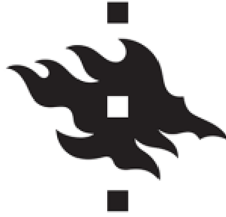

**University of Helsinki**

**Master's Thesis**

# Production of bioethanol and enzyme activities by a white-rot fungus on lignocelluloses under variant cultivation atmospheres

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Tiivistelmä – Referat – Abstract


In this research, lignocellulose decomposition and bioethanol production potentiality of the white rot fungus *Phlebia radiata* 79 was studied at different atmospheric conditions on several solid substrate mixtures containing spent brewery barley mash (SBBM), barley straw, spruce wood sawdust, and birch wood sawdust. The fungus was capable of growing on all substrate mixtures, subsequently converting them into fermentable sugars like glucose, producing various primary metabolites involving ethanol, acetate, and glycerol. Ethanol accumulation was always dominant under nitrogen flushed anaerobic conditions as well as semi-aerobic conditions.

The highest concentration of ethanol accumulated on the second week of cultivation on all solid substrate mixtures. Under anaerobic conditions, the detected amount of ethanol was 88 mmol/l, 87 mmol/l, and 108 mmol/l, after two weeks of cultivation of the fungus on lignocellulose substrates containing SBBM mixed with barley straw, spruce wood sawdust, or birch wood sawdust, respectively. Under semi-aerobic conditions, corresponding concentrations of ethanol - 90 mmol/l, 61 mmol/l, and 105 mmol/l - accumulated in the cultures after two weeks of cultivation on the same substrate mixtures, respectively. Under aerobic conditions, only small amounts of ethanol were detected during the first two weeks of cultivation.

Another part of the study was to establish an enzyme assay method for pectin degradation and conversion, in order to measure the activities of specific carbohydrate-active enzymes (CAZymes) involving pectinase, as well as cellulolytic β-glucosidase and cellobiohydrolase (CBH) activities. The highest β-glucosidase activity (5.2 nkat/ml) was observed under aerobic conditions in cultures on the substrate mixture of SBBM and barley straw. CBH activity was also prominent under aerobic conditions, and the maximal activity (0.7 nkat/ml) was detected on the substrate mixture of SBBM and spruce wood sawdust, while elevated pectinase activity (83 nkat/ml) was recorded under aerobic conditions on substrate mixtures containing SBBM and barley straw.

Thus, the conclusions are that composition of the solid waste lignocellulose substrate mixture affected enzyme production by the fungus, whereas production of ethanol was mainly controlled by the cultivation atmosphere. Interestingly, both anaerobic and semi-aerobic atmospheric conditions supported similar bioconversion efficiency resulting with similar high levels of bioethanol production in the fungal cultures within two weeks.



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Lignocellulose, Wood-decay fungi, Consolidated bioprocessing, CAZyme activity, Bioethanol, Bioconversion


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**Abbreviations**

| | |
|---|---|
| ACS | acetyl-CoA synthetase, acetate-CoA ligase |
| Adh | alcohol dehydrogenase |
| Ald | aldehyde dehydrogenase |
| ATP | adenosine triphosphate |
| BGL | β-glucosidase |
| BM | barley mash |
| BS | barley straw |
| BW | birch wood |
| BXL | β-xylosidase |
| CBH | cellobiohydrolase |
| CDH | cellobiose dehydrogenase |
| CBP | consolidated bioprocessing |
| CAZyme | carbohydrate-active enzyme |
| DNS | 3, 5- dinitrosalicylic acid |
| dw | dry weight |
| EG | endoglucanase |
| FFV | Flexi-Fuel Vehicles |
| GH | glycoside hydrolase |
| h | hours |
| D | days |
| LiP | lignin peroxidase |
| LPMO | lytic polysaccharide monooxygenase |
| MnP | manganese peroxidase |
| NADH | nicotinamide adenine dinucleotide (NAD) + hydrogen (H) |
| NC | negative control |
| FAO | Food and Agricultural Organization of the United Nations |
| FBCC | Fungal Biotechnology Culture Collection |
| MEA | malt extract agar |
| MULac | 4-methylumbelliferyl-beta D-lactoside |
| OXPHOS | oxidative phosphorylation |

| | |
|---|---|
| Pdc | pyruvate decarboxylase |
| Pdh | pyruvate dehydrogenase |
| PGA | polygalacturonic acid |
| PG | polygalacturonase |
| PL | pectate lyase |
| SBBM | spent brewery barley mash |
| SSF | simultaneous saccharification and fermentation |
| SW | spruce wood |
| TCA | tricarboxylic acid cycle |
| VP | versatile peroxidase |
| XLN | β-1,4-endoxylanase |

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## 1. Background and introduction to the study

The amount of underground fossil fuels is decreasing day by day, and the energy crisis is one of the main concerns of our modern times. Besides, frequent use of fossil fuels, especially raw oil and coal, is responsible for climate change (accumulation of greenhouse gases like carbon dioxide) and environmental pollution. The creation of sustainable energy sources might be a potential way to save our planet's nature as well as to fulfill the increasing global energy demand.

Production of biofuels is a promising alternative to fossil fuels for transportation. According to FAO (Food and Agricultural Organization of the United Nations) definition, biofuels are "fuels such as fuelwood, charcoal, bioethanol, biodiesel, biogas or bio-hydrogen produced directly or indirectly from biomass" (FAO, 2019). Regarding transportation, both renewable and sustainable fuel alternatives such as bioethanol, renewable diesel and biogas, are presently available. Based on feedstocks and conversion technology used for production, biofuels and especially, production of bioethanol is divided into the first, second, third and fourth generation biofuels (Aro, 2016).

### 1.1 Production of biofuels, especially bioethanol

Food crop feedstock including sugarcane, corn, whey, barley, potato waste, sugar beets, and vegetable oils are known as the starting materials for first-generation biofuels (Mohr & Raman, 2013). Second-generation bioethanol, also called cellulosic bioethanol is produced from non-food feedstocks, such as wood and lignocellulose biomass, municipal solid wastes, and agricultural and forest residues. Third-generation biofuels are based on aquatic, light-energy fixing microbial feedstock like algal biomass and considered as an emerging biofuel production technology with great production potentiality (Saladini et al., 2016). Production of the fourth-generation biofuels comprises the construction of synthetic living factories and designing microorganisms for efficient carbon dioxide fixing ability and conversion of solar energy to biomass and fuel. Electrobiofuels are an example of an emerging technology for the efficient production and storage of liquid fuels (Aro, 2016).

Bioconversion of sugars and starchy materials into bioethanol is, however, not sustainable due to negative impact on our food chain and supply (Mohr & Raman, 2013). Therefore, the use of lignocellulosic materials such as wood waste, agricultural residues, and by-products from paper and pulp industries are gaining more acceptance to produce second-generation bioethanol. However, the use of lignocellulose is challenging because of the complex and rigid structure of

the raw material (Saini et al., 2015). Utilization of second-generation raw materials usually requires physico-chemical and enzymatic pretreatments which in turn complicate the processes and increase the overall production costs.

An advantage for second-generation bioethanol production is that lignocelluloses derived from plant biomass are locally available in vast quantities, and globally around $2*10^{11}$ tons of plant biomass is produced in each year (Lin & Tanaka, 2006). Sources of lignocelluloses include agricultural, forestry, and industrial wastes as well as biomass from non-edible energy crops. Use of biofuels are environmentally more sustainable, and compared to fossil fuels, reduction from 60% to 90% of $CO_2$ emissions are accomplished (Wang et al., 2007). Among different biofuels, bioethanol gets more commercial acceptance globally. Bioethanol production has notably elevated in the past ten years with increasing estimations from current total production of $120*10^9$ liters (in 2017) to $131*10^9$ liters by 2027, while global biodiesel production is predicted to stay at the current level of about $40*10^9$ liters of annual production (OECD-FAO, 2018).

## 1.2 Current state of biofuel production

Number of vehicles are increasing globally and unless new renewable fuel sources are created, current number of vehicles ($1.2*10^9$) will depend on natural oil based fuels for their operation (Kazemi et al., 2019). The use of bioethanol as liquid transportation fuel is currently gaining more acceptance due to its sustainable and renewable nature. Countries including mainly Brazil, and the United States have been using bioethanol as part of transportation fuel from 1980s. Bioethanol is mainly utilized in Flexi-Fuel Vehicles (FFV) by blending it with fossil gasoline ranging in mixtures between 10% (E-10 fuel) and 85% (E-85 fuel). In Europe, bioethanol or biodiesel are mixed with fossil petrol or diesel fuels, respectively, in different percentages. For instance, in Norway, 7% bioethanol in octane petrol and 10% biodiesel in diesel oil, respectively, mixtures are in use for transportation (Sundvor & López-Aparicio, 2014).

The selection and use of suitable microorganisms with the potentiality of fermenting lignocelluloses without pretreatments might overcome the raw material influenced and other current obstacles in more sustainable production of bioethanol. Recently, genetically modified yeasts and bacteria have been constructed together with research on natural microbial consortia in order to enhance lignocellulose utilization and fermentation to ethanol and other alcohols (Kricka et al., 2015).

However, most of the fermenting microorganisms, for instance, baker's yeast *Saccharomyces cerevisiae*, is not capable of direct fermentation of cellulose due to the lack of generation of extracellular enzymes necessary for the hydrolysis of cellulose. More precisely, yeast is unable to ferment pentose sugars such as xylose and arabinose (Kricka et al., 2015) that are predominant in many hemicelluloses. On the other hand, wood-decaying fungi possess wide metabolic and plant biomass degrading abilities, for instance, the fungal isolate *Phlebia radiata* 79 can decompose lignocellulosic biomass without pretreatment and thereby produce bioethanol through fermentation (Mattila et al., 2017).

Fungal bioconversion of wood biomass is regulated by various factors such as atmosphere and aeration, incubation temperature, substrate size, light, moisture, and other culture conditions connected to growth and production of proper enzyme activity. The selection of suitable fungi and optimization of the fermentation conditions will contribute to the industrial production of second-generation bioethanol. Also, enzyme activity assays will help to determine the fungal decomposition efficiency of the wood-based substrates.

### 1.3 Fungi, their importance and ecological diversity

Fungi are multi- or unicellular, heterotrophic eukaryotic organisms which can be classified as saprotrophic, pathogenic, or symbiotic for plants and animals, and associated with algae or cyanobacteria in lichens (Deacon, 2009). Among Eukarya, fungi form their own phylogenetic lineage within Opisthokonta with nucleariids as their sister lineage (Steenkamp et al., 2006). Fungi are distributed worldwide and can be found from the temperate zones to the polar zones as well as in tropical habitats (Mohanta & Bae, 2015). Besides their ecological importance as major degrader of especially plant-based organic matters, different fungi such as mushrooms and truffles are consumed as human food. Fungi are also capable of producing proteins and enzymes, together with antibiotics, mycotoxins, alkaloids, polyketides and other industrially important chemical compounds (Barke et al., 2010).

Based on morphological and other characteristics and phylogenetic studies, the kingdom fungi is classified into several (seven to thirteen) major phyla consisting of Ascomycota, Basidiomycota, Chytridiomycota, Monoblepharidomycota, Neocallimastigomycota, Blastocladiomycota, Glomeromycota, Entomophthoromycota, and others (Hibbett et al., 2007; Tedersoo et al., 2018). A large number of species from the phyla Ascomycota and Basidiomycota play a remarkable role in the decomposition of plant biomass based materials (Hibbett et al., 2007).

Huge diversity and long evolutionary history of this kingdom provide a great opportunity to study their various activities in different ecosystems. Wood-decaying fungi are filamentous Basidiomycota and a few Ascomycota species with various strategies for inhabitation, decomposition and nutritional utilization of dead wood biomass (Lundell et al., 2014). These fungi are characterized as white rot, brown rot or soft rot according to the physical and visual properties of the degraded wood.

White rot fungi are common in decayed hardwood, which is softened to fibrous, white to yellow matter often containing dark manganese deposits ( Hatakka & Hammel, 2010; Lundell et al., 2014). Brown rot fungi decompose wood polysaccharides mainly through a non-enzymatic attack including Fenton chemistry. Brown rot fungi leave the decayed wood usually dry, brown, powdery, and cracking to cubicles. Soft rot fungi are Ascomycota species characterized by colonized green or dark mold colonies and zones mainly on the wood surface. Many soft rot fungi are efficient decomposers of plant litter and also can decompose cellulose-hemicellulose parts of wounded or cut wood surfaces (Lundell et al., 2014). Litter-decomposing fungi of Basidiomycota are another type of plant biomass decomposing and mushroom forming fungi. Litter decomposing fungi are often capable of producing pectinolytic enzymes and participate significantly in the global carbon cycle by acting on non-woody plant tissues (Rytioja et al., 2014).

White rot fungi can enzymatically degrade the lignocellulose main polymers cellulose, hemicellulose, and lignin efficiently, whereas brown rot fungi decompose cellulose and hemicellulose but modify lignin only partially (Lundell et al., 2014). Therefore, between white rot fungi and brown rot fungi, fundamental and applied research has been performed extensively on white rot fungi to discover the best isolates for biotechnological and industrial purposes. Wood-decaying fungi have both ecological and economic importance such as in the pulp and paper industry (Gutiérrez et al., 2011).

### 1.4 *Phlebia radiata* isolate 79

The Basidiomycota class Agaricomycetes order Polyporales in the phlebioid clade can be phylogenetically divided into sub-clades of the genera *Phlebia*, *Phanerochaete*, and *Byssomerulius* (Kuuskeri et al., 2015; Floudas & Hibbett, 2015). *Phlebia radiata* is a phlebioid white rot species of the order Polyporales (Justo et al., 2017) and *P. radiata* 79 is a Finnish isolate capable of degrading a wide range of plant biomasses (Kuuskeri, 2016). This fungus expresses and secretes a set of carbohydrate-active enzymes (CAZymes) and oxidoreductases necessary for the

bioconversion of wood lignocelluloses (Kuuskeri et al., 2016; Mäkinen et al., 2019). The fungus is capable of hydrolyzing lignocellulose biomass without harsh pretreatments and can ferment both pentose and hexose sugars into bioethanol (Mattila et al., 2020). This simultaneous saccharification and fermentation (SSF) process adopting a single organism may be characterized as a simplified modification of consolidated bio-processing (Mattila et al., 2017; Mattila et al., 2018).

### 1.5 Decomposition of wood lignocelluloses

Global carbon cycle is directly connected to decomposition of organic matter like lignocelluloses in terrestrial ecosystems. Trees and grasses are the largest sources of these lignocelluloses (Crowther et al., 2015). Recycling of carbon in lignocellulose is difficult because of the rigid structure of the plant cell wall as well as the presence of recalcitrant compounds such as lignin. Saprotrophic fungi have a vital role in biodegradation of lignocelluloses and ultimately participate in the carbon cycle actively. Wood-decaying white rot fungi secrete sets of extracellular enzymes to degrade wood biomass, and thus reduces the energy and chemical demand in the pulp and paper industry (Rodríguez, 2018). Lignocellulosic material is composed of the bio-polymers cellulose, hemicellulose, and lignin. Hydrolysis of cellulose and hemicellulose results in the formation of fermentable sugars consisting of hexoses such as glucose, galactose, and mannose, and pentoses like xylose and arabinose (Balat & Balat, 2008). These sugars are then assimilated and converted to bioethanol through fermentation by yeasts, filamentous fungi or bacteria (Mussatto et al., 2012).

#### 1.5.1 Structure of lignocellulosic biomass

Lignocellulosic biomass is composed of the carbohydrate polymers cellulose, hemicellulose, pectin, and of the aromatic heteropolymer lignin (Rytioja et al., 2014). Softwood (coniferous tree wood) and hardwood (deciduous tree wood) both contain mainly cellulose (40-45% of the dry mass), then hemicelluloses (about 30% of the dry mass), and lignin (20-30% of the dry mass) and extractives (2-3% of the dry mass) (Sjöström, 1993). However, these proportions and the chemical structure of hemicelluloses and lignins vary between plant species and woody tissues (Boerjan et al., 2003).

Presence of lignin together with cellulose, hemicellulose, and pectin form the rigid, complex and functional lignocellulose structure of the plant cell wall (Figure 1), which also protects plants from external attacks. Cellulose is composed of glucose units linked by β-1,4-

glycosidic linkages. Linear cellulose chains are linked together by hydrogen bonds to create crystalline structures called microfibrils at the lignified plant cell wall (Fernandes et al., 2011).

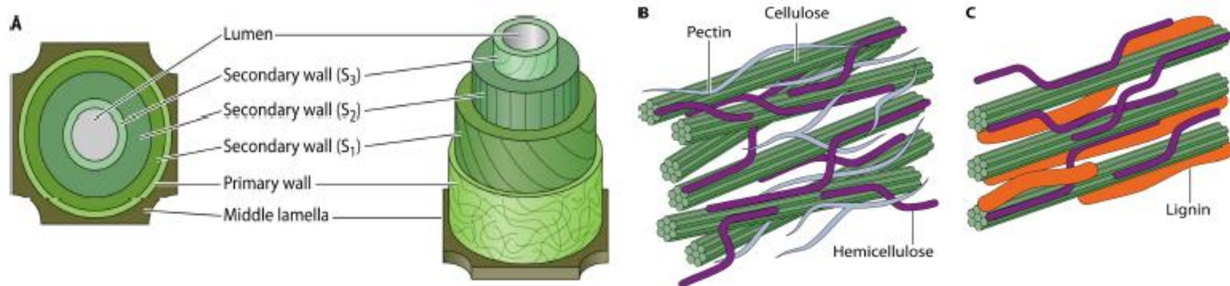


**Figure 1.** Simplified model of plant cell wall structure. (A) The structure consists of several layers: the middle lamella attached to primary wall and secondary walls. (A and B) The main polysaccharides and lignin which form the wood cell wall lignocellulose are present at the primary (B) and the secondary (C) cell walls. Picture copied from publication (Rytioja et al., 2014; Microbiology Molecular Biology Reviews, doi:10.1128/MMBR.00035-14) with allowance from the publisher (American Society of Microbiology).

Hemicellulose is composed of mainly xylan, xyloglucan or mannan backbones (van den Brink & de Vries, 2011), comprising 20% to 30% of plant dry weight. Hemicelluloses are known for their structural support of the cellulose microfibrils embedded in both primary and secondary cell walls (Sjöström, 1993).

Lignin is the third main biopolymer of wood composed of phenylpropanoid residues and is partially covalently bound via ether and benzyl ester linkages to the carboxyl groups of hemicellulose (Boerjan et al., 2003). Lignin contains three types of aromatic subunits, which are as *p*-hydroxyphenyl, guaiacyl and syringyl monolignols. Various linkages and subunits in lignin units with attachment and linkages to both cellulose and hemicellulose provides rigidity and toughness of woody cell walls, which ultimately give resistance to chemical and enzymatic degradation of wood (Halpin, 2013).

Pectin is the non-cellulosic polysaccharide of many plant fruits as well as plant primary cell walls and composed of the galacturonic acid backbone. Pectins have variable branching linkages containing also rhamnose units, and may create additional cross-links between the cellulose and hemicellulose polymers to strengthen the rigid wood structure (Caffall & Mohnen, 2009). In woody tissue, for instance, the primary cell walls and middle lamellae contains pectin (Figure 1).

### 1.5.2 Enzymatic breakdown of lignocelluloses

For the degradation of lignocellulosic feedstock, groups of enzymes are essential for the modification of cellulose and hemicelluloses to get monomeric carbohydrates and especially sugars (Rytioja et al., 2014). Lignin and pectin modifying enzymes are essential to break down the basic structure of the lignocellulose. Subsequently, free sugars are converted into ethanol through microbial fermentation.

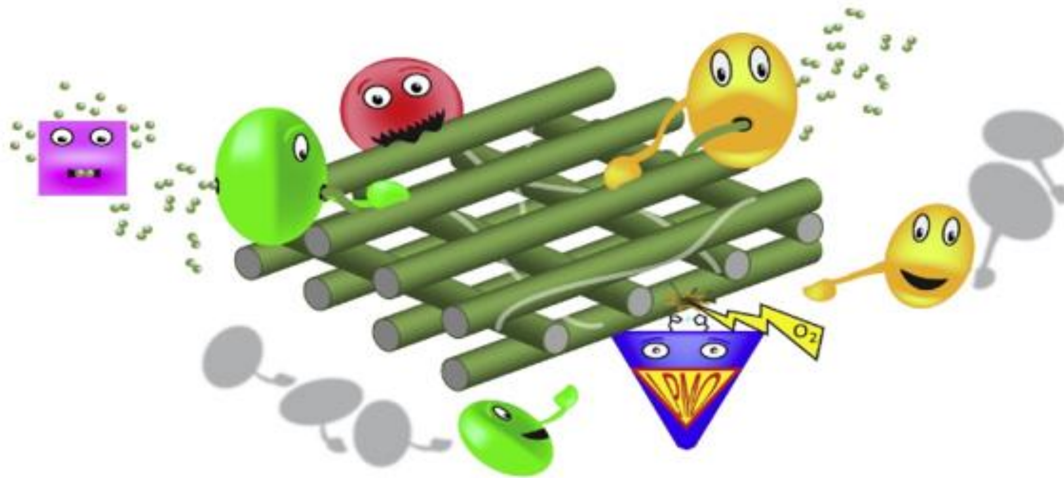


**Figure 2.** Schematic representation of enzymatic cellulose degradation, while exo-action of reducing-end (orange) and non-reducing-end (green) cellulases generate disaccharide cellobiose units from cellulose. Subsequently, cellobiose units are converted into glucose by β-glucosidases (pink). Hydrolytic action of endo-acting cellulases (red) breaks cellulose chain for further modification by exo-acting cellulases. Oxidative action of LPMOs (blue) participate in breakage of cellulose chains which are further degraded by processive cellulases. Picture copied from publication (Hemsworth et al., 2015; Trends in Biotechnology) with a licence from the publisher, Elsevier Ltd.

For cellulose breakdown, a set of hydrolytic cellulases including endoglucanases (EG) (CAZy class GH-5), cellobiohydrolases (CBH) (GH-6 & GH-7), and β-glucosidases (BGL) (GH-3) are essential (Figure 2; Table 1). The hydrolytic cellulases act synergistically to degrade crystalline as well as non-crystalline cellulose chain regions into its monomeric units which is glucose. In addition, auxiliary oxidoreductive enzymes like cellobiose dehydrogenase (CDH) and lytic polysaccharide monooxygenases (LPMO) are involved in effective biodegradation and modification of cellulose.

EG releases glucooligosaccharides by cleaving cellulose chains and CBHs release cellobiose from the ends of the cellulose chains (Figure 2; Table 1). Two types of CBHs (GH-6 & GH-7) are responsible for the sequential hydrolysis of reducing and nonreducing ends of cellulose chains, respectively. BGLs act on shorter oligosaccharides of cellulose and release glucose units. CDH acts by oxidizing cellobiose and cello-oligosaccharides to the corresponding lactones. These enzymes may also contribute to lignin modification for instance by producing hydroxyl radicals according to Fenton chemistry, and may ultimately help in LPMO catalyzed cellulose depolymerization by cleaving glycosidic bonds releasing monomeric sugars (Henriksson et al., 2000).

EG enzymes together with other cellulases work synergistically to hydrolyze β-1,4 linkages in cellulose chains consequently breaking the polymer into sugar monomers through the involvement of different catalytic modules. BGLs are involved in cellobiose degradation by binding nonreducing glucose units through its pocket shaped active site to release glucose monomers (Zhang & Zhang, 2013; Rytioja et al., 2014).

**Table 1.** Carbohydrate active enzyme (CAZyme) activities experimentally studied in the research. Classification and abbreviation is depicted according to (http://www.cazy.org/); EC numbering is following the classification of ExplorEnz- The Enzyme Database (https://www.enzyme-database.org/). GH= Glycoside hydrolases; PL= Pectate lyases.

| CAZy enzyme | Activity | EC number | Main substrate |
|---|---|---|---|
| GH-3 | β-glucosidase; BGL | 3.2.1.21 | Cellobiose, β-D-glucosides |
| GH-5 | Endo-β-1,4-glucanase (endo-glucanase, cellulase); EG | 3.2.1.4 | Cellulose |
| GH-6, GH-7 | Cellobiohydrolase, reducing end-acting cellobiohydrolase; CBH | 3.2.1.91 and 3.2.1.176 | Cellulose |
| GH-11 | Endo-β-1,4-xylanase; XLN | 3.2.1.8 | Xylan hemicellulose |
| PL-1, PL-2, PL-3 | Pectate lyase; PL | 4.2.2.2 | Pectin |

A specific set of carbohydrate-active enzymes is essential to get monomeric sugars from hemicelluloses, which have a branching structure. β-1,4-endoxylanase (XLN), β-1,4-xylosidase, β -1,4-endomannanase and β-1,4-mannosidase are the major hemicellulose-active enzymes (van

den Brink & de Vries, 2011). XLN acts on xylan by clipping the polymer backbone into oligomers, whereas β-1,4-xylosidase performs the hydrolysis of xylobiose into its monomeric units and discharges D-xylose from the nonreducing terminal of larger xylooligosaccharides. Similarly, mannan hemicelluloses (galactoglucomannans) are cleaved to produce mannooligosaccharides and further modified to D-mannose. Hydrolysis of xyloglucan is conducted by EGs, CBHs, and BGLs, and is similar to the cellulose hydrolysis. Besides, LPMOs may have the potentiality of cleaving xyloglucan, β-glucan, and even glucomannan with varying efficiency (Agger et al., 2014).

The amount of lignin is quite high in lignocellulose (up to 30% of dry mass; Sjöström, 1993); Boerjan et al., 2003) and its modification is essential for the optimal release of monomeric sugars from cellulose and hemicellulose components (Ruiz-Dueñas & Martínez, 2009). Various heme-including, fungal secreted peroxidases including lignin peroxidases (LiPs), manganese peroxidases (MnPs), and versatile peroxidases (VPs) together with laccases are capable of depolymerizing lignin polymers (Hofrichter et al., 2010; Lundell et al., 2010).

Polygalacturonic acid is the backbone of pectin, and a group of enzymes are required for the degradation of this polymer. Pectin hydrolysis also enables the proper release of sugar contents from cellulose and hemicellulose parts. Pectin degrading enzymes include mainly endo-and exo-polygalacturonases, pectate lyases (for instance classes PL-1, PL-2, PL-3, PL-9 & PL-10), and rhamnogalacturonan lyases (for instance classes PL-4 & PL-11) (carbohydrate Active Enzyme database- http://www.cazy.org; Lombard et al., 2014)). The cumulative effect of these enzymes degrades pectin and releases monomers like galacturonic acid, while favors in loosening the structure of lignocellulosic feedstock (van den Brink & de Vries, 2011).

### 1.6 Accumulation of bioethanol and natural by-products during lignocellulose decomposition

Fungal saccharification and fermentation may be a simultaneous process with suitable microorganisms like wood-decaying fungi, while bioethanol accumulates during fermentation of available sugars generated by lignocellulose decomposition by fungal enzymatic activity (Mattila et al., 2018). Accumulated sugars can be utilized by fungal hyphal intake either through respiration under aerobic conditions or through fermentation under oxygen-limited to anaerobic conditions. In ethanol fermentation by the baker's yeast *Saccharomyces cerevisiae*, glucose is converted to pyruvate through glycolysis, and acetaldehyde is generated by the action of pyruvate decarboxylase (Pdc) (Figure 3). Finally, alcohol dehydrogenase (Adh) acts on acetaldehyde by reducing it into ethanol (Pfeiffer & Morley, 2014).

Besides ethanol, this process generally facilitates the generation of various industrially important chemicals such as acetate and glycerol. Glycerol is the natural by-product of ethanol fermentation by *S. cerevisiae* and other yeasts (Karimi et al., 2006), and can also be used for the generation of fuels and chemicals. Also, glycerol might be used by fungi and bacteria to produce glycolytic intermediates such as pyruvate from which ethanol will be generated (Yazdani & Gonzalez, 2007). Usually, some acetate accumulates during ethanol fermentation. Through ethanol fermentation, NADH is consumed and NAD+ is generated through reduction of acetaldehyde to ethanol by Adh enzymes (Wei et al., 2013).

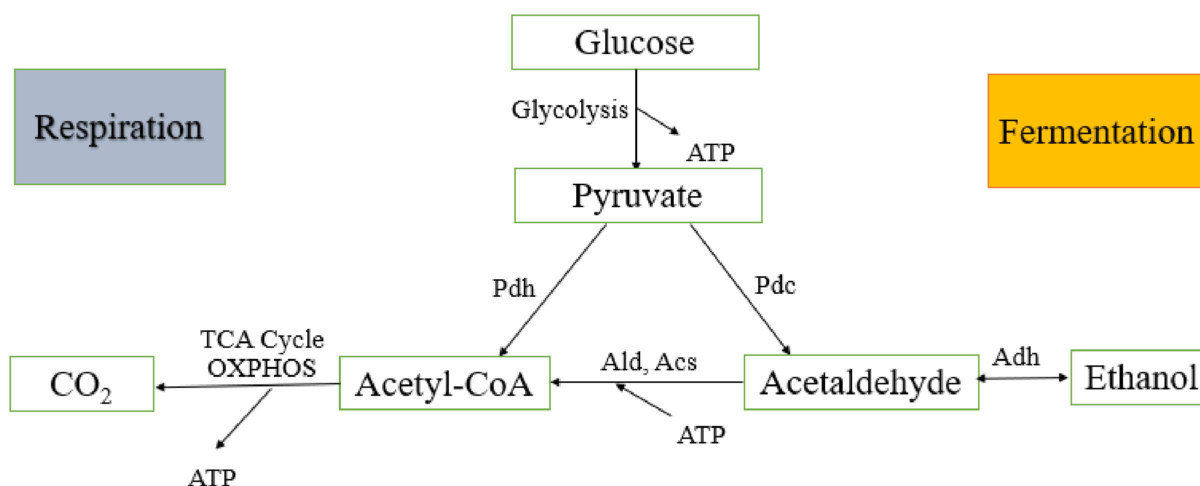


**Figure 3.** Generalized pathways of glucose utilization and ethanol formation by the baker's yeast fungus *Saccharomyces cerevisiae*. Glucose is converted into pyruvate through glycolysis, and pyruvate will be further converted into acetyl-coenzyme A to enter TCA cycle in respiration and for biosyntheses or fermented into ethanol through acetaldehyde formation. ATP=adenosine triphosphate; Pdh= pyruvate dehydrogenase; Pdc= pyruvate decarboxylase; Adh= alcohol dehydrogenase; Ald= aldehyde dehydrogenase; Acs= acetyl-CoA synthetase; TCA= tricarboxylic acid cycle; OXPHOS= oxidative phosphorylation. Illustrated according to a published scheme (Pfeiffer & Morley, 2014).

From lignocellulose starting materials for ethanol fermentation, lignin may also serve as a source for useful by-products. For instance, the production of bio-adhesives and second-generation bioplastics from lignin might be an interesting addition in the global market. Moreover, 10% to 20% of lignin can be converted into valuable aromatic compounds such as guaiacol, cathecol, and phenol (Beauchet et al., 2012). Lignin can also be chemically converted into valuable transportation diesel oil fuels like jet fuels (Cheng & Brewer, 2017).

## 2. Aims and hypotheses of the study

The CBP (consolidated bioprocessing) ability of the filamentous fungus *Phlebia radiata* isolate 79 was conducted on different mixtures of lignocellulose substrates including barley straw, spruce wood sawdust, and birch wood sawdust, with spent brewery barley mash. This thesis aimed to identify how atmospheric conditions affect enzymatic biodegradation of lignocellulose and metabolic activity, especially production of ethanol by the fungus. Another aim of the study was to establish an assay method for enzymatic pectin degradation and conversion.

In this study, *P. radiata* isolate 79 was cultivated on solid lignocellulose mixtures under three different atmospheric conditions: anaerobic ($N_2$ gas flushed), semi-aerobic (partially $N_2$ gas flushed) and aerobic (air flushed). The effect of the culture gas phase was monitored by measuring activities of lignocellulose degrading enzymes and bioethanol production by the fungus.

**Research hypotheses were:**

1. Change of atmosphere to oxygen-depleted, fermentative conditions will affect the production of fungal extracellular enzymes, expectably negatively by suppressing their production and detectable activities.
2. Semi-aerobic culture atmosphere would allow the fungal mycelium to grow and produce more enzymes against the lignocellulose substrates, thereby improving the bioconversion and ethanol production.
3. Together with enzymatic degradation of cellulose and hemicellulose, enzymatic degradation of pectin is an important activity for the fungus during growth and bioconversion of lignocellulose, in order to produce ethanol under fermentative conditions.

## 3. Materials and methods

### 3.1 Microorganism

In this study, the Basidiomycota fungus *Phlebia radiata* isolate 79 (FBCC0043) (Kuuskeri et al., 2015; Mäkinen et al., 2019) stored at the FBCC sub-collection of the Microbial Domain Biological Resource Centre HAMBI of the Helsinki Institute of Life Science, University of Helsinki, Helsinki, Finland, was used for bioconversion of the lignocellulosic materials into ethanol. This filamentous fungus was selected also to monitor the activity of different enzymes secreted by the organism to modify lignocelluloses during cultivations.

### 3.2 Chemicals

Chemicals used during the experimental part are given below in Table 2.

**Table 2.** Purity and manufacturer of various chemicals used in the study

| Chemical | Purity grade | Manufacturer |
|---|---|---|
| Sodium hydroxide (NaOH) | 98.8% | VWR(BDH), Prolabo chemicals, Belgium |
| Hydrochloric acid (HCl) | n.k.[1)] | Merck, Darmstadt, Germany |
| *p*-nitrophenyl beta-D-glucopyranoside | n.k.[1)] | Calbiochem, China |
| Citric acid | 99.5% | Sigma-Aldrich, China |
| Sodium carbonate ($Na_2CO_3$) | 99.6% | Acros Organics, Spain |
| 4-methylumbelliferyl-betad-lactoside (MULac) | n.k.[1)] | Biokemis, Russia |
| Glucose | 99.5% | Sigma-Aldrich, USA |
| Polygalacturonic acid | 90% | Sigma-Aldrich, Switzerland |
| D-galacturonic acid | 97% | Sigma-Aldrich, Slovakia |
| KNa-tartrate | 99.9% | VWR (BDH) chemicals, Germany |

[1)]n.k. not known

### 3.3 Malt extract agar medium

Malt extract agar (MEA) medium containing 20 g/L of malt extract (Biokar Diagnostics, Beuvais Cedex, France) and agar-agar, 20 g/L (Amresco, Solon, Ohio, USA) was prepared for the maintenance and cultivation of the fungus. The medium was autoclaved (121 °C, 15 minutes, 1 atm) prior pouring into sterile plastic petri dishes (50 mL MEA in each). pH of the medium was 5.5 after autoclaving.

### 3.4 Preparation of lignocellulose substrates

Solid-state cultures of mixed lignocellulose substrates including spent brewery barley mash (SBBM) (obtained from prof. Per-Erik Saris group microbrewery, Department of Microbiology, Viikki Campus, University of Helsinki), barley straw (straw from *Hordeum vulgare* crop harvest of summer 2017, Viikki, Helsinki, Finland; Veloz Villavicencio et al., 2020), spruce wood (*Picea abies* sawdust; Mali et al., 2019) and birch wood (*Betula pendula* sawdust; Veloz Villavicencio et al., 2020) were used as carbon sources for bioethanol production by *P. radiata* 79 at different atmospheric conditions. Air-dried barley straw was cut into small pieces (about 2 cm), and spent brewery barley mash was milled with IKA A 11 basic analytical laboratory mill (IKA-Werke GmbH & Co. KG, Staufen, Germany) after drying in oven (Memmert GmbH & Co. KG, Schwabach, Germany) at 70 °C overnight. Spruce wood and birch wood sawdust pieces were dried in oven overnight at 70 °C and sieved (through a metal sieve) to separate ≤ 2 mm sized pieces. Three combinations of substrate mixtures were prepared in 100 ml glass Erlenmeyer flasks: a) a mixture of 1.5 g (dry weight, dw) barley straw and 0.5 g (dw) milled barley mash, b) a mixture of 2.5 g (dw) spruce wood sawdust and 0.5 g (dw) milled barley mash, and c) a mixture of 2.5 g (dw) birch wood sawdust and 0.5 g (dw) milled barley mash, into each 100 ml Erlenmeyer flask. All flasks containing lignocellulose substrate mixtures were closed with cellulose stoppers and aluminium caps, then autoclaved (dry program at 121℃ for 15 min). After autoclaving, 20 ml of sterile Milli-Q water was added into each flask and mixed well, aseptically inside the laminar hood. pH of the water phase was adjusted to 3.0 by adding sterile 5.0 M HCl solution, and the substrate flasks were kept at room temperature overnight to stabilize.

### 3.5 Solid-state cultivations of the fungus on the lignocellulose mixtures

One mycelial agar plug (diameter 6 mm) of *P. radiata* 79 cultivated on MEA medium was added into each flask at the top of the lignocellulosic substrate mixture. Three different atmospheric conditions in the culture flasks were generated by 1) selection of closing of the cultivation flasks,

and 2) sequential flushing of the gas phase with either air (aerobic cultures) or 100% nitrogen gas (semi-aerobic and anaerobic fermentative cultures). Anaerobic and semi-aerobic flasks were closed with tight rubber plugs containing an inlet and valve system (Mattila et al., 2017) while the aerobic flasks were closed with ventilating cellulose plugs covered with a loose aluminium cap. All flasks were incubated in four biological replicates as stationary cultures (non-agitated) at 25 °C in a laboratory incubator, and cultivations were continued for four weeks in the dark. Negative controls (four replicates of lignocellulose mixtures without fungus) were also incubated under the same cultivation conditions.

### 3.6 Nitrogen gas flushing and treatments

Three atmospheric conditions were chosen to identify the effect of the gas phase for lignocellulose bioconversion, enzyme activities, and ethanol accumulation by *P. radiata* 79. The average volume of the cultivation Erlenmeyer flasks was 139 ml, where solid barley straw blended with SBBM along with Milli-Q water (20 ml) occupied 26 ml of space. The remaining gas space was estimated to take 113 ml in volume. All flasks from the anaerobic and semi-aerobic groups were flushed with nitrogen gas ten times through the stealing plug inlet valve system, each time with 35 ml of pure nitrogen, thereby summing to over 3x the total gaseous phase volume. To get semi-aerobic conditions, 50% (54 ml) of the fermentation gas phase was taken out from the culture flask by syringe through the inlet valve system, and replaced with an equal volume of laminar hood air by pushing the air aseptically into the flask through a sterile membrane filter and the valve. For the culture flasks containing spruce wood sawdust blended with SBBM as well as birch wood sawdust blended with SBBM, remaining gas space was 114 ml, and therefore, the same gas exchange procedures as described above were followed to get anaerobic and semi-aerobic conditions. Nitrogen gas treatments were conducted once a week after collection of the liquid phase sample from each culture flask.

### 3.7 Sample collection and HPLC analysis

Liquid samples (1 ml/flask) were aseptically collected from all flasks once a week (every seventh day of cultivation time) and equal volume (1 ml) of sterile Milli-Q water was added to maintain the humidity and constant water content in the solid-state cultures. HPLC samples were processed by centrifuging the solids of the liquid samples in sterile plastic (Eppendorf) tubes at 13000 rpm for 5 minutes at room temperature. After centrifugation, 0.1 ml of the liquid sample was transferred to HPLC vial by filtering through 0.2 µm pore size Acrodisc GHP syringe filters (Pall Laboratory,

Pall Corporation, via VWR Finland). HPLC analysis was performed by Hans Mattila using the Waters Alliance e2695 HPLC coupled with photodiode array and refractive index detectors (Department of Food and Nutrition, Faculty of Agriculture and Forestry, Viikki Campus, University of Helsinki) adopting the chromatographic method applied previously (Mattila et al., 2018; Mattila et al., 2020). The aim was to identify and quantify the expected fermentation products such as ethanol, glycerol, and acetic acid, together with analysis of the released sugars, especially glucose and xylose. Remaining liquid samples were stored at -20 °C for further analysis and enzyme activity assays.

### 3.8 Enzyme activity assays

Activities of β-glucosidase, cellobiohydrolase (CBH) and pectinase at cultivation time points (four weeks) were measured by using the -20 °C stored liquid samples of all cultivations. Frozen samples were thawed at room temperature and vortexed before measuring the enzyme activity. Activity of β-glucosidase and CBH were measured by the 96-well plate assay methods optimized previously (Rytioja et al., 2014; Kuuskeri et al., 2015; Mäkinen et al., 2018) and measured with the Spark M200 multimode microplate reader (Tecan, Switzerland) and VICTOR fluorescence plate reader (PerkinElmer Inc., USA), respectively.

For β-glucosidase assay, 1.0 mM 4-nitrophenyl β-D-glucopyranoside (Table 2) was used as substrate, and activity was determined by measuring the amount of *p*-nitrophenol released at 400 nm (Kuuskeri et al., 2015). CBH activity was measured in 96-well plastic microtiter black plates by using the VICTOR fluorescence plate reader and 2 mM 4-methylumbelliferyl-β-D-lactoside (MULac, Table 2) as substrate (Mäkinen et al., 2018). For both β-glucosidase and CBH assay, 50 mM Na-citrate (pH 5) was used as buffer and incubation temperature was 45 ºC (Rytioja et al., 2014), while various incubation temperatures were tested for the pectinase activity assay. In all cases, incubation time was 10 minutes, while the pectinase assay required additional boiling for 5 minutes before transferring the cooled reaction mixtures (150 µl each portion) to the NUNC F plastic microwell plates for measurement.

### 3.9 Pectin degradation and conversion assay

Development of pectinase (pectin degrading) enzyme activity assay was established as part of the thesis project by using the Tecan Spark M200 multimode microplate reader and SparkControl software (Tecan Switzerland), and plastic transparent 96-well plates. Polygalacturonic acid (PGA) was used as substrate by adding 0.5 g of polygalacturonic acid into 100 ml of 50 mM sodium

citrate buffer (pH 5) and dissolved with a magnetic stirrer. DNS (dinitrosalicylic acid) was used as the reagent selective for released, dissolved sugars with reducing end reactivity for coupling with the reagent (Sumner & Somers, 1949). Coupled DNS methods are generally adopted for respective polysaccharide cleaving activity assays for cellulose degradation (endocellulase, endoglucanase) and hemicellulose degradation (xylanase) (Rytioja et al., 2014; Kuuskeri et al., 2015; Mali et al., 2017). DNS reagent was prepared in the laboratory as containing 10 g of 3, 5-dinitrosalicylic acid, 16 g NaOH, and 300 g KNa-tartrate in 1000 ml of DNS reagent.

In pectinase assay, 20 mM (20 µmol/ml) D-galacturonic acid was used as reference for standard regression calculation. 4.2 g of D-galacturonic acid was dissolved into 50 ml of 50 mM sodium citrate buffer using a magnetic stirrer. Five standard solutions, St4 (20 µmol/ml), St3 (10 µmol/ml)**,** St2 (6.7 µmol/ml)**,** St1 (4.0 µmol/ml), and St0 (only buffer) were used for the assay, in three replicate reactions, respectively.

### 3.9.1 Change of incubation time and temperature

This experimental part aimed to establish a 96-well mediated pectinase assay. As part of the optimization process, different incubation temperatures between 30 °C and 45 °C with several incubation times ranging from 2.5 to 30 minutes were tested. For these purposes, liquid samples from the solid-state cultivations of the fungus on barley straw mixed with SBBM were used. In the primary trial, 60 µl of the substrate solution was used for 30 µl of sample solution and incubation temperature was 30 °C, 35 °C, 40 °C and 45 °C, respectively (Figure 13), whereas incubation time in the primary trial was 5 minutes, 10 minutes and 15 minutes, respectively. Afterwards, the sample amount was reduced to 20 µl and two incubation temperatures were selected (30 °C and 45 °C) with an extended range of incubation time (2.5 to 30 minutes) (Figure 14). Subsequently, 20 mM (20 µmol/ml) D-galacturonic acid was used as reference for product formation to calculate the relative activity of pectinase. Pectinase activity was measured at 35 °C and 40 °C with incubation time of 15 minutes and 10 minutes, respectively. Liquid samples from anaerobic, semi-aerobic, aerobic and their corresponding negative controls (lignocellulose substrates without fungus) from different (four weeks) time-points were used as sample (20 µl) for 60 µl of substrate solution. Eventually, the accepted incubation time was either 10 minutes at 40 °C or 15 minutes at 35 °C and the sample amount was 20 µl, while 60 µl of substrate solution was added in each well (Figure 15).

### 3.9.2 Optimized protocol for pectinase assay

In the optimized pectinase assay protocol, 60 µl of the substrate was transferred to a 96-well polypropylene plate (three technical replicas) with a multichannel pipette and the substrate solution was pre-warmed to 40 °C. Subsequently, samples (20 µl/well) were transferred to polypropylene plate and incubated for 10 minutes at 40 °C. Incubation time started immediately when the samples were added and therefore the samples were pipetted for example three (3) columns (1-3, then 7-9, and so on, and rows A-F) at a time with a multichannel pipette (e.g. 30 sec interval) and covered with plastic plate seal. After 10 minutes incubation, reaction was stopped by adding 100 µl DNS with a multichannel pipette first to the wells with samples in the same order and with the same intervals as when starting the reactions (1-3, then 7-9, so on and rows A-H) and then to the rest of the wells (4-6, then 10-12, so on and rows A-H). Later, 20 µl of samples were transferred to enzyme zero wells (4-6, then 10-12, so on and rows A-F) and 20 µl of each standard dilution (st1-st4), and buffer (st0) was added into standard wells (row G and H). The plate containing reaction mixture was boiled for 5 minutes in a water bath by covering with a plastic foil. After boiling, the plate was cooled immediately in an ice containing water bath and an equal amount of cooled reaction mixtures (150 µl each portion) were transferred to the plastic NUNC F microwell plates for measurement. Absorbances were measured spectrophotometrically by Spark (absorbance at 540 nm) microtiter plate reader by selecting the program DNS-Endoglucanase. Standard curve and standard deviations of parallel samples were checked to calculate the results from excel.

**Calculation of the pectinase activity:**

The absorbance value of the sample was converted to enzyme activity units (nkat/ml) using the following equation according to the standard curve, where absorbances in Y-axis and nkat/ml in X-axis,

$$\text{activity} = \frac{10\ \mu mol/ml}{600\ s} = 0.0167\ \mu\frac{mol}{ml} \times s = 16.7 nkat/ml$$

Standard zero was subtracted from the standards before drawing the standard curve, and standard zero (point 0,0) was also included in the curve. Individual enzyme activities were calculated by dividing the absorbances with slope of the standard curve and then multiplying with the dilution factor.

## 4. Results

### 4.1 Bioconversion of the lignocellulose substrate mixtures by the fungus

*Phlebia radiata* 79 was cultivated on a mixtures of lignocellulose substrates to evaluate fungal ability to use the lignocellulosic waste materials for hyphal growth as carbon, nutrient and energy source, and to evaluate the fungal bioconversion efficiency to valuable end products, especially ethanol.

#### 4.1.1 Mixture of barley straw and spent brewery barley mash

The fungus was capable of growing on barley straw and spent brewery barley mash subsequently converting them into fermentable sugars like glucose and producing various metabolites involving ethanol, glycerol, and acetate (Figure 4 and Figure 5). In anaerobic cultures, ethanol accumulation was 34 mmol/l, 88 mmol/l, 68 mmol/l and 67 mmol/l on cultivation weeks 1, 2, 3, and 4, respectively (Figure 4).

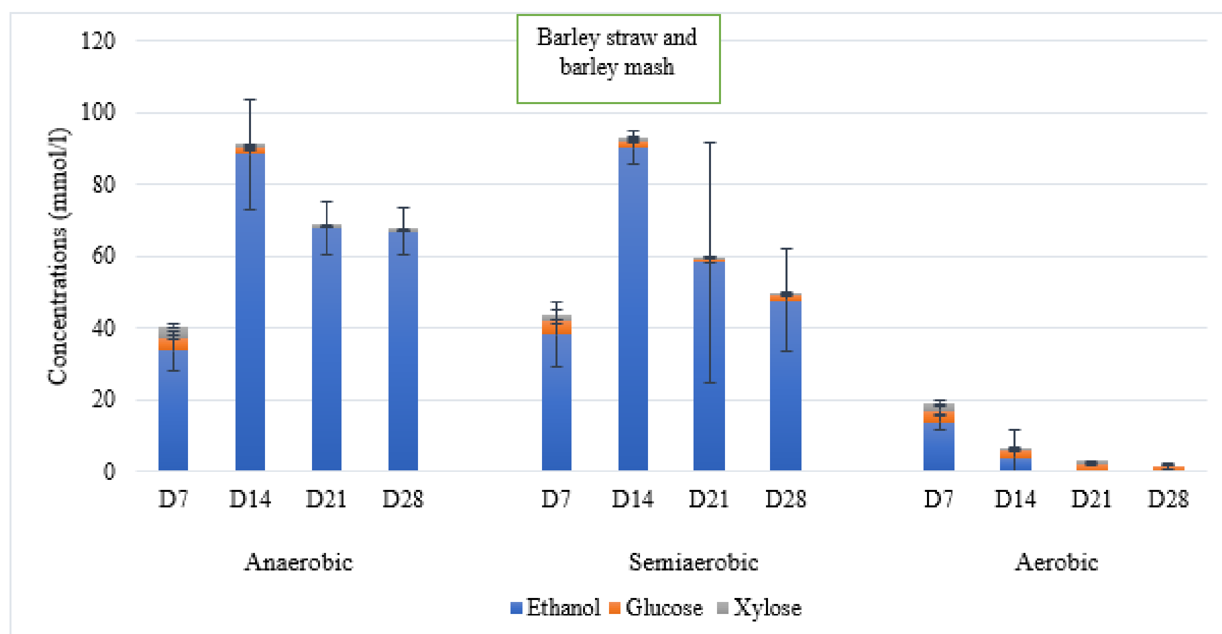


**Figure 4.** Accumulation of ethanol, glucose, and xylose by *P. radiata* 79 in anaerobic, semi-aerobic and aerobic solid-state cultures. Solid substrate was a mixture of barley straw and spent brewery barley mash. Mean values of four replicate culture flasks with standard deviation (bars) are presented. D, cultivation day.

In semi-aerobic cultures, detected ethanol amounts were 38 mmol/l, 90 mmol/l, 58 mmol/l, and 48 mmol/l, at the corresponding four time-points, while not more than 14 mmol/l and 4 mmol/l of ethanol accumulated during the two first weeks of cultivation in the aerobic cultures. It is notable

that both under anaerobic and semi-aerobic conditions, the highest concentration of ethanol was reached on the second week of cultivation (Figure 4).

Glucose and xylose were released to the liquid phase from the lignocellulosic substrates by fungal metabolic activity. The highest amount of both sugars was detected on the first week of cultivation in all atmospheric conditions. However, glucose concentrations were lower than 4 mmol/l in all cases (3.5 mmol/l, 3.7 mmol/l and 3.4 mmol/l at the day-7 time-point), and xylose concentration was slightly lower (3.4 mmol/l, 2.0 mmol/l and 2.1 mmol/l), under anaerobic, semi-aerobic and aerobic cultivation conditions, respectively. Sugar concentration decreased on the second week of cultivation in all atmospheric conditions, and only low concentrations of free sugars were detected at the end of the cultivation (Figure 4).

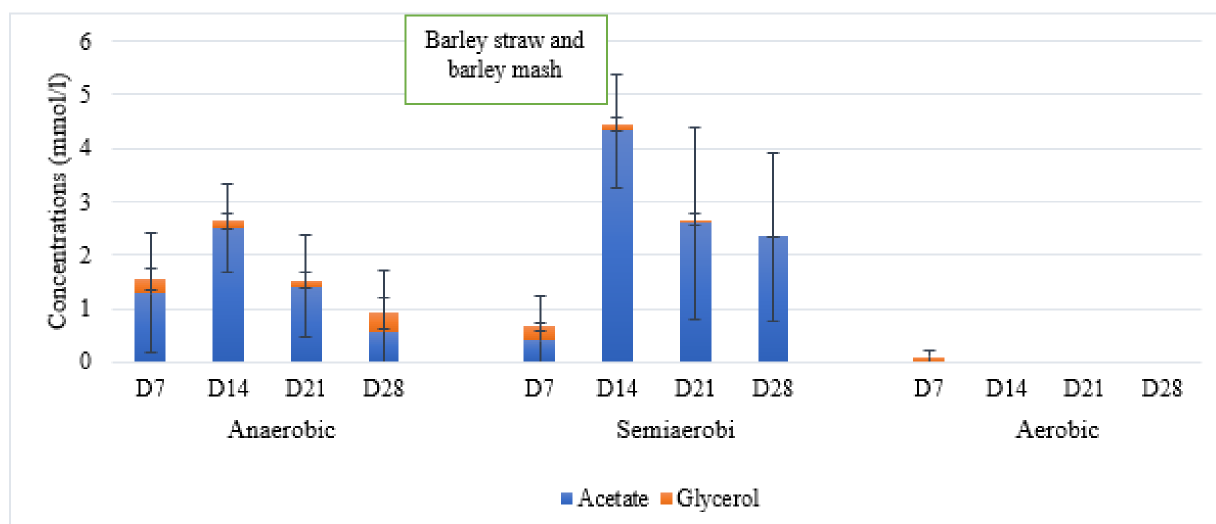


**Figure 5.** Conversion of the mixture of barley straw and spent brewery barley mash by *P. radiata* 79 in solid-state cultures into acetate and glycerol under anaerobic, semi-aerobic and aerobic cultivation conditions. Mean values of four replicate culture flasks with standard deviation (bars) are presented. D, cultivation day.

Acetate (as free acetic acid) was detected in the anaerobic and semi-aerobic culture flasks, already after the first week of cultivation (Figure 5). However, no acetate accumulated in aerobic culture flasks. Maximal production of acetate was detected on day-14 in both anaerobic (2.5 mmol/l) and semi-aerobic conditions (4.3 mmol/l). Production of glycerol was limited to hardly detectable levels, for instance on day-7 in anaerobic (0.3 mmol/l) and semi-aerobic conditions (0.2 mmol/l), with the highest concentration of glycerol (0.4 mmol/l) detected after four weeks of

cultivation under anaerobic conditions. Under aerobic conditions, no more detectable amounts of glycerol could be recorded after day-7, while production of acetic acid was absent throughout the cultivation period (Figure 5).

### 4.1.2 Mixture of spruce wood sawdust and spent brewery barley mash

On the substrate mixture of spruce wood sawdust and spent brewery barley mash, the highest ethanol concentrations were detected under anaerobic cultivation conditions, peaking on day-14 time-point to 87 mmol/l, staying at the level of over 70 mmol/l until the end of cultivation (Figure 6). In the semi-aerobic cultures, a similar production pattern for ethanol was observed, and about 2/3 of the ethanol concentrations accumulated in comparison to the anaerobic conditions. In accordance, the maximum 61 mmol/ml of ethanol accumulation was detected after two weeks of cultivation, with about 40 mmol/l still present in the end of the cultivation (Figure 6).

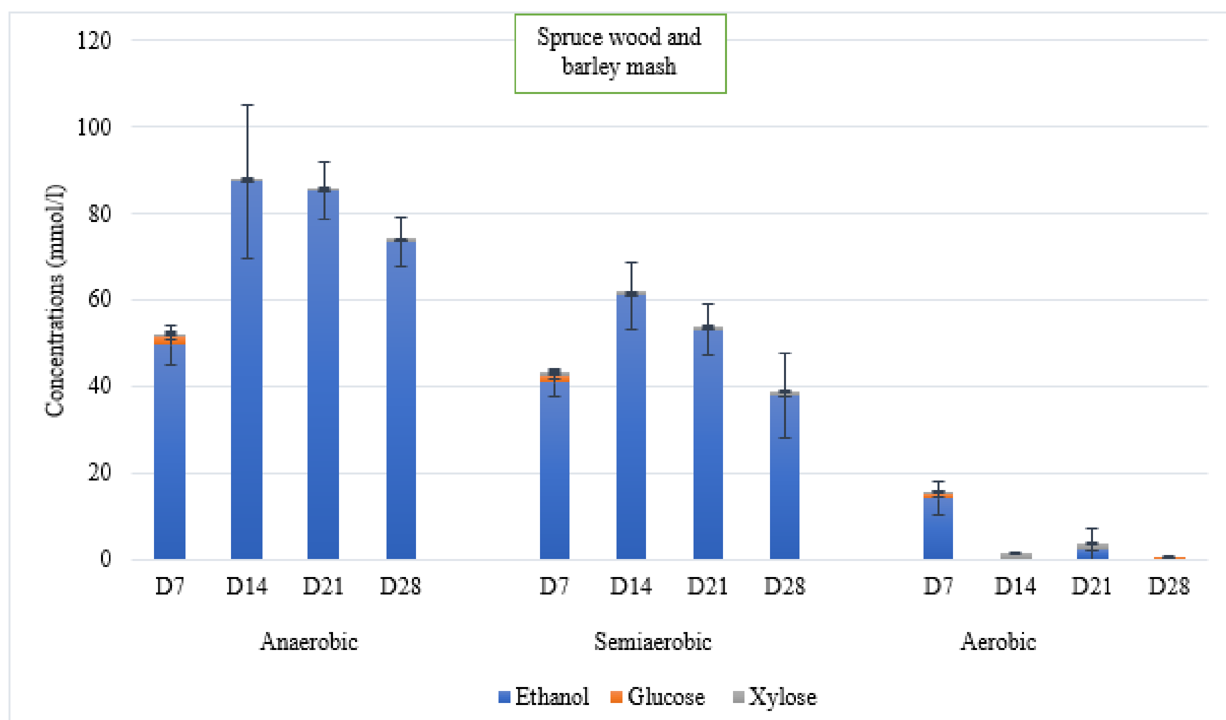


**Figure 6.** Accumulation of ethanol, glucose, and xylose by *P. radiata* 79 in solid-state cultures on a mixture of spruce wood sawdust and spent brewery barley mash under different cultivation atmospheric conditions. Mean values of four replicate culture flasks with standard deviation (bars) are presented. D, cultivation day.

Notable is that some ethanol production was detected also in the aerobic culture flasks on the substrate mixture containing spruce wood sawdust. Detected ethanol concentrations were 14 mmol/l and 2.4 mmol/l on day-7 and day-21 in aerobic cultivation (Figure 6).

In comparison to the bioconversion of the substrate mixture of barley straw and spent brewery barley mash, less free glucose and xylose was detected on the mixture of spruce wood sawdust and spent brewery barley mash, under all atmospheric conditions. Glucose concentrations less than 2 mmol/l were detectable after the first week of cultivations under aerobic, semi-aerobic and aerobic conditions, while almost the same very low amount of xylose (0.7 mmol/l) was observed during the anaerobic cultivation (Figure 6).

Accumulation of acetate and glycerol was detected only in anaerobic and semi-aerobic conditions of spruce wood sawdust blended spent brewery barley mash cultivation. Similar to the pattern of acetate production on the substrate mixture containing barley straw, the highest concentrations of acetic acid to the levels of over 2.6 mmol/l (up to 3.2 mmol/l on day 28$^{th}$) were detected under semi-aerobic conditions (Figure 7).

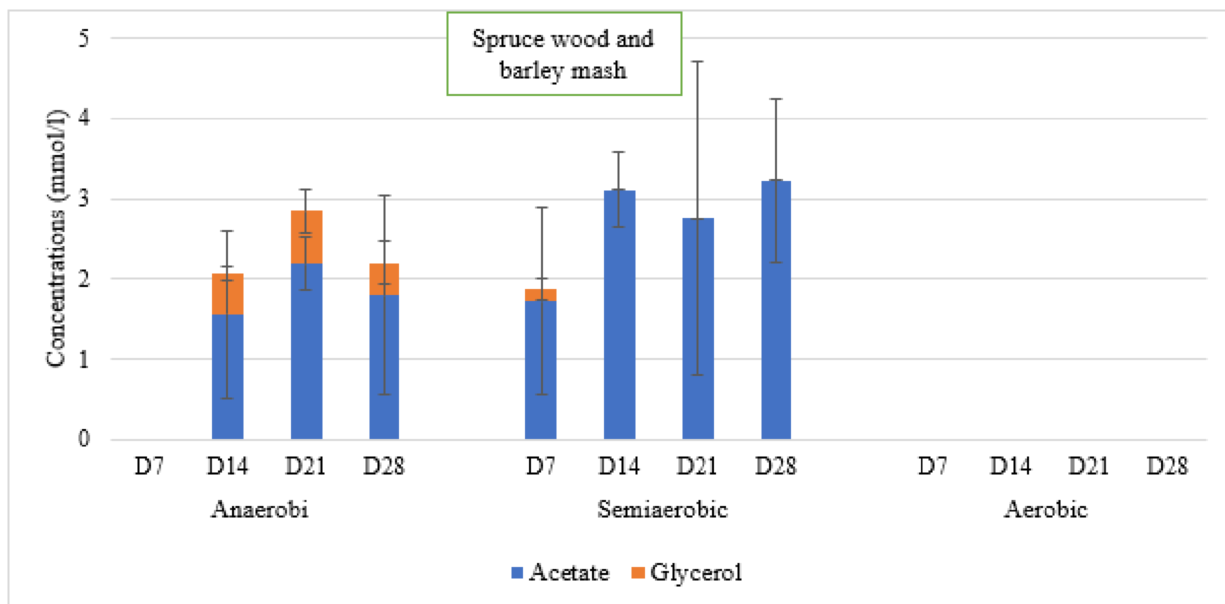


**Figure 7.** Conversion of the substrate mixture of spruce wood sawdust and spent brewery barley mash by *P. radiata* 79 in solid-state cultures into acetate and glycerol under three atmospheric conditions. Mean values of four replicate culture flasks with standard deviation (bars) are presented. D, cultivation day. Under aerobic conditions, concentrations were below the detection limit.

Under anaerobic conditions, however, more glycerol accumulated on this substrate mixture to 0.5 mmol/l, 0.7 mmol/l, and 0.4 mmol/l after 1, 2 and 3 weeks of cultivation, respectively. Under aerobic conditions, no detectable amounts of acetic acid or glycerol could be recorded (Figure 7).

### 4.1.3 Mixture of birch wood sawdust and spent brewery barley mash

The highest concentrations of ethanol production were detected on the substrate mixture of birch wood sawdust and spent brewery barley mash, up to the concentrations of about 108 mmol/l after the first and second week of cultivation under anaerobic conditions, and after the second week of cultivation under semi-aerobic conditions (Figure 8). Under anaerobic conditions, ethanol concentration also stayed at a high level (over or near to 80 mmol/l) until the end of cultivation. Under semi-aerobic conditions, similarly about 50 % of this amount of ethanol was still present in the cultures after four weeks. In aerobic cultures, 21 mmol/l and 8.2 mmol/l ethanol production was observed on day-7 and day-14, respectively (Figure 8).

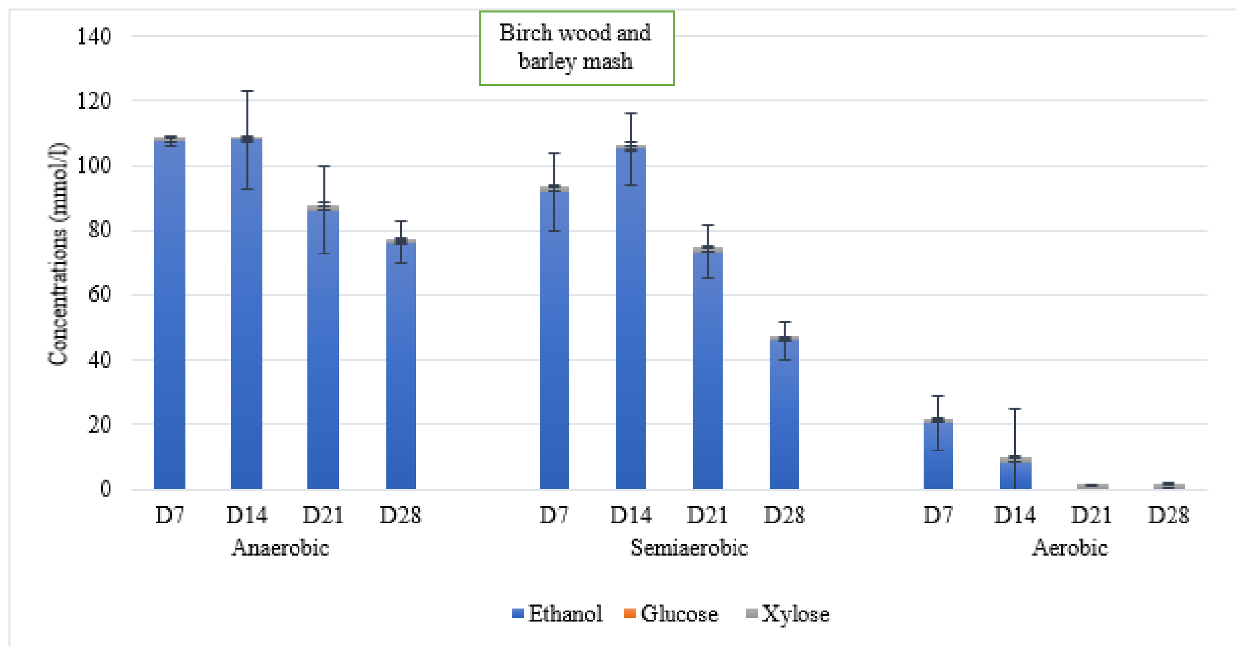


**Figure 8.** Accumulation of ethanol, glucose, and xylose by *P. radiata* 79 in solid-state cultures on substrate mixture of birch wood sawdust and spent brewery barley mash under different cultivation atmospheric conditions. Mean values of four replicate culture flasks with standard deviation (bars) are presented. D, cultivation day.

Regarding the released sugars, very small amounts of glucose (less than 0.3 mmol/l) were detected in the culture fluids (Figure 8). Compared to glucose, more xylose (from 1.1 to 1.8 mmol/l) was detected under aerobic and semi-aerobic conditions. Under aerobic conditions, xylose concentration was 1.3 mmol/l, 1.8 mmol/l, 1.5 mmol/l and 1.6 mmol/l on day-7, day-14, day-21 and day-28, respectively, while no glucose was recorded (Figure 8).

Similar to bioconversion of the substrate mixtures containing either barley straw or spruce wood sawdust, accumulation of some acetic acid and glycerol were detected in anaerobic and semi-aerobic cultures. Acetate concentrations of about 2.5 mmol/l at all-time points were detected under anaerobic conditions, while glycerol accumulated to 0.8 mmol/l on day-7, then to 0.2 mmol/l on day-28 (Figure 9). Under semi-aerobic conditions, acetate production fluctuated between 2.1 mmol/l (day-7) and 3.5 mmol/l (day-28), whereas very low amount (0.2 mmol/l) of glycerol was detected only on day-7. As before, no production of acetic acid or glycerol were detected under aerobic conditions (Figure 9).

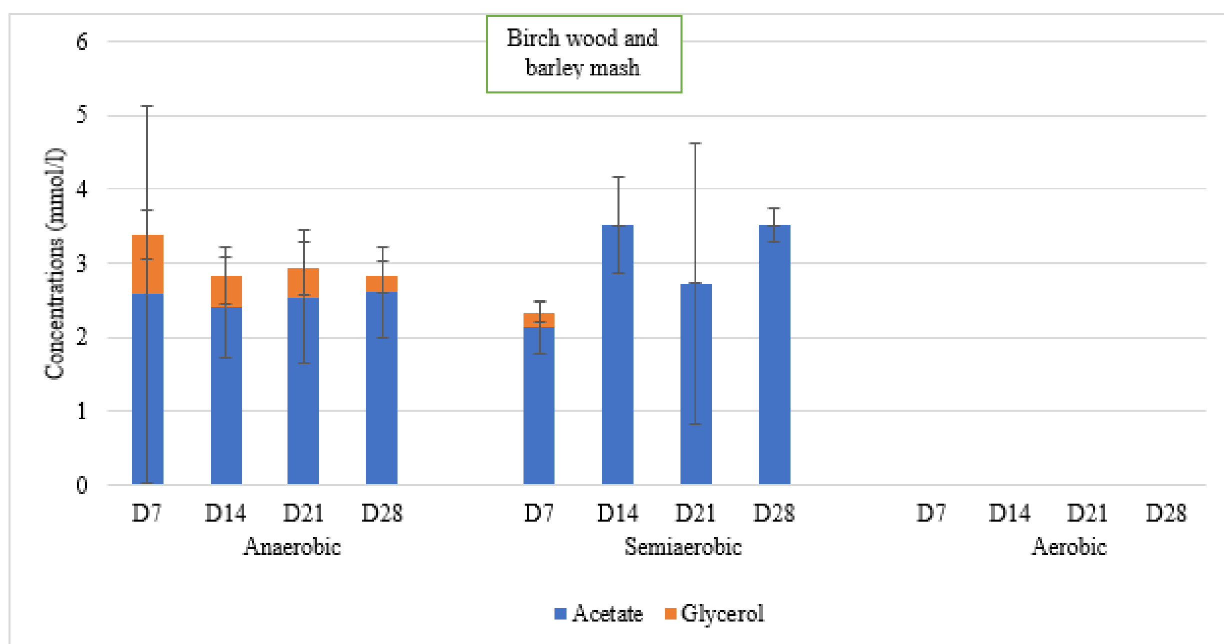


**Figure 9.** Conversion of the substrate mixture of birch wood and spent brewery barley mash by *P. radiata* 79 in solid-state cultures into acetate and glycerol under different cultivation atmospheric conditions. Mean values of four replicate culture flasks with standard deviation (bars) are presented. D, cultivation day. Under aerobic conditions, concentrations were below the detection limit.

### 4.2 Enzyme activities produced on the lignocellulose substrate mixtures

Samples for enzyme activity assays were collected four times during fungal growth on the solid lignocellulose substrate mixtures to monitor extracellular enzyme activities produced by the fungus under the three different atmospheric conditions. During this research project activity of β-glucosidase, cellobiohydrolase and pectinase were measured.

### 4.2.1 Activities on barley straw-spent brewery barley mash

The activity of β-glucosidase increased almost linearly until the end of the culture (day-28) under anaerobic and semi-aerobic conditions. β-glucosidase activity was 0.2 nkat/ml, 1.7 nkt/ml, 2.0 nkat/ml and 2.3 nkat/ml after week 1, 2, 3, and 4, respectively under anaerobic conditions. Under semi-aerobic cultivation, the recorded activity was 0.5 nkat/ml, 1.7 nkat/ml, 2.5 nkat/ml and 2.6 nkat/ml after four respective time points. β-glucosidase activity fluctuated between 0.7 nkat/ml (day-7) and 5.2 nkat/ml (day-28), under aerobic conditions. Activity of β-glucosidase fluctuated between 0.1 nkat/ml and 0.3 nkat/ml in all negative controls of different atmospheric conditions (Figure 10 A).

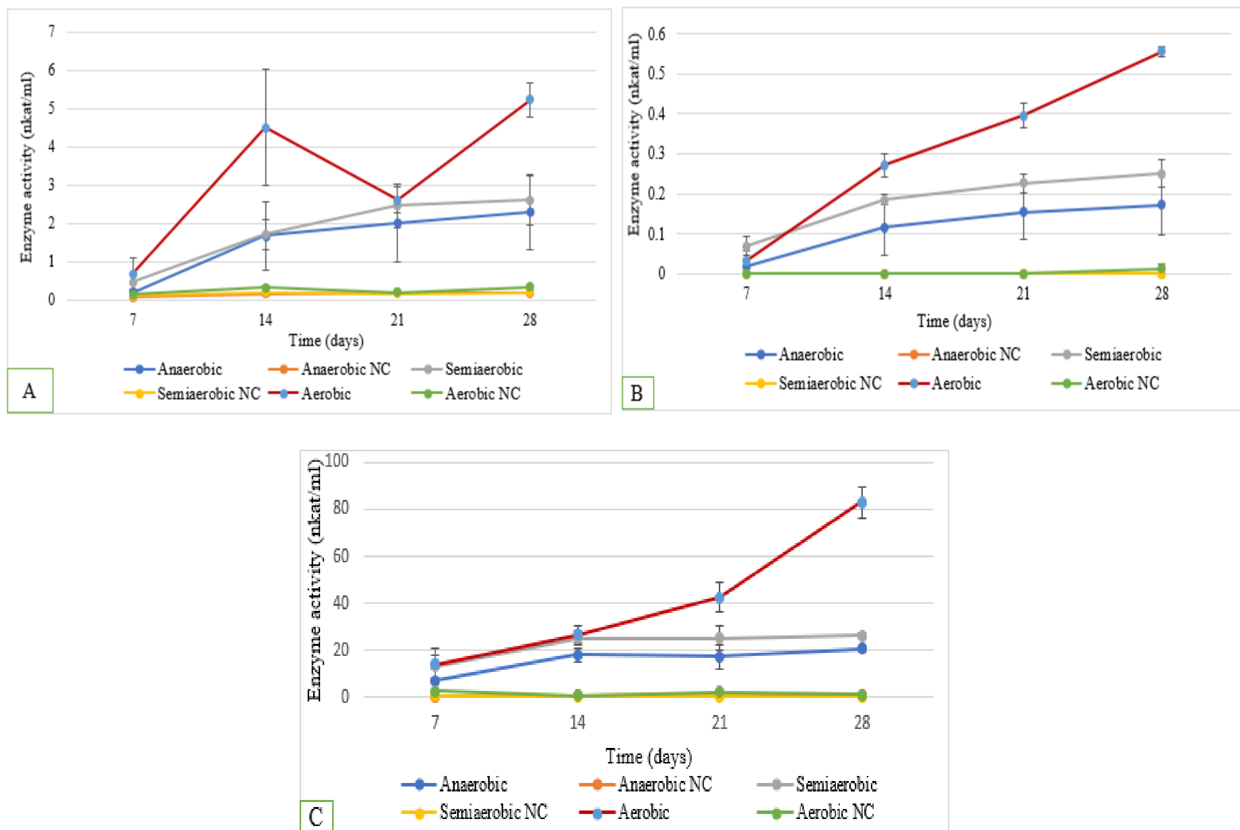


**Figure 10.** Production of enzyme activities on the substrate mixture of barley straw and spent brewery barley mash under different atmospheric conditions during four weeks of cultivation. Activities of A) β-glucosidase, B) cellobiohydrolase, and C) pectinase, are presented. Mean values of four replicate culture flasks with standard deviation (bars) are presented. NC= negative control; culture substrate flasks without the fungus.

CBH activity enhanced linearly until the end time point (day-28) of all cultivations and CBH activity under aerobic conditions was always dominant. Under anaerobic conditions, CBH activity was between 0.02 nkat/ml (day-7) and 0.2 nkat/ml (day-28), while highest activity was 0.3 nkat/ml after 4 weeks of cultivation under semi-aerobic conditions. CBH activity was 0.03 nkat/ml, 0.3 nkat/ml, 0.4 nkat/ml and 0.6 nkat/ml after week 1, 2, 3, and 4, respectively, under aerobic conditions (Figure 10 B).

The trend of pectinase activity was similar to the result of CBH activity on barley straw and spent brewery barley mash substrate, and the highest activity was observed under aerobic cultivations of all time points. Under anaerobic cultivations, pectinase activity was 6.8 nkat/ml, 17.9 nkat/ml, 17.4 nkat/ml and 20.6 nkat/ml, on day-7, day-14, day-21, and day-28, respectively. Under semi-aerobic conditions, activity was between 13.3 nkat/ml (day-7) and 26.3 nkat/ml (day-28). Pectinase activity was 14.0 nkat/ml, 26.6 nkat/ml, 42.3 nkat/ml, and 83.1 nkat/ml in corresponding four time points under aerobic cultivations. The recorded pectinase activity of negative controls (lignocellulose substrates without fungus) in all cultivation conditions were between 0.4 nkat/ml and 2.6 nkat/ml (Figure 10 C).

**4.2.2 Activities on spruce wood sawdust-spent brewery barley mash**

β-glucosidase activity was very low in spruce wood sawdust cultivation and fluctuated from 0.1 nkat/ml to 0.2 nkat/ml in all culture conditions. The highest activity 0.22 nkat/ml was recorded on day-14 under anaerobic cultivation, which reduced to 0.14 nkat/ml on day-21 and again increased to 0.18 nkat/ml. In semi-aerobic cultivation, except day-21 (0.17 nkat/ml) β-glucosidase activity was around 0.1 nkat/ml in all other time points. Surprisingly, activity was much lower under aerobic cultivations with the highest value of 0.16 nkat/ml on day-28 (Figure 11 A).

No detectable CBH activity was recorded during the first and second weeks of cultivations in all conditions. On day-21, measured activity was 0.03 nkat/ml in both anaerobic and semi-aerobic cultivations, while detected CBH activity was 0.04 nkat/ml in aerobic cultivation. CBH activity was increased rapidly to 0.7 nkat/ml under aerobic conditions on day-28 (Figure 11 B).

The trend of pectinase activity was similar to the previous cultivation with barley straw, and pectinase activity was dominant under aerobic conditions. The activity was between 2.3 nkat/ml and 5.4 nkat/ml in both anaerobic and semi-aerobic conditions during the whole cultivation period. Pectinase activity was 12.1 nkat/ml, 30.3 nkat/ml, 40.3 nkat/ml and 81.6

nkat/ml on day-7, day-14, day-21 and day-28, respectively, under aerobic conditions (Figure 11 C).

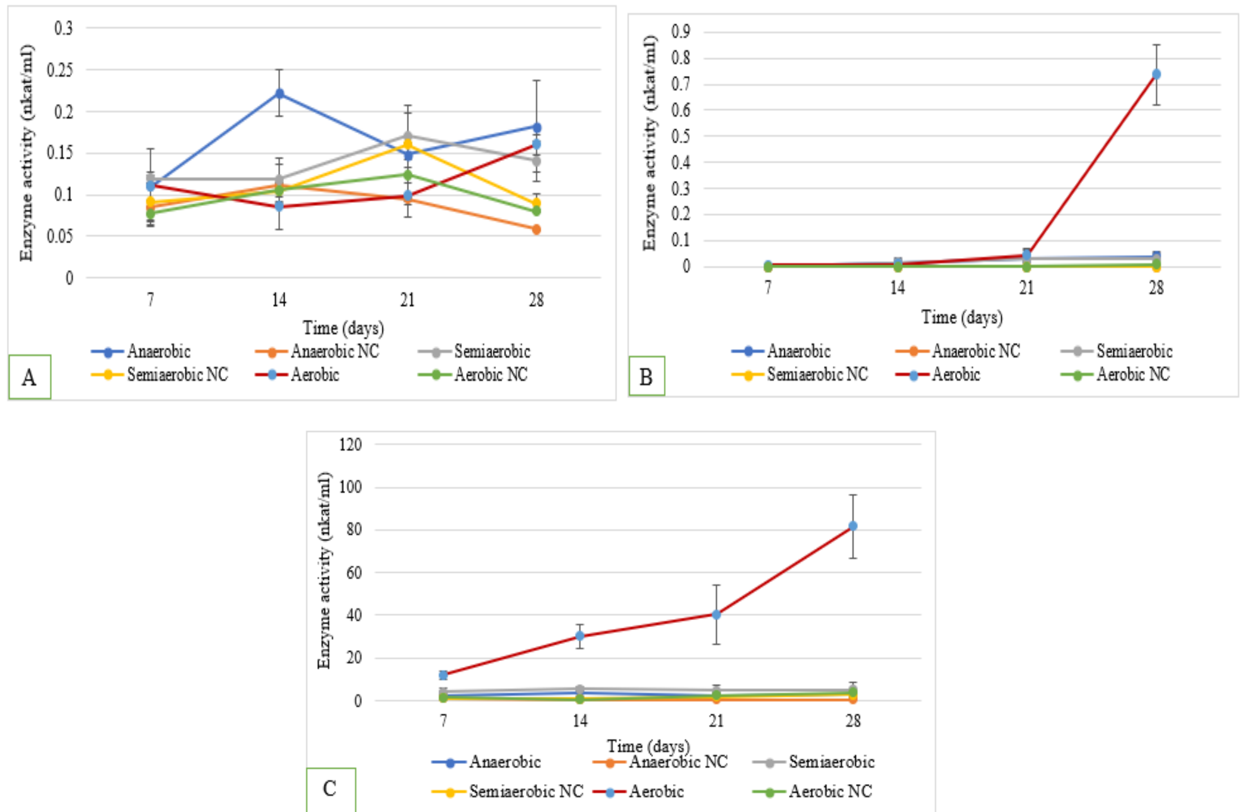


**Figure 11.** Production of enzyme activities on the substrate mixture of spruce wood sawdust and spent brewery barley mash under different atmospheric conditions during four weeks of cultivation. Activities of A) β-glucosidase, B) cellobiohydrolase, and C) pectinase, are presented. Mean values of four replicate culture flasks with standard deviation (bars) are presented. NC= negative control; culture substrate flasks without the fungus.

### 4.2.3 Activities on birch wood sawdust-spent brewery barley mash

The activity of β-glucosidase on birch wood sawdust and spent brewery barley mash was remarkably low and was reduced during the first two weeks of cultivation in all conditions. In anaerobic cultivation, activity was about 0.13 nkat/ml on day-7 and day-14, which dropped to 0.05 nkat/ml on day-21 and again raised to 0.09 nkat/ml on day-28. β-glucosidase activity was about 0.1 nkat/ml during the whole semi-aerobic cultivation, except a certain drop on day-28 (0.04 nkat/ml). under aerobic cultivation, β-glucosidase activity fluctuated between 0.12 (day-7) and 0.03 nkat/ml (day-21) (Figure 12 A).

Among all three cultivations, the lowest CBH activity was recorded from birch wood sawdust blended spent brewery barley mash cultures. In anaerobic condition, CBH activity was about 0.02 nkat/ml in all-time point except time-point day-7 (0.01 nkat/ml). Highest activity 0.05 nkat/ml was recorded on day-21 under semi-aerobic cultivation, while both in the second and fourth week of cultivation, the activity was 0.03 nkat/ml. Lowest CBH activity was observed under aerobic cultivation, which vacillated between 0.001 nkat/ml and 0.01 nkat/ml (Figure 12 B).

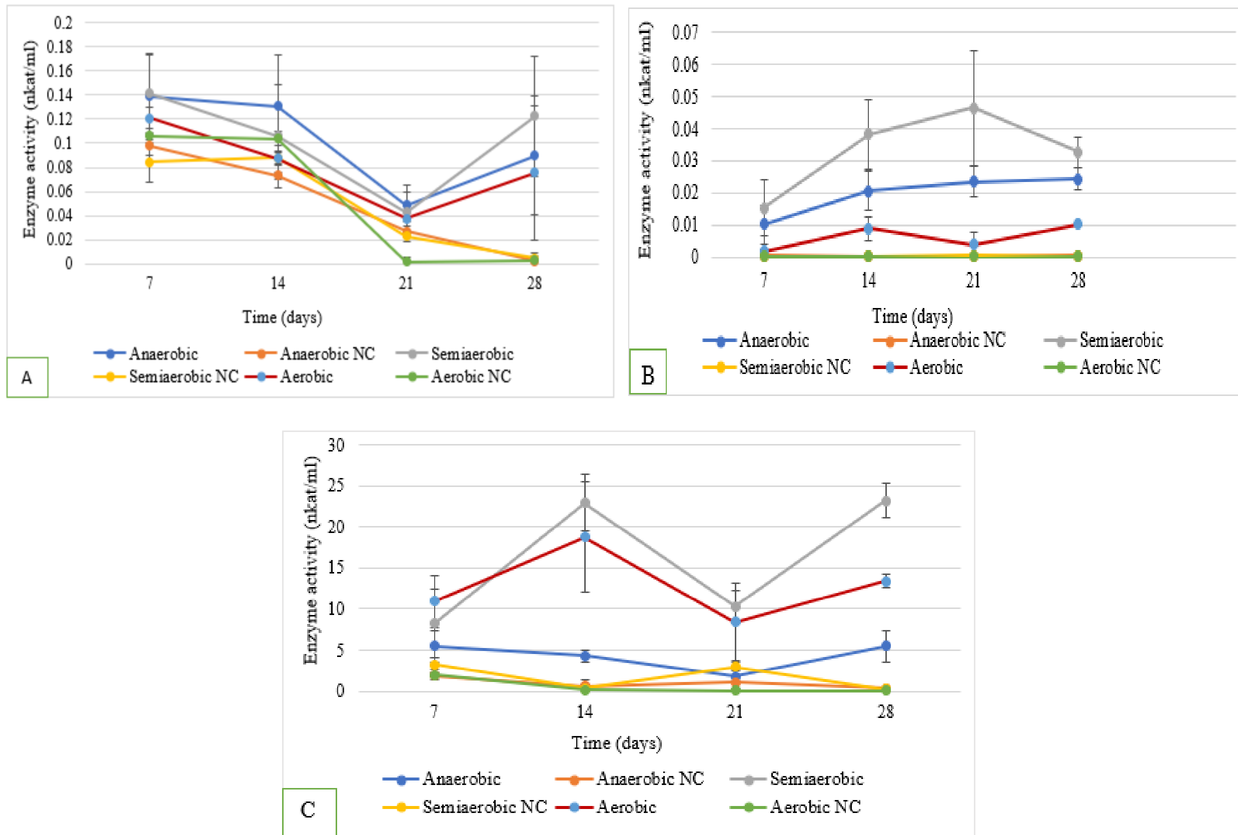


**Figure 12.** Production of enzyme activities on the substrate mixture of birch wood sawdust and spent brewery barley mash under different atmospheric conditions during four weeks of cultivation. Activities of A) β-glucosidase, B) cellobiohydrolase, and C) pectinase. Mean values of four replicate culture flasks with standard deviation (bars) are presented. NC= negative control; culture substrate flasks without the fungus.

Compared to β-glucosidase and CBH, pectinase activity was prominent on birch wood sawdust and spent brewery barley mash substrate. Pectinase activity was about 5.5 nkat/ml under anaerobic condition during the whole cultivation period with a drop of activity to 1.8 nkat/ml on day-21. Highest pectinase activity 23.2 nkat/ml was observed under semi-aerobic conditions and activity fluctuated between 8.3 (day-7) nkat/ml and 23.2 nkat/ml (day-28). Pectinase activity was

10.9 nkat/ml, 18.7 nkat/ml, 8.4 nkat/ml and 13.3 nkat/ml on day-7, day-14, day-21 and day-28, respectively, under aerobic cultivation (figure 12 C).

### 4.3 Optimization of pectin degradation assay on 96-microwell plate scale

Different reaction temperatures with various incubation times were tested, and absorbances were recorded for the optimization of pectin degradation enzyme (pectinase) activity using polygalacturonic acid (PGA) as substrate. The highest absorbance values were observed samples taken from aerobic, semi-aerobic and anaerobic cultures, after incubating the reaction mixtures for 15 minutes at 30 °C (Figure 13 A).

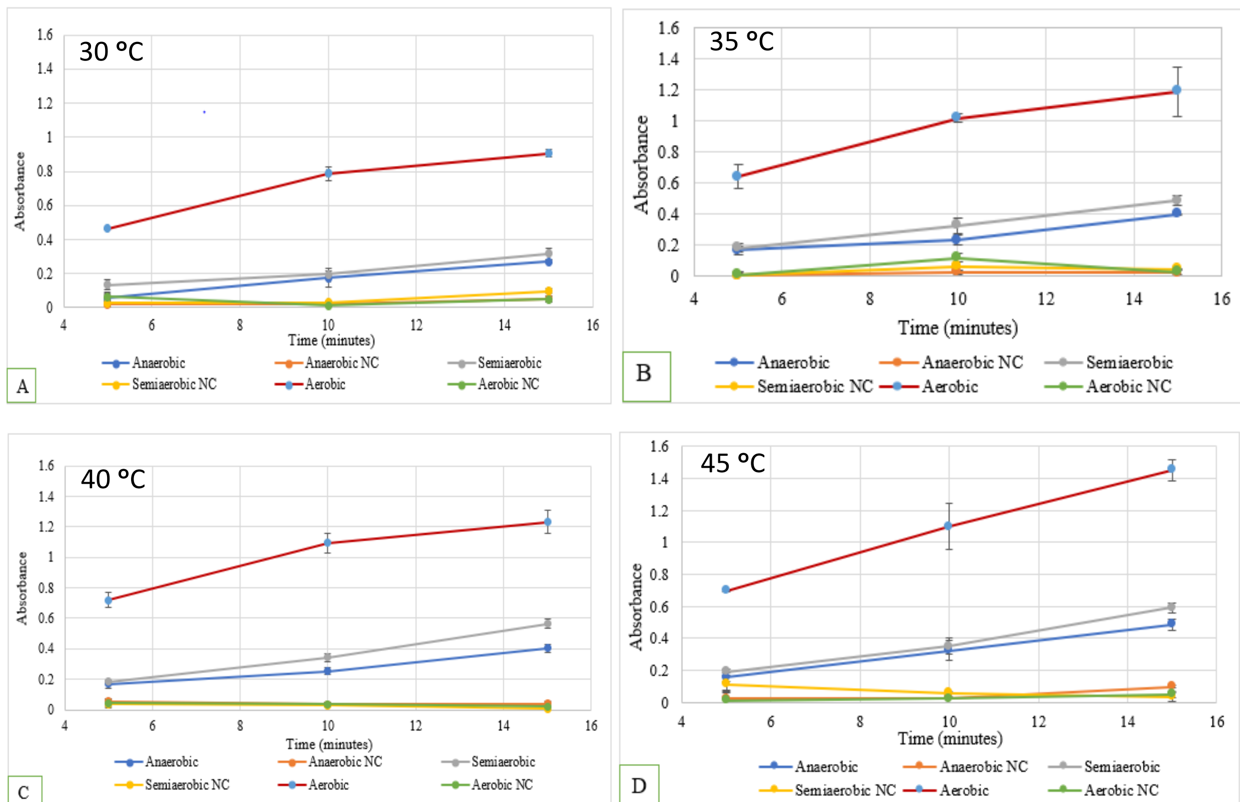


**Figure 13.** Optimization of incubation time and temperature of pectinase assay with 60 µl substrate and 30 µl of each sample. Samples are from barley straw and spent brewery barley mash cultivation of day-28 time point, incubated under the three variant atmospheric conditions. (A= incubation temperature 30 °C; B= incubation temperature 35 °C; C= incubation temperature 40 °C and D= incubation temperature 45 °C). NC= negative control, culture substrate flasks without the fungus.

After 10 minutes incubation, the absorbances were 0.8, 0.2 and 0.17, respectively in aerobic, semi-aerobic and anaerobic samples. Reaction temperature was increased to 35 °C, 40 °C and 45 °C with same incubation time (15 minutes) to monitor the changes in absorbances and

elevated absorbance values were recorded in all cases. The highest absorbances 1.5, 0.6 and 0.5 were recorded samples taken from aerobic, semi-aerobic and anaerobic cultures, respectively after incubating them for 15 minutes at 45 °C (Figure 13 D). In all these four cases, 60 µl substrate and 30 µl of the sample solution was used (Figure 13).

Later, the sample amount was reduced to 20 µl and used only samples from aerobic condition with extended incubation time (2.5 to 30 minutes) and the substrate amount was 60 µl (Figure 14). Absorbances were 0.3 and 0.7 after 5 minutes incubation, while absorbances were 0.8 and 1.1 after 15 minutes incubation at 30 °C and 45 °C, respectively. Highest absorbance 1.4 and 2.2 were recorded after incubating for 30 minutes at 30 °C and 45 °C, whereas absorbances were 1.1 and 1.8, for 25 minutes incubation at 30 °C and 45 °C, respectively. These absorbances were measured without standard solution (Figure 14).

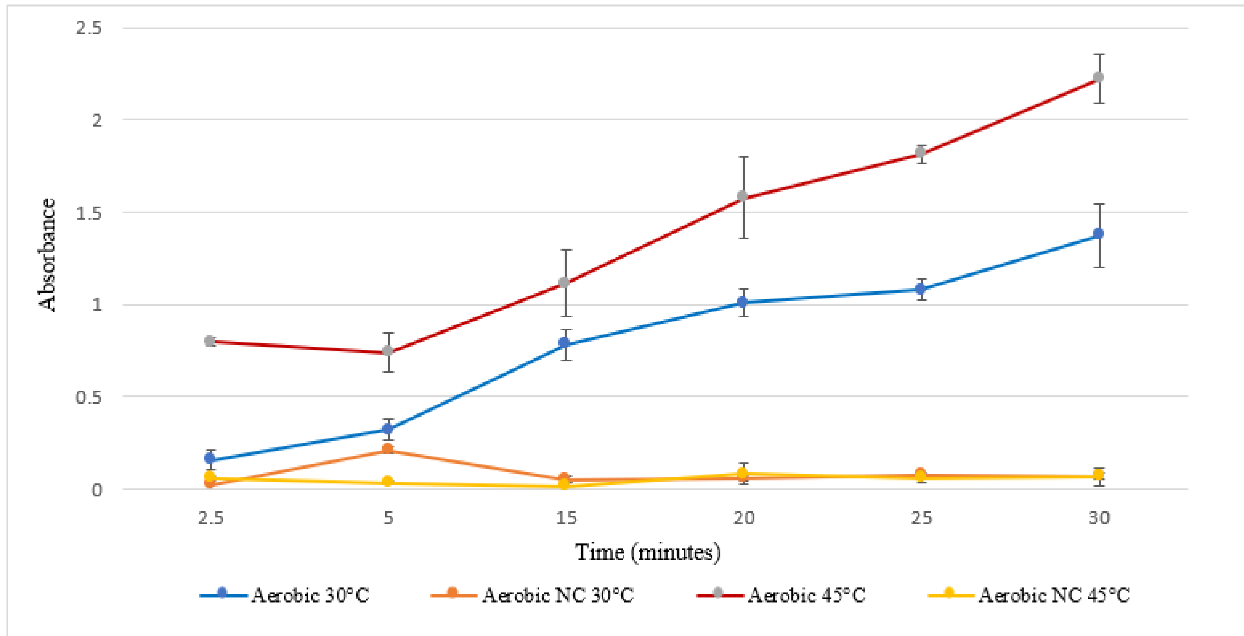


**Figure 14.** Optimization of incubation time and temperature (30 °C and 45 °C) of pectinase assay with 60 µl substrate and 20 µl of the sample. Samples are from barley straw and spent brewery barley mash cultivation of day 28, incubated under aerobic conditions. NC= negative control, culture substrate flasks without the fungus.

Subsequently, pectinase activity was measured using the standard solution at 30 °C and 40 °C with incubation time 15 minutes and 10 minutes, respectively (Figure 15). For 15 minutes incubation at 35 °C detected pectinase activity was 19 nkat/ml, 32 nkat/ml and 77 nkat/ml for samples taken from aerobic cultivations on day-14, day-21 and day-28, respectively (Figure 15 A).

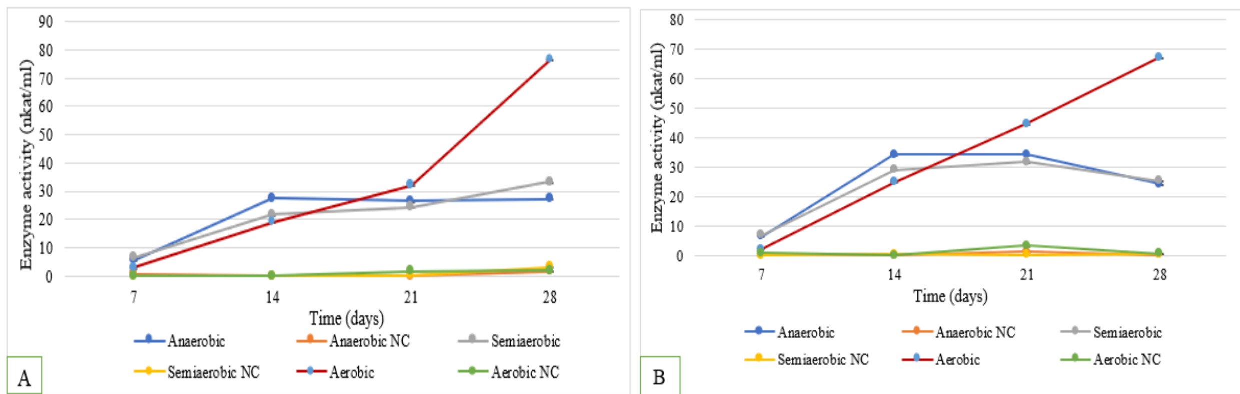


**Figure 15.** Activity of pectinase with different incubation times and temperatures. Samples are from barley straw and spent brewery barley mash cultivation of different time-points. (A= 15 minutes incubation at 35 °C and B= 10 minutes incubation at 40 °C). NC= negative control, culture substrate flasks without the fungus.

Pectinase activity increased linearly from 7 nkat/ml (day-7) to 33 nkat/ml (day-28) in semi-aerobic condition, while activity was between 6 nkat/ml (day-7) and 28 nakt/ml (day-21) in anaerobic culture samples (Figure 15 A).

For 10 minutes incubation at 40° C, pectinase activity was 25 nkat/ml, 45 nkat/ml and 67 nkat/ml for samples taken from aerobic cultivations on day-14, day-21 and day-28, respectively. Similar trends of pectinase activity were detected in semi-aerobic and anaerobic culture samples (Figure 15 B). Notable is the steady increase in enzyme activity until the end of cultivations, indicating on-going or even promoted enzyme expression in the fungus (Figure 15).

## 5. Discussion

This study was performed to evaluate the potential of second-generation bioethanol production at different atmospheric conditions from waste lignocelluloses by the white-rot fungus *Phlebia radiata* isolate 79. Second-generation bioethanol production was chosen because feedstock for this approach is sustainable and available as waste material in large quantities compared to first-generation feedstocks. In addition, production and utilization of second-generation bioethanol is environment-friendly as well as ethically accepted because of the non-edible nature of the lignocellulose biomass (Farrell et al., 2006; Lennartsson et al., 2014).

First-generation bioethanol is produced by direct fermentation of starchy materials including sugarcane, corn, barley, potato waste, and other food crop feedstock (Buijs et al., 2013). On the other hand, second-generation bioethanol is produced by applying non-food and non-feed plant biomass-based feedstocks, such as wood and non-wood lignocelluloses and agricultural wastes, which are at first subjected to pretreatments like steam explosion and chemical hydrolysis prior to fermentation of the released carbohydrates. Generally, external cellulases and other enzymes are used for commercial hydrolysis processes of lignocellulose substrates, and the generated sugars are fermented by yeasts or bacteria in bioethanol production (Kricka et al., 2014). This two-step process hinders the industrial goal to produce bioethanol using single microorganisms capable of producing biomass hydrolyzing enzymes and fermenting the released sugars to bioethanol through consolidated bioprocessing (CBP) (Kricka et al., 2015).

CBP was the chosen strategy for bioconversion in this study as it does not require the pretreatment steps which may lead to sugar losses and lower yield of ethanol produced as well as increase in the reactor and associated equipment costs (Alvira et al., 2010). Physical size reduction of the solid substrate materials increases microbial accessibility and thus, decreases the crystallinity of the substrate which favors the overall fermentation process (Chiaramonti et al., 2012). Therefore, the lignocellulose biomasses adopted in this study (barley straw, and spruce and birch wood sawdust) were cut or sawn into small pieces, and the additional substrate (SBBM, spent brewery barley mash) was dried and milled to fine powder to increase the accessible surface area.

Lignocellulose hydrolysis generates various hexose and pentose sugars, while only a small number of yeast species can metabolize pentoses such as xylose and arabinose. In addition, wild-type and industrially used *Saccharomyces* species isolates grow very slowly on a medium containing xylose or arabinose as the only carbon source (Wenger et al., 2010). Therefore, the

filamentous Basidiomycota fungus *P. radiata* 79 was selected as the fermentative organism instead of yeast for CBP. Several fungal species such as the Ascomycota yeasts of the genera *Candida* and *Pichia*, as well as the species *Arxula adeninivorans* and *Debaryomyces hansenii* can metabolize pentoses like arabinose (Madhavan et al.,2012). Accordingly, the yeast species *Pichia stipitis, Candida shehatae*, and *Pachysolen tannophilus* can metabolize xylose naturally (Agbogbo et al., 2006).

The baker's yeast *S. cerevisiae* is not able to secrete an adequate array of lignocellulosic biomass hydrolyzing enzymes to generate sugars. Addition of a little amount of glucose at the beginning of fermentation usually accelerates the growth of *S. cerevisiae* to produce cellulases. However, cellulase encoding genes are repressed by high concentrations of glucose (Kricka et al., 2015). As studied recently, the selected fungus *P. radiata* 79 does not require any external sugar for its growth and development upon CBP on lignocelluloses and lignocellulosic wastes (Mattila et al., 2017; Mattila et al., 2018). This fungus is capable of hydrolyzing lignocellulosic biomass without any pretreatment, and thereby, it produces significant amounts of ethanol through fermentation of the released sugars (Table 3).

**Table 3.** Ethanol production potentiality by decomposition and fermentation of different lignocellulosic substrates performed by phlebioid white rot fungi. Present study was compared to published studies with similar cultivation conditions and approaches. BS= barley straw; BW= birch wood sawdust; SBBM= spent brewery barley mash; SW= spruce wood sawdust.

| Substrate | Ethanol yield/ Substrate (g/100 g) | Fungal isolate | Duration (days) | Reference |
|---|---|---|---|---|
| BS+SBBM | 5.3 | *P. radiata* 79 | 14 | Present study |
| SW+SBBM | 3.4 | *P. radiata* 79 | 14 | Present study |
| BW+SBBM | 4.1 | *P. radiata* 79 | 14 | Present study |
| BW+SBBM | 10.1* | *P. radiata* 79 | 14 | (Mattila et al., 2018) |
| Core board | 10.4 | *P. radiata* 79 | 9 | (Mattila et al., 2017) |
| Newspaper | 20 | *Phlebia* sp. MG-60 | 9 | (Kamei et al., 2012) |
| Rice straw | 9.9 | *Phanerochaete chrysosporium* | 19 | (Bak et al., 2009) |

* concentration was given in g/l

Oxygen is essential for respiration and growth of wood-decay fungi, and it is also assumed that aeration and oxygen availability favor enzymatic radical-based degradation reactions during lignocellulose decomposition ( Kirk & Farrell, 1987; Lundell et al., 2014). In this research project, three conditions involving anaerobic, semi-aerobic and aerobic cultivations were chosen to investigate the effects of atmospheric conditions on lignocellulose decomposition and ethanol production by the white rot fungus *P. radiata* 79.

Proteome and transcriptome of the fungus *P. radiata* 79 was previously examined on spruce wood, while capability of lignin attacking and CAZyme activities were also detected (Kuuskeri et al., 2015; Kuuskeri et al., 2016). In addition, the fungus is able to produce a significant amount of ethanol on different lignocellulose substrates also in larger scale bioreactors (Mattila et al., 2018). In this research project, the CBP ability of the fungus was conducted on solid-state cultures of mixtures of spent brewery barley mash (SBBM, 1/4 of the substrate total mass) blended with several lignocellulosic waste materials (Table 3).

Compared to previous results, less ethanol accumulated in yield (30% to 50% of the previous yield and concentrations) in this study than previously with the same fungus (Table 3). This experiment was conducted to find out the suitable lignocellulose substrate combinations for ethanol production. The substrate mixture containing barley straw (3/4 of the total mass) and spent brewery barley mash (1/4 of the total mass) was the best solid substrate combination for *P. radiata* 79 in this respect. Birch wood with spent brewery barley mash was also a potential mixture for ethanol production by the fungus (Table 3).

*P. radiata* 79 was capable of decomposing the waste lignocellulose mixtures under different atmospheric conditions (nitrogen gas flushed anaerobic, semi-aerobic, and aerobic atmospheres). HPLC results demonstrated that changes in the atmospheric conditions generated by nitrogen gas flushing caused direct effects on production of bioethanol and fermentation by-products (acetate and glycerol). One assumption was that the presence of oxygen would allow activity of oxidoreductive enzymes secreted by the fungus, thus favoring more efficient decomposition of wood lignocellulose components under semi-aerobic and aerobic conditions.

In this study, ethanol production under anaerobic conditions was always dominant compared to semi-aerobic or aerobic conditions and ethanol accumulation started to decline or dropped on the third week of fermentation. These findings imply that ethanol production and fermentation occurred until third week of cultivation under above-mentioned cultivation

conditions, while the presence of atmospheric oxygen levels affected fermentation negatively. Most likely, the presence of oxygen under aerobic conditions initiated oxidation of the produced ethanol to acetate ultimately decreasing ethanol yield. It may be assumed that enzymatic decomposition of solid lignocelluloses and the consumption of released soluble sugars by wood-decay fungi is a simultaneous process. Therefore, interpretation of sugar consumption and bioethanol accumulation was challenging.

More acetate with a little amount of glycerol was detected under anaerobic and semi-aerobic conditions on all substrate mixtures during the cultivations. Through ethanol fermentation, yeast (*S. cerevisiae*) cells re-generate NAD+ by reducing acetaldehyde to ethanol, while the opposite reaction oxidation of acetaldehyde to acetate will produce NAD(P)H thus leading to redox imbalance under anaerobic and fermentative conditions (Wei et al., 2013). Most likely, a certain amount of ethanol was converted to acetate and therefore, ethanol accumulation decreased after the third week of cultivation. On the other hand, glycerol is generated naturally by *S. cerevisiae* yeast cells during fermentation to oxidize excess NADH to NAD+ and thus support the cellular redox balance (Bideaux et al., 2006). Little amount of glycerol was also detected under anaerobic and semi-aerobic cultivations in this study. This by-product can also be used for the generation of fuels and chemicals, and for instance, glycerol may serve as fermentation substrate for microbes to produce ethanol (Yazdani & Gonzalez, 2007).

Activity of enzymes on wood degradation and accumulation of fermentation metabolites by *P. radiata* 79 was examined at different atmospheric oxygen concentrations to identify suitable cultivation conditions for ethanol production. Addition of atmospheric oxygen favors oxidative processes and ultimately increases lignin degradation by white rot fungi (Kamei et al., 2014). In this study, elevated activity of β-glucosidase, cellobiohydrolase, and pectinase were recorded under aerobic conditions on all substrate mixtures, which indicates that presence of oxygen may increase the production of fungal secreted enzymes.

White rot Polyporales species of fungi have the potentiality to produce cellulose-degrading enzymes (activities including β-glucosidase, endoglucanase and CBH), and are able to degrade lignocellulose substrates (Kuuskeri et al., 2016; Zhu et al., 2016). Highest β-glucosidase and CBH activities were detected on the mixture of barley straw and SBBM under aerobic conditions. On spruce wood and birch wood sawdust mixed with SBBM, however, activities of β-glucosidase and CBH were quite low. These lower activities may be either due to limited expression and secretion

of the enzymes or due to inhibition of cellulases by lignin degradation products (Berlin et al., 2006; Rahikainen et al., 2013). One explanation may be glucose repression suppressing transcriptional expression of the respective CAZyme encoding genes (Niku-Paavola et al., 1990; Mäkinen et al., 2018). In addition, *P. radiata* 79 showed lower activities of cellulolytic enzymes compared to lignin-modifying oxidoreductases in solid-state cultures on spruce wood slices (Kuuskeri et al., 2016).

Another part of this research and study was to establish an enzyme assay method for pectin degradation and conversion. Optimization of this assay was conducted by following the established DNS-based enzyme assay methods. After optimization, this 96-well mediated assay was used for pectinase activity measurement of liquid samples from all cultivations. The non-cellulosic polysaccharide pectin which is characterized by its galacturonic acid backbone, is mainly located at wood primary cell walls and middle lamellae (Caffall & Mohnen, 2009). Basidiomycota fungi are a potential source of novel pectinases with unique properties such as polygalacturonase, β-1,4-endogalactanase, rhamnogalacturonan hydrolase, pectin methyl esterase and rhamnogalacturonan lyase, all enzymes required for complete pectin modification and degradation (Rytioja et al., 2014).

In this study, *P. radiata* demonstrated significant enzymatic activity of pectin degradation. The highest pectinase activity (83 nkat/ml) was recorded under aerobic conditions on barley straw-SBBM substrate mixture. In comparison, the Ascomycota fungus *Fusarium moniliforme* NCIM 1276 produces increased amounts of polygalacturonase and pectate lyase enzymes in solid-state cultures compared to liquid cultivations (Niture & Pant, 2004). Interestingly and accordingly, pectin-degrading enzyme activity of *P. radiata* 79 was detected on all lignocellulose substrate mixtures.

As part of the pectinase assay optimization, initial goal was to detect the highest temperature to reach saturated plateau phase of enzyme activity within the 15 minutes incubation time. Different temperatures ranging between 30 °C and 45 °C were tested. It is known that fungal secreted polygalacturonases perform better catalytic activity on neutral or acidic conditions with an incubation temperature between 40 °C and 60 °C (Pedrolli et al., 2009). For instance, PG enzymes from *Aspergillus niger* (Dinu et al., 2007) and *F. moliniforme* (Endo-PG II) (Niture & Pant, 2004) show optimal activity at 40 °C. Endo-PG secreted by *Mucor flavus* (Gadre et al., 2003) and *Rhizopus oryzae* also perform better pectinolytic activity at 45 °C (Saito et al., 2004).

In addition, 40 °C incubation temperature was used in a previous study to measure PG activity (Dinu et al., 2007). Therefore, in this study, 40 °C was selected as the incubation temperature for *P. radiata* 79 secreted pectinase activity assay. Sample amount was decreased from 30 µl to 20 µl, mainly to dilute potential inhibitors and background sugars present in the samples, thus also allowing more of available substrates for the enzymes. Pectinase activity was increasing linearly at 40 °C during the first 10 minutes of incubation. Most likely this incubation time and temperature were adequate for enzyme-substrate saturation before reaching the stationary phase. Therefore, these experimental parameters (temperature 40 °C and incubation time 10 minutes) were chosen for pectinase activity assay throughout the study.

Finally, it can be concluded that composition of the solid waste lignocellulose substrate mixture affected enzyme production by the fungus, whereas production of ethanol was mainly controlled by the cultivation atmosphere.

## 6. Conclusions

The results of this research demonstrated that *P. radiata* 79 can grow on different lignocellulosic substrates under different atmospheric conditions, while converting the substrates into fermentable sugars to produce various metabolites involving ethanol, glycerol, and acetate through fermentation. In all cases, ethanol accumulation was always dominant under anaerobic conditions as well as under semi-aerobic conditions. In addition, this fungus is capable of producing a wide number of carbohydrate active enzymes (CAZyme) with varying activities on different substrates. Maximal activity of β-glucosidase, cellobiohydrolase, and pectinase was recorded under aerobic conditions compared to anaerobic and semi-aerobic conditions on all substrate mixtures. From overall findings, anaerobic conditions favored accumulation of fermentation metabolites, especially ethanol, and presence of oxygen under aerobic conditions accelerated production of CAZyme activities which supports the initial hypotheses of this research. It might be concluded that an optimal amount of oxygen is needed for proper CAZyme activity secreted by fungus for both lignocellulose decomposition and bioethanol production. Significant amount of ethanol accumulation and enzyme activity detection under semi-aerobic conditions also supports our hypotheses. Fruitful outcome of these experiments will contribute to the industrial production of second-generation biofuels. However, further research is recommended for the biotechnological application of this fungus for bioethanol production and optimization of oxygen requirements for proper enzyme activity.

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## 8. References


Agbogbo, F. K., Coward-Kelly, G., Torry-Smith, M., & Wenger, K. S. (2006). Fermentation of glucose/xylose mixtures using *Pichia stipitis*. Process Biochemistry, 41(11), 2333–2336.

Agger, J. W., Isaksen, T., Varnai, A., Vidal-Melgosa, S., Willats, W. G. T., Ludwig, R., & Westereng, B. (2014). Discovery of LPMO activity on hemicelluloses shows the importance of oxidative processes in plant cell wall degradation. Proceedings of the National Academy of Sciences, 111(17), 6287–6292.

Alvira, P., Tomás-Pejó, E., Ballesteros, M., & Negro, M. J. (2010). Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: A review. Bioresource Technology, 101(13), 4851–4861.

Annele Hatakka & Kenneth E. Hammel. (2010). Fungal biodegradation of lignocelluloses, Treesearch. In Springer-Verlag (pp. 319–340).

Aro, E. M. (2016). From first generation biofuels to advanced solar biofuels. Ambio, 45, 24–31.

Bak, J. S., Ko, J. K., Choi, I.G., Park, Y. C., Seo, J. H., & Kim, K. H. (2009). Fungal pretreatment of lignocellulose by *Phanerochaete chrysosporium* to produce ethanol from rice straw. Biotechnology and Bioengineering, 104(3), 471–482.

Balat, M., & Balat, H. (2008). Progress in bioethanol processing. Progress in Energy and Combustion Science, 34, 551–573.

Barke, J., Seipke, R. F., Grüschow, S., Heavens, D., Drou, N., Bibb, M. J., & Hutchings, M. I. (2010). A mixed community of actinomycetes produce multiple antibiotics for the fungus farming ant *Acromyrmex octospinosus*. BMC Biology, 8, 109.

Beauchet, R., Monteil-Rivera, F., & Lavoie, J. M. (2012). Conversion of lignin to aromatic-based chemicals (L-chems) and biofuels (L-fuels). Bioresource Technology, 121, 328–334.

Berlin, A., Balakshin, M., Gilkes, N., Kadla, J., Maximenko, V., Kubo, S., & Saddler, J. (2006). Inhibition of cellulase, xylanase and β-glucosidase activities by softwood lignin preparations. Journal of Biotechnology, 125(2), 198–209.

Bideaux, C., Alfenore, S., Cameleyre, X., Molina-Jouve, C., Uribelarrea, J. L., & Guillouet, S. E. (2006). Minimization of glycerol production during the high-performance fed-batch ethanolic fermentation process in *Saccharomyces cerevisiae*, using a metabolic model as a prediction tool. Applied and Environmental Microbiology, 72(3), 2134–2140.

Boerjan, W., Ralph, J., & Baucher, M. (2003). Lignin biosynthesis. Annual Review of Plant Biology, 54(1), 519–546.

Buijs, N. A., Siewers, V., & Nielsen, J. (2013). Advanced biofuel production by the yeast *Saccharomyces cerevisiae*. Current Opinion in Chemical Biology, 17(3), 480-488.

Caffall, K. H., & Mohnen, D. (2009). The structure, function, and biosynthesis of plant cell wall pectic polysaccharides. Carbohydrate Research, 344(14), 1879–1900.

Cheng, F., & Brewer, C. E. (2017). Producing jet fuel from biomass lignin: Potential pathways to alkyl-benzenes and cycloalkanes. Renewable and Sustainable Energy Reviews. Elsevier Ltd. 72(C), 673-722.

Chiaramonti, D., Prussi, M., Ferrero, S., Oriani, L., Ottonello, P., Torre, P., & Cherchi, F. (2012). Review of pretreatment processes for lignocellulosic ethanol production, and development of an innovative method. Biomass and Bioenergy, 46, 25–35.

Crowther, T. W., Glick, H. B., Covey, K. R., Bettigole, C., Maynard, D. S., Thomas, S. M., & Bradford, M. A. (2015). Mapping tree density at a global scale. Nature, 525(7568), 201–205.

Dinu, D., Nechifor, M. T., Stoian, G., Costache, M., & Dinischiotu, A. (2007). Enzymes with new biochemical properties in the pectinolytic complex produced by *Aspergillus niger* MIUG 16. Journal of Biotechnology, 131(2), 128–137.

FAO (2019). Energy and Bioenergy. Food and Agriculture Organization of the United Nations. http://www.fao.org/energy/bioenergy/en/

Farrell, A. E., Plevin, R. J., Turner, B. T., Jones, A. D., O'Hare, M., & Kammen, D. M. (2006). Ethanol can contribute to energy and environmental goals. Science, 311(5760), 506–508.

Fernandes, A. N., Thomas, L. H., Altaner, C. M., Callow, P., Forsyth, V. T., Apperley, D. C., & Jarvis, M. C. (2011). Nanostructure of cellulose microfibrils in spruce wood. Proceedings of the National Academy of Sciences of the United States of America, 108(47).

Floudas, D., & Hibbett, D. S. (2015). Revisiting the taxonomy of Phanerochaete (Polyporales, Basidiomycota) using a four gene dataset and extensive ITS sampling. Fungal Biology, 119(8), 679–719.

Gadre, R. V., Van Driessche, G., Van Beeumen, J., & Bhat, M. K. (2003). Purification, characterisation and mode of action of an endo-polygalacturonase from the psychrophilic fungus *Mucor flavus.* Enzyme and Microbial Technology, 32(2), 321–330.

Gutiérrez, A., del Río, J. C., & Martínez, Á. T. (2011). Fungi and their enzymes for pitch control in the pulp and paper industry. In Industrial Applications (pp. 357–377). Springer Berlin Heidelberg.

Halpin, C. (2013). Cell biology: Up against the wall. Current Biology, Cell Press. Volume 23, Issue 23, R1048-R1050.

Hemsworth, G. R., Johnston, E. M., Davies, G. J., & Walton, P. H. (2015). Lytic polysaccharide monooxygenases in biomass conversion. Trends in Biotechnology. Elsevier Ltd. 33(12), 747-761.

Henriksson, G., Johansson, G., & Pettersson, G. (2000). A critical review of cellobiose dehydrogenases. Journal of Biotechnology, 78(2), 93–113.

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

Hofrichter, M., Ullrich, R., Pecyna, M. J., Liers, C., & Lundell, T. (2010). New and classic families of secreted fungal heme peroxidases. Applied Microbiology and Biotechnology. Springer Verlag. 87, 871-897.

J. W. Deacon. (2009). Fungal Biology, 4th edition. Blackwell publishing Ltd.

Justo, A., Miettinen, O., Floudas, D., Ortiz-Santana, B., Sjökvist, E., Lindner, D., & Hibbett, D. S. (2017). A revised family-level classification of the Polyporales (Basidiomycota). Fungal Biology, 121(9), 798–824.

Kamei, I., Hirota, Y., & Meguro, S. (2014). Direct fungal production of ethanol from high-solids pulps by the ethanol-fermenting white-rot fungus *Phlebia* sp. MG-60. BioResources, 9(3), 5114-5124.

Kamei, I., Hirota, Y., Mori, T., Hirai, H., Meguro, S., & Kondo, R. (2012). Direct ethanol production from cellulosic materials by the hypersaline-tolerant white-rot fungus *Phlebia* sp. MG-60. Bioresource Technology, 112, 137–142.

Karimi, K., Emtiazi, G., & Taherzadeh, M. J. (2006). Ethanol production from dilute-acid pretreated rice straw by simultaneous saccharification and fermentation with *Mucor indicus*, *Rhizopus oryzae,* and *Saccharomyces cerevisiae*. Enzyme and Microbial Technology, 40(1), 138–144.

Kazemi Shariat Panahi, H., Tabatabaei, M., Aghbashlo, M., Dehhaghi, M., Rehan, M., & Nizami, A. S. (2019). Recent updates on the production and upgrading of bio-crude oil from microalgae. Bioresource Technology Reports, 7, 100216.

Kirk, T. K., & Farrell, R. L. (1987). Enzymatic "Combustion": The microbial degradation of lignin. Annual Review of Microbiology, 41(1), 465–505.

Kricka, W., Fitzpatrick, J., & Bond, U. (2014). Metabolic engineering of yeasts by heterologous enzyme production for degradation of cellulose and hemicellulose from biomass: a perspective. Frontiers in Microbiology, 5, 174.

Kricka, W., Fitzpatrick, J., & Bond, U. (2015). Challenges for the production of bioethanol from biomass using recombinant yeasts. Advances in Applied Microbiology, 92, 89–125.

Kuuskeri, J., Häkkinen, M., Laine, P., Smolander, O. P., Tamene, F., Miettinen, S., & Lundell, T. (2016). Time-scale dynamics of proteome and transcriptome of the white-rot fungus *Phlebia radiata*: growth on spruce wood and decay effect on lignocellulose. Biotechnology for Biofuels, 9(1), 192.

Kuuskeri, J., Mäkelä, M. R., Isotalo, J., Oksanen, I., & Lundell, T. (2015). Lignocellulose-converting enzyme activity profiles correlate with molecular systematics and phylogeny grouping in the incoherent genus Phlebia (Polyporales, Basidiomycota). BMC Microbiology, 15, 217.

Kuuskeri, J. T. (2016). Genomics and systematics of the white-rot fungus *Phlebia radiata*: special emphasis on wood-promoted transcriptome and proteome. Doctoral dissertation, ISBN: 978-951-51-2420-3.

Lennartsson, P. R., Erlandsson, P., & Taherzadeh, M. J. (2014). Integration of the first and second generation bioethanol processes and the importance of by-products. Bioresource Technology, 165, 3–8.

Lin, Y., & Tanaka, S. (2006). Ethanol fermentation from biomass resources: current state and prospects. Applied Microbiology and Biotechnology, 69(6), 627–642.

Lombard, V., Golaconda Ramulu, H., Drula, E., Coutinho, P. M., & Henrissat, B. (2014). The carbohydrate-active enzymes database (CAZy) in 2013. Nucleic Acids Research, 42(D1).

Lundell, T. K., Mäkelä, M. R., de Vries, R. P., & Hildén, K. S. (2014). Genomics, lifestyles and future prospects of wood-decay and litter-decomposing basidiomycota. In Advances in Botanical Research (Vol. 70, pp. 329–370). Academic Press Inc.

Lundell, T. K., Mäkelä, M. R., & Hildén, K. (2010). Lignin-modifying enzymes in filamentous basidiomycetes- ecological, functional and phylogenetic review. Journal of Basic Microbiology, 50(1), 5–20.

Madhavan, A., Srivastava, A., Kondo, A., & Bisaria, V. S. (2012). Bioconversion of lignocellulose-derived sugars to ethanol by engineered *Saccharomyces cerevisiae*. Critical Reviews in Biotechnology, 32(1), 22–48.

Mäkinen, M. A., Risulainen, N., Mattila, H., & Lundell, T. K. (2018). Transcription of lignocellulose-decomposition associated genes, enzyme activities and production of ethanol upon bioconversion of waste substrate by *Phlebia radiata*. Applied Microbiology and Biotechnology, 102(13), 5657–5672.

Mäkinen, M., Kuuskeri, J., Laine, P., Smolander, O. P., Kovalchuk, A., Zeng, Z., & Lundell, T. (2019). Genome description of *Phlebia radiata* 79 with comparative genomics analysis on lignocellulose decomposition machinery of phlebioid fungi. BMC Genomics, 20(1), article number 430.

Mali, T., Kuuskeri, J., Shah, F., & Lundell, T. K. (2017). Interactions affect hyphal growth and enzyme profiles in combinations of coniferous wood-decaying fungi of Agaricomycetes. PLOS ONE, 12(9), e0185171.

Mali, T., Mäki, M., Hellén, H., Heinonsalo, J., Bäck, J., & Lundell, T. (2019). Decomposition of spruce wood and release of volatile organic compounds depend on decay type, fungal interactions and enzyme production patterns. FEMS Microbiology Ecology, 95(9), fiz135.

Mattila, H., Mäkinen, M., & Lundell, T. (2020). Hypoxia is regulating enzymatic wood decomposition and intracellular carbohydrate metabolism in filamentous white rot fungus. Biotechnology for Biofuels 13, article number: 26 (2020).

Mattila, H., Kačar, D., Mali, T., & Lundell, T. (2018). Lignocellulose bioconversion to ethanol by a fungal single-step consolidated method tested with waste substrates and co-culture experiments. AIMS Energy, 6(5), 866–879.

Mattila, H., Kuuskeri, J., & Lundell, T. (2017). Single-step, single-organism bioethanol production and bioconversion of lignocellulose waste materials by phlebioid fungal species. Bioresource Technology, 225, 254–261.

Mohanta, T. K., & Bae, H. (2015). The diversity of fungal genome. Biological Procedures Online, 17(1), 8.

Mohr, A., & Raman, S. (2013). Lessons from first generation biofuels and implications for the sustainability appraisal of second generation biofuels. Efficiency and Sustainability in Biofuel Production: Environmental and Land-Use Research, 63, 114-122.

Mussatto, S. I., Machado, E. M. S., Carneiro, L. M., & Teixeira, J. A. (2012). Sugars metabolism and ethanol production by different yeast strains from coffee industry wastes hydrolysates. Applied Energy, 92, 763–768.

Niku-Paavola, M. L., Karhunen, E., Kantelinen, A., Viikari, L., Lundell, T., & Hatakka, A. (1990). The effect of culture conditions on the production of lignin modifying enzymes by the white-rot fungus *Phlebia radiata.* Journal of Biotechnology, 13(2–3), 211–221.

Niture, S. K., & Pant, A. (2004). Purification and biochemical characterization of polygalacturonase II produced in semi-solid medium by a strain of *Fusarium moniliforme*. Microbiological Research, 159(3), 305–314.

OECD-FAO (2018). Chapter 9. Biofuels. OECD-FAO, Agricultural Outlook 2018-2027. http://www.fao.org/3/i9166e/i9166e_Chapter9_Biofuels.pdf

Pedrolli, D. B., Monteiro, A. C., Gomes, E., & Carmona, E. C. (2009). Pectin and pectinases: production, characterization and industrial application of microbial pectinolytic enzymes. The Open Biotechnology Journal, 3(1), 9–18.

Pfeiffer, T., & Morley, A. (2014). An evolutionary perspective on the Crabtree effect. Frontiers in Molecular Biosciences, 1(17).

Rahikainen, J. L., Evans, J. D., Mikander, S., Kalliola, A., Puranen, T., Tamminen, T., & Kruus, K. (2013). Cellulase-lignin interactions-The role of carbohydrate-binding module and pH in non-productive binding. Enzyme and Microbial Technology, 53(5), 315–321.

Rodríguez, S. (2018). Industrial and environmental applications of white-rot fungi. Mycosphere 8(3), 456–466.

Ruiz-Dueñas, F. J., & Martínez, Á. T. (2009). Microbial degradation of lignin: how a bulky recalcitrant polymer is efficiently recycled in nature and how we can take advantage of this. Microbial Biotechnology, 2(2), 164–177.

Rytioja, J., Hildén, K., Hatakka, A., & Mäkelä, M. R. (2014). Transcriptional analysis of selected cellulose-acting enzymes encoding genes of the white-rot fungus *Dichomitus squalens* on spruce wood and microcrystalline cellulose. Fungal Genetics and Biology, 72, 91–98.

Rytioja, J., Hildén, K., Yuzon, J., Hatakka, A., de Vries, R. P., & Mäkelä, M. R. (2014). Plant-polysaccharide-degrading enzymes from Basidiomycetes. Microbiology and Molecular Biology Reviews, 78(4), 614–649.

Saini, J. K., Saini, R., & Tewari, L. (2015). Lignocellulosic agriculture wastes as biomass feedstocks for second-generation bioethanol production: concepts and recent developments. 3 Biotech, 5(4), 337–353.

Saito, K., Takakuwa, N., & Oda, Y. (2004). Purification of the extracellular pectinolytic enzyme from the fungus *Rhizopus oryzae* NBRC 4707. Microbiological Research, 159(1), 83–86.

Saladini, F., Patrizi, N., Pulselli, F. M., Marchettini, N., & Bastianoni, S. (2016). Guidelines for emergy evaluation of first, second and third generation biofuels. Renewable and Sustainable Energy Reviews, 66 (C), 221-227.

Sjöström, E. (1993). Wood chemistry: fundamentals and applications, 2nd edition. San Diego, California ; London, England : Academic Press, Inc.

Steenkamp, E. T., Wright, J., & Baldauf, S. L. (2006). The protistan origins of animals and fungi. Molecular Biology and Evolution, 23(1), 93–106.

Sundvor, I., & López-Aparicio, S. (2014). Impact of bioethanol fuel implementation in transport based on modelled acetaldehyde concentration in the urban environment. Science of the Total Environment, 496, 100–106.

Tedersoo, L., Sánchez-Ramírez, S., Kõljalg, U., Bahram, M., Döring, M., Schigel, D., & Abarenkov, K. (2018). High-level classification of the fungi and a tool for evolutionary ecological analyses. Fungal Diversity, 90(1), 135–159.

van den Brink, J., & de Vries, R. P. (2011). Fungal enzyme sets for plant polysaccharide degradation. Applied Microbiology and Biotechnology, 91(6), 1477–1492.

Veloz Villavicencio, Mali, Mattila, & Lundell. (2020). Enzyme cctivity profiles produced on wood and straw by four fungi of different decay strategies. Microorganisms, 8(1), 73.

Wang, M., Wu, M., & Huo, H. (2007). Life-cycle energy and greenhouse gas emission impacts of different corn ethanol plant types. Environmental Research Letters, 2(2), 024001.

Wei, N., Quarterman, J., Kim, S. R., Cate, J. H. D., & Jin, Y. S. (2013). Enhanced biofuel production through coupled acetic acid and xylose consumption by engineered yeast. Nature Communications, 4, 2580.

Wenger, J. W., Schwartz, K., & Sherlock, G. (2010). Bulk segregant analysis by high-throughput sequencing reveals a novel xylose utilization gene from *Saccharomyces cerevisiae*. PLOS Genetics, 6(5), e1000942.

Yazdani, S. S., & Gonzalez, R. (2007). Anaerobic fermentation of glycerol: a path to economic viability for the biofuels industry. Current Opinion in Biotechnology, 18(3), 213–219.

Zhang, X. Z. & Zhang, Y. H. P. (2013). Chapter 8, Cellulases: characteristics, sources, production, and applications. Bioprocessing Technologies in Biorefinery for Sustainable Production of Fuels, Chemicals, and Polymers (pp. 131–146). Hoboken, NJ, USA: John Wiley & Sons, Inc.

Zhu, N., Liu, J., Yang, J., Lin, Y., Yang, Y., Ji, L., & Yuan, H. (2016). Comparative analysis of the secretomes of Schizophyllum commune and other wood-decay basidiomycetes during solid-state fermentation reveals its unique lignocellulose-degrading enzyme system. Biotechnology for Biofuels, 9(1), 42.