Polysaccharides from Finnish Fungal Resources

GABRIELE BELTRAME

Food Chemistry and Food Development
Department of Life Technologies

Polysaccharides from Finnish Fungal Resources

GABRIELE BELTRAME

Food Chemistry and Food Development
Department of Life Technologies
University of Turku, Finland

Fróðr er hverr fregnvíss

TABLE OF CONTENTS

ABSTRACT

Mushrooms are the second largest edible biomass available from Finnish forests. Their main components are non-digestible polysaccharides such as β-glucans, mannans, and galactans. These molecules have been shown to possess a large variety of biological activities. These activities are determined by their physicochemical properties, like sugar composition, glycosidic linkage and molecular weight, which are significantly influenced by the fungal species and extraction methods. Currently, the low collection of natural basidiomycete production leaves the edible mushroom biomass largely unused. Development of β-glucan-oriented isolation and purification schemes can increase the value of Finnish mushrooms, meeting the rapidly growing international market for functional foods and nutraceuticals.

Usually, β-glucans are extracted from fungi with hot water. Higher yield and purity of these molecules can be achieved by pre-treatments and harsher extracting conditions. For a complete understanding of influence of processing parameters on composition and properties of polysaccharides, thorough characterization of molecular properties of the obtained polysaccharides is also required. Seasonal variation, slow growth and logistic problems might hamper the mushroom industry. Currently, two strategies are studied to overcome these issues: cultivation of fungal mycelium in liquid medium (submerged cultivation) or on solid substrate (solid-state cultivation). The produced biomasses can be readily used as source of fungal polysaccharides. Submerged cultivation is further favored by low cost and fast production. Moreover, the use of lignocellulosic, such as agricultural sidestreams, and liquid, such as oils, supplements to the medium has been shown to increase mycelial yield.

The aim of the current work was to extract polysaccharides from Finnish fungal resource and determine their yield and properties utilizing colorimetric, chromatographic, and spectroscopic techniques. The cultivated *Agaricus bisporus*, wild *Craterellus tubaeformis*, and rare and slow-growing *Inonotus obliquus* were investigated as polysaccharide resource. The effects of a pretreatment with ethanol-hydrochloric acid mixture, prior to sequential hot water extractions, were investigated using *A. bisporus*. Cell wall polysaccharides were extracted from *C. tubaeformis* with a sequential protocol of increasing harshness using hot water and alkaline solutions of different concentrat. Cultivated mycelium of *I. obliquus* was considered as an alternative of the sterile conk, commonly known as Chaga, produced by this species as polysaccharide source. Sea buckthorn (*Hippophaë rhamnoides*) berry press cake, a fibrous side stream with high oil content, was supplemented to the submerged cultivation medium of *I. obliquus*. Exopolysaccharides (EPS) from culture media and

intracellular polysaccharides (IPS) extracted from mycelia were investigated. Moreover, the potential of the birch heart rot, resulted from infection by this species, as polysaccharide source has been assessed in this work for the first time. Polysaccharides from wild sterile conk, cultivated mycelium, and heart rot were extracted, characterized, and compared.

The pretreatment with acid and the use of alkali during the extraction resulted in disruption of the cell wall, which facilitated the solvent access to its deeper layers. The harsher methods increased extraction yields and the β-glucan contents in the extracts, while decreasing the molecular weight of the polysaccharides. In case of *C. tubaeformis*, hot water was an inefficient extraction medium for β-glucans. Structural investigation assigned to these polymers a backbone of →6)-β-Glc-(1→ units with short and frequent branches in O-3 position. The thermal stability of fungal polysaccharides was drastically reduced only after strong alkali extraction. Supplementation with press cake increased the cultivation yield of *I. obliquus*. The supplementation positively affected the yield and molecular weight of EPS. However, at higher dosages, the supplementation reduced content and the molecular weight of IPS. The pectins in the sea buckthorn press cake affected to small extent the monomer composition of both IPS and EPS. The polysaccharides extracted from cultivated mycelium showed high abundance of glycogen, absence of phenolic compounds, and polydispersed polymer populations, while the opposite was observed for sterile conk polysaccharides. Structurally similar β-glucan was identified in sterile conk and mycelium extracts. On the other hand, heart rot polysaccharides were mainly composed of hemicelluloses. However, fungal polysaccharides were identified as well, particularly in the hot water extract, showing potential also for the birch leftover after sterile conk harvesting.

The results of the present doctoral thesis offer valuable information for the exploitation of Finnish fungal species as sources of bioactive polysaccharide (in particular β-glucan) aimed to the nutraceutical market. Moreover, this thesis could be a starting point for more in-depth studies on the effect of liquid culture supplementation on the macromolecular properties of mycelial polysaccharides.

SUOMENKIELINEN ABSTRAKTI

Sienet ovat Suomen metsien toiseksi suurin syötävä biomassa. Niiden pää-komponentit ovat sulamattomia polysakkarideja, kuten β-glukaaneja, mannaa-neja ja galaktaaneja. Näillä molekyyleillä on osoitettu olevan laaja valikoima biologisia aktiivisuuksia. Nämä toiminnot määräytyvät niiden fysikaalis-kemiallisten ominaisuuksien, kuten sokerikoostumuksen, glykosidisidosten ja molekyylipainon perusteella, joihin sienilajit ja uuttomenetelmät vaikuttavat merkittävästi. Suurin osa syötävien sienten biomassasta jää tällä hetkellä käyttämättä, koska kantasieniä ei kerätä riittävästi. Suomalaisten sienien arvoa voi lisätä β-glukaanipohjaisten eristämis- ja puhdistusjärjestelmien kehittäminen, jolloin voidaan tuottaa raaka-aineita nopeasti kasvaville kansainvälisille funktionaalisten elintarvikkeiden ja ravintoaineiden markkinoille.

Yleensä β-glukaanit uutetaan sienistä kuumalla vedellä. Esikäsittelyillä ja ankarammilla uutto-olosuhteilla voidaan saavuttaa näiden molekyylien suurempi saanto ja puhtaus. Jotta ymmärrettäisiin täysin käsittelyparametrien vaikutus polysakkaridien koostumukseen ja ominaisuuksiin, vaaditaan myös saatujen polysakkaridien molekulaaristen ominaisuuksien perusteellista karakterisointia. Kausivaihtelut, hidas kasvu ja logistiikkaongelmat saattavat haitata sieni-teollisuutta. Tällä hetkellä tutkitaan kahta strategiaa näiden ongelmien ratkaisemiseksi: sienirihman viljely nestemäisessä väliaineessa (upotusviljely) tai kiinteällä alustalla (*solid state*-viljely). Tuotettua biomassaa voidaan helposti käyttää sienipolysakkaridien lähteenä. Upotettua viljelyä suosivat lisäksi edulliset kustannukset ja nopea tuotanto. Lisäksi lignoselluloosaa sisältävien maatalouden sivuvirtojen, ja nesteiden, kuten öljyjen, käyttö kasvatusalustan ravintolisänä on osoittanut lisäävän sienirihmaston satoa.

Tämän työn tavoitteena oli uuttaa polysakkarideja suomalaisista sieni-resursseista ja määrittää niiden saanto ja ominaisuudet käyttämällä kolori-metrisiä, kromatografisia ja spektroskooppisia tekniikoita. Polysakkaridien lähteenä tutkittiin viljeltyä *Agaricus bisporusta* (herkkusieni), villiä *Craterellus tubaeformista* (suppilovahvero) ja harvinaista ja hitaasti kasvavaa *Inonotus obliquusta* (pakurikääpä). Esikäsittelyn vaikutuksia etanoli-suolahapposeoksella ennen peräkkäisiä kuumavesiuuttoja tutkittiin käyttämällä *A. bisporusta*. Soluseinän polysakkarideja uutettiin *C. tubaeformista* menetelmällä, jonka voimakkuutta lisättiin käyttämällä peräkkäin kuumaa vettä ja eri konsentraation emäksisiä liuoksia. *I. obliquusi*n viljeltyä rihmastoa pidettiin vaihtoehtoisena polysakkaridilähteenä steriilille kasvustolle, joka tunnetaan yleisesti nimellä pakuri. Tyrnin (*Hippophaë rhamnoides*) -marjapuristuskakulla, joka on kuitumainen sivuvirta ja jolla on korkea öljypitoisuus, täydennettiin *I. obliquusin* nestemäistä kasvatusainetta. Kasvatusaineen eksopolysakkarideja (EPS) ja

sienirihmastosta uutettuja solunsisäisiä polysakkarideja (IPS) tutkittiin. Lisäksi tämän lajin tartunnasta syntyvän koivun ydinlahon potentiaali polysakkaridien lähteenä on arvioitu tässä työssä ensimmäistä kertaa. Luonnonvaraisen steriilin kasvuston, viljellyn sienirihmaston ja ydinlahon polysakkarideja uutettiin, karakterisoitiin ja niiden koostumusta vertailtiin.

Esikäsittely hapolla ja emäksen käyttö uuton aikana johti soluseinän hajoamiseen, mikä helpotti liuottimen pääsyä sen syvempiin kerroksiin. Rajummat menetelmät lisäsivät uuton saantoa ja uutteiden β-glukaanipitoisuutta samalla kun polysakkaridien molekyylipaino pieneni. Kuuma vesi oli tehotonta β-glukaanin uuttamiseksi *C. tubaeformista*. Rakenteellinen tutkimus osoitti näiden polymeerien koostuvan →6)-β-Glc-(1→ -yksiköistä, joilla on lyhyet ja usein esiintyvät haarat O-3-asemassa. Sienipolysakkaridien lämpövakaus heikkeni jyrkästi vasta voimakkaan alkaliuuton jälkeen. Tyrnin puristekakku lisäsi *I. obliquusin* kasvua viljelyssä. Täydennys vaikutti positiivisesti EPS:n saantoon ja molekyylipainoon. Kuitenkin suuremmilla annoksilla lisäravinteet pienensivät IPS:n pitoisuutta ja molekyylipainoa. Puristekakun pektiinit vaikuttivat hieman sekä IPS:n että EPS:n monomeerikoostumukseen. Viljellystä sienirihmastosta uutetuissa polysakkarideissa esiintyi runsaasti glykogeenia, mutta ei fenolisia yhdisteitä ja polymeeripopulaatiot olivat polydispergoituneita, kun taas steriilin kasvuston polysakkarideilla havaittiin päinvastainen. Rakenteellisesti samankaltaista β-glukaania tunnistettiin steriilistä kasvustosta ja sienirihmastouutteista. Toisaalta sydänlahon polysakkaridit koostuivat pääasiassa hemiselluloosista. Kuitenkin myös sienipolysakkarideja tunnistettiin, etenkin kuumavesiuutteessa, mikä osoittaa potentiaalia myös koivujätteille steriilin kasvuston keräämisen jälkeen.

Tämän väitöskirjan tulokset tarjoavat arvokasta tietoa suomalaisten sienilajien hyödyntämisestä bioaktiivisen polysakkaridin (erityisesti β-glukaanin) lähteinä funktionaalisten elintarvikkeiden ja lisäravinteiden markkinoille. Lisäksi tämä opinnäytetyö voisi olla lähtökohta perusteellisemmille tutkimuksille nestemäisen viljelmän täydentämisen vaikutuksesta sienipolysakkaridien makromolekulaarisiin ominaisuuksiin.

LIST OF ABBREVIATIONS

ANOVA   analysis of variance
Ara   arabinose
ATR   attenuated total reflection
BRM   biological response modifier
C/N   carbon/nitrogen
CAT   catalase
COSY   correlation spectroscopy
COX   cyclo-oxigenase
CR   complement receptor
d.w.   dry weight
DB   degree of branching
DHN   1,8-dihydroxynaphtalene
DMSO   dimethylsulfoxide
DOPA   3,4-dihydroxyphenylalanine
EPS   exocellular polysaccharide
FID   flame-ionization detector
FT-IR   Fourier-transform infrared spectroscopy
Fuc   fucose
Gal   galactose
GalA   galacturonic acid
GalN   galactosamine
GALT   gut-associated lymphoid tissue
GC   gas chromatography
Glc   glucose
GlcA   glucuronic acid
GlcN   glucosamine
GPI   glycosylphosphatidylinositol
GSH   glutathione peroxidase
HMBC   heteronuclear multiple bond correlation
HMDS   hesamethyldisilazane
HPSEC   high performance size-exclusion chromatography
HSQC   heteronuclear single quantum coherence
IL   interleukin
IPS   internal polysaccharide
LPS   lipopolysaccharide
Man   mannose
MAPK   mitogen-activated protein kinase
MS   mass spectrometry
NK   natural killer

NMR	nuclear magnetic resonance
NO	nitric oxide
NOESY	nuclear overhauser effect spectroscopy
NOS	NO synthase
POD	peroxidase
Ref.	reference
Rha	rhamnose
RID	refractive index detector
ROS	reactive oxygen species
SCFA	short chain fatty acid
SOD	superoxide dismutase
sp.	species
spp.	several species
TCA	trichloroacetic acid
Th	T-helper
TLR	toll-like receptor
TMSC	trimethylsilylchloride
TNF	tumor necrosis factor
TOCSY	total correlation spectroscopy
v/v	volume/volume ratio
w/w	weight/weight ratio
Xyl	xylose

LIST OF ORIGINAL PUBLICATIONS

I. Trygg, J.; Beltrame, G.; Yang, B. Rupturing fungal cell walls for higher yield of polysaccharides: Acid treatment of the basidiomycete prior to extraction. *Innov. Food Sci. Emerg.* **2019**, 57, 102206.

II. Beltrame, G.; Trygg, J.; Rahkila, J.; Leino, R.; Yang, B. Structural investigation of cell wall polysaccharides extracted from wild Finnish mushroom *Craterellus tubaeformis* (Funnel Chanterelle). *Food Chem.* **2019**, 301, 125255.

III. Beltrame, G.; Hemming, J.; Yang, H.; Han, Z.; Yang, B. Effects of supplementation of sea buckthorn press cake on mycelium growth and polysaccharides of *Inonotus obliquus* in submerged cultivation. *J. Appl. Microbiol.* **2021**, 131 (3), 1318.

IV. Beltrame, G.; Trygg, J.; Hemming, J.; Han, Z.; Yang, B. Comparison of Polysaccharides Extracted from Cultivated Mycelium of *Inonotus obliquus* with Polysaccharide Fractions Obtained from Sterile Conk (Chaga) and Birch Heart Rot. *J. Fungi* **2021**, 7, 189.

1   INTRODUCTION

Mushrooms are the fruiting bodies produced by fungi belonging to basidiomycete and ascomycete phyla. They have been consumed as a food and medicinal source for millennia throughout the world. Mushrooms are appreciated for their texture and flavor. They are rich in dietary fiber and their proteins contain all the essential ammino acids, while they have low fat and caloric content[1]. The inclusion of mushrooms in the diet has a continuous tradition in Asian countries, where the first written source on their utilization is also attested. China leads the world mushroom production, with 8×10^6 tonnes reported in 2019, followed by European Union, Japan, and United States[2].

The traditional utilization of mushrooms and mushroom extracts as medicine is strongly attested in Asia and Eastern Europe, with particular use of the species *Ganoderma lucidum* (Lingzhi in Chinese and Reishi in Japanese) and *Lentinula edodes* (Shiitake in Japanese) in the earlier and *Inonotus obliquus* (*Inonotus obliquus* (Ach. ex Pers.) Pilát) (commonly called Chaga) and *Fomitopsis* spp. (different species of bracket fungi). The research on the chemistry of mushroom extracts started from their traditional uses, particularly against cancer. The evidence of the biological properties of compounds extracted from mushrooms has lead the attention of scientific research to the species of interest for the western countries, such as the cultivated *Agaricus bisporus* (*Agaricus bisporus* (J.E. Lange) Imbach) (white button) or the mushrooms that can be collected from the forests.

Polysaccharides, the main components of mushrooms, are the molecular class that have received the most attention from the research. The main fungal polysaccharides are the β-glucans, which are polymers of mainly →3)-β-Glc-(1→ and →6)-β-Glc-(1→ units. Other important fungal polysaccharides are the heterosaccharides and the mannans, with great variety in structure and constituting monomers. Most of mushroom polysaccharides have structural roles in the fungal cell wall, while others constitute energy reserve. Polysaccharides are also secreted outside the fungal cell as defensive or colonization mechanism. These molecules are not digested by the human gastrointestinal tract. Numerous biological activities have been assigned to these molecules, such as immunomodulatory, hypocholesterolemic, hypoglycemic, and prebiotic properties. The biological activities of mushroom polysaccharides are correlated to their chemical properties, such as monomer composition, glycosidic linkage, conformation, molecular weight, and presence of covalently-linked moyeties. These properties are influenced by the mushroom species and the extraction method used[3].

Nowadays, mushrooms are considered functional food and an attractive source of fractions with bioactive properties for the dietary supplement market.

The reported market value of dietary supplements obtained from mushrooms is 18 billion dollars[4]. Finland, whose surface is 70% covered by forests, has a large production of forest mushrooms. Between 2015 and 2019, the market volume of wild mushrooms has oscillated in the approximate range $2-6 \times 10^2$ tonnes[5]. In contrast, the forest production has been estimated between $8-25 \times 10^5$ tonnes[6]. The fungal biomass of the Finnish forests is therefore left largely unused. The utilization of this forest biomass for the extraction of polysaccharides would increase their value and stimulate the harvesting.

The forest mushroom yield is subject to seasonal variations. Some species of interest, such as the birch obligate parasite *I. obliquus*, grow slowly and/or are less frequent. The research on the cultivation of this species on dedicated birch stands is still in its infancy. A more common strategy is the cultivation of the fungal mycelium in liquid medium (submerged cultivation). The biomass swiftly produced with this procedure could be readily utilized as a source of polysaccharides. Moreover, the liquid medium allows the use of supplements to increase the cultivation yield. The utilization of agro-forestry sidestreams as supplements for fungal cultivation is a strategy to increase their value and support circular economy.

The most common method to extract bioactive polysaccharides from fungi is hot water, with generally low yields. However, the utilization of different solvents and the use of pre-treatments can improve yield and purity of the polysaccharides. On the other hand, different extraction methods would have an influence on the molecular properties influencing the biological activities.

The present doctoral dissertation focused on investigation on alternative sustainable methods for producing and extracting bioactive polysaccharides from Finnish mushrooms. The starting materials were three selected fungal species, as representative of three arbitrary categories: 1. *Agaricus bisporus*, for mushrooms cultivated in large scale; 2. *Craterellus tubaeformis* (*Craterellus tubaeformis* (Fr.) Quél.) (Funnel chanterelle) for mushrooms largely available from the forests but underutilized; 3. *Inonotus obliquus* for rare and slow-growing fungi. These three species represent also three distinct ecological groups: saprotrophic, mycorrhizal, and wood-rotting, respectively. The aim of the research project is to develop strategies for the production of polysaccharide-rich fractions. For categories 1 and 2, the research investigated extraction methods from the mushroom biomass. For category 3, the utilization of a Finnish agricultural sidestream was tested for the production of mycelium aimed to polysaccharide production. In addition, the birch stem colonized by *I. obliquus*, sidestream from harvesting and cultivation, has also been investigated as potential source of polysaccharides.

2 REVIEW OF THE LITERATURE

2.1 Mushrooms

Fungi are eukaryotic (the cell has a membrane-enveloped nucleus) and heterotrophic organisms possessing a chitinaceus cell wall and lacking chloroplasts. They reproduce both sexually and asexually through spores and form filamentous structures called hyphae. Multiple hyphae produce a network called mycelium. They have different ecological roles, such as symbiotic, saprotrophic, or parasitic. The ability of fungi to degrade dead organic material (saprotrophic nutrition) is important for the carbon cycle. Fungal symbiosis with plants (mycorrhiza) facilitates nutrition uptake by the plant from the soil, while the fungus receives nutrients from plant.

Fungi represent one of the taxonomic kingdoms of organisms. The kingdom, according to Hibbett and coauthors, is divided in seven phyla: Chytridiomycota, Neocallimastigomycota, Blastocladiomycota, Microsporidia, Glomeromycota, Ascomycota, and Basidiomycota. The latter two are grouped in a subkingdom called Dikarya[7] and are the ones of interest for the present work. The structures producing the spores responsible of Dikarya sexual reproduction are carried on specialized structures called sporocarps or fruiting bodies. The sporocarp of Ascomycota is called ascocarp and the spore-producing structure is called ascus, which has typically a bag-like shape. Here, usually eight spores are produced after karyogamy (two nuclei of the ascus cell fuse), meiosis, and mitosis. The parallel of the ascus for basidiomycetes is the basidium, which produces typically four spores, after karyogamy and meiosis, kept on an external structure[8]. Both ascocarp and basidiocarp exist in a plethora of different shapes.

Table 1. Taxonomic classification of the fungal species studied in this dissertation.

English name	Button mushroom	Funnel chanterelle	Chaga
Finnish name	Herkkusieni	Suppilovahvero	Pakuri
Kingdom	Fungi	Fungi	Fungi
Phylum	Basidiomycota	Basidiomycota	Basidiomycota
Class	Agaricomycetes	Agaricomycetes	Agaricomycetes
Order	Agaricales	Cantharellales	Hymenochaetales
Family	Agaricaceae	Cantharellaceae	Hymenochaetaceae
Genus	*Agaricus*	*Craterellus*	*Inonotus*
Species	*A. bisporus*	*C. tubaeformis*	*I. obliquus*

Mushrooms have been defined as "macrofungus with distinctive fruiting body which can be hypogeous or epigeous, large enough to be seen with the naked eye and picked by hand"[9]. A corollary followed: "mushrooms need not to be basidiomycetes, nor fleshy, nor edible"[9]. Mushrooms have commonly an umbrella-shape structure, with cap and stem. Caps can assume different shapes

and stems can have a ring and/or a cup. However, mushroom can have structures totally different from umbrella. Some fungal species belonging to Ascomycota phylum produce fruiting bodies commonly considered mushrooms, for example those of the genus *Morchella*. On the other hand, some basidiomycetes do not produce fruiting bodies and are single-cell forms, like the Cryptococci.

In 2008, the number of fungal species has been estimated around 90000. Of these, about 30000 were basidiomycetes and most of the remaining were ascomycetes[8]. More recently, the number of fungal species has been estimated in the range 2.2-3.8 million[10]. The Catalogue of Life reports about 146000 species, of which 63% are ascomycetes and 35% basidiomycete. Most of the fruiting body-producing species belong to the class Agaricomycetes of the phylum Basidiomycota, representing about 38000 species[11]. All Agaricomycetes are fruiting body producers, with great variety in shape and structure[12]. The taxonomic classifications of the species under the focus of the present doctoral dissertation are reported in **Table 1**. The phylogenetic relationships between different orders of Agaricomycetes with relevance to this dissertation are reported schematically in **Figure 1**. On the ecological point of view, this class contains saprotrophic species, such as the cultivated *A. bisporus* and *L. edodes* or wild and rare *Ganoderma* spp., and ectomycorrhizal *Boletus* spp. and *Cantherellus* spp. Typical wood-decaying mushrooms such as *Fomes* and *Fomitopsis* spp. belong to this class as well. Others, such as *Phellinus* spp., can be considered plant pathogens[13].
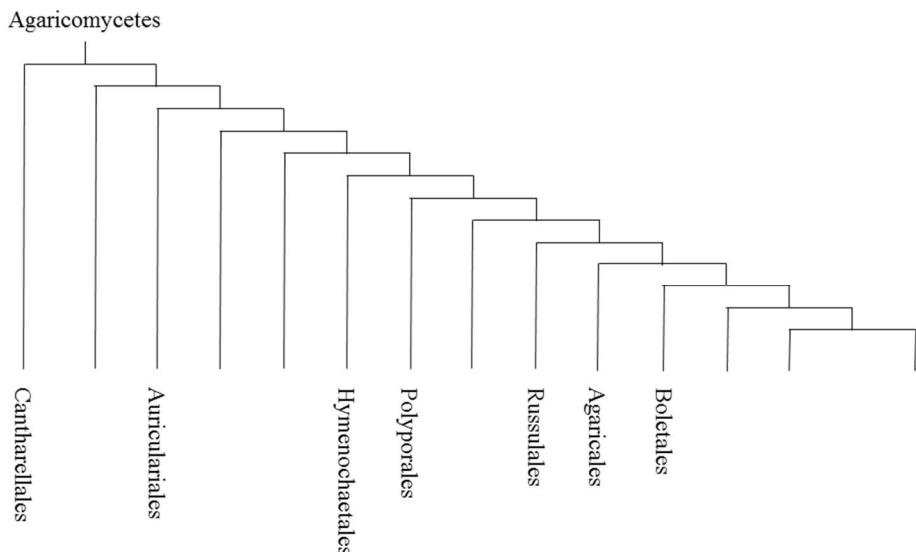
Figure 1. Phylogenetic relationship of different orders of Agaricomycetes with relevance to this doctoral dissertation. Adapted from[14].

2.1.1 The uses of mushrooms

Mushrooms have been traditionally used as food and medicine for millennia. Reportedly, the first archeological record of mushroom consumption was found in Chile[15]. The ancient utilization of mushroom is however most attested in the Eurasian landmass. Fragments of *Fomitopsis betulina* (birch polypore) and *Fomes fomentarius* (tinder fungus) were carried by the Ötzi mummy (3500 BC), found at the Italian-Austrian border in the Alps, although their purpose is not clear [16]. First written record of wild mushroom consumption is found in China[15]. In western culture, the consumption of mushrooms goes back to the Classical era, when they were considered "food of the Gods" [1]. Mushrooms were highly valued by the Roman ruling class, as indicated by the name of *Amanita cesarea* (Caesar's mushroom) or by Epigrams of the poet Martial, in which mushrooms are referred as valuable food (Book 13) and offerings (Book 3)[17]. While in Asia mushroom consumption has a continuous tradition, Western Europe became mycophobic between the fall of the Roman Empire and the onset of Middle Ages and its mushroom consumption increased more recently[16]. On the other hand, Easter Europe, Italy, and France remained more mycophilic[15,16]. On the worldwide scale, mushroom consumption is increasing. Only as example, both value and volume of mushroom sales have increased in the United States since 2013[18]. Nevertheless, as Boa stated, "(Globally) the use of wild edible fungi is both extensive and intensive, though patterns of use do vary". Nowadays, mushroom harvesting constitutes a source of income for developing countries and local farming practices all over the world[15].

Mushrooms are commonly consumed as food and food flavoring agent. They are appreciated by consumers for their aroma and texture. Moreover, their nutritional properties make them suitable for vegetarian and vegan diets. The main component of fresh mushrooms is water, between 82-92 w/w%[19], making their dry matter content low. In this way, the overall caloric intake of mushrooms is also low[1]. The main components of mushrooms are carbohydrates and proteins, generally followed by ashes and lipids. The proximate chemical composition of selected mushroom species is reported in **Table 2**. There are noticeable differences in contents between different species. The composition is also influenced by growth medium, growth stage and analyzed mushroom part (whole, cap, or stipe). Moreover, comparison of different reports shows noticeable variation within the same species[19–21].

Table 2. Proximate composition of selected mushroom species.

Species	Dry matter	Crude protein	Lipids	Ash	Carbohydrates	Energy	Ref.
	g/kg f.w.			g/kg d.w.		kcal/kg f.w.	
Agaricus bisporus	77.0	271.0	43.0	43.0	584.0	270	[22]
Agaricus campestris	118.3	185.7	1.1	231.6	581.6	364	[19]
Armillaria mellea	117.3	163.8	55.6	67.8	712.8	470	[19]
Boletus edulis	108.5	210.7	24.5	55.3	709.5	423	[19]
Cantharellus cibarius	n.r.[a]	357.9	14.7	64.2	563.2	n.r. [a]	[19]
Craterellus tubaeformis	n.r.	296.0	53.8	127.9	432.3	n.r. [a]	[23]
Flammulina velutipes	93.2	178.9	18.4	94.2	708.5	346	[19]
Lentinula edodes	84.0	214.0	37.0	58.0	690.0	300	[22]
Lactarius deliciosus	n.r. [a]	202.0	80.2	71.5	646.3	n.r. [a]	[19]
Pleurotus ostreatus	n.r. [a]	132.3	35.8	80.8	751.1	n.r. [a]	[19]
Russula delica	133.1	505.9	9.1	229.3	255.7	416	[19]
Suilus variegatus	92.3	175.7	33.1	153.6	637.6	328	[19]

[a]not reported

Ulziijargal and Mau reviewed the proximate composition of different mushrooms, differentiating the fractions constituting the total carbohydrate compositions of fungi, i.e. reducing sugars, soluble polysaccharides, and insoluble fiber. For selected mushrooms, **Table 3** reports the relative amounts of these carbohydrate components as percentage of total carbohydrate. **Table 3** includes also the total carbohydrate content, as reported by Ulziijargal and Mau[24]. Noticeably, some species have higher content of insoluble fiber (like *G. lucidum*), while others have more soluble polysaccharides. Only a small fraction of mushroom polysaccharides is glycogen, in the range 50-100 g/kg d.w. Most of the polysaccharides include cell wall structural components, such as β-glucans, mannans, and chitin[1,19]. These polymers are not digested by the human gastro-intestinal tract and therefore posses the ability to act as dietary fiber[22].

Reducing sugars were quantified spectrophotometrically. Therefore, the amounts reported do not necessarily coincide with the major free sugars present in mushrooms, which include mannitol and trehalose (α-Glc-(1→1)-α-Glc), besides glucose and mannose[19]. Trehalose and mannitol are the most abundant free carbohydrates in mushrooms, although their amounts vary considerably both among different species and within the same species. For example, reported amounts of mannitol and trehalose in *B. edulis* were 24.5 and 58.8 g/kg d.w. and 124.0 and 32.7 g/kg d.w., respectively. On the other hand, reported amounts in

cultivated *A. bisporus* were 641 and 18.3 g/kg d.w. for mannitol and trehalose, respectively[19]. Nevertheless, it has been reported that, generally, mycorrhizal mushrooms have higher total sugar content than saprotrophic ones[25].

Table 3. Carbohydrate composition of selected mushroom species.

Species	Reducing sugars	Soluble polysaccharides	Insoluble fiber	Total carbohydrate g/100 g d.w.
		relative carbohydrate content w/w%		
A. bisporus	22.4%	44.8%	32.9%	62.2
B. edulis	11.9%	68.5%	19.6%	69.9
F. velutipes	21.6%	53.5%	24.9%	64.2
G. lucidum	17.8%	12.8%	69.5%	85.2
Grifola frondosa	15.6%	69.8%	14.6%	68.8
L. edodes	38.0%	53.7%	8.3%	67.9
P. ostreatus	29.6%	62.4%	8.0%	66.4

Table data elaborated from[24].

The importance of mushrooms in traditional medicine is strongly attested in China, with the use of *G. lucidum* going back to thousands of years[13]. Wasser estimated that there are about 700 mushroom species known to possess pharmacological properties[26], while Boa estimated the number of mushroom species currently utilized for both food and medicinal purposes to be close to 250, and 133 species used solely for medicinal purpose[15]. The commercial interest for Chinese traditional remedies originating from mushrooms have sparked interest also in traditional uses outside China. The traditional use of mushrooms is nowadays attested all over the world. Noticeably, many of these traditional medicines of different cultures incorporate members of the Polyporales order, possibly due to a connection between perennial nature and human wisdom[27]. The cultivation of a wide array of Agaricomycetes for nutritional and medicinal purposes is mentioned in the Korean "History of the Three Kingdoms" of 1145[18]. Mushrooms are part of the traditional medicine of the Khanty, an indigenous population of West Siberia speaking an Ugric language (Uralic language family). Saar has reported different mushrooms as members of their traditional medicine including *I. obliquus*, *Fomes fomentarius*, *Amanita muscaria*, and *Phellinus nigricans*. They are consumed as tea, smoked, or applied as powder. Mushrooms were used to cure and prevent diseases, cleaning, teeth problems, and bleeding[28]. Recently, an excursus on the mushrooms of European traditional medicine has been published. Among the different mushrooms and the many reported uses, *Auricularia auricula* was used for treating sore throat, *F. fomentarius* for wound dressing and gastric disorders, *F. betulina* for cancer and as an immune system enhancer, and *Fomitopsis officinalis* (the name itself attests medicinal use) for fever, nausea, and weakness[16].

The research on the biological activities of mushrooms started from the aforementioned evidences. Multiple extracts have been produced from

mushrooms and tested in their biological activities. Extensive research has been performed on the ability of the extracts to exert anti-cancer and cytotoxic activities, in relation to the traditional use of mushrooms such as *G. lucidum* and *F. betulina* against various forms of cancer. Different molecules of small and large molecular weight exerting anti-oxidant, cytotoxic, and immunomodulative activities have been isolated utilizing polar and apolar solvents. Extracts and molecules possessing antimicrobial properties have also been obtained with different solvents. The antibacterial compound class of pleuromutilins has been discovered from Agaricomycetes, and some compounds derived from these are nowadays on the pharmaceutical market for human and animal use. Other compound classes are still in the research phase[29]. Moreover, anti-hypercholesterolemia and anti-diabetic effects have been associated with mushroom extracts[30].

Due to the presence of multiple components exercising beneficial effects on the human body, mushrooms are nowadays considered as nutraceuticals or starting material for production of nutraceuticals. Nutraceuticals are foods, part of foods, or food extracts, which are consumed "to promote well-being, through the prevention and/or treatment of diseases/disorders"[30]. In this sense, they have attracted the attention of the market and, as mentioned before, an increasing interest for the consumers, as can be seen by the sale volume of mushrooms and mushroom products. Medicinal mushrooms, i.e. mushroom consumed for their nutraceutical properties, represented 38% of the mushroom marked in 2013, meaning 24 billion USD[31]. The market value of mushroom extracts was reported as 18 billion USD per year[4].

As indicated in **Table 2** and discussed above, the main components of mushrooms are carbohydrates and, more specifically, polysaccharides. Most of them constitute the mushroom cell wall. After the discovery of the immunomodulating properties of polysaccharides extracted with water from mushrooms, great research efforts have been spent on these molecules. Polysaccharides are the most studied mushroom molecules, overshadowing all the other compound classes (Wasser, 2017, oral communication). Polysaccharides are polymers of carbohydrate units, with great variability in type, position and configuration linkage, and degree of polymerization. These properties are crucial, as it will be discussed in the following sections, for their biological activities and for their applications.

2.1.2   Cultivated and wild mushrooms in Finland

Finland is an interesting meeting point of mycophilic and mycophobic cultures. In western Finland, mushroom picking has been generally neglected until French cuisine became popular in Sweden, from where it spread with the aid of the Swedish-speaking upper class. In Eastern Finland, due to the centuries-old

interactions with Russian culture, mycophilic traditions have always prevailed. This divided attitude towards mushrooms was still present in the previous century, due also to the immigration of Karelians from the territories ceded to Soviet Union after WWII[32]. This divide has gradually reduced, but it still exists[33]. A line starting from the city of Helsinki and passing the towns of Jämsä (Central Finland), Muhos (Northern Ostrobothnia), and Kuusamo (Northern Ostrobothnia) would, with approximation, divide the western and eastern attitudes towards mushrooms (Marnila, 2017, personal communication).

Finland is a Nordic country, whose surface consists for about 70% of forests. The south of the country has a humid continental climate, while most of the country has a subarctic climate. The forest dominant type is boreal, with *Picea abies* (Norway spruce) and *Pinus sylvestris* (Scots pine) as dominant trees. Although the tree species favor the yields in mycorrhizal mushrooms, temperature and moisture are the most determining factors influencing the mushroom yield every year[34]. Forest thinning has also an impact on mushroom production, especially regarding mycorrhizal species[35]. On the other hand, impact of forest characteristics on the growth of saprotrophic mushrooms is less evident[34].

In 1947, the production of Finnish forest mushrooms has been estimated to range between $15\text{-}50 \times 10^5$ tonnes, depending on the season. Less than a quarter of this amount would be edible and easily collected[32]. More recently, a range between $8\text{-}25 \times 10^5$ tonnes has been proposed, taking into account seasonal variation and, on a lesser degree, geographical differences. Yield oscillations of one magnitude have been considered normal[6]. Ohenoja and Koistinen estimated that, at least in Northern Finland and during their study (1976-1978), less than the 3% of forest mushrooms have been harvested each year[36].

The annual amounts of forest edible mushrooms collected and sold in the Finnish market are recorded by the Finnish Food Authority and published in the Marsi report[5]. **Figure 2a** reports the forest mushroom amounts sold in the years 2015-2019 and the total revenue for the most relevant species. As can be seen, the market is dominated by *B. edulis*, *Lactarius trivialis*, *Lactarius rufus*, and *Cantharellus cibarius*. Species such as *Craterellus tubaeformis* are considered of lower value, commonly picked and used in Finnish households although less frequently than other species[37]. The Finnish forest mushroom revenue (1.5×10^6 € in 2016 and 4.9×10^5 € in 2019, **Figure 2a**) is dominated by *B. edulis*, *L. trivialis*, and *C. cibarius*. The first species represented 40-70% of total revenue in the year range 2015-2019, while *C. tubaeformis* the 2-10%[5]. The best season yield observed in the studied interval was in 2016, with about 6.0×10^5 kg (6.0×10^2 tonnes) of mushroom (fresh weight) collected and sold (**Figure 2a**), which contrasts with the estimated mushroom yield (even the one for poor seasons). It is noticeable that the mushroom output of Lapland (the largest and northernmost

region of Finland) was almost none and that Eastern Finland had by far the largest output[5]. Nevertheless, a multipurpose forest management, taking into account yields of marketed edible mushrooms (mycosilviculture), would grant up to 25% of the rotation period income, due to the synergy between mushroom and timber harvest[35,38].

Finland has also a mushroom cultivation industry. Its output in terms of kilograms of products (fresh weight) is showed in **Figure 2b**. The production (1.33×10^6 kg – 1.33×10^3 tonnes in 2019) is completely dominated by *A. bisporus*. Other species, *Lentinula edodes* and *Pleurotus ostratus*, constitute 1% or less of the recorded Finnish output[39].
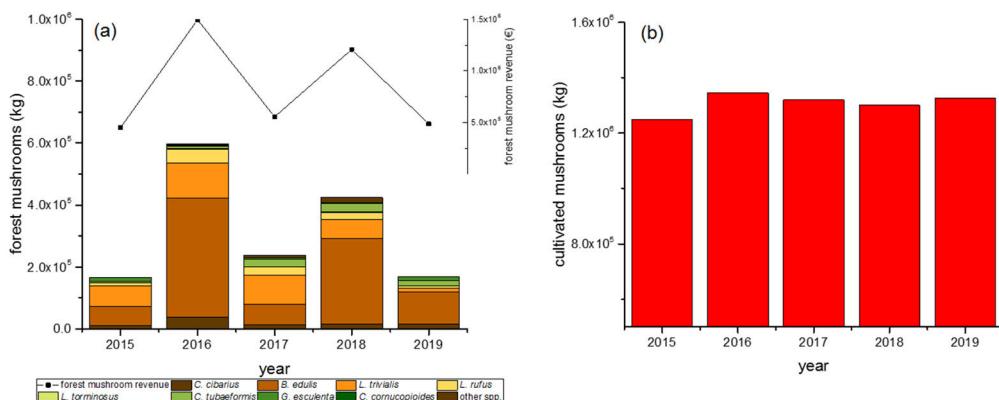
Figure 2. Annual harvests of wild forest (a) and cultivated (b) mushrooms, reported in kg. In (a), the annual revenues from mushroom market are reported in euro. Data for (a) is from Finnish Food Authority[5] and data for (b) is from Natural Resource Institute Finland[39].

For the purpose of this doctoral research, Finnish mushrooms have been divided into three broad and arbitrary categories: cultivated, wild, and rare. While the first two are intuitive and have been already discussed, the third one has been defined utilizing the Finnish Biodiversity Information Facility[40]. The number of observations of *Cantharellus* spp. has been selected as the threshold of frequency below which a genus could be considered rare. The number of observations for relevant species found in the Finnish forests are reported in **Table 4**. The presence of *Craterellus* spp. in the rare category should be attributed solely to under-reporting, as can be seen from its market volume.

Table 4. Number of observations reported for relevant mushroom genera and species of the Finnish forests.

Genus	Observations (2020)	Most observed species of the genus[a]	Observations (2020)	Relevant species	Relevance of the species	Relevance reference
Lactarius spp.	1712	*L. rufus*	172	*L. trivialis, L. rufus, L. turminosus*	culinary	[32]
Russula spp.	1557	*R. aurigena*	99	*R. vinosa, R. paludosa*	culinary	[32]
Phellinus spp.	1265	*P. ferrugineofuscus*	139	*P. nigricans, P. linteus*	medicinal	[28,41]
Fomes spp.	927	*F. fomentarius*	917	*F. fomentarius*	medicinal	[28]
Leccinum spp.	872	*L. scabrum*	259	*L. versipelle, L. auranticum*	culinary	[32]
Tricholoma spp.	815	*T. equestre*	66	*T. matsutake*	medicinal	[41]
Trametes spp.	683	*T. ochracea*	245	*T. versicolor*	medicinal	[41]
Suillus spp.	624	*S. luteus*	220	*S. luteus*	culinary	[32]
Boletus spp.	442	*B. edulis*	320	*B. edulis*	culinary	[32]
Cantharellus spp.	377	*C. cibarius*	371	*C. cibarius*	culinary	[32]
Inonotus spp.	364	*I. obliquus*	218	*I. obliquus*	medicinal	[28,41]
Craterellus spp.	357	*C. tubaeformis*	268	*C. tubaeformis, C. cornucopioides*	culinary	[32]
Ganoderma spp.	308	*G. applanatum*	261	*G. lucidum*	medicinal	[41]
Armillaria spp.	242	*A. borealis*	78	*A. mellea*	culinary	[32]
Flammulina spp.	124	*F. velutipes*	87	*F. velutipes*	medicinal	[41]
Albatrellus spp.	115	*A. ovinus*	79	*A. ovinus*	culinary	[32]
Schizophyllum spp.	70	*S. commune*	70	*S. commune*	medicinal	[41]
Grifola spp.	7	*G. frondosa*	7	*G. frondosa*	medicinal	[41]

[a]The source doesn't confirm that the most observed species of the genus was the most observed species in 2020; source of the data:[40]

2.2   The peculiar case of Chaga

Inonotus obliquus, commonly called Chaga (Pakuri in Finnish) is a peculiar case of the Nordic forests. It is one of the most famous fungal traditional remedies. It appears as a charcoal-black, cracked, and brittle mass, protruding from tree stems. It is usually found on birch trees, although it is also able to colonize alder and beech, of which it is an obligate parasite. The wood decay it causes has been classified as white-rot type. The utilization of this black protrusion as a traditional remedy is first attested in the 16th century and it is found in the traditions of Russia, Poland, Finland, Baltic countries, and Siberia. It is traditionally used as cancer remedy and for illness[42]. In Finland, it was used as a substitute for coffee during WWII. The Khanty people traditionally use tea from powdered Chaga for treating all kinds of diseases, in particular for stomach, heart, and liver problems, tuberculosis, and cancer. An extract made from burnt Chaga is used for body hygiene and after-birth cleaning[28].

Chaga grows in the Northern Hemisphere, above the 40th parallel. Besides the aforementioned locations, it grows also in North America, Canada, and Northern China. It is not the fruiting body of *I. obliquus*, but a sterile conk, consisting of fungal and wood tissues. Fruiting bodies are produced only once in the life cycle, at the death of the host tree. Fruiting bodies are resupinate, yellow-brown and appear below the cracked bark[43]. *I. obliquus* penetrates the tree through open wounds and the sterile conk is formed at the site of the infection. The formation mechanism is not fully understood. However, it is the result of the pressure exercised by a wedge of fungal tissue, once it has established itself in the tree periderm, on the outer bark. The pressure cracks open the bark, forming a protrusion of necrotic tissue[44]. Chaga grows slowly, about 1-2 cm/year. Its growth is the result of the compartmentalization attempt of the host. Lignified wood tissue is formed around the infection site but is penetrated or circumvented annually by the hyphae. Therefore, the sterile conk is made of different necrotic wood tissues. Fungal hyphae are more aggregated in the wood outer cortex and inner periderm (i.e., where the fungus has started the infection), while less connected in the surrounding necrotic area. This is reflected in the two different structures characterizing Chaga, visible to naked-eyes. The outer, brown-black, has pseudosclerotial plates, i.e. layers of branched, thick-walled hyphae incorporating wood tissue. The inner, brown-yellow, has more compact and granular hyphal tissue[43–46].

Mycelium, while exercises pressure towards the outer bark, continues to invade the wood in the opposite direction, towards the sapwood and heartwood. The most decayed wood is found in proximity to the sterile conk formation point (**Figure 3**). The hyphae use the wood vessels to colonize new tissue, slowly degrading the occlusions formed as a response. Presence of other bacteria/fungi is usually required for basidiomycetes to counteract the reaction of wood to

fungal infection (such as secretion of phenolics, suberization, and lignification). While this seems not to be the case for *I. obliquus*, the exact reason is not clear[46].

Chaga extracts and molecules of different classes have been the subject of extensive research. The main triterpenoid of Chaga, inotodiol, has showed potential as anti-cancer compound, inducing apoptosis of human cervical[47] and lung [48] cancers and by positively affecting mice papilloma[49]. Different phenolic compounds belonging to the class of the hispidins and possessing different degrees of radical-scavenging capacity have been isolated from Chaga[50]. In addition to these, anti-tumoral, immunomodulatory, and antioxidant activities of aqueous Chaga extracts have been reported[50]. In particular, polysaccharides have been isolated from these extracts and proven to possess immunomodulatory and anti-oxidant properties[51–54].
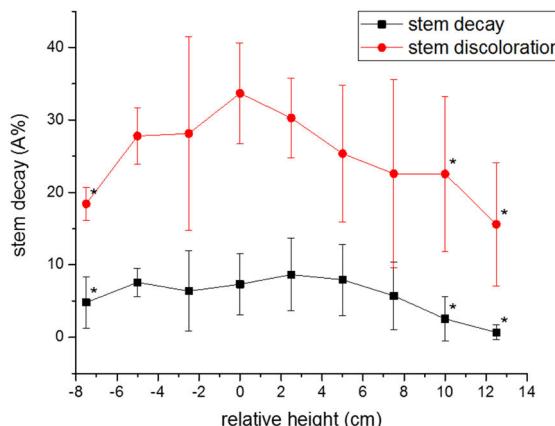
Figure 3. Birch stem decay and discoloration (stem area%) caused by *I. obliquus* measured at different distances from the sterile conk. *number of trees <4. Data from[46].

The commercial interest of Chaga has increased in recent years. Nevertheless, the slow growth and the logistical problems connected to its harvesting make it not industrially sustainable. In addition, concerns of overexploitations have been raised for more accessible areas[55]. A strategy to overcome these issues would be the cultivation of the sterile conk in birch stands, by inoculating the cultivated strain in the stem. An inoculation experiment has been published recently by Ka and coauthors. After 9 years of inoculation, sterile conks have been formed, with strain-specific volumes. The longest size obtained was 10 cm in lenght[56]. The first large-scale inoculation experiment has been performed in Finland and reported very recently. Data was collected 4-5 years after inoculation. Out of 679 inoculated trees, only 30 developed one or more sterile conks. The volume of these conks had a very wide range (1-294 cm³). The relative amount of birches

per stand bearing sterile conks had a wide range among the different stands (0-72%). Stand density and inoculation timing positively influenced the development of conks. Nevertheless, 79% of trees developed sign of infection. Noticeably, *Betula pubescens* (white birch), which is less commercially valuable, was found to be more vulnerable to *I. obliquus* [57].

The data reported in **Figure 3**, elaborated from the findings of Blanchette[46], indicated with approximation that, at the moment of the tree felling, about 10% of the wood stem volume would be decayed. This bulk material, after harvesting the sterile conks, can be defined as spent mushroom substrate, in the same manner as the exhausted substrate for the cultivation of *P. eryngii*. However, while 1 kg of *P. eryngii* would generate 5-6 kg of exhausted substrate[58], the ratio would be, in the case of *I. obliquus*, probably much lower. This sidestream, probably destined to incineration or fertilization[59], could be further valorized with the extraction of fungal compounds of interests, such as polysaccharides[60,61] or enzymes[62].

2.3   Fungal cell wall

The fungal cell wall is a dynamic organelle involved in a number of important processes of the fungal cell. It provides mechanical strength, plasticity for the cell life cycle and it is responsible of the interactions with the cell environment. It regulates cell permeability, mediates the adhesion between cells and to the substratum and has a role in the activation cell signal transduction. Therefore, for the fungal cell, the role of the wall is not only defensive and structural but also aggressive, in its support of fungal colonization[63,64]. The disruption of the cell wall structure has a profound effect on the growth and morphology of the fungal cell, often making it susceptible to lysis and death. Despite the importance of the fungal cell wall for the pharmaceutical (target for drugs to be used against human pathogenic species, mainly ascomycetes), agricultural (fungal plant pathogens cause crop losses and are a threat to both human and animal health), and food (fungal cell walls are main components also of edible fungi) sectors, the structure of the fungal cell wall is not the focus of research attention as much as the plant cell wall. In particular, the investigation of the cell wall of non-pathogenic species, such as edible mushrooms, has received little attention.

Fungal cell wall differs greatly from cellulose-based plant cell wall. The architecture of the cell wall differs among different phyla, genera, and, in some cases, even among different species. Some researchers believe the composition and the structure of the cell wall can be used for taxonomic classification. At the same time, the composition of the cell wall is influenced by growth environment and nutrients[63]. Nevertheless, fungal cell walls are all comprised of proteins, glucans, and chitin. Cellulose is scarcely present in the fungal kingdom and

hemicellulose-like polymers are absent. Polysaccharides constitute 80-90% of the cell wall, while proteins constitute up to 20% (dry weight basis)[65]. Melanins are also present in the cell walls of some fungal species. The proteins present in the cell wall are extensively modified with both *N*- and *O*-linked carbohydrates and, in many instances, contain a glycosylphosphatidylinositol (GPI) anchor as well[66]. Multiple carbohydrates have been identified from cell wall lysates (**Table 5**). However, the most abundant monomer units of carbohydrate polymers in most fungi are glucose, mannose, and N-acetylglucosamine. Examples of the most common polysaccharide structures are shown in **Figure 4**.

Table 5. Carbohydrate composition of the fungal cell wall of Ascomycota and Basidiomycota.

Old taxonomic group	Glc	Gal	GlcN	Man	GalN	GlcA	Rha	Fuc	Xyl	Rha
				relative % of total cell wall sugar						
Hemiascomycota[a]	>21	1-5	1-5	>21	0	0	0	0	0	0
Euascomycota[b]	>21	6-20	6-20	1-5	1-5	1-5	<1	0	tr.[e]	tr.[e]
Heterobasidiomycota[c]	>21	tr.[e]	1-5	6-20	0	1-5	0	tr.[e]	tr.[e]	tr.[e]
Homobasidiomycota[d]	>21	1-5	6-20	1-5	tr.[e]	0	1-5	1-5	1-5	1-5

[a]Ascomycetes lacking an ascocarp; [b]Ascomycetes having the asci borne in or on an ascocarp; [c]Basidiomycetes with septate basidia; [d]Basidiomycetes with aseptate basidia. *A. bisporus*, *C. tubaeformis*, and *I. obliquus* are Homobasidiomycota. Homobasidiomycota is considered an old analogue of the taxonomic class Agaricomycetes[13]; [e]traces. Source of the table:[63].
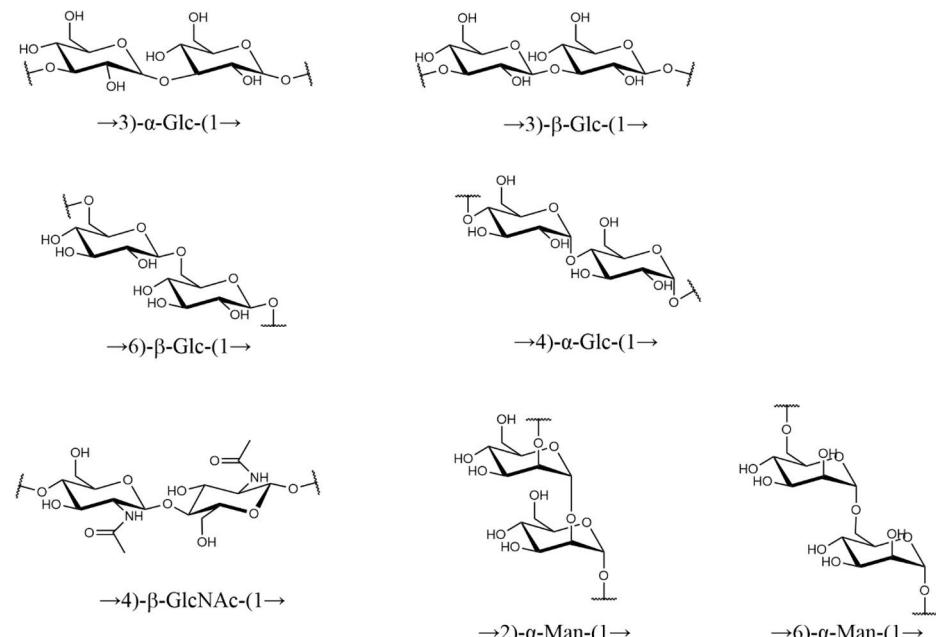
Figure 4. Structures of polysaccharides commonly found in the fungal cell wall.

2.3.1 Glucans

The most abundant polysaccharides of the fungal cell walls are the glucans. These are classified basing on their linkage (α- or β-glycosidic), the position of the linkage on the carbohydrate moiety (O-3, O-4, or O-6) and the presence of branching on the backbone. The degree of branching and the molecular weight further distinguishes the polymers.

   The α-glucans found in the fungal cell wall are mainly constituted of →3)-α-Glc-(1→ units. Linear, water-insoluble, and crystalline (1→3)-α-glucans chains are the main α-glucan of the fungal cell wall[63]. They are covalently bound to each other by →4)-α-Glc-(1→ residues situated in the backbone[67]. Nigeran, a linear polymer of →3)-α-Glc-(1→4)-α-Glc-(1→ units, has been found in the cell wall of two ascomycetes, *Aspergillus* spp. and *Penicillum* spp.[68]. There is some evidence indicating that some portions of the nigeran backbone are constituted of →3)-α-Glc-(1→ sequences separated by 4)-α-Glc-(1→ units, making nigeran rather a peculiar type of →3)-α-Glc-(1→ polymer than a different polymer[69]. The position, as well as the amount, of the →3)-α-Glc-(1→ polymers in the cell wall structure is believed to depend on species and growth stage[70]. There is multiple evidence connecting (1→3)-α-glucans to the virulence of fungal pathogens[68]. Nevertheless, these polymers has been isolated also from basidiomycetes such as *F. betulina* and *Armillaria mellea*[68], *Ganoderma lucidum*[71], and *Wolfiporia cocos*[72]. Noticeably, the stiff fruiting bodies of the Polyporales *Laetiporus sulphureus* and *F. betulina* contain high amounts of →3)-α-Glc-(1→ polymers[70]. Other types of α-glucans have been isolated from fungi. Linear (1→6)-α-glucans have been isolated from basidiomycetes such as *Termitomyces eurhizus*[73], although their role in the fungal cell wall is not reported. Polymers containing →4)-α-Glc-(1→, both linear and branched, have been isolated as well from multiple fungi. Glycogen, (1→4)-α-glucan with (1→6)-α branches, is situated in the cytoplasm of the fungal cell, where it exerts energy storage function.

   With the exception of Zygomycota, the most abundant polysaccharides in the cell wall of all fungal species are the β-glucans[63]. Glucans can constitute up to 60% of the dry weight of the cell wall[66]. These polymers of glucose are composed of →3)-β-Glc-(1→ and →6)-β-Glc-(1→ units, although linear polymers composed solely of →3)-β-Glc-(1→ or →6)-β-Glc-(1→ have been isolated from both ascomycetes and basidiomycetes[63]. The core of the fungal cell wall, in which →3)-β-Glc-(1→ would constitute the most abundant unit, is generally thought to be a network of →3)-β-Glc-(1→ linear chains with →6)-β-Glc-(1→ branches. This core is water-insoluble, generally believed to be organized in microfibrils, and covalently bound to chitin. It is the main polymer responsible of the tensile strength and elasticity of the cell wall and the inhibition of the β-glucan synthesis leads to cell death[74]. The length of the →6)-β-Glc-(1→ side-

chain is highly variable. However, it is commonly believed that, except for some ascomycetes, the →6)-β-Glc-(1→ polymers are responsible of the cross-linking of the other cell wall components. In their work on the cell wall of *Candida glabrata*, Lowman and coworkers proposed several roles of this sidechain in the cell wall matrix: cross-link of two →3)-β-Glc-(1→ chains; non-covalent bond of two →3)-β-Glc-(1→ chains via hydrogen bonds of two sidechains; non-covalent bond of two →3)-β-Glc-(1→ chains via hydrogen bonds of backbone and sidechain; "hooking" of crosslink with sidechain; anchoring of glycoproteins to a β-Glc-(1→3) triple helix via GPI bound to the sidechain[75].

2.3.2 Chitin

Chitin, a long linear homopolymer of β-(1,4)-linked N-acetylglucosamine, accounts for only maximum 20% of the dry weight of basidiomycetes[76], and maximum 15% for ascomycetes[74]. Despite its low amount, it is structurally fundamental for the fungal cell wall: if chitin biosynthesis is artificially hampered, the resulting fungal cell walls are not rigid and cells are osmotically unstable. Chitin chains are organized in microfibrils, giving rise to three different arrangements: β-chitin, where all chains are parallel (i.e., same reducing-nonreducing end orientation); α-chitin, where chains are antiparallel (opposite end orientation); γ−chitin, where two parallel chains alternate with one antiparallel chain. While these three forms of chitin are present in nature, α-chitin is the most abundant in the fungal kingdom, and γ-chitin the rarest. The α-chitin arrangement is the stiffest, making this form extremely insoluble and with high tensile strength[63]. There is general agreement on placing the chitin microfibrils close to the plasma membrane and on the covalent nature of the bond between this polymer and β-glucan. However, the exact nature of the linkage is still unclear[77].

2.3.3 Glycoproteins and heterosaccharides

The proteinaceus components of the cell wall are tightly interconnected with the structural matrix created by chitin and glucan. Nevertheless, most of the proteins are located in the outer layers of the cell wall[78,79]. Compared to ascomycetes, basidiomycete cell wall proteins were only generally located in the outer layers, since some exceptions, like the human pathogen *Cryptococcus neoformans*, have been observed[80]. The roles of the cell wall proteins are ensuring cell shape, mediation of adhesion and absorption of molecules, recognition of foreign substances, cellular signaling, and synthesis and remodeling of cell wall components[66]. The protein composition of the cell wall is influenced by growth stage, cell cycle, environmental conditions, presence of stress factors, and development stage[80]. The fungal cell wall contains non-covalent and covalent

bound proteins[63,81]. Most of the research attention, with the notable exception of hydrophobins[82], has focused on the latter. Some of these are covalently bound to cell wall components, while others are bound to the plasma membrane[74]. Most, if not all, fungal cell wall proteins are glycoproteins (also some hydrophobins are glycosylated[83]), extensively modified with *N*-linked (through Asp residue) or *O*-linked oligo- or polysaccharides (through Ser or Thr residues). Hampering the glycosylation of the cell wall proteins results in cell death[63]. Compared to *N*-linked chains, *O*-linked carbohydrates are shorter and often consisting of galactomannan[63,80].

While glucans and proteins are enzymatically cross-linked once secreted in cell wall space, proteins are glycosylated in the endoplasmic reticulum and in the Golgi apparatus. The structures of the carbohydrate chains attached to these glycoproteins differ amongst fungi. However, the glycosylation with α-mannan chains with linkages in positions O-2, O-3, and/or O-6 is widespread in the fungal kingdom.As examples, the mannans isolated from *Saccharomyces* sp. and *Candida albicans* are reported in **Figure 5**. Proteins are also glycosylated with mannogalactans, rhamnose, glucose, and fucose. Most of the molecular weight of these glycoproteins arises from their carbohydrate chains[63].
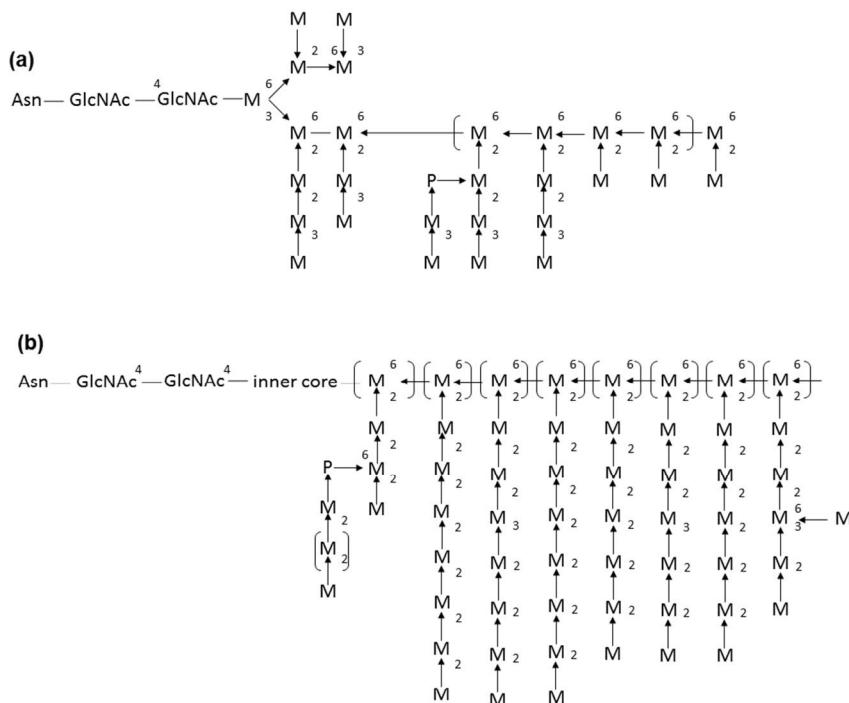
Figure 5. Schematic representation of cell wall mannans. (a) cell wall mannan of *Saccharomyces* sp. (adapted from[63]); (b) cell wall mannan of *C. albicans* (adapted from[84]).

Ruiz-Herrera[63] classifies the covalently-bound glycoproteins in: proteins bound with GPI anchor; proteins with internal repeats (Pir-proteins); proteins attached to other proteins via S-S bridges. The characteristics of the protein classes are summarized in **Table 6**. They are not distinguished by their catalytic activity. In their review on fungal cell wall proteins, De Groot and coauthors[80] do not consider disulfide-bound proteins as a separate class. Moreover, they suggest Pir-proteins should be renamed and their class include all proteins whose bond is alkali-cleaved. On the other hand, Bowman and Free rather classify the fungal cell wall proteins based on the absence or presence of catalytic activity. Such classification would be supported by *in silico* studies, which highlight the eventual presence of catalytic sites[66]. Due to the disagreements present in the research, **Table 6** should be considered only a general description.

Reportedly, more than half of the cell wall proteins have a GPI-anchor[74]. The presence of part of the GPI anchor carbohydrate moiety (α-Man-(1$\rightarrow$3)-α-Man-(1$\rightarrow$2)-α-Man-(1$\rightarrow$2,6)-α-Man-(1$\rightarrow$6)-α-Man-(1$\rightarrow$, with phosphate group in position 6) in a $\rightarrow$6)-β-Glc-(1$\rightarrow$ lysate obtained from *Saccharomyces cerevisiae* highlighted the use of a "trimmed" GPI (Glycosylphosphatidylinositol) anchor (defined as GPI-remnant,[80]) for the covalent bond between protein and β-glucan[85]. However, the use of GPI-remnant for the anchorage of proteins to β-glucans is disputed for basidiomycetes[63]. For ascomycetes, it has been suggested that GPI-proteins are anchored to the cell membrane only prior to maturation[80]. Some authors assign to proteins covalently bound to glucans mainly structural functions[74], while others suggest they are involved in cell-cell interactions[64]. Moreover, mutations in the genes responsible of the GPI anchoring have resulted in cell death in experiments with *S. cerevisiae* and *C. albicans*[74].

Pir-proteins, differently from GPI-proteins, are found also in deeper layers of the cell wall. They can contain one or multiple repeated amino acid sequences and these sequences are likely involved in the covalent bond of the protein. It is not clear whether proteins classifiable as Pir-proteins are present in basidiomycetes[63].

Table 6. Overview of fungal cell wall proteins.

Protein Type	Common aa	Glycosylation	Covalent bond	Amount in fungal species	Protein-polysaccharide linkage
GPI-protein	Ser, Thr, Val, Ile, Leu	*N,O*-glycosylation	$\rightarrow$6)-β-Glc-(1$\rightarrow$	Variable	GPI-remnant[a]
Pir-protein[a]	Gln-[Ile-Val]-X-Asp-Gly-Gln-[Ile-Val-Pro]-Gln[b]	*O*-glycosylation	$\rightarrow$3)-β-Glc-(1$\rightarrow$	Variable but lower than GPI	Glu-Glc ester
Disulfide-bound	n.r. [c]	*O*-glycosylation	-S-S- with another protein	low[86]	absent

[a]disputed in basidiomycetes; [b]internal repeat consensus sequence; [c]not reported

2.3.4   Melanins

Melanins are the most common pigments present in the fungal cell wall and, for some species, they can constitute up to 20% of its dry weight[63]. It is believed that all fungal species are able to produce melanins, even though not all species have constitutively a melanized cell wall[87]. Chemically, they could be defined as "network of aliphatic and aromatic structures"[88]. All melanins are amorphous polyphenolic compounds assembled in structures which are paramagnetic and with some degree of chemical stability[87,89]. Even though their structures are poorly understood, their two main biosynthetical pathways are known. One type of fungal melanin is defined as eumelanin, or DOPA-melanin, and is generated by the polymerization of L-dihydroxyphenylalanine by tyrosinase and/or laccase. The second pathway 1,8-dihydroxynaphtalene (DHN), to generate allomelanin, or DHN-melanin, using laccase. While DOPA is synthetized from tyrosine, DHN is synthetized by the polyketide pathway[88]. The polymerization of melanin precursors occurs via radical mechanism and formation of different covalent bonds, making the structure difficult to elucidate. Melanin protects the fungus from UV radiation[74], oxidation, and hydrolytic enzymes[88], such as β-glucanase[80]. It has been widely acknowledged that the presence of melanin is crucial for the pathogenicity of some fungal species, for example *A. fumigatus* (DHN-melanin) or *C. neoformans* (DOPA-melanin), although the exact role in the pathogenicity itself is not always clear[79,87]. Melanins are also present in the cell walls of basidiomycetes such as *A. bisporus* (DOPA-melanin[90]), *A. auricula* (DOPA-melanin[91]), and *Inonotus hispidus* (DOPA-melanin[92]). Moreover, melanins of unidentified nature were also detected in the cultured hyphal tissues of several basidiomycetes, including *Lentinula edodes*, *Armillaria mellea*, and *Lactarius rufus*[93].

A noticeable accumulation of melanin is observed in the cell wall of fungal sclerotium. As defined by Money, a fungal sclerotium is a "hardened mass of hyphae that serves as survival structure for ascomycetes and basidiomycetes"[94]. Melanin is considered one of the major components of the sterile conk produced by the basidiomycete *Inonotus obliquus*[43,95]. Melanins could be located in the pseudosclerotial plates of the sterile conk. In contrast, the mycelium of *I. obliquus* has been considered an unefficient melanin producer in submerged cultivation[96].

2.3.5   The mushroom cell wall model

The research on the fungal cell wall has mainly focused on ascomycetes fungi and human pathogens. Therefore, the elaborated model of the fungal cell wall has been considered generally valid also for basidiomycetes. As mentioned previously, glucans are the major structural polysaccharide of the fungal cell wall.

Their amounts have been measured in different basidiomycetes. The cell wall preparation of *A. bisporus* was consituted of glucans by 41% d.w.[97]. On the other hand, these polymers constituted up to 80% of dry weight of cell wall fractions produced from different strains of *Pleurotus ostreatus*[76]. More variable amounts of glucans (31-99% d.w.) were found in different cell wall fractions produced from *Pleurotus tuber-regium*[98].

Michalenko and coworkers proposed a mushroom three-layers cell wall model based on their results: an outer layer of water-soluble amorphous "mucilage" composed of glucans and proteins; a "loose" middle layer of alkali-soluble glucans; a compact inner layer of randomly oriented chitin fibrils and interfibrillar β-glucan. The authors found proteins present in all the three layers and rejected the presence of single-component layers in the mushroom cell wall[97]. Later studies have confirmed the position of the chitin in the mushroom cell wall of *A. bisporus* and showed the trasverse arraignment of their microfibrils[99]. More recently, Chen and Cheung proposed another model, based on their results on *P. tuber-regium*. In their model, based on results summarized in **Figure 6**, the mushroom cell wall would be composed of four layers from outer layers to inner layers: proteins and glycoproteins linked to other cell wall components with non-covalent bonds; heteropolysaccharides and proteins non-covalently bound to each other; main layer of β-glucans; chitin and β-glucan covalently bound. They reported absence of proteins in the deeper layers of the cell wall of *P. tuber-regium*[98].
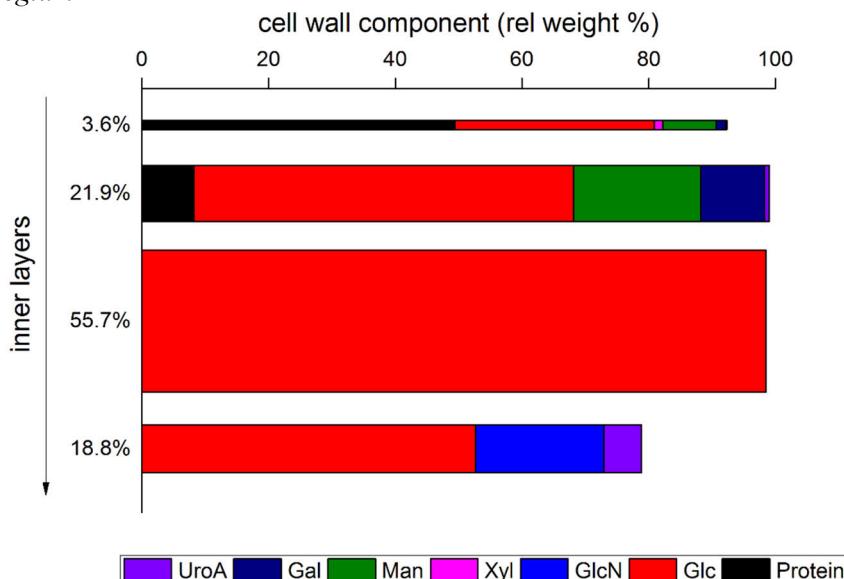
Figure 6. Schematic representation of the cell wall of *P. tuber-regium*, based on which Chen and Cheung proposed the mushroom cell wall model. Percentage on the left represents the relative amount of the layer on the total cell wall. Data from[98].

Recently, a new model of the basidiomycete cell wall has been proposed using a combination of chemical and NMR spectroscopic methods, with *Schizophyllum commune* as model species[100]. The authors propose the core of the cell wall to be constituted of a rigid network of chitin, →3,6)-β-Glc-(1→ polymers, →3)-α-Glc-(1→ chains, and heterosaccharides such as →3)-α-Fuc-(1→ chains. The chitin chains would be covalently linked to the glucan polymers, in particular to the β-glucan chains, via linkages mainly with →4,6)-β-GlcNAc-(1→ units. The network of →3,6)-β-Glc-(1→ polymers becomes less compact and flexible towards the outer end of the cell wall, in which α-glucans and →2,3,6)-α-Man-(1→ chains would be situated. All protein NMR signals disappeared after the removal of the outer layer of the cell wall. However, the authors indicated this absence could also be connected to experimental limitations. Therefore, their suggestion of the presence of proteins solely in the outer layer of the cell wall was cautious [100]. The near absence of covalently linked proteins in the cell wall of *P. tuber-regium* at different maturation stages would anyway support their observation[101].

The research on fungal, and in particular mushroom, cell wall polysaccharides has been mainly performed by extracting and analyzing the different cell wall layers from homogenized material, utilizing solvents of increasing harshness. However, the fungal cell wall is a dynamic element of the organism[63] and undergoes changes depending on multiple factors, such as cell development stage and environment. Elongation is one of the peculiar aspects of mushroom development and maturation and such process is strongly connected to the cell wall, due to its role in balancing cell turgor and plasticity. Mushroom elongation is connected to elongation of preexisting cells, rather than production of new cells. Therefore, as cells elongate, cell wall has to lose rigidity to allow the insertion of *de novo*-synthesized structural components, which are required to adapt the cell wall to the new turgor pressure and to cover the expanded surface[102]. Mushroom elongation is mainly located in the apical region of the stipe, with intensity and duration highly species-specific. The process is based on the action of chitinases, which partially dismantle chitin microfibrils and allow their network to expand, creating space for the insertion of newly synthesized polymers. Crucially, chitinases are adjuvated by glucanases, which disrupt the cell wall network by hydrolyzing β-(1→3) or β-(1→6) glucose linkages[103]. Therefore, the amounts of the different β-glucan units would be dependent of the elongation stage of the mushroom. Moreover, experiments with the mushrooms *Coprinopsis cinerea* have indicated that the specific hydrolysis of →6)-β-Glc-(1→ chains could be involved in the cessation of the elongation in the basal side of the stipe[103].

2.4 Structural features of mushroom polysaccharides

As described in the previous section, mushrooms contain polysaccharides differing in monomers, glycosidic linkage types, and position of the linkage on the unit. The number of monomers composing the polysaccharides determines the molecular weight of the polymer. Moreover, some polysaccharides, such as mannans, are covalently bound to proteins. The differences in structural properties could be connected to differences in starting material (strain, cultivation conditions, growth stage, etc.) but also to differences in extraction and purification methods[3]. It is therefore not surprising that polysaccharides with variations in structural properties have been isolated from mushrooms, even from the mushrooms produced by the same species. A throughout review of structural features of mushroom polysaccharides would be beyond the scope of this doctoral dissertation. The focus of the presented research work is to study the polysaccharides extractable from Finnish fungi. Therefore, this section will focus on the structural features of polysaccharides isolated from cultivated, wild, and rare species, which could be found in Finland. **Tables 7** and **8** summarize the structural features of β-glucans and heterosaccharides extracted from mushrooms belonging to these three categories, respectively.

2.4.1 β-glucans

Polymers of →3)-β-Glc-(1→ and →6)-β-Glc-(1→ units are the most abundant components of the mushroom cell wall. Branched glucans are the most common polysaccharides extracted from mushrooms. Nevertheless, linear →6)-β-Glc-(1→ polymers have been isolated as well, for example from *Agaricus bisporus* or *Lentinula edodes*. The most common branched β-glucan consists of a backbone of →3)-β-Glc-(1→ units and branches in position O-6. Polymers with branches in O-2 or O-4 positions are less frequent. The branching units could be single β-glucose units or short chains of →3)-β-Glc-(1→ or →6)-β-Glc-(1→ units. The degree of branching (DB) of the β-glucan depends, like the other chemical properties, on the starting material and on the extraction method. Therefore, it is not surprising that different branching degrees have been observed for polysaccharides extracted from the same fungi. An example of variation in the BD of →3,6)-β-Glc-(1→, taken from the review of Novak and Vetvicka[104], is showed in **Figure 7**. Indicatively, DB values of 0.2, 0.25, and 0.33 indicate the presence of a branching every 5, 4, and 3 backbone units, respectively.
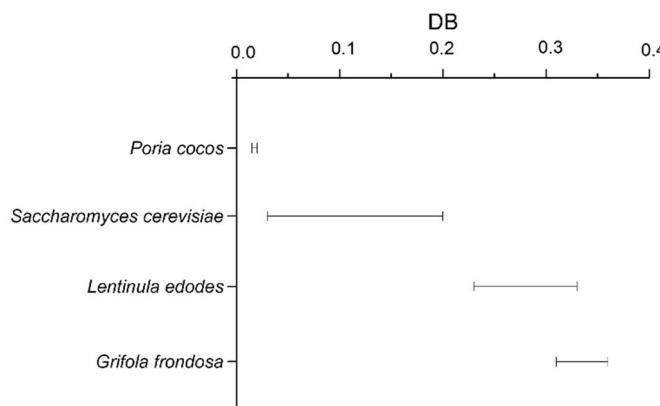
Figure 7. Intervals in the degree of branching (DB) of β-glucans isolated from different fungal species. Data taken from the review of Novak and Vetvicka[104].

Both linear and branched β-glucans were isolated from *A. bisporus* (**Table 7**). The linear →6)-β-Glc-(1→ polymer was isolated using hot water and by treating with DMSO the insoluble polysaccharides[105], while the branched polymer was isolated with alkali, together with heterosaccharides by Pires and coworkers[106]. In the later work, the presence of O-2 linkages, together with the presence of units branching in both O-2 and O-4 was reported. The branched β-(1→3)-glucan isolated from *L. edodes*, called "lentinan", is one of the most well-known mushroom polysaccharides. It is a →3)-β-Glc-(1→ polymer with branches consisting of a single β-Glc-(1→6) unit. More recently, linear and branched →6)-β-Glc-(1→ polymers have been isolated from the same mushroom, with the utilization of alkali, as reported by different research groups. Jeff and coauthors isolated linear β-(1→6) glucan and heterosaccharides from this mushroom[107] from the hot water extract after column chromatography. The first polymer was obtained also by alkali extraction, together with lentinan [108]. With the utilization of alkali, Li and coworkers extracted a β-(1→6) branched in position O-3 with short β-(1→3) chains or single β-(1→3) glucose units[109]. A linear β-(1→6) glucan with the unusual methylation in position O-4 was isolated from wild *Boletus edulis* utilizing hot water, together with heterosaccharides (see next section), by Zhang and coworkers[110]. The →6)-4-*O*-Me-β-Glc-(1→ unit was present in very low amounts in the polymer and the authors refrain from speculating on the biological role of methylation of the polysaccharide. Besides from this peculiar case, in **Table 7** are reported examples of branched β-glucans isolated from mushrooms frequently found in the Finnish forests. Two →3)-β-Glc-(1→ polymers have been isolated from *Lactarius rufus* by Ruthes and coworkers utilizing hot water. These two polymers differed in their degree of branching of O-6 position and in their solubility (DB approximately 0.48 for the soluble against 0.25 for the insoluble).

Both polysaccharides were decorated with single β-glucose units or, in the case of the soluble glucan, short β-(1→3) chains (maximum 3 units)[111]. Noticeably, branched β-glucans with backbones constituted by →6)-β-Glc-(1→ or →3)-β-Glc-(1→ were both extracted from *Cantharellus cibarius*. Nyman utilized alkali to extract a →6)-β-Glc-(1→ with branches in position O-3 constituted by single glucose units or short β-(1→3) chains. The previous hot water extraction step led to the isolation of mannans[112]. On the other hand, Villares and coworkers utilized as well alkali, subsequently to hot water, but the polysaccharide they isolated had a →3)-β-Glc-(1→ backbone and branching in O-6 with two β-glucose units, connected to each other by β-(1→6) linkage[113].

The polysaccharides extractable from the sterile conk of the rare fungus *I. obliquus* have been throughly investigated only very recently. Moreover, a peculiar β-glucan, together with galactan (see next section), has been isolated during the process. While this polymer was mainly linear, with O-6 branches of single glucose units, the presence of both →6)-β-Glc-(1→ and →3)-β-Glc-(1→ units in the backbone created what the authors have defined as "kinks" in the structure[54]. To the best of our knowledge, there are no other examples of mixed-linkage β-glucans isolated from fungi besides lichenan, β-(1→3),(1→4) found in lichenized fungi[114]. A β-glucan similar to the ones previously discussed has been isolated from the rare mushroom *Grifola frondosa*. It consisted of a →6)-β-Glc-(1→ polymer with frequent branching in O-3 position, and the branches constituted of single glucose units, or di-trisaccharides connected with β-(1→3) linkages[115]. However, the polysaccharide, commonly called "grifolan", differs from the polymer reported by Nanba, since it consisted of a →3)-β-Glc-(1→ backbone, decorated in position O-6 with a single glucose unit[116]. Grifolan is the polysaccharide extractable from *G. frondosa* with the DB in the interval 0.31-0.36 (**Figure 7**). Another polysaccharide, schizophyllan, has been extracted from a rare mushroom, *Schizophyllum commune*. Like grifolan, it has a backbone of →3)-β-Glc-(1→ units with branching in position of O-6 constituted by a single β-glucose unit[117].

Table 7. Selected examples of β-glucans isolated from basidiomycetes.

Species	Polysaccharide	Branching position	Molecular weight	Extraction solvent	Ref.
Cultivated mushrooms					
A. bisporus	→6)-β-Glc-(1→	none	2.9×10^4	Hot water	[105]
	→6)-β-Glc-(1→	O-2, O-4	1.8×10^4	KOH 2%	[106]
	→2)-β-Glc-(1→				
	→2,4,6)-β-Glc-(1→				
L. edodes	→6)-β-Glc-(1→	none	75.8×10^4	Hot water	[107]
	→3,6)-β-Glc-(1→	O-3	9.6×10^4	NaOH 0.1 M	[109]
	→3,6)-β-Glc-(1→	O-6	not reported	not reported	[118]
Forest mushrooms					
B. edulis	→6)-β-Glc-(1→	none	1.8×10^4	Hot water	[110]
	→6)-4-*O*-Me-β-Glc-(1→				
L. rufus	→3,6)-β-Glc-(1→	O-6	11.3×10^4	Hot water	[111]
	→3,6)-β-Glc-(1→	O-6	not reported	Hot water	[111]
C. cibarius	→3,6)-β-Glc-(1→	O-6	12.0×10^4	NaOH 1 M	[113]
	→3,6)-β-Glc-(1→	O-3	not reported	NaOH 1 M	[112]
Rare mushrooms					
I. obliquus	→6)-β-Glc-(1→	O-6	6.0×10^4	Hot water	[54]
	→3)-β-Glc-(1→				
G. frondosa	→3,6)-β-Glc-(1→	O-3	18.0×10^4	Hot water	[115]
	→3,6)-β-Glc-(1→	O-6	not reported	not reported	[116]
S. commune	→3,6)-β-Glc-(1→	O-6	430×10^4 (estimated)	not reported	[117]

2.4.2 Heterosaccharides and mannans

Even though the most studied mushroom polysaccharides are the β-glucans, heterosaccharides isolated from fruiting bodies have been extensively studies as well. Their presence in mushroom extracts is not surprising, since they are components of the mushroom cell wall. This section includes mannans that are not heteropolysaccharides *sensu stricto*, i.e. they are composed of only one monomer, in this case mannose. Since mannans and heteromannans are, as discussed above, important components of the mushroom cell wall, and they have been isolated from multiple mushrooms, they are included together with other polymers.

Fucogalactans are polymers of →6)-α-Gal-(1→ decorated with single α-Fuc-(1→ units in position O-2. They have been identified in extracts produced from multiple mushrooms, both cultivated and wild. In some instances, they have been extracted together with β-glucans or mannans. Fucogalactans have been identified in extracts obtained from species such as *A. bisporus*[119], *L. rufus*[120], and *I. obliquus*[54]. In the case of *B. edulis*, the galactan has been discovered to be decorated with α-Ara-(1→ units [121]. A galactan decorated with either α-Fuc-(1→2) or β-Man-(1→2) was extracted with hot water from *L. edodes* [122]. The mannogalactan isolated from *A. bisporus* has been proposed to be decorated with →2)-β-Man-(1→ chains in addition to single β-mannose[106]. Moreover, the

mannogalactan isolated from *B. edulis* showed further branching in position O-6 of the β-mannose sidechain[110]. The presence of O-3 methylated galactose units in the →6)-α-Gal-(1→ chain has been observed for galactans extracted from *A. bisporus*, *L. rufus*, and *I. obliquus* (**Table 8**). Noticeably, the galactan isolated by Ruthes from *A. bisporus* was methylated[119], while the one isolated by Pires lacked methylated galactose[106]. The earlier was extracted with cold water, the latter with alkali. The methylation of galactan has been observed also in *Pleurotus pulmonarius*, *Pleurotus eryngii*, and *Agaricus bisporus* var. *hortensis*[123–125]. Nevertheless, the biological role of polysaccharide methylation in fungi is still unknown[126].

Mannoglucans have been isolated from *L. edodes*, *B. edulis*, and *G. frondosa* (**Table 8**). In these polymers, the mannan chains constitute the branching of the β-glucan backbone [127]. In the case of the mannoglucan extracted from *L. edodes*, the branching of the →3,6)-β-Glc-(1→ would be replaced by either →3,6)-α-Man-(1→ or →2,4)-α-Man-(1→ branched sidechains [107]. In the case of *B. edulis* however, no NMR signals were found to prove linkages between mannose and glucose chains [128]. On the other hand, such linkage was proposed by Yu and coworkers, which have suggested their isolated mannoglucan to be composed of →3)-β-Glc-(1→ backbone with branching in O-6 composed of single glucose units or →3)-α-Man-(1→ chains, branched in position O-6 with β-Glc-(1→3)-α-Man-(1→ disaccharides [129].

Mannans have been isolated from the forest mushrooms *Lactarius deliciosus*, *Lactarius volemus*, and *C. cibarius* (**Table 8**). The mannan isolated from the first had the backbone of →6)-α-Man-(1→ decorated with a single xylose unit through a α-Man-(2→3)-α-Xyl linkage[130]. The other mannans reported here were constituted solely of α-Man units. The mannan isolated from *L. volemus* had a backbone of →4)-α-Man-(1→ substituted in position O-6 with single α-Man residues[131]. Conversely, the mannan isolated from *C. cibarius* had a backbone of →6)-α-Man-(1→ and O-2 branching with either single α-Man or α-(1→2)-Man disaccharides [112]. The mannan from *L. volemus* had a DB of 0.30, against the DB of 0.38 proposed for the *C. cibarius* mannan.

Table 8. Selected examples of heterosaccharides and mannans isolated from basidiomycetes.

Species	Polysaccharide	Branching position	Molecular weight	Extraction solvent	Ref.
Cultivated mushrooms					
A. bisporus	→6)-α-Gal-(1→ →6)-3-*O*-Me-α-Gal-(1→ →2,6)-α-Gal-(1→ α-Fuc-(1→	O-2	37.1×10^4	Cold water (4 ºC)	[119]
	→6)-α-Gal-(1→ →2,6)-α-Gal-(1→ →2)-β-Man-(1→ β-Man-(1→	O-2	1.8×10^4	KOH 2%	[106]
L. edodes	→6)-α-Gal-(1→ →2,6)-α-Gal-(1→ β-Man-(1→ α-Fuc-(1→	O-2	1.6×10^4	Cold water (4 ºC)	[122]
	→3,6)-β-Glc-(1→ →3,6)-α-Man-(1→ →2,4)-α-Man-(1→ α-Glc-(1→	not reported	2.1×10^4	Hot water	[107]
Forest mushrooms					
B. edulis	→6)-α-Gal-(1→ →2,6)-α-Gal-(1→ α-Ara-(1→	O-2	11.3×10^4	Hot water	[121]
	→6)-α-Gal-(1→ →2,6)-α-Gal-(1→ α-Fuc-(1→ →2,6)-α-Man-(1→	O-2	1.8×10^4	Hot water	[110]
	→3)-α-Glc-(1→ →3)-α-Man-(1→	not reported	85.0×10^4 (estimated)	NaOH 1 M	[128]
L. rufus	→6)-α-Gal-(1→ →6)-3-*O*-Me-α-Gal-(1→ →2,6)-α-Gal-(1→ α-Fuc-(1→	O-2	1.4×104	Cold water (4 ºC)	[120]
L. deliciosus	→6)-α-Man-(1→ →2,6)-α-Man-(1→ (2→3)-α-Xyl	O-2	1.1×10^4	Hot water	[130]
L. volemus	→4)-α-Man-(1→ →4,6)-α-Man-(1→ α-Man-(1→	O-6	1.7×10^4	Hot water	[131]
C. cibarius	→6)-α-Man-(1→ →2,6)-α-Man-(1→ α-Man-(1→	O-2	67.1×10^4	Hot water	[112]
Rare mushrooms					
I. obliquus	→6)-α-Gal-(1→ →6)-3-*O*-Me-α-Gal-(1→	none	6.0×10^4	Hot water	[54]
G. frondosa	→3,6)-β-Glc-(1→ →3)-α-Man-(1→ →3,6)-α-Man-(1→ β-Glc-(1→3)-α-Man- (1→6)	O-6	64.5×10^4	Cold water (30 ºC)	[129]

2.5 Bioactivities of mushroom polysaccharides

2.5.1 Immunomodulating activity

Ruiz-Herrera indicated that β-glucans were first recognized as immunomodulators due to their recognition by immune system receptors after studies on the ascomycete cell wall isolates. The β-glucans were found to be capable of trigger and amplify inflammatory response, in some cases anaphylactic shock[63]. Nevertheless, it is reported that the first experiment on the bioactivity of polysaccharides from basidiomycete was performed in 1957, when it was found that a polysaccharide fraction extracted from *B. edulis* was able to inhibit the growth of Sarcoma S-180 cells[41,132]. Due to the traditional use of mushrooms in Asian medicine, the research on mushroom components and their bioactivities has a longer history. The first report on the immunomodulation properties of their polysaccharides was published in 1969[104]. The research on the topic of the immunomodulating activity of mushroom polysaccharides has grown ever since.

The variables that have been found to influence the immunostimulant activity of mushroom polysaccharides are: monomer composition; water solubility; molecular weight; branching degree; conformation. The correlation between these variables and biological activity are not fully understood. While at first it was believed that only β-glucans were able to interact with the immune system, there is concrete evidence nowadays that other cell wall components have such property[127]. In general, it is believed that high molecular weight polymers have the strongest interaction with the immune system[26]. However, for certain β-glucans, the activity was retained after depolymerization by mild hydrolysis or oxidation, while, in other experiments, it was shown that polymers with different molecular weight have similar activity[132]. The more recent review of Zhang and coauthors suggested an optimum range of 10-200 kDa but their scope covers all immunomodulating polysaccharides and only one receptor of the immune system[133]. The polysaccharide conformations in water, which have shown immunomodulation activities, are single helix, triple helix, and random coil. Some authors believe that single elix and random coil are the same conformation, called single chain[134]. Of the three conformations, the triple helix is believed the most important for the activity[26]. In acqueous medium, without denaturating conditions, polysaccharides assume single chain conformation below 25-40 kDa, a fact that might influence their biological activity[134]. Regarding the branching degree, the comparison of →3,6)-β-Glc-(1→ polymers extracted from *Tricholoma matsutake*, *L. edodes*, and *Sparassis crispa* has shown, regarding the immunostimulation activity, an optimal degree of branching of 29-32%[135], while lower or higher branching degree resulted in a loss of activity. Moreover, →3,6)-β-Glc-(1→ with lower branching degree are also less soluble in water.

Polysaccharides possessing immunomodulating activities are commonly defined as biological response modifiers (BRMs) because they do not cause damage nor stress to the organism but they support its response to stresses, in untargeted and noninvasive manner. Immunomodulators are able to increase or reduce the activity of the immune system [104], depending on the host conditions. The loss of activity of these molecules in animals with artificially knocked-down immune system or thymectomyzed indicated that T-cells and thymus are required for their effect, which consists in the modulation of the response of T-cells and macrophages to cytokines[26].

Mushroom polysaccharides have been administrated intraperitoneally to mice bearing tumor cells lines such as Sarcoma 180, Erlich solid cancer, Sarcoma 37, Yoshida Sarcoma, and Lewis lung carcinoma. An increase in blood concentration of cytokines such as TNF-α, IL-1, IL-6, and interferons has been observed, resulting in maturation, differentiation, and proliferation of immunocompetent cells [41]. While most of these studies have been performed with intraperitoneal, intravenous, or subcutaneous injection, the oral absorption of these molecules has been proved by administration to mice of labeled lentinan and quantification of serum fluorescence. The absorption of β-glucans is believed to be mediated by the gut-associated lymphoid tissue (GALT) and by the epithelial cells[136]. The β-glucans interact with macrophages, and, after receptor binding, phagocitized, hydrolyzed, and transported to spleen, lymph nodes, and bone marrow, where the fragments are recognized by other immune system cells [137].

The immunomodulation properties of mushroom polysaccharides have been attributed to their binding to innate and adaptive immunity cell receptors, such as Toll-like receptors (TLR), Complement Receptor 3 (CR3), Dectin-1 receptor, scavenger receptor, and lactosilceramide receptor[132]. Dectin-1 receptor is expressed by macrophages, dendritic cells, neutrophils, and eosinophils[138]. It promotes the innate immune response by the activation of phagocytosis, ROS production, and cytokine production. Its signal cascade has a pathway in synergy with TLR which leads to the production of IL-2, IL-12, and TNF-α, and another pathway mediated by syk kinase, leading to the production of MIP2 (Macrophage Inflammatory Protein 2) and IL-2[132]. The TLR is a family glycoprotein expressed by dendritic cells, phagocytes, and endothelial cells, which, after binding with the molecule, homo- or heterodimerizes. The TRL which recognizes β-glucans is the heterodimer TLR2/TLR6, responsible for the activation of transcription factor NF-κB, which leads to the expression of genes codifying cytokines. The homodimer TLR4 is likely involved in the recognition of β-glucans as well and it is also activated by fungal mannans[137,139]. It is nowadays believed that TLR4 is the most important receptor responsible of the immunomodulatory properties of mushroom polysaccharides[140]. According to

Zhang and coauthors, and differently from →3,6)-β-Glc-(1→ and mannans, it is unknown whether linear →6)-β-Glc-(1→ is able to bind TLR4 receptor[133]. However, this polymer is proven to bind Dectin-1 receptor[141]. The CR3 receptor is expressed by neutrophils and NK cells, while it is absent in macrophages. It has two binding sites, one recognizing β-glucans and the other recognizing the complement protein fragment iC3b. The binding of both sites is required for the activation of this receptor [132]. The scavenger receptor is located on mieloid and endothelial cells and on macrophages, while the lactosylceramide receptor is located on neutrophils and endothelial cells. Both receptors activate a signal pathway leading to the activation of MAPK (mitogen-activated protein kinase), among other kinases, and, for the scanvenger receptor, a nitric oxide synthase[132]. According to Chan and coauthors, polysaccharides are presented to naive T-helper cells (Th0), possibly via class II major histocompatibility complex (MHC-II), and this will determine the development of a naive T-helper (Th0), i.e. whether it becomes a type 1 T-helper (Th1), type 2 (Th2), or others[137]. For example, Th1 produces INFγ and IL-2. The first is important for the activation of macrophages, the second has a role in T-cell memory. Th2 and Th17, on the other hand, have a role in the activation and class switching of B cells[142]. Therefore, the interaction between mushroom polysaccharides and the immune cell receptors leads to the modification of both innate and adaptive immunity.

As summarized by Thompson and coauthors[143], there is wide range of *in vitro* evidence of the ability of mushroom polysaccharides, and in particular β-glucans, to modulate the immune system and to stimulate the leukocyte phagocytosis, ROS production, and secretion of cytokynes. There are also evidence obtained *in vivo* showing the stimulation of antibodies production, T-cells and NK-cells activity, phagocytosis, and cytokine production in mice fed with mushroom β-glucans[143].

Table 9. Overview of immunomodulatory effect of basidiomycete polysaccharides.

Species	Activity	Polysaccharide	Reference
Cultivated mushrooms			
A. bisporus	(*in vivo*) decrease of formalin-induced nociception	fucogalactan	[125]
	(*in vivo*) decrease of iNOS and COX-2 expression in induced sepsis	fucogalactan	[119]
L. edodes	(*in vivo*) increased IL-2, TNF-α, IL-6	not elucidated	[144]
	(*in vivo*) decreased TNF-α, IL-6, IL-1 after LPS stimulation	not elucidated	[145]
	decreased TNF-α, IL-6, IL-1 after LPS stimulation	→3,6)-β-Glc-(1→	[108]
	increased NO production, TNF-α and IL-6	→3,6)-β-Glc-(1→	[146]
	(*in vivo*) increased NK and macrophage activity	not elucidated	[147]
	increased proliferation of rat thymocytes	not elucidated	[148]
	(*in vivo*) increase proliferation of lymphocites and mononuclear cells	→3,6)-β-Glc-(1→	[149]
Forest mushrooms			
B. edulis	(*in vivo*) increased IL-2, TNF-α, and proliferation of NK	→6)-β-Glc-(1→ and arabinogalactan	[121]
C. cibarius	increased IL-6, NO production	→3,6)-β-Glc-(1→ (speculated)	[150]
L. rufus	(*in vivo*) decrease of formalin-induced nociception	→3,6)-β-Glc-(1→	[111]
L. volemus	increased white blood cell count	mannan	[131]
R. albonigra	increased NO production	mixture of glucan, galactan, and mannan	[151]
Rare mushrooms			
I. obliquus	Enhanced lymphocyte proliferation; increased TNF-α	not elucidated	[51]
	(*in vivo*) increase NO production	not elucidated	[152]
	increased NO production	→3,6)-β-Glc-(1→ and galactan	[54]
	increased IL-1, IL-2, TNF-α, and IFN-γ	not elucidated	[153]
G. frondosa	(*in vivo*) decreased NO production	not elucidated	[154]
	increased VEGF and TNF-α	not elucidated	[155]
	(*in vivo*) increased NK and macrophage activity	not elucidated	[147]
	increased IL-1, IL-6, TNF-α	→3,6)-β-Glc-(1→	[156]
S. commune	decreased NO production, iNOS, 5-LOX	→3,6)-β-Glc-(1→ protein-bound	[157]
	(*in vivo*) enhanced NK proliferation, ROS production	not elucidated	[158]
	(*in vivo*) increased antibody production after viral infection	→3,6)-β-Glc-(1→	[159]

While the main application of this research has been cancer therapy, there is *in vivo* evidence that the stimulation of the immune system is able to provide host resistance against microbial infections caused by species such as *Staphylococcus aureus* and *C. albicans*[143]. The immunomodulation comprises also inhibition of the immune system cells, in addition to stimulation. Wide evidence is available in literature indicating that mushroom polysaccharides are able to decrease the activity of an already stimulated immune system (by LPS, for example). Reduction of the production of TNF-α, IL-1, IL-6, IL-8, and NO synthase activity has been reported after *in vitro* and *in vivo* experiments[140]. Noticeably, when macrophages were treated with linear →6)-β-Glc-(1→, significant increase of cytokine genes expression was observed (in the case of IL-1, even higher than positive control LPS), while the cotreatment with →6)-β-Glc-(1→ and LPS resulted in cytokine expression levels significantly lower than treatment with LPS alone[105]. A brief overview of the immunomodulatory effect of cultivated, wild, and rare mushrooms is reported in **Table 9**.

2.5.2 Prebiotic activity

Mushroom polysaccharides are not digested by the human gastro-intestinal tract. Therefore, they can act as dietary fiber and enhance the growth of bacteria species beneficial for the human gut, such as *Bifidobacterium* or *Lactobacillus*, due to their fermentability. The fermentation of dietary fibers leads to the generation of short chain fatty acids (SCFA) which could act as energy source for the epithelium and for beneficial microflora species. SCFA have been also correlated to decrease in ammonia production in the gut and interference with cholesterol and glucose metabolism[160]. This area of research has received attention more recently, compared to the immunomodulatory activity. The results published so far would support that mushroom polysaccharides have, as required by the definition of prebiotic reported by Gibson and coauthors, "beneficial effect through their selective metabolism" by "selective stimulation of the growth and/or activity of intestinal bacteria associated with health and wellbeing"[161]. The review of Gibson has no referencing to mushroom polysaccharides (nor β-glucans in general) and the research on the field is still ongoing. In the research community, mushrooms or mushroom polysaccharides are not considered yet prebiotics, although there is wide acknowledgment of their potential.

A polysaccharide fraction from *Trametes versicolor* stimulated fecal bacterial growth and SCFA production in the same manner as fructo-oligosaccharides[162]. A →3,6)-β-Glc-(1→ polymer isolated from *P. tuber-regium* stimulated in vitro the growth of *Bifidobacterium* spp. strains similarly to inulin and barley and seaweed β-glucans. Similar findings were roperted on the production of SCFA[163]. In another set of in vitro experiments, linear →3)-β-Glc-(1→ from different

sources stimulated the growth of *Bifidobacterium* similarly to inulin, barley and xylo-oligosaccharides[164]. The oral subministration to rats of a polysaccharide extracted from *L. edodes* has shown improvement in the gut microflora, with the increase of beneficial populations such as *Lactobacillus* and *Bifidobacterium*[144]. A recent clinical trial has showed changes in the subjects microflora after consumption of meals enriched with *L. edodes* β-glucan, which correlated to with cholesterol metabolism markers[165].

Purified polysaccharides from *Pleurotus eryngii* showed better growth stimulant properties for *Lactobacillus* strain Lac A than the ones extracted from *Pleurotus ostreatus*. Moreover, polysaccharides extracted with hot water showed worse performance than the polymers extracted with alkali. On the other hand, the polymers from P. *ostreatus* favored the growth of *Bifidobacterium* strain Bifi A and hot water extracts performed better than the alkali. The fractions differed in monomer composition, ratio of glucose $(1{\rightarrow}3)$ and $(1{\rightarrow}6)$ linkages, and molecular weight [76]. The influence of the branching degree on the fermentability of mushroom polysaccharides was noticed by Cantu-Jungles and coworkers, who reported a different growth stimulation profile of linear water-insoluble β-$(1{\rightarrow}3)$-glucan, compared to the branched, water-soluble $\rightarrow$3,6)-β-Glc-(1$\rightarrow$ polymer [166].

A screening of raw polysaccharide fractions produced from different Polish forest mushrooms has showed different degrees of *Lactobacillus acidophilus* and *Lactobacillus rhamnosus* growth stimulation. Among the 53 species tested, *Rhodocollybia maculata*, *Paxillus involutus*, and *Panellus stypticus* favored the earlier, while *Psilocybe capnoides*, *Laccaria laccata*, and *Pholiota mutabilis* favored the latter. The differences in the growth stimulation between the screened fractions could be due to differences in monomer composition, glycosidic linkage, and molecular weight[167]. The authors abstained from discussing the little market value of the aforementioned species: they are inedible or not recommended, except *P. involutus*, which is toxic[168]. However, while toxic species should be avoided, the results indicate that low-value forest mushrooms could be used as a source of polysaccharides with prebiotic activity. Raw polysaccharides extracted from the discarded parts of *L. edodes*, *P. eryngii*, and *Flammulina velutipes* resulted in prolonged viability of *Lactobacillus acidophilus* cultures during cold storage and in simulated bile and gastric juices[169].

2.5.3   Anti-oxidative activity

Anti-oxidant activity of molecules has been defined as "limitation of the oxidation of proteins, lipids, DNA or other molecules that occurs by blocking the propagation stage in oxidative chain reactions" [170]. The anti-oxidant activity of mushroom polysaccharides has been widely researched. On one hand, the

definition of anti-oxidant activity in vivo has been connected to reduction in ROS and NO production, which is, as discussed above, correlated to the capacity of mushroom polysaccharides to interact with macrophages and other immune system cells. In addition, anti-oxidant activity has been correlated with the increase in expression of enzymes such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), and glutathione peroxidase (GSH). These enzymes protect the cell from oxidative stress. A raw polysaccharide fraction produced from *A. auricula* significantly increased mice serum SOD and GSH levels, in addition to, noticeably, thymus index [171]. Mice blood serum SOD was also increased after feeding with a polysaccharide extracted from Tricholoma lobayense. At the same time, the production of IL-6 was modulated [172]. Similar increase in anti-oxidant enzymes were obtained with raw polysaccharides extracted from *Agrocybe aegerita* [173] and *G. frondosa* [154]. The capability of mushroom polysaccharides to exert radical-scavenging activity in vitro has been widely reported. Among the multiple polysaccharide fractions tested, scavenging capacity was demonstrated for extracts produced from the cultivated *L. edodes* [108,174], *A. bisporus* [175], *Calocybe indica* [176], and *P. eryngii* [58]; from the wild *A. aegerita* [173], *T. lobayense* [172], *Russula vinosa* [177], and *Russula virescens* [178]; for the rare *I. obliquus* [179] and *Hericium erinaceus* [174]. It is nowadays believed that the moieties responsible for such activity are the remaining or covalent-bound proteins (in particular, the aromatic amino acid residues) or phenolic compounds. As examples, the FT-IR spectra of the polysaccharide fractions showed the vibration ascribable to aromatic compounds or proteins in the case of *A.bisporus* [175] and proteins in the case of *C. indica* [176], *R. virescens* [178], and *T. lobayense* [172]. The fractions produced from *I. obliquus* mentioned above showed, in another work, the presence of proteins and aromatic compounds, for mycelium and sterile conk extracts, respectively [153]. The authors report that deproteinization affected the radical scavenging capacity of the fractions, although some fractions observed an increase rather than a decrease [153]. The decrease of radical scavenging capacity of mushroom polysaccharide fractions after different deproteinization steps was already noticed [180]. Despite this, it has been suggested that also the carbohydrate chain can scavenge radicals, through the generation of carbon radical in position C1, which would be stabilized by reaction with another monomer unit [181]. It has been suggested that the geometry of the glycosidic linkage would then influence the scavenging capacity of the polysaccharides [182]. The capability of mushroom polysaccharides to perform in metal ion reduction essays, which has been observed for example in *A. aegerita* [173], *C. indica* [176], *I.obliquus* [179], *Trametes orientalis* [183], and *R. vinosa* [177], has been attributed to the polysaccharide chelating properties [177] and to the presence of proteins in the extract [179]. The chelating properties of mushroom polysaccharides could be mainly ascribed to the presence of uronic

acid or other charged moieties in the chain [181], although Lo suggests polymer side-chains might have a positive effect in the chelation properties of polysaccharides [182].

2.6   Food applications

Compared to the market volume of mushrooms and mushroom extracts, the applications of mushroom polysaccharides as food ingredients are scarce and mainly in the research and development phase. Also, compared to the research on the biological activities, the research on the use of mushroom polysaccharides as ingredients is very limited. Moreover, the studies on "realistic food matrices" for the creation of mushroom polysaccharides-based functional foods with scientifically demonstrated positive effects on the consumer health are scarce [3]. Most of the studies concern the incorporation of mushroom powder or mushroom polysaccharide in food matrices. These ingredients have been incorporated in liquid or semisolid formulations, such as creams [165,184] or yoghurts [169,185], and they have been studied as well as meat replacer [186]. However, the main focus of the research has been their incorporation in flour-based foods [3,187].

As recently reviewed, powdered forms of mushrooms such as *L. edodes* and *Pleurotus pulmonarius* have been incorporated in muffins, extruded snacks, and cookies. In addition, these powders have been incorporated in flours such as wheat, brown rice, potato, rice, and yam [187]. In his review regarding wheat flour-based products, Salehi suggested a maximum dosage of 15% mushroom powder as flour replacement [188], due to its effect on the technological properites and quality of the fortified food. The addition of *L. edodes* powder to muffin formulation caused an increase in hardness and decrease in expansion [189]. Addition of powder originating from the same mushroom to pasta increased tensile strength without affecting significantly other properties [190]. Bread preparation was added with different dosages of powdered *L. edodes*, *A. bisporus*, or *B. edulis*. Negative effects on technological properties of bread were observed, although differed between mushroom species[191]. The addition of the same mushroom powders to semolina extruded snacks altered the structure of product and starch digestibility [192]. Positive, although preliminary, results on the overall approval of brown rice extruded snacks containing *P. pulmonarius* powder were recently obtained [193]. Powdered mycelia of *Antrodia camphorata*, *Agaricus brasiliensis*, *H. erinaceus* and *Phellinus linteus* were used as replacement for bread wheat flour. The dosage of 5% was deemed acceptable after technological analysis and sensory evaluation, except for *A. camphorata*, which was the most disliked by the panel [194].

The incorporation of mushroom polysaccharides has been mainly tested in dough systems. After their experiments with the addition of *F. velutipes*

polysaccharides, Nie and cowriters recommend the addition of low levels of polysaccharides due to their ability to disrupt the gluten network and the increased hardness of the final product [195]. On the other hand, Sulieman and coauthors found that the addition of *A. bisporus* polysaccharides had a general improvement of dough technological properties, reporting also a decrease in dough hardness. Moreover, the product was acceptable for consumers [196]. A decrease in hardness was also observed after the addition to yam starch of *A. auricula* polysaccharides. The authors indicated the potential of these polysaccharides for the incorporation in jelly foods [197]. The conflicting results could be explained by differences in molecular weight of the polysaccharides: longer chains would create a network between starch granules able to prevent their aggregation [187,197]. Recent evidence showed that polysaccharides of higher molecular weight tend to coat less efficiently starch granules than polymers with lower molecular weight [198], making this explanation plausible.

2.7 Extraction technologies

The most common extraction medium used to obtain mushroom polysaccharides is hot water, usually at 100 ºC. From the extract, polysaccharides are purified utilizing ethanol precipitation, deproteinization (performed with Sevag solvent, protease hydrolysis, or TCA precipitation), and dialysis. Eventually, column chromatography fractionation is performed to purify polysaccharides basing on charge (anion exchange chromatography) or molecular weight (size exclusion chromatography) [199]. The properties of the polysaccharides are highly dependent on the medium with which they are obtained. On the other hand, the same medium can be modified to improve the extraction procedure and increase the yield. The balance between desired polymer properties and extraction yield is one of the key aspects of the research on mushroom polysaccharides. Different physical and chemical methodologies can be applied to modify the aqueous extraction medium to improve the extraction yield. Outlining the influence of the utilized method on properties such as monomer composition, molecular weight, glycosidic linkage, and presence of protein, is crucial, since these properties influence the potential of the obtained polysaccharides for their application. Moreover, the polymers with most interest from the food and nutraceutical industry are the β-glucans. As reviewed in Section 2.3, these polymers are located in the middle and lower layers of the fungal cell wall. Their position reduces their accessibility to hot water during extraction. Therefore, different extraction technologies are applied to increase the yield of these polymers.

2.7.1   Pressurized hot water extraction

Pressurized hot water extraction has been also called pressurized water, pressurized liquid, hydrothermal extraction, or subcritical water extraction. As pretreatment in biorefinery, it is mainly called hydrothermal processing or autohydrolysis. The aqueous extraction medium, with the aid of pressure, is kept at temperatures above the boiling point, in a range 100-374 ºC, and below the critical point (374 ºC, 22.1 MPa) [200,201]. The difference of the dielectric constant of subcritical water compared to water at atmospheric pressure is believed to have an impact on the solubility of polysaccharides. For example, at 20 MPa and about 150 ºC, water has the dielectric constant of dimethyl sulphoxide (DMSO) at 25 ºC and 0.1 MPa [202] and DMSO is a common solvent for water-insoluble polysaccharides (for example in [203]). However, pressurized hot water exercises a positive effect on polysaccharide extraction mainly due to the increase in the ionization constant of water, i.e. in the acidification of the medium and the consequent hydrolysis of the cell wall structure [201]. Biomass is reported to collapse at about 250 ºC and extracted polysaccharides are hydrolyzed in the medium at temperature above 170-190 ºC. Pressure, on the other hand, is considered to have a negligible effect on the hydrolysis [204].

The forest mushroom *C. tubaeformis* was submitted to autohydrolysis process at different temperatures (80-240 ºC). Yield of unpurified extracts ranged between 40-66%, with protein contents in the range 9-11%. Raw yield increased from about 50% to about 63% when the temperature was raised to 170 ºC, and afterwards oscillated between that and the maximum value. Noticeably, free glucose had maximum concentration at 100 ºC, while the concentration of polymeric glucose remained almost stable until 170 ºC, after which it dramatically increased. The β-glucan content increased from about 2% of the extract to about 4.5% at 170 ºC, and stabilized around 14% at 210 and onwards ºC [205]. In another report, after the extraction of polysaccharides from *P. ostreatus* and *G. lucidum* with pressurized hot water, the influence of water temperature on the yield was stronger than the extraction time. The tested extraction temperatures were 50, 115, and 180 ºC and the tested extraction times were 5, 17.5, and 30 minutes. The extractions, even at suboptimal parameters, granted polysaccharide yields, after EtOH precipitation, higher than that obtained with standard hot water. The polysaccharides extracted with optimized parameters showed the presence of α- and β-glucans and heterosaccharides[206]. Powdered *L. edodes* has been extracted with hot water in subcritical conditions with different temperatures in the range 100-150 ºC for 15 minutes. The polysaccharide yield, measured after deproteinization and dialysis, increased until 120 ºC, after which it decreased. The polysaccharides were composed mainly of glucose, and its relative content increased proportionally to the extraction temperature. The molecular weight of the main population of polysaccharides increased until 130

ºC and then decreased. On the other hand, the effect of the extraction conditions on the structure of the polysaccharides was not fully elucidated [207]. Morales and coworkers compared different methodologies to extract *L. edodes* polysaccharides. The comparison of different hot water extractions (100 ºC) and subcritical water extractions (200 ºC, after [208]) carried in parallel with increasing time (in the range 15-60 min) showed, on one hand, lack of significant increase in yield with classical hot water and a continuous decrease in yield with subcritical water. Nevertheless, the subcritical water granted, compared to classical extraction, yields higher by two folds or more. However, the β-glucan content of the extracts was clearly inversely proportional to the extraction time and, after 60 minutes of extraction, was about 1/3 of the content obtained with traditional hot water[209].

2.7.2   Ultrasonic-assisted extraction

Compared to pressurized hot water extraction, the utilization of ultrasounds to increase polysaccharide yields is much more common in literature. The disruptive effect of ultrasound on the cell wall structure is attributed to the formation of bubbles, whose collapse generates shear force. In this way, higher mass transfer is enabled. In a typical ultrasound extraction apparatus, an ultrasonic probe is immersed in the extraction medium. In this way, multiple extraction parameters can be tuned: ultrasonic frequency; ultrasonic power; liquid temperature; extraction time; duration of ultrasound treatment. Besides the parameter optimization is required to avoid sonic depolymerization, most of the apparatuses available have an open extraction chamber or are not connected to a refrigerator, therefore solvent losses are possible, especially at higher temperature [200].

   With the application of ultrasounds at room temperature, the extraction yield from *G. lucidum* almost doubled, compared to standard hot water extraction of the same duration. Noticeably, there were no significant differences in the β-glucan content of the extracts. Nevertheless, scanning electron microscopy of the extraction residue showed a disruption of the hyphal structure much more intense compared to hot water extraction [210]. The optimization study of ultrasonic-assisted extraction of polysaccharides from *T. orientalis* showed that, increasing ultrasonic power, extraction yield reaches a maximum and then decreases (the same was found for extraction temperature), while with the increase of extraction time the yield reaches a plateau. Parameters were optimized with Box-Behnken design and they were tested together with hot water extraction, performed with the same solid-liquid ratio at 80 ºC (compared to the optimized 40.2 ºC) and for 120 min (compared to the optimized 42 min). Compared to traditional extraction, the ultrasound extraction granted an yield increase of about 19% [183]. On the other hand, the optimized ultrasonic-assisted extraction granted, for *Panellus serotinus*

polysaccharides and compared to hot water extraction, a yield increase of 63%, with little influence on their monomer composition. The authors in their report mentioned differences in molecular weight profile, without further details [211]. Morales and coauthors, in their comparison of different extraction methods applied to *L. edodes*, reported no significant influence of the extraction time on the polysaccharide yield of ultrasound-assisted extraction (50 ºC). However, all the yields were higher than the ones obtained with traditional extraction. Noticeably, 15 min of ultrasound-assisted extraction granted a yield 141% higher than 60 min of hot water extraction without ultrasonication. The β-glucan content of the extract was proportional to the extraction time in both methodologies. In addition, the content of ultrasound extracts was at least 50% higher than the content of hot water extracts[209].

The depolymerization effect of ultrasounds has been documented with a polysaccharide extracted from the mycelium of *P. linteus*, whose degradation profile fitted a second-order rate law $1/M_t = 1/M_0 + k \times t$ $(0.988 < R^2 < 0.997)$, with the rate constant k proportional to the ultrasound power[212].

2.7.3 Microwave-assisted extraction

In a similar way to ultrasounds, the yield improvement of microwave-assisted extraction, compared to use of hot aqueous medium, has been attributed to the generation of internal pressure in the fungal material and the disruption of the cell wall. The pressure would be caused by the cell water, whose temperature (i.e. molecular vibration) is increased by the microwaves. The main advantage of microwave-assisted extraction is the short extraction time (usually in the range 5-30 minutes) and the flexibility of the apparatus, while its main drawback is the inhomogeneous heating [200]. Since microwave might cause depolymerization of the fungal polysaccharides, optimization of the extraction parameters is usually carried out. Microwave power, extraction temperature and time are the parameters usually optimized. With specific equipment, extraction temperatures above water boiling point can also be used.

Gil-Ramirez and cowriters reported a polysaccharide yield, after microwave-assisted extraction of *L. edodes*, substantially higher than conventional hot water extraction, with the clear advantage of extraction time (19% obtained after 30 min at 180 ºC against 5% after 3 h at 100 ºC) [213]. Similar results were obtained with *P. ostreatus* and *G. lucidum* [206]. The microwave-assisted extraction of polysaccharides from *F. velutipes* granted, compared to conventional hot water, a yield increase by 31%, with a decrease in polysaccharide content by 10%. The extraction times were 90 min and 10 min for conventional and microwave-assisted extractions, respectively, and the extraction temperatures were almost the same (95 and 110 ºC, respectively) [214]. Similar results were observed in another study on *F. velutipes* polysaccharides, again comparing conventional hot

water and microwave-assisted extraction [215]. Since the utilization of microwaves improved both yield and sugar content also of *Clitocybe maxima* extract [216] and other screened species [213], it could be inferred that trends are species-specific. While no significant differences in yields were found between microwave-assisted and hydrothermal extractions of *G. lucidum*, the latter granted higher yield, compared to the earlier, when applied to *P. ostreatus* [206]. In the screening performed by Gil-Ramirez and coworkers, extraction yields ranged between 13-18%, with the notable exception of *A. auricula*, whose extraction yield was 44% [213].

It is possible to combine microwave- and ultrasound-assisted extraction in a single apparatus. In the works examined, the ultrasound frequency and power have been kept constant. Huang and Ning extracted polysaccharides from *G. lucidum* by screening different microwave powers, extraction time, and liquid/solid ratios. The optimized extraction conditions provided a yield (3.3%) higher of 28%, compared to ultrasonic-assisted extraction, and of 116%, compared to traditional extraction [217]. Zheng and coworkers reported a limited yield increase when the different techniques were applied to *T. orientalis*: ultrasound/microwave-assisted , ultrasound-assisted, microwave-assisted, and traditional extractions had yields of 7.52%, 7.49%, 6.25%, and 6.48%, respectively [218]. At the conditions tested by Huang and Ning, a clear decrease in yield was observed with prolonged extraction times, rather than with the increase in microwave power [217]. On the other hand, the optimization of the ultrasound/microwave-assisted extraction of *Tricholoma mongolicum* granted a yield of 35.4% and showed that increase in microwave energy at constant temperature causes decrease in polysaccharide yield [219].

2.7.4 Acidic and alkaline extraction

The fungal cell wall layer removal with alkali has been well documented and widely used for the study of the cell wall itself [97]. The disruption of the fungal cell wall has been classically achieved with the use of alkali or acidic solutions. These extractions are typically performed after the classical hot water extraction and are widely reported in literature. The yield increase is attributed to the hydrolysis of covalent bonds and disruption of the non-covalent bond network between cell wall components. In this way, aqueous medium has easier access to the lower layers of the cell wall. Therefore, these methodologies have been widely used to obtain fungal β-glucan and heterosaccharides enriched fractions. Despite this, increase in yield has not been observed universally.

The well-known limitation of the use of acid or alkali for the extraction of polysaccharide is its hydrolytic effect. Alkali solutions "peel" polysaccharides starting from the reducing end monomer, through isomerization (depending on the glycosidic linkage) and β-alkoxy elimination. Glycans linked (1→2) and

(1→6) are reportedly more stable than (1→3) and (1→4). Branches situated in position O-2 of (1→4) and branches situated in position O-3 stop the peeling process[220]. In addition to this, polysaccharides can also be hydrolyzed in alkali due to the deprotonation of C2 hydroxyl [220], which would react with the glycosydic C1 of the same monomer and cleave the linkage via a C1 carbanion intermediate [221]. This second reaction could occur in any position of the polysaccharide chain. However, reaction kinetics are dependent on the position of the glycosidic linkage and the monomers involved, since the position of the hydroxyl groups has a stabilization effect on the linkage [221]. In acidic environment, acid catalyzes the hydrolysis of glycosidic linkages. While this could theoretically happen in any position on the chain, with a mechanism equal for all polysaccharides, depolymerization rates are dependent, besides temperature, on conformation flexibility of the linkage and steric effect of substitutes on the monomeric unit of the polysaccharides. The research on the elaboration of models to explain the polysaccharide depolymerization is still ongoing [222].

Typical alkali solutions used to extract fungal polysaccharides are KOH and NaOH. A list of polysaccharides extracted with these solutions would be excessively long, therefore only some examples are given. Hot 1 M NaOH were used to extract a β-glucan from *P. ostreatus* [223]; cold 1 M NaOH was used to extract β-glucans from *P. eryngii* [76]; cold and hot 10% NaOH extracted β-glucans from *A. brasiliensis* [141]; hot 2% KOH and 25% KOH extracted from *F. velutipes* a β-glucan and a xylomannan, respectively [203]; hot 0.1 M NaOH extracted a β-glucan from *L. edodes* [109]; hot 1 M NaOH extracted a β-glucan from *C. cibarius* [112]; room temperature 1 M NaOH extracted a glucomannan from *B. edulis* [128]; hot 2% KOH extracted an heterosaccharide from *A. bisporus* [106]; hot 1 M NaOH extracted a mixture of polysaccharides from the internal tissues of Chaga [54]. Yield results are reported in **Table 10**. As can be observed, there is no systematic addressing in literature on the influence of the use of alkali on yield improvements and molecular weight of the polysaccharides.

Recently, the stepwise fractionation of polysaccharides via their precipitation with increasing concentrations of EtOH, a known procedure in hemicellulose chemistry, has been applied to a mushroom alkali extract, obtained without prior hot water extraction. *Agrocybe cylindracea* was extracted with 0.5 M NaOH. The extract was deproteinized and dialyzed, and then added with anhydrous EtOH to reach ethanol concentrations of 10%, 30%, 50%, and 80% v/v. Although some fractions required further column chromatography purification, the authors have noticed that the fraction precipitated with 10% EtOH was richer in heterosaccharides, while 50% and 80% fractions were richer in β-glucan [224].

Table 10. Selected examples of yields obtained with sequential hot water and alkali extraction of basidiomycete polysaccharides.

Species	Hot water	Alkali 1[c]	Alkali 2[c]	Reference
		yield (w/w%)		
Cultivated mushrooms				
A. bisporus	3.9	6.0 (KOH 2%)	n.p.[a]	106
L. edodes	1.1	6.2 (NaOH 0.1 M)	n.p.[a]	109
A. brasiliensis	10.8	2.2 (cold NaOH 10%)	1.8 (hot NaOH 10%)	141
P. ostreatus	1.5	4.5 (NaOH 1 M)	n.p.[a]	223
Forest mushrooms				
B. edulis	n.r.[b]	n.r.[b] (Na$_2$CO$_3$ 5%)	2.0 (NaOH 1 M)	128
C. cibarius	1.9	6.0 (NaOH 1 M)	n.p.[a]	112
F. velutipes	n.r.[b]	3.6 (KOH 2%)	0.7 (KOH 25%)	203
Rare mushrooms				
G. frondosa	14.9	6.3 (cold NaOH 10%)	4.5 (hot NaOH 10%)	225
I. obliquus	1.4	1.0 (NaOH 1 M)	n.p.[a]	54

[a]not performed; [b]not reported; [c]alkali medium reported in brackets.

Hydrochloric acid is the typical acid used for the extraction of polysaccharides. In addition, the use of ammonium oxalate and formic acid has been also reported[200]. Polysaccharides have been sequentially extracted from *P. linteus* mycelium utilizing hot water, 1% ammonium oxalate, and 1.25 M NaOH. The respective yields were 19.5%, 17.0%, and 9.1%. Nevertheless, the sugar content of the extracts increased from 65% to 78% and 85%, respectively. Alkali extracted an heterosaccharide, while the other extracts were rich in glucans[226]. The mycelium of *P. cocos* has been sequentially extracted as well, utilizing hot water, 0.5 M NaOH, and 88% formic acid. Hot water extract was rich in mannose, galactose, and proteins, while the others were mainly glucose and had >90% sugar content, although the formic acid extract had high content of galactose. Polymers extracted with formic acid had M_w of one magnitude higher than the ones extracted with alkali and also higher than the M_w of the hot water polymers[227]. In the case of *P. linteus*, ammonium oxalate extracted polysaccharides with M_w slightly higher than hot water and alkali, although populations of lower M_w were more abundant[226]. Szwengiel and Stachowiak compared a classical hot water extraction of *P. ostreatus* to optimized hydrochloric acid extraction aiming to maximize polysaccharide content (measured as area of polysaccharide population in HPSEC) and minimize the protein content (since the protein-polysaccharide covalent bond is hydrolysable).

They concluded that 3.8% HCl extraction carried at 30 ºC for 5 h was able to preserve the most the three polymer populations observed and remove all bound proteins[228].

2.7.5   Enzymatic-assisted extraction

Another way to disrupt the fungal cell wall to increase its permeability to the solvent is through enzymatic hydrolysis. Compared to the aforementioned methodologies, it has lower energy requirements and it is more environmental-friendly. Moreover, if proper enzymes and extraction conditions are used, there is no depolymerization of the target compound, nor the denaturation of its tertiary structure. The disadvantages of this technique are the high cost and the difficulties in scaling-up the process. Besides the enzyme type, pH, temperature, and reaction time are the parameters investigated in optimization studies. Typical enzymes employed in the process are cellulase, papain, pectinase, and trypsin, used alone (generally cellulase) or in combination[200].

Cellulase alone has been used for the extraction of polysaccharides from *Hohenbuelia serotina* [211], *F. velutipes* [214], and *T. mongolicum* [229]. In the latter work, extraction conditions were also optimized by Box-Behnken design. However, significant extraction yield increase, compared to classical extraction, was reported only for the first mushroom. Polysaccharides from both mycelium and fruiting body of *H. erinaceus* were extracted utilizing enzyme mixtures. For the earlier, the mixture was cellulase:pectinase:papain 2:1:1, while for the latter it was cellulase:pectinase:trypsin 2:1:1. Experimental conditions were also optimized. Extraction yields were 13.9% and 13.5%, respectively [230,231]. Compared to hot water extraction, the use of enzyme mixture increased the yield from *H. erinaceus* fruiting body of 68% [230]. A mixture of cellulase:pectinase:papain 1:1.3:1, with optimized extraction conditions, has been used to obtain polysaccharides from *L. edodes*. In comparison with hot water extraction, the yield increase was 47%[232]. Viscozyme L, a mixture of different carbohydrases, has been applied for the extraction of different components of *I. obliquus*. The content of total glucan in the extract was higher than traditional extraction and similar to autoclave extraction, while the β-glucan content after enzymatic extraction was the highest [233]. Snailase, a complex mixture of hydrolytic enzymes extracted from snail gastro-intestinal tract, has been used, with extraction parameter optimization, to obtain polysaccharides from *A. bisporus*. The extraction yield from the first species was 5.3% [234], which was comparable or lower than optimized hot water extraction [235].

2.8 Submerged cultivation of mycelium

The seasonal growth and yield fluctuations typical of wild mushroom harvesting have been solved, for some species, with cultivation. Traditionally, mushrooms have been cultivated on solid substrates (solid-state cultivation or solid-state fermentation). The oldest example reported is the cultivation of *A. auricula-judae* in Asia, which goes back to about 600 A.D. Different substrates have been used to cultivate fruiting bodies after inoculation, such as wood logs and boxes or bags containing substrate[236]. A typical mushroom cultivated on wood logs is *L. edodes*. Wood sawdust-based substrates pressed in bags (sometimes called "artificial log") are used for the cultivation of, for example, *L. edodes*, *F. velutipes*, and *A. auricula-judae*[237]. Examples of other substrates are chicken or horse manure mixed with straw for the cultivation of *A. bisporus* or straw or sawdust for *P. ostreatus*[236]. The cultivation of this latter species is favored by its ability to grow on multiple agricultural and forest residues, therefore enhancing their value[238]. Despite the agro-forestry waste valorization and its relevance for circular economy, the major drawback of solid-state cultivation is the cultivation time. The first appearance of immature fruiting bodies (primordia) requires: up to 1 year in natural log cultivation[237]; up to 4-6 weeks in oak sawdust bags[237] or different lignocellulosic residues[238].

There is an alternative to the solid-state cultivation of mushroom. The large-scale cultivation in liquid medium (submerged cultivation) of ascomycetes for the production of antibiotics sparked the interest for the cultivation of basidiomycetes as well. Compared to solid-state cultivation, submerged cultivation is faster (although basidiomycetes are noticeably slower than ascomycetes) and easier to carry out, monitor, and scale-up in larger bioreactors. The advantages and disvantages of solid-state and liquid-state cultivation are reported in **Table 11**.

Table 11. Comparison of advantages and disvantages of solid-state and liquid-state cultivation of basidiomycetes.

Cultivation method	Advantages	Disadvantages
Solid-state	easy conversion of agro-forestry sidestreams; low energy consumption	Slow; compound isolation from cultivation medium not straightforward
Liquid-state	fast; easy to control; compound isolation from cultivation medium straightforward	high energy consumption; difficult commercial scale-up

While not necessarily a disadvantage, the mycelium obtained with submerged cultivation is not identical with the mushroom obtained from solid-state cultivation. The review on the proximate composition of different mycelia and

fruiting bodies published by Ulziijargal and Mau[24] is able to provide some (at least approximative) information. In this work, the proximate composition of both mushroom and mycelium was reported for 11 species. The heatmap reported in **Figure 8** shows the variation from fruiting body to mycelium in terms of contents of ash, reducing sugars, soluble polysaccharides, insoluble fiber, total fiber, total carbohydrate, fat, and proteins. General decrease in ash and general increase in fat and protein contents can be observed. Regarding carbohydrates, besides reducing sugars and fiber contents, general decreasing trends can be observed. The decrease in soluble polysaccharides was quite generally close to 50%, while there were no general decreasing trends for the others. In the case of *I. obliquus*, the differences can be explained by the nature of the compared materials: Chaga is a sterile conk, containing wood tissue, and therefore wood fibers, such as hemicelluloses and cellulose. In **Figure 8**, the most striking increases were the protein content *of I. obliquus* (+1595%) and the fat content of *G. frondosa* (+695%). In the whole dataset, it can be noticed that, in general, mycelia had significant higher amounts of fat and protein and significant lower amounts of soluble polysaccharides, total fiber, and total carbohydrates. On the other hand, the contents of reducing sugars and insoluble fiber between fruiting bodies and mycelia do not differ significantly[24].
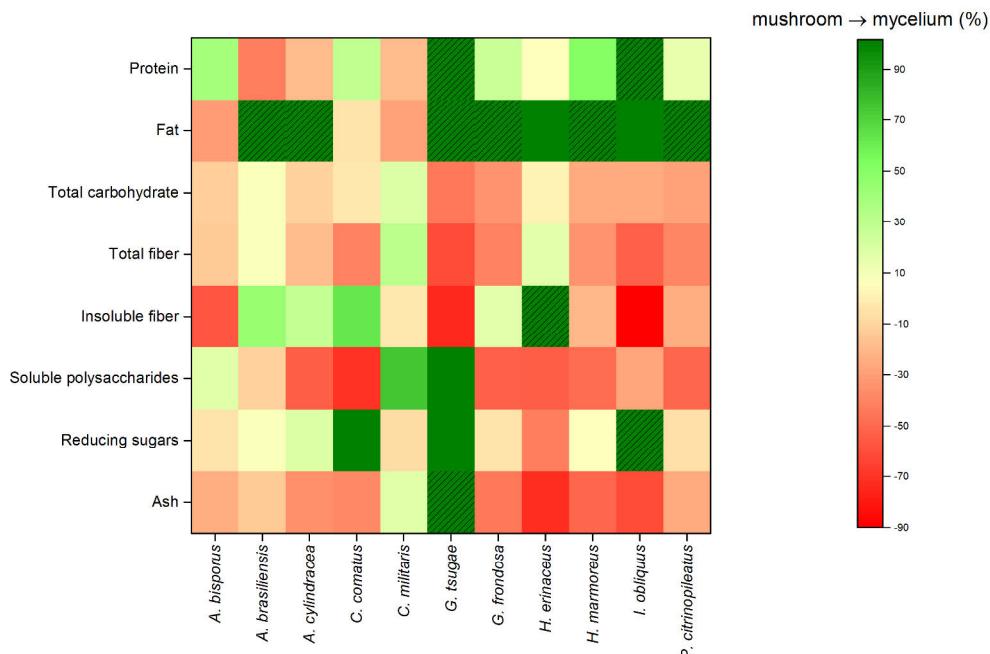

Figure 8. Heatmap representing the change in proximate composition of cultivated mycelia components (weight%) respective to the fruiting body of the same species. Line pattern represents an increase above 200%. Data elaborated from[24].

Submerged cultivation has been defined by Fazenda and cowriters as "intrinsically less problematic" than solid-state cultivation, due to the achievable higher heat and mass (oxygen and nutrients) transfers and culture homogeneity[239], making studies on the cultivation conditions easier to perform. On the other hand, the authors complained about the lack of research on the biochemical aspects of mycelium growth in liquid medium and how they are effect by the different cultivation conditions. This complaint has been reiterated more recently[240]. Most of the studies in literature concern optimization of cultivation conditions and liquid medium components, with the aim to increase cultivation yield and content of molecules of interest. The investigated factors have been classified as physical, such as temperature, agitation, and aeration; chemical, such as pH, carbon source, nitrogen source, and C/N ratio in the cultivation medium; biological, such as inoculum and mycelium morphology.

2.8.1 Physical factors

The cultivation temperature affects mycelium yield, growth rate, and formation of the compound of interest. It also affects the amount of oxygen dissolved in the liquid medium. An increase in temperature generally results in metabolism stimulation until an optimum value, while the concentration of oxygen is negatively affected. Optimal temperatures are usually in the range 25-36 ºC. However, the optimal temperature for mycelium growth does not necessarily correspond to the temperature of higher internal (IPS) or exocellular (EPS) polysaccharide production[241].

The agitation rate influences the mixing of medium components and mass and heat transfer. Without proper agitation rate, the mass transfer gradient could negatively affect mycelium growth and metabolism by ineffective nutrition, oxygenation, and removal of catabolites from cell environment. On the other hand, agitation creates shear forces in the medium, which could as well hamper mycelium growth and cause cell damage. It has been noticed that the agitation rate resulting in highest mycelium growth could be different than the agitation rate for highest exopolysaccharide yield [239]. Both higher and lower agitation rate, compared to optimal mycelium yield, have been reported[239,241].

Fungal metabolism is aerobic. Therefore, the supply of oxygen affects all the results of the cultivation process. In most of the published studies, aeration of submerged culture is controlled by modification of agitation rates. Studies measuring the oxygen transfer from gaseous phase to liquid phase are available and indicated that cell growth and IPS, and EPS production have different oxygen requirements. Moreover, the pellet critical size is directly proportional to oxygen concentration in the medium and inversely proportional to oxygen consumption rate [239].

The cultivation time determines the amount of mycelium and polysaccharides collectable from the liquid medium. At prolonged cultivation times, nutrients are depleted, and mycelium secretes enzymes for nutrient production, such as β-glucanases. The cultivation time granting highest EPS yield does not necessarily correspond to the cultivation time granting highest mycelium nor IPS yields. However, mycelium and EPS are usually obtained simultaneously, at the stationary phase.

2.8.2   Chemical factors

The pH of the medium can affect fungal membrane functionality, solubility of medium components, uptake and metabolism of nutrients, and formation of bicarbonate from dissolved CO_2 [239]. Optimal pH values for mycelium growth and compound production are species- and strain-specific, making generalizations difficult. Most of the studies on medium pH have monitored only initial pH, even though secretion of catabolites would cause shifts in its value throughout the cultivation[241]. Moreover, optimal pH is also dependent on medium composition[239].

Carbon and nitrogen sources are the major cultivation medium components. There is no universal medium for optimal submerged cultivations. However, some general statements have been formulated by Fazenda and cowriters: higher concentration of carbohydrates generate higher mycelium yield; C/N ratio influences compound production, mycelial yield and protein and lipid contents; complex media like lignocellulose or waste generally requires nitrogen and mineral addition; carbon source affects mycelial polysaccharide properties; protein content is affected also by the nature of the nitrogen source; supplementation of lipids generally stimulates mycelium growth[239].

Carbon sources could be simple carbohydrates such as glucose, xylose, sucrose, or maltose, or complex, such as starch, syrup, or agro-forestry wastes. Different fungal species utilize carbon sources differently. Moreover, different carbon source concentrations could lead to different effects, especially if the carbon source is complex. The optimal carbon source concentration for mycelium growth does not necessarily coincide with the optimal concentration for EPS or IPS production. For example, increasing glucose concentration in liquid medium increased mycelium yield and EPS production of *Ganoderma applanatum*, but not IPS production[242]. Moreover, Elisashvili reported that *Trametes maxima* and *Phellinus igniarius* had mannitol as optimal C source, while highest EPS production was achieved with maltose[241].

Submerged cultivation can utilize both inorganic and organic nitrogen sources. Examples of earlier are nitrate and ammonium salts, while the latter are represented by single amino acids, casein, peptone, or yeast extract. As with the carbon source, the nitrogen source for optimal cultivation is species-dependent,

although some preference for organic nitrogen can be noticed[243–245]. Moreover, nitrogen source or concentration are not necessarily the same for optimal mycelium yield and polysaccharide production[241]. There is no general agreement on the effect of the C/N ratio to the mycelium growth: increase of C/N determined increase in yield for *G. applanatum* [242] and *A. cinnamomea* [246], while the opposite was observed for *C. versicolor* [247]. Increase of IPS, followed by decrease, were observed while decreasing C/N ratio were observed for both *G. applanatum* [242] and *Cordyceps gunnii* [248].

2.8.3   Biological factors

Differently to the submerged cultivation of ascomycetes, basidiomycetes require the utilization of mycelium as inoculum. Most commonly, the inoculum consists of pieces of agar on which the mycelium was grown. In bigger scale procedures, such as bioreactors, a seed culture is used. The agar inoculum causes a longer lag phase, compared to seed culture, assuming that bioreactor medium is the same of the seed culture. The optimal inoculum concentration (v/v) is dependent on the experimental set-up. A concentration higher than the optimal causes drastic decreases in mycelium growth[239].

The morphology of the mycelium (i.e., the appearance and shape of the hyphal mass) is affected by inoculum concentration, physical, and chemical factors. Basidiomycetes generally grow as filaments, pellets (up to 2 cm diameter), or as shapes in between[249]. While it seems that inoculum concentration affects only mycelial pellet size, the other factors produce results that are more complex. Moreover, the rheology of the cultivation medium is influenced not only by the mycelium concentration, but also by its shape. As mentioned before, pellets have a critical size, related to oxygen concentration and consumption, above which the mycelium has a "non-homogeneus physiological state"[239]. Nevertheless, the pellet form should be the one favored while cultivating the mycelium, since it generates lower medium viscosity, and therefore better mass transfer. Literature is contradictory on the effect of oxygen concentration on morphology, with some reports indicating even no influence at all. There is no clear relationship also between morphology and pH[239]. Moreover, to date there is no general relationship between morphology and polysaccharides (or, in general, fungal compounds) production[250]. For example, it was found that pellet form grants higher EPS production for *G. lucidum*[249], while, for *G. frondosa*, the filamentous mycelia had highest production of EPS[251].

2.8.4   Lignocellulosic medium

Basidiomycetes are capable to produce enzymes to degrade lignocellulose and utilize it as a carbon source in liquid medium. In fact, submerged cultivation has

been considered a strategy to produce lytic enzymes such as cellulase, xylanase, laccase, and peroxidase. Lignin degrading enzymes are used by fungi to gain access to carbohydrates in the substrate, rather than for energy production per se. Multiple reports are available indicating a stimulated production of these enzymes from the mycelium when cultured in liquid medium supplemented with, for example, wheat or barley bran [252], tree leaves, and fruit peels [253]. These enzymes have application potential in biopulping, energy production, and bioremediation [254]. Mycelia are capable of utilizing these materials as nutrients due to their degradation enzyme array. Another aspect of supplementation of agro-forestry waste materials and sidestreams to liquid medium is therefore the increase in cultivation yield. The mycelium so obtained can be utilized as a source of polysaccharides. Different agro-forestry residues and sidestreams have been utilized to enhance mycelium cultivation yield. Some examples are reported in **Table 12**.

Table 12. Lignocellulosic agricultural side-streams utilized for the submerged cultivation of mycelium. The effect on the mycelium yield is relative to control unless otherwise stated.

Species	Supplement	Dosage	Mycelium yield[f]	Reference
Cordyceps sinensis[a]	rice bran	2% w/v	~30%[b]	[258]
G. applanatum	oat seed cake	60 g/L	85.7%	[257]
G. applanatum	soybean cake	60 g/L	80.7%	[257]
G. frondosa	linseed cake	60 g/L	70.5%	[257]
G. lucidum	oat seed cake	60 g/L	75.4%	[257]
H. erinaceus	wheat bran[c]	30 g/L	not reported	[259]
I. obliquus	corn straw	3% w/v	6.7%	[255]
I. obliquus	rosehip fruit cake	60 g/L	55.0%	[257]
I. obliquus	wheat straw	30 g/L	~15%	[256]
L. edodes	wheat straw	0.5% w/v	161.9%	[260]
Lasiodiplodia theobromae[a]	sugarcane straw[c]	40 g/L[d]	not reported	[261]
P. ostreatus	grape pomace	40 g/L	25.0%[e]	[262]
Pleurotus dryinus	xylan	60 g/L	283.3%	[263]
T. versicolor	camelina seed cake	60 g/L	74.7%	[257]
T. versicolor	oat seed cake	60 g/L	89.9%	[257]
T. versicolor	rapeseed cake	60 g/L	51.9%	[257]
T. versicolor	soybean cake	60 g/L	146.8%	[257]
T. versicolor	sunflower seed cake	60 g/L	68.4%	[257]

[a]Ascomycete; [b]biomass reported as glucosamine content; [c]supplement was pretreated; [d]supplement dosage is reported as glucose equivalent; [e]submerged cultivation yield was compared to solid-state cultivation; [f]the symbol ~ indicates a yield not reported by the authors but estimated from reported graphics.

The most common lignocellulose supplements are straws. These have been supplied at different concentrations to different fungal species. Corn and wheat straw have been supplemented to *I. obliquus* submerged cultivation medium, with respective yield increases of 6.7% and about 15%. In addition to the enhanced mycelium growth, the authors have reported an increase in the EPS

yield from the cultivation medium of 26.6% and 91.4%, respectively. Sugar content and monomer composition of the EPS were significantly affected by the presence of lignocellulose supplement. In particular, a drastic increase in the relative content of mannose was observed after both supplementations[255,256]. Wheat straw was the only lignocellulosic supplement positively affecting the IPS content of *I. obliquus*. At the same time, the IPS relative glucose amount increased, compared to IPS obtained without supplementation[256]. Higher mycelium yield increase, compared to the results mentioned above for *I. obliquus*, was obtained by Krupodorova and Barshteyn after supplementation of rosehip fruit cake[257]. These authors have showed that different seed cakes have positive effects on the cultivation yield of species such as *G. applanatum*, *G. frondosa*, *G. lucidum*, and *T. versicolor*. With the same dosages, the most effective supplements for these species were respectively oat seed, linseed, oat seed, and soybean cakes.

2.8.5 Oil and fatty acid supplementation

The addition of specific compound to stimulate ascomycete mycelium growth and compound production in liquid medium has been extensively studied. Moreover, the discovery of the positive effect of the supplementation of plant oil to the liquid medium for the submerged cultivation of mycelium goes back to the 1970s[264]. The supplementation of oil and fatty acids has been studied with different basidiomycetes, especially with rare species (as in the case of Finland, **Table 4**) such as *G. frondosa* or *G. lucidum*. It has been suggested that these additives, due to their surfactant properties, would alter cell permeability and allow higher intake of nutrients [241]. Moreover, the investigation of *P. sajor-caju* lipid metabolism showed increased extracellular lipase activity after supplementation of oils. In addition, as demonstrated by radiolabeling, mycelium degraded free fatty acids to acetate, which was utilized for the synthesis of polar and apolar lipids[265]. In the case of more complex additives, such as oils, other components could exert a direct or indirect influence on the gene expression of the fungus. The genome analysis of basidiomycetes cultivated in liquid medium with the supplementation of olive mill wastewater, an oil-containing sidestream, revealed modifications in the expression of β-glucan synthase. These modifications were likely related to the oxidative stress caused by the phenolic compounds of the supplement or by radicals generated during their degradation. The lipidic compounds had a less clear effect on gene expression, although contributed to the mycelium growth[266].

Examples of effects of lipid supplementation on mycelium, EPS, and IPS yields in submerged culture are reported in **Table 13**. The influence of the supplement seems to be more related to the fungal species than to the nature of the supplement itself (i.e. the length of the fatty acid carbon chain). The

supplementation of oleic acid to *I. obliquus* had noticeably lower positive effect on mycelium and EPS yield compared to *G. lucidum*. Oleic acid slightly negatively affected the IPS content of *I. obliquus*. Palmitic acid was supplemented to *G. lucidum* and *I. obliquus*. For the latter fungus, the dosage had no substantial effect on mycelium and EPS yields, although it decreased the IPS content (about -20%), quite similarly to oleic acid but at lower dosage. Palmitic acid was supplemented to *G. lucidum* in two different experiment sets. The same dosage (1 g/L) resulted in similar mycelium yield increases (91% and 83%) but in different EPS enhancements (23% and about 45%). At higher dosage, yield enhancement decreased from 91% to 48%, while EPS enhancement increased from 23% to 62%. The effect of stearic acid supplementation to *I. obliquus* was similar to palmitic acid, with higher positive effects.

Table 13. Relative effect of oil and fatty acid supplemented to mycelium liquid cultivation medium to mycelium, EPS, and IPS yields, compared to control.

Species	Supplement	Dosage[a]	Mycelium yield[b]	EPS yield[b]	IPS yield[b]	References
G. frondosa	olive oil	0.1%	31.5%	-2.0%	-66.6%	[267]
G. frondosa	olive oil	0.5%	139.3%	12.2%	-70.9%	[267]
G. frondosa	olive oil	1%	238.4%	26.9%	-50.9%	[267]
G. frondosa	sunflower oil	0.1%	35.8%	-20.0%	-63.5%	[267]
G. frondosa	sunflower oil	0.5%	145.1%	-49.0%	-55.8%	[267]
G. frondosa	sunflower oil	1%	236.4%	-30.7%	-29.8%	[267]
G. lucidum	corn oil	1%	51.5%	23.0%	n.r.[c]	[268]
G. lucidum	oleic acid	1.5 g/L	100.0%	23.1%	n.r.[c]	[269]
G. lucidum	oleic acid	1.5 g/L	130.0%	~50%	n.r.[c]	[268]
G. lucidum	olive oil	1%	42.6%	23.1%	n.r.[c]	[269]
G. lucidum	olive oil	1%	62.4%	26.2%	n.r.[c]	[268]
G. lucidum	palmitic acid	1 g/L	91.3%	23.1%	n.r.[c]	[269]
G. lucidum	palmitic acid	2.5 g/L	47.8%	61.5%	n.r.[c]	[269]
G. lucidum	palmitic acid	1 g/L	82.6%	~45%	n.r.[c]	[268]
G. lucidum	safflower oil	1%	20.9%	38.5%	n.r.[c]	[269]
G. lucidum	safflower oil	1%	37.4%	44.4%	n.r.[c]	[268]
G. lucidum	sunflower oil	1%	45.3%	24.6%	n.r.[c]	[268]
I. obliquus	oleic acid	0.05%	~0%	16.3%	-5.1%	[270]
I. obliquus	oleic acid	0.1%	~16%	16.0%	1.6%	[270]
I. obliquus	oleic acid	0.5%	23.7%	26.4%	-12.4%	[270]
I. obliquus	palmitic acid	0.1%	~16%	~0%	~-20%	[270]
I. obliquus	stearic acid	0.1%	27.1%	~10%	~-20%	[270]

[a]percentage represents v/v%; [b]the symbol ~ indicates a yield not reported by the authors but estimated from reported graphics; [c]not reported.

When olive oil was supplemented to *G. frondosa* and *G. lucidum*, the same dosage had remarkably different effects on the mycelium yield: for the earlier, the yield increase was 238%, for the latter it was 43% and 62% (two different experiment sets). However, the effect of olive oil on EPS yield was similar (increases of 27%, 23%, and 26%, respectively). Olive had a clear negative effect on the IPS content of *G. frondosa*. Safflower oil was supplemented to *G. lucidum* in two different experiments, using the same dosage, obtaining similar results on

mycelium yield (increases of 21% and 37%) and EPS yield (increases of 39% and 44%). Sunflower oil was supplemented to *G. frondosa* and *G. lucidum*. The same dosage, like with olive oil, favored the growth of *G. frondosa* (236%) rather than *G. lucidum* (45%). Sunflower oil had as well remarked negative effect on *G. frondosa* IPS content, and, differently from olive oil, on EPS yield. The EPS yield from *G. lucidum* cultivation medium was positively affected by sunflower oil in a similar manner to olive oil. The utilization of corn oil, on the other hand, had positive effect on *G. lucidum* mycelium yield, in between olive oil and sunflower oil. Regarding the cultivation yield of this fungus, safflower oil had the lowest enhancing effect.

2.9   Summary of the literature review

Mushroom is the common name of the specialized spore-bearing structure produced by fungi belonging to the phyla Basidiomycota and, to a lesser extent, Ascomycota. Most of the species of interest belong to the class Agaricomycetes of the phylum Basidiomycota. The position (respective to soil), structure, and shape of mushrooms vary greatly. These organisms can be saprotrophic, ectomycorrhizal, or plant pathogens.

Mushrooms have been consumed as a food and medicine for millennia, with traditional uses attested all over the world. They have a low dry matter content, which on the other hand is rich in dietary fibers and proteins and poor in lipids. One of the most consumed mushrooms is the cultivated *Agaricus bisporus*. In addition to their culinary properties, mushrooms and mushroom extracts have been used as medicine, especially in Asia and Eastern Europe. Examples of species used for medicinal purposes are *G. lucidum*, *L. edodes*, *Fomitopsis officinalis*, and *Inonotus obliquus*. *Inonotus obliquus* is a species used as traditional remedy and is found as wild resource in the forests of Fennoscandia, Baltic countries, Russia, United States, Canada, and northern China. The part used for decoction is the sterile conk produced by the fungi, rather than the fruiting body. Great research efforts have been spent to investigate the biological properties of mushroom extracts and compounds. Molecules of varying molecular weights have been proven to possess multiple biological properties, such as anti-oxidant, cytotoxic, immunomodulative, antimicrobial, anti-hypercholestolemic, and anti-diabetic.

Polysaccharides are polymers of carbohydrate units, with great variability in type, position and configuration linkage, and degree of polymerization. They are the main components (dry weight) of mushrooms. The main polysaccharides present in mushroom are the β-glucans, i.e. polymers of →3)-β-Glc-(1→ and →6)-β-Glc-(1→ units. Other polysaccharides, such as mannans, galactans, and heterosaccharides, are also present. The investigation of the extracts produced

from mushrooms has assigned to these polymers many of the biological activities as mentioned previously. In this sense, and due to their low toxicity, they have been considered biological response modifiers (BRM). In particular, there is a large body of evidence assigning immunomodulating properties to these polymers. Examples of immunomodulating properties are the modification of immune cell proliferation, citokyne release from immune system cells, and production of ROS from macrophages. Their ability to exert prebiotic properties is a more recent but promising field of investigation. Their anti-oxidant activity, demonstrated in vitro, has been connected to covalently linked moieties, such as proteins.

The biological activities of mushroom polysaccharides has sparked the interest of the dietary supplement market. Mushroom extracts are mainly commercialized as such. However, there is increasing research on the incorporation of mushroom powder and mushroom extracts in food, in particular flour-based formulations, such as bread, cookies, and extruded snacks. Noticeably, the experimental evidence shows that the technological properties of mushroom polysaccharides are influenced by their chemical and physic-chemical properties, which are influenced by starting material and extraction method.

The traditional method for mushroom polysaccharide extraction is hot water, followed by polymer precipitation with ethanol. In general, the extraction yield is low. Throughout the years, different methodologies have been applied to increase the yield. Examples are physical methods, such as the increase of temperature and pressure and utilization of ultrasounds, microwaves, or both, and chemical, such as addition of enzymes or modification of the medium pH. Modification of extraction method has a clear effect of monomer composition, type of glycosidic linkage, and molecular weight of the obtained polysaccharides. The balance between desired polymer properties and extraction yield is one of the key aspects to be considered when the extraction methods of mushroom polysaccharides are optimized.

Finland is a Nordic country, whose land is on its large part covered with forest, with large amounts of unpicked mushrooms available every year. These have great potential as starting material for polysaccharide extraction. However, their industrial utilization is hampered by the fluctuation in seasonal growth of wild mushrooms and by the logistic problems connected to forest harvesting. These issues can be overcome with cultivation. Basidiomycetes have a vast array of lignocellulose-degrading enzymes, meaning that multiple species of interest can be cultivated utilizing agricultural waste and side-streams. The cultivation of mushrooms on solid substrate is a worldwide practice. A faster alternative is the cultivation of fungal mycelium in liquid medium (submerged cultivation). Mycelium can be utilized as a polysaccharide source, taking into account that it

is not interchangeable with the mushroom obtained from solid-state cultivation, due to its different composition in protein, lipids, and fibers. Noticeably, with submerged cultivation, polysaccharides can be obtained directly from the medium with relatively simple purification. The utilization of solid-state cultivation medium as polysaccharide source has been considered much less in research. Liquid state cultivation allows the supplementation of lignocellulosic medium, although in lower amounts compared to solid-state cultivation, since as such the medium has to be acqueous. On the other hand, the supplementation of chemical compounds in liquid form, such as fatty acids, oils, growth stimulants, and surfactants, has shown positive effects on cultivation yields. Supplementation also has an impact on polysaccharides obtained from the cultivation medium and from the mycelium. Currently, most of the studies focus solely on cultivation and extraction yields, while research on effects on polysaccharide properties is lacking.

3   AIMS OF THE STUDY

The aim of the research was to characterize polysaccharides in mushroom species relevant for Finland and to investigate the effect of different extraction and production methodologies on the yield and molecular properties. For this purpose, fungal species were divided in three categories: mushrooms cultivated in large scale; underutilized forest mushrooms; rare and slow-growing species. One fungal species was utilized as representative of these categories. The objectives of the studies included in this dissertation were:

To investigate the effect of ethanol-hydrochloric acid pretreatment of cultivated *Agaricus bisporus* on the yield and the macromolecular properties of polysaccharides extracted with sequential hot water extractions. (**Publication I**)

To characterize the polysaccharides extractable from the cell wall of wild *Craterellus tubaeformis* utilizing sequential hot water and alkali extractions. (**Publication II**)

To study the effect of sea buckthorn press cake supplementation on yield and properties of polysaccharides obtained from cultivation medium and mycelium of *Inonotus obliquus* in submerged cultivation. (**Publication III**)

To compare polysaccharides obtained from sterile conk and cultivated mycelium of *Inonotus obliquus* and to investigate the polysaccharides extractable from the heart rot of birch infected by *Inonotus obliquus*. (**Publication IV**)

4 MATERIALS AND METHODS

4.1 Starting material for polysaccharide extraction

4.1.1 *Agaricus bisporus* (Publication I)

Fresh fruiting bodies of *Agaricus bisporus* were supplied by Mykora Oy (Kiukainen, Finland). The batch was cut to <1 cm slices, freeze-dried, and the slices were ground with a with a Grindomix GM 300 knife mill (Retsch GmbH, Germany) for 1 min at 1000 rpm. Dry content of the mushroom powder was >97%.

4.1.2 *Craterellus tubaeformis* (Publication II)

Fresh fruiting bodies of *C. tubaeformis* were purchased from Sienestä Oy (Kontiomäki, Finland). Fresh mushrooms were dried in an oven for 8 h at 60 °C, to obtain 100 g of dried sample of *C. tubaeformis*.

4.1.3 Submerged cultivaton of *Inonotus obliquus* mycelium (Publications III & IV)

Dried sea buckthorn (*Hippophae rhamnoides*) press cake was obtained from Polarforma Oy (Tornio, Finland). The press cake was ground and passed through a 30-mesh sieve. Particles with size below 30 mesh were collected and used as supplement in submerged cultivation. The press cake had a total lipid content of 39.5 g/100 g and a total phenolic content of 42.5 mg/100 g[271]. The content of hemicellulose was 13.2 g/100 g and the content of acid-insoluble matters was 47.3 g/100 g. The acid-insoluble matters were estimated as difference between the defatted press cake and hemicellulose.

The mycelium of *I. obliquus* was isolated from wild sclerotia collected from the birch forest in Yichun (Heilongjiang province, China) and maintained on potato dextrose agar slants. The strain was deposited at the Institute of Microbiology of the Heilongjiang Academy of Sciences. Slants were inoculated, incubated at 30 ºC for 7 days and thereafter stored at 4 ºC until further use. The mycelium was subcultured every three months. About 1 cm length of agar slant, free from aerial hyphae, was cut, smashed and transferred to a 500 mL Erlenmeyer flask containing 200 mL of aqueous cultivation medium, previously autoclaved at 120 ºC for 30 minutes. The cultivation medium contained (g/L): glucose 15; maltose 15; peptone 2; beef extract 1.3; MgSO4·7 H_2O 1.5; KH_2PO_4 3; vitamin B1 0.01. The inoculated flasks were incubated in a rotary shaker at 27 ºC, with a rotation speed of 140 rpm. Parallel to the control flask, the medium of the treatment flasks was supplemented with the different dosages of sea

buckthorn press cake (g/L): 2.5; 5; 10; 30. For each dosage, two cultivation times were investigated: 200 h and 250 h. Each cultivation was performed in triplicate.

4.1.4 Sterile conk of *Inonotus obliquus* and birch heart rot (Publications IV)

Milled powder of wild Chaga harvested from the Finnish forests was obtained from Eevia Oy (Kauhajoki, Finland). Birch stem infected with *I. obliquus* was obtained from a private forest in the municipality of Lieto, South-West Finland. The heart rot of the birch stem was carved and ground. A milled sample of wild Chaga from the forest of northern China was obtained from Yichun (Heilongjiang province, China).

4.2 Extraction of polysaccharides from *A. bisporus* (Publication I)

4.2.1 Ethanol-acid pretreatment

Ground *A. bisporus* powder was refluxed in round-bottom flask with preheated mixture of 92.5% w/w ethanol and 37% hydrochloric acid. The ratio of ethanol and hydrochloric acid 20:1[272]. Consistency was about 10%, temperature was 75 °C and the treatment was 1 h long. After the treatment, the mixture was cooled down in an ice-water bath and neutralized with sodium bicarbonate. Solid fraction was separated by centrifuging at $14334 \times g$ for 15 min. Solids were separated and stored at 4 °C for further extraction of polysaccharides.

4.2.2 Polysaccharide extraction

Approximately 75–80 g (dry mass) of acid-treated (solid residue of the ethanol-acid pretreatment) and untreated mushroom were extracted three times with 900 mL of technical ethanol for 6 h to remove alcohol soluble substances. Ethanol was removed by centrifugation. After the ethanol extractions, polysaccharides were extracted from the remaining solids by refluxing with 750 mL of boiling water for 6 h. The treated and untreated mushrooms were extracted three times and the supernatants were separated from the solid residues by centrifugation. Polysaccharides were precipitated by dropping technical ethanol to the extracts while stirring and by overnight storage at 4 °C. Polysaccharides were filtered, rinsed with acetone and dried. Yield was measured gravimetrically. The precipitates were labelled as AB1-3 (untreated *A. bisporus*) and tAB1-3 (treated *A. bisporus*), respectively.

4.3 Extraction of polysaccharides from *C. tubaeformis* (Publication II) and *I. obliquus* (Publications III & IV)

4.3.1 Polysaccharide extraction (Publications II, III, & IV)

Dried starting material was ground in a mortar (particle size <3 mm). Fats and phenolic compounds were removed from the mushroom powder by extraction with 500 mL of ethanol (95% v/v purity) performed three times, each extraction time lasting for 6 h. The solid residue was subsequently extracted three times with 500 mL of deionized water by solvent reflux (100 °C) for 6 h during each extraction. Extraction mixtures were then filtered, and the combined. After filtration, the residue of the hot water extraction was extracted with 500 mL of 2% KOH solution. The extraction was carried out three times at 80 °C, for 3 h each time[273]. After filtration, the residue was subsequently extracted three times with 500 mL of 25% KOH by refluxing for 3 h during each extraction[203]. The supernatants were combined after filtration. After extraction, the supernatants of the alkali extractions were neutralized with acetic acid. After evaporation, proteins were removed from the concentrated extracts using the Sevag method and collecting the supernatant after centrifugation at $1225 \times g$ for 10 min. All the extracts were dialyzed against running tap water for 24 h using a cellulose membrane (cut-off 12–14 kDa). After dialysis, the polysaccharides were precipitated by addition of cold ethanol (3:1 v/v) and overnight storage at 4 °C. The polysaccharides were collected by centrifugation ($13000 \times g$ for 20 min at 4 °C). Water soluble and insoluble polymers were separated using the freeze-thawing process[274] three times. Soluble and insoluble polymers were finally separated by centrifugation ($13000 \times g$ for 20 min at 4 °C), with insoluble polysaccharides being precipitated after centrifugation. The collected supernatants, which contained the soluble polysaccharides were freeze-dried. Yields were measured gravimetrically.

4.3.2 Anion-exchange chromatography (Publication II)

The soluble polysaccharides from the hot water and alkali extractions of *C. tubaeformis* were purified using anion-exchange chromatography. An aliquot of the fractions was dissolved in a minimum amount of deionized water and then loaded on a column (30 cm, 2.2 cm i.d.) packed with DEAE-cellulose, previously equilibrated with deionized water. The column was eluted with potassium chloride (0.05 M) solution and fractions, 5 mL each, were collected. To follow the elution of polysaccharides, the sugar content of each fraction was monitored with the phenol–sulfuric acid method (Section 4.5.1). The fractions were collected based on the sugar content and profile, dialyzed (12–14 kDa as cut-off molecular weight) against deionized water for 24 h, changing the water every 4

h, and freeze-dried after dialysis. The fractions were coded as Crat HW1, Crat 2%1, and Crat 25%1, respectively, and were used in further investigation and characterization of polysaccharides.

4.4 Separation of *I. obliquus* mycelium and isolation of EPS from cultivation liquid (Publication III)

At the end of the cultivation time, the medium was filtered with a 30-mesh sieve. Remaining particles of press cake were not retained. After extensive washing with distilled water, the collected mycelium was oven dried at 80 ˚C for 1 h and the yield was measured gravimetrically. The obtained cultivation liquid was further filtrated and concentrated to 50 mL. Technical ethanol (3 volumes) was added to precipitate the EPS. After overnight storage at 4 ˚C, the precipitates were collected with ultracentrifugation ($9000 \times g$ for 20 min at 4 ˚C). Precipitates were recovered with fresh technical ethanol and ultracentrifuged again to remove free sugars. Thereafter, the precipitates were redissolved in deionized water. Insoluble material was removed from EPS with freeze-thawing cycle and soluble EPS were freeze-dried. Finally, the yield of EPS was measured gravimetrically.

4.5 Composition analyses

4.5.1 Sugar, protein, phenolic, and glucan contents

Sugar content of the polysaccharide fractions was measured with the phenol–sulfuric acid method adapted for microplate[275]. Protein content of polysaccharide fractions was measured with a modified Lowry method[276] (Publications II, III, & IV) and with Bradford method[277] (Publication I). The total amount of phenolic compounds was measured according to Folin-Ciocalteu method[278]. The phenolic content was calculated as gallic acid equivalents.

Total, α- and β-glucan contents were determined with commercial Mushroom and Yeast β-glucan Assay Procedure (Megazyme Int.). Briefly, total glucan content was measured by hydrolysing the polysaccharides with sulphuric acid and enzymatically breaking the remaining oligomers to glucose units with glucosidase. Glucose was then quantified with a colourimetric assay based on glucose oxidase-peroxidase. α-Glucans were measured using the same colourimetric analysis after stirring in alkaline solution and enzyme hydrolysis. The amount of β-glucans were computed by reducing the α-glucans from the total glucans. The method was validated using yeast β-glucan standard provided in the analysis kit.

4.5.2 Monomer composition of polysaccharides

For determination of the monosaccharide composition, the obtained polysaccharides were hydrolyzed with 2M TFA at 100 °C for 6 h, in test tubes with a Teflon screw cap. Hydrolysates were filtered through a 0.45 μm regenerated cellulose membrane and pipetted into autosampler vials. Myo-inositol solution was added as standard in each vial and the solutions were evaporated to dryness with nitrogen flow and heating. The vial content was silylated by adding 500 μL of TriSil (Thermo Scientific, Bellefonte, PA, USA), shaking for 7 min, and heating at 70 °C for 1 h. After silylation, 1 μL was injected in a Shimadzu GC-2010 Plus gas chromatograph equipped with a flame ionization detector and a SPB-1 column. After injection (split mode 1:15), the initial column temperature was held at 150 °C for 2 min, then increased to 210 °C with a rate of 4 °C/min and to 275 °C with a rate of 40 °C/min and kept at 275 °C for 5 min. The injector and FID temperatures were 210 °C and 290 °C, respectively. Helium was used as carrier gas. Sugar standards (glucose, mannose, galactose, xylose, rhamnose, arabinose, glucuronic acid, galacturonic acid, glucosamine and fucose) were silylated and analyzed in the same way for identification and quantification purposes (calculation of correction factors).

4.5.3 Monomer composition of *I. obliquus* mycelium (Publication III)

The monomer composition of dried mycelium, ground press cake, autoclaved press cake extract and autoclaved press cake residue were analyzed with methanolysis[279]. Briefly, about 10 mg of dried mycelium sample was mixed with 2 mL HCl 2 M in MeOH and hydrolyzed at 105 ˚C for 5 h. After cooling down, neutralization with pyridine, addition of internal standard (resorcinol in MeOH), and sedimentation, an aliquot of 1.5 mL of clear phase was transferred into a test tube and dried with nitrogen flow. After the recovery of the hydrolysate with pyridine, the samples were silylated overnight with 150 μL of HMDS and 70 μL of TMSC. The clear phases containing sylilated sugars and uronic acids were transferred into autosampler vials and analyzed with GC-FID. Arabinose, fucose, galactose, galacturonic acid, glucose, glucuronic acid, mannose, rhamnose, and xylose were used as standards for identification and quantification (calculation of correction factors).

4.6 Structural analyses

4.6.1 Molecular weight

Molecular weight and molecular weight distribution of polysaccharides were determined by high performance size exclusion chromatography (HPSEC), using

a Waters 2690 system equipped with a TSK-GMPW column (30 cm×7.5 mm i.d.) and coupled with a Waters 2487 UV detector and a Shimadzu 20A refractive index detector. An aqueous solution of sodium nitrate (0.1 M) was used as mobile phase. A calibration curve was obtained with a series of standard pullulans with molecular weight ranging from 708 kDa to 0.3 kDa (Pullulan Kit, Polymer Standards Service, Germany). Polysaccharide samples and pullulan standards were dissolved in the mobile phase at a concentration of 1 mg/mL and 50 μL of sample or pullulan standard were injected. The system temperature was kept at 40 °C and the flow rate at 0.5 mL/min.

4.6.2   Methylation analysis (Publication II)

Methylation of Crat 2%1 and Crat 25%1 was performed according to literature[280]. The permethylated polysaccharides were hydrolyzed with HCl 2M in MeOH at 100 °C for 3 h, silylated and analyzed with GC–MS[281].

4.6.3   ATR-FT-IR (Publications I, II, & IV)

Fourier transform infrared spectroscopy was performed with a Bruker Vertex 70 spectrometer equipped with a single bounce (angle of incidence 45˚) attenuated total reflection accessory (VideoMVP, Harrick), employing a diamond hemisphere and a sampling surface of 0.5 mm^2. Spectra were recorded directly on the freeze-dried polysaccharides, sampling the region 5000–450 cm^{-1} with a resolution of 2 cm^{-1}. Samples were subjected to 128 scans. For Publication I, spectra baseline was corrected and the absorbance values were normalized to the highest value, i.e. the wavenumber around 1020 cm^{-1}. In Publications II, III, and IV, the spectra absorbances were ATR-corrected with the instrument software Opus (Bruker).

4.6.4   Thermogravimetric analysis (Publication II)

Thermal analyses of the semi-purified mushroom polysaccharides were carried out with a thermogravimeter (STA 449C, Netzsch Instruments, Germany), coupled to a mass spectrometer (QMS 403C Aëolos, Pfeiffer Vacuum Technology, Germany) for the detection of the evolving gases. Approximately 9 mg of freeze-dried samples were loaded in open aluminum oxide crucibles and heated from 25 °C to 600 °C, first with a heating rate of 10 °C/min until 190 °C, then at a lower rate of 2 °C/min until the end of the analysis. Starch and bovine serum albumin were used as reference compounds for pure polysaccharide and pure protein, respectively, and were analyzed in the same way. Inert atmosphere was granted by a nitrogen flow of 50 mL/min. A helium flow of 40 mL/min was used as protective gas. The gas transfer line (1.5 m long) was kept at 240 °C for preventing condensation of the evolved gases.

4.6.5 NMR (Publications II & IV)

The samples were prepared by dissolving 10 mg of polysaccharide fraction in 600 µL D_2O (99.9% D). A small drop of acetone was added as internal reference (δ^1H=2.23 ppm, $\delta^{13}C$=29.6 ppm). All ^1H and 2D NMR spectra were recorded on a Bruker AVANCE III spectrometer operating at 600.20 MHz (^1H) and 150.92 MHz (^{13}C) equipped with a Prodigy TCI inverted CryoProbe optimized for proton detection. ^{13}C NMR spectra were recorded on a Bruker AVANCE III spectrometer operating at 500.20 MHz (^1H) and 125.78 MHz (^{13}C) equipped with a Prodigy BBO CryoProbe. The experiment set consisted in: ^1H, ^{13}C, double-quantum filtered correlated spectroscopy (DQF-COSY), total correlation spectroscopy (TOCSY, 80 ms mixing time), nuclear overhauser effect spectroscopy (NOESY, 300 ms mixing time), heteronuclear single quantum coherence (HSQC, multiplicity edited, CH/CH_3 positive and CH_2 negative) and heteronuclear multiple bond correlation (HMBC, with one-bond correlation suppression). All NMR spectra were recorded at 308 K.

4.7 Statistical analysis (Publications I & III)

Statistical analysis was performed with softwares SPSS (SPSS Inc., Chicago, IL) (Publication I) and RStudio[282] (Publication III). Shapiro-Wilk test was used to assess normality distribution of the data. Significance of difference in mean was tested with t-test. One-way ANOVA with Levene test was used to analyze the variance among samples. Tukey-HSD and Games-Howell were used as post hoc tests. Correlation among variables was assessed with Pearson or Spearman methods. Significance was assigned at $p < 0.05$.

5   RESULTS AND DISCUSSION

5.1   Submerged cultivation of *I. obliquus* mycelium (Publication III)

5.1.1   Submerged cultivaton yield

The supplementation of sea buckthorn press cake had a noticeable enhancing effect on the cultivation yield of *I. obliquus*. The cultivation yields, expressed as dry weight of mycelium per liter of medium (g/L), are reported in **Figure 9**. When sea buckthorn was added to the culture medium, both the dosage of sea buckthorn addition and the cultivation time showed a significant impact on the yield of mycelium. At cultivation time of 200 h only the addition of 5 g/L of supplement led to a significant ($p < 1 \times 10^{-4}$) increase in yield by 71% compared to the control. Press cake addition at higher or lower dosages did not produce any significant impact on the mycelium yield compared to the control. Conversely, the addition of press cake had more noticeable positive effect on yield obtained after 250 h (**Figure 9**). At this cultivation time, significant ($p < 1 \times 10^{-4}$) and the highest yield increase was obtained with the addition of press cake at 5 g/L or 10 g/L (increase by 122% from the control). Also, addition of the press cake at 2.5 g/L resulted in significant increase (94%).
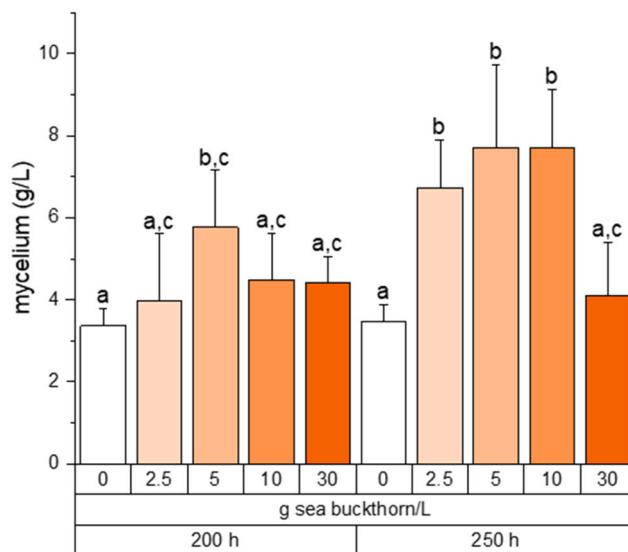

Figure 9. Mycelium yield of the submerged cultivation of *I. obliquus*. Press cake dosages are grouped by cultivation time. Different letters mark significant difference (p<0.05).

The increase of cultivation time from 200 h to 250 h had no significant effect on the mycelium growth in absence of sea buckthorn press cake. The influence of the cultivation time was most evident at the addition of 10 g/L and 2.5 g/L of press cake, where the increase of cultivation time resulted in yield increase by 72% and 69%, respectively. However, addition of the press cake at 30 g/L had no significant effect on the cultivation yield, at either of the cultivation times.

Sea buckthorn press cake has been used for the first time as supplement for the submerged cultivation of the mycelium of *I. obliquus*. The increase of the cultivation time granted a significant increase in yield, while the increase in different supplement dosages granted yields statistically equal to each other, except that the highest dosage of sea buckthorn press cake (30 g/L) had no effect on the cultivation yield. At this dosage, the supplement might have formed physical barrier limiting the mycelium from access to oxygen. A difference in particle size of the supplemented material could explain the contrast with the results obtained by Xu[256], where the addition of 30 g/L of wheat straw to the medium resulted in a significant increases in cultivation yield of *I. obliquus* mycelium at 216 h and 240 h (about 15% and 10%, respectively) of cultivation.

The supplementation of sea buckthorn press cake provided, at the tested experimental conditions, greater yield enhancements than those obtained with the supplementation of fatty acids or oils. Xu[270] reported a yield increase for *I. obliquus* mycelium of 27% and approximately 15% after the addition (0.1% v/v) of stearic and oleic acid, respectively. Yang[268] reported, for *G. lucidum*, an increase in cultivation yield of 62% and 52% after the supplementation (1%) of olive and corn oil, respectively. Huang[283] reported a yield increase of about 100% after a 2% supplementation of corn oil to *G. lucidum* cultivation medium, which is close to the increase observed in the current study with sea buckthorn press cake. The fatty acid composition of sea buckthorn press cake, dominated by palmitic and palmitoleic acids[271], was different from all the aforementioned supplements. The absence of clear correlation between fatty acid chain length of the supplement material and cultivation yield of mycelia has already been reported[240].

Fibrous biomass and lipids used separately have been proven to act as efficient growth enhancers[259,283]. Therefore, their simultaneous presence in the sea buckthorn press cake could explain the obtained results. Previously, it was reported[257] that supplementations of press cakes of oilseeds, such as camelina or rapeseed, failed to enhance the mycelium growth of *I. obliquus*. Similar finding was observed in the cultivation of other white-rot basidiomycetes, such as *T. versicolor*[257]. Moreover, the supplementation of olive mill wastewater (containing sugars, free fatty acids, phenolic compounds, and lipids) to mycelium liquid cultivation medium of *G. lucidum* granted a yield increase of 72%, while failed to enhance the yield of multiple *Pleurotus* strains[284,285]. On the

other hand, the effects of the supplementation of pectin to the medium of mycelium submerged cultivation have received little attention. Also, the pectinolytic activity of *I. obliquus* has never been subject of investigation. However, strong pectinase activity has been measured from the culture of *Inonotus rickii*[286]. Moreover, Kruporodova and Barshteyn incremented the cultivation yield of *I. obliquus* by 55%[257] with the supplementation of rose hip fruit, which contains about 1% w/w pectin[287]. Hence, the pectin present in sea buckthorn press cake could have been used as carbon source by *I. obliquus* and stimulated its mycelial growth. However, further studies are required to verify the pectinolytic activity and the effect of supplementation of pectins on the submerged cultivation yield of *I. obliquus*.

5.1.2   Monomer composition of the mycelium

Cultivated mycelium of *I. obliquus* was subjected to methanolysis, in order to investigate the possible impact of supplementation of sea buckthorn press cake and the cultivation time on the content and monomer composition of mycelium polysaccharides. The monomer concentrations (mg/g of mycelium) are reported in **Figure 10**. The monomer composition of sea buckthorn press cake was analyzed with methanolysis before and after autoclaving for comparison with the monomer composition of mycelium. While the press cake polysaccharides were mainly composed of galacturonic acid (35%) and xylose (20%) before autoclaving, the polymers extracted by the medium during autoclaving were mainly pectin (50.2% of total sugars were galacturonic acid). On the other hand, xylose represented 39.8% of total sugars in the polysaccharides retained by the press cake after autoclaving, indicating hemicelluloses were the major components of the autoclave residue.
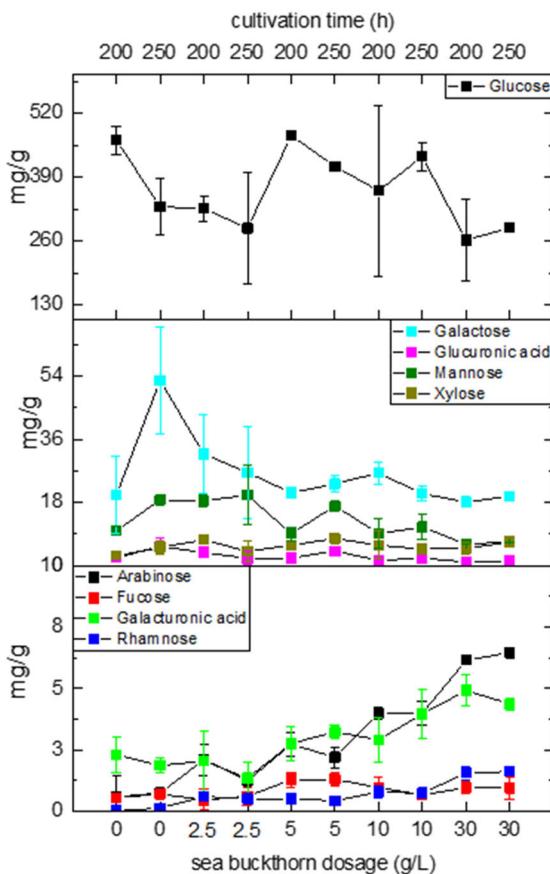
Figure 10. Monomer composition of the cultivated mycelium reported as concentration of monomers (mg/g mycelium). Samples are identified by combination of press cake dosage (bottom x-axis) and cultivation time (upper x-axis).

As shown in **Figure 10**, glucose was the main monomer released by the mycelium during methanolysis, with a concentration between 263−475 mg/g mycelia (75-93% w/w of the total sugars), at least one magnitude higher than all the other monomers. The most abundant monomers after glucose were galactose and mannose, with ranges of relative abundance of 3-16% and 1-9% w/w, respectively, of the total sugars. Glucose, fucose, and xylose were not affected by cultivation conditions. On the other hand, there was a significant increase in arabinose, rhamnose, and galacturonic acid in mycelia cultivated with higher dosage of sea buckthorn press cake (dosage 10 and 30 g/L medium).

Table 14. Spearmann correlation values between *I. obliquus* mycelium monomers, hydrolysis yield and cultivation condition.

	SB conc.	cult. time	Ara	Fuc	Gal	GalA	Glc	GlcA	Man	Rha	Xyl	hydr. yield[a]
SB conc.	1.00	0.00	0.96*	0.42	-0.46*	0.83*	-0.28	-0.58*	-0.71*	0.89*	0.20	-0.33
cult. time	0.00	1.00	-0.07	0.02	0.10	0.02	-0.21	0.26	0.36	-0.06	0.10	-0.19
Ara	0.96*	-0.07	1.00	0.37	-0.37	0.82*	-0.24	-0.54*	-0.73*	0.92*	0.23	-0.29
Fuc	0.42	0.02	0.37	1.00	-0.05	0.46*	-0.19	-0.06	-0.15	0.24	0.29	-0.17
Gal	-0.46*	0.10	-0.37	-0.05	1.00	-0.52*	-0.03	0.32	0.56*	-0.37	0.06	0.05
GalA	0.83*	0.02	0.82*	0.46*	-0.52*	1.00	-0.22	-0.29	-0.63*	0.74*	0.25	-0.26
Glc	-0.28	-0.21	-0.24	-0.19	-0.03	-0.22	1.00	0.15	-0.14	-0.48*	-0.34	0.99*
GlcA	-0.58*	0.26	-0.54*	-0.06	0.32	-0.29	0.15	1.00	0.64*	-0.58*	0.47*	0.22
Man	-0.71*	0.36	-0.73*	-0.15	0.56*	-0.63*	-0.14	0.64*	1.00	-0.61*	0.23	-0.09
Rha	0.89*	-0.06	0.92*	0.24	-0.37	0.74*	-0.48*	-0.58*	-0.61*	1.00	0.22	-0.52*
Xyl	0.20	0.10	0.23	0.29	0.06	0.25	-0.34	0.47*	0.23	0.22	1.00	-0.31
hydr. yield[a]	-0.33	-0.19	-0.29	-0.17	0.05	-0.26	0.99*	0.22	-0.09	-0.52*	-0.31	1.00

[a]methanolysis yield (w/w%); *$\rho < 0.05$

The concentration of galacturonic acid was drastically lower in mycelium, compared to that in sea buckthorn press cake, and also in the mycelium cultivated at highest supplement dosage. Nevertheless, Spearman correlation test showed strong positive correlation between arabinose, rhamnose, and galacturonic acid and press cake dosage ($0.8 < \rho < 0.9$, $p < 1 \times 10^{-4}$) (**Table 14**). On the other hand, glucuronic acid and mannose had a negative correlation with press cake dosage ($\rho = -0.58$, $p = 0.008$ and $\rho = -0.71$, $p < 1 \times 10^{-4}$, respectively), while a weak negative correlations was found between press cake dosage and galactose. The increase in cultivation time showed no significant effect to the monomer composition.

The results of the hydrolysis of the *I. obliquus* mycelium in the present study were in partial agreement with the monomer composition of cultivated mycelium of *Pleurotus pulmonarius*[288]. While the latter had mannose as second most abundant monomer, the *I. obliquus* hydrolysates in our current study showed a higher amount of galactose. In the study on *P. pulmonarius*, the carbon source had a significant effect on the relative amount of glucose in mycelium[288]. Conversely, and in agreement with our study, olive mill wastewater supplementation had no effect on the mycelial glucan of *G. lucidum*[285]. However, this study reported biomass content of glucose of 5%, while the w/w content of glucose of *I. obliquus* was in the range 21-49%. This difference could be attributed to the species examined or to the difference in the analytical method.

The strong correlations of arabinose, rhamnose and galacturonic acid with the press cake dosage suggested a partial retention of pectin by the mycelium. Interestingly, the increment of arabinose was more pronounced than galacturonic acid, despite the higher amount of the latter in the medium. The contents of arabinose and rhamnose in *I. obliquus* polysaccharides increased with the supplementation of wheat straw[256,289]. The relationships between concentration of galacturonic acid and arabinose in the mycelium and amounts supplemented with sea buckthorn press cake (considered as a whole, irrespective of autoclave extraction) are outlined in **Figure 11**. The increase in mycelium monomer amount followed a linear trend, with adjusted R^2 between 0.860 and 0.956, except for the trend of galacturonic acid after 250 h of cultivation (adj. R^2 0.501, slope $p > 0.05$).
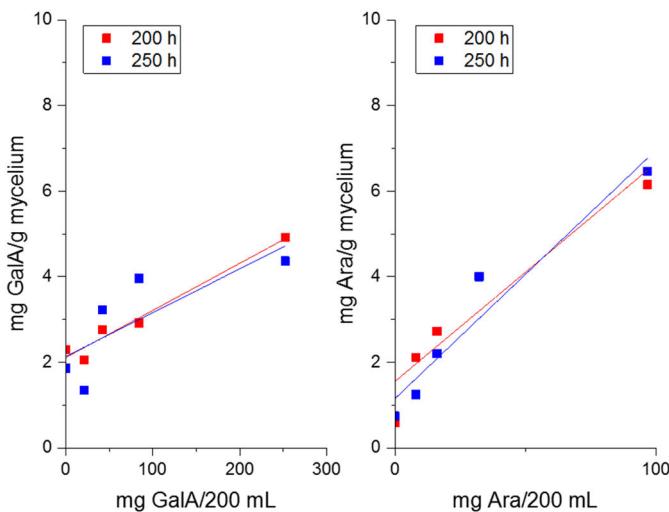
Figure 11. Amounts of mycelial galacturonic acid (left) and arabinose (right), reported mg/g mycelium, in relation to the amounts of the same monomers in the cultivation medium.

5.2   Yield and composition of polysaccharide fractions

5.2.1   *A. bisporus* polysaccharides (Publication I)

Dried and powdered *A. bisporus* was extracted thrice with hot water, with and without ethanol-acid pretreatment. Total sugar content of the extraction liquids and precipitates was measured by phenol-sulfuric acid method. AB2 had a similar value as AB1, while the amount of sugars and polysaccharides in AB3 clearly decreased. The amount of sugars and polysaccharides in the extraction liquids of treated mushroom were higher than from untreated mushroom, especially for the first hot water extract (tAB1). The higher sugar amounts in solution compared to reported yields of AB3 and tAB1-3 indicates carbohydrates did not precipitate with ethanol, probably due to their low molecular weight.

Total yield for AB (AB1+AB2+AB3) was 7.7 and for tAB (tAB1+tAB2+tAB3) 16.2 g per 100 g of dry mushroom. Noticeably, tAB1 was higher than AB extractions combined. While extraction yield decreased from AB1 to AB3, sugar content of the precipitates increased from 11% to 31%. The same was observed with tAB fractions. It could be inferred that the cell wall rupturing caused by pretreatment improved the solvent access and extractability of the polysaccharides.

Table 15. Yield, sugar, and protein content of the polysaccharide fractions produced in this doctoral dissertation.

Starting material	Extraction medium	Fraction label	Yield (%)	Sugar content in the extracts (w/w %)	Protein content in the extracts (w/w %)
A. bisporus (Publication I)	Hot water	AB1	4.1	10.6 ± 0.2	n.a.[a]
	Hot water	AB2	2.5	19.2 ± 2.7	39.4 ± 5.3
	Hot water	AB3	1.1	30.5 ± 1.0	42.2 ± 14.4
A. bisporus EtOH-HCl pretreatment	Hot water	tAB1	13.2	25.3 ± 0.6	23.0 ± 5.0
	Hot water	tAB2	2.2	38.2 ± 0.6	21.3 ± 2.4
	Hot water	tAB3	0.8	62.6 ± 2.3	21.6 ± 2.7
C. tubaeformis (Publication II)	Hot water	Crat HW1	1.1[b]	73.4 ± 2.2	31.6 ± 1.6
	2% KOH	Crat 2%1	4.3[b]	87.9 ± 5.5	< 2
	25% KOH	Crat 25%1	3.0[b]	> 95	< 2
I. obliquus mycelium (Publication III)	Hot water	IPSsb0 HW	0.9	85.3 ± 5.3	15.6 ± 1.1
I. obliquus mycelium – pooled batches 0 and 2.5 g/L supplement dosages (Publication IV)	Hot water	IPSsb0-2.5 HW	2.2	80.0 ± 2.2	23.4 ± 1.7
	2% KOH	IPSsb0-2.5 2%	4.1	76.0 ± 1.4	33.4 ± 4.6
I. obliquus mycelium – pooled batches 5 and 10 g/L supplement dosages (Publication III)	Hot water	IPSsb5-10 HW	1.1	74.3 ± 2.6	22.8 ± 0.8
	2% KOH	IPSsb5-10 2%	1.0	58.9 ± 2.2	34.4 ± 0.9
I. obliquus sterile conk (Publication IV)	Hot water	F-Chaga HW	1.1	27.6 ± 0.2	n.a.[a]
	2% KOH	F-Chaga 2%	3.7	31.9 ± 2.7	n.a.[a]
	Hot water	C-Chaga HW	0.9	26.1 ± 1.7	n.a.[a]
Birch heart rot (Publication IV)	Hot water	Heart Rot HW	2.2	19.5 ± 1.1	n.a.[a]
	2% KOH	Heart Rot 2%	3.5	44.2 ± 5.0	n.a.[a]

[a]not analyzed; [b]prior to DEAE-cellulose column.

5.2.2   *C. tubaeformis* **polysaccharides (Publication II)**

After removal of more apolar compounds with ethanol, the mushroom material was sequentially extracted with hot water, KOH 2% and KOH 25%. The three raw fractions produced underwent the same purification process, resulting in three semi-purified polysaccharide fractions. The yields (w/w %) of semi-purified extracts from the hot water, KOH 2% and KOH 25% extractions were 1.12%, 4.25% and 3.04%, respectively (**Table 15**). The hot water extraction yield was in agreement with reported yields from *Pleurotus tuber-regium* (1.6%)[98] and from *C. cibarius* (1.9%)[112]. The sequential use of mild alkali granted a yield similarly reported in the reference work (4%)[273], higher than in the report about *F. velutipes* (1%)[203] but lower than the one obtained with *C. cibarius* (6%)[112]. Conversely, the yield of the polysaccharide extraction with 25% KOH was higher than the yield of the reference work (0.9%)[203]. The sugar contents (w/w %) of the semipurified fractions, measured with the phenol–sulfuric acid method, were 32.4%, 64.4% and 67.5%, respectively. Their protein contents (w/w %) were 39.7%, 13.4%, and 2.7%, respectively. After DEAE-cellulose, the protein content was 38.6% for the hot water fraction and below 2% for both alkali fractions, while their sugar content was 73.4%, 87.9%, and above 95%, respectively (**Table 15**). The higher amount of protein in the hot water extract is coherent with the reported amount of the same extract from *P. tuber-regium*[98], while, differently from our result, the hot water extract from *C. cibarius* had a protein content of 6%[112]. The presence of protein in the mild alkali fraction was in agreement with the presence of a layer of glycoproteins between the outer layer and the β-glucan layer, as indicated by the mushroom cell wall model of Chen and Cheung[98]. The use of KOH 2% solution as extraction media after the hot water extraction almost quadrupled the final yield of the semi-purified polysaccharides and doubled the sugar content compared to the hot water extraction.

5.2.3   *I. obliquus* **mycelium polysaccharides (Publications III & IV)**

Polysaccharides were extracted with hot water from mycelium cultivated without supplement (IPSsb0), from mycelium pool obtained without supplement and with dosage 2.5 g/L (IPSsb0-2.5 HW), and from mycelium pool obtained with supplement dosages of 5 and 10 g/L (IPSsb5-10 HW). Alkali extraction was performed with the mycelium pools (IPSsb0-2.5 2% and IPSsb5-10 2%). The cultivated mycelium of *I. obliquus* granted similar or higher extraction yields, compared to the sterile conk (**Table 15**). The sugar contents of the produced fractions were 80% and 76% w/w% for hot water and alkali extracts, respectively, noticeably higher compared to corresponding extracts from the sterile conk. If

mycelium was cultivated with higher dosages of sea buckthorn press cake, sugar content were decreased but still higher than sterile conk. The decrease in the sugar content of the alkali extract, compared to hot water, has been observed also with mycelium cultivated with both mycelium pools. Moreover, the sugar content of the mycelial hot water extract (IPSsb0-2.5 HW) was in agreement with the hot water extract of mycelium cultivated without supplement (**Table 15**). The observed decrease in production of polysaccharides by the mycelium might be connected to the presence of press cake. This was in agreement with the methanolysis results, which showed negative Spearman correlations between press cake content and hydrolysis yield (ρ = -0.33, **Table 14**). Conversely, the use of alkali resulted in an increase in the protein content of the fractions.

Xu and coworkers have reported a comparison of polysaccharides obtained with hot water extraction from cultivated mycelium and sterile conk of *I. obliquus*[153]. Compared to our results, they report higher sugar content in the sterile conk fraction (40.5%), and lower sugar content of the mycelial extract (64.0%). Our results have showed, for the pool obtained at lower supplement dosages, IPS contents (17.2 mg/g and 30.8 mg/g starting material, respectively) in agreement with IPS production previously reported for *I. obliquus*[270] and other basidiomycetes[290,291]. However, it has to be taken into account that in the mentioned studies, dialysis was not performed.

5.2.4 Sterile conk of *I. obliquus* (Publication IV)

The hot water extraction of *I. obliquus* sterile conks obtained from the Finnish and Chinese forests granted similar yields (F-Chaga HW and C-Chaga HW, **Table 15**). The slight difference could be due to the shorter extraction time used for the Chinese sample. On the other hand, the sugar contents of the two extracts were almost identical (27.6% and 26.1% w/w). The alkali extraction of the Finnish sterile conk (F-Chaga 2%) granted higher yield (9.20% w/w) compared to the hot water extraction. However, despite the freeze-thawing step, the main substances in the fraction were insoluble in water. Therefore, a second freeze-thawing cycle was performed and the actual yield decreased to 3.7%. This value was higher than most of mycelium extractions except IPSsb0-2.5 2%. Despite the use of alkali, the sugar content of the extract from the alkali extraction was only slightly higher (31.9% w/w) than the content in the water extract (27.6%).

5.2.5 Birch heart rot caused by *I. obliquus* (Publication IV)

The yield of hot water extraction of birch heart rot (Heart Rot HW) was in agreement with the yields obtained after hot water extraction of birch core[292] and subcritical water extraction of birch sawdust[293]. The sugar contents of Heart Rot HW and subsequently produced alkali extract (Heart Rot 2%) were 19.5% and

44.2% (w/w), respectively. Overall, the final contents of water and alkali-extractable polysaccharides in the heart rot were 4.4 mg/g and 15.4 mg/g, respectively. There is little data available on the polysaccharides extracted from lignocellulosic substrate colonized by the mycelium. The water extraction of corn biomass colonized by *G. frondosa* mycelium resulted in lower yield, compared to our study[294]. The polysaccharide contents resulting from birch heart rot were lower than those obtained with water extraction from the oak sawdust-based substrate infected with different white-rot fungi[295]. Conversely, similar contents were obtained from beech sawdust colonized by *G. lucidum* mycelium[296] and from birch sawdust after subcritical extraction[293]. The later finding is worth noticing because the hemicellulose content of lignocellulosic material should decrease after white-rot mycelium colonization[238]. This finding could be explained by the preferred secretion of ligninolytic enzymes by *I. obliquus* during wood degradation, at least in submerged cultivation conditions[62]. The yield and sugar content of colonized substrate might therefore be specific to substrate itself and fungal species, in addition to the extraction method. However, it should be noted again that the dialysis step performed in the present work, responsible for the removal of simple sugars and small oligomers, was not included in the polysaccharide extraction protocols of the aforementioned previous works.

5.3   Monomer composition and molecular weight of polysaccharides

5.3.1   *A. bisporus* polysaccharides (Publication I)

As can be observed from **Table 16**, the relative amount of glucose increased in the hydrolysates from AB1 to AB3 and from tAB1 to tAB3 similarly as the total glucan amounts (**Table 18**). The purity of the glucans increased when the solvent gets access to the inner layers of the cell wall, which was in accordance to the cell wall model. The relative amounts of arabinose and rhamnose remained almost constant throughout the process, regardless of the ethanol-acid treatment or the number of the extraction. On the contrary, galactose, mannose, and fucose decreased in each batch along with the extractions, which indicated a decreasing gradient when moving inwards the cell wall layers or the difference may arise from the relative increment of glucose. The presence of higher amounts of galactose and mannose in the outer cell wall layers was in accordance with the cell wall model. Polymers of mannose and galactose are covalently bound to proteins.

Table 16. Monomer composition (rel mol %) of the polysaccharide fractions produced in the present doctoral dissertation.

	Starting material	Xyl	Gal	Rha	Glc	Man	Ara	Fuc	GalA	GlcA	3-O-Me-Gal	4-O-Me-GlcA	Gal/3-O-Me-Gal
						relative mol %							mol/mol
AB1	*A. bisporus*	2.59 ± 1.10	23.74 ± 2.32	n.d.[*]	57.95 ± 0.68	9.45 ± 0.79	3.14 ± 0.20	3.14 ± 0.44	n.a.[a]	n.a.[a]	n.a.[a]	n.a.[a]	n.a.[a]
AB2		3.96 ± 0.23	18.52 ± 1.06	1.22 ± 0.06	63.54 ± 0.36	8.16 ± 0.97	3.01 ± 0.60	1.59 ± 0.11	n.a.[a]	n.a.[a]	n.a.[a]	n.a.[a]	n.a.[a]
AB3		9.39 ± 4.49	19.14 ± 1.07	0.75 ± 0.44	55.1 ± 7.58	9.05 ± 2.13	5.09 ± 2.38	1.48 ± 0.13	n.a.[a]	n.a.[a]	n.a.[a]	n.a.[a]	n.a.[a]
tAB1		6.89 ± 0.20	12.33 ± 1.46	0.21 ± 0.07	73.06 ± 1.15	3.55 ± 0.50	2.88 ± 0.35	1.07 ± 0.11	n.a.[a]	n.a.[a]	n.a.[a]	n.a.[a]	n.a.[a]
tAB2		1.09 ± 0.03	12.27 ± 0.24	0.08 ± 0.01	76.53 ± 0.62	6.77 ± 0.74	2.72 ± 0.06	0.54 ± 0.03	n.a.[a]	n.a.[a]	n.a.[a]	n.a.[a]	n.a.[a]
tAB3		2.37 ± 0.04	9.20 ± 0.05	0.19 ± 0.01	79.24 ± 0.56	5.25 ± 0.56	3.39 ± 0.03	0.35 ± 0.02	n.a.[a]	n.a.[a]	n.a.[a]	n.a.[a]	n.a.[a]
Crat HW1	*C. tubaeformis*	21.95 ± 0.26	17.82 ± 0.63	0.80 ± 0.03	11.94 ± 0.46	32.61 ± 0.56	0.80 ± 0.04	14.04 ± 0.33	n.d.[b]	n.d.[b]	n.a.[a]	n.a.[a]	n.a.[a]
Crat 2%1		5.67 ± 0.04	4.14 ± 0.24	0.11 ± 0.00	68.47 ± 0.07	17.83 ± 0.8	2.48 ± 0.07	1.30 ± 0.09	n.d.[b]	n.d.[b]	n.a.[a]	n.a.[a]	n.a.[a]
Crat 25%1		7.62 ± 0.13	12.21 ± 1.15	0.21 ± 0.06	45.61 ± 0.46	24.22 ± 0.29	1.88 ± 0.03	8.25 ± 0.57	n.d.[b]	n.d.[b]	n.a.[a]	n.a.[a]	n.a.[a]

IPSsb0 HW	*I. obliquus* (mycelium)	3.42 ± 0.14	4.98 ± 0.24	0.74 ± 0.09	66.02 ± 0.66	20.13 ± 0.24	2.34 ± 0.07	1.26 ± 0.11	0.56 ± 0.10	n.d.[b]	0.56 ± 0.10	n.d.[b]	9 ± 1
IPSsb0-2.5 HW		1.44 ± 0.02	12.78 ± 0.4	0.17 ± 0.00	62.9 ± 0.46	20.2 ± 0.18	0.45 ± 0.05	1.16 ± 0.08	0.44 ± 0.02	n.d.[b]	1.3 ± 0.01	n.d.[b]	10 ± 0
IPSsb0-2.5 2%		1.68 ± 0.03	15.47 ± 0.21	0.22 ± 0.01	55.09 ± 0.36	23.42 ± 0.16	0.76 ± 0.04	1.27 ± 0.03	0.20 ± 0.02	n.d.[b]	1.85 ± 0.04	n.d.[b]	8 ± 0
IPSsb5-10 HW		0.84 ± 0.02	11.49 ± 0.14	0.35 ± 0.08	64.07 ± 0.13	17.59 ± 0.14	1.21 ± 0.01	1.02 ± 0.02	1.96 ± 0.07	n.d.[b]	1.43 ± 0.04	n.d.[b]	8 ± 0
IPSsb5-10 2%		6.66 ± 0.06	10.73 ± 0.24	0.52 ± 0.02	49.16 ± 0.26	26.57 ± 0.37	1.95 ± 0.04	1.54 ± 0.02	1.17 ± 0.06	n.d.[b]	1.61 ± 0.01	n.d.[b]	7 ± 0
F-Chaga HW	*I. obliquus* (sterile conk)	14.58 ± 0.18	15.76 ± 0.2	3.60 ± 0.11	34.68 ± 0.04	11.84 ± 0.19	8.55 ± 0.01	2.64 ± 0.25	6.75 ± 0.27	0.50 ± 0.01	1.11 ± 0.10	n.d.[b]	14 ± 1
F-Chaga 2%		5.11 ± 0.15	5.60 ± 0.11	1.20 ± 0.18	62.27 ± 1.42	11.72 ± 0.17	7.26 ± 0.18	1.50 ± 0.16	4.43 ± 1.98	0.52 ± 0.13	0.40 ± 0.10	n.d.[b]	15 ± 3
C-Chaga HW		10.91 ± 0.69	16.88 ± 0.24	2.33 ± 0.1	38.33 ± 0.85	5.61 ± 0.07	12.51 ± 0.2	5.49 ± 0.25	3.44 ± 0.25	0.29 ± 0.08	4.2 ± 0.23	n.d.[b]	4 ± 0
Heart Rot HW	*I. obliquus* (birch heart rot)	26.76 ± 0.21	12.15 ± 0.26	3.87 ± 0.05	18.18 ± 0.13	11.84 ± 0.69	7.78 ± 0.13	4.16 ± 0.35	5.39 ± 0.56	0.17 ± 0.07	3.42 ± 0.04	2.96 ± 0.30	4 ± 0
Heart Rot 2%		77.45 ± 1.57	3.63 ± 0.07	n.d.[*]	7.01 ± 0.06	1.88 ± 0.05	4.50 ± 0.10	n.d.[*]	2.02 ± 0.02	n.d.[*]	0.47 ± 0.03	6.28 ± 0.10	8 ± 1

[a] not analyzed; [b] not detected.

Table 17. Molecular weight (Da) and relative area of the polysaccharide populations composing the fractions analyzed in the present dissertation.

Fraction	Molecular weight (Da)		Area % (Peak 1 : Peak 2)
	Peak 1	**Peak 2**	
AB1	1.4×10^4	1.8×10^3	53 : 30
AB2	6.5×10^5	1.4×10^4	40 : 60
AB3	3.2×10^5	1.7×10^4	22 : 78
tAB1	2.3×10^5	1.3×10^4	24 : 73
tAB2	2.2×10^5	4.5×10^4	41 : 59
tAB3	1.9×10^5	5.8×10^4	51 : 49
Crat HW1	4.0×10^5	1.7×10^4	84 : 16
Crat 2%1	5.1×10^5	1.7×10^4	60 : 40
Crat 25%1	5.4×10^5	8.7×10^3	8 : 92
IPSsb0 HW	3.8×10^5	4.8×10^3	58 : 14
IPSsb0-2.5 HW	3.6×10^5	1.0×10^4	55 : 45
IPSsb0-2.5 2%	2.2×10^5	1.7×10^4	26 : 74
IPSsb5-10 HW	2.0×10^5	6.5×10^4	19 : 80
IPSsb5-10 2%	2.0×10^5	1.5×10^4	30 : 70
F-Chaga HW		8.3×10^3	100
F-Chaga 2%		6.3×10^3	100
C-Chaga HW		1.3×10^4	100
Heart Rot HW	3.2×10^5	9.3×10^3	18 : 82
Heart Rot 2%	3.6×10^5	9.3×10^3	4 : 96

Higher amounts of mannose and galactose were found in fractions with higher content of proteins (Table 15) and were higher in untrated extracts than in treated extracts. The amount of xylose was noticeably higher in AB3, compared to earlier extractions of the untreated sample. On the contrary, tAB1 had the highest amount of xylose, with significant decrease in the following fractions. It could be speculated that xylans are present in deeper layers of the cell wall and were extracted all at once in tAB1.
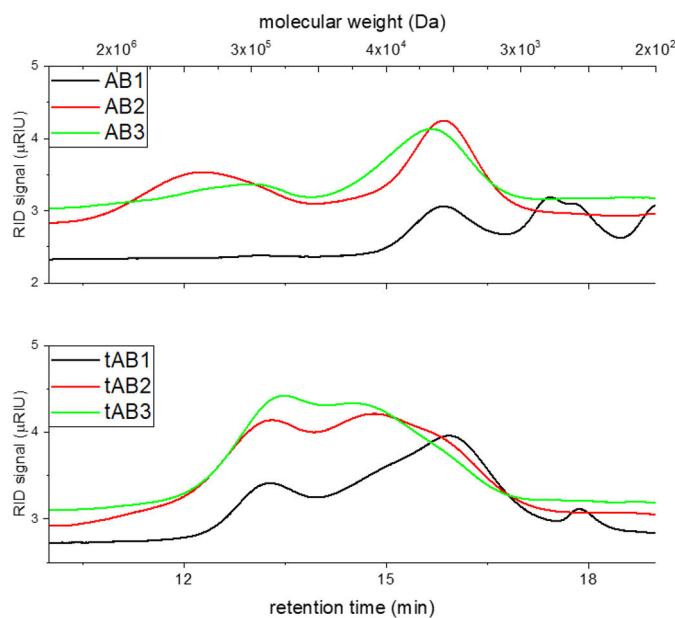
Figure 12. HPSEC-RID chromatograms of polysaccharides extracted from untreated (top) and treated (bottom) *A. bisporus*.

HPSEC chromatograms of AB and tAB fractions are reported in **Figure 12**. Polysaccharides with molecular weight $\log M_p \geq 5$ were detected in AB2 and AB3, but not in AB1. The solubility of glucans decreases at the increase of the molecular weight, therefore absence of high-molecular weight polymers in AB1 could be explained by their early precipitation during cold storage, before ethanol addition to the extract. Polysaccharides with $\log M_p \leq 3.5$ were present only in AB1 (**Figure 12**). These polysaccharides were oligomers with ≤ 20 glucose units which are highly soluble and therefore were extracted at the first step. Similar results was noticed in tAB-samples (**Figure 12**, bottom). Ethanol-acid treatment had a significant effect on the molecular weight distribution. The largest polysaccharides, i.e. $\log M_p$ about 5.8 and 5.5 in AB fractions, were degraded and they formed new populations with $\log M_p$ about 5.2–5.5 and 4.6–4.8 in tAB fractions. It is also noteworthy that the lack of high molecular weight glucans correlated with the absence and the low amounts of α-glucans in the samples (**Table 18**).

Table 18. Glucan amount of *A. bisporus* fractions as measured with enzymatic kit.

Fraction	Total glucan	α-glucan	β-glucan
		(w/w %)	
AB1	7.2 ± 0.0	0.3 ± 0.0	7.0 ± 0.0
AB2	19.3 ± 0.5	14.4 ± 0.5	4.9 ± 1.0
AB3	20.2 ± 1.2	4.4 ± 0.1	15.8 ± 4.4
tAB1	30.9 ± 0.8	7.9 ± 0.1	23.0 ± 0.8
tAB2	36.2 ± 0.3	9.0 ± 0.0	27.2 ± 0.3
tAB3	n.a.[*]	n.a.[*]	n.a.[*]

[*]not analyzed

5.3.2 *C. tubaeformis* polysaccharides (Publication II)

The monomer compositions of the Crat HW1, Crat 2%1 and Crat25%1 fractions, expressed as relative molar percentages, are reported in **Table 16**. Crat HW1 consisted mainly of mannose (34.4%), while Crat 2%1 contained glucose as the major component (69.5%). The predominance of glucose in Crat 2%1 indicates that this hexose is the main monomer of both the polymer populations (**Table 16**) of this fraction. Glucose was the main component of Crat 25%1 as well (46.7% of the total sugars), followed by mannose (24.8%). The monomer composition of Crat HW1 indicates that hot water is an inefficient medium for extracting glucans from *C. tubaeformis*, and harsher conditions are needed for disrupting the cell wall in order to improve the extractability of these polymers. The high amounts of mannose, galactose and fucose in Crat HW1 indicate that hot water extracts mainly proteoglycans and heterosaccharides of the outer layer of the cell wall. Polysaccharides with high contents of mannose and galactose have been isolated from the hot water extract of multiple mushrooms[127]. These results are in agreement with the generally accepted mushroom cell wall model[101] and with our results in the study on the disruption of the cell wall of *A. bisporus* as shown in **Publication I**. However, the increased presence of mannose and galactose in Crat 25%1 suggests the presence of heterosaccharides in the lowest layer of the cell wall of *C. tubaeformis*. Polymers with high content of galactose and mannose have been already isolated from fungi with alkali, for example from *F. velutipes*[203] and *Cordyceps militaris*[297].
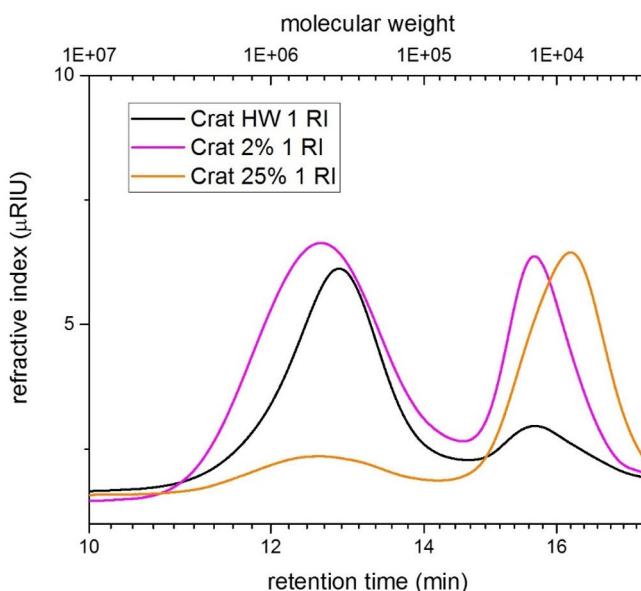
Figure 13. HPSEC-RID chromatograms of Crat HW1, Crat 2%1, and Crat 25%1.

The HPSEC-RID chromatograms of Crat HW1, Crat 2%1 and Crat25%1 fractions are reported in **Figure 13**. All purified fractions consisted of two polysaccharide populations. Peak molecular weight values are reported in **Table 17**. Relevant peaks below the penetration limit of the column were not observed. Different from Crat HW1 and Crat 25%1, the mild alkali fraction was composed of two polymer populations with close peak areas (**Table 17**). The use of alkali as extraction media resulted in polymer populations with molecular weights lower by one order of magnitude, compared to the population of high M_p. This may have been caused by alkali depolymerization of polysaccharides of higher molecular weights.

5.3.3   *I. obliquus* mycelium polysaccharides (Publications III & IV)

Glucose, mannose, and galactose were the main monomers of the polysaccharides extracted from the mycelium of *I. obliquus*, with the marked predominance of the first (**Table 16**), regardless of the presence of sea buckthorn press cake. Compared to our results, lower abundance of glucose and higher proportion of mannose and galactose were reported by Xu and coworkers from the different fractions fractionated from cultivated mycelium[153]. Dominance of glucose over other sugar monomers was also previously observed in polysaccharides extracted from the cultivated mycelia of *I. obliquus*[152,256] and other basidiomycetes[298]. The prevalence of glucose over the other monomers of the extracted IPS was in agreement with previous results on *I. obliquus*, which also have shown scant influence of lignocellulose supplementation on the monomer composition [256].

The use of alkali decreased the relative molar percentage of glucose, and increased the relative content of other monomers, in particular mannose. Galactose, on the other hand, had a less clear trend but it was less abundant in IPSsb0 HW, compared to the other fractions. The monomer composition analysis showed the presence of galacturonic acid in the extracts, whose amounts increased noticeably at higher press cake dosages. The increase of this monomer could be attributed to the pectin in the cultivation medium, as shown by the monomer composition of the mycelium.

Our analysis has shown a decrease in the sugar content of the fractions obtained after supplementation. The negative trend in mycelial polysaccharide production is in agreement with Xu[270], who has reported a negative effect on the extraction yield of IPS from *I. obliquus* after the supplementation of fatty acids. However, the supplementation of different lignocellulose materials to *I. obliquus* had little or negative effects on the IPS production of the mycelium[256]. On the other hand, supplementation of plant oils had positive effect on the IPS production of *Antrodia cinnamomea*[290]. Differences in mycelial IPS content at the variation in concentration of nutrients and medium components have been attributed also to changes in mycelium metabolism and morphology[251,299]. However, the nature of this relationship is still a matter of debate. This aspect was not investigated further in the present doctoral thesis.
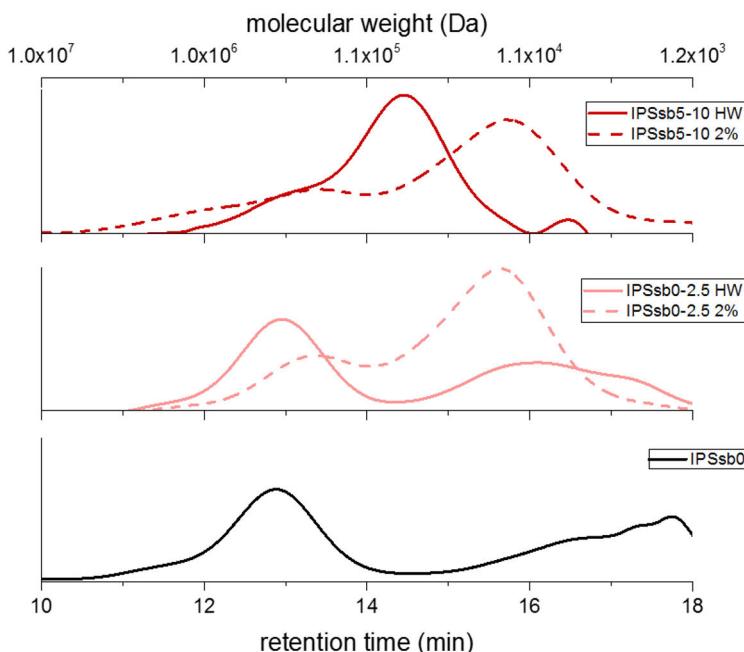
Figure 14. HPSEC-RID chromatograms of IPS fractions produced from *I. obliquus* mycelium cultivated without (bottom) or with sea buckthorn press cake.

The molecular weight of IPS polysaccharides is reported in **Table 17**. The HPSEC chromatograms are reported in **Figure 14**. No relevant peaks were observed at the penetration limit of the column. The M_p of the polysaccharides extracted from *I. obliquus* mycelium was similar to molecular weights of IPS obtained from the same species, although with different population abundances[153]. In IPSsb5-10 HW, almost 80% of the polymers extracted with hot water had M_p of 65 kDa, while the 70% of the polymers extracted with alkali had Mp of 15 kDa. The decrease in molecular weight of the main population can be attributed to the hydrolyzing effect of the alkali solution. The chromatogram of IPSsb0-2.5 2% showed the decrease in M_p of the population of higher molecular weight, while the population of lower size increased in abundance (from 45% to 74% of total area), possibly due to the alkaline depolymerization of the high M_p population.

The HPSEC chromatograms showed also that both IPSsb5-10 fractions contained polymers with a Mp of 2.0×10^2 kDa at lower percentages (18.5% and 21.4% for hot water and alkali fractions, respectively). This population was present also in IPSsb0, however it was the most abundant (58% of total area). In the fraction IPSsb0-2.5 HW, the 55% of polymers had a molecular weight of 3.6×10^2 kDa. Nevertheless, IPSsb5-10 2% contained (about 9% of the total area) a polysaccharide population of high molecular weight (1.4×10^3 kDa) that was not observed in the IPSsb5-10 HW chromatogram. Basing on the increase in the relative amount of mannose, the 1.4×10^3 kDa population of IPSsb5-10 2% could be attributed to the heterosaccharides that are present in the lower layers of the fungal cell wall[64,300]. The population of 65 kDa of IPSsb5-10 HW was absent from IPSsb0 HW and from IPSsb0-2.5 HW. The differences might be attributed to the presence of the press cake. Oil supplementation has been proven already to influence the expression of mycelial polysaccharide biosynthesis enzymes[266].

5.3.4 Sterile conk of *I. obliquus* (Publication IV)
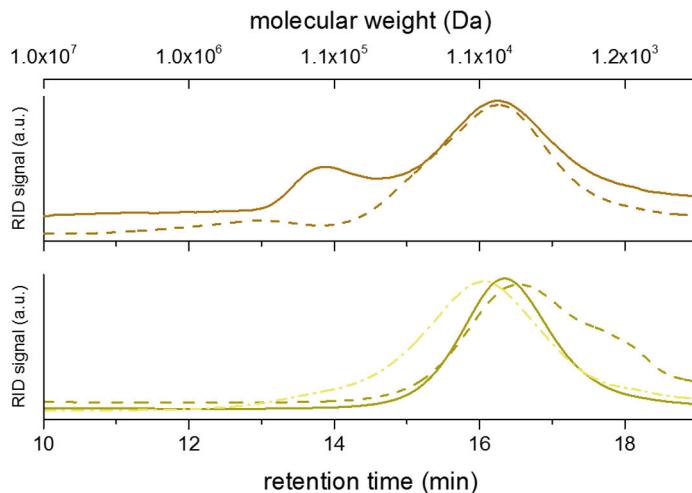
Figure 15. HPSEC-RID chromatograms of Heart Rot fractions (top) and Chaga fractions (bottom). Continous line marks hot water extract, dash line marks the alkali extract. C-Chaga HW is distinguished by point and dash line.

The Chaga HW fractions contained xylose at higher proportions (14.6% and 10.9% from Finnish and Chinese Chaga, respectively), compared to mycelium polysaccharides (**Table 16**). Xylose was found in very low percentage of the total sugars in F-Chaga 2% (5%). Noticeably, no 4-*O*-Me-glucuronic acid was detected in the Chaga fractions. This monomer is mainly found as side-chain of xylan, therefore its reduction in the content or even total absence could be attributed to fungal debranching and degradation of hemicelluloses [301]. The most abundant monomer of the Chaga polysaccharides was glucose, whose relative amount increased from about 36.5% in the hot water extracts to 62% in F-Chaga 2%. This increase is in agreement with the monomer composition of alkali extract of Chaga previously reported by Wold[54]. The monomer composition of the hot water extracts from Finnish and Chinese Chaga (F-Chaga HW and C-Chaga HW, respectively) was in agreement with that of polysaccharide fraction from a water extract of Chinese Chaga sterile conk reported by Xu and coworkers[153], although no fractionation was performed in the present work. The C-Chaga HW monomer composition differed from the report mainly in the arabinose content (12.5% against 5.2%). The water extracts from the Finnish and the Chinese Chaga differed in the content of multiple monomers, most noticeably mannose.

Compared to the fractions previously discussed, our results indicated that polysaccharides in hot water extracts of Chaga (F-Chaga HW and C-Chaga HW) consisted of a single population (**Figure 15** and **Table 17**). Interestingly, the

polysaccharides extracted from Finnish and Chinese Chaga differed, although marginally, in molecular size (8.3 and 13.1 kDa, respectively). As could be expected, the use of alkali resulted in polymers of lower M_p (6.3 kDa). Our observed values were lower than the M_w values reported by Xu and coworkers (32 kDa[153]) and Wold and coworkers (60 kDa[54]). However, in both works HPSEC-MALLS-RID analysis was performed (absolute molecular weight measurement), while HPSEC-RID was utilized in this doctoral dissertation.

5.3.5   Birch heart rot caused by *I. obliquus* (Publication IV)

The monomer composition of the polysaccharides extracted from birch infected by *I. obliquus* is reported in **Table 16**. Compared to the Chaga extracts, the heart rot fractions were clearly distinguished by the predominance of xylose among the monomers (29.8% and 78.0% for hot water and alkali fractions, respectively). Glucose was the second most abundant monomer for both (**Table 16**). The relative molar concents of xylose and galactose were higher in Heart Rot HW in comparison with the corresponding contents in hemicelluloses extracted sequentially from birch sawdust with subcritical water. The monomer composition of the combined second and subsequent birch sawdust extracts was characterized by the dominance of xylan, which was in general similar with the composition of Heart Rot 2%. However, galactose (3.6%) and particularly glucose (7.0%) contents of Heart Rot 2% were higher than in the birch sawdust extracts. Heart Rot HW had higher content of 4-*O*-Me-glucuronic acid, compared to the first birch sawdust extract, while, for Heart Rot 2%, it was in agreement with the combined second and following extracts[293]. The increase in the content of glucose and galactose compared to the amounts in sawdust could be connected to the presence of fungal polysaccharides in Heart Rot HW. The monomer composition of water extracts of debarked birch biodegraded with *G. lucidum* showed increase in the relative contents of glucose and mannose. After the longest colonization time, the glucose and mannose contents of the extract were similar to the levels detected in Heart Rot HW, while the reported galactose content was higher[301]. The polysaccharides extracted by Liu and coworkers from the spent solid substrate from the cultivation of *F. velutipes* showed a prevalence of glucose and galactose in their monomer composition, and, compared to our monomer composition results, a higher content of uronic acids. Moreover, their water extract had higher sugar content (about 88%, against 20% w/w). However, the nature of the starting material was not reported[60].

  The polysaccharides extracted with hot water from the heart rot of birch had two distinct populations (3.2×10^2 kDa and 9.3 kDa, respectively), the one of lower molecular weight being more abundant (82% of total peak area). This population was observed also in Heart Rot 2%, where the lower M_p population constituted the 96% of the polymer area (**Table 17**). In addition to the monomer

composition, also the molecular weight of Heart Rot 2% was in agreement with the values reported for birch xylan. Moreover, the larger polymers of Heart Rot HW had M_p in agreement with wood hemicelluloses[302]. Chaga and birch heart rot polysaccharides showed a noticeable similarity in molecular weight. The similarity could be partially attributed to the presence of wood tissue in Chaga, which was also highlighted by the xylose content of the water extracts. However, glucans were more abundant than xylans in the sterile conk fractions. While a coincidental result cannot be ruled out, it is also possible that production of high molecular weight polymers is specific to the mycelium, while pseudosclerotial tissue would produce low molecular weight glucans. It has to be taken into account that polymers with low M_p were found also in IPS fractions.

5.4 Effect of cultivation conditions on EPS from *I. obliquus* growth medium (Publication III)

The effects of supplementation of sea buckthorn press cake to liquid medium and of two different cultivation times have been investigated also regarding macromolecular properties of the EPS isolated from the growth medium. The concentrations of EPS in the culture medium (measured gravimetrically as g/L) and its sugar content (w/w %) are reported in **Table 20**. The production of exo-polysaccharide, expressed as mg/g mycelium, was calculated from these values. The highest supplement dosage (30 g/L) granted highest EPS concentration in the culture medium and sugar content in EPS fraction. With the two tested cultivation times, the minimum concentration of EPS was obtained with supplementation at dosages of 5 g/L and 2.5 g/L, respectively.

The sugar content of the EPS positively correlated with the dosage of sea buckthorn press cake (r = 0.48, p = 0.006). However, as can be observed in **Table 20**, with the increase of the supplement dosage, EPS content in the medium showed, with both cultivation times, a negative trend with a turning point followed by an increase. At lower concentration of supplement, EPS content was higher at lower cultivation times. Interestingly, this was observed also by Shih after supplementation of peanut and soy oils to *G. frondosa* mycelium[291]. The trends observed in **Table 20** fitted a second order polynomial regression function, as reported in **Equations 1** and **2**:

(1) $EPS_{200h} = 0.61658 - 0.06393 \times [SB] + 0.00283 \times [SB]^2$ $R^2 = 0.774$

(2) $EPS_{250h} = 0.37278 - 0.00721 \times [SB] + 0.00243 \times [SB]^2$ $R^2 = 0.854$

where EPS_{200h} and EPS_{250h} represent the EPS concentration (g/L) after 200 h and 250 h of cultivation, respectively, and [SB] represents the dosage of sea buckthorn press cake (g/L) in the cultivation medium. This trend was in contrast with the almost continuous increasing trend observed by Xu, when oleic acid concentration was increased in the medium[270]. The quadratic relationship of the

production of exo-polysaccharides (mg/g mycelium) with the supplementation of sea buckthorn press cake was more evident, as reported in **Equations 3** and **4**:

(3) $P_{200h} = 9.91238 + 0.05939 \times [SB] + 0.02896 \times [SB]^2$ $\qquad R^2 = 0.983$

(4) $P_{250h} = 7.71521 - 0.72105 \times [SB] + 0.08179 \times [SB]^2$ $\qquad R^2 = 0.995$

where P_{200h} and P_{250h} represent the production of exo-polysaccharide (mg/g mycelium) and [SB] represents the dosage of sea buckthorn press cake (g/L) in the cultivation medium. These quadratic trends could be explained by the findings of Huang and colleagues[283], who reported a quadratic relationship between EPS concentration in the medium and carbon source (glucose, corn oil, or both) consumption. The comparison of the relationship of EPS content with press cake dosage (**Equations 1** and **2**) and with glucose only and glucose&corn oil consumptions (obtained from[283]) is reported in **Figure 16**.
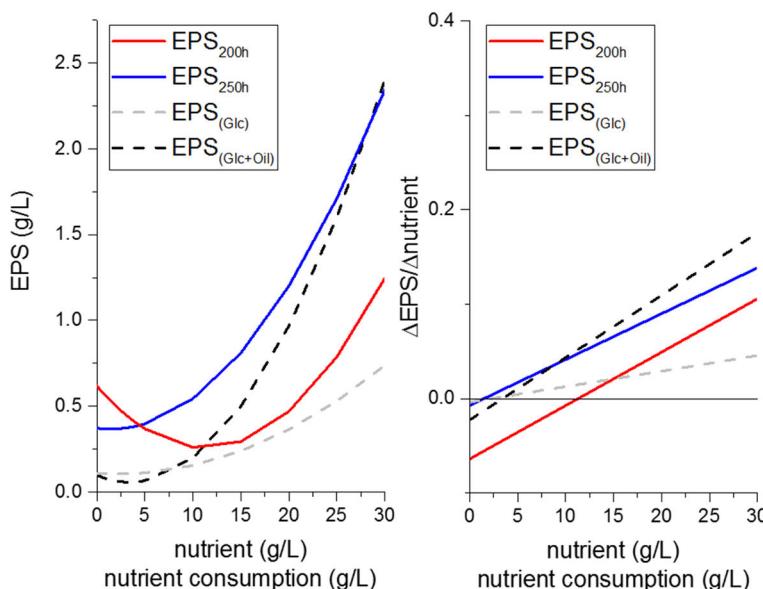
Figure 16. Comparison of **Equations 1** and **2** with the equations relating EPS content and nutrient consumption reported in[283] (respectively marked as $EPS_{(Glc)}$ and $EPS_{(Glc+Oil)}$). On the right, the computed first derivates of the respective equations.

The monomer composition of the EPS is reported in **Table 21**. The most abundant monomers were glucose (23-46 mol%), mannose (14-28% mol%), and galacturonic acid (7-26% mol%). Noticeably, only arabinose, rhamnose, 3-*O*-Me-galactose, and the galactose/3-*O*-Me-galactose ratio significantly ($p < 0.05$) differed in the cultivation conditions. Pearson correlation heatmap between monomer composition, cultivation conditions, molecular weight, and polydispersity index (PDI), is reported in **Figure 17**. The Pearson correlation

values, separated by cultivation times, of relevant variables can be found in **Table 22**.
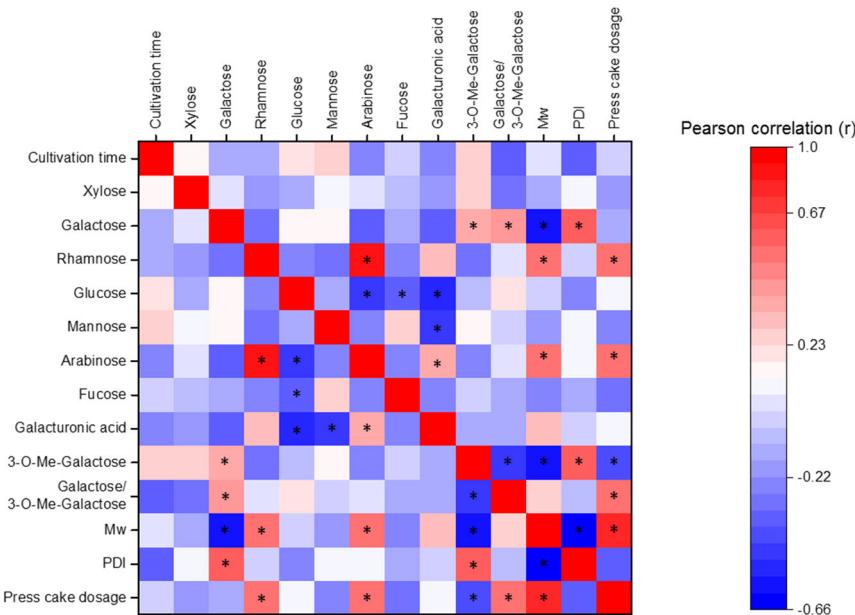
Figure 17. Pearson correlation heatmap between EPS monomers, Mw, PDI, and cultivation conditions. Symbol marks statistical significance ($p < 0.05$).

The relative contents of arabinose and rhamnose correlated with press cake dosage ($r = 0.55$, $p = 0.002$). The concentration of press cake had a negative effect on the amount of 3-*O*-Me-galactose ($r = -0.40$, $p = 0.03$), without effecting galactose, which explained the significance in the increase of the galactose/3-*O*-Me-galactose ratio at higher dosages ($r = 0.51$, $p = 0.004$). The monomer composition analysis has shown absence of correlation between the galacturonic acid content and the amount of supplement. Despite the abundance of galacturonic acid in the extract of sea buckthorn press cake, the relative amount of this monomer had no significant correlation with supplement dosage. This was in contrast with the monomer composition of IPS, which showed increase in the relative content of galacturonic acid at the increase of dosage. The presence of galacturonic acid in EPS even in absence of press cake indicated that this monomer was present in the polysaccharides secreted into the medium by *I. obliquus* mycelium. Polysaccharides containing galacturonic acid have been already isolated from the submerged cultivation medium of basidiomycetes[303]. Nevertheless, a retention of pectin in the EPS could be hinted by the weak positive correlation between galacturonic acid and arabinose ($r = 0.38$, $p = 0.036$), although the ratio between the two monomers was unaffected by the concentration of press cake ($p > 0.05$).

While the ability of *I. obliquus* to depolymerize hemicelluloses in liquid medium has been already proven[62,255], the present results suggested that *I. obliquus* would be able to depolymerize also pectin and possibly use galacturonic acid as carbon source. Differences in the nature of the lignocellulose supplement had significant effects on the monomer composition of EPS isolated from *I. obliquus*, as it was shown in the work of Xu[256] (**Table 19**). The use of different hexoses and pentoses as carbon sources influenced the monomer composition of EPS produced by *P. pulmonarius*. In particular, the use of pentoses resulted in a disappeareance of of 3-*O*-Me-galactose[288]. On the other hand, the use of different dosages of the same carbon source had no noticeable effect on the monomer composition of EPS produced by *G. lucidum*[304].

Table 19. Monosaccharide composition of EPS isolated from *I. obliquus* cultivation medium supplemented with different lignocellulose materials.

Supplement	Rha	Ara	Xyl	Man	Glc	Gal
				rel mol%		
Control	11.4	7.4	8.7	19.0	40.0	13.5
Wheat straw	10.5	9.1	14.5	27.6	27.0	11.3
Rice straw	6.5	7.8	8.6	26.8	35.9	14.4
Sugarcane bagasse	7.2	7.3	6.6	22.6	41.2	15.1

Source of the table:[256]

Table 20. EPS yield, sugar content, exo-polysaccharide production, weight-average molecular mass (M_w) and polydispersity index (PDI) of the isolated EPS fractions. Different letters mark significant difference ($p<0.05$).

Sea buckthorn (g/L)	Cultivation time (h)	EPS concentration (g EPS/L medium)	EPS sugar content (w/w%)	Production of polysaccharide (mg/g mycelium)	M_w (kDa)	PDI (M_w/M_n)
0	200	0.63 ± 0.07 [a]	5.47 ± 3.09 [a]	9.99	4.06 ± 0.03 [a,b]	1.28 ± 0.02 [a,b]
0	250	0.55 ± 0.19 [a]	7.40 ± 5.38 [a]	9.65	4.07 ± 0.10 [a,b]	1.37 ± 0.01 [a,b]
2.5	200	0.46 ± 0.37 [a]	12.37 ± 4.51 [a,b]	11.69	4.15 ± 0.10 [a,b]	1.30 ± 0.06 [a,b]
2.5	250	0.33 ± 0.21 [a]	8.47 ± 2.49 [a,b]	3.78	4.19 ± 0.06 [a]	1.25 ± 0.00 [a]
5	200	0.33 ± 0.07 [a]	14.97 ± 2.06 [a,b]	8.36	4.20 ± 0.01 [a]	1.31 ± 0.00 [b]
5	250	0.40 ± 0.00 [a]	11.39 ± 1.00 [a,b]	5.87	4.19 ± 0.05 [a,b]	1.25 ± 0.00 [a]
10	200	0.67 ± 0.28 [a]	10.27 ± 3.18 [a,b]	14.52	4.22 ± 0.10 [a]	1.32 ± 0.00 [b]
10	250	0.60 ± 0.10 [a]	12.72 ± 1.82 [a,b]	9.77	4.24 ± 0.01 [b]	1.25 ± 0.00 [a]
30	200	1.10 ± 0.67 [a,b]	17.08 ± 4.74 [b]	37.70	4.34 ± 0.10 [b,c]	1.26 ± 0.02 [a,b]
30	250	2.04 ± 0.85 [b]	12.30 ± 1.32 [a,b]	59.60	4.30 ± 0.03 [c]	1.25 ± 0.01 [a]

Table 21. Monomer composition of the exopolysaccharides fractions isolated from the cultivation medium of *I. obliquus*. Different letters mark significant difference ($p < 0.05$).

Sea buckthorn (g/L)	Cultivation time (h)	Xyl	Gal	Rha	Glc	Man	Ara	Fuc	GalA	3-*O*-Me-Gal	Gal/ 3-*O*-Me-Gal
				(rel mol%)			(mol/mol)				
0	200	4.54 ± 1.99 [a]	7.93 ± 1.33 [a]	1.26 ± 0.80 [a,b]	39.35 ± 27.34 [a]	13.45 ± 2.84 [a]	3.12 ± 2.33 [a]	16.21 ± 13.51 [a]	12.44 ± 12.4 [a]	1.70 ± 0.7 [a,b]	5.31 ± 2.76 [a,b]
0	250	7.16 ± 3.07 [a]	12.17 ± 4.15 [a]	0.71 ± 0.24 [a]	35.72 ± 5.11 [a]	20.65 ± 4.54 [a]	3.22 ± 2.44 [a]	6.28 ± 6.66 [a]	9.72 ± 9.38 [a]	4.35 ± 2.21 [a,b]	3.00 ± 0.61 [a]
2.5	200	8.64 ± 3.12 [a]	13.17 ± 8.04 [a]	1.90 ± 0.33 [a,b]	34.07 ± 6.17 [a]	19.86 ± 1.15 [a]	5.17 ± 0.74 [a]	6.29 ± 3.16 [a]	9.76 ± 2.72 [a]	1.15 ± 0.34 [a]	13.00 ± 10.96 [a,b,c]
2.5	250	7.70 ± 5.5 [a]	5.47 ± 0.43 [a]	1.27 ± 0.54 [a,b]	29.22 ± 14.16 [a]	27.56 ± 8.02 [a]	4.45 ± 0.36 [a]	15.32 ± 13.81 [a]	8.25 ± 6.43 [a]	0.76 ± 0.24 [a,b]	8.05 ± 3.82 [a,b]
5	200	6.08 ± 0.84 [a]	6.14 ± 1.09 [a]	3.26 ± 0.25 [b]	24.33 ± 2.66 [a]	20.96 ± 1.88 [a]	11.53 ± 2.5 [b]	1.53 ± 0.48 [a]	24.51 ± 5.03 [a]	1.67 ± 0.19 [a]	3.68 ± 0.65 [a,b]
5	250	5.14 ± 1.85 [a]	8.89 ± 1.77 [a]	1.31 ± 0.56 [a,b]	45.98 ± 6.97 [a]	22.04 ± 2.13 [a]	3.30 ± 1.69 [a]	5.26 ± 2.73 [a]	6.80 ± 2.75 [a]	1.28 ± 0.52 [a,b]	8.54 ± 6.04 [a,b,c]
10	200	4.75 ± 0.58 [a]	7.30 ± 2.00 [a]	4.25 ± 0.99 [b]	22.51 ± 4.39 [a]	17.33 ± 2.94 [a]	16.37 ± 2.83 [b]	6.38 ± 5.58 [a]	20.24 ± 11.83 [a]	0.87 ± 0.04 [a]	8.36 ± 1.95 [a,b]
10	250	3.48 ± 0.18 [a]	6.48 ± 0.44 [a]	3.48 ± 0.61 [b]	33.11 ± 7.46 [a]	16.20 ± 0.26 [a]	8.43 ± 1.53 [b]	1.82 ± 0.92 [a]	25.94 ± 4.27 [b]	1.06 ± 0.08 [a]	6.15 ± 0.61 [b]
30	200	2.03 ± 0.22 [b]	9.64 ± 2.74 [a]	2.71 ± 0.72 [a,b]	36.86 ± 3.24 [a]	18.58 ± 5.54 [a]	9.29 ± 2.64 [b]	4.02 ± 5.18 [a]	16.55 ± 13.38 [a]	0.32 ± 0.06 [b]	29.86 ± 4.26 [c]
30	250	7.81 ± 7.10 [a]	6.25 ± 0.28 [a]	4.50 ± 2.65 [a,b]	37.27 ± 9.79 [a]	15.85 ± 0.68 [a]	13.51 ± 1.99 [b]	3.06 ± 2.89 [a]	10.61 ± 7.76 [a]	1.14 ± 0.35 [a,b]	5.88 ± 1.83 [a,b]

Table 22. Pearson correlation values of selected variables, distinguished by cultivation time. Symbol marks statistical significance ($p<0.05$)

Variables		200 h	250 h
Sugar content (w/w%)	Press cake dosage (g/L)	0.54*	0.44
Ara (rel mol%)	"	0.30	0.90*
Rha (rel mol%)	"	0.28	0.75*
Xyl (rel mol%)	"	-0.63*	0.08
Gal (rel mol%)	"	0.03	-0.39
Glc (rel mol%)	"	0.07	0.06
Man (rel mol%)	"	0.14	-0.52*
Fuc (rel mol%)	"	-0.29	-0.31
GalA (rel mol%)	"	0.10	0.11
3OMeGal (rel mol%)	"	-0.77*	-0.34
Gal/3OMeGal (mol/mol)	"	0.81*	0.00
GalA/Ara (mol/mol)	"	-0.26	-0.35
M_w (Da)	"	0.75*	0.73*
GalA (rel mol%)	Ara (rel mol%)	0.46	0.19
M_w (Da)	"	0.49	0.65*
"	3OMeGal (rel mol%)	-0.45	-0.82*
"	Gal (rel mol%)	-0.40	-0.84*
"	Rha (rel mol%)	0.50	0.61*
"	Xyl (rel mol%)	-0.24	-0.07
"	Glc (rel mol%)	-0.14	0.17
"	Man (rel mol%)	0.14	-0.46
"	Fuc (rel mol%)	-0.41	-0.08
"	GalA (rel mol%)	0.39	0.20
"	Sugar content (w/w%)	0.57*	0.80*

The molecular weight analysis focused on the major peak found in the chromatograms. The weight-average molecular mass (M_w) and polydispersity index (PDI) of the main EPS population for the different cultivation conditions are reported in **Table 20**. The average M_w of the EPS main population was 4.20 ± 0.10 kDa. A polysaccharide population of similar magnitude of M_w was found in the cultivation medium of *Antrodia camphorata*[305] and *Rigidoporus ulmarius*[306]. However, these values are more commonly found for EPS produced by ascomycetes such as *Botryosphaeria rhodina*[307] and *Phoma herbarum*[308]. Galactose (r = -0.56, p = 0.001) and 3-*O*-Me-galactose (r = -0.58, p = 0.001) had a significant negative correlation with M_w. The relationship was more marked at 250 h of cultivation (**Table 22**). The results would hence suggest galactans of EPS fractions had low molecular weight. On the other hand, a positive correlation between M_w and arabinose and rhamnose was found (r = 0.53 and r = 0.52, respectively, p = 0.003). The correlation between M_w and press cake dosage (r = 0.73, p < 1×10^{-4}) was more evident. For the latter relationship, experimental data suggested an asymptotic exponential trend, as reported in **Equations 5** and **6**:

(5) $M_{w(200h)} = 4348.14 - 275.05 \times 0.91^{[SB]}$ $\qquad$ $R^2 = 0.566$

(6) $M_{w(250h)} = 4292.17 - 212.22 \times 0.84^{[SB]}$ $\qquad$ $R^2 = 0.648$

where $M_{w(200h)}$ and $M_{w(200h)}$ represent the M_w (Da) of the EPS population and [SB] the dosage of sea buckthorn press cake (g/L) in the medium. Cultivation

time, on the other hand, showed no significant influence on M_w of EPS and no cultivation parameter significantly affected their PDI. A similar trend can be observed in the data published by Shu and Lung, relating $logM_n$ of fungal EPS and culture pH[305]. However, due to the high complexity of sea buckthorn press cake, it cannot be deduced that pH alone would be responsible of the observed trend. Another similar trend was observed in the data of *R. ulmarius*. The M_w of the EPS population of low M_w fitted as well an asymptotic exponential equation. However, only three concentrations of carbon source were tested, therefore the trend would be uncertain[306].

5.5 ATR-FT-IR spectroscopy of polysaccharide fractions

5.5.1 *A. bisporus* polysaccharides (Publication I)
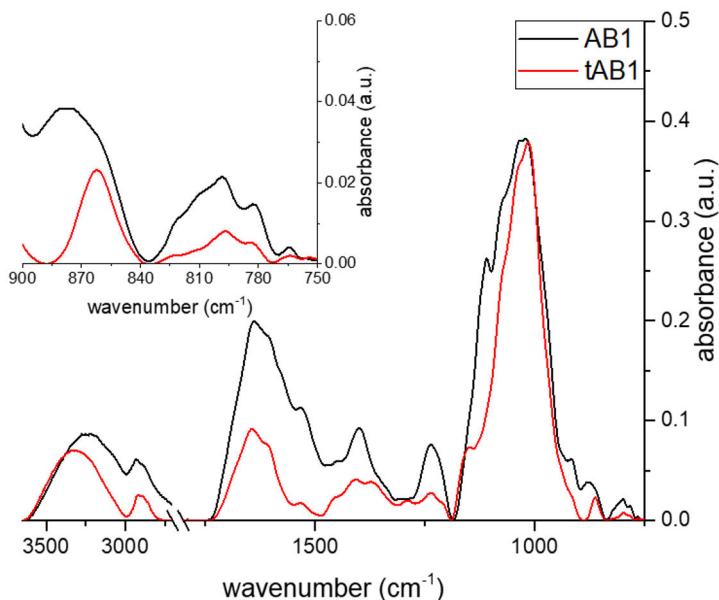
Figure 18. ATR-FT-IR of AB1 and tAB1 fractions, with an expansion of the anomeric region (900-750 cm^{-1}).

ATR-FT-IR spectroscopy was used to analyze the structure of the extracted polysaccharides. The spectra of AB1 and tAB1 are reported as representatives of the dataset in **Figure 18**. Characteristic strong O–H stretching of hydroxyl groups and bound water was observed in the spectra of AB1 and tAB1 at the range 2500–3500 cm^{-1} (**Figure 18**). The peak of O–H stretching in AB1 was clearly shifted towards lower energy to 3215 cm^{-1} compared to the signals in

other samples at 3283–3325 cm^{-1}. Stretching of CH$_2$ group were observed at 2855, 2887, and 2925 cm^{-1}. The tAB fractions had notably weaker vibrations than AB in the region of amide and amine vibrations, i.e. at 1639, 1523, 1398, and 1236 cm^{-1}[309], most likely due to hydrolytic effect of the pretreatment.

The stretchings C–O–C and C–O of the pyranose ring are visible in the range 1000–1200 cm^{-1}. The differences between the two fractions are probably due to the near absence of α-glucans in AB1 (**Table 18**). Therefore, the trends of α/β-ratio and O–H and C–O stretching wavenumbers have been investigated. The patterns are reported in **Figure 19**.
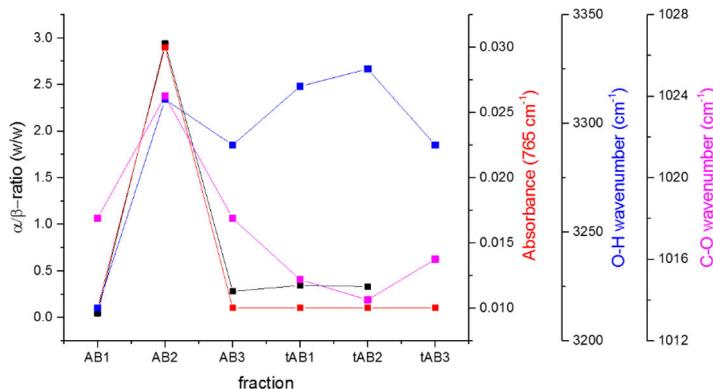
Figure 19. α/β-ratio (w/w) as measured with enzymatic kit (black) in comparison with 765 cm^{-1} wavenumber absorbance (red), wavenumber of the O–H stretching (blue), and wavenumber of the C–O stretching (purple).

Compared to the inspected wavenumber values, the intensity of wavevumber 765 cm^{-1}, which can be assigned to α-glucan basing on the Raman study of De Gussem[310], showed a noticeable correlation with the ratio between α- and β-glucans. Due to the low yield of tAB3, it was not possible to measure the glucan composition with Megazyme kit. However, the absorbance at 765 cm^{-1} hinted to similar or slightly higher ratios of α- and β-glucans as in samples tAB1 and tAB2 (**Figure 19**). The wavenumbers of the O–H (3290 cm^{-1}) would suggest a decrease in α-glucan content. Other wavenumbers gave less clear trends.

5.5.2   *C. tubaeformis* **polysaccharides (Publication II)**
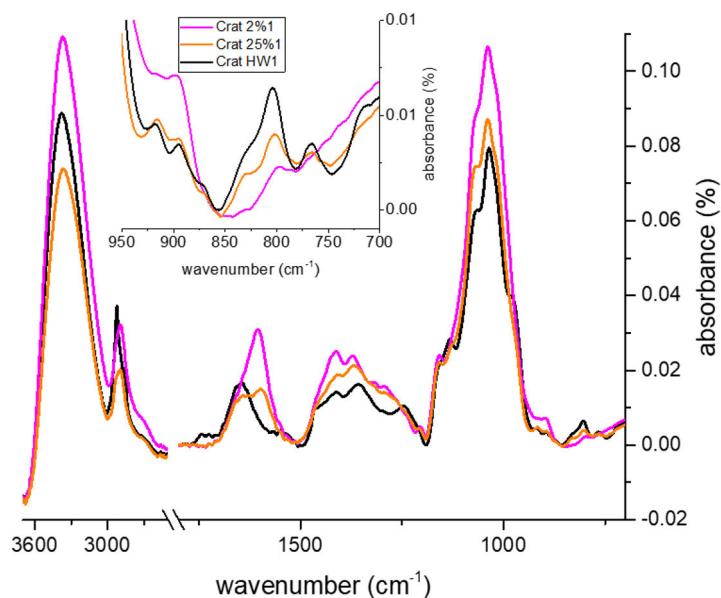
Figure 20. ATR-FT-IR spectra of Crat HW1, Crat 2%1, and Crat 25%1. An expansion of the anomeri region (900–700 cm^{-1}) is reported.

The ATR-FT-IR spectra of the three polysaccharide fractions Crat HW1, Crat 2%1 and Crat 25%1 are shown in **Figure 20**. The figure shows the vibration bands in the area 3600–935 cm^{-1} and, in the inserted expansion, the area of the recorded spectra between 1000 and 700 cm^{-1}. The broad band around 3320 cm^{-1} was assigned to the O–H stretching, due to both polysaccharide hydroxyl groups and sample moisture. The absorption bands between 2920 and 2850 cm^{-1} were, in turn, assigned to C–H (pyranoid ring and CH$_2$) bond stretching. The strong and overlapping bands in the area 1160–935 cm^{-1} represent the stretching of the C–O and C–O–C (glycosidic) bonds, typical of polysaccharide backbones. The IR bands between 1650 and 1590 cm^{-1} originated from C=O stretching of protein amides. In the case of Crat 2%1, this vibration band overlapped with the stretching vibration of carboxylates. The spectrum of Crat HW1 showed a small band at 1743 cm^{-1}, assigned to the stretching of the ester group. The bands in the area 1470–1160 cm^{-1} of the spectrum were due to C–H bending, CO–H bending, O–H bending and C–C[309]. Additionally, the carboxylate anion vibrations suggest the presence of uronic acid units in Crat 2%1 and Crat 25%1 fractions.

The low absorbance bands in the region 950–800 cm^{-1} was produced by the C–H bending of the anomeric proton[311]. In particular, vibrations around 890 cm^{-1} were produced by β-anomeric configurations, while those between 830 and 800

cm[-1] by α-configurations[312]. Bands around 920 cm^{-1} were attributed to α-glycosidic linkages as well. The α-linkage vibration at 800 cm^{-1} (**Figure 20**, expansion) was relatively more intense in the spectra of the polysaccharides extracted with hot water, and was assigned to the α-mannan chain[309]. The anomeric C–H vibration around 865 cm^{-1} was assigned to a α-galactan[76,313]. As indicated by the cell wall model, the outer layer of the mushroom cell wall is rich in proteoglycans with mannose and galactose as major carbohydrate monomers. Therefore, we can conclude that hot water mainly extracted the polysaccharides from the outer layer of the cell wall. The vibration at 892 cm^{-1} had the highest intensity in the anomeric region of the spectrum of Crat 2%1, and it was assigned to the β-linkage, suggesting that the β-glucans were more abundant in this fraction.

5.5.3 *I. obliquus* and birch heart rot polysaccharides (Publication IV)
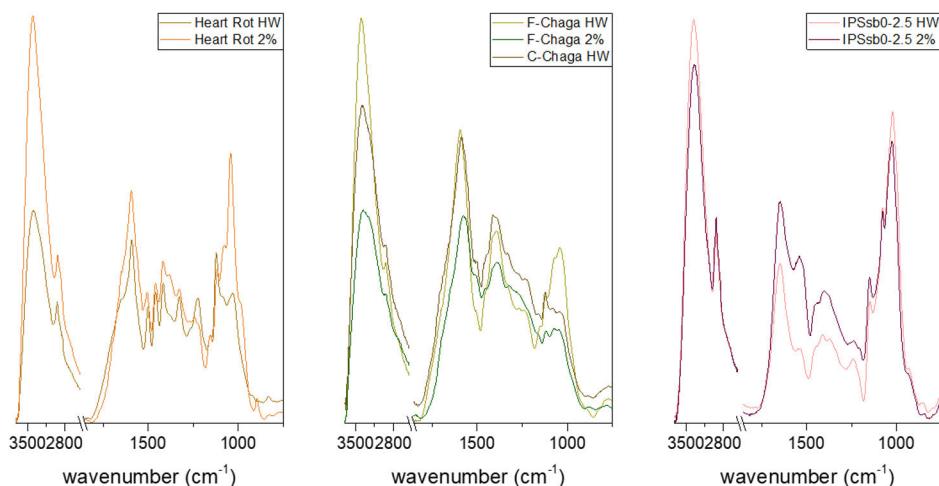
Figure 21. ATR-FT-IR spectra of heart rot, sterile conk, and cultivated mycelium polysaccharides.

The ATR-FT-IR spectra of the heart rot, sterile conk, and cultivated mycelium polysaccharide fractions are reported in **Figure 21**. The stretching of the O–H bond produced the broad bands 3300–3400 cm^{-1}, while the signal at 2930–2940 cm^{-1} was assigned to C–H stretching. For Heart Rot and Chaga extracts, C–O and C–O–C bonds vibrations were found around 1040 cm^{-1}, while the polysaccharide backbone of IPS fractions produced vibrations around 1020 cm^{-1}. Noticeably, similar values were reported for xyloglucan and starch, respectively[313]. Differently from the other fractions, the spectra produced by mycelial polysaccharides had strong signals at 1646 cm^{-1} and 1542 cm^{-1}, which were assigned to protein amide vibrations.

Only in IPS and Heart Rot 2% spectra the C–O and C–O–C vibration group was the most intense signal of the mid-IR region. In the others, the band around 1590 cm^{-1} was the most intense. As indicated above, this signal can be attributed to carboxylate stretching. Besides it, the FT-IR spectra of heart rot extracts were in good agreement with those reported of hemicelluloses[313]. This signal would be explained by the presence of aromatic compounds and uronic acids. Aromatic C=C vibrations could contribute to this band as well. Presence of aromatic moieties was further confirmed by the signal around 1120 cm^{-1}, which was ascribable to aromatic C–H deformation[314]. Moreover, the shoulder at 1650 cm^{-1}, most prominent in Heart Rot HW but visible also in Heart Rot 2% and partially in F-Chaga 2%, has been observed in fungal-degraded hemicelluloses and assigned to the C=O vibration of lignin bound to hemicelluloses[301].

The FT-IR spectra of Chaga fractions shared some similarities with the Chaga melanin spectrum reported by Wold and coworkers, who have suggested 1,8-dihydroxynaphtalene as monomer unit[315]. In both spectra, the bands around 1590 cm^{-1}, 1510 cm^{-1} (C=C), 1450 cm^{-1} (C–H), 1400 cm^{-1} (CO–H), and 1320 cm^{-1} (C–C, C–O aliphatic and aromatic) were observed. In their report, the signal around 1400 cm^{-1}, which could be due to the presence of polysaccharides, was much less intense. The bands between 1280–1230 cm^{-1} observed in Chaga fractions were absent [315]. The FT-IR spectra of synthetic 1,8-dihydroxynaphtalene (DHN) polymers showed 1611 cm^{-1}, 1402 cm^{-1}, and 1284 cm^{-1} bands[316], which would be in partial agreement with the our spectra and the report from Wold. However, bands around 1510 cm^{-1}, 1450 cm^{-1}, and 1320 cm^{-1} were observed also in lignin-polysaccharide complexes[314]. Therefore, it is difficult to determine whether the phenolics in Chaga fractions are DHN melanin or lignin.

The fractions Heart Rot 2%, C-Chaga HW and F-Chaga 2% had a clear β-anomeric bond (around 890 cm^{-1}) signal. In F-Chaga HW spectrum, this band was less visible. On the other hand, signals ascribable to α-glycosidic linkages were observed in Heart Rot HW spectrum (830 cm^{-1}) and in both mycelial polysaccharide spectra (850–860 cm^{-1}). A shoulder ascribable to α-mannans was also found in the IPS fractions around 800 cm^{-1}[309]. No clear β-linkage signal was visible in these spectra, indicating the prominence of α-polysaccharides in these fractions.

5.6 TGA-MS analysis of *C. tubaeformis* polysaccharides (Publication II)

Thermal and thermomechanical treatment of food, such as extrusion, causes physicochemical changes to the material. While this is a common approach for food processing or fortification with the addition of dietary fibers in products, the process could implicate undesired thermal degradation of the food

components or chemical reactions, such as Maillard reaction[317]. Thermogravimetric analysis was carried out to investigate the effect of the mushroom cell wall disruption with alkaline solution on the thermal stability of the extracted polysaccharides. The analysis was performed using a low temperature gradient until complete degradation (600 °C) of the polymers, in order to thoroughly investigate the effect of alkali on the polysaccharides. The thermogravimetric profiles and the DTGA curves of the extracted polysaccharides are illustrated in **Figure 22**.
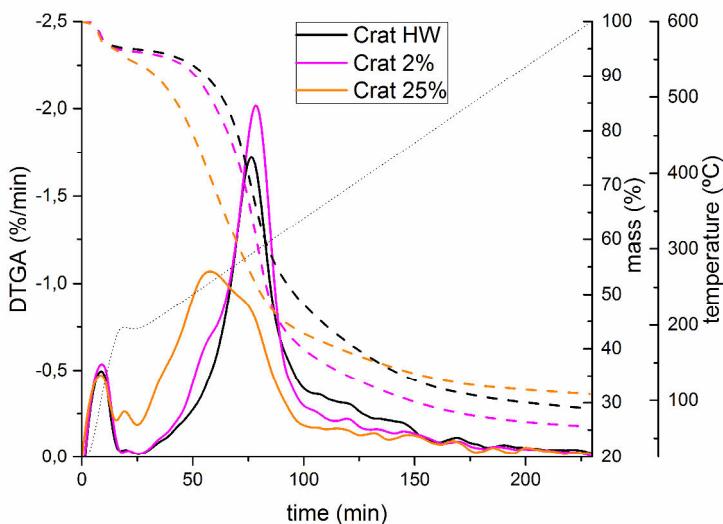
Figure 22. TGA and DTGA profiles of Crat HW, Crat 2%, and Crat 25%. Continuous line, dashed line, and black dotted line represent DTGA, TGA, and temperature program, respectively.

The polysaccharides decomposed in a relatively narrow temperature range, starting at about 195 °C, after the loss of moisture and volatile compounds at approximately 100 °C. The degradation range was very similar for all polysaccharides studied, ending at approximately 350 °C, while minor degradations occurred until 441 °C. The main mass loss of Crat HW and Crat 2% polysaccharides took place in the same temperature range, 226–348 °C (43–104 min), while Crat 25% polysaccharides exhibited a wider range (196–348 °C, 25–103 min) and the lowest mass loss rate. Crat HW and Crat 2% fractions had comparable maximum rates of decomposition, at very close temperatures (292 °C and 298 °C, respectively). The Crat HW decomposition process exhibited a sharp DTGA peak, while Crat 2% polysaccharides DTGA peak showed a shoulder at 260 °C. The polysaccharides extracted with KOH 25% showed, on the other hand, a faster degradation process, starting with higher mass loss rate at lower temperatures (i.e., the DTGA shoulder at around 210 °C),

and with earlier mass loss peak at 256 °C. Interestingly, this temperature was close to the Crat 2% DTGA shoulder.

The differential thermal analysis of *C. tubaeformis* polysaccharides (**Figure 23**) indicated, during the thermal degradation, the presence of two broad endothermal events, with temperature ranges of 25–196 °C and 196–295 °C, respectively. The first one could be attributed to water desorption, the second one to the first step of the degradation of polysaccharides. After 295 °C, in all the profiles, the curve has an inflection and an increase, suggesting the presence of an exothermal event, which could be assigned to the main thermal degradation. Such endothermal-exothermal inflection at the DTGA peak is similar to the one reported for starch[318] and for agar[319].
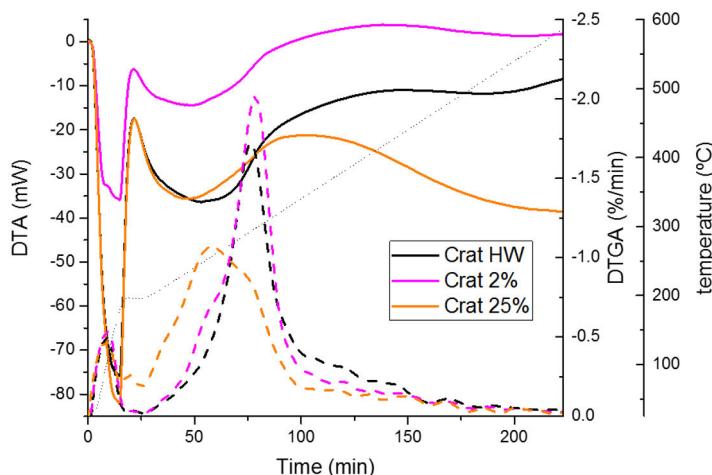
Figure 23. Differential thermal analysis (DTA) and DTGA profiles of Crat HW, Crat 2%, and Crat 25%. Continuous line, dashed line, and black dotted line represent DTA, DTGA, and temperature program, respectively.

The thermal profile of of the polysaccharides extracted from *C. tubaeformis* were in agreement with previous thermal stability studies of fungal polysaccharides. A mannan isolated from *Agaricus brasiliensis* with hot water had a maximum degradation rate at 301 °C, with a DTGA profile similar to Crat HW. A branched β-glucan, isolated from the fruiting bodies of the same fungus with the same method, showed a similar DTGA profile, with a maximum degradation rate at 314 °C[320]. Polysaccharide fractions of different molecular weight and different β-glucan contents, produced from *A. brasiliensis* fruiting bodies, showed different DTGA profiles with maximum degradation rates in the range 270-315 ˚C. It was noticed that the degradation events of the different fractions happened approximately at same temperature, however with different degradation rates[321]. The same was noticed during thermal analysis of Crat fractions. The thermogravimetric analysis of a complex β-glucan, predominantly

→6)-β-Glc-(1→, isolated from culture broth of the ascomycete *Lasiodiplodia theobromae* had a maximum degradation rate around 290 °C[322].

The presence of thermal degradation earlier than 290 °C could be due to the presence in the fractions of polymers with lower molecular weight, since glycosidic linkage of terminal units breaks at lower temperatures[222,323]. Crat 25% DTGA showed also, after the loss of volatiles, a consistent peak in the DTGA in the beginning of the slow temperature program (197 °C), which could be attributed to the degradation of monosaccharides or oligosaccharides. The great reduction in thermal stability of Crat 25% could be explained as well by the increased amounts of terminal units, due to the reduction in molecular weight consequent to alkali hydrolysis of the polysaccharide chain. A decrease in thermal stability of polysaccharide fractions obtained in a stepwise process of increasing harshness was noticed by Sun and Sun[324] and Sun and coworkers[325] during the extraction of hemicelluloses from barley straw and sweet sorghum stem, respectively. The authors correlated the decrease in thermal stability to the decrease in molecular weight of the polysaccharides. It would be difficult to establish whether molecular weight has higher or lower influence on the thermal stability of polysaccharides, compared to monomer or glycosidic linkage composition.

5.7 Structures of *C. tubaeformis* polysaccharides (Publication II)

5.7.1 Crat HW1

The Crat HW1 fraction had a complex pattern of anomeric proton in the region 4.95–5.35 ppm. These signals were assigned basing on multiple NMR experiments. The anomeric proton producing the signal at 4.95 ppm correlated, in the HSQC spectrum (**Figure 24**), with the carbon resonating at 98.4 ppm. In the COSY spectrum, the anomeric proton interacted with a proton signal at 3.85 ppm (H1–H2 interaction), and this proton produced an HSQC interaction with a carbon at 72.0 ppm, whose chemical shift was ascribed to C2 of glucose. The HMBC correlation between the anomeric signal at 4.95 ppm and the C6 signal at 66.8 ppm was assigned to a →6)-α-Glc-(1→chain. The HSQC interaction between the carbon signal at 66.8 ppm and proton at 4.17 ppm (H6′) further proved the α-(1→6) linkage. The doublet at 4.98 ppm had a corresponding C1 signal resonating at 98.8 ppm. The HMBC correlations of this proton with the carbon at 70.9 ppm and at 67.0 ppm were interpreted as H1–C2 and H1–C6 interactions, respectively. These correlations were produced by the terminal non-reducing unit of the→6)-α-Glc-(1→ chain[73]. This assignment was confirmed by the NOESY spectrum, which showed H1–H6 and H1–H6′ interactions and a

strong H1–H3 interaction at 4.98/3.74 ppm (confirmed by HSQC interaction 3.74/73.2) in the terminal unit.
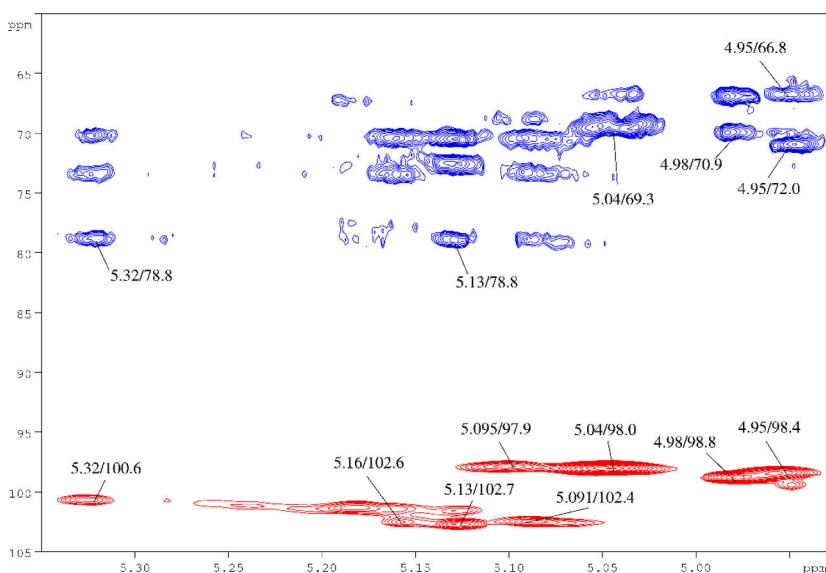
Figure 24. HSQC (red) and HMBC (blue) NMR spectra of Crat HW1. NMR spectra were recorded in D_2O at 308 K. See **Table 24** for signal assignments.

The proton signal at 5.32 ppm (**Figure 24**), corresponding to a C1 at 100.6 ppm, gave an HMBC interaction with a C2 carbon 78.8 ppm. Such interactions and the chemical shift of the proton signal were in agreement with a→2)-α-Man-(1→unit. The NOE spectrum showed a strong correlation between the proton at 5.32 ppm and the signal at 4.12 ppm. This proton was confirmed to be O-substituted H2 by the HSQC spectrum. The HSQC-TOCSY showed an intra-residue interaction between the H1 and the O-substituted C2, further confirming the 5.32 ppm signal assignment[326].

The H1 at 5.13 ppm had an HSQC correlation with a C1 at 102.7 ppm and an HMBC interaction with a carbon at 78.8 ppm. The COSY interaction with an O-substituted H2 indicated that the 5.13 ppm signal was produced by the monomer of a→2)-α-Man-(1→chain[327].

The most intense H1 signal of the ^{1}H spectrum α-anomer region was at 5.04 ppm. It interacted with a C1 signal resonating at 98.0 ppm and it produced HMBC interactions with an O-substituted C6 (69.3 ppm). The presence of two HSQC-TOCSY H1–C6 signals under the 5.04 signal and the separation of the HMBC peak (**Figure 24**) suggested that the 5.04 ppm signal was an overlapping of two α-(1→6) H1 signals. Both →6)-α-Gal-(1→and→6)-α-Man-(1→ could be responsible for such signal pattern[297].

The proton peak at 5.16 ppm had HMBC and HSQC-TOCSY correlations with an O-substituted C6 carbon (70.3 ppm). This anomeric proton had a COSY interaction with O-substituted H2 (4.12 ppm), which, on the other hand, produced a NOESY correlation with the H1 of the →2)-Man-(1→ unit at 5.32 ppm. These correlations and the agreement of chemical shifts with the literature indicated that the proton resonating at 5.16 ppm belonged to a→2,6)-α-Man-(1→unit[297].

The proton signal at 5.09 ppm was due to the overlapping of two different anomeric signals, at 5.095 ppm and 5.091 ppm. The proton signal at 5.095 ppm (corresponding to a C1 at 97.9 ppm) had a NOESY correlation at 5.095/4.03 ppm, which could be assigned to a H1–H6′ inter-residue interaction (HSQC correlation 4.03/70.2 ppm). The COSY spectrum showed a 5.095/3.88 ppm peak, the latter being the chemical shift of an unsubstituted H2. Such H1 was assigned to a →6)-α-Man-(1→ residue. The proton at 5.091 ppm, with a corresponding C1 at 102.4 ppm, showed a 5.091/4.19 NOESY correlation, which was assigned to a H1–H2 inter-residue interaction. The COSY spectrum showed a weak 4.19/5.23 ppm correlation, with the latter signal assigned to H1 of→2)-α-Gal-(1→ (confirmed by weak HSQC correlation 5.23/101.3). The H1 at 5.091 ppm was then assigned to a α-L-Fuc-(1→ branching unit of→2)-α-Gal-(1→[119,326,328]. The α-L-Fuc-(1→ unit was further confirmed by the deoxyhexopyranose C6 1.33/18.0 HSQC correlation.

5.7.2   Crat 2%1

The polysaccharides of Crat 2%1 were investigated using different NMR spectroscopy experiments. The HSQC spectrum (**Figure 25**) showed four broad correlations: 4.55/104.0; 4.52/104.2; 4.73/104.2; 4.77/103.9. They were assigned to →3,6)-β-Glc-(1→, →6)-β-Glc-(1→, β-Glc-(1→ , and →3)-β-Glc-(1→ units, respectively. The signal assignment was provided by HSQC interactions at 3.75/86.1 ppm, 4.22/70.3 ppm and 3.86/70.3, which were assigned to O-substituted H3/C3, H6′/C6 and H6/C6, respectively. The typical splitting of the proton of unsubstituted position 6 was visible as well, with the signal pair at 3.92/62.0 ppm and 3.74/62.0 ppm. The HMBC spectrum showed a H1–C6 correlation broad signal at 4.52/70.3 ppm and H6′–C1 at 4.22/104.2 ppm and H6–C1 at 3.86/104.2 ppm. The HMBC spectrum showed as well a 4.55/86.1 correlation, which indicated an intra-residue interaction between the H1 and the O-substituted C3, proving the→3,6)-β-Glc-(1→ unit. This was further confirmed by the HSQC-TOCSY spectrum, which showed an intra-unit correlation between the H1 at 4.55 pm and an O-substituted C3 together with an O-substituted C6. Moreover, in this spectrum, the intra-unit correlation between H1 at 4.77 ppm and O-substituted C3 was visible as well, confirming the assignment of this H1 to a →3)-β- Glc-(1→ unit. The assignments were in agreement with the HSQC-

TOCSY spectrum, which showed H1 correlations with O-substituted C3 only for 4.55 ppm and 4.77 ppm signals.
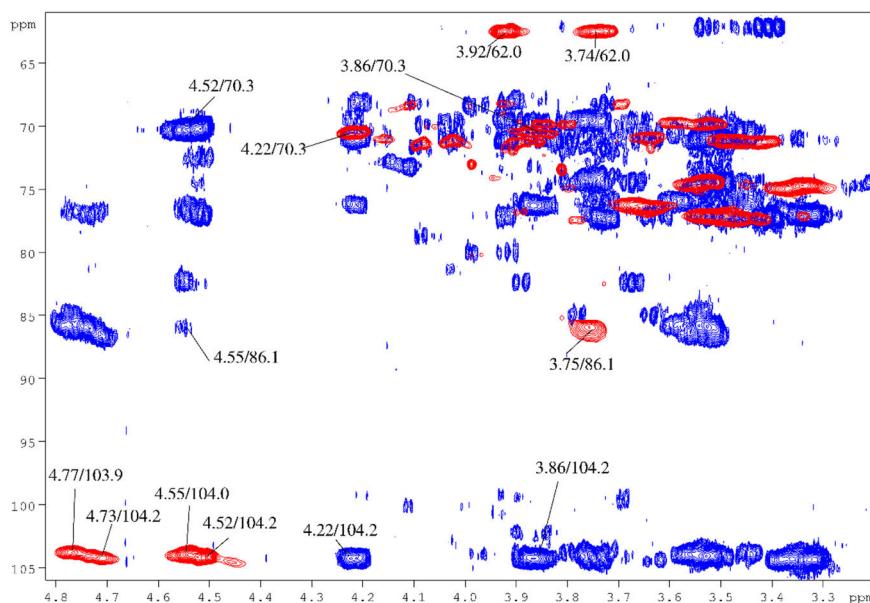

Figure 25. HSQC (red) and HMBC (blue) spectra of Crat 2%1. NMR spectra were recorded in D_2O at 308 K. See **Table 23** for signal assignments.

To further investigate the structure of the β-glucan present in Crat 2%1, the fraction was submitted to methylation, hydrolysis, and GC-MS analysis of the silylated permethylated glucose units. The results of the linkage analysis were in agreement with the signal integration of the ^{1}H spectrum: the ratio between →6)-Glc-(1→ and →3)-Glc-(1→ was 1:1.09, and the ratio between →6)-Glc-(1→ and →3,6)-Glc-(1→ was 1:0.63. The ratio between →6)-Glc-(1→ and terminal glucose unit was 1:0.06. Due to the dominance of glucose and of →6)-Glc-(1→ and→ 3)-Glc-(1→ units in the polysaccharides of Crat 2%1, it can be assumed that both polymer populations would contain the glycosidic units identified with NMR spectroscopy. It can be speculated that this low molecular weight population likely has different ratios between the two types of β-Glc linkage units, compared to the population of high molecular weight. In particular, a lower ratio between →6)-Glc-(1→ and →3,6)-Glc-(1→ would be expected, due to the shorter backbone length consequent to alkali hydrolysis, compared with the population of high molecular weight.

5.7.3 Crat 25%1

The NMR spectra of Crat 25%1 had patterns similar to the Crat 2%1 spectra. However, the linkage analysis, performed as well for this fraction, indicated an increase of the ratio between →6)-Glc-(1→ and →3)-Glc-(1→ units (1:0.73), which was also seen in the integration of the ^{1}H NMR signals. In contrast, the ratios between →6)-Glc-(1→ and terminal glucose and →6)-Glc-(1→ and →3,6)-Glc-(1→ decreased to 1:0.22 and 1:0.9, respectively. These differences could be ascribed to alkali depolymerization.

The two proton peaks at 5.44 ppm and 5.89 ppm marked the clear difference between Crat 2%1 and Crat 25%1. The proton at 5.44 ppm gave an HSQC correlation with a carbon signal at 100.8 ppm, ascribable to a glycosidic C1 (**Figure 26**). Basing on its chemical shift and the coupling constant (4.93 Hz), it was assigned to a β-isomer. The proton at 5.89 ppm gave a HSQC correlation with a carbon at 107.4 ppm and HMBC correlations with carbons at 144.7 ppm and 169.8 ppm.
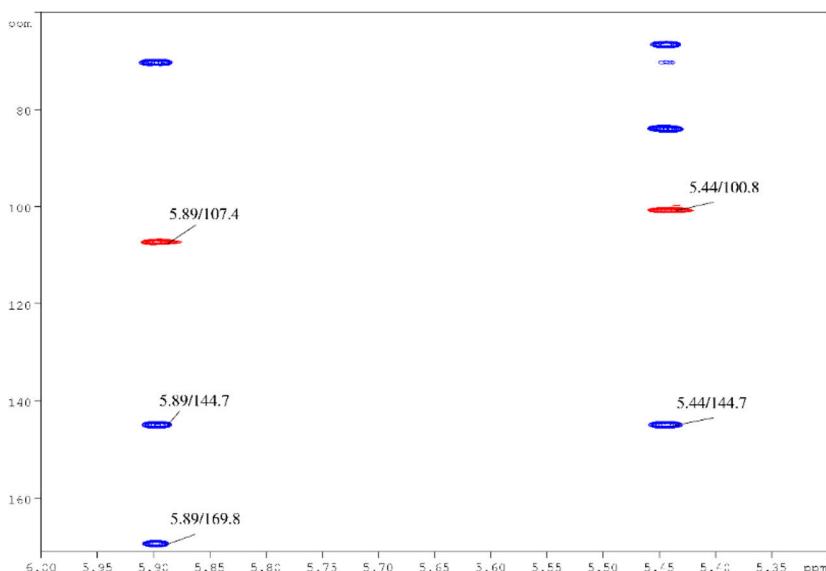
Figure 26. HSQC (red) and HMBC (blue) of Crat 25%1, focusing on the region 5.30–6.00 ppm of the ^{1}H spectrum. NMR spectra were recorded in D$_2$O at 308 K. See **Table 23** for signal assignments.

These signals were ascribed to a double bond and a carboxyl group, respectively. The anomeric proton had a HMBC correlation with the carbon at 144.7 ppm, similar to the proton at 5.86 ppm. The proton signal at 5.89 ppm was assigned to the β-position of an α,β-unsaturated uronic acid[329,330]. The COSY

spectrum of Crat 25%1 showed short-range correlations at 5.89/4.26 ppm and 5.44/3.94 ppm, which allowed the assignment of 3.94 ppm to H2, 4.26 to H3 and 5.89 to H4. The HSQC spectrum allowed the assignments of C2 and C3 positions to 70.4 ppm and 66.7 ppm, respectively. The HSQC-TOCSY spectrum further confirmed the assignments.

The H1 of the uronic acid gave an HMBC signal with a carbon at 84.0 ppm, indicating that the uronic acid formed a β-(1→3) linkage. This was further confirmed by the HMBC correlation of the C1 position with a proton at 3.88 ppm, which, on the other hand, produced an HSQC interaction signal with a carbon at 84.0 ppm (O-substituted C3). The proton at 3.88 ppm gave an HSQC-TOCSY interaction with the carbon signal at 103.9 ppm and a TOCSY correlation with the H1 signal at 4.77. Such correlations were interpreted as a glycosidic linkage between uronic acid and→3)-β-Glc-(1→ side chain of the β-glucan. The ^{1}H signal ratio between the uronic acid and the→6)-β-Glc-(1→was 1:12, indicating a seldom presence of the former unit in the polysaccharide chain.

Table 23. ^{13}C and ^{1}H chemical shifts (ppm) of the β-glucan extracted from *C. tubaeformis*.

Type of linkage	C1/H1	C2/H2	C3/H3	C4/H4	C5/H5	C6/H6
→6)-β-Glc-(1→	104.2	74.4	76.9	71.4	76.3	70.3
	4.52	3.33	3.51	3.43	3.63	3.86/4.22
→3,6)-β-Glc-(1→	104.0	74.1	86.1	71.4	76.2	70.3
	4.55	3.52	3.75	3.43	3.68	3.86/4.22
β-Glc-(1→	104.2	74.7	77.1	70.7	76.2	62.0
	4.73	3.36	3.42	3.55	3.68	3.74/3.92
→3)-β-Glc-(1→	103.9	74.4	86.1	69.4	76.2	62.0
	4.77	3.54	3.75	3.57	3.68	3.74/3.92
β-4,5-en-UroA-(1→	100.8	78.7	67.6	107.4	144.7	169.8
	5.44	3.94	4.26	5.89	-	-

5.7.4   Comparison of the structures with other fungi

Table 24 reports the identified anomeric signals of the different polysaccharides extracted from *C. tubaeformis*. Monomer composition analysis, ATR-FT-IR and NMR spectroscopies confirmed that the polysaccharides extracted with hot water were mainly α-heterosaccharides. The elucidation of the anomeric protons of Crat HW1 indicated that such polymers were mainly →6)-α-Man-(1→ and →6)-α-Gal-(1→. The α-mannan was branched in the C2 position, possibly to another mannan chain, while the α-galactan was branched in the C2 position with single L-fucose. These two polymers have been already reported as cell wall components extractable with hot water, for example the fucogalactan from *A. bisporus*[119] and *Coprinus comatus*[328] and the mannan from *C. cibarius*[112]. →6)-α-Man-(1→ chains have been extracted from ascomycetes such as *Kluyveromyces marxianus*[326] and *Aspergillus fumigatus*[331]. The monomer

composition analysis indicated a relevant amount of xylose among the monomers of Crat HW1. However, NMR spectra lacked in clear xylose signals.

NMR spectroscopy confirmed the results obtained with gas chromatography and infrared spectroscopy, i.e. that the main component of the alkali fractions was a β-glucan. The ^{1}H and ^{13}C chemical shifts of its units are reported in **Table 23**. A glucan with→3)-β-Glc-(1→ backbone and→6)-β-Glc-(1→ branches have been isolated from *Pleurotus pulmonarius* with KOH 2%[273]. Such β-glucan has been isolated also from *F. velutipes*, using KOH 2% as well[203]. Differently from these mushrooms, alkali solutions extracted a →6)-β-Glc-(1→ backbone with branches in the C-3 position from *C. tubaeformis*. Such branches consisted of single β-Glc-(1→ units or short →3)-β-Glc-(1→ chains. The spectra of Crat 25%1 highlighted the β-4,5-enuronic acid sparsely constituting the terminal unit of the side chain.

Table 24. Anomeric ^{13}C and ^{1}H chemical shifts (ppm) assignments of polysaccharides isolated from *C. tubaeformis*.

H1/C1 assignment	^{13}C δ (ppm)	^{1}H δ (ppm)	NOESY (ppm)	HMBC (ppm)
→6)-β-Glc-(1→	104.2	4.52	4.52/4.22	4.52/70.3
→3,6)-β-Glc-(1→	104.0	4.55	4.55/4.22	4.55/70.3
β-Glc-(1→	104.2	4.73	4.73/3.75	4.73/86.1
→3)-β-Glc-(1→	103.9	4.77	4.77/3.75	4.77/86.1
→6)-α-Glc-(1→	98.4	4.95	4.95/3.85	4.95/66.8
α-Glc-(1→	98.8	4.98	4.98/3.85	4.98/67.0
→6)-α-Man-(1→	98.0	5.04	5.04/3.89	5.04/69.3
→6)-α-Gal-(1→				
α-L-Fuc-(1→	103.8	5.091	5.091/4.19	5.091/78.9
→6)-α-Man-(1→	102.3	5.095	5.095/4.03	5.095/70.2
→2)-α-Man-(1→	102.7	5.13	5.13/4.14	5.13/78.8
→2,6)-α-Man-(1→	102.6	5.16	5.16/4.03	5.16/70.3
→2)-α-Gal-(1→	101.3	5.23	a	5.23/70.0[b]
→2)-α-Man-(1→	100.6	5.32	5.32/4.12	5.32/78.8
β-4,5-en-UroA-(1→	100.8	5.44	5.44/3.89	5.44/84.0

[a]assignment based on COSY (5.23/4.19 H1–H2); [b]weak signal

The β-glucan extracted from *C. tubaeformis* shared some similarities with the one extracted from *C. cibarius*[112], such as the short length of the side chain (1–2 residues). The β-glucan present in Crat 2%1 differed from the β-glucans extracted from *C. cibarius* and from the yeast basidiomycete *Malassezia restricta*[77] in the degree of branching and by the presence of an uronic acid residue. Nyman proposed a backbone/branching points ratio of 8:2 in the repeating unit, while Stalhberger reported a variable frequency of branching every 2–10 units. Our results suggest an average frequency of branching every 2 units. The differences in the ratios between →3)-β-Glc-(1→ and →6)-β-Glc-(1→ units of Crat 2%1 and Crat 25%1 were ascribed to the harsher extraction conditions of Crat 25%1, which reduced the branch length and broke down the backbone. The presence of β-4,5-enuronic was due to the alkali β-elimination reaction of a β-uronic acid unit.

5.8 Structure of polysaccharides from *I. obliquus* mycelium, Chaga, and birch heart rot (Publication IV)

### 5.8.1 *I.obliquus* cultivated mycelium
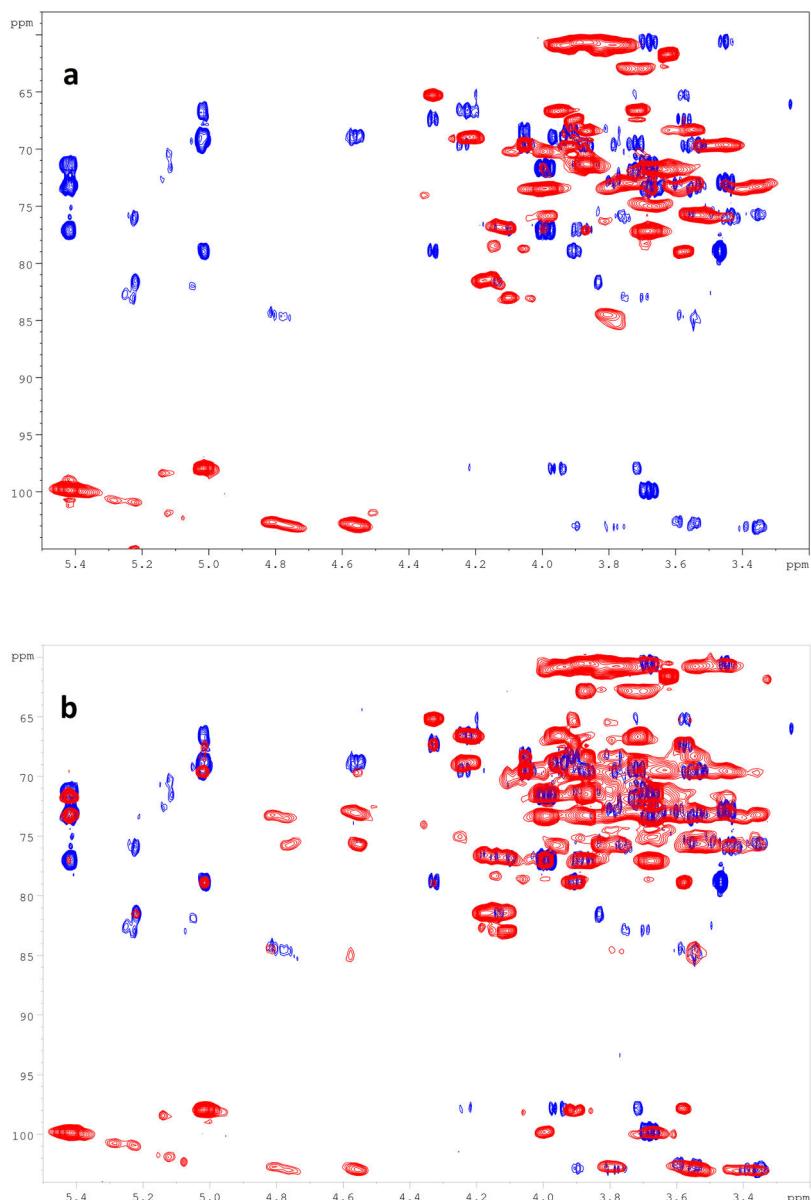
Figure 27. (a) HSQC (red) and HMBC (blue) spectra of IPSsb0-2.5 2%; (b) HSQC-TOCSY (red) and HMBC (blue) spectra of IPSsb0-2.5 2%. NMR spectra were recorded in D_2O at 308 K. See **Table 25** for signal assignments.

The polysaccharide fractions produced from mycelium of *I. obliquus* were investigated with NMR spectroscopy. Spectra of the fraction IPSsb0-2.5 HW were not recorded further than ^{1}H and HSQC, due to their high similarity with IPSsb0-2.5 2%. The HSQC and HMBC spectra of IPSsb0-0.5 2% are reported in **Figure 27a**. The structural assignments of IPS polysaccharides are reported in **Table 25**.

In the IPS spectra, the strongest anomeric proton signal was found at 5.42 ppm. The HSQC correlation of this proton was found at 99.8 ppm, suggesting a α-configuration. The TOCSY spectrum showed ^{1}H–^{1}H correlations between this anomeric proton and signals at 3.99 ppm and 3.87 ppm. In addition, the spectrum showed a wide correlation around 3.70 ppm, in which different signals were identified with the aid of HSQC and HMBC spectroscopy, and a correlation around 3.45 ppm. In particular, the HMBC correlation 3.68/99.8 ppm allowed the identification of H4 and subsequently of C4 (HSQC correlation 3.68/77.1). The HMBC spectrum showed a correlation 5.42/77.1 ppm cross-confirmed the assignment. Moreover, the HSQC spectrum showed the correlation of protons 3.99 ppm and 3.78 ppm with the broad carbon signal at 60.6 ppm, which was then assigned to C6. Overall, the signal at 5.42 ppm and the identified correlations were in good agreement with glycogen[332]. The predominance of glycogen among the other polysaccharides of IPS fractions could explain the higher intensity of the α-anomeric signal in the FT-IR spectra.

The second most intense anomeric signal of IPSsb0-0.5 2% ^{1}H spectrum resonated at 5.01 ppm. In the HSQC spectrum, it correlated with a carbon signal at 97.9 ppm, suggesting α-configuration. This proton was part of a spin system identified with the combination of TOCSY, 1D-TOCSY, and HSQC spectra. The correlations 4.33/65.3, 4.05/69.5, 3.91/67.3, 3.87/68.4, 3.58/78.9, 3.95/66.5, and 3.72/66.5 ppm were found. This anomeric signal was attributed to the overlapping of the H1 of →6)-α-Gal-(1→6) and →6)-α-3-*O*-Me-Gal-(1→6) units (**Table 25**). In particular, the correlations 4.33/65.3 and 3.58/78.9 were respectively assigned to H4/C4 and H3/C3 of 3-*O*-Me-Gal unit[124]. In addition, the HSQC spectrum showed the correlation 3.48/56.2, which was assigned to the methoxy group of 3-*O*-Me-Gal and further confirmed by the NOESY correlation 3.48/4.33 ppm (OMe/H4).

Two broad proton signals were found in the HSQC spectrum of IPSsb0-2.5 2%, between 4.85 ppm and 4.50 ppm, and they were further investigated. The HMBC correlations 4.81/84.5 (H1/O-substituted C3) and 4.56/69.7 (H1/O-substituted C6) visible in Figure 3a ascribed these broad proton signals to anomers of β-Glc-(1→3) and β-Glc-(1→6) linkages, respectively. The HSQC-TOCSY correlations of these signals are reported in **Figure 27b**. The two correlations 4.81/84.5 ppm and 4.57/85.0 ppm can be assigned to two different H1/O-substituted C3, respectively belonging to β-Glc-(1→3) and β-Glc-(1→6)

units. While the HSQC-TOCSY signal 4.56/69.7 ppm confirms the →6)-β-Glc-(1→6) unit, the correlation 4.76/69.7 ppm indicated the O-substitution of C6 of the β-Glc-(1→3) unit. The H6 and H6' signals of the units at 4.56 and 4.76 are assigned to 4.25 ppm and 3.89 ppm with the aid of HMBC (3.89/102.9, H6'/C1), HSQC (H6/C6 and H6'/C6), and 1D-TOCSY (4.56 ppm irradiation). The proton and carbon of position 2 of the β-Glc units were assigned using COSY H1/H2 correlations 4.56/3.35 ppm, 4.57/3.55 ppm, 4.76/3.40 ppm, and 4.81/3.59 ppm, which were cross-confirmed with C1/H2 correlations in the HMBC spectrum. The structural assignments are summarized in **Table 25**.

Table 25. ^{13}C and ^{1}H chemical shifts (ppm) assignments of polysaccharides isolated from mycelium from *I. obliquus*.

Type of linkage	C1/H1	C2/H2	C3/H3	C4/H4	C5/H5	C6/H6	O-CH₃
→6)-β-Glc-(1→6)	102.9	73.1	75.8	68.3	73.2	69.7	
	4.56	3.35	3.53	3.62	3.71	4.25/3.89	
→3)-β-Glc-(1→6)	102.7	72.9	85.0	68.2	71.3	60.6	
	4.57	3.55	3.77	3.54	3.67	3.94/3.76	
→6)-β-Glc-(1→3)	103.0	73.6	75.8	68.3	73.2	69.7	
	4.76	3.40	3.53	3.62	3.71	4.25/3.89	
→3)-β-Glc-(1→3)	102.7	73.2	84.5	68.2	71.3	60.6	
	4.81	3.59	3.81	3.54	3.67	3.94/3.76	
→6)-α-3-*O*-Me-Gal-(1→6)	97.9	67.3	78.9	65.3	68.4	66.5	56.2
	5.02	3.91	3.58	4.33	3.87	3.95/3.72	3.48
→6)-α-Gal-(1→6)	97.9	67.3	69.5	n.a.ᵃ	68.4	66.5	
	5.02	3.91	4.05		3.87	3.95/3.72	
→4)-α-Glc-(1→4)	99.8	69.7	73.2	77.1	71.5	60.6	
	5.42	3.45	3.99	3.67	3.87	3.94/3.76	

ᵃnot assigned

Terminal β-Glc-(1→3) and β-Glc-(1→6) would be expected to have the H1/C1 HSQC signals overlapping with the →6)-β-Glc-(1→3) and →6)-β-Glc-(1→6), respectively. Noticeably, the HMBC spectrum lacked clear signals ascribable to the →3,6)-β-Glc-(1→ branching point unit. The H1/O-substituted C3 correlation was observed only in the HSQC-TOCSY. However, only the →3,6)-β-Glc-(1→6) branching point can be clearly excluded. If the β-glucan contained →3,6)-β-Glc-(1→3) branching point units, they would be in little amount. Therefore, it could be inferred from the reported assignments that the β-glucan produced by *I. obliquus* is a mainly linear chain of β-Glc-(1→3) and β-Glc-(1→6) units. Our results were hence in agreement with the results obtained by Wold and coworkers on the polysaccharides extracted from Chaga[54]. The integration of the HSQC anomeric signals suggested a ratio between β-Glc-(1→3) and β-Glc-(1→6) units of 0.8:1. The ratio between glycogen anomeric signal and total β-Glc anomeric signals was 1.7:1.

## 5.8.2   Birch heart rot
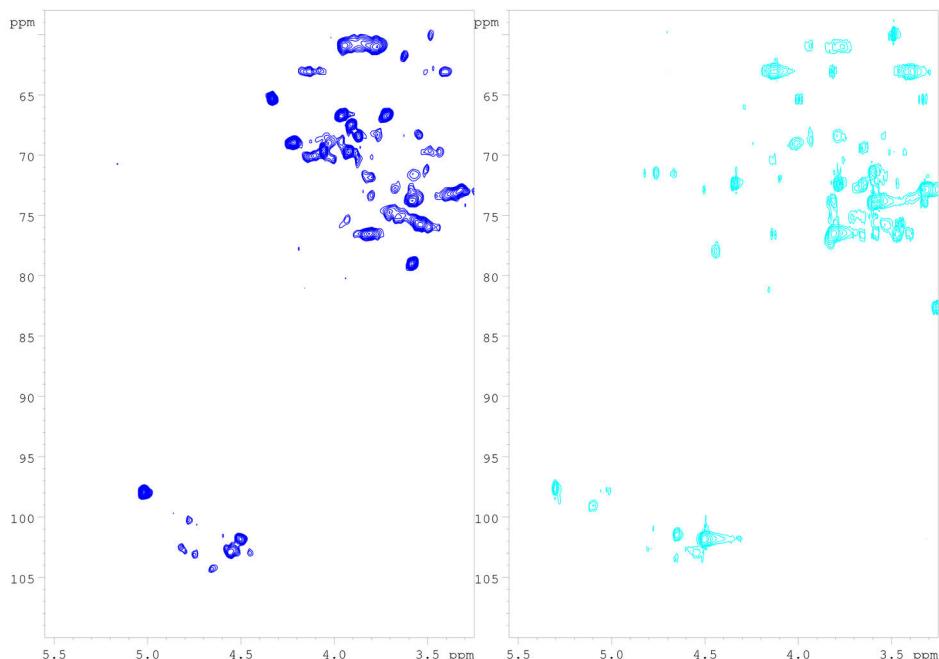
Figure 28. HSQC spectra of Heart Rot HW (blue) and Heart Rot 2% (light blue). NMR spectra were recorded in D_2O at 308 K. See **Table 26** for signal assignments.

The HSQC spectra of Heart Rot HW and Heart Rot 2% are reported in **Figure 28** and their structural assignments are summarized in **Table 26**. In the Heart Rot HW spectrum, the H/C correlation ascribable to anomeric proton of xylan units was found at 4.50/101.7, which was assigned to 4)-β-Xyl-(1→4) unit[333]. The assignment was also confirmed with a xylan standard. The HSQC spectrum of Heart Rot HW showed partially overlapping HQSC correlations around 4.50-4.60/103 ppm. Of these, the correlation 4.53/102.6 was assigned to →4)-β-Glc-(1→4) with the aid of the literature[334]. This glucose unit constitutes the backbone of the hemicellulose glucomannan, which has been extracted previously from birch[335]. Further confirmation of the presence of this polysaccharide in the fraction was provided by the anomeric signal 4.66/104.1, which was assigned to →4)-β-Man-(1→4)[335]. The presence of acetylated →4)-β-Man-(1→4) units was confirmed by the weak HSQC signals 5.56/72.6 ppm (O-acetylated C2) and 5.02/75.9 ppm (O-acetylated C3). The corresponding H1/C1 correlations were assigned with the aid of literature[335] and confirmed with a galactoglucomannan reference compound. The presence of acetyl group was supported by the HSQC correlation 2.19/20.6 ppm.

Differently from the hot water extract, the main anomeric signals found in the HSQC spectrum of Heart Rot 2% were ascribable to xylan. The HMBC spectrum of Heart Rot 2% showed the H1/C4 correlation 4.50/76.5, while the HSQC spectrum showed the H4/C4 correlation 3.81/76.5, confirming also the 4.50/101.7 correlation assignment. The correlations 4.65/101.4 ppm and 5.30/97.6 ppm were assigned to →4)-2-OR-β-Xyl-(1→4) and 4-*O*-Me-α-GlcA-(1→ units, respectively. The substitution in C2 of the xylan unit was mainly ascribable to the methylated glucuronic acid moiety. However, the presence of acetylated 4)-β-Xyl-(1→4) units was hinted by the HSQC signals 4.65/103.3 and 4.61/79.2 ppm, which were tentatively assigned to H1/C1 and H3/C3 of →4)-3-*O*-Ac-β-Xyl-(1→4) unit[333,336]. The low intensity of acetylated unit signals could be due to the hydrolytic effect of the alkali extraction medium. The anomeric signal 5.11/99.0 ppm was assigned to the α-reducing end of xylan[336]. The assignment of this unit to xyloglucan was excluded due to absence of α-Xyl-(1→6) H1/C6 correlation in Heart Rot 2% HMBC spectrum.

The superimposition of the collected HSQC spectra showed the presence of the mycelial β-glucan signals also in the birch heart rot fractions, particularly in Heart Rot HW. The superimposition of the anomeric regions of Heart Rot HW, IPSsb0-2.5 2% and standard galactoglucomannan is reported in **Figure 29**. Indicatively, based on the anomeric signals, the HSQC spectra showed a ratio between glucomannan and mycelial β-glucan of 1.2:1 in Heart Rot HW, while in Heart Rot 2% the ratio between xylan and β-glucan was 7.3:1. Moreover, in Heart Rot HW spectrum, galactose and glucose signals had a ratio close to one, which decreased to 0.2:1 for Heart Rot 2%. The observed trends were in partial agreement with the results of monomer quantification. The ratio between the mycelial glucan units was 0.7:1 ((1→3):(1→6)) in Heart Rot HW, therefore lower than IPSsb0-2.5 fractions. The signal of the β-Glc-(1→3) had too low intensity in Heart Rot 2% to measure a reliable ratio.
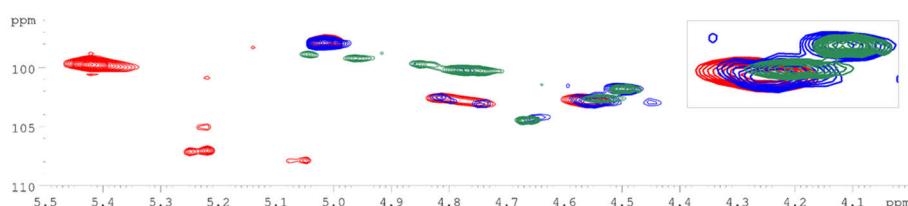

Figure 29. Anomeric region of the HSQC spectra of IPSsb0-2.5 HW (red), Heart Rot HW (blue), and galactoglucomannan standard (olive). NMR spectra were recorded in D_2O at 308 K. See **Tables 25** and **26** for signal assignment.

Table 26. ^{13}C and ^{1}H chemical shifts (ppm) for the polysaccharides extracted from birch heart rot caused by *I. obliquus*.

Fraction	Type of linkage[a]	C1/H1	C2/H2	C3/H3	C4/H4	C5/H5	C6/H6	O-CH₃
HW (2%)	→6)-α-3-*O*-Me-Gal-(1→6)	97.9	67.3	78.9	65.3	68.4	66.5	56.2
		5.02	3.91	3.58	4.33	3.87	3.95/3.72	3.48
HW	→4)-β-Glc-(1→4)	102.6	72.8	n.a.[b]	76.4	n.a.[b]	60.6	
		4.53	3.23		3.82		3.94/3.76	
HW	→4)-β-Man-(1→4)	104.1	70.1	n.a.$^{\$}$	76.4	n.a.[b]	60.6	
		4.66	4.13		3.82		3.94/3.76	
HW	→4)-3-*O*-R-β-Man-(1→4)	100.1	69.1	75.9	n.a.[b]	n.a.[b]	60.6	
		4.79	4.22	5.02			3.94/3.76	
HW	→4)-2-*O*-R-β-Man-(1→4)	99.7	72.6	n.a.[b]	n.a.[b]	n.a.[b]	60.6	
		4.87	5.56				3.94/3.76	
HW, 2%	→4)-β-Xyl-(1→4)	101.7	72.8	73.7	76.5	62.8		
		4.50	3.33	3.59	3.81	4.13/3.40		
2%	→4)-2-*O*-R-β-Xyl-(1→4)	101.4	72.8	n.a.[b]	76.1	62.8		
		4.65	4.50		3.83	4.13/3.40		
2%	→4)-3-*O*-R-β-Xyl-(1→4)[c]	103.3	n.a.[b]	79.2	n.a.[b]	62.8		
		4.65		4.61		4.13/3.40		
2%	→4)-α-Xyl	99.0	n.a.[b]	n.a.[b]	76.5	62.8		
		5.11			3.81	4.13/3.40		
2%	4-*O*-Me-α-GlcA-(1→	97.6	71.5	72.3	82.5	72.3		56.1
		5.30	3.60	3.79	3.26	4.35		3.47

[a]R indicates acetyl group (OAc 2.19/20.6 ppm). In the case of →4)-2-OR-β-Xyl-(1→4), it indicates 4-*O*-Me-α-GlcA-(1→2); [b]not assigned; [c]tentative assignment.

5.8.3   *I. obliquus* sterile conk
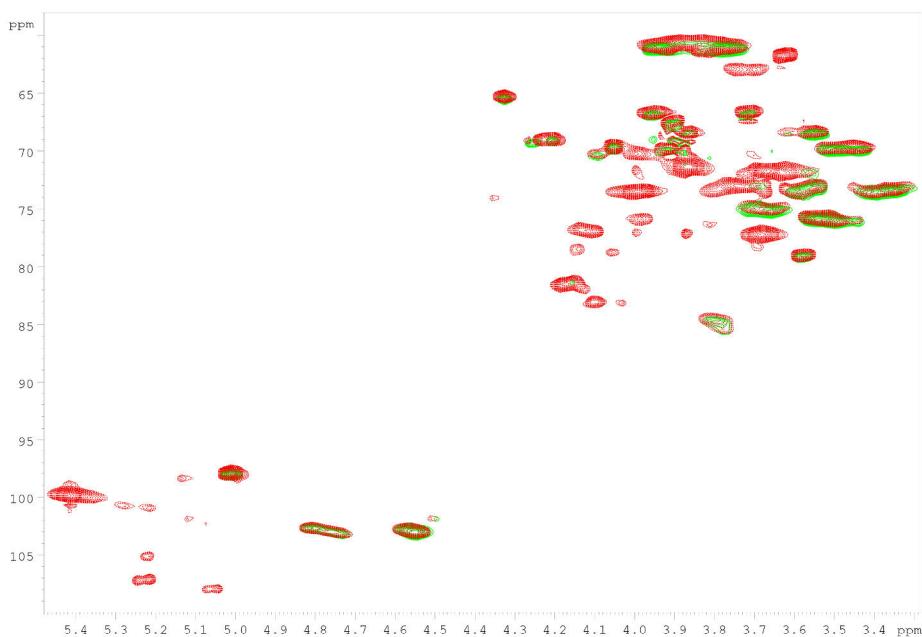
Figure 30. HSQC spectra of F-Chaga HW (green) and IPSsb0-2.5 2% (red). NMR spectra were recorded in D_2O at 308 K. See **Table 25** for signal assignments.

The polysaccharide fractions produced from mycelium of *I. obliquus* sterile conk were investigated with NMR spectroscopy. Spectra of the fraction C-Chaga HW were not recorded further than ^1H and HSQC, due to their high similarity with F-Chaga HW. Our results showed a complete overlapping in the HSQC spectra of the β-glucan signals of IPS and the anomeric signals of the F-Chaga fractions (**Figure 30**). The HSQC signal 5.02/97.9 ppm was present in both F-Chaga HW and C-Chaga HW. The preponderance of β-glucan signals in F-Chaga 2% was explained by the preponderance of glucose in the fraction (63% relative mol%). The HSQC spectra of Chaga fractions showed also the 4)-β-Xyl-(1→4) unit anomeric signal 4.50/101.7 ppm. Noticeably, no glycogen signal was observed in the Chaga extracts, despite the presence of hyphae in the sterile conk. This could be explained by the sclerotium development, during which mycelial glycogen is converted into soluble sugars[337]. The ratio between β-Glc-(1→3) and β-Glc-(1→6) units in Chaga extracts was 0.6:1, irrespective of starting material and extraction method, which was almost the same as the ratio reported by Wold (0.5:1)[54]. This ratio was lower than the ones observed in IPSsb0-2.5 2% (0.8:1) and in Heart Rot HW (0.7:1) HSQC spectra.

Heart Rot HW showed the presence of the anomeric signal of 3-*O*-methylated →6)-α-Gal-(1→6) unit. The signal was weak in the Heart Rot 2% HSQC spectrum. The identity between the 5.02/97.9 signals was confirmed by 1D-TOCSY (irradiation frequency of 5.02 ppm), as can be observed in **Figure 31**.
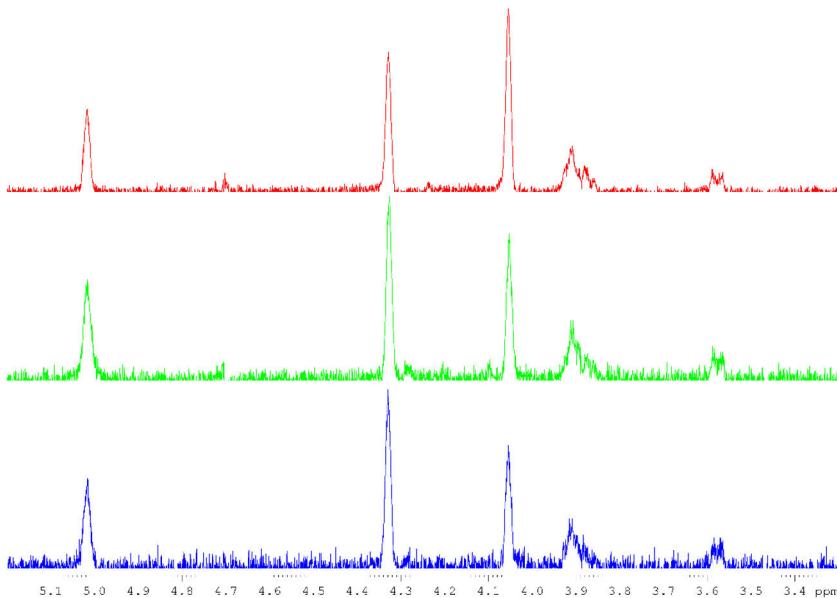

Figure 31. 1D-TOCSY of IPSsb0-2.5 HW (red), F-Chaga HW (green), and Heart Rot HW (blue). NMR spectra were recorded in D_2O at 308 K, with an irradiation frequency of 5.02 ppm. See **Tables 25** and **26** for signal assignments.

Anomeric signals in the sterile conk fractions HSQC spectra had too low intensities for any attempt of identification, except for F-Chaga HW. Signals ascribable to phenolic compounds were reported in **Figure 32**.

The broad signal 3.76/56.0 ppm (not shown) was ascribable to the methoxyl group of aromatic rings. The overlapping signal 3.93/56.3 ppm could be assigned to the methoxyl group of condensed aromatic units. It could be concluded that the phenolics present in Chaga extracts had a high degree of methoxylation. The assignments of the HSQC correlations observed in **Figure 32** were only tentative. While the signals (**Table 27**) could not unambigously determine the nature of the phenolic compounds covalently bound to the polysaccharides, they suggested the presence of hydroxypropyl units, which could be ascribed to lignin or lignans. Interestingly, this evidence was in agreement with the results of Wold and coworkers[315]. They reported a mixture of organic acids and phenolic and methoxyl-substituted compounds as degradation product of the Chaga melanin. The same mixture can be observed after lignin degradation[338]. Nevertheless, to

the best of our knowledge, the precise nature of the phenolic compounds covalently bound to Chaga polysaccharides is still unclear. Further purification and detailed structural analysis would be needed to determine whether melanin or lignin/lignans are covalently bound to Chaga polysaccharides.
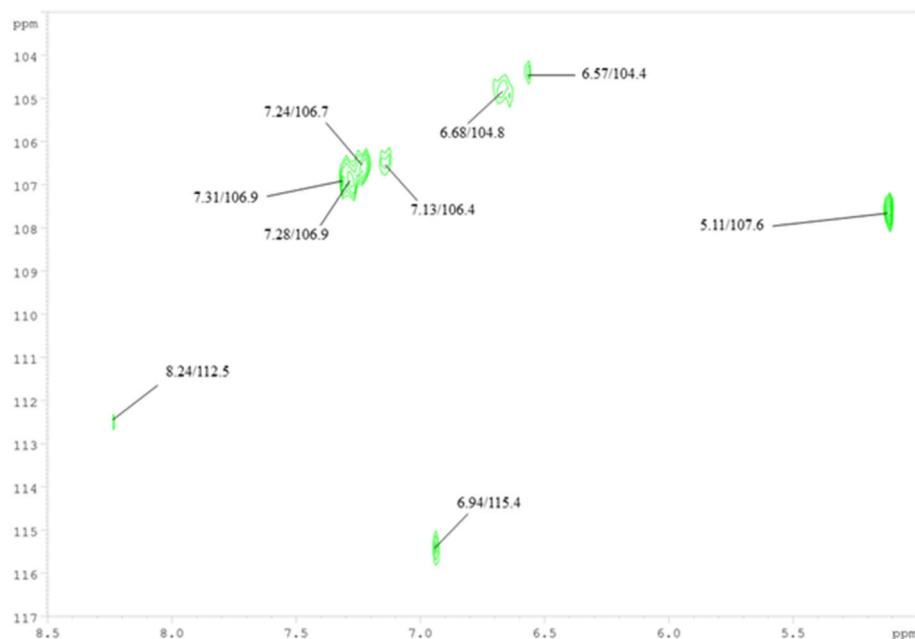
Figure 32. Aromatic region of the HSQC spectrum of F-Chaga HW. NMR spectra were recorded in D_2O at 308 K. See **Table 27** for signal assignments.

Table 27. Tentative ^{13}C and ^{1}H assignments of the lignin-correlated signals found in the HSQC spectrum of F-Chaga HW.

^{1}H	^{13}C	Assignment	Structure of Figure 33
3.76	56.0	OMe	1
3.93	56.3	OMe condensed aromatic units	2
3.69	60.7	Hγ/Cγ of β-O-4 substituted units	3
4.8	72.7	Hα/Cα of β-O-4 substituted units	4
6.57	104.4	H2/C2 of free 4-OH syringyl units	5
6.68	104.8	H2/C2 of syringyl units	6
7.31	106.9	H2/C2 of α oxidized syringyl units	7
5.11	107.6	H/C aryl enol ether	8
7.67	113.7	H2/C2 of α oxidized guaiacyl units	9
6.94	115.4	H2/C2 of guaiacyl units	10
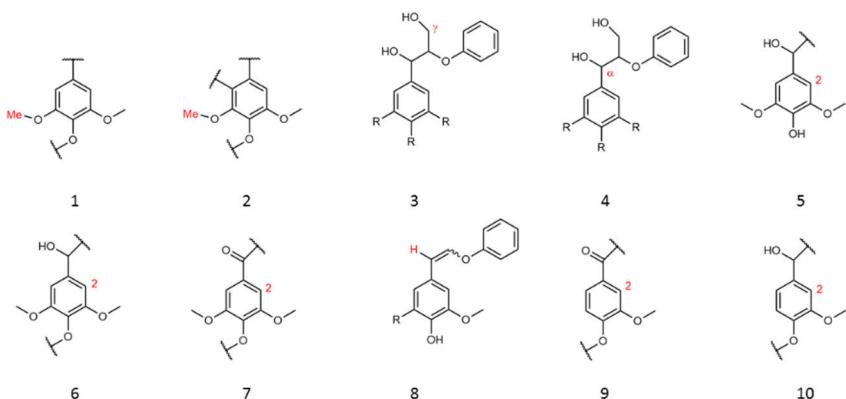
Figure 33. Tentative assignments of the lignin-correlated signals (marked in red) found in the HSQC spectrum of F-Chaga HW. See **Table 27**.

5.9 Effect of pretreatment on *A. bisporus* polysaccharide fractions (Publication I)

The utilization of EtOH-HCl as pretreatment had a noticeable effect on the glucan content of the polysaccharide fractions produced from *A. bisporus*. The rupturing of the cell wall by hydrolysis increased the content of the β-glucans in these fractions. The utilization of enzymatic kit revealed the presence of both α- and β-glucans. The earlier polymers are present in the mushroom cell wall as water-insoluble, linear →3)-α-Glc-(1→ polymers, and in the cell lumen as energy storage polymers (glycogen). The β-glucans have, on the other hand, mainly cell wall structural function. (1→3)-α-glucan can be extracted with alkali from fungi but they are unsoluble at neutral conditions[339]. Therefore, the AB and tAB fractions would contain mainly α-glucans extracted from the cytoplasm. The α- and β-glucan contents of the produced fractions were reported in **Table 18**. After AB1 extraction, a precipitate was formed during the cold storage. This precipitate was removed and labeled AB1p. This precipitate was rich in α-glucans (12% w/w) (see **Publication I** for details). Considering the higher α-glucan content of AB1p and AB2, compared to AB3, it can be inferred that α-glucans were depleted after the second hot water extraction (and not observed in AB1 due to early precipitation). Noticeably, there was no relevant precipitation of α-glucans after tAB1 extraction. This indicates that, with the disruption of the fungal structure, cytoplasm α-glucans were dissolved, possibly due to hydrolysis. Unfortunately, fraction tAB3 was produced in insufficient amount for the enzymatic measurement. However, the ATR-FT-IR investigation (**Figure 19**) hinted to a similar or only slightly higher α/β-ratio, compared to tAB2. Therefore, also after pretreatment the second fraction had the highest α-glucan content and fungal material was most likely depleted. It can be speculated that globally the

pretreatment resulted in loss of cytosolic α-glucans, in addition to increase in cell wall β-glucans.

Although the yield of tAB3 was low, it was noticeably the fraction richest in sugars (63% w/w). From the relationship between total glucan content measured with enzymatic kit and the total glucose content measured with GC-FID (**Figure 34**), it can be inferred that tAB3 would have the highest relative total glucan content (about 50% w/w). The trend observed in **Figure 34** was is agreement with the correlation reported in[76].

A polysaccharide fraction produced by Kozarski from *A. bisporus* with hot water had a total sugar content of 74.4% and β- and α-glucan contents of 58.2% and 5.6%, respectively[340]. These amounts were not obtained in the present work with hot water alone. The comparison with our results indicates that dialysis and/or other purification steps would increase the content of β-glucans. At the same time, the utilization of EtOH-HCl pretreatment would bring the sugar content of tAB fractions, if pooled, to 28%, which would be almost completely glucans, with a β-glucan content of 22% (enzymatic kit), with only ethanol precipitation as purification step.
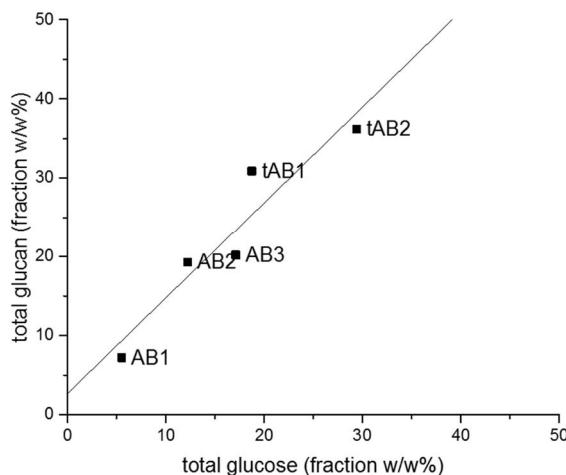
Figure 34. Correlation between the total glucan content of AB fractions (measured with enzymatic kit) and the total glucose content (measured with GC-FID).

Sari and coworkers have utilized the enzymatic kit to measure the glucan content of commercially cultivated *A. bisporus*. They reported for the mushroom caps a total glucan content of 10.05 g/100g and α- and β-glucan contents of 1.55 g/100g and 8.61 g/100g, respectively[341]. They also point out that β-glucans constitute the 85.64% of the total glucans of *A. bisporus*. The results of the

comparison of total AB and tAB fractions with these values is reported in **Figure 35**. The tAB3 fraction was not taken into account, due to absence of enzymatic kit data. Noticeably, after the pretreatment almost 50% of the total glucans of *A. bisporus* were extracted, against the 10% of untreated mushroom. Interestingly, almost all α-glucans (80%) were extracted after pretreatment. It could be speculated that even more was extracted but was also hydrolyzed. The efficiency of the pretreatment for the extraction of β-glucans is clearly visible, with 42% of *A. bisporus* extracted with two extractions, against the 7% of three.
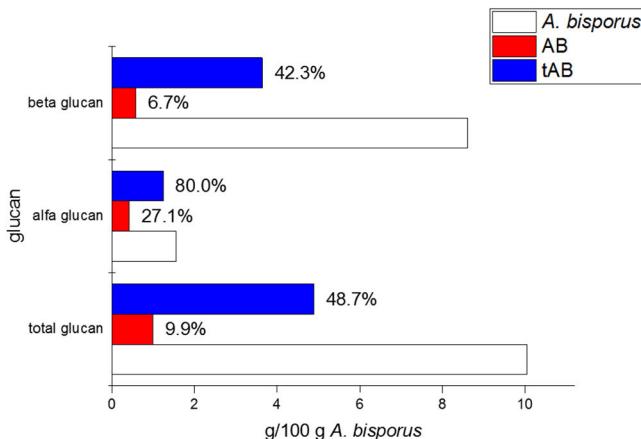
Figure 35. Comparison of glucan contents (g/100 g *A. bisporus*) of pooled AB and tAB fractions with the glucan contents of the whole fruiting body cap (data taken from[341]). On the right side of the bar, percentage of extracted glucan relative to whole cap.

5.10   Effect of cultivation conditions on polysaccharides extracted from *I. obliquus* mycelium (Publications III & IV)

In this dissertation, mycelia have been pooled according to the supplement dosage and IPS have been extracted and their content measured gravimetrically. Traditionally, IPS are extracted with hot water. Mycelium cultivated without sea buckthorn press cake, pool of 0 and 2.5 g/L dosages, and pool of 5 and 10 g/L dosages had hot water extraction yields of 0.9%, 2.2%, and 1.1%, respectively. It can be immediately noticed that sea buckthorn had, at lower concentrations, a positive effect on the yield. The polysaccharide concentration in the mycelium (mg/g) were respectively 7.7, 17.2, and 8.2. Except for IPSsb0-2.5 HW, these values were lower than reported literature values, obtained without supplement, for *I. obliquus* (32 mg/g)[270] and *G. frondosa* (oscillating around 77 mg/g)[291],

while they were more similar to *Antrodia cinnamomea* (5-7 mg/g)[290]. Remarkably, the used IPS extraction protocols differed significantly from our work (i.e., in the dialysis step). When soybean oil was supplemented, in the case of *A. cinnamomea*, two different cultivation times were tested (240 h and 336 h). With the lower, the supplementation followed the same trend observed with supplementation *I. obliquus* (oleic acid in the reference work and in the present dissertation), meaning an increase followed by a decrease. With longer cultivation time, the production trend of *A. cinnamomea* has been of a constant increase[270,290].

In the present work, the IPS contents after single cultivation conditions were not investigated. Some conclusions could be drawn from the results of the mycelium methanolysis. It can be expected that monomers analyzed during methanolysis represented the sum of free sugars and hydrolysable polysaccharides. To the best of our knowledge, there is no analysis of the free sugars of *I. obliquus* mycelium available in the literature. Ulzijargal reported a reducing sugar content of 26.2 w/w% for *I. obliquus* mycelium, which translated in the 40.0 w/w% of the total carbohydrate content reported for this species[24]. There is no knowledge on the influence of different cultivation conditions on the reducing sugar content, although there is evidence of their influence on fruiting body carbohydrate composition[238,342]. The major reducing sugar would be glucose, as mannose and galactose represent monomers of cell wall polysaccharides[127] and they are absent from reports on free sugars in mushrooms or mycelium[19,343]. **Figure 36** reports, for each cultivation condition, the hydrolysis yield of *I. obliquus* mycelium and the relative percentage of monomers different from glucose (dominated by mannose and galactose). Our data showed no clear correlation between hydrolysis yield and supplement dosage (ρ = -0.33, p > 0.05) nor with cultivation time (ρ = -0.19, p > 0.05). An effect of sea buckthorn press cake could be hinted by the negative correlation between hydrolysis yield and rhamnose (ρ = -0.52, p = 0.02) (**Table 14**). Cultivation conditions had an even less clear influence on the relative amounts of monomers different from glucose. Nevertheless, if the supplement dosage is converted in sea buckthorn oil dosage (using the sea buckthorn oil density value reported in[344]), the trends in total amount of monomers obtained with methanolysis reveal some similarity with the IPS production trends commented above (**Figure 37**). In particular, the decrease followed by increase at lower dosages was observed also with *I. obliquus* supplemented with oleic acid and with *G. frondosa* supplemented with soybean oil. Interestingly, oleic acid accounts for only about 18% of all the fatty acids of soybean and sea buckthorn press cake oils[345,346].

Most of the research on mycelial IPS has focused on the effects of supplements and cultivation conditions on their content or on structural

elucidation of polysaccharides extracted from large pools of mycelium. Therefore, there is limited data available on the effect of supplementation on the monomer composition of mycelium IPS.
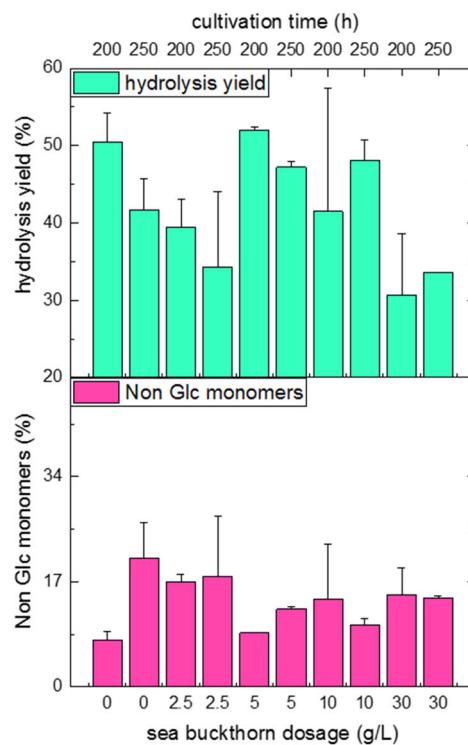

Figure 36. Mycelium methanolysis yield (w/w %) (above) and relative amount of monomers different from glucose on the total hydrolysate monomers (w/w %) (below). Results are distinguished by supplement dosage (lower x-axis) and cultivation time (upper x-axis).
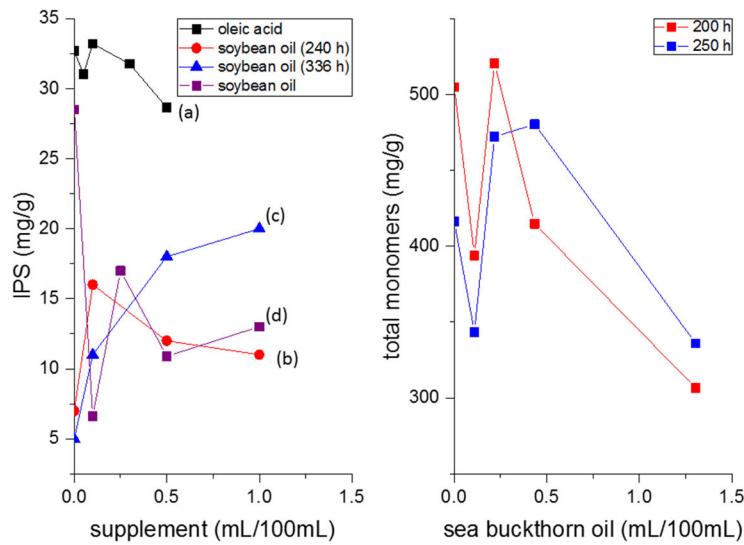
Figure 37. Comparison between IPS content of mycelia of different species cultivated with different concentrations of supplement (left) and total monomers obtained after methanolysis of cultivated mycelium of *I. obliquus* (right). The supplement dosage is reported as medium concentration of sea buckthorn oil. Data for (a) is obtained from[270], data for (b) and (c) is obtained from[290], and data for (d) is obtained from[267].

5.11 Comparison of polysaccharides from *A. bisporus*, *I. obliquus*, and *C. tubaeformis*

Polysaccharide fractions have been produced from cultivated *A. bisporus*, wild *C. tubaeformis*, wild sterile conk of *I. obliquus*, and cultivated mycelium of *I. obliquus*. **Table 28** summarizes the total polysaccharide yields and total glucan yields obtained from the different starting materials.

Table 28. Total polysaccharide and total glucan yields obtained from *A. bisporus*, *I. obliquus*, and *C. tubaeformis*.

Species	Fraction	g final polysaccharide /100 g starting material	g final glucan /100 g starting material
A. bisporus	AB[a]	1.24	0.71
	tAB[a]	4.66	3.50
C. tubaeformis	Crat HW	0.36	0.10 (0.06[b])
	Crat 2%	2.74	1.30 (1.27[b])
	Crat 25%	2.05	1.13 (1.04[b])
I. obliquus (conk)	F-Chaga HW	0.31	0.12
	F-Chaga 2%	2.93	1.93
	C-Chaga HW	0.22	0.09
I. obliquus (birch heart rot)	Heart Rot HW	0.42	0.08
	Heart Rot 2%	1.55	0.11
I. obliquus (mycelium)	IPSsb0 HW	0.77	0.51
	IPSsb0-2.5 HW	1.72	1.10
	IPSsb0-2.5 2%	3.44	1.92
	IPSsb5-10 HW	0.79	0.51
	IPSsb5-10 2%	0.50	0.25
	EPSsb0	0.39	0.15
	EPSsb0-2.5[c]	0.80	0.28
	EPSsb5-10[c]	1.03	0.28

[a]assuming AB fractions and tAB fractions are pooled together; [b]after DEAE chromatography purification; [c]assuming the EPS are pooled together in the same manner as the mycelium for IPS extraction.

5.11.1 Comparison of polysaccharide and glucan yields and properties from *A. bisporus* and *C. tubaeformis*

The comparison between the two fruiting bodies indicated *A. bisporus* was a more efficient starting material for the extraction of polysaccharides and of glucans, compared to *C. tubaeformis*. The hot water extraction of *C. tubaeformis* produced polysaccharides rich in mannose, galactose, and xylose. In contrast, three hot water extractions of *A. bisporus* produced mainly glucans (average

glucose content of 59% against 12% in Crat HW1, **Table 16**). Nevertheless, the polymers extracted from *A. bisporus* were mainly (by percentage of total chromatogram area) of molecular weight (M_p) lower than the polysaccharides extracted from *C. tubaeformis* (**Table 17**). It could be hypothesized that an eventual dialysis step during the purification of the AB fractions could have produced results more in agreement with the properties of Crat HW1.

The disruption of the cell wall with the utilization of acidic ethanol and alkali had a noticeable effect on the polysaccharide yield. The acidic ethanol treatment increased the combined yield of polysaccharides to 276% in the subsequent hot water extractions. Moreover, the use of three sequential KOH 2% extractions produced a polysaccharide yield increase of 661%, compared to the previous hot water extraction step. The polysaccharides extracted after treatment and with alkali extraction were all mainly composed of glucose (average of 76% against 69% of Crat 2%1, **Table 16**). However, the amount of glucan produced from *A. bisporus* was 2.7 times the amount produced from *C. tubaeformis* (**Table 28**). Nevertheless, the utilization of alkali produced a polysaccharide fraction with a population of about 5×10^2 kDa (60% of total area), which was not observed in tAB fractions. Moreover, the polysaccharide populations of higher M_p constituted 50% or less of total chromatogram area and had Mp below 2.5×10^2 kDa.

The results of the enzymatic kit analysis (**Table 18**) indicated β-glucans were about three times more abundant than α-glucans in tAB1 and tAB2 fractions. Enzymatic kit analysis was not performed for Crat HW1 and Crat 2%1. However, the comparison of the FT-IR spectra of Crat 2%1 and tAB1 indicated a clearer β-glucan signal around 890 cm^{-1} in the Crat 2%1 spectrum, compared to tAB1. Moreover, the ^{1}H NMR spectrum of Crat 2%1 showed an integrated signal ratio between the α-glucan (4.95 and 4.98 ppm) and β-glucan (4.52, 4.55, 4.73, and 4.77 ppm) signals in Crat 2%1 of 1:17. Therefore, the ratio between β- and α-glucans would be higher in Crat2%1 than that in tAB.

The ethanol-acid pretreatment was performed on *A. bisporus* with an ethanol−hydrocholic acid 37% 20:1 mixture for 1 h at 75 ºC and with a consistency of about 10%. After neutralization, the pretreatment was followed by three hot water extractions lasting 6 h. The mild alkali extraction of *C. tubaeformis* was performed with a KOH 2% solution for 3 h at 80 ºC and with a consistency of about 17%. The extraction was performed three times. The mild alkali extraction solubilized directly the polysaccharides, which were obtained after neutralization. This would be an advantage, compared to the pretreatment. In the present doctoral dissertation, the two methods were not applied to the same fungal species. Therefore, it would not be possible to conclude with method would be the best for increasing the polysaccharide yield. However, it can be noticed that the tAB pool would be obtained after a total extraction time of 19 h,

including pretreatment, while Crat 2% was obtained after a total extraction time of 9 h. These extraction times translated in polysaccharide productions of 0.25 g/h for *A. bisporus* and 0.30 g/h for *C. tubaeformis*. On the other hand, the glucan production was 0.18 g/h for *A. bisporus* and 0.14 g/h for *C. tubaeformis*. The application of the ethanol-hydrochoric acid pretreatment to *C. tubaeformis* and other fungal species, in particular to fruiting bodies with stiff structure such as Polyporales, would be required to assess the industrial potential of this protocol.

From the data presented so far, it would not be possible to conclude which fruiting body studied in the present doctoral dissertation would be the best starting material for the production of β-glucan fractions. A thorough screening of the biological activities of the fractions would provide important information guiding the selection of both starting materials and extraction methods. The availability of the starting material should also be taken into consideration.

5.11.2 Comparison of yields and properties of polysaccharides and glucans from sterile conk and mycelium of *I. obliquus*

The sterile conks of *I. obliquus* originating from Finnish and Chinese forests had polysaccharide yields, after hot water extraction, of 0.31 and 0.22 g/100 g starting material. The difference in yield after hot water extraction could be due to the different protocols used, rather than geographical origin. Only Finnish Chaga was extracted with alkali and the yield increased from 0.31 to 2.93 g/100 g (**Table 28**). Compared to Finnish Chaga, the birch heart rot caused by *I. obliquus* had higher polysaccharide yield after hot water extraction (0.42 g/100g) but lower yield after alkali extraction (1.55 g/100g). Noticeably, the glucan yields after hot water extraction of sterile conks and birch heart rot resulted similar (0.12, 0.9, and 0.8 g/100g, respectively). The use of alkali had no remarkable effect on the glucan yield from heart rot (0.11 g/100 g). On the contrary, the increase of sterile conk glucan yield after alkali extraction was ten-fold (**Table 28**).

The yields of hot water and alkali extractions reported in **Table 28** showed that the cultivated mycelium was, in general, a better source of polysaccharides, compared to the sterile conk. Without supplementation, the polysaccharide yield after mycelium hot water extraction increased of 148%, while the glucan yield quadruplicated. The use of sea buckthorn press cake as supplement (control and 2.5 g/L dosage pool) increased the glucan content, compared to the hot water extract of mycelium cultivated without supplement, of 116% and 277% for, respectively, hot water and alkali extract. Noticeably, the utilization of alkali resulted almost in the same glucan yield from both sterile conk and cultivated mycelium. However, it is worth noticing that the use of KOH 2% solution increased the glucan yield more than 15 times, whereas the yield increase by 2% alkaline from cultivated mycelium was 76% from cultivated mycelium.

The amounts of polysaccharides collected from the cultivation medium of *I. obliquus* (produced by 100 g of mycelium) were higher than the amounts extracted from sterile conk and comparable to the amounts extracted from the mycelium, considering the hot water extracts. The amount of glucan extracted from cultivation medium without supplementation (EPSsb0) was 25% and 66% higher than F-Chaga HW and C-Chaga HW, respectively. On the other hand, the glucan amount was lower than IPS HW fractions. Noticeably, while the amount of polysaccharides was higher from the pools obtained after higher supplement dosages, the amount of glucan remained constant (0.28 g glucan /100 g mycelium, about double the amounts produced with hot water from sterile conk). As reported in Section 5.4, the experiments showed no significant influence of sea buckthorn press cake on the relative glucose content in the isolated EPS. On the other hand, there are reports in literature showing the influence of medium dosages on the EPS glucose. For example, a regression equation for EPS glucose amount in relation to medium pH, glucose, and peptone was elaborated for *G. lucidum*[304]. The experimental data reported by the authors shows clear differences in the glucan content of the produced EPS in relation to the medium pH and glucose.

If we do not take into account the pooling of the EPS, the trend of the glucan production was similar to the one reported in **Equations 3** and **4**. The extrapolation of the reported experimental data of Xu and coworkers[270] showed that when *I. obliquus* cultivation medium was supplemented with increasing concentrations of Tween 80, the amounts of EPS and glucan (g/100g of mycelium) increased with lowest dosage followed by a decrease, therefore in opposition with our results (**Figure 38**). It has been proposed that surfactants such as Tween 80 improve the mycelium cultivation yield due to a slow release of oleic acid[267] or due to changes in the permeability of the membrane and the increased secretion of enzymes[289].
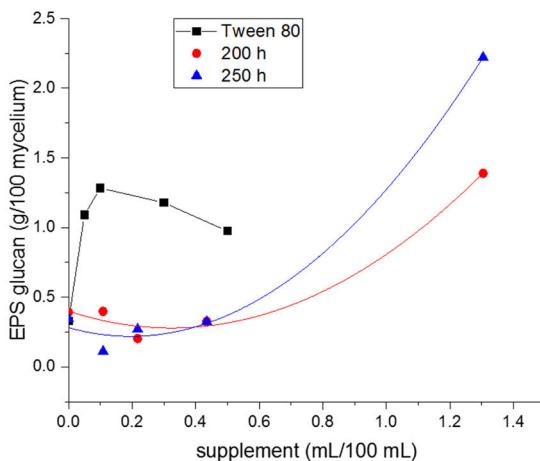
Figure 38. Comparison of EPS glucan (g/100 g of mycelium) produced by *I. obliquus* at different supplement dosage and EPS glucan obtained after supplementation of Tween 80 at different dosages. Data after Tween 80 supplementation was obtained from[270].

The utilization of NMR spectroscopy indicated that β-glucans in sterile conk and cultivated mycelium are structurally similar. However, the spectrophotometric and spectroscopic investigation of the *I. obliquus* fractions highlighted the high content of phenolic compounds in the sterile conk fractions, in particular in F-Chaga 2%. This fraction had a gallic acid equivalent content of 0.69 g/100 g starting material, indicating a ratio between glucans and phenolics of 2.8:1 (w/w). No phenolic compounds were detected in the cultivated mycelium extracts. Therefore, while considering the utilization of cultivated mycelium as replacement of the sterile conk for the production of polysaccharide fractions, not only the consumer acceptance of the mycelium extract should be taken into account (issue raised by Money in[347]), but also the impact of the presence of covalent-bound phenolics on the biological activities of the polysaccharides.

Differently from the sterile conk, the structural investigation of the mycelial glucans indicated a high amount of glycogen in the extracts. As mentioned in Section 5.8.1, the ^{1}H spectrum of IPSsb0-2.5 2% showed an integration ratio between the glycogen signal and the β-glucan signals of 1.7:1. It could be speculated that the glycogen is the main polymer of the population of higher M_p, given the molecular weight values of the mycelial glycogen reported in literature[348,349]. Moreover, the ratios between the two populations in the hot water extracts were 1.37:1 (Populations 2 and 3 combined) and 1.22:1, for IPSsb0 and IPSsb0-2.5 mycelia respectively. The differences between the population ratios

measured with HPSEC and the NMR data could hint to the presence of →4)-α-Glc-(1→4) units also in the populations of lower molecular weight. Glycogen signals have been found in polysaccharide population of lower molecular weight produced from *P. eryngii*[350].
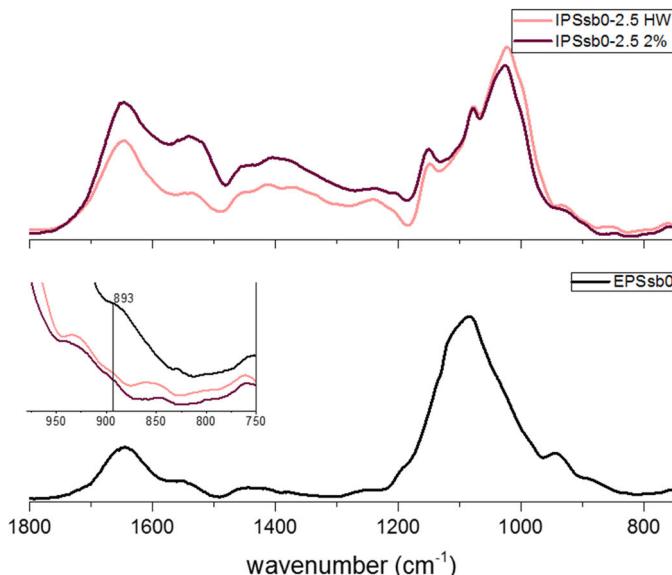

Figure 39. Comparison between the ATR-FT-IR spectra of IPSsb0-2.5 polysaccharides (top) with EPSsb0 (EPS obtained without supplement) (bottom). An expansion of the anomeri region (950–750 cm^{-1}) is reported, with indication of the β-anomeric vibration.

In the present doctoral dissertation, no structural investigation of the produced EPS was performed. However, a comparison of the FT-IR spectra of the EPS collected from medium not supplemented with sea buckthorn press cake (EPSsb0) and IPSsb0-2.5 fractions is reported in **Figure 39**. The intensity of the C−O and C−O−C bond vibrations differed noticeably, with the most intense of EPSsb0 being the 1084 cm^{-1} vibration, in opposition to the 1020 cm^{-1} vibration for IPS fractions. The EPS spectrum showed the presence of protein amide (1644 and 1542 cm^{-1}) and carboxyl group (1708 cm^{-1}) vibrations. Most importantly, the expansion of the anomeric region highlights the higher intensity of the β-anomeric signal (893 cm^{-1}) of EPSsb0, compared to the IPS fractions. The ^{1}H spectrum of *I. obliquus* EPS isolated by Xue and coworkers[351] showed both α- and β-linkage signals, with higher intensity for the latter.

The α-glucan polymers would not exert the same biological role of the mushroom β-glucans in the organism, since they are susceptible to enzymatic digestion by amylase. However, there are reports in literature on the biological

activities *in vitro* and *in vivo* of glycogen-like polymers extracted from fungi. A branched →4)-α-Glc-(1→4) polymer, covalently bound to protein, extracted from the mycelium of *Tricholoma matsutake*, has showed immunomodulatory properties *in vivo*[352]. A polysaccharide fraction rich in →4)-α-Glc-(1→4) units, extracted from *A. bisporus* fruiting body, stimulated NO production of macrophages in cell lines experiments[353]. A glycogen-like polysaccharide extracted from the mycelium of the ascomycote *Pseudallescheria boydii* stimulated the production of cytokines from macrophages[354]. On the other hand, no clear effect on the human immune system has been observed *in vivo* during a human study where α-glucan was administrated[355]. Moreover, the comparison of polysaccharide fractions produced from *A. bisporus* and *Agaricus brasiliensis* performed by Smiderle and coworkers led the authors to conclude that the presence of α-glucan in the studied extracts interfered with the dose-dependent bioactivity of the extracts[356]. Therefore, further experiments and the purification of the different polymer populations in the cultivated mycelium extracts would be required to quantify the amount of this polymer and verify its influence on the biological activity of the extracts.

6   SUMMARY AND CONCLUSION

The experiments described in the present dissertation investigated the effects of extraction methods on the properties of fungal polysaccharides obtained from species with relevance for Finland.

In order to improve the yield of the hot water extraction in terms of polysaccharides, in particular β-glucans, the cultivated *A. bisporus* was treated with ethanol-hydrochloric acid. Polysaccharides were extracted with three sequential hot water extraction from treated and untreated mushroom powder and analyzed after ethanol precipitation. In both cases, the first extraction was the most significant in terms of yield. The treatment not only caused an increase of the total extraction yield but also granted higher yield than the total yield from untreated mushroom with only the first hot water extraction. Glucose was the main monomer of all the polysaccharide fractions produced. The use of pretreatment caused a disruption of the cell wall network and increased the content in β-glucans of the extracts. The applied treatment could therefore be of industrial interest. On the other hand, a clear hydrolytic effect of the pretreatment was observed, as the molecular weight of the polysaccharides decreased and large polymer populations drastically reduced.

The cell wall polysaccharides of the wild and underutilized *C. tubaeformis* were extracted utilizing sequentially hot water, KOH 2%, and KOH 25% and characterized. The polysaccharides extracted with hot water were mainly composed of polymers with →6)-α-Man-(1→6) and →6)-α-Gal-(1→6) units, with a higher abundancy of the earlier. These polymers were covalently bound to proteins. The polymers extracted with alkali were constituted mainly by a →6)-β-Glc-(1→6) glucan with branches in O-3 position of single β-Glc or β-Glc-(1→3)-β-Glc-(1→3) dimers. As revealed by spectroscopical studies, the polymer was seldomly branched also with a β-uronic acid unit. For the utilization of *C. tubaeformis* as a source of β-glucans, hot water is an unefficient extraction medium. The utilization of alkali caused a reduction of branch and backbone length, as observed with methylation. Size-exclusion chromatography showed a bimodal distribution of the KOH 2% polysaccharides, while the fraction produced with KOH 25% had only low molecular weight polymers. The TGA study of the produced fractions indicated that only the use of KOH 25% caused a drastic decrease of the thermal stability of the polysaccharides extracted from *C. tubaeformis*.

In the present dissertation, sea buckthorn press cake, a sidestream from juice pressing rich in fibers and oil, has been investigated for the first time as supplement for the cultivation in liquid medium of the rare basiomycete *I. obliquus*. The mycelium yield of the submerged cultivation of *I. obliquus* can be significantly increased with the supplementation of sea buckthorn press cake.

Methanolysis of the mycelium highlighted little retention of pectin after cultivation. The amount of press cake influenced, with a trend fitting a second-order polynome, the production of EPS but reduced the production of IPS, which were obtained with sequential hot water and KOH 2% extraction. The EPS contained relative amounts of pectic monomers (arabinose and rhamnose, but not galacturonic acid) in proportion to the supplement. However, the molecular weight of the main polymer population was only slightly increased after supplementation. The IPS, on the other hand, had a little retention of press cake pectin, as suggested by the increase in their galacturonic acid content. The molecular weight profile was influenced by the use of alkali as extracting solvent. On the other hand, at higher dosages of supplement, the size-exclusion chromatogram showed a reduction of the main polymer population observed after lower dosages and the appearance of a population of lower molecular weight.

The spectroscopical investigation of the polysaccharides extracted from the mycelium and the sterile conk of *I. obliquus* showed that the same β-glucan was extracted from both starting materials. Fractions produced from sterile conk were rich in phenolic compounds. While this was not the case of IPS, their investigation showed that glycogen was more abundant than the β-glucan. IPS and sterile conk polysaccharides also differed in the polymer population profiles, since the latter were monodispersed. Galactan was identified as a polymer present in both IPS and sterile conk polysaccharides.

In addition to liquid state cultivation of *I. obliquus* mycelium, this doctoral dissertation took into account also the sidestream of the obtainment of the sterile conk of this species. Here, the birch heart rot caused by *I. obliquus* has been considered as a potential source of polysaccharides for the first time. The fractions produced with hot water and KOH 2% from birch heart rot were mainly constituted by xylan. Nevertheless, the hot water extract contained also the same galactan and β-glucan found in IPS and sterile conk, while traces were seen in the alkali fraction with NMR spectroscopy.

The biological activities of fungal polysaccharides, which have attracted industrial attention, are influenced by their macromolecular properties. Therefore, it is important to outline how these properties are affected by different starting materials and extraction methods. The balance between desired properties and desired yield is of industrial interest. This doctoral thesis has provided new information on how different methods increasing polysaccharide yields affect their properties. This thesis has outlined the potential of a common and underutilized Finnish mushroom as starting material and provided new information on the utilization and improvement of cultivated mycelium of *I. obliquus* as replacement of its sterile conk.

This dissertation indicated also the presence of grey areas in the field of fungal polysaccharides. Compared to ascomycetes, the elaboration of a detailed model of the cell wall has received too little attention. Moreover, little importance has been attributed to the influence of growth stage and environmental conditions on the detailed structure of the cell wall components. The communication between these areas and the research efforts dedicated to isolation and identification of fungal polysaccharides seems inadequate. The nutraceutical market has strong need of standardization in terms of biological properties and, therefore, chemical and physico-chemical properties of the polysaccharide fractions produced from fungi. Detailing the influence of the starting material on these properties could fill this gap. Regarding the biological properties of fungal polysaccharides, prebiotic activity is probably the most recent area of study. Structure-prebiotic activity relationship studies focusing on purified fungal polysaccharides are still very few and inadequate in uniformity[357]. However, the whole field of fungal polysaccharides is lacking in *in vivo* studies, particularly in clinical trials. These are required for the development of safe and effective products. Stronger research efforts are also required for the utilization of cultivated mycelium as replacement of fruiting body as polysaccharide resource. Detailed studies on the chemical composition of mycelia are lacking. Also, there is too little research on the influence of cultivation conditions, particularly with the use of supplements, on the properties of mycelial polysaccharides. Despite the large breadth of the field of fungal polysaccharides, the amount of challenges and open questions is also vast.

ACKNOWLEDGEMENTS

REFERENCES

1. Cateni, F.; Gargano, M. L.; Procida, G.; Venturella, G.; Cirlincione, F.; Ferraro, V. Mycochemicals in wild and cultivated mushrooms: nutrition and health. *Phytochemistry Reviews*, **2021**, *2*.

2. FAOSTAT http://www.fao.org/faostat/en/#data/QC/visualize (accessed June 28, 2021).

3. Giavasis, I. Bioactive fungal polysaccharides as potential functional ingredients in food and nutraceuticals. *Current Opinion in Biotechnology*, **2014**, *26*, 162–173.

4. Wasser, S. P. Medicinal mushroom science: Current perspectives, advances, evidences, and challenges. *Biomedical Journal*, **2014**, *37* (6), 345–356.

5. Ruokavirasto. Marsi report 2019. https://www.ruokavirasto.fi/globalassets/viljelijat/tuet-ja-rahoitus/marsi-2019-raportti.pdf (accessed May 31, 2021).

6. Salo, P.; Niemelä, T.; Nummela-Salo, U.; Ohenoja, E. *Suomen Helttasienten Ja Tattien Ekologia, Levinneisyys Ja Uhanalaisuus*; Suomen ympäristökeskus, Helsinki, 2005.

7. Hibbett, D. S.; Binder, M.; Bischoff, J. F.; Blackwell, M.; Cannon, P. F.; Eriksson, O. E.; Huhndorf, S.; James, T.; Zhang, N.; et al. A higher-level phylogenetic classification of the Fungi. *Mycological Research*, **2007**, *111* (5), 509–547.

8. Kirk Paul; Cannon Paul; Minter D; Stalpers J (Editors). *Dictionary of the Fungi*, 10th edition; CABI, Oxon, 2008.

9. Chang, S.; Miles, P. Mushroom biology — A new discipline. *Mycologist*, **1992**, *6* (2), 64–65.

10. Hawksworth, D. L.; Lücking, R. Fungal Diversity Revisited: 2.2 to 3.8 Million Species. In *The Fungal Kingdom*; ASM Press, Washington, DC, USA, 2017; pp. 79–95.

11. Catalogue of Life - Fungi https://www.catalogueoflife.org/data/taxon/F.

12. Kües, U.; Navarro-González, M. How do Agaricomycetes shape their fruiting bodies? 1. Morphological aspects of development. *Fungal Biology Reviews*. Elsevier Ltd June 1, 2015, pp. 63–97.

13. de Mattos-Shipley, K. M. J.; Ford, K. L.; Alberti, F.; Banks, A. M.; Bailey, A. M.; Foster, G. D. The good, the bad and the tasty: The many roles of mushrooms. *Studies in Mycology*, **2016**, *85* (Litten 1975), 125–157.

14. McLaughlin, D. J.; Spatafora, J. W. *Systematics and Evolution: Part A: Second Edition*; 2014.

15. Boa, E. *Wild Edible Fungi a Global Overview of Their Use and Importance to People*; 2008.

16. Gründemann, C.; Reinhardt, J. K.; Lindequist, U. European medicinal mushrooms: Do they have potential for modern medicine? – An update. *Phytomedicine*, **2020**, *66* (August 2019), 153131.

17. Martial. Epigrams. https://www.tertullian.org/fathers/martial_epigrams_book03.htm (accessed May 28, 2021).

18. Yoo, Y. B.; Oh, M. J.; Oh, Y. L.; Shin, P. G.; Jang, K. Y.; Kong, W. S. Development trend of the mushroom industry. *Journal of Mushroom*, **2016**, *14* (4), 142–154.

19. Kalač, P. A review of chemical composition and nutritional value of wild-growing and cultivated mushrooms. *Journal of the Science of Food and Agriculture*, **2013**, *93* (2), 209–218.

20. Kalač, P. Chemical composition and nutritional value of European species of wild growing mushrooms: A review. *Food Chemistry*, **2009**, *113* (1), 9–16.

21. Koutrotsios, G.; Tagkouli, D.; Bekiaris, G.; Kaliora, A.; Tsiaka, T.; Tsiantas, K.; Chatzipavlidis, I.; Zoumpoulakis, P.; Kalogeropoulos, N.; Zervakis, G. I. Enhancing the nutritional and functional properties of *Pleurotus citrinopileatus* mushrooms through the exploitation of winery and olive mill wastes. *Food Chemistry*, **2022**, *370* (March 2021), 131022.

22. Mattila, P.; Salo-Väänänen, P.; Könkö, K.; Aro, H.; Jalava, T. Basic composition and amino acid contents of mushrooms cultivated in Finland. *Journal of Agricultural and Food Chemistry*, **2002**, *50* (22), 6419–6422.

23. Li, X.; Guo, Y.; Zhuang, Y.; Qin, Y.; Sun, L. Nonvolatile taste components, nutritional values, bioactive compounds and antioxidant activities of three wild Chanterelle mushrooms. *International Journal of Food Science and Technology*, **2018**, *53* (8), 1855–1864.

24. Ulziijargal, E.; Mau, J. Nutrient

Compositions of Culinary-Medicinal Mushroom Fruiting Bodies and Mycelia. *International Journal of Medicinal Mushrooms*, **2011**, *13* (4), 343–349.

25. Grangeia, C.; Heleno, S. A.; Barros, L.; Martins, A.; Ferreira, I. C. F. R. Effects of trophism on nutritional and nutraceutical potential of wild edible mushrooms. *Food Research International*, **2011**, *44* (4), 1029–1035.

26. Wasser, S. Medicinal mushrooms as a source of antitumor and immunomodulating polysaccharides. *Applied Microbiology and Biotechnology*, **2003**, *60* (3), 258–274.

27. Grienke, U.; Zöll, M.; Peintner, U.; Rollinger, J. M. European medicinal polypores - A modern view on traditional uses. *Journal of Ethnopharmacology*, **2014**, *154* (3), 564–583.

28. Saar, M. Fungi in khanty folk medicine. *Journal of Ethnopharmacology*, **1991**, *31* (2), 175–179.

29. Stadler, M.; Hoffmeister, D. Fungal natural products-the mushroom perspective. *Frontiers in Microbiology*, **2015**, *6* (FEB), 1–4.

30. Reis, F. S.; Martins, A.; Vasconcelos, M. H.; Morales, P.; Ferreira, I. C. F. R. Functional foods based on extracts or compounds derived from mushrooms. *Trends in Food Science and Technology*, **2017**, *66*, 48–62.

31. Royse, D. J.; Baars, J.; Tan, Q. Current Overview of Mushroom Production in the World. *Edible and Medicinal Mushrooms*, **2017**, *2010*, 5–13.

32. Härkönen, M. Uses of mushrooms by Finns and Karelians. *International journal of circumpolar health*, **1998**, *57* (1), 40–55.

33. Pikkarainen, E. Sienten ja marjojen käyttötavat Suomessa, Italiassa ja Saksassa, University of Helsinki, 2006.

34. Collado, E.; Bonet, J. A.; Camarero, J. J.; Egli, S.; Peter, M.; Salo, K.; Martínez-Peña, F.; Ohenoja, E.; Martín-Pinto, P.; Primicia, I.; Büntgen, U.; Kurttila, M.; Oria-de-Rueda, J. A.; Martínez-de-Aragón, J.; Miina, J.; de-Miguel, S. Mushroom productivity trends in relation to tree growth and climate across different European forest biomes. *Science of the Total Environment*, **2019**, *689* (June), 602–615.

35. Tahvanainen, V.; Miina, J.; Kurttila, M.;

Salo, K. Modelling the yields of marketed mushrooms in *Picea abies* stands in eastern Finland. *Forest Ecology and Management*, **2016**, *362*, 79–88.

36. Ohenoja, E.; Koistinen, R. Fruit body production of larger fungi in Finland. 2. Edible fungi in northern Finland 1976-78. *Annales Botanici Fennici*, **1984**, *21* (4), 357–366.

37. Rantala, M.; Salmi, L.; Sarkapalo, T.; Visala, L. Utilization of mushrooms in Pirkanmaa. *Karstenia*, **1978**, *18*, 112–119.

38. Tahvanainen, V.; Miina, J.; Pukkala, T.; Kurttila, M. Optimizing the joint production of timber and marketed mushrooms in *Picea abies* stands in eastern Finland. *Journal of Forest Economics*, **2018**, *32* (September 2017), 34–41.

39. Horticultural Statistics | Luonnonvarakeskuksen tilastot https://stat.luke.fi/en/tilasto/138 (accessed June 1, 2021).

40. Finnish Biodiversity Information Facility https://laji.fi/en (accessed May 31, 2021).

41. Zhang, M.; Cui, S. W.; Cheung, P. C. K.; Wang, Q. Antitumor polysaccharides from mushrooms: a review on their isolation process, structural characteristics and antitumor activity. *Trends in Food Science and Technology*, **2007**, *18* (1), 4–19.

42. Lee, M.-W.; Hur, H.; Chang, K.-C.; Lee, T.-S.; Ka, K.-H.; Jankovsky, L. Introduction to Distribution and Ecology of Sterile Conks of *Inonotus obliquus*. *Mycobiology*, **2008**, *36* (4), 199–202.

43. Balandaykin, M. E.; Zmitrovich, I. V. Review on Chaga Medicinal Mushroom, *Inonotus obliquus* (Higher Basidiomycetes): Realm of Medicinal Applications and Approaches on Estimating its Resource Potential. *International Journal of Medicinal Mushrooms*, **2015**, *17* (2), 95–104.

44. Shigo, A. L. How *Poria obliqua* and *Polyporus glomeratus* incite canker. *Phytopathology*, **1969**, *59*, 1164–1165.

45. Blanchette, R.; Biggs, R. *Defense Mechanisms of Woody Plants Against Fungi*; Blanchette, R. A., Biggs, A. R. (Editors); Springer Series in Wood Science; Springer Berlin Heidelberg, Berlin, Heidelberg, 1992.

46. Blanchette, R. A. Progressive stages of

discoloration and decay associated with the canker-rot fungus, *Inonotus obliquus*, in Birch. *Phytopathology*. 1982, pp. 1272–1277.

47. Zhang, S. D.; Yu, L.; Wang, P.; Kou, P.; Li, J.; Wang, L. T.; Wang, W.; Yao, L. P.; Zhao, X. H.; Fu, Y. J. Inotodiol inhibits cells migration and invasion and induces apoptosis via p53-dependent pathway in HeLa cells. *Phytomedicine*, **2019**, *60*, 152957.

48. Zhong, X. H.; Wang, L. B.; Sun, D. Z. Effects of inotodiol extracts from *Inonotus obliquus* on proliferation cycle and apoptotic gene of human lung adenocarcinoma cell line A549. *Chinese Journal of Integrative Medicine*, **2011**, *17* (3), 218–223.

49. Nakata, T.; Yamada, T.; Taji, S.; Ohishi, H.; Wada, S. ichi; Tokuda, H.; Sakuma, K.; Tanaka, R. Structure determination of inonotsuoxides A and B and in vivo anti-tumor promoting activity of inotodiol from the sclerotia of *Inonotus obliquus*. *Bioorganic and Medicinal Chemistry*, **2007**, *15* (1), 257–264.

50. Zheng, W.; Miao, K.; Liu, Y.; Zhao, Y.; Zhang, M.; Pan, S.; Dai, Y. Chemical diversity of biologically active metabolites in the sclerotia of *Inonotus obliquus* and submerged culture strategies for up-regulating their production. *Applied Microbiology and Biotechnology*, **2010**, *87* (4), 1237–1254.

51. Fan, L.; Ding, S.; Ai, L.; Deng, K. Antitumor and immunomodulatory activity of water-soluble polysaccharide from *Inonotus obliquus*. *Carbohydrate Polymers*, **2012**, *90* (2), 870–874.

52. Huang, S. quan; Ding, S.; Fan, L. Antioxidant activities of five polysaccharides from *Inonotus obliquus*. *International Journal of Biological Macromolecules*, **2012**, *50* (5), 1183–1187.

53. Niu, H.; Song, D.; Mu, H.; Zhang, W.; Sun, F.; Duan, J. Investigation of three lignin complexes with antioxidant and immunological capacities from *Inonotus obliquus*. *International Journal of Biological Macromolecules*, **2016**, *86*, 587–593.

54. Wold, C. W.; Kjeldsen, C.; Corthay, A.; Rise, F.; Christensen, B. E.; Duus, J. Ø.; Inngjerdingen, K. T. Structural characterization of bioactive heteropolysaccharides from the medicinal fungus *Inonotus obliquus* (Chaga). *Carbohydrate Polymers*, **2018**, *185* (September 2017), 27–40.

55. Thomas, P. W.; Elkhateeb, W. A.; Daba, G. M. Chaga (*Inonotus obliquus*): a medical marvel but a conservation dilemma? *Sydowia*, **2020**, *72* (May), 123–130.

56. Ka, K.-H.; Jeon, S.-M.; Park, H.; Lee, B.-H.; Ryu, S.-R. Growth of Chaga Mushroom (*Inonotus obliquus*) on Betula platyphylla var. japonica. *The Korean Journal of Mycology*, **2017**, *45* (3), 241–245.

57. Miina, J.; Peltola, R.; Veteli, P.; Linnakoski, R.; Escribano, M. C.; Haveri-Heikkilä, J.; Mattila, P.; Marnila, P.; Pihlava, J.-M.; Hellström, J.; Sarjala, T.; Silvan, N.; Kurttila, M.; Vanhanen, H. Inoculation success of *Inonotus obliquus* in living birch (*Betula* spp.). *Forest Ecology and Management*, **2021**, *492*, 119244.

58. Ma, G.; Yang, W.; Fang, Y.; Ma, N.; Pei, F.; Zhao, L.; Hu, Q. Antioxidant and cytotoxicites of *Pleurotus eryngii* residue polysaccharides obtained by ultrafiltration. *LWT*, **2016**, *73*, 108–116.

59. Antunes, F.; Marçal, S.; Taofiq, O.; Morais, A. M. M. B.; Freitas, A. C.; Ferreira, I. C. F. R.; Pintado, M. Valorization of Mushroom By-Products as a Source of Value-Added Compounds and Potential Applications. *Molecules (Basel, Switzerland)*. NLM (Medline) June 9, 2020, p. 2672.

60. Liu, Y.; Zhang, B.; Ibrahim, S. A.; Gao, S. S.; Yang, H.; Huang, W. Purification, characterization and antioxidant activity of polysaccharides from *Flammulina velutipes* residue. *Carbohydrate Polymers*, **2016**, *145*, 71–77.

61. Zhu, H.; Sheng, K.; Yan, E.; Qiao, J.; Lv, F. Extraction, purification and antibacterial activities of a polysaccharide from spent mushroom substrate. *International Journal of Biological Macromolecules*, **2012**, *50* (3), 840–843.

62. Xu, X.; Xu, Z.; Shi, S.; Lin, M. Lignocellulose degradation patterns, structural changes, and enzyme secretion by *Inonotus obliquus* on straw biomass under submerged fermentation. *Bioresource Technology*, **2017**, *241*, 415–423.

63. Ruiz-Herrera, J. *Fungal Cell Wall*, Second Edi.; CRC Press, 2016.

64. Latgé, J. P. The cell wall: A carbohydrate armour for the fungal cell. *Molecular Microbiology*, **2007**, *66* (2), 279–290.

65. Bartnicki-Garcia, S. Cell Wall Chemistry, Morphogenesis, and Taxonomy of Fungi. *Annual Review of Microbiology*, **1968**, *22* (1), 87–108.

66. Bowman, S. M.; Free, S. J. The structure and synthesis of the fungal cell wall. *BioEssays*, **2006**, *28* (8), 799–808.

67. Grün, C. H.; Hochstenbach, F.; Humbel, B. M.; Verkleij, A. J.; Sietsma, J. H.; Klis, F. M.; Kamerling, J. P.; Vliegenthart, J. F. G. The structure of cell wall α-glucan from fission yeast. *Glycobiology*, **2005**, *15* (3), 245–257.

68. Ruiz-Herrera, J.; Ortiz-Castellanos, L. Cell wall glucans of fungi. A review. *Cell Surface*. Elsevier B.V. December 1, 2019, p. 100022.

69. Synytsya, A.; Novák, M. Structural diversity of fungal glucans. *Carbohydrate Polymers*, **2013**, *92* (1), 792–809.

70. Złotko, K.; Wiater, A.; Waśko, A.; Pleszczyńska, M.; Paduch, R.; Jaroszuk-Ściseł, J.; Bieganowski, A. A Report on Fungal (1→3)-α-d-glucans: Properties, Functions and Application. *Molecules*, **2019**, *24* (21), 3972.

71. Wiater, A.; Paduch, R.; Choma, A.; Pleszczyńska, M.; Siwulski, M.; Dominik, J.; Janusz, G.; Tomczyk, M.; Szczodrak, J. Biological study on carboxymethylated (1→3)-α-d-glucans from fruiting bodies of *Ganoderma lucidum*. *International Journal of Biological Macromolecules*, **2012**, *51* (5), 1014–1023.

72. Huang, Q.; Zhang, L. Preparation, chain conformation and anti-tumor activities of water-soluble phosphated (1 → 3)-α-d-glucan from *Poria cocos* mycelia. *Carbohydrate Polymers*, **2011**, *83* (3), 1363–1369.

73. Mondal, S.; Chakraborty, I.; Pramanik, M.; Rout, D.; Islam, S. S. Structural studies of water-soluble polysaccharides of an edible mushroom, *Termitomyces eurhizus*. A reinvestigation. *Carbohydrate Research*, **2004**, *339* (6), 1135–1140.

74. Free, S. J. Fungal Cell Wall Organization and Biosynthesis. In *Advances in Genetics*; 2013; Vol. 81, pp. 33–82.

75. Lowman, D. W.; West, L. J.; Bearden, D. W.; Wempe, M. F.; Power, T. D.; Ensley, H. E.; Haynes, K.; Williams, D. L.; Kruppa, M. D. New insights into the structure of (1→3,1→6)-β-D-glucan side chains in the *Candida glabrata* cell wall. *PLoS ONE*, **2011**, *6* (11).

76. Synytsya, A.; Míčková, K.; Synytsya, A.; Jablonský, I.; Spěváček, J.; Erban, V.; Kováříková, E.; Čopíková, J. Glucans from fruit bodies of cultivated mushrooms *Pleurotus ostreatus* and *Pleurotus eryngii*: Structure and potential prebiotic activity. *Carbohydrate Polymers*, **2009**, *76* (4), 548–556.

77. Stalhberger, T.; Simenel, C.; Clavaud, C.; Eijsink, V. G. H.; Jourdain, R.; Delepierre, M.; Latge, J. P.; Breton, L.; Fontaine, T. Chemical organization of the cell wall polysaccharide core of *Malassezia restricta*. *Journal of Biological Chemistry*, **2014**, *289* (18), 12647–12656.

78. Muszewska, A.; Piłsyk, S.; Perlińska-Lenart, U.; Kruszewska, J. S. Diversity of cell wall related proteins in human pathogenic fungi. *Journal of Fungi*, **2018**, *4* (1), 6.

79. Geoghegan, I.; Steinberg, G.; Gurr, S. The Role of the Fungal Cell Wall in the Infection of Plants. *Trends in Microbiology*, **2017**, *25* (12), 957–967.

80. De Groot, P. W. J.; Ram, A. F.; Klis, F. M. Features and functions of covalently linked proteins in fungal cell walls. *Fungal Genetics and Biology*, **2005**, *42* (8), 657–675.

81. Ruiz-Herrera, J.; Ortiz-Castellanos, L.; Martínez, A. I.; León-Ramírez, C.; Sentandreu, R. Analysis of the proteins involved in the structure and synthesis of the cell wall of *Ustilago maydis*. *Fungal Genetics and Biology*, **2008**, *45* (SUPPL. 1), S71–S76.

82. Wösten, H. A. B. Hydrophobins: Multipurpose proteins. *Annual Review of Microbiology*. Annual Reviews 4139 El Camino Way, P.O. Box 10139, Palo Alto, CA 94303-0139, USA October 28, 2001, pp. 625–646.

83. Wösten, H. A. B.; Van Wetter, M. A.; Lugones, L. G.; Van der Mei, H. C.; Busscher, H. J.; Wessels, J. G. H. How a fungus escapes the water to grow into the air. *Current Biology*, **1999**, *9* (2), 85–88.

84. Shibata, N.; Suzuki, A.; Kobayashi, H.; Okawa, Y. Chemical structure of the cell-wall mannan of *Candida albicans* serotype A and its difference in yeast

and hyphal forms. *Biochemical Journal*, **2007**, *404* (3), 365–372.

85. Kollár, R.; Reinhold, B. B.; Petráková, E.; Yeh, H. J. C.; Ashwell, G.; Drgonová, J.; Kapteyn, J. C.; Klis, F. M.; Cabib, E. Architecture of the Yeast Cell Wall. *Journal of Biological Chemistry*, **1997**, *272* (28), 17762–17775.

86. Ruiz-Herrera, J.; Martínez, A. I.; Sentandreu, R. Determination of the stability of protein pools from the cell wall of fungi. *Research in Microbiology*, **2002**, *153* (6), 373–378.

87. Cordero, R. J. B.; Casadevall, A. Functions of fungal melanin beyond virulence. *Fungal Biology Reviews*, **2017**, *31* (2), 99–112.

88. Toledo, A. V.; Franco, M. E. E.; Yanil Lopez, S. M.; Troncozo, M. I.; Saparrat, M. C. N.; Balatti, P. A. Melanins in fungi: Types, localization and putative biological roles. *Physiological and Molecular Plant Pathology*, **2017**, *99*, 2–6.

89. Beltrán-García, M. J.; Prado, F. M.; Oliveira, M. S.; Ortiz-Mendoza, D.; Scalfo, A. C.; Pessoa, A.; Medeiros, M. H. G.; White, J. F.; Di Mascio, P. Singlet molecular oxygen generation by light-activated DHN-melanin of the fungal pathogen *Mycosphaerella fijiensis* in black sigatoka disease of bananas. *PLoS ONE*, **2014**, *9* (3), e91616.

90. Weijn, A.; Bastiaan-Net, S.; Wichers, H. J.; Mes, J. J. Melanin biosynthesis pathway in *Agaricus bisporus* mushrooms. *Fungal Genetics and Biology*, **2013**, *55*, 42–53.

91. Wu, Z.; Zhang, M.; Yang, H.; Zhou, H.; Yang, H. Production, physico-chemical characterization and antioxidant activity of natural melanin from submerged cultures of the mushroom *Auricularia auricula*. *Food Bioscience*, **2018**, *26* (February), 49–56.

92. Hou, R.; Liu, X.; Xiang, K.; Chen, L.; Wu, X.; Lin, W.; Zheng, M.; Fu, J. Characterization of the physicochemical properties and extraction optimization of natural melanin from *Inonotus hispidus* mushroom. *Food Chemistry*, **2019**, *277*, 533–542.

93. Siletti, C. E.; Zeiner, C. A.; Bhatnagar, J. M. Distributions of fungal melanin across species and soils. *Soil Biology and Biochemistry*, **2017**, *113*, 285–293.

94. Money, N. P. Fungal Cell Biology and Development. In *The Fungi*; Elsevier, 2016; pp. 37–66.

95. Shashkina, M. Y.; Shashkin, P. N.; Sergeev, A. V. Chemical and medicobiological properties of chaga (review). *Pharmaceutical Chemistry Journal*, **2006**, *40* (10), 560–568.

96. Ribera, J.; Panzarasa, G.; Stobbe, A.; Osypova, A.; Rupper, P.; Klose, D.; Schwarze, F. W. M. R. Scalable Biosynthesis of Melanin by the Basidiomycete *Armillaria cepistipes*. *Journal of Agricultural and Food Chemistry*, **2019**, *67* (1), 132–139.

97. Michalenko, G. O.; Hohl, H. R.; Rast, D. Chemistry and Architecture of the Mycelial Wall of *Agaricus bisporus*. *Journal of General Microbiology*, **1976**, *92* (2), 251–262.

98. Chen, L.; Cheung, P. C. K. Mushroom Dietary Fiber from the Fruiting Body of *Pleurotus tuber-regium*: Fractionation and Structural Elucidation of Nondigestible Cell Wall Components. *Journal of Agricultural and Food Chemistry*, **2014**, *62* (13), 2891–2899.

99. Mol, P. C.; Vermeulen, C. A.; Wessels, J. G. H. Diffuse extension of hyphae in stipes of *Agaricus bisporus* may be based on a unique wall structure. *Mycological Research*, **1990**, *94* (4), 480–488.

100. Ehren, H. L.; Appels, F. V. W.; Houben, K.; Renault, M. A. M.; Wösten, H. A. B.; Baldus, M. Characterization of the cell wall of a mushroom forming fungus at atomic resolution using solid-state NMR spectroscopy. *The Cell Surface*, **2020**, *6*, 100046.

101. Chen, L.; Zhang, B. B.; Cheung, P. C. K. Comparative proteomic analysis of mushroom cell wall proteins among the different developmental stages of *Pleurotus tuber-regium*. *Journal of Agricultural and Food Chemistry*, **2012**, *60* (24), 6173–6182.

102. Niu, X.; Liu, Z.; Zhou, Y.; Wang, J.; Zhang, W.; Yuan, S. Stipe cell wall architecture varies with the stipe elongation of the mushroom *Coprinopsis cinerea*. *Fungal Biology*, **2015**, *119* (10), 946–956.

103. Liu, C.; Bi, J.; Kang, L.; Zhou, J.; Liu, X.; Liu, Z.; Yuan, S. The molecular mechanism of stipe cell wall extension for mushroom stipe elongation growth. *Fungal Biology Reviews*. Elsevier Ltd March 1, 2020, pp. 14–26.

104. Novak, M.; Vetvicka, V. β-glucans,

history, and the present: Immunomodulatory aspects and mechanisms of action. *Journal of Immunotoxicology*, **2008**, *5* (1), 47–57.

105. Smiderle, F. R.; Alquini, G.; Tadra-Sfeir, M. Z.; Iacomini, M.; Wichers, H. J.; Van Griensven, L. J. L. D. *Agaricus bisporus* and *Agaricus brasiliensis* (1 → 6)-β-d-glucans show immunostimulatory activity on human THP-1 derived macrophages. *Carbohydrate Polymers*, **2013**, *94* (1), 91–99.

106. Pires, A. do R. A.; Ruthes, A. C.; Cadena, S. M. S. C.; Iacomini, M. Cytotoxic effect of a mannogalactoglucan extracted from *Agaricus bisporus* on HepG2 cells. *Carbohydrate Polymers*, **2017**, *170*, 33–42.

107. Jeff, I. B.; Yuan, X.; Sun, L.; Kassim, R. M. R.; Foday, A. D.; Zhou, Y. Purification and in vitro anti-proliferative effect of novel neutral polysaccharides from *Lentinus edodes*. *International Journal of Biological Macromolecules*, **2013**, *52* (1), 99–106.

108. Morales, D.; Rutckeviski, R.; Villalva, M.; Abreu, H.; Soler-Rivas, C.; Santoyo, S.; Iacomini, M.; Smiderle, F. R. Isolation and comparison of α- and β-D-glucans from shiitake mushrooms (*Lentinula edodes*) with different biological activities. *Carbohydrate Polymers*, **2020**, *229* (July 2019), 115521.

109. Li, J.; Cai, C.; Zheng, M.; Hao, J.; Wang, Y.; Hu, M.; Fan, L.; Yu, G. Alkaline extraction, structural characterization, and bioactivities of (1→6)-β-d-glucan from *Lentinus edodes*. *Molecules*, **2019**, *24* (8).

110. Zhang, A.; Liu, Y.; Xiao, N.; Zhang, Y.; Sun, P. Structural investigation of a novel heteropolysaccharide from the fruiting bodies of *Boletus edulis*. *Food Chemistry*, **2014**, *146*, 334–338.

111. Ruthes, A. C.; Carbonero, E. R.; Córdova, M. M.; Baggio, C. H.; Santos, A. R. S.; Sassaki, G. L.; Cipriani, T. R.; Gorin, P. A. J.; Iacomini, M. *Lactarius rufus* (1→3),(1→6)-β-d-glucans: Structure, antinociceptive and anti-inflammatory effects. *Carbohydrate Polymers*, **2013**, *94* (1), 129–136.

112. Nyman, A. A. T.; Aachmann, F. L.; Rise, F.; Ballance, S.; Samuelsen, A. B. C. Structural characterization of a branched (1 → 6)-α-mannan and β-

glucans isolated from the fruiting bodies of *Cantharellus cibarius*. *Carbohydrate Polymers*, **2016**, *146*, 197–207.

113. Villares, A.; García-Lafuente, A.; Guillamón, E.; Mateo-Vivaracho, L. Separation and characterization of the structural features of macromolecular carbohydrates from wild edible mushrooms. *Bioactive Carbohydrates and Dietary Fibre*, **2013**, *2* (1), 15–21.

114. Carbonero, E. R.; Montai, A. V.; Mellinger, C. G.; Eliasaro, S.; Sassaki, G. L.; Gorin, P. A. J.; Iacomini, M. Glucans of lichenized fungi: Significance for taxonomy of the genera *Parmotrema* and *Rimelia*. *Phytochemistry*, **2005**, *66* (8), 929–934.

115. Nanba, H.; Hamaguchi, A.; Kuroda, H. The Chemical Structure of an Antitumor Polysaccharide in Fruit Bodies of *Grifola frondosa* (Maitake). *Chemical and Pharmaceutical Bulletin*, **1987**, *35* (3), 1162–1168.

116. Tada, R.; Adachi, Y.; Ishibashi, K. ichi; Ohno, N. An unambiguous structural elucidation of a 1,3-β-d-glucan obtained from liquid-cultured *Grifola frondosa* by solution NMR experiments. *Carbohydrate Research*, **2009**, *344* (3), 400–404.

117. Tabata, K.; Ito, W.; Kojima, T.; Kawabata, S.; Misaki, A. Ultrasonic degradation of schizophyllan, an antitumor polysaccharide produced by *Schizophyllum commune* fries. *Carbohydrate Research*, **1981**, *89* (1), 121–135.

118. Sasaki, T.; Takasuka, N. Further study of the structure of lentinan, an anti-tumor polysaccharide from *Lentinus edodes*. *Carbohydrate Research*, **1976**, *47* (1), 99–104.

119. Ruthes, A. C.; Rattmann, Y. D.; Malquevicz-Paiva, S. M.; Carbonero, E. R.; Córdova, M. M.; Baggio, C. H.; Santos, A. R. S.; Gorin, P. A. J.; Iacomini, M. *Agaricus bisporus* fucogalactan: Structural characterization and pharmacological approaches. *Carbohydrate Polymers*, **2013**, *92* (1), 184–191.

120. Ruthes, A. C.; Rattmann, Y. D.; Carbonero, E. R.; Gorin, P. A. J.; Iacomini, M. Structural characterization and protective effect against murine sepsis of fucogalactans from *Agaricus bisporus* and *Lactarius rufus*. *Carbohydrate Polymers*, **2012**,

87 (2), 1620–1627.

121. Wang, D.; Sun, S.-Q.; Wu, W.-Z.; Yang, S.-L.; Tan, J.-M. Characterization of a water-soluble polysaccharide from Boletus edulis and its antitumor and immunomodulatory activities on renal cancer in mice. *Carbohydrate Polymers*, **2014**, *105* (1), 127–134.

122. Carbonero, E. R.; Gracher, A. H. P.; Komura, D. L.; Marcon, R.; Freitas, C. S.; Baggio, C. H.; Santos, A. R. S.; Torri, G.; Gorin, P. A. J.; Iacomini, M. *Lentinus edodes* heterogalactan: Antinociceptive and anti-inflammatory effects. *Food Chemistry*, **2008**, *111* (3), 531–537.

123. Smiderle, F. R.; Olsen, L. M.; Carbonero, E. R.; Marcon, R.; Baggio, C. H.; Freitas, C. S.; Santos, A. R. S.; Torri, G.; Gorin, P. A. J.; Iacomini, M. A 3-O-methylated mannogalactan from *Pleurotus pulmonarius*: Structure and antinociceptive effect. *Phytochemistry*, **2008**, *69* (15), 2731–2736.

124. Yan, J.; Meng, Y.; Zhang, M.; Zhou, X.; Cheng, H.; Sun, L.; Zhou, Y. A 3-O-methylated heterogalactan from *Pleurotus eryngii* activates macrophages. *Carbohydrate Polymers*, **2019**, *206* (November 2018), 706–715.

125. Komura, D. L.; Carbonero, E. R.; Gracher, A. H. P.; Baggio, C. H.; Freitas, C. S.; Marcon, R.; Santos, A. R. S.; Gorin, P. a J.; Iacomini, M. Structure of *Agaricus* spp. fucogalactans and their anti-inflammatory and antinociceptive properties. *Bioresource Technology*, **2010**, *101* (15), 6192–6199.

126. Staudacher, E. Methylation-an uncommon modification of glycans. *Biological Chemistry*, **2012**, *393* (8), 675–685.

127. Ruthes, A. C.; Smiderle, F. R.; Iacomini, M. Mushroom heteropolysaccharides: A review on their sources, structure and biological effects. *Carbohydrate Polymers*, **2016**, *136*, 358–375.

128. Choma, A.; Nowak, K.; Komaniecka, I.; Waśko, A.; Pleszczyńska, M.; Siwulski, M.; Wiater, A. Chemical characterization of alkali-soluble polysaccharides isolated from a *Boletus edulis* (Bull.) fruiting body and their potential for heavy metal biosorption. *Food Chemistry*, **2018**, *266* (May), 329–334.

129. Yu, J.; Ji, H. yu; Liu, C.; Liu, A. jun. The structural characteristics of an acid-soluble polysaccharide from *Grifola frondosa* and its antitumor effects on H22-bearing mice. *International Journal of Biological Macromolecules*, **2020**, *158*, 1288–1298.

130. Ding, X.; Hou, Y.; Hou, W. Structure feature and antitumor activity of a novel polysaccharide isolated from *Lactarius deliciosus* Gray. *Carbohydrate Polymers*, **2012**, *89* (2), 397–402.

131. Huang, Y.; Yu, J.; Huang, J.; Liu, D.; Liang, M. Structural characterization of a novel *Lactarius volemus* Fr. polysaccharide and its immunity activity in BALB/c mice. *RSC Advances*, **2020**, *10* (51), 30254–30264.

132. Ren, L.; Perera, C.; Hemar, Y. Antitumor activity of mushroom polysaccharides: a review. *Food & Function*, **2012**, *3* (11), 1118.

133. Zhang, X.; Qi, C.; Guo, Y.; Zhou, W.; Zhang, Y. Toll-like receptor 4-related immunostimulatory polysaccharides: Primary structure, activity relationships, and possible interaction models. *Carbohydrate Polymers*, **2016**, *149*, 186–206.

134. Sletmoen, M.; Stokke, B. T. Higher order structure of (1,3)-β-D-glucans and its influence on their biological activities and complexation abilities. *Biopolymers*, **2008**, *89* (4), 310–321.

135. Bae, I. Y.; Kim, H. W.; Yoo, H. J.; Kim, E. S.; Lee, S.; Park, D. Y.; Lee, H. G. Correlation of branching structure of mushroom β-glucan with its physiological activities. *Food Research International*, **2013**, *51* (1), 195–200.

136. Dalonso, N.; Goldman, G. H.; Gern, R. M. M. β-(1→3),(1→6)-Glucans: medicinal activities, characterization, biosynthesis and new horizons. *Applied Microbiology and Biotechnology*, **2015**.

137. Chan, G. C.-F.; Chan, W. K.; Sze, D. M.-Y. The effects of beta-glucan on human immune and cancer cells. *Journal of hematology & oncology*, **2009**, *2*, 25.

138. Volman, J. J.; Ramakers, J. D.; Plat, J. Dietary modulation of immune function by β-glucans. *Physiology and Behavior*, **2008**, *94* (2), 276–284.

139. Abbas, A.; Lichtman, A.; Pillai, S. *Cellular and Molecular Immunology*, 9th edition; Elsevier, 2017.

140. Mingyi, Y.; Belwal, T.; Devkota, H. P.; Li, L.; Luo, Z. Trends of utilizing mushroom polysaccharides (MPs) as potent nutraceutical components in food and medicine: A comprehensive

140. review. *Trends in Food Science & Technology*, **2019**, *92* (October 2018), 94–110.

141. Yamanaka, D.; Tada, R.; Adachi, Y.; Ishibashi, K. I.; Motoi, M.; Iwakura, Y.; Ohno, N. *Agaricus brasiliensis*-derived β-glucans exert immunoenhancing effects via a dectin-1-dependent pathway. *International Immunopharmacology*, **2012**, *14* (3), 311–319.

142. Zhu, J.; Paul, W. E. CD4 T cells: fates, functions, and faults. *Blood*, **2008**, *112* (5), 1557–1569.

143. Thompson, I. J.; Oyston, P. C. F.; Williamson, D. E. Potential of the β-glucans to enhance innate resistance to biological agents. *Expert Review of Anti-Infective Therapy*, **2010**, *8* (3), 339–352.

144. Xu, X.; Yang, J.; Ning, Z.; Zhang, X. *Lentinula edodes*-derived polysaccharide rejuvenates mice in terms of immune responses and gut microbiota. *Food and Function*, **2015**, *6* (8), 2653–2663.

145. Ren, Z.; Li, J.; Song, X.; Zhang, J.; Wang, W.; Wang, X.; Gao, Z.; Jing, H.; Li, S.; Jia, L. The regulation of inflammation and oxidative status against lung injury of residue polysaccharides by *Lentinula edodes*. *International Journal of Biological Macromolecules*, **2018**, *106*, 185–192.

146. Xu, X.; Yan, H.; Zhang, X. Structure and immuno-stimulating activities of a new heteropolysaccharide from *Lentinula edodes*. *Journal of Agricultural and Food Chemistry*, **2012**, *60* (46), 11560–11566.

147. Kurashige, S.; Akuzawa, Y.; Endo, F. Effects of *Lentinus edodes*, *Grifola frondosa* and *Pleurotus ostreatus* administration on cancer outbreak, and activities of macrophages and lymphocytes in mice treated with a carcinogen, N-butyl-N-butanolnitrosoamine. *Immunopharmacology and Immunotoxicology*, **1997**, *19* (2), 175–183.

148. Israilides, C.; Kletsas, D.; Arapoglou, D.; Philippoussis, A.; Pratsinis, H.; Ebringerová, A.; Hříbalová, V.; Harding, S. E. In vitro cytostatic and immunomodulatory properties of the medicinal mushroom *Lentinula edodes*. *Phytomedicine*, **2008**, *15* (6–7), 512–519.

149. Wang, K. ping; Wang, J.; Li, Q.; Zhang, Q. lin; You, R. xu; Cheng, Y.; Luo, L.; Zhang, Y. Structural differences and conformational characterization of five bioactive polysaccharides from *Lentinus edodes*. *Food Research International*, **2014**, *62*, 223–232.

150. Chen, L.; Peng, X.; Lv, J.; Liao, S.; Ou, S.; Shen, Y. Purification and Structural Characterization of a Novel Water-Soluble Neutral Polysaccharide from *Cantharellus cibarius* and Its Immunostimulating Activity in RAW264.7 Cells. *International Journal of Polymer Science*, **2017**, *2017*.

151. Nandi, A. K.; Samanta, S.; Sen, I. K.; Devi, K. S. P.; Maiti, T. K.; Acharya, K.; Islam, S. S. Structural elucidation of an immunoenhancing heteroglycan isolated from *Russula albonigra* (Krombh.) Fr. *Carbohydrate Polymers*, **2013**, *94* (2), 918–926.

152. Kim, Y. O.; Han, S. B.; Lee, H. W.; Ahnb, H. J.; Yoonb, Y. D.; Jungb, J. K.; Kimb, H. M.; Shin, C. S. Immuno-stimulating effect of the endo-polysaccharide produced by submerged culture of *Inonotus obliquus*. *Life Sciences*, **2005**, *77* (19), 2438–2456.

153. Xu, X.; Li, J.; Hu, Y. Polysaccharides from *Inonotus obliquus* sclerotia and cultured mycelia stimulate cytokine production of human peripheral blood mononuclear cells in vitro and their chemical characterization. *International Immunopharmacology*, **2014**, *21* (2), 269–278.

154. Chen, Z.; Tang, Y.; Liu, A.; Jin, X.; Zhu, J.; Lu, X. Oral administration of *Grifola frondosa* polysaccharides improves memory impairment in aged rats via antioxidant action. *Molecular Nutrition & Food Research*, **2017**, *61* (11), 1700313.

155. Matsui, K.; Kodama, N.; Nanba, H. Effects of maitake (*Grifola frondosa*) D-Fraction on the carcinoma angiogenesis. *Cancer Letters*, **2001**, *172* (2), 193–198.

156. ADACHI, Y.; OKAZAKI, M.; OHNO, N.; YADOMAE, T. Enhancement of Cytokine Production by Macrophages Stimulated with (1.RAR.3)-.BETA.-D-Glucan, Grifolan (GRN), Isolated from *Grifola frondosa*. *Biological and Pharmaceutical Bulletin*, **1994**, *17* (12), 1554–1560.

157. Du, B.; Yang, Y.; Bian, Z.; Xu, B. Characterization and anti-inflammatory

potential of an exopolysaccharide from submerged mycelial culture of *Schizophyllum commune*. *Frontiers in Pharmacology*, **2017**, *8* (MAY), 252.

158. YONEDA, K.; UETA, E.; YAMAMOTO, T.; OSAKI, T. Immunoregulatory effects of Sizofiran (SPG) on lymphocytes and polymorphonuclear leukocytes. *Clinical & Experimental Immunology*, **2008**, *86* (2), 229–235.

159. Hak Hotta; Katsushi Hagiwara; Kengo Tabata; Wataru Ito; Morio Homma. Augmentation of protective immune responses against sendai virus infection by fungal polysaccharide schizophyllan. *International Journal of Immunopharmacology*, **1993**, *15* (1), 55–60.

160. Slavin, J. Fiber and prebiotics: Mechanisms and health benefits. *Nutrients*, **2013**, *5* (4), 1417–1435.

161. Gibson, G. R.; Probert, H. M.; Loo, J. Van; Rastall, R. A.; Roberfroid, M. B. Dietary modulation of the human colonic microbiota: updating the concept of prebiotics. *Nutrition Research Reviews*, **2004**, *17* (2), 259–275.

162. Yu, Z. T.; Liu, B.; Mukherjee, P.; Newburg, D. S. *Trametes versicolor* Extract Modifies Human Fecal Microbiota Composition In vitro. *Plant Foods for Human Nutrition*, **2013**, *68* (2), 107–112.

163. Zhao, J.; Cheung, P. C. K. Fermentation of β-glucans derived from different sources by bifidobacteria: Evaluation of their bifidogenic effect. *Journal of Agricultural and Food Chemistry*, **2011**, *59* (11), 5986–5992.

164. Lam, K.-L.; Cheng, W.-Y.; Su, Y.; Li, X.; Wu, X.; Wong, K.-H.; Kwan, H.-S.; Cheung, P. C.-K. Use of random forest analysis to quantify the importance of the structural characteristics of beta-glucans for prebiotic development. *Food Hydrocolloids*, **2020**, *108*, 106001.

165. Morales, D.; Shetty, S. A.; López-Plaza, B.; Gómez-Candela, C.; Smidt, H.; Marín, F. R.; Soler-Rivas, C. Modulation of human intestinal microbiota in a clinical trial by consumption of a β-d-glucan-enriched extract obtained from *Lentinula edodes*. *European Journal of Nutrition*, **2021**.

166. Cantu-Jungles, T. M.; Ruthes, A. C.; El-Hindawy, M.; Moreno, R. B.; Zhang, X.; Cordeiro, L. M. C.; Hamaker, B. R.; Iacomini, M. In vitro fermentation of *Cookeina speciosa* glucans stimulates the growth of the butyrogenic *Clostridium* cluster XIVa in a targeted way. *Carbohydrate Polymers*, **2018**, *183* (November 2017), 219–229.

167. Nowak, R.; Nowacka-Jechalke, N.; Juda, M.; Malm, A. The preliminary study of prebiotic potential of Polish wild mushroom polysaccharides: the stimulation effect on *Lactobacillus* strains growth. *European Journal of Nutrition*, **2018**, *57* (4), 1511–1521.

168. The Mushroom Database https://www.mushroom.world/mushrooms/search (accessed April 28, 2021).

169. Chou, W. T.; Sheih, I. C.; Fang, T. J. The applications of polysaccharides from various mushroom wastes as prebiotics in different systems. *Journal of Food Science*, **2013**, *78* (7).

170. Huang, D.; Boxin, O. U.; Prior, R. L. The chemistry behind antioxidant capacity assays. *Journal of Agricultural and Food Chemistry*. American Chemical Society March 23, 2005, pp. 1841–1856.

171. Wu, Q.; Tan, Z.; Liu, H.; Gao, L.; Wu, S.; Luo, J.; Zhang, W.; Zhao, T.; Yu, J.; Xu, X. Chemical characterization of *Auricularia auricula* polysaccharides and its pharmacological effect on heart antioxidant enzyme activities and left ventricular function in aged mice. *International Journal of Biological Macromolecules*, **2010**, *46* (3), 284–288.

172. Pan, W. J.; Ding, Q. Y.; Wang, Y.; Wang, D. D.; Lu, Y. M.; Yang, W. W.; Cai, Z. N.; Cheng, X. Du; Zhang, W. N.; Chen, Y. A bioactive polysaccharide TLH-3 isolated from *Tricholoma lobayense* protects against oxidative stress-induced premature senescence in cells and mice. *Journal of Functional Foods*, **2018**, *42*, 159–170.

173. Jing, H.; Li, J.; Zhang, J.; Wang, W.; Li, S.; Ren, Z.; Gao, Z.; Song, X.; Wang, X.; Jia, L. The antioxidative and anti-aging effects of acidic- and alkalic-extractable mycelium polysaccharides by *Agrocybe aegerita* (Brig.) Sing. *International Journal of Biological Macromolecules*, **2018**, *106*, 1270–1278.

174. Chen, P.; Yong, Y.; Gu, Y.; Wang, Z.; Zhang, S.; Lu, L. Comparison of antioxidant and antiproliferation

activities of polysaccharides from eight species of medicinal mushrooms. *International Journal of Medicinal Mushrooms*, **2015**, *17* (3), 287–295.

175. Tian, Y.; Zeng, H.; Xu, Z.; Zheng, B.; Lin, Y.; Gan, C.; Lo, Y. M. Ultrasonic-assisted extraction and antioxidant activity of polysaccharides recovered from white button mushroom (*Agaricus bisporus*). *Carbohydrate Polymers*, **2012**, *88* (2), 522–529.

176. Govindan, S.; Johnson, E. E. R.; Christopher, J.; Shanmugam, J.; Thirumalairaj, V.; Gopalan, J. Antioxidant and anti-aging activities of polysaccharides from *Calocybe indica* var. APK2. *Experimental and Toxicologic Pathology*, **2016**, *68* (6), 329–334.

177. Liu, Q.; Tian, G.; Yan, H.; Geng, X.; Cao, Q.; Wang, H.; Ng, T. B. Characterization of Polysaccharides with Antioxidant and Hepatoprotective Activities from the Wild Edible Mushroom *Russula vinosa* Lindblad. *Journal of Agricultural and Food Chemistry*, **2014**, *62* (35), 8858–8866.

178. Sun, Y. X.; Liu, J. C.; Yang, X. D.; Kennedy, J. F. Purification, structural analysis and hydroxyl radical-scavenging capacity of a polysaccharide from the fruiting bodies of *Russula virescens*. *Process Biochemistry*, **2010**, *45* (6), 874–879.

179. Xu, X.; Wu, Y.; Chen, H. Comparative antioxidative characteristics of polysaccharide-enriched extracts from natural sclerotia and cultured mycelia in submerged fermentation of *Inonotus obliquus*. *Food Chemistry*, **2011**, *127* (1), 74–79.

180. Chen, Y.; Xie, M.; Li, W.; Zhang, H.; Nie, S.; Wang, Y.; Li, C. An effective method for deproteinization of bioactive polysaccharides extracted from lingzhi (*Ganoderma atrum*). *Food Science and Biotechnology*, **2012**, *21* (1), 191–198.

181. Wang, J.; Hu, S.; Nie, S.; Yu, Q.; Xie, M.; Wang, J.; Hu, S.; Nie, S.; Yu, Q.; Xie, M. Reviews on Mechanisms of *In Vitro* Antioxidant Activity of Polysaccharides. *Oxidative Medicine and Cellular Longevity*, **2016**, *2016*, 1–13.

182. Lo, T. C. T.; Chang, C. A.; Chiu, K. H.; Tsay, P. K.; Jen, J. F. Correlation evaluation of antioxidant properties on the monosaccharide components and glycosyl linkages of polysaccharide with different measuring methods. *Carbohydrate Polymers*, **2011**, *86* (1), 320–327.

183. Zheng, Y.; Li, Y.; Wang, W. D. Optimization of ultrasonic-assisted extraction and in vitro antioxidant activities of polysaccharides from *Trametes orientalis*. *Carbohydrate Polymers*, **2014**, *111*, 315–323.

184. Petrović, J.; Glamočlija, J.; Stojković, D.; Ćirić, A.; Barros, L.; Ferreira, I. C. F. R.; Soković, M. Nutritional value, chemical composition, antioxidant activity and enrichment of cream cheese with chestnut mushroom *Agrocybe aegerita* (Brig.) Sing. *Journal of Food Science and Technology*, **2015**, *52* (10), 6711–6718.

185. Huang, Y.; Zhao, S.; Yao, K.; Liu, D.; Peng, X.; Huang, J.; Huang, Y.; Li, L. Physicochemical, microbiological, rheological, and sensory properties of yoghurts with new polysaccharide extracts from *Lactarius volemus* Fr. using three probiotics. *International Journal of Dairy Technology*, **2020**, *73* (1), 168–181.

186. Choe, J.; Lee, J.; Jo, K.; Jo, C.; Song, M.; Jung, S. Application of winter mushroom powder as an alternative to phosphates in emulsion-type sausages. *Meat Science*, **2018**, *143*, 114–118.

187. Tu, J.; Brennan, M.; Brennan, C. An insight into the mechanism of interactions between mushroom polysaccharides and starch. *Current Opinion in Food Science*. Elsevier Ltd February 1, 2021, pp. 17–25.

188. Salehi, F. Characterization of different mushrooms powder and its application in bakery products: A review. *International Journal of Food Properties*, **2019**, *22* (1), 1375–1385.

189. Olawuyi, I. F.; Lee, W. Y. Quality and antioxidant properties of functional rice muffins enriched with shiitake mushroom and carrot pomace. *International Journal of Food Science & Technology*, **2019**, *54* (7), 2321–2328.

190. Lu, X.; Brennan, M. A.; Serventi, L.; Mason, S.; Brennan, C. S. How the inclusion of mushroom powder can affect the physicochemical characteristics of pasta. *International Journal of Food Science and Technology*, **2016**, *51* (11), 2433–2439.

191. Lu, X.; Brennan, M. A.; Serventi, L.; Brennan, C. S. Incorporation of

mushroom powder into bread dough—effects on dough rheology and bread properties. *Cereal Chemistry*, **2018**, *95* (3), 418–427.

192. Lu, X.; Brennan, M. A.; Narciso, J.; Guan, W.; Zhang, J.; Yuan, L.; Serventi, L.; Brennan, C. S. Correlations between the phenolic and fibre composition of mushrooms and the glycaemic and textural characteristics of mushroom enriched extruded products. *Lwt*, **2020**, *118* (September 2019), 108730.

193. Tepsongkroh, B.; Jangchud, K.; Jangchud, A.; Chonpracha, P.; Ardoin, R.; Prinyawiwatkul, W. Consumer perception of extruded snacks containing brown rice and dried mushroom. *International Journal of Food Science and Technology*, **2020**, *55* (1), 46–54.

194. Ulziijargal, E.; Yang, J. H.; Lin, L. Y.; Chen, C. P.; Mau, J. L. Quality of bread supplemented with mushroom mycelia. *Food Chemistry*, **2013**, *138* (1), 70–76.

195. Nie, Y.; Jin, Y.; Deng, C.; Xu, L.; Yu, M.; Yang, W.; Li, B.; Zhao, R. Rheological and microstructural properties of wheat dough supplemented with *Flammulina velutipes* (mushroom) powder and soluble polysaccharides. *CyTA - Journal of Food*, **2019**, *17* (1), 455–462.

196. Sulieman, A. A.; Zhu, K.-X.; Peng, W.; Hassan, H. A.; Obadi, M.; Siddeeg, A.; Zhou, H.-M. Rheological and quality characteristics of composite gluten-free dough and biscuits supplemented with fermented and unfermented *Agaricus bisporus* polysaccharide flour. *Food Chemistry*, **2019**, *271*, 193–203.

197. Zhou, R.; Kang, Y. H. Synergistic interaction of *Auricularia auricula-judae* polysaccharide with yam starch: effects on physicochemical properties and in vitro starch digestibility. *Food Science and Biotechnology*, **2018**, *27* (6), 1579–1588.

198. Feng, T.; Shui, M.; Chen, Z.; Zhuang, H.; Wang, W.; Yang, Y.; Zhang, J.; Ye, R. *Hericium Erinaceus* β-glucan modulates in vitro wheat starch digestibility. *Food Hydrocolloids*, **2019**, *96* (May), 424–432.

199. Shi, L. Bioactivities, isolation and purification methods of polysaccharides from natural products: A review. *International Journal of Biological Macromolecules*, **2016**, *92*, 37–48.

200. Leong, Y. K.; Yang, F. C.; Chang, J. S. Extraction of polysaccharides from edible mushrooms: Emerging technologies and recent advances. *Carbohydrate Polymers*, **2021**, *251* (August 2020), 117006.

201. Ruiz, H. A.; Rodríguez-Jasso, R. M.; Fernandes, B. D.; Vicente, A. A.; Teixeira, J. A. Hydrothermal processing, as an alternative for upgrading agriculture residues and marine biomass according to the biorefinery concept: A review. *Renewable and Sustainable Energy Reviews*, **2013**, *21*, 35–51.

202. Herrero, M.; Castro-Puyana, M.; Mendiola, J. A.; Ibañez, E. Compressed fluids for the extraction of bioactive compounds. *TrAC - Trends in Analytical Chemistry*, **2013**, *43* (2), 67–83.

203. Smiderle, F. R.; Carbonero, E. R.; Mellinger, C. G.; Sassaki, G. L.; Gorin, P. A. J.; Iacomini, M. Structural characterization of a polysaccharide and a ??-glucan isolated from the edible mushroom *Flammulina velutipes*. *Phytochemistry*, **2006**, *67* (19), 2189–2196.

204. Prado, J. M.; Lachos-Perez, D.; Forster-Carneiro, T.; Rostagno, M. A. Sub- and supercritical water hydrolysis of agricultural and food industry residues for the production of fermentable sugars: A review. *Food and Bioproducts Processing*, **2016**, *98*, 95–123.

205. Rodríguez-Seoane, P.; González-Muñoz, M. J.; Falqué, E.; Domínguez, H. Pressurized hot water extraction of β-glucans from *Cantharellus tubaeformis*. *ELECTROPHORESIS*, **2018**, *39* (15), 1892–1898.

206. Smiderle, F. R.; Morales, D.; Gil-Ramírez, A.; de Jesus, L. I.; Gilbert-López, B.; Iacomini, M.; Soler-Rivas, C. Evaluation of microwave-assisted and pressurized liquid extractions to obtain β-D-glucans from mushrooms. *Carbohydrate Polymers*, **2017**, *156*, 165–174.

207. Zhang, J.; Wen, C.; Gu, J.; Ji, C.; Duan, Y.; Zhang, H. Effects of subcritical water extraction microenvironment on the structure and biological activities of polysaccharides from *Lentinus edodes*. *International Journal of Biological Macromolecules*, **2019**, *123*, 1002–1011.

208. Palanisamy, M.; Aldars-García, L.; Gil-Ramírez, A.; Ruiz-Rodríguez, A.; Marín, F. R.; Reglero, G.; Soler-Rivas, C. Pressurized water extraction of β-glucan enriched fractions with bile acids-binding capacities obtained from edible mushrooms. *Biotechnology Progress*, **2014**, *30* (2), 391–400.

209. Morales, D.; Smiderle, F. R.; Villalva, M.; Abreu, H.; Rico, C.; Santoyo, S.; Iacomini, M.; Soler-Rivas, C. Testing the effect of combining innovative extraction technologies on the biological activities of obtained β-glucan-enriched fractions from *Lentinula edodes*. *Journal of Functional Foods*, **2019**, *60* (July), 103446.

210. Alzorqi, I.; Sudheer, S.; Lu, T. J.; Manickam, S. Ultrasonically extracted β-D-glucan from artificially cultivated mushroom, characteristic properties and antioxidant activity. *Ultrasonics Sonochemistry*, **2017**, *35*, 531–540.

211. Li, X.; Wang, L. Effect of extraction method on structure and antioxidant activity of *Hohenbuehelia serotina* polysaccharides. *International Journal of Biological Macromolecules*, **2016**, *83*, 270–276.

212. Yan, J. K.; Wang, Y. Y.; Ma, H. Le; Wang, Z. Bin. Ultrasonic effects on the degradation kinetics, preliminary characterization and antioxidant activities of polysaccharides from *Phellinus linteus* mycelia. *Ultrasonics Sonochemistry*, **2016**, *29*, 251–257.

213. Gil-Ramírez, A.; Smiderle, F. R.; Morales, D.; Iacomini, M.; Soler-Rivas, C. Strengths and weaknesses of the aniline-blue method used to test mushroom (1→3)-β-D-glucans obtained by microwave-assisted extractions. *Carbohydrate Polymers*, **2019**, *217* (January), 135–143.

214. Zhang, Z.; Lv, G.; He, W.; Shi, L.; Pan, H.; Fan, L. Effects of extraction methods on the antioxidant activities of polysaccharides obtained from *Flammulina velutipes*. *Carbohydrate Polymers*, **2013**, *98* (2), 1524–1531.

215. Chen, X.; Fang, D.; Zhao, R.; Gao, J.; Kimatu, B. M.; Hu, Q.; Chen, G.; Zhao, L. Effects of ultrasound-assisted extraction on antioxidant activity and bidirectional immunomodulatory activity of *Flammulina velutipes* polysaccharide. *International Journal of Biological Macromolecules*, **2019**,

216. Chen, J.; Lai, P.; Shen, H.; Zhen, H.; Fang, R. Effect of extraction methods on polysaccharide of *Clitocybe maxima* stipe. *Advance Journal of Food Science and Technology*, **2013**, *5* (3), 370–373.

217. Huang, S. quan; Ning, Z. xiang. Extraction of polysaccharide from *Ganoderma lucidum* and its immune enhancement activity. *International Journal of Biological Macromolecules*, **2010**, *47* (3), 336–341.

218. Zheng, Y.; Cui, J.; Chen, A. H.; Zong, Z. M.; Wei, X. Y. Optimization of Ultrasonic-Microwave Assisted Extraction and Hepatoprotective Activities of Polysaccharides from *Trametes orientalis*. *Molecules*, **2019**, *24* (1), 147.

219. You, Q.; Yin, X.; Zhang, S.; Jiang, Z. Extraction, purification, and antioxidant activities of polysaccharides from *Tricholoma mongolicum* Imai. *Carbohydrate Polymers*, **2014**, *99*, 1–10.

220. Whistler, R.; BeMiller, J. Alkaline Degradation of Polysaccharides. *Advances in Carbohydrate Chemistry*, **1958**, *13*, 289–329.

221. Berglund, J.; Azhar, S.; Lawoko, M.; Lindström, M.; Vilaplana, F.; Wohlert, J.; Henriksson, G. The structure of galactoglucomannan impacts the degradation under alkaline conditions. *Cellulose*, **2019**, *26* (3), 2155–2175.

222. Dumitriu, S. *Polysaccharides: Structural Diversity and Functional Versatility*, 2nd edition; Dumitriu, S. (Editor); Marcel Dekker, New York, 2005.

223. Palacios, I.; García-lafuente, A.; Guillamón, E.; Villares, A. Novel isolation of water-soluble polysaccharides from the fruiting bodies of *Pleurotus ostreatus* mushrooms. *Carbohydrate Research*, **2012**, *358*, 72–77.

224. Wang, Y.-X.; Zhang, T.; Xin, Y.; Huang, X.-J.; Yin, J.-Y.; Nie, S.-P. Comprehensive evaluation of alkali-extracted polysaccharides from *Agrocybe cylindracea*: Comparison on structural characterization. *Carbohydrate Polymers*, **2021**, *255*, 117502.

225. OHNO, N.; ADACHI, Y.; SUZUKI, I.; SATO, K.; OIKAWA, S.; YADOMAE, T. Characterization of the antitumor glucan obtained from liquid-cultured

Grifola frondosa. Chemical and Pharmaceutical Bulletin, **1986**, *34* (4), 1709–1715.

226. Wang, Z. Bin; Pei, J. J.; Ma, H. Le; Cai, P. F.; Yan, J. K. Effect of extraction media on preliminary characterizations and antioxidant activities of *Phellinus linteus* polysaccharides. *Carbohydrate Polymers*, **2014**, *109*, 49–55.

227. Huang, Q.; Jin, Y.; Zhang, L.; Cheung, P. C. K.; Kennedy, J. F. Structure, molecular size and antitumor activities of polysaccharides from *Poria cocos* mycelia produced in fermenter. *Carbohydrate Polymers*, **2007**, *70* (3), 324–333.

228. Szwengiel, A.; Stachowiak, B. Deproteinization of water-soluble ß-glucan during acid extraction from fruiting bodies of *Pleurotus ostreatus* mushrooms. *Carbohydrate Polymers*, **2016**, *146*, 310–319.

229. Zhao, Y. M.; Song, J. H.; Wang, J.; Yang, J. M.; Wang, Z. B.; Liu, Y. H. Optimization of cellulase-assisted extraction process and antioxidant activities of polysaccharides from *Tricholoma mongolicum* Imai. *Journal of the science of food and agriculture*, **2016**, *96* (13), 4484–4491.

230. Zhu, Y.; Li, Q.; Mao, G.; Zou, Y.; Feng, W.; Zheng, D.; Wang, W.; Zhou, L.; Zhang, T.; Yang, J.; Yang, L.; Wu, X. Optimization of enzyme-assisted extraction and characterization of polysaccharides from *Hericium erinaceus*. *Carbohydrate Polymers*, **2014**, *101* (1), 606–613.

231. Qin, Y.; Zhang, Z.; Song, T.; Lv, G. Optimization of enzyme-assisted extraction of antitumor polysaccharides from *Hericium erinaceus* mycelia. *Food Science and Technology Research*, **2017**, *23* (1), 31–39.

232. Zhao, Y. ming; Wang, J.; Wu, Z. gang; Yang, J. ming; Li, W.; Shen, L. xia. Extraction, purification and anti-proliferative activities of polysaccharides from *Lentinus edodes*. *International Journal of Biological Macromolecules*, **2016**, *93*, 136–144.

233. Hwang, A. Y.; Yang, S. C.; Kim, J.; Lim, T.; Cho, H.; Hwang, K. T. Effects of non-traditional extraction methods on extracting bioactive compounds from chaga mushroom (*Inonotus obliquus*) compared with hot water extraction. *Lwt*, **2019**, *110* (December 2018), 80–84.

234. Li, S.; Liu, M.; Zhang, C.; Tian, C.; Wang, X.; Song, X.; Jing, H.; Gao, Z.; Ren, Z.; Liu, W.; Zhang, J.; Jia, L. Purification, in vitro antioxidant and in vivo anti-aging activities of soluble polysaccharides by enzyme-assisted extraction from *Agaricus bisporus*. *International Journal of Biological Macromolecules*, **2018**, *109*, 457–466.

235. Liu, G.; Ye, J.; Li, W.; Zhang, J.; Wang, Q.; Zhu, X. ai; Miao, J. yin; Huang, Y. hui; Chen, Y. jiao; Cao, Y. Extraction, structural characterization, and immunobiological activity of ABP Ia polysaccharide from *Agaricus bisporus*. *International Journal of Biological Macromolecules*, **2020**, *162*, 975–984.

236. Junior Letti, L. A.; Destéfanis Vítola, F. M.; Vinícius de Melo Pereira, G.; Karp, S. G.; Pedroni Medeiros, A. B.; Ferreira da Costa, E. S.; Bissoqui, L.; Soccol, C. R. Solid-State Fermentation for the Production of Mushrooms. In *Current Developments in Biotechnology and Bioengineering*; Elsevier, 2018; pp. 285–318.

237. Bruce, A.; Palfreyman, J. (Editors). *Forest Products Biotechnology*; CRC Press, 1997; Vol. 74.

238. Koutrotsios, G.; Mountzouris, K. C.; Chatzipavlidis, I.; Zervakis, G. I. Bioconversion of lignocellulosic residues by *Agrocybe cylindracea* and *Pleurotus ostreatus* mushroom fungi-Assessment of their effect on the final product and spent substrate properties. *Food Chemistry*, **2014**, *161*, 127–135.

239. Fazenda, M. L.; Seviour, R.; McNeil, B.; Harvey, L. M. Submerged Culture Fermentation of "Higher Fungi": The Macrofungi. In *Advances in Applied Microbiology*; 2008; Vol. 63, pp. 33–103.

240. Berovic, M.; Podgornik, B. B. Cultivation of Medicinal Fungi in Bioreactors. In *Mushroom Biotechnology: Developments and Applications*; Petre, M. (Editor); Academic Press, 2016; pp. 155–171.

241. Elisashvili, V. Submerged cultivation of medicinal mushrooms: Bioprocesses and products (review). *International Journal of Medicinal Mushrooms*, **2012**, *14* (3), 211–239.

242. Lee, W. Y.; Park, Y.; Ahn, J. K.; Ka, K. H.; Park, S. Y. Factors influencing the production of endopolysaccharide and exopolysaccharide from *Ganoderma applanatum*. *Enzyme and Microbial*

Technology, **2007**, *40* (2), 249–254.

243. Kim, H. O.; Lim, J. M.; Joo, J. H.; Kim, S. W.; Hwang, H. J.; Choi, J. W.; Yun, J. W. Optimization of submerged culture condition for the production of mycelial biomass and exopolysaccharides by *Agrocybe cylindracea*. *Bioresource Technology*, **2005**, *96* (10), 1175–1182.

244. Chang, M. Y.; Tsai, G. J.; Houng, J. Y. Optimization of the medium composition for the submerged culture of *Ganoderma lucidum* by Taguchi array design and steepest ascent method. *Enzyme and Microbial Technology*, **2006**, *38* (3–4), 407–414.

245. Lin, E. S.; Sung, S. C. Cultivating conditions influence exopolysaccharide production by the edible Basidiomycete *Antrodia cinnamomea* in submerged culture. *International Journal of Food Microbiology*, **2006**, *108* (2), 182–187.

246. Lin, E.-S.; Chen, Y.-H. Factors affecting mycelial biomass and exopolysaccharide production in submerged cultivation of *Antrodia cinnamomea* using complex media. *Bioresource technology*, **2007**, *98* (13), 2511–2517.

247. Jo, W.-S.; Kang, M.-J.; Choi, S.-Y.; Yoo, Y.-B.; Seok, S.-J.; Jung, H.-Y. Culture Conditions for Mycelial Growth of *Coriolus versicolor* . *Mycobiology*, **2010**, *38* (3), 195.

248. Liu, X. C.; Li, H.; Kang, T.; Zhu, Z. Y.; Liu, Y. L.; Sun, H. Q.; Pan, L. C. The effect of fermentation conditions on the structure and anti-tumor activity of polysaccharides from: *Cordyceps gunnii*. *RSC Advances*, **2019**, *9* (32), 18205–18216.

249. Wagner, R.; Mitchell, D. A.; Sassaki, G. L.; Amazonas, M. A. L. de A. Links between morphology and physiology of *Ganoderma lucidum* in submerged culture for the production of exopolysaccharide. *Journal of Biotechnology*, **2004**, *114* (1–2), 153–164.

250. Veiter, L.; Rajamanickam, V.; Herwig, C. The filamentous fungal pellet—relationship between morphology and productivity. *Applied Microbiology and Biotechnology*, **2018**, *102* (7), 2997–3006.

251. Cui, F. J.; Chen, X. X.; Liu, W. M.; Sun, W. J.; Huo, S.; Yang, Y. Control of *Grifola frondosa* Morphology by Agitation and Aeration for Improving

Mycelia and Exo-Polymer Production. *Applied Biochemistry and Biotechnology*, **2016**, *179* (3), 459–473.

252. Elisashvili, V.; Kachlishvili, E. Physiological regulation of laccase and manganese peroxidase production by white-rot Basidiomycetes. *Journal of Biotechnology*, **2009**, *144* (1), 37–42.

253. Elisashvili, V.; Penninckx, M.; Kachlishvili, E.; Tsiklauri, N.; Metreveli, E.; Kharziani, T.; Kvesitadze, G. *Lentinus edodes* and *Pleurotus* species lignocellulolytic enzymes activity in submerged and solid-state fermentation of lignocellulosic wastes of different composition. *Bioresource Technology*, **2008**, *99* (3), 457–462.

254. Singh, A. P.; Singh, T. Biotechnological applications of wood-rotting fungi: A review. *Biomass and Bioenergy*, **2014**, *62*, 198–206.

255. Chen, H.; Yan, M.; Zhu, J.; Xu, X. Enhancement of exo-polysaccharide production and antioxidant activity in submerged cultures of *Inonotus obliquus* by lignocellulose decomposition. *Journal of Industrial Microbiology and Biotechnology*, **2011**, *38* (2), 291–298.

256. Xu, X.; Hu, Y.; Quan, L. Production of bioactive polysaccharides by *Inonotus obliquus* under submerged fermentation supplemented with lignocellulosic biomass and their antioxidant activity. *Bioprocess and Biosystems Engineering*, **2014**, *37* (12), 2483–2492.

257. Krupodorova, T.; Barshteyn, V. Alternative substrates for higher mushrooms mycelia cultivation. *Journal of BioScience and Biotechnology*, **2015**, *1* (4), 339–347.

258. Choi, J. W.; Ra, K. S.; Kim, S. Y.; Yoon, T. J.; Yu, K. W.; Shin, K. S.; Lee, S. P.; Suh, H. J. Enhancement of anti-complementary and radical scavenging activities in the submerged culture of *Cordyceps sinensis* by addition of citrus peel. *Bioresource Technology*, **2010**, *101* (15), 6028–6034.

259. Wolters, N.; Schabronath, C.; Schembecker, G.; Merz, J. Efficient conversion of pretreated brewer's spent grain and wheat bran by submerged cultivation of *Hericium erinaceus*. *Bioresource Technology*, **2016**, *222*, 123–129.

260. Matjuškova, N.; Okmane, L.; Zala, D.; Rozenfelde, L.; Puke, M.; Kruma, I.; Vedernikovs, N.; Rapoport, A. Effect of

lignin-containing media on growth of medicinal mushroom *Lentinula edodes*. *Proceedings of the Latvian Academy of Sciences, Section B: Natural, Exact, and Applied Sciences*, **2017**, *71* (1), 38–42.

261. Abdeshahian, P.; Ascencio, J. J.; Philippini, R. R.; Antunes, F. A. F.; dos Santos, J. C.; da Silva, S. S. Utilization of sugarcane straw for production of β-glucan biopolymer by *Lasiodiplodia theobromae* CCT 3966 in batch fermentation process. *Bioresource Technology*, **2020**, *314*, 123716.

262. Papadaki, A.; Kachrimanidou, V.; Papanikolaou, S.; Philippoussis, A.; Diamantopoulou, P. Upgrading grape pomace through *Pleurotus* spp. Cultivation for the production of enzymes and fruiting bodies. *Microorganisms*, **2019**, *7* (7).

263. Elisashvili, V.; Penninckx, M.; Kachlishvili, E.; Asatiani, M.; Kvesitadze, G. Use of *Pleurotus dryinus* for lignocellulolytic enzymes production in submerged fermentation of mandarin peels and tree leaves. *Enzyme and Microbial Technology*, **2006**, *38* (7), 998–1004.

264. Schisler, L. C.; Volkoff, O. The Effect of Safflower Oil on Mycelial Growth of Boletaceae in Submerged Liquid Cultures. *Mycologia*, **1977**, *69* (1), 118.

265. NAIR, N. G.; HOLLEY, M. P.; SONG, C. H.; CHO, K. Y. Lipid metabolism of *Pleurotus sajor caju*. *Annals of Applied Biology*, **1990**, *116* (3), 455–462.

266. Reverberi, M.; Di Mario, F.; Tomati, U. β-Glucan synthase induction in mushrooms grown on olive mill wastewaters. *Applied Microbiology and Biotechnology*, **2004**, *66* (2), 217–225.

267. Hsieh, C.; Wang, H. L.; Chen, C. C.; Hsu, T. H.; Tseng, M. H. Effect of plant oil and surfactant on the production of mycelial biomass and polysaccharides in submerged culture of *Grifola frondosa*. *Biochemical Engineering Journal*, **2008**, *38* (2), 198–205.

268. Yang, F.-C.; Ke, Y.-F.; Kuo, S.-S. Effect of fatty acids on the mycelial growth and polysaccharide formation by *Ganoderma lucidum* in shake flask cultures. *Enzyme and Microbial Technology*, **2000**, *27* (3–5), 295–301.

269. Wagner, R.; Mitchell, D. A.; Sassaki, G. L.; De Almeida Amazonas, M. A. L.; Berovič, M. Current Techniques for the Cultivation of *Ganoderma lucidum* for the Production of Biomass, Ganoderic Acid and Polysaccharides. *Food Technology and Biotechnology*, **2003**, *41* (4), 371–382.

270. Xu, X.; Quan, L.; Shen, M. Effect of chemicals on production, composition and antioxidant activity of polysaccharides of *Inonotus obliquus*. *International Journal of Biological Macromolecules*, **2015**, *77*, 143–150.

271. Damerau, A.; Kakko, T.; Tian, Y.; Tuomasjukka, S.; Sandell, M.; Hopia, A.; Yang, B. Effect of supercritical CO2 plant extract and berry press cakes on stability and consumer acceptance of frozen Baltic herring (*Clupea harengus membras*) mince. *Food Chemistry*, **2020**, *332*, 127385.

272. Trygg, J.; Yildir, E.; Kolakovic, R.; Sandler, N.; Fardim, P. Anionic cellulose beads for drug encapsulation and release. *Cellulose*, **2014**, *21* (3), 1945–1955.

273. Smiderle, F. R.; Olsen, L. M.; Carbonero, E. R.; Baggio, C. H.; Freitas, C. S.; Marcon, R.; Santos, A. R. S.; Gorin, P. A. J.; Iacomini, M. Anti-inflammatory and analgesic properties in a rodent model of a (1→3),(1→6)-linked β-glucan isolated from *Pleurotus pulmonarius*. *European Journal of Pharmacology*, **2008**, *597* (1–3), 86–91.

274. Gorin, P. A. J.; Iacomini, M. Polysaccharides of the lichens *Cetraria islandica* and *Ramalina usnea*. *Carbohydrate Research*, **1984**, *128* (1), 119–132.

275. Masuko, T.; Minami, A.; Iwasaki, N.; Majima, T.; Nishimura, S.-I.; Lee, Y. C. Carbohydrate analysis by a phenol-sulfuric acid method in microplate format. *Analytical biochemistry*, **2005**, *339* (1), 69–72.

276. Markwell, M. A.; Haas, S. M.; Bieber, L. L.; Tolbert, N. E. A Modification of the Lowry Procedure to Simplify Protein Determination in Membrane and Lipoprotein Samples. *Analytical Biochemistry*, **1978**, *87*, 206–210.

277. Redmile-Gordon, M. A.; Armenise, E.; White, R. P.; Hirsch, P. R.; Goulding, K. W. T. A comparison of two colorimetric assays, based upon Lowry and Bradford techniques, to estimate total protein in soil extracts. *Soil Biology and Biochemistry*, **2013**, *67*, 166–173.

278. Magalhães, L. M.; Santos, F.; Segundo, M. A.; Reis, S.; Lima, J. L. F. C. F. C. Rapid microplate high-throughput

methodology for assessment of Folin-Ciocalteu reducing capacity. *Talanta*, **2010**, *83* (2), 441–447.

279. Sundberg, A.; Sundberg, K.; Lillandt, C.; Holmbom, B. Determination of hemicelluloses and pectins in wood and pulp fibres by acid methanolysis and gas chromatography. *Nordic Pulp and Paper Research Journal*, **1996**, *4*, 216–219.

280. Ciucanu, I.; Kerek, F. A simple and rapid method for the permethylation of carbohydrates. *Carbohydrate Research*, **1984**, *131* (2), 209–217.

281. Laine, C.; Tamminen, T.; Vikkula, A.; Vuorinen, T.; Vilkkula, a; Vuorinen, T. Methylation Analysis as a Tool for Structural Analysis of Wood Polysaccharides. *Holzforschung*, **2002**, *56* (6), 607–614.

282. RStudio. RStudio: Integrated Development for R. RStudio, Inc., Boston, MA URL http://www.rstudio.com/. **2020**.

283. Huang, H. C.; Chen, C. I.; Hung, C. N.; Liu, Y. C. Experimental analysis of the oil addition effect on mycelia and polysaccharide productions in *Ganoderma lucidum* submerged culture. *Bioprocess and Biosystems Engineering*, **2009**, *32* (2), 217–224.

284. Ntougias, S.; Gaitis, F.; Katsaris, P.; Skoulika, S.; Iliopoulos, N.; Zervakis, G. I. The effects of olives harvest period and production year on olive mill wastewater properties – Evaluation of *Pleurotus* strains as bioindicators of the effluent's toxicity. *Chemosphere*, **2013**, *92* (4), 399–405.

285. Zerva, A.; Papaspyridi, L. M.; Christakopoulos, P.; Topakas, E. Valorization of Olive Mill Wastewater for the Production of β-glucans from Selected Basidiomycetes. *Waste and Biomass Valorization*, **2017**, *8* (5), 1721–1731.

286. Xavier-Santos, S.; Carvalho, C. C.; Bonfá, M.; Silva, R.; Capelari, M.; Gomes, E. Screening for pectinolytic activity of wood-rotting basidiomycetes and characterization of the enzymes. *Folia Microbiologica*, **2004**, *49* (1), 46–52.

287. Ognyanov, M.; Remoroza, C.; Schols, H. A.; Georgiev, Y.; Kratchanova, M.; Kratchanov, C. Isolation and structure elucidation of pectic polysaccharide from rose hip fruits (Rosa canina L.). *Carbohydrate Polymers*, **2016**, *151*, 803–811.

288. Smiderle, F. R.; Olsen, L. M.; Ruthes, A. C.; Czelusniak, P. A.; Santana-Filho, A. P.; Sassaki, G. L.; Gorin, P. A. J.; Iacomini, M. Exopolysaccharides, proteins and lipids in *Pleurotus pulmonarius* submerged culture using different carbon sources. *Carbohydrate Polymers*, **2012**, *87* (1), 368–376.

289. Xu, X.; Wu, P.; Wang, T.; Yan, L.; Lin, M.; Chen, C. Synergistic effects of surfactant-assisted biodegradation of wheat straw and production of polysaccharides by *Inonotus obliquus* under submerged fermentation. *Bioresource Technology*, **2019**, *278* (November 2018), 43–50.

290. Shih, I. L.; Pan, K.; Hsieh, C. Influence of nutritional components and oxygen supply on the mycelial growth and bioactive metabolites production in submerged culture of *Antrodia cinnamomea*. *Process Biochemistry*, **2006**, *41* (5), 1129–1135.

291. Shih, I. L.; Chou, B. W.; Chen, C. C.; Wu, J. Y.; Hsieh, C. Study of mycelial growth and bioactive polysaccharide production in batch and fed-batch culture of *Grifola frondosa*. *Bioresource Technology*, **2008**, *99* (4), 785–793.

292. Wang, L.-X.; Lu, Z.-M.; Geng, Y.; Zhang, X.-M.; Xu, G.-H.; Shi, J.-S.; Xu, Z.-H. Stimulated production of steroids in *Inonotus obliquus* by host factors from birch. *Journal of Bioscience and Bioengineering*, **2014**, *118* (6), 728–731.

293. Martínez-Abad, A.; Giummarella, N.; Lawoko, M.; Vilaplana, F. Differences in extractability under subcritical water reveal interconnected hemicellulose and lignin recalcitrance in birch hardwoods. *Green Chemistry*, **2018**, *20* (11), 2534–2546.

294. Švagelj, M.; Berovič, M.; Boh, B.; Menard, A.; Simčič, S.; Wraber, B. Solid-state cultivation of *Grifola frondosa* (Dicks: Fr) S.F. Gray biomass and immunostimulatory effects of fungal intra- and extracellular β-polysaccharides. *New Biotechnology*, **2008**, *25* (2–3), 150–156.

295. Sánchez, Ó. J.; Montoya, S. Assessment of Polysaccharide and Biomass Production from Three White-Rot Fungi by Solid-State Fermentation Using Wood and Agro-Industrial Residues: A Kinetic Approach. *Forests*,

2020, *11* (10), 1055.

296. Habijanič, J.; Berovič, M. The Relevance of Solid-state Substrate Moisturing on *Ganoderma lucidum* Biomass Cultivation. *Food Technology and Biotechnology*, **2000**, *38* (3), 225–228.

297. Smiderle, F. R.; Sassaki, G. L.; Van Griensven, L. J. L. D.; Iacomini, M. Isolation and chemical characterization of a glucogalactomannan of the medicinal mushroom *Cordyceps militaris*. *Carbohydrate Polymers*, **2013**, *97* (1), 74–80.

298. Li, T.; Chen, L.; Wu, D.; Dong, G.; Chen, W.; Zhang, H.; Yang, Y.; Wu, W. The Structural Characteristics and Biological Activities of Intracellular Polysaccharide Derived from Mutagenic *Sanghuangporous sanghuang* Strain. *Molecules*, **2020**, *25* (16), 3693.

299. Tao, T. L.; Cui, F. J.; Chen, X. X.; Sun, W. J.; Huang, D. M.; Zhang, J.; Yang, Y.; Wu, D.; Liu, W. M. Improved mycelia and polysaccharide production of *Grifola frondosa* by controlling morphology with microparticle Talc. *Microbial Cell Factories*, **2018**, *17* (1), 1–10.

300. Bernabé, M.; Salvachúa, D.; Jiménez-Barbero, J.; Leal, J. A.; Prieto, A. Structures of wall heterogalactomannans isolated from three genera of entomopathogenic fungi. *Fungal Biology*, **2011**, *115* (9), 862–870.

301. Xiao, L.; Shi, Z.; Bai, Y.; Wei, W.; Zhang, X.-M.; Run-Cang, S. Biodegradation of Lignocellulose by White-Rot Fungi: Structural Characterization of Water-Soluble Hemicelluloses. **2013**.

302. Sun, R. C.; Tomkinson, J. Physicochemical Characterization of Hemicelluloses from Steamed Aspen and Birchwood. *International Journal of Polymer Analysis and Characterization*, **1999**, *5* (2), 181–193.

303. Li, J.; Jia, X.; Yao, Y.; Bai, Y. Characterization of exopolysaccharide produced by *Phellinus vaninii* (Agaricomycetes) and antioxidant potential for meat batter. *International Journal of Medicinal Mushrooms*, **2019**, *21* (5), 459–468.

304. Fraga, I.; Coutinho, J.; Bezerra, R. M.; Dias, A. A.; Marques, G.; Nunes, F. M. Influence of culture medium growth variables on *Ganoderma lucidum* exopolysaccharides structural features. *Carbohydrate Polymers*, **2014**, *111*, 936–946.

305. Shu, C.-H.; Lung, M.-Y. Effect of pH on the production and molecular weight distribution of exopolysaccharide by *Antrodia camphorata* in batch cultures. *Process Biochemistry*, **2004**, *39* (8), 931–937.

306. Chao, C. H.; Wu, H. J.; Lu, M. K. Promotion of fungal growth and underlying physiochemical changes of polysaccharides in *Rigidoporus ulmarius*, an edible Basidiomycete mushroom. *Carbohydrate Polymers*, **2011**, *85* (3), 609–614.

307. Selbmann, L.; Stingele, F.; Petruccioli, M. Exopolysaccharide production by filamentous fungi: the example of *Botryosphaeria rhodina*. *Antonie van Leeuwenhoek*, **2003**, *84* (2), 135–145.

308. Selbmann, L.; Onofri, S.; Fenice, M.; Federici, F.; Petruccioli, M. Production and structural characterization of the exopolysaccharide of the Antarctic fungus *Phoma herbarum* CCFEE 5080. *Research in Microbiology*, **2002**, *153* (9), 585–592.

309. Mohaček-Grošev, V.; Božac, R.; Puppels, G. J. Vibrational spectroscopic characterization of wild growing mushrooms and toadstools. *Spectrochimica Acta - Part A: Molecular and Biomolecular Spectroscopy*, **2001**, *57* (14), 2815–2829.

310. De Gussem, K.; Vandenabeele, P.; Verbeken, A.; Moens, L. Raman spectroscopic study of *Lactarius* spores (Russulales, Fungi). *Spectrochimica Acta - Part A: Molecular and Biomolecular Spectroscopy*, **2005**, *61* (13–14), 2896–2908.

311. Synytsya, A.; Novak, M. Structural analysis of glucans. *Annals of translational medicine*, **2014**, *2* (2), 17.

312. Novák, M.; Synytsya, A.; Gedeon, O.; Slepička, P.; Procházka, V.; Synytsya, A.; Blahovec, J.; Hejlová, A.; Čopíková, J. Yeast β(1-3),(1-6)-d-glucan films: Preparation and characterization of some structural and physical properties. *Carbohydrate Polymers*, **2012**, *87* (4), 2496–2504.

313. Kacuráková, M.; Capek, P.; Sasinková, V.; Wellner, N.; Ebringerová, A. FT-IR study of plant cell wall model compounds: Pectic polysaccharides and

hemicelluloses. *Carbohydrate Polymers*, **2000**, *43* (2), 195–203.

314. Singh, R.; Singh, S.; Trimukhe, K. D.; Pandare, K. V.; Bastawade, K. B.; Gokhale, D. V.; Varma, A. J. Lignin-carbohydrate complexes from sugarcane bagasse: Preparation, purification, and characterization. *Carbohydrate Polymers*, **2005**, *62* (1), 57–66.

315. Wold, C. W.; Gerwick, W. H.; Wangensteen, H.; Inngjerdingen, K. T. Bioactive triterpenoids and water-soluble melanin from *Inonotus obliquus* (Chaga) with immunomodulatory activity. *Journal of Functional Foods*, **2020**, *71*, 104025.

316. Zhou, X.; McCallum, N. C.; Hu, Z.; Cao, W.; Gnanasekaran, K.; Feng, Y.; Stoddart, J. F.; Wang, Z.; Gianneschi, N. C. Artificial Allomelanin Nanoparticles. *ACS Nano*, **2019**, *13* (10), 10980–10990.

317. Santillán-Moreno, A.; Martínez-Bustos, F.; Castaño-Tostado, E.; Amaya-Llano, S. L. Physicochemical Characterization of Extruded Blends of Corn Starch-Whey Protein Concentrate-Agave tequilana Fiber. *Food and Bioprocess Technology*, **2011**, *4* (5), 797–808.

318. Hornung, P. S.; Ávila, S.; Lazzarotto, M.; da Silveira Lazzarotto, S. R.; de Andrade de Siqueira, G. L.; Schnitzler, E.; Ribani, R. H. Enhancement of the functional properties of Dioscoreaceas native starches: Mixture as a green modification process. *Thermochimica Acta*, **2017**, *649*, 31–40.

319. Ouyang, Q. Q.; Hu, Z.; Li, S. D.; Quan, W. Y.; Wen, L. L.; Yang, Z. M.; Li, P. W. Thermal degradation of agar: Mechanism and toxicity of products. *Food Chemistry*, **2018**, *264* (April), 277–283.

320. Cardozo, F. T. G. S.; Camelini, C. M.; Cordeiro, M. N. S.; Mascarello, A.; Malagoli, B. G.; Larsen, I. v.; Rossi, M. J.; Nunes, R. J.; Braga, F. C.; Brandt, C. R.; Simões, C. M. O. Characterization and cytotoxic activity of sulfated derivatives of polysaccharides from *Agaricus brasiliensis*. *International Journal of Biological Macromolecules*, **2013**, *57*, 265–272.

321. Hong, J. H.; Choi, Y. H. Physico-chemical properties of protein-bound polysaccharide from *Agaricus blazei* Murill prepared by Ultrafiltration and spray drying process. *International Journal of Food Science and Technology*, **2007**, *42* (1), 1–8.

322. Kagimura, F. Y.; Da Cunha, M. A. A.; Theis, T. V.; Malfatti, C. R. M.; Dekker, R. F. H.; Barbosa, A. M.; Teixeira, S. D.; Salomé, K. Carboxymethylation of (1 → 6)-β-glucan (lasiodiplodan): Preparation, characterization and antioxidant evaluation. *Carbohydrate Polymers*, **2015**, *127*, 390–399.

323. Collard, F. X.; Blin, J. A review on pyrolysis of biomass constituents: Mechanisms and composition of the products obtained from the conversion of cellulose, hemicelluloses and lignin. *Renewable and Sustainable Energy Reviews*, **2014**, *38*, 594–608.

324. Sun, R. C.; Sun, X. F. Fractional and structural characterization of hemicelluloses isolated by alkali and alkaline peroxide from barley straw. *Carbohydrate Polymers*, **2002**, *49* (4), 415–423.

325. Sun, S.-L. L.; Wen, J.-L. L.; Ma, M.-G. G.; Sun, R.-C. C. Successive alkali extraction and structural characterization of hemicelluloses from sweet sorghum stem. *Carbohydrate Polymers*, **2013**, *92* (2), 2224–2231.

326. Galinari, É.; Sabry, D. A.; Sassaki, G. L.; Macedo, G. R.; Passos, F. M. L.; Mantovani, H. C.; Rocha, H. A. O. Chemical structure, antiproliferative and antioxidant activities of a cell wall α-D-mannan from yeast *Kluyveromyces marxianus*. *Carbohydrate Polymers*, **2017**, *157*, 1298–1305.

327. Ustyuzhanina, N. E.; Kulakovskaya, E. V.; Kulakovskaya, T. V.; Menshov, V. M.; Dmitrenok, A. S.; Shashkov, A. S.; Nifantiev, N. E. Mannan and phosphomannan from *Kuraishia capsulata* yeast. *Carbohydrate Polymers*, **2018**, *181* (August 2017), 624–632.

328. Fan, J.; Zhang, J.; Tang, Q.; Liu, Y.; Zhang, A.; Pan, Y. Structural elucidation of a neutral fucogalactan from the mycelium of *Coprinus comatus*. *Carbohydrate Research*, **2006**, *341* (9), 1130–1134.

329. Adorjan, I.; Jääskeläinen, A. S.; Vuorinen, T. Synthesis and characterization of the hexenuronic acid model methyl 4-deoxy-β-l-threo-hex-4-enopyranosiduronic acid. *Carbohydrate Research*, **2006**, *341* (14), 2439–2443.

330. Jongkees, S. A. K.; Withers, S. G. Glycoside cleavage by a new

mechanism in unsaturated glucuronyl hydrolases. *Journal of the American Chemical Society*, **2011**, *133* (48), 19334–19337.

331. Henry, C.; Fontaine, T.; Heddergott, C.; Robinet, P.; Aimanianda, V.; Beau, R.; Beauvais, A.; Mouyna, I.; Prevost, M. C.; Fekkar, A.; Zhao, Y.; Perlin, D.; Latgé, J. P. Biosynthesis of cell wall mannan in the conidium and the mycelium of *Aspergillus fumigatus*. *Cellular Microbiology*, **2016**, *18* (12), 1881–1891.

332. Dais, P.; Perlin, A. S. High-field, 13C-N.M.R. spectroscopy of β-d-glucans, amylopectin, and glycogen. *Carbohydrate Research*, **1982**, *100* (1), 103–116.

333. Giummarella, N.; Lawoko, M. Structural Basis for the Formation and Regulation of Lignin-Xylan Bonds in Birch. *ACS Sustainable Chemistry and Engineering*, **2016**, *4* (10), 5319–5326.

334. Willför, S.; Sjöholm, R.; Laine, C.; Roslund, M.; Hemming, J.; Holmbom, B. Characterisation of water-soluble galactoglucomannans from Norway spruce wood and thermomechanical pulp. *Carbohydrate Polymers*, **2003**, *52* (2), 175–187.

335. Teleman, A.; Nordström, M.; Tenkanen, M.; Jacobs, A.; Dahlman, O. Isolation and characterization of O-acetylated glucomannans from aspen and birch wood. *Carbohydrate Research*, **2003**, *338* (6), 525–534.

336. Teleman, A.; Lundqvist, J.; Tjerneld, F.; Stålbrand, H.; Dahlman, O. Characterization of acetylated 4-O-methylglucuronoxylan isolated from aspen employing 1H and 13C NMR spectroscopy. *Carbohydrate Research*, **2000**, *329* (4), 807–815.

337. Wong, K.-H.; Cheung, P. C. K. Sclerotia: Emerging Functional Food Derived from Mushrooms. In *Mushrooms as Functional Foods*; John Wiley & Sons, Inc., Hoboken, NJ, USA, 2008; pp. 111–146.

338. Azarpira, A.; Ralph, J.; Lu, F. Catalytic Alkaline Oxidation of Lignin and its Model Compounds: A Pathway to Aromatic Biochemicals. *Bioenergy Research*, **2014**, *7* (1), 78–86.

339. Choma, A.; Wiater, A.; Komaniecka, I.; Paduch, R.; Pleszczyńska, M.; Szczodrak, J. Chemical characterization of a water insoluble (1 → 3)-α-d-glucan from an alkaline

extract of *Aspergillus wentii*. *Carbohydrate Polymers*, **2013**, *91* (2), 603–608.

340. Kozarski, M.; Klaus, A.; Niksic, M.; Jakovljevic, D.; Helsper, J. P. F. G.; Van Griensven, L. J. L. D. Antioxidative and immunomodulating activities of polysaccharide extracts of the medicinal mushrooms *Agaricus bisporus*, *Agaricus brasiliensis*, *Ganoderma lucidum* and *Phellinus linteus*. *Food Chemistry*, **2011**, *129* (4), 1667–1675.

341. Sari, M.; Prange, A.; Lelley, J. I.; Hambitzer, R. Screening of beta-glucan contents in commercially cultivated and wild growing mushrooms. *Food Chemistry*, **2017**, *216*, 45–51.

342. Koutrotsios, G.; Patsou, M.; Mitsou, E. K.; Bekiaris, G.; Kotsou, M.; Tarantilis, P. A.; Pletsa, V.; Kyriacou, A.; Zervakis, G. I. Valorization of olive by-products as substrates for the cultivation of *Ganoderma lucidum* and *Pleurotus ostreatus* mushrooms with enhanced functional and prebiotic properties. *Catalysts*, **2019**, *9* (6).

343. Heleno, S. A.; Barros, L.; Martins, A.; Queiroz, M. J. R. P.; Santos-Buelga, C.; Ferreira, I. C. F. R. Fruiting body, spores and in vitro produced mycelium of *Ganoderma lucidum* from Northeast Portugal: A comparative study of the antioxidant potential of phenolic and polysaccharidic extracts. *Food Research International*, **2012**, *46* (1), 135–140.

344. Munkhbayar, D.; Ariuntungalag, J.; Delgersuuri, G.; Badamkhand, D. Enzymatic technology for Sea buckthorn oil extraction and its biochemical analysis. *Mongolian Journal of Chemistry*, **2014**, *15* (41), 62–65.

345. Clemente, T. E.; Cahoon, E. B. Soybean oil: Genetic approaches for modification of functionality and total content. *Plant Physiology*, **2009**, *151* (3), 1030–1040.

346. Yang, B.; Kallio, H. P. Fatty Acid Composition of Lipids in Sea Buckthorn (*Hippophaë rhamnoides* L.) Berries of Different Origins. *Journal of Agricultural and Food Chemistry*, **2001**, *49* (4), 1939–1947.

347. Money, N. P. Are mushrooms medicinal? *Fungal Biology*. Elsevier B.V. April 1, 2016, pp. 449–453.

348. Komura, D. L.; Ruthes, A. C.;

Carbonero, E. R.; Gorin, P. A. J.; Iacomini, M. Water-soluble polysaccharides from *Pleurotus ostreatus var. florida* mycelial biomass. *International Journal of Biological Macromolecules*, **2014**, *70*, 354–359.

349. Kojima, H.; Akaki, J.; Nakajima, S.; Kamei, K.; Tamesada, M. Structural analysis of glycogen-like polysaccharides having macrophage-activating activity in extracts of *Lentinula edodes* mycelia. *Journal of Natural Medicines*, **2010**, *64* (1), 16–23.

350. Abreu, H.; Zavadinack, M.; Smiderle, F. R.; Cipriani, T. R.; Cordeiro, L. M. C.; Iacomini, M. Polysaccharides from *Pleurotus eryngii*: Selective extraction methodologies and their modulatory effects on THP-1 macrophages. *Carbohydrate Polymers*, **2021**, *252* (September 2020), 117177.

351. Xue, J.; Tong, S.; Wang, Z.; Liu, P. Chemical Characterization and Hypoglycaemic Activities In Vitro of Two Polysaccharides from *Inonotus obliquus* by Submerged Culture. *Molecules*, **2018**, *23* (12), 3261.

352. Hoshi, H.; Yagi, Y.; Iijima, H.; Matsunaga, K.; Ishihara, Y.; Yasuhara, T. Isolation and Characterization of a Novel Immunomodulatory α-Glucan−Protein Complex from the Mycelium of *Tricholoma matsutake* in Basidiomycetes. *Journal of Agricultural and Food Chemistry*, **2005**, *53* (23), 8948–8956.

353. Volman, J. J.; Mensink, R. P.; van Griensven, L. J. L. D.; Plat, J. Effects of α-glucans from *Agaricus bisporus* on ex vivo cytokine production by LPS and PHA-stimulated PBMCs; a placebo-controlled study in slightly hypercholesterolemic subjects. *European Journal of Clinical Nutrition*, **2010**, *64* (7), 720–726.

354. Bittencourt, V. C. B.; Figueiredo, R. T.; da Silva, R. B.; Mourão-Sá, D. S.; Fernandez, P. L.; Sassaki, G. L.; Mulloy, B.; Bozza, M. T.; Barreto-Bergter, E. An α-Glucan of *Pseudallescheria boydii* Is Involved in Fungal Phagocytosis and Toll-like Receptor Activation. *Journal of Biological Chemistry*, **2006**, *281* (32), 22614–22623.

355. Volman, J. J.; Helsper, J. P. F. G.; Wei, S.; Baars, J. J. P.; van Griensven, L. J. L. D.; Sonnenberg, A. S. M.; Mensink, R. P.; Plat, J. Effects of mushroom-derived β-glucan-rich polysaccharide extracts on nitric oxide production by bone marrow-derived macrophages and nuclear factor-κB transactivation in Caco-2 reporter cells: Can effects be explained by structure? *Molecular Nutrition & Food Research*, **2010**, *54* (2), 268–276.

356. Smiderle, F. R.; Ruthes, A. C.; van Arkel, J.; Chanput, W.; Iacomini, M.; Wichers, H. J.; Van Griensven, L. J. L. D. Polysaccharides from *Agaricus bisporus* and *Agaricus brasiliensis* show similarities in their structures and their immunomodulatory effects on human monocytic THP-1 cells. *BMC Complementary and Alternative Medicine*, **2011**, *11*, 58.

357. Ruthes, A. C.; Cantu-Jungles, T. M.; Cordeiro, L. M. C.; Iacomini, M. Prebiotic potential of mushroom D-glucans: implications of physicochemical properties and structural features. *Carbohydrate Polymers*, **2021**, *262* (March), 117940.

APPENDIX: ORIGINAL PUBLICATIONS

I.   Reprinted from *Innov. Food Sci. Emerg.* 2019, 57, 102206 with permission from Elsevier Ltd.

II.   Reprinted from *Food Chem.* 2019, 301, 125255 with permission from Elsevier Ltd.

III. Reprinted from *J. Appl. Microbiol.* 2021, 131 (3), 1318, an open access article published under the terms of the Creative Commons (CC-BY 4.0) license.

IV.   Reprinted from *J. Fungi* 2021, 7, 189, an open access article published under the terms of the Creative Commons (CC-BY 4.0) license.

DOCTORAL THESES IN FOOD SCIENCES AT THE UNIVERSITY OF TURKU

1. **REINO R. LINKO (1967)** Fatty acids and other components of Baltic herring flesh lipids. (Organic chemistry).
2. **HEIKKI KALLIO (1975)** Identification of volatile aroma compounds in arctic bramble, *Rubus arcticus* L. and their development during ripening of the berry, with special reference to *Rubus stellatus* SM.
3. **JUKKA KAITARANTA (1981)** Fish roe lipids and lipid hydrolysis in processed roe of certain *Salmonidae* fish as studied by novel chromatographic techniques.
4. **TIMO HIRVI (1983)** Aromas of some strawberry and blueberry species and varieties studied by gas liquid chromatographic and selected ion monitoring techniques.
5. **RAINER HUOPALAHTI (1985)** Composition and content of aroma compounds in the dill herb, *Anethum graveolens* L., affected by different factors.
6. **MARKKU HONKAVAARA (1989)** Effect of porcine stress on the development of PSE meat, its characteristics and influence on the economics of meat products manufacture.
7. **PÄIVI LAAKSO (1992)** Triacylglycerols – approaching the molecular composition of natural mixtures.
8. **MERJA LEINO (1993)** Application of the headspace gas chromatography complemented with sensory evaluation to analysis of various foods.
9. **KAISLI KERROLA (1994)** Essential oils from herbs and spices: isolation by carbon dioxide extraction and characterization by gas chromatography and sensory evaluation.
10. **ANJA LAPVETELÄINEN (1994)** Barley and oat protein products from wet processes: food use potential.
11. **RAIJA TAHVONEN (1995)** Contents of lead and cadmium in foods in Finland.
12. **MAIJA SAXELIN (1995)** Development of dietary probiotics: estimation of optimal *Lactobacillus* GG concentrations.
13. **PIRJO-LIISA PENTTILÄ (1995)** Estimation of food additive and pesticide intakes by means of a stepwise method.
14. **SIRKKA PLAAMI (1996)** Contents of dietary fiber and inositol phosphates in some foods consumed in Finland.
15. **SUSANNA EEROLA (1997)** Biologically active amines: analytics, occurrence and formation in dry sausages.
16. **PEKKA MANNINEN (1997)** Utilization of supercritical carbon dioxide in the analysis of triacylglycerols and isolation of berry oils.
17. **TUULA VESA (1997)** Symptoms of lactose intolerance: influence of milk composition, gastric emptying, and irritable bowel syndrome.
18. **EILA JÄRVENPÄÄ (1998)** Strategies for supercritical fluid extraction of analytes in trace amounts from food matrices.
19. **ELINA TUOMOLA (1999)** *In vitro* adhesion of probiotic lactic acid bacteria.
20. **ANU JOHANSSON (1999)** Availability of seed oils from Finnish berries with special reference to compositional, geographical and nutritional aspects.
21. **ANNE PIHLANTO-LEPPÄLÄ (1999)** Isolation and characteristics of milk-derived bioactive peptides.
22. **MIKA TUOMOLA (2000)** New methods for the measurement of androstenone and skatole – compounds associated with boar taint problem. (Biotechnology).
23. **LEEA PELTO (2000)** Milk hypersensitivity in adults: studies on diagnosis, prevalence and nutritional management.
24. **ANNE NYKÄNEN (2001)** Use of nisin and lactic acid/lactate to improve the microbial and sensory quality of rainbow trout products.
25. **BAORU YANG (2001)** Lipophilic components of sea buckthorn (*Hippophaë rhamnoides*) seeds and berries and physiological effects of sea buckthorn oils.
26. **MINNA KAHALA (2001)** Lactobacillar S-layers: Use of *Lactobacillus brevis* S-layer signals for heterologous protein production.
27. **OLLI SJÖVALL (2002)** Chromatographic and mass spectrometric analysis of non-volatile oxidation products of triacylglycerols with emphasis on core aldehydes.
28. **JUHA-PEKKA KURVINEN (2002)** Automatic data processing as an aid to mass spectrometry of dietary triacylglycerols and tissue glycerophospholipids.
29. **MARI HAKALA (2002)** Factors affecting the internal quality of strawberry (*Fragaria x ananassa* Duch.) fruit.
30. **PIRKKA KIRJAVAINEN (2003)** The intestinal microbiota – a target for treatment in infant atopic eczema?
31. **TARJA ARO (2003)** Chemical composition of Baltic herring: effects of processing and storage on fatty acids, mineral elements and volatile compounds.
32. **SAMI NIKOSKELAINEN (2003)** Innate immunity of rainbow trout: effects of opsonins, temperature and probiotics on phagocytic and complement activity as well as on disease resistance.
33. **KAISA YLI-JOKIPII (2004)** Effect of triacylglycerol fatty acid positional distribution on postprandial lipid metabolism.
34. **MARIKA JESTOI (2005)** Emerging *Fusarium*-mycotoxins in Finland.
35. **KATJA TIITINEN (2006)** Factors contributing to sea buckthorn (*Hippophaë rhamnoides* L.) flavour.
36. **SATU VESTERLUND (2006)** Methods to determine the safety and influence of probiotics on the adherence and viability of pathogens.
37. **FANDI FAWAZ ALI IBRAHIM (2006)** Lactic acid bacteria: an approach for heavy metal detoxification.
38. **JUKKA-PEKKA SUOMELA (2006)** Effects of dietary fat oxidation products and flavonols on lipoprotein oxidation.
39. **SAMPO LAHTINEN (2007)** New insights into the viability of probiotic bacteria.
40. **SASKA TUOMASJUKKA (2007)** Strategies for reducing postprandial triacylglycerolemia.

41. **HARRI MÄKIVUOKKO (2007)** Simulating the human colon microbiota: studies on polydextrose, lactose and cocoa mass.
42. **RENATA ADAMI (2007)** Micronization of pharmaceuticals and food ingredients using supercritical fluid techniques.
43. **TEEMU HALTTUNEN (2008)** Removal of cadmium, lead and arsenic from water by lactic acid bacteria.
44. **SUSANNA ROKKA (2008)** Bovine colostral antibodies and selected lactobacilli as means to control gastrointestinal infections.
45. **ANU LÄHTEENMÄKI-UUTELA (2009)** Foodstuffs and medicines as legal categories in the EU and China. Functional foods as a borderline case. (Law).
46. **TARJA SUOMALAINEN (2009)** Characterizing *Propionibacterium freudenreichii* ssp. *shermanii* JS and *Lactobacillus rhamnosus* LC705 as a new probiotic combination: basic properties of JS and pilot *in vivo* assessment of the combination.
47. **HEIDI LESKINEN (2010)** Positional distribution of fatty acids in plant triacylglycerols: contributing factors and chromatographic/mass spectrometric analysis.
48. **TERHI POHJANHEIMO (2010)** Sensory and non-sensory factors behind the liking and choice of healthy food products.
49. **RIIKKA JÄRVINEN (2010)** Cuticular and suberin polymers of edible plants – analysis by gas chromatographic-mass spectrometric and solid state spectroscopic methods.
50. **HENNA-MARIA LEHTONEN (2010)** Berry polyphenol absorption and the effect of northern berries on metabolism, ectopic fat accumulation, and associated diseases.
51. **PASI KANKAANPÄÄ (2010)** Interactions between polyunsaturated fatty acids and probiotics.
52. **PETRA LARMO (2011)** The health effects of sea buckthorn berries and oil.
53. **HENNA RÖYTIÖ (2011)** Identifying and characterizing new ingredients *in vitro* for prebiotic and synbiotic use.
54. **RITVA REPO-CARRASCO-VALENCIA (2011)** Andean indigenous food crops: nutritional value and bioactive compounds.
55. **OSKAR LAAKSONEN (2011)** Astringent food compounds and their interactions with taste properties.
56. **ŁUKASZ MARCIN GRZEŚKOWIAK (2012)** Gut microbiota in early infancy: effect of environment, diet and probiotics.
57. **PENGZHAN LIU (2012)** Composition of hawthorn (*Crataegus* spp.) fruits and leaves and emblic leafflower (*Phyllanthus emblica*) fruits.
58. **HEIKKI ARO (2012)** Fractionation of hen egg and oat lipids with supercritical fluids. Chemical and functional properties of fractions.
59. **SOILI ALANNE (2012)** An infant with food allergy and eczema in the family – the mental and economic burden of caring.
60. **MARKO TARVAINEN (2013)** Analysis of lipid oxidation during digestion by liquid chromatography-mass spectrometric and nuclear magnetic resonance spectroscopic techniques.
61. **JIE ZHENG (2013)** Sugars, acids and phenolic compounds in currants and sea buckthorn in relation to the effects of environmental factors.
62. **SARI MÄKINEN (2014)** Production, isolation and characterization of bioactive peptides with antihypertensive properties from potato and rapeseed proteins.
63. **MIKA KAIMAINEN (2014)** Stability of natural colorants of plant origin.
64. **LOTTA NYLUND (2015)** Early life intestinal microbiota in health and in atopic eczema.
65. **JAAKKO HIIDENHOVI (2015)** Isolation and characterization of ovomucin – a bioactive agent of egg white.
66. **HANNA-LEENA HIETARANTA-LUOMA (2016)** Promoting healthy lifestyles with personalized, *APOE* genotype based health information: The effects on psychological-, health behavioral and clinical factors.
67. **VELI HIETANIEMI (2016)** The *Fusarium* mycotoxins in Finnish cereal grains: How to control and manage the risk.
68. **MAARIA KORTESNIEMI (2016)** NMR metabolomics of foods – Investigating the influence of origin on sea buckthorn berries, *Brassica* oilseeds and honey.
69. **JUHANI AAKKO (2016)** New insights into human gut microbiota development in early infancy: influence of diet, environment and mother's microbiota.
70. *WEI YANG (2017)* Effects of genetic and environmental factors on proanthocyanidins in sea buckthorn (*Hippophaë rhamnoides*) and flavonol glycosides in leaves of currants (*Ribes* spp.).
71. **LEENAMAIJA MÄKILÄ (2017)** Effect of processing technologies on phenolic compounds in berry products.
72. **JUHA-MATTI PIHLAVA (2017)** Selected bioactive compounds in cereals and cereal products – their role and analysis by chromatographic methods.
73. **TOMMI KUMPULAINEN (2018)** The complexity of freshness and locality in a food consumption context
74. **XUEYING MA (2018)** Non-volatile bioactive and sensory compounds in berries and leaves of sea buckthorn (*Hippophaë rhamnoides*)
75. **ANU NUORA (2018)** Postprandial lipid metabolism resulting from heated beef, homogenized milk and interesterified palm oil.
76. **HEIKKI AISALA (2019)** Sensory properties and underlying chemistry of Finnish edible wild mushrooms.
77. **YE TIAN (2019)** Phenolic compounds from Finnish berry species to enhance food safety.
78. **MAIJA PAAKKI (2020)** The importance of natural colors in food for the visual attractiveness of everyday lunch.
79. **SHUXUN LIU (2020**) Fermentation with non-*Saccharomyces* yeasts as a novel biotechnology for berry wine production.

80. **MARIKA KALPIO (2020)** Strategies for analyzing the regio- and stereospecific structures of individual triacylglycerols in natural fats and oils.
81. **JOHANNA JOKIOJA (2020)** Postprandial effects and metabolism of acylated anthocyanins originating from purple potatoes.
82. **NIINA KELANNE (2021)** Novel bioprocessing for increasing consumption of Nordic berries.
83. **NIKO MARKKINEN (2021)** Bioprocessing of berry materials with malolactic fermentation.
84. **GABRIELE BELTRAME (2021)** Polysaccharides from Finnish fungal resources.