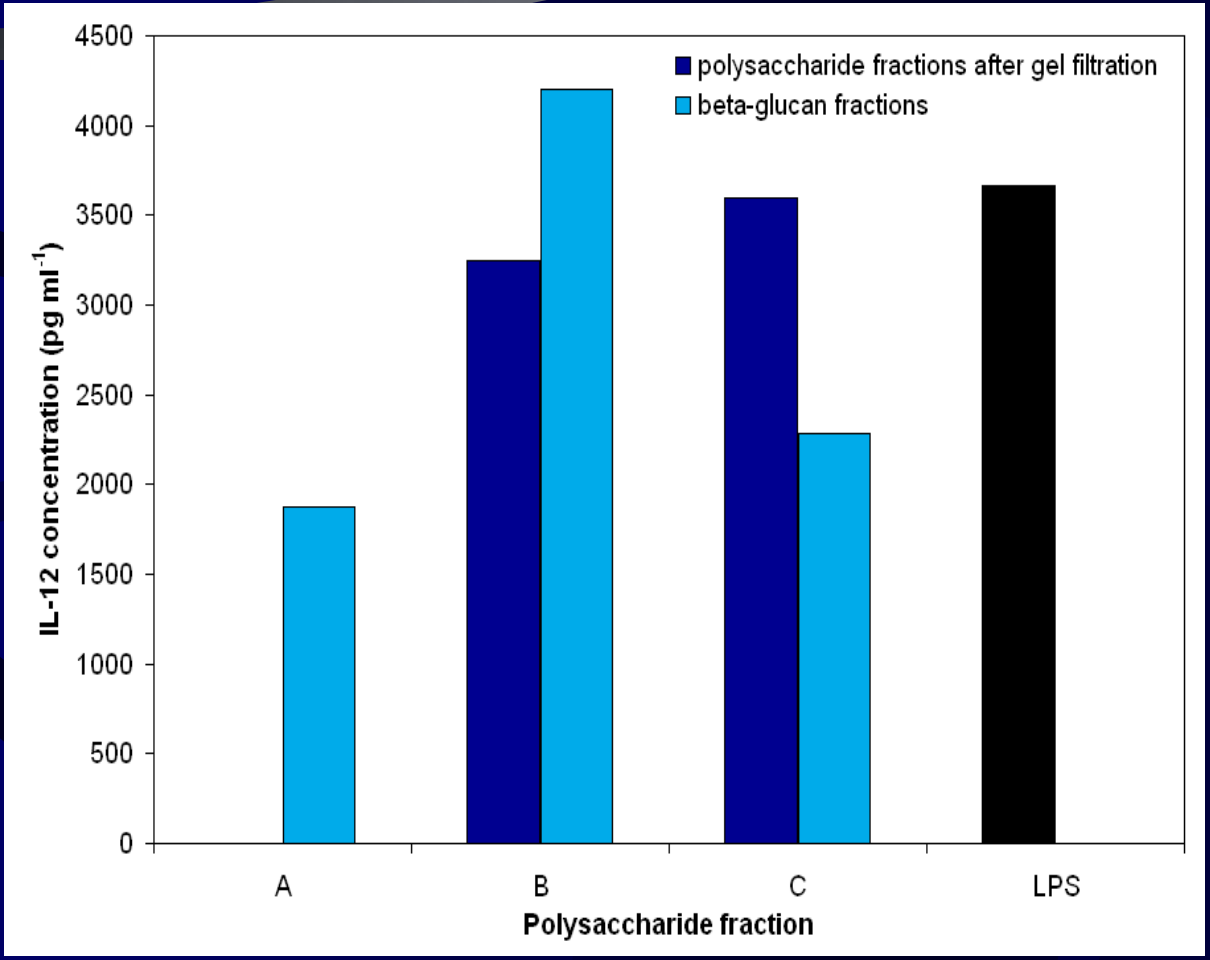
All three fractions obtained after gel filtration demonstrate capacity below the detection limit.

B fraction , Intracellular polysaccharides **demonstrates the highest capacity among all fractions.**

A fraction , Extracellular polysaccharides

The lowest capacity was observed .

IL-12 response



Intracellular polysaccharides (Fraction B and C)

induce higher amounts of cytokine IL-12

Compared with

Extracellular polysaccharides (Fraction A).

Fraction B

The highest IL-12 inducing capacity

(3664 pg ml^{-1})

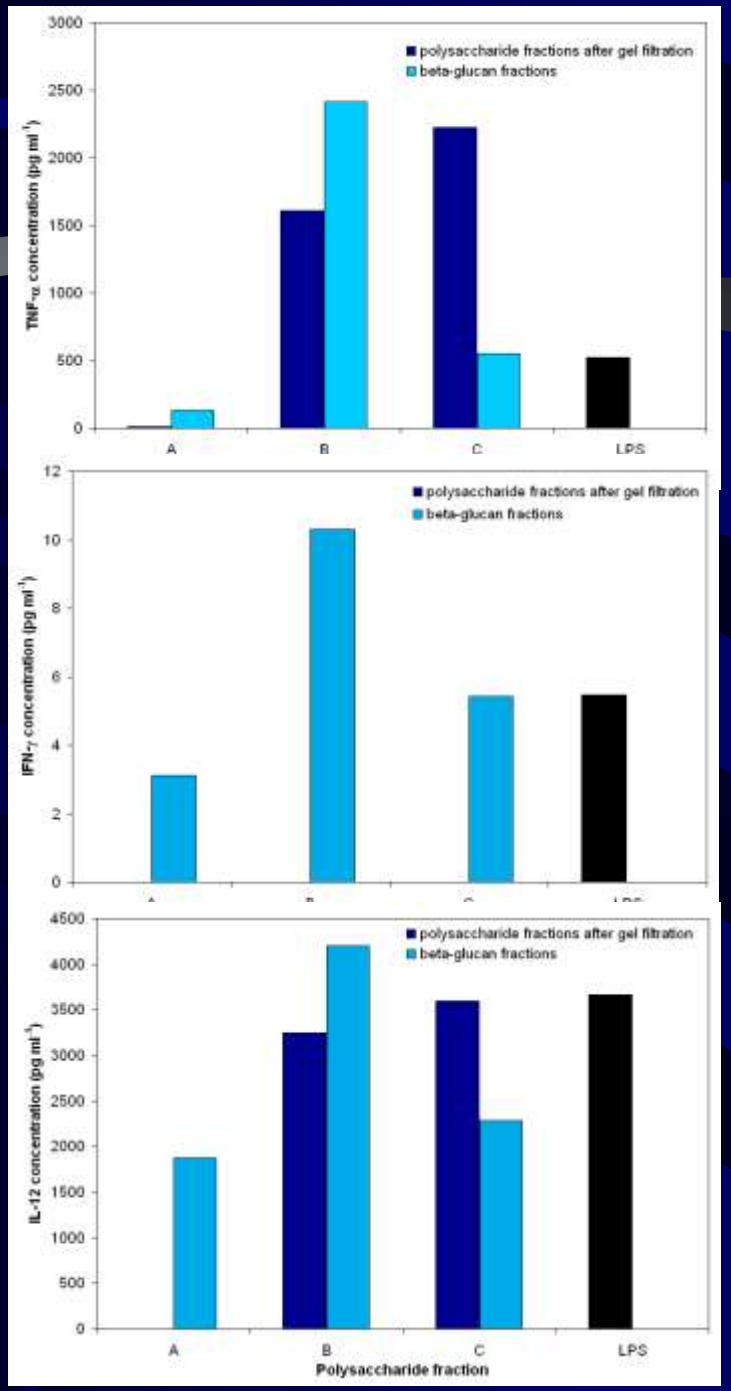
followed by Fraction C obtained after gel filtration, compared to inducing capacity of LPS.

Effect of polysaccharides on primary inflammatory immune response - summary

Intracellular polysaccharides (Fractions B , 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).

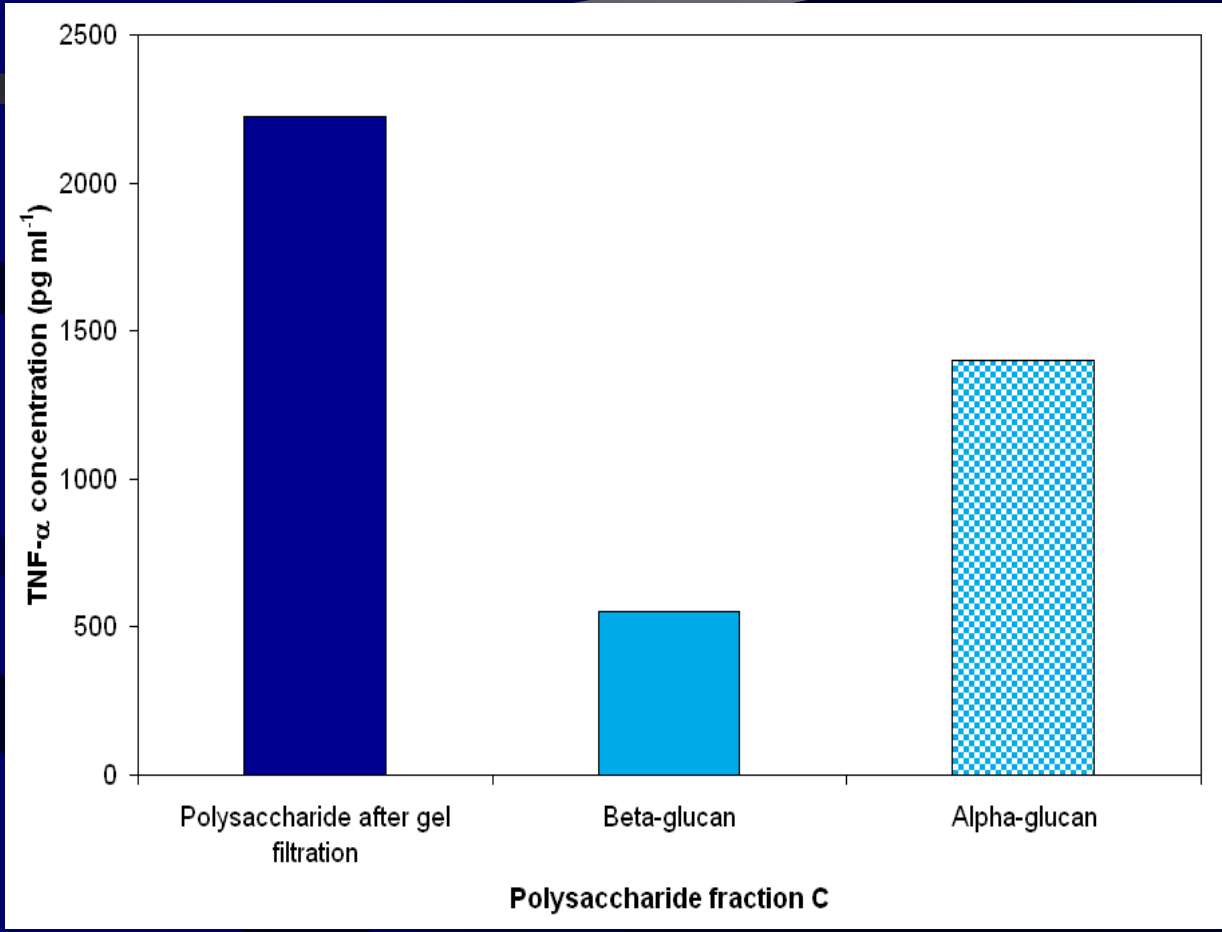
In general β-glucan fractions induce stronger inflammatory response than fractions obtained after gel filtration. Exceptions are observed at fractions C in case of TNF-α and IL-12 response.



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 C fraction 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 C fraction α-glucan in concentration 100 μg ml-1, the supernatants were screened for the content of TNF-α after a 4-h incubation.

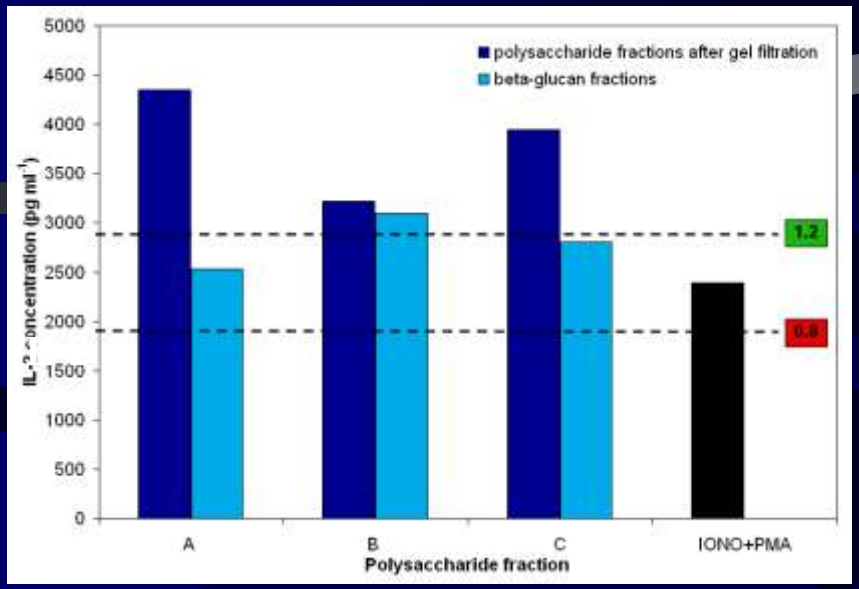
TNF-α inducing capacity of Fraction C α-glucan

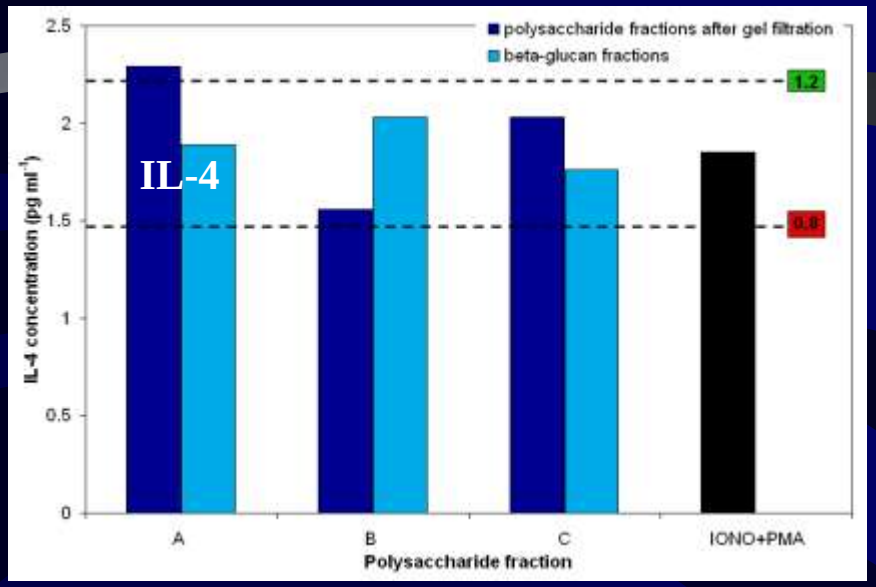


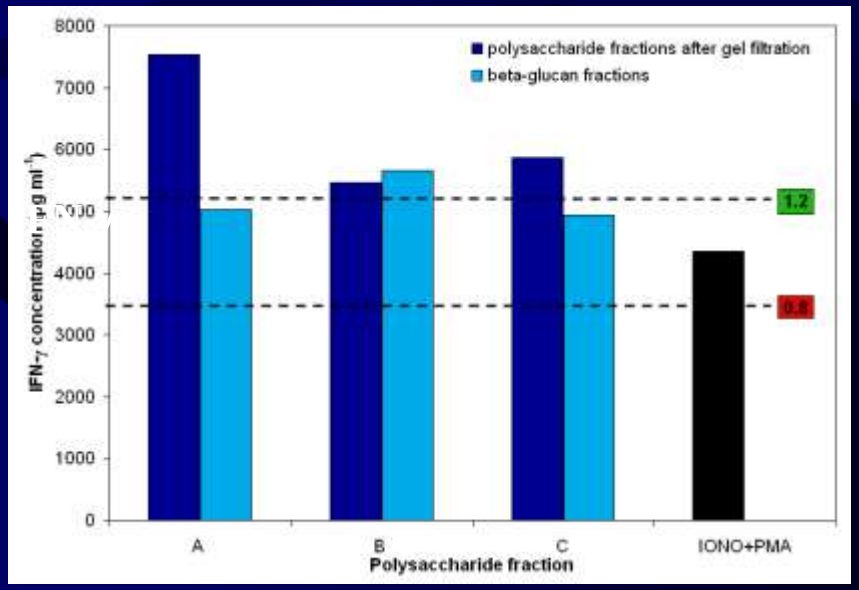
α-glucan demonstrates 2.5-higher capacity to induce TNF-α response than β-glucan.

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

Th1 vs. Th2 immuno response







Polysaccharides direct lymphocyte response into Th1

- IL-2 and IFN-γ positive response and

Polysaccharides do not direct lymphocyte response into Th2

- IL-4 negative to neutral response

+ IMMUNOMODULATION WITH POLYSACCHARIDES A,B,C
POLYCLONAL ACTIVATION:
PMA – Phorbal miristate acetat
IONO – Ionomycine

Conclusions

Original strains of *Ganoderma lucidum* (MZKI G97) was isolated from Slovenian forests.

Submerged and solid state cultivations were applied.

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.

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

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) then 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 intracellular polysaccharides. The highest effect is observed with fractions obtained after gel filtration.
- Polysaccharides direct lymphocyte response into Th1 reponse.

Research Team

Research Team

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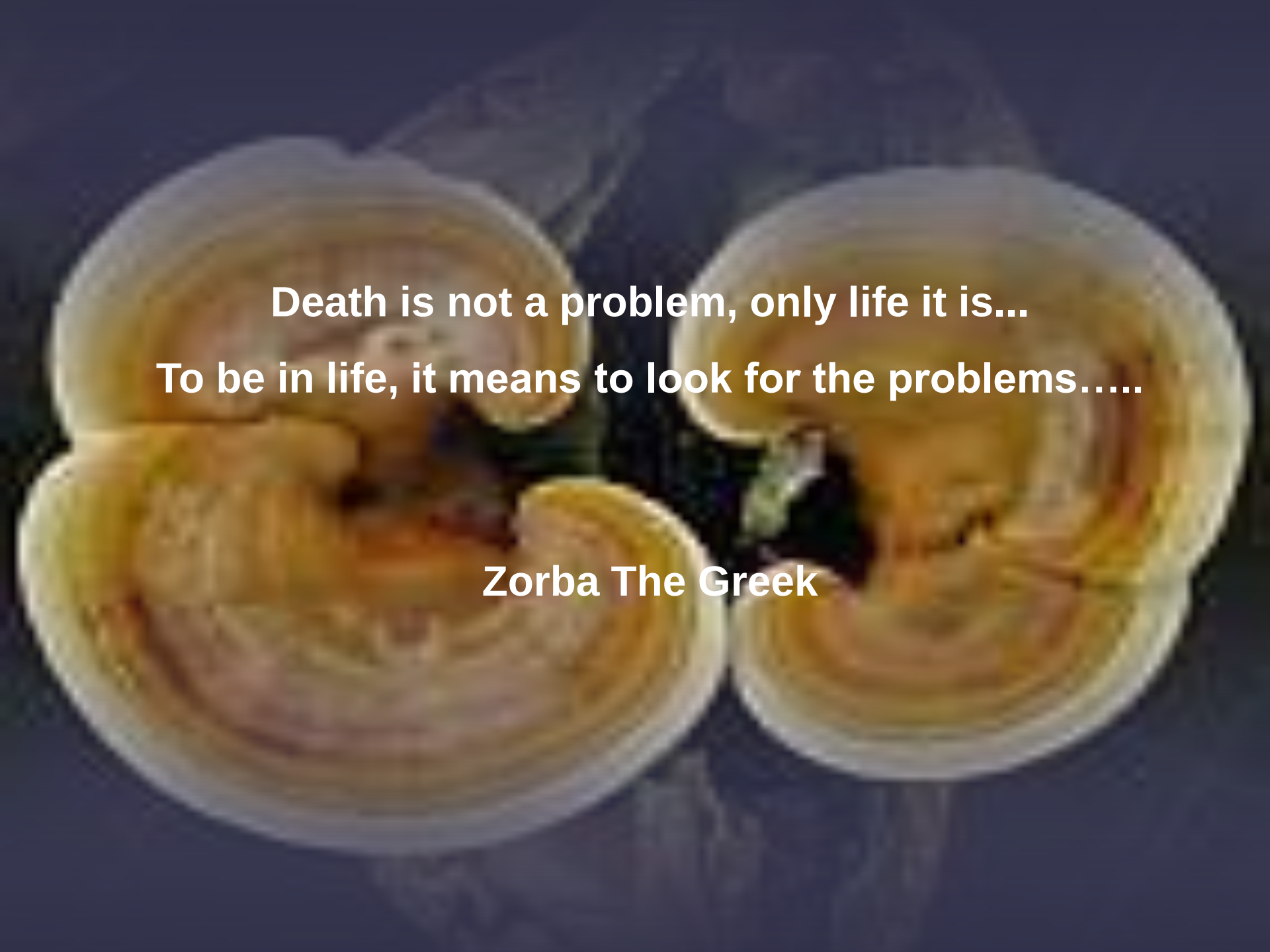
Death is not a problem, only life it is...
To be in life, it means to look for the problems…..
Zorba The Greek

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 imunski odziv. T celice