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Combined Production of Edible Mushrooms and Biofuels from Lignocellulosic Residues

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Abstract

Forest residues, wood-processing by-products and other lignocellulosic materials are promising renewable resources for bioconversion to advanced biofuels such as cellulosic ethanol. Increased production of biofuels from lignocellulosic materials would allow reducing environmental problems caused by the use of fossil resources while supporting development of bioeconomy.

Enzymatic saccharification of cellulose is crucial in the production of cellulosic ethanol, since it produces the carbohydrates needed for microbial fermentation. However, due to feedstock recalcitrance, enzymatic saccharification of raw lignocellulose results in low rates and yield. In this thesis, the possibility of pretreating lignocellulosic biomass with edible white-rot fungi for integrated production of edible mushroom and cellulosic ethanol, as primary products, were investigated.

This thesis was able to conclude that the effectiveness of fungal pretreatment, using shiitake as a model species, varied with substrate composition. Shiitake (*Lentinula edodes*) cultivation on wood-based substrates resulted in major degradation of lignin and hemicelluloses, while cellulose was degraded to a minor extent. Nitrogen content of the substrate was an important factor since low nitrogen content resulted in increased delignification and minimal cellulose consumption but compromised the production of mushroom fruiting bodies. Compared to the impact of nitrogen content, the choice of hardwood species had a less significant role, as indicated by the comparable performance in mushroom yield or delignification, across substrates composed of birch, alder and aspen and their combinations. Differences in chemical features between stemwood and bark were significant, which had a major effect on the degree of lignocellulose degradation by shiitake. Delignification by shiitake was correlated with degradation of syringyl lignin unit, as indicated by a significant reduction of the syringyl-to-guaiacyl ratio during cultivation. Shiitake cultivation did not cause statistically significant changes in the substrate crystallinity.

Cellulose contained in the spent mushroom substrate (SMS) resulting from shiitake cultivation displayed higher susceptibility to enzymatic saccharification than cellulose contained in the raw woody material of initial substrate. The enhancement of the enzymatic saccharification of cellulose was related to the low mass fractions of lignin and hemicelluloses in the SMS, and it was neither affected by the relative content of lignin phenylpropane units nor by substrate crystallinity.

Considerable formation of phenolic compounds was resulted from lignin degradation during fungal cultivation. Formed phenols ended up in the SMS hydrolysate after enzymatic saccharification. Acetic acid was hardly detectable in substrates, but it was found in the SMS hydrolysates as result of the hydrolysis of remaining hemicelluloses during enzymatic saccharification. The concentrations of phenolic compounds and acetic acid in the enzymatic hydrolysates were low and, consequently, their inhibitory effect on fermentation was limited.

Fungal pretreatment caused accumulation of nitrogen, including soluble forms, in the substrates. The nitrogen source was further enriched in the SMS hydrolysates, which ensured high ethanol yield in fermentation by *Saccharomyces cerevisiae* even without supplementing additional nutrients.

Furthermore, studies on the potential of wood ear (*Auricularia auricular-judae*) and summer oyster (*Pleurotus pulmonarius*) for food and biofuel production suggested that the both species adapted well to the hot-air pasteurization based novel mushroom cultivation system. Comparable production of fruiting bodies and lignocellulose degradation were achieved for each studied mushroom, regardless pasteurization regimes. However, before applying these two species for the food and cellulosic ethanol concept, further studies are needed to investigate the factors that may improve their efficiency of fungal pretreatment while obtaining acceptable quantity mushroom fruiting bodies.

The solid leftover generated after SMS enzymatic saccharification, being one major side-stream, showed high thermal energy value and promising combustion characteristics, demonstrating a plausibility to be recycled as solid fuel for self-supporting energy system and space heating.

Keywords: Fungal pretreatment, Enzymatic saccharification, Ethanolic fermentation, Utilization of side streams, Hot-air pasteurization, *Lentinula edodes*, *Pleurotus pulmonarius*, *Auricularia auricular-judae*.

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Dedication

To my family

Contents

List of Publications

This thesis is based on work contained in the following papers:

I. Chen, F., Martín, C., Lestander, T.A., Grimm, A., Xiong, S. 2022. Shiitake cultivation as biological preprocessing of lignocellulosic feedstocks – Substrate changes in crystallinity, syringyl/guaiacyl lignin and degradation-derived by-products. Bioresource Technology, 344.

II. Chen, F., Xiong, S., Gandla, M.L., Stagge, S., Martín, C. 2022. Spent mushroom substrates for ethanol production – effect of chemical and structural factors on enzymatic saccharification and ethanolic fermentation of *Lentinula edodes* pretreated hardwood. Bioresource Technology. https://doi.org/10.1016/j.biortech.2021.126381

III. Chen, F., Martín, C., Finell, M., Xiong, S.J. 2020. Enabling efficient bioconversion of birch biomass by *Lentinula edodes*: regulatory roles of nitrogen and bark additions on mushroom production and cellulose saccharification. Biomass Conversion and Biorefinery. https://doi.org/10.1007/s13399-020-00794-y

IV. Chen, F., Grimm, A., Eilertsen, L., Martín, C., Arshadi, M., Xiong, S. 2021. Integrated production of edible mushroom (*Auricularia auricular-judae*), fermentable sugar and solid biofuel. Renewable Energy, 170, 172-180.

V. Chen, F., Xiong, S., Sundelin, J., Martín, C., Hultberg, M. 2020. Potential for combined production of food and biofuel: Cultivation of *Pleurotus pulmonarius* on soft- and hardwood sawdusts. Journal of Cleaner Production, 266, 122011.

Papers I−IV are open access publications; Paper V is reproduced with the permission of the publishers.

The contributions of Feng Chen to the papers included in this thesis are as follows:

I. Design of the experiments, performing practical and analytical work, and writing the manuscript draft. Corresponding author.

II. Design of the experiments, performing practical and analytical work, and writing the manuscript draft. Corresponding author.

III. Design of the experiments, performing practical and analytical work, and writing the manuscript draft.

IV. Performing practical and analytical work, and writing the manuscript draft. Corresponding author.

V. Performing practical and analytical work, and writing the manuscript draft. Corresponding author.

Abbreviations

CCF	Central Composite Face Design
CrI	Crystallinity Index of cellulose
DM	Dry mass
HMF	5-hydroxymethylfurfural
HPLC	High-Performance Liquid Chromatography
MLR	Multiple linear regression
PCA	Principal compound analysis
PLS	Partial least squares
SMS	Spent mushroom substrate
XRD	X-ray diffraction analysis
HTP	Hydrothermal pretreatment

1. Introduction

1.1 Food and Fuel: from competition to synergism using edible white-rot fungi as pretreatment of lignocellulosic residues

The world's population is expected to approach 10 billion by 2050, and 69% of that amount is prognosed to be concentrated in urban areas (FAO, 2018). That demography dynamics pushes upwards the demands on food and energy sources. At the same time, fossil fuels are expected to become scarce, while the emission of greenhouse gases caused by human activities is expected to continue increasing if no effective actions are taken. Although EU sets an appropriate abatement objective aiming to reduce greenhouse gas emissions by 80–95% below 1990 levels by 2050, the energy demand is projected to increase by 12% from 2005 to 2050 (EC, 2019). To be able to deal with such challenges, the development of resource- and energy-efficient biotechnology for the sustainable production of CO_2-neutral fuels is an urgent task.

Over past decades, so-called first-generation (1G) bioethanol has received considerable attention as an alternative to fossil-based transportation fuels. Large-scale production based on fermentation of field crops (including, but not limited to, corn, sugarcane, wheat and sweet sorghum), which are rich in starch or sucrose, has been dominating on the world market. In 2020, corn and sugarcane ethanol accounted for around 90% of the global bioethanol market. United States and Brazil are the main producers, and they dominate more than 80% of the world market. However, 1G bioethanol has been subjected to criticism, and is considered not favor a sustainable biobased strategy on the long-term prospect. That is related to the competition on land usage for either food crops or energy crops, which ignites the “food vs fuel” debate. When field crops are used for conventional biofuel production, the input of water and fertilizers, and its implications on additional energy input and carbon footprint, has also to be considered. Therefore, development of second-generation (2G) bioethanol, or cellulosic ethanol, i.e. the ethanol is produced using lignocellulose materials such as forest and wood-processing residues, is important.

Lignocellulosic biomass is currently the most important source available at large-scale in the world for producing cellulosic ethanol. Forestry activities and wood-processing industries generate large amounts of by-products such as logging residues and sawdust, which are so far underutilized. Only in Sweden, around 15 million tons forest residues are generated yearly (Forest-Statistics, 2019), today they are largely used for direct combustion to generate heat and electric power. The use of wood by-products for direct combustion is not resource efficient, since they are high-quality biomass sources containing valuable components that can be converted into value-added biobased products. In an efficient bio-economy, the energetic use of wood residues should be restricted to the fractions remaining after their potential for value-added applications has been appropriately exploited. Cellulose is the main structural component of lignocellulosic biomass, and it accounts for around 40% of wood dry mass (Zhu and Pan, 2010). Cellulose contained in forest residues can be converted to 2G ethanol by biochemical conversion

processes, including enzymatic saccharification and fermentation. However, the conversion of cellulose into fermentable sugar is a major challenge, compared to 1G ethanol production. Enzymatic saccharification of raw lignocellulose results in low sugar release due to feedstock recalcitrance (Fig. 1), while in 1G ethanol processes, sugars are either available for direct fermentation in saccharine substrates or can be made available without complicated saccharification processes from starch-containing substrates.

Lignocellulosic biomass had a complex structure and composition, which contributes to its intrinsic recalcitrance (Fig. 1). Lignin and hemicelluloses, which are two major structural lignocellulosic components, beside cellulose, typically represent around 25–35% and 15–30%, respectively, in wood (Zhu and Pan, 2010). Structurally, lignin is covalently connected to hemicellulose, and they composes a 3D structure together with cellulose. Hemicelluloses and lignin block the access of enzymes to cellulose, and that is a major reason behind the low efficient enzymatic saccharification of cellulose (Shirkavand et al., 2016). The main building blocks of wood lignin are the phenylpropane structures known as syringyl (S) and guaiacyl (G) units (Shirkavand et al., 2016). Their relative amounts, which vary largely between different tree species, affect enzymatic saccharification (Guo et al., 2014). In addition the high degree of crystallinity is believed to increase the recalcitrance to enzymatic saccharification (Shirkavand et al., 2016; Zhu et al., 2008).

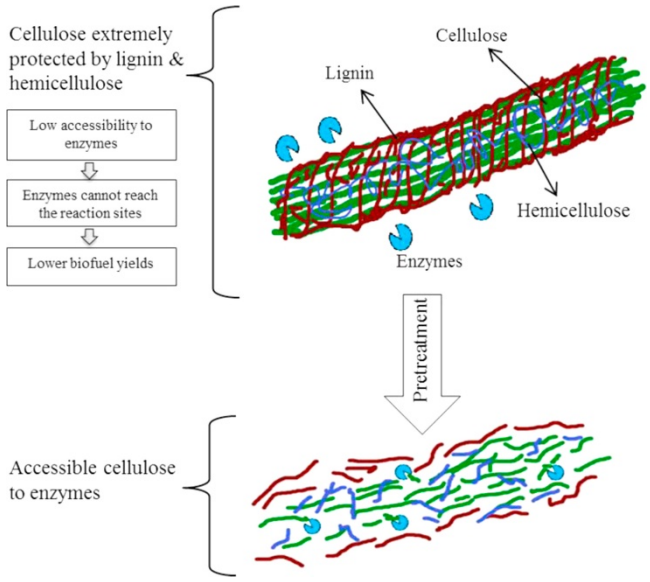

Figure 1. Architecture of lignocellulose and the schematic role of pretreatment. The picture was adapted from (Shirkavand et al., 2016).

In order to reduce the recalcitrance of lignocellulosic biomass and to facilitate enzymatic saccharification of cellulose, pretreatment must be implemented (Fig. 1) (Jönsson and Martín, 2016; Taherzadeh and Karimi, 2008; Zhu and Pan, 2010). Many pretreatment approaches have been investigated (Galbe and Wallberg, 2019). Different hydrothermal pretreatment (HTP) approaches are

among the most attractive methods for industrial implementation (Table 1). In HTP, moist feedstocks are subjected to high temperature for a certain time, and an auto-catalyzed process is pushed by organic acids released from the biomass. In HTP, hemicelluloses are solubilized, and the enzymatic digestibility of cellulose remaining in the pretreated solids is enhanced. HTP effectivity can be increased by either including an explosion at the end of the holding period (known as steam explosion) or by setting initial pH values far from neutrality. Low starting pH, is achieved by adding an acidic compound, such as sulfuric acid or sulfur dioxide, while high starting pH is typical of alkaline treatments (Table 1). HTP deconstructs the compact structure of lignocellulose and remove hemicelluloses and/or lignin. At acidic pH, the main effect is hydrolysis of hemicelluloses, while alkaline pH promotes the dissolution of lignin (Galbe and Wallberg, 2019). Since HTP methods clear away the physical and chemical barriers affecting bioconversion and they result in significant improvement of cellulose saccharification, they are the option of choice in many upscaling initiatives. However, HTP often suffers from high energy demand for reaching the work temperatures, chemicals inputs and corrosion problems of equipment (Zhu and Pan, 2010). Furthermore, side reactions resulting in formation of bioconversion inhibitors, such as phenolics, furan aldehydes and aliphatic acids (Jönsson and Martín, 2016), is a major issue affecting downstream processes.

Table 1. Simplified overview of some relevant pretreatment methods for lignocellulosic feedstocks

Methods	Primary action	Feedstocks	Used chemicals	Temperature	Pretreatment time	References
Acid-based	Hydrolysis of hemicelluloses	Aspen, Birch, Spruce	H_2SO_4	165–180 °C	5–10 min	1
		Spruce	SO_2	195–215 °C	7 min	2
		Sugarcane bagasse, Spruce	H_2SO_4	190–205 °C	5–14 min	3
Alkali-based	Delignification, partial solubilization of hemicelluloses	corn stover	NaOH	~100 °C	~120 min	4
		Rice Straw	NaOH	37 °C	3 h	5
		Rice husk	NaOH	121 °C	20–60 min	6

1, (Wang et al., 2018a); 2, (Wang et al., 2018b); 3, (Ilanidis et al., 2021b); 4, (Chen et al., 2013); 5, (Sabeeh et al., 2020); 6, (Shahabazuddin et al., 2018).

To be of interest, a pretreatment method should enhance the enzymatic digestibility and be effective for different feedstocks, in addition to being environmentally friendly, resource efficient and cost effective. Biological pretreatment, using lignin-degrading microorganisms, such as white-rot fungi and some bacteria, has attracted researchers' interest because it has lower input of energy and chemicals than conventional methods. However, the slow rate and the occurrence of cellulose losses are major drawbacks that have excluded biological pretreatment from the methods with potential for industrial application (Shirkavand et al., 2016; Wan and Li, 2012). Recent studies, showing the possibility of

combining biological pretreatment with production of edible fungi bring back the attention to this method (Lin et al., 2015; Xiong et al., 2019). During the vegetative growth of edible fungi, lignin is highly degraded by the oxidoreductases secreted from mycelium, such as laccases and peroxidases. The lignin degradation allows increased access of fungal mycelium to hemicellulose and cellulose, and will be followed by the partial digestion of carbohydrates (Kuijk, 2016). Overall, the mushroom cultivation process results in a significant decrease of lignin amount and changes in physical and biochemical characteristics of the wood substrate.

The example by shiitake (*L. edodes*), an edible white-rot fungus, which is the most cultivated mushroom in the world (Royse et al., 2017), is rather explicit. After growing shiitake on wood, the content of lignin and xylan in the spent mushroom substrate (SMS) corresponds to less than half of the amount contained in the initial substrates as a result of the fungal activity during cultivation (Wei et al., 2020). Due to its low lignin and xylan content, SMS is less recalcitrant to bioconversion than the initial substrate, and its enzymatic saccharification results in high cellulose conversion (Lin et al., 2015; Xiong et al., 2019). The produced hydrolysate can be then used for producing cellulosic ethanol through fermentation. It should also be pointed out that the edible mushroom industry is developing fast (Royse et al., 2017), which is due to the acceptance of edible mushrooms as protein-rich food produced on local non-food biomass using in-house and vertical cultivation. Furthermore, differently from field crops, edible mushroom and their SMS are harvested around the year. The SMS generated from shiitake along could reach 12.5 million tons per year globally (Wei et al., 2020). SMS has traditionally been discarded as waste or combusted directly, but the massive amounts that are been generated today deserve been used in a more economic and sustainable way. The approach of integrated production of bioenergy (cellulosic ethanol) and protein food (edible mushroom) using the lignocellulosic residues suits very well the bio-based circular economy concept (Fig. 2).

However, the concept is currently at laboratory scale and many challenges remain to be overcome before large-scale industrial application can be set. Pointing in that direction, the overall objectives of this research were to tackle scientific issues of major relevance regarding fungal pretreatment as a platform for production of cellulosic ethanol and food.

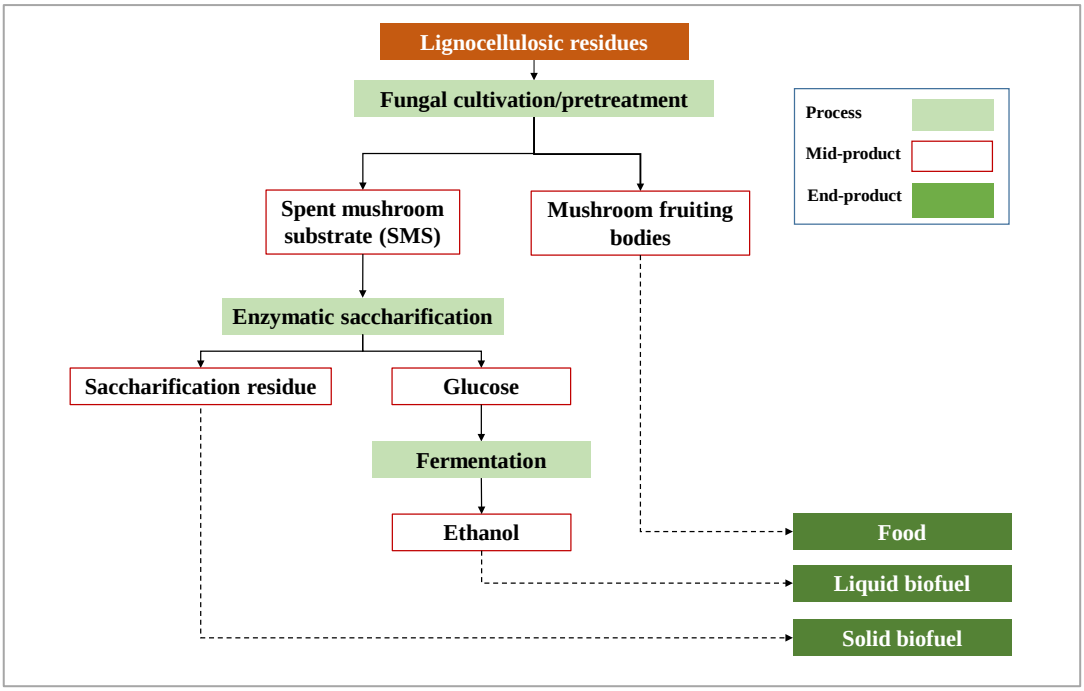

Figure 2. Schematic illustration of the concept and major processes for combined production of edible mushroom and biofuels from lignocellulosic residues.

1.2 Processes from mushroom cultivation to cellulosic ethanol using shiitake as a model species

Before an industrial integration development is implemented, intense research efforts are required towards (1) setting fungal cultivation operations that can lead to both good edible mushroom production and effective pretreatment of wood-based substrate, and (2) ensuring efficient enzymatic saccharification resulting in hydrolysates that are readily fermentable to ethanol. Efficient reduction of lignocellulose recalcitrance, short cultivation/pretreatment time, and minimal loss of cellulose are of central importance for an integrated mushroom production and SMS biorefining process. Apart from the used fungal species, the effectiveness of a mushroom-based pretreatment of lignocellulosic biomass is closely associated with the cultivation process, including substrate compositions and cultivation environment. In order to achieve competitive pretreatment methods towards industrial implementation, basic understanding on the fundamental mechanisms behind the fungal pretreatment is necessary, and optimal culture conditions need to be established. That knowledge is still lacking. Furthermore, the suitability of SMS hydrolysates for microbial fermentation, including their potential content of both nutrients and inhibitors, needs to be investigated in order to get a correct assessment of the downstream processing required to make the end product (cellulosic ethanol).

In the previous studies (Lin et al., 2015; Xiong et al., 2019), shiitake was found to be able to remove up to 75% of initial lignin mass during the cultivation, while achieving a good yield of fruiting bodies (~60% biological efficiency). As a reference, Shirkavand et al. (2016) (more references therein) found, from 12 published cases covering 8 white-rot fungi (including edible *Pleurotus ostreatus*)

growing on different substrates, that the ratio of delignification by fungal pretreatment was between 12% and 46%. Thus, shiitake was used as a model species to develop the food and cellulosic ethanol concept in this research.

1.2.1 Substrate composition and fungal pretreatment

1.2.1.1 Biomass species/assortments for mushroom substrate

In a previous study (Xiong et al., 2019), high lignin degradation (75–80% of initial mass) was achieved after shiitake cultivation on a birch-based substrate, and the method was shown to be of interest as pretreatment for facilitating enzymatic saccharification of cellulose. Sawdust and wood chips used for industrial mushroom production originates from sawmills, where wood from different tree species is processed. From the point of view at sustainable development of bioeconomy, it is important to investigate the effects of using different tree species on the effectivity of shiitake cultivation as biomass pretreatment.

Lignocellulose properties/compositions vary considerably between wood species and assortments. Fungal species may respond differently to different substrates material and thus might end up diverse outcomes in terms of the fungal production and/or the efficiency of pretreatment. For example, lignin is one of three main components of lignocellulosic biomass together with cellulose and hemicelluloses, but its content and composition are the biomass species/assortment dependent (Shirkavand et al., 2016). Chemical pretreatments (Santos et al., 2012; Wang et al., 2018a) could alter the composition ratio of lignin phenylpropane units, namely, syringyl (S) and guaiacyl (G) and p-hydroxyphenyl (H). There are few studies, if any, showing the relations between substrate property and efficiency of fungal pretreatment with shiitake, and how that affects enzymatic saccharification of cellulose.

Tree bark, a major by-product of sawmills, has antioxidant and antimicrobial properties, which may inhibit mycelium growth (Tascioglu et al., 2013; Vane et al., 2006). Bark constitutes around 10–25% of the dry mass of a tree stem, depending on the position and growth stage/age, and contain lower carbohydrate contents but higher contents of lignin than stemwood (Lestander et al., 2012; Miranda et al., 2013). In the aspect of efficient use of wood-processing byproducts and forest thinning residues, the effect of adding different amounts of bark on mushroom production and on changes in substrate composition should be understood, but it has rarely been addressed in the literature.

1.2.1.2 Nitrogen supplementation to the mushroom substrate

Fungal pretreatment is performed by lignin-degrading enzymes secreted by the fungi that colonize lignocellulose (Janusz et al., 2013). Depending on cultivation conditions, including the nutritional status, fungi can secrete different types and quantities of enzymes, which results in different outcomes in terms of lignocellulose degradation. Shiitake growth on birch-based substrates containing 0.5% nitrogen leads to considerable degradation of lignin (up to 70%) and hemicelluloses (up to 75%), together with 50% preservation of cellulose (Xiong et al., 2019). It might agree with the finding of reports (D'Agostini et

al., 2011; Mikiashvili et al., 2006) that nitrogen-limited conditions enhance the production of lignin-degrading enzymes and thus induce the more delignification. However, nitrogen supplementation is considered a key factor in substrate formulation for industrial mushroom cultivation to allow an optimized fruiting body production (Carrasco et al., 2018). Supplying nitrogen of good quantity and bioavailability is an important topic for both research and industrial practice for mushroom production, and the effects on the cultivation process vary with the ratio and source (Koutrotsios et al., 2014). Considering that nitrogen limitation might stimulate lignin-degrading enzymes (D'Agostini et al., 2011; Mikiashvili et al., 2006), the issue of nitrogen supplementation is important not only for production of edible mushroom bodies, but also for the lignocellulose degradation and cellulosic ethanol production.

1.2.2 Enzymatic saccharification

An issue to be understood is how SMS chemical and structural differences, either caused by the fungal growth or intrinsic to the nature of the used wood species, impact the enzymatic saccharification. For example, the share of lignin phenylpropane units and the crystallinity in SMS differed from the initial substrate species (Shirkavand et al., 2016) and might change after fungal pretreatment in parallel with removal of lignin and xylan. Changes of lignin content, composition and structure, as well as cellulose crystallinity have often been associated with the improvement of susceptibility to enzymatic saccharification observed after pretreatment (Santos et al., 2012; Wang et al., 2018a). It would be of interest to know if a similar pattern is also valid for biological pretreatment using shiitake.

1.2.3 Fermentation of SMS hydrolysates to ethanol

1.2.3.1 Nutrient supplementation

In fermentation of cellulosic hydrolysates, nutrient supplementation is required for ensuring the metabolic activity of the ethanolic microorganism, usually baker's yeast (*S. cerevisiae*). Nitrogen is an important element that is required, and it is typically supplied as either urea, ammonium sulfate or peptone (Liu et al., 2014; Martín et al., 2002). The situation might be different if SMS hydrolysates are used. Nitrogen is a major element for the fungi and is incorporated in the mycelia as well as fruiting bodies (Koutrotsios et al., 2014; Parchami et al., 2021). After the harvest of mushroom fruiting bodies, considerable amount of mycelia were remained in the SMS, which combined with the nitrogen remaining in the partly degraded substrate might be released to the liquid phase during hydrolysis, and serve as nutrient in the fermentation of the hydrolysate. That would minimize supplementation requirements, thus resulting in a decrease of the fermentation cost and environmental impact. However, differently from nitrogen contained in the nutrient supplements, which effectively solubilize in fermentation media, the solubilization of SMS nitrogen and the forms of solubilized nitrogen, as well as its use during fermentation process is still an open question worth being explored.

1.2.3.2 Potential inhibitory components

The potential inhibition of cellulolytic enzymes and fermenting microorganisms by lignocellulose degradation by-products is another issue of interest. A drawback of hydrothermal pretreatment approaches is the occurrence of side reactions resulting in formation of by-products, such as furan aldehydes, aliphatic acids and phenolic compounds, which are potential inhibitors of enzymes and fermenting organisms (Jönsson and Martín, 2016; Kim et al., 2013). Since mushroom cultivation is performed under mild conditions, most inhibitors formed by sugar degradation during hydrothermal treatments might not be expected. Anyway, formation of phenolics and acetic acid might result from the degradation of lignin and hemicelluloses, respectively. Furthermore, heating used for pasteurization, which is essential for substrate preparation, might lead to formation of some inhibitors. Since the potential toxicity of by-products of fungal pretreatment has so far been well underestimated, investigating whether and to what extent they affect bioconversion is a relevant question to be answered, especially considering that SMS from different tree species might have divergent characteristics regarding inhibitory compounds.

1.3 Utilization of side streams

To develop a sustainable production chain, a possibility of valorization of side streams have to be considered. A major solid leftover is a residue after enzymatic saccharification of SMS. Theoretically, the leftover contains a relatively high ratio of lignin, with a higher calorific value than other components (Demirbas, 2001), which could be used as fuel for production of heat by combustion. Heat is needed for pasteurization of mushroom substrate and for space heating for the rest of the cultivation processes. It would be good to recycle the hydrolysis solid leftovers for a self-supporting energy system and thereby, reduce the costs associated with the mushroom production process. However, a prerequisite is to understand fuel characteristics. Among others, the content of ash and ash-forming elements (such as Ca, Mg, K, Na, Si, P, Al, and Cl) of the substrate increased after mushroom cultivation (Wei et al., 2020), due to fungal metabolism. They may be further enriched in the leftovers, because a conversion of cellulose to soluble sugar and an addition of buffer chemicals during enzymatic saccharification. It is well known that ash composition in the fuel may has a crucial role in the combustion in terms of whether slagging, corrosion, and particle emissions could be induced. The characteristics of the solid leftover from the enzymatic saccharification of SMS is worth being explored.

1.4 Objectives

The overall objective of this thesis was to develop an integrated approach for production of edible mushroom and biofuel from residual lignocellulosic biomass. The concept is shown schematically in Fig. 2.

The following specific goals should be fulfilled to reach the overall objective (Table 2):

- To evaluate production of shiitake mushroom, as a model species, in three different hardwood based substrates, parallel with studying lignocellulose degradation and identifying degradation/metabolic by-products (Paper I)
- To study the regulatory roles of nitrogen supplementations and lignocellulosic biomass assortments on shiitake mushroom production and the consequent fungal pretreatment (Paper I and III)
- To investigate saccharification of shiitake SMS by both analytic and preparative enzymatic saccharification, and to study the fermentation of the obtained hydrolysate for cellulosic ethanol production (Paper II)
- To build mass balances for entire production chain, using shiitake as model species, from wood substrates to mushroom and cellulosic ethanol (Paper I and II)
- To characterize the solid leftover after enzymatic saccharification of SMS and to study its potential for direct combustion (Paper II and IV)
- To assess the adaptation of oyster and wood ear mushrooms to hot-air pasteurization based new approach for the production of food and fuels (Paper IV and V)

2. Methods and materials

A summary of edible mushroom species, substrates ingredients and substrate pasteurization methods studied in this thesis for Paper I-V is briefed in Table 3.

2.1 Mushroom production

2.1.1 Substrate preparation

For preparation of the mushroom substrates, forest residues from the hardwoods (birch (*Betula pubescens Ehrh.*), alder (A*lnus incana (L.)* Moench) and aspen (*Populus tremula L.*)) were obtained from natural forest areas in Sweden. The tree materials were debarked, and stemwood was then milled to < 4 mm, and used as the major ingredient in the substrate for mushroom cultivation in papers I, III, IV and V (Table 3). Birch bark was also prepared and used as substrate ingredient in paper III. Wheat (*Triticum aestivum L.*) bran and barley (*Hordeum vulgare L.*) grain were supplied for the basic nutrient sources in the substrates in Paper III and IV. Wheat bran is the only basic nutrient source in Paper I and V. Whey powder (Whey-100, HSNG AB, Sweden) was used as additional nitrogen source in Paper I and III.

The substrates treatment in Paper I was designed in D-optimal model (Eriksson et al., 2008) with combined formula and quantitative factors. Firstly, a formulation factor 'sawdust species' referring to the mass fraction of birch, alder and aspen sawdust was created. Secondly, additions of 0%, 1% and 2% whey as quantitative factor were used to adjust the nitrogen content in the substrates. The design incorporated five replicated center points using blends containing equal proportions of all three tree species, with 1% whey (Table 2 in Paper I).

In Paper III, a central composite face (CCF) design (Eriksson et al., 2008) with two independent factors (bark and nitrogen addition to the substrate) was used, each of them at three different levels. The designed addition of bark were from 0%, 10% and 20%. Additions of 0%, 1% and 2% whey were used to adjust the nitrogen content in the substrates. Three replicated center points were included, containing 10% bark and 1% whey (Table 2 in Paper IV).

Table 2. Overview of the specific objectives for the work described in Paper I-V.

Paper	Mushroom production	Fungal pretreatment				Enzymatic saccharification of SMS		Ethanolic fermentation
		Major lignocellulose components*	S:G ratio	Crystallinity	By-products**	Glucan digestibility	Solid leftover	
I	×	×	×	×	×			
II						×		×
III	×	×				×		
IV	×	×				×	×	
V	×	×				×		

* Including lignin, xylan, glucan, ** Including Furfural, HMF, levulinic acid, formic acid, acetic acid and soluble phenolic compounds.

Table 3. Overview of the studied mushroom species, substrate ingredients and pasteurisation methods described in Paper I-V.

Paper	Mushroom Species	Mushroom substrates ingredients					Pasteurization Methods
		Stemwood	Bark	Wheat bran	Wheat grain	Nitrogen additives	
I*	Shiitake	birch/aspen/alder 80%	-	20%	-	Whey 0, 1, 2%	Hot air at 85 °C
II**	Shiitake	birch/aspen/alder 80%	-	20%	-	-	Hot air at 85 °C
III*	Shiitake	birch 60-80%	20-0%	10%	10%	Whey 0, 1, 2%	Hot air at 85 °C
IV	Wood ear	birch 80%	-	10%	10%	-	Steam autoclaving 121 °C Hot air at 75/85/100 °C
V	Summer oyster	alder/spruce 73%	-	24.6%	-	-	Steam autoclaving 121 °C Hot air at 65 °C

* The experiments in the Paper I and III were designed in D-optimal and Central Composite Face model, respectively. ** The SMSs for the fermentation study in Paper II were collected from Paper I.

The pH of the substrates was adjusted to around 6.3–6.5 by adding $CaCO_3$ (1–2%). Water was added to adjust the moisture content of the substrate to around 65%. After blending all the required ingredients (Table 3), the substrates were packed into polypropylene boxes/bags with inbuilt micro-porous filters as gas exchange windows (Fig. 3).

Figure 3. Mushroom cultivation boxes and bags.

2.1.2 Substrate pasteurization

For the study on shiitake (Papers I and III), novel process of hot-air pasteurization at 85 °C was used for deactivation of competitive microorganisms in the substrates, based on previous findings and novelty (Xiong et al., 2019). In Papers IV and V, the feasibility of hot-air pasteurization for cultivation of summer oyster (*Pleurotus pulmonarius*) and wood ear (*Auricularia auricularia*) to the hot-air pasteurization was studied. The substrates for wood ear and summer oyster were sterilized or pasteurized using either autoclave (steam at 121 °C and 2 bar) or hot-air oven (100–65 °C, under atmospheric pressure). See Table 3. When the required treatment time at the work temperature was reached, sterilization/pasteurization was terminated. The substrate containers were then left overnight in the autoclave or oven to cool to room temperature.

2.1.3 Mushroom cultivation and sampling

The inoculation process was performed in a laminar-flow cabinet. Fungal spawn, shiitake, wood ear and summer oyster were used (Table 3). During the incubation period, the containers were placed in a dark room but the temperature was controlled at around 19–22 °C. When the entire block was fully covered with mycelia, the colonization period was considered complete. When the mushroom fruiting bodies emerged, the boxes/bags were opened, the temperature was lowered to 18 °C, humidity was

increased to 90% and some light (< 500 lx) was induced in the climate chamber until the harvest was completed. The mushroom fruiting bodies were harvested manually, and the entire SMS block was collected immediately after mushroom harvest.

2.2 Chemical analysis

2.2.1 Compositional analysis of the substrates

The content of extractive compounds of substrates was determined by successive extraction according to an NREL protocol (Sluiter et al., 2005). Extractive-free materials were then air-dried and stored for the determination of structural components by analytical acid hydrolysis (Sluiter et al., 2008). Klason lignin was determined gravimetrically, and acid-soluble lignin was determined spectrophotometrically at 240 nm (Shimadzu, Kyoto, Japan). The amount of carbohydrates was determined by high-performance liquid chromatography (HPLC, Shimadzu, Kyoto, Japan) or/and gas chromatography in combination with mass spectrometry (GC-MS instruments Agilent Technologies 6890N GC connected to MSD 5973).

2.2.2 Pyrolysis-gas chromatography/mass spectrometry (Py-GC/MS) analysis

For the work of Paper I, Py-GC/MS was used to determine the relative ratio of dimethoxylated (syringyl, S) and monomethoxylated (guaiacyl, G) phenylpropane lignin units in raw substrate ingredients, initial substrates and SMSs. The analysis was performed at Umeå Plant Science Centre (Umeå, Sweden) according to a previously established method (Gerber et al., 2012).

2.2.3 X-ray diffraction (XRD) analysis

To determine the crystallinity index (CrI) of cellulose in the studied samples of Paper I, powder X-ray diffraction (XRD) analyses were performed using a Malvern Panalytical X'Pert3 Powder diffractometer equipped with an Empyrean Cu LFF HR X-ray generator and a X'Celerator detector. The patterns were acquired by exposing the samples to Cu Kα1 radiation (1.54056 Å). The instrument was operated at 1.8 kW and the samples were scanned in the 2 θ range between 5–45°. The crystallinity index was calculated based on the height ratio between the intensity of the crystalline peak and the total intensity after the background signal (non-crystalline) using the expression

$$\mathrm{CrI} = \frac{I_{200} - I_{non-cr.}}{I_{200}} \times 100$$

where I200 is the maximum intensity of the peak corresponding to the plane in the sample with the Miller indices 200 at a 2 θ between 22–24°; Inon-cr is the intensity of the non-crystalline material, which is taken at an angle of aproximatelly 18° 2 θ in the valley between the peaks (Kumar et al., 2009).

2.2.4 Determination of degradation-derived by-products

In the study described in Paper I, for determining the possible formation of degradation products, aliquots of the substrates were suspended in in 50 mM sodium citrate buffer (pH 5.2) at a 10%

consistency in the same way as used for enzymatic saccharification (Gandla et al., 2018). The suspensions were vortexed and then incubated for 2 h. After that, the liquid phase was separated by centrifugation, acetic acid, formic acid and levulinic acid were determined by HPAEC-PAD as previously indicated (Martín et al., 2019). Possible presence of the furan aldehydes furfural and 5-hydroxymethylfurfural (HMF) was evaluated with an HPLC system. For determination of soluble phenolics, boiling water extraction was performed for 3 h. Soluble phenolic compounds in the extracts were determined by Folin Ciocalteu's method (Singleton et al., 1999) using vanillin as calibration standard.

2.3 Analytical enzymatic saccharification

For each substrate sample, 50 mg was suspended in 50 mM sodium citrate buffer (pH 5.2) at a 5% consistency. After that, the commercial enzyme preparation Cellic CTec2 (acquired from Sigma-Aldrich Chemie GmbH, Steinheim, Germany), a blend of cellulases, β-glucosidases and hemicellulases, was added at a load of 100 CMCase units/g biomass. After adding the enzyme blend, the flasks containing the reaction mixture were incubated for 72 h in an Ecotron orbital incubator (INFORS HT, Bottmingen, Switzerland) at 45 °C and 170 rpm. At the end of the hydrolysis, the tubes were centrifuged, glucose in the supernatants was analyzed by HPLC and used for calculating the enzymatic convertibility of cellulose.

2.4 Preparative enzymatic saccharification

In Paper II, birch-, alder- and aspen-based SMSs generated in Paper I were investigated regarding their potential for downstream fermentation process for bioethanol production. Preparative enzymatic saccharification of the SMS was performed in order to produce hydrolysates to be used in the fermentation experiment. Larger volumes, and higher biomass load and enzyme dosage (200 CMCase units/g biomass) were used compared to those of the analytical enzymatic saccharification. The experimental conditions were based in the method described in analytical enzymatic hydrolysis (Gandla et al., 2018). At the end of hydrolysis, the hydrolysate was separated by centrifugation for the following fermentation experiment, and the solid leftover was air-dried until 90% DM content and stored for further analysis.

Preparative enzymatic saccharification of SMS was also performed in wood ear study in Paper IV, and after that the solid leftover was collected for fuel characterization analysis (see section 2.7).

2.5 Nitrogen solubilization test

In the study of Paper II, the solubilization of nitrogen contained in the substrates was examined. Aliquotes of 5 g initial substrates and SMSs were suspended in 50 mM sodium citrate buffer (pH 5.2), at 10% solids content, for 72 hours, mimicking the environment of the preparative enzymatic saccharification, but without adding enzymes. After that, the liquid phase was separated by centrifugation. Samples of the supernatants, in parallel with samples of SMS preparative enzymatic

hydrolysates that collected from section 2.4, were analyzed by an accredited laboratory (EUROFINS, Sweden) for the contents of ammonium nitrogen (NH_4^+-N), nitrate nitrogen (NO_3^--N), nitrite nitrogen (NO_2^--N) and total nitrogen.

2.6 Fermentation of SMS hydrolysates

The potential for ethanolic fermentation of SMS hydrolysate collected in section 2.4 was explored in Paper II. It was hypothesized that the high nitrogen content and the possible presence of other substances originated from fungal biomass remnants in SMS hydrolysates could contribute to the nutrient requirements of fermenting organisms.

The fermentation media consisted of 92.4% (v/v) filter-sterile SMS hydrolysate, 5.6% (v/v) yeast inoculum (*S. cerevisiae Ethanol Red*) and nutrient solution in four different doses (2%, 1%, 0.5% and 0%). The raw nutrient solution, corresponding to total nitrogen content of 30.9 g/L, contained 150 g/L yeast extract, 75 g/L $(NH_4)_2HPO_4$, 3.75 g/L $MgSO_4 \cdot 7\ H_2O$ and 238.2 g/L $NaH_2PO_4 \cdot H_2O$ as previously described (Martín et al., 2018). The 2% dosage corresponded to the full nutrient supplementation reported previously in fermentation of lignocellulosic hydrolysates (Martín et al., 2018), while the 1% and 0.5% corresponded to 50% and 25% charges. The experiment included also two reference fermentations, one with full nutrient charge (Reference 1) and one with no nutrients (Reference 2), of a glucose medium with concentration around 35 g/L. The pH of all media was adjusted to 5.5.

Filter-sterile SMS hydrolysates and reference media were mixed under aseptic conditions with the required amount of nutrient solution in 25-mL bottles with a working volume of 18 mL. One mL of the yeast inoculum cell suspension was added, and the flasks were sealed with cotton plugs to allow the release of CO_2 formed during fermentation. The fermentation media were incubated in Ecotron orbital incubator at 35 °C and 180 rpm under oxygen-limited conditions. Samples were taken at the beginning of the fermentation, and after 4, 8, 12 and 16 h. The concentrations of glucose, xylose and acetic acid in the enzymatic hydrolysates and in fermentation samples, as well as ethanol in fermentation samples, were determined by HPLC.

2.7 Fuel characterization of the solid leftover from enzymatic saccharification

In the wood ear study (Paper IV), to investigate whether the solid leftovers after enzymatic saccharification of SMS can be used as fuel for heat production, samples of the solid leftovers were analyzed for their fuel characteristics. The content of ash, main energy elements (C, H, O, N, S), and main ash forming elements as well as the calorific values were determined of solid leftover samples were analysed by a certified laboratory (EUROFINS, Sweden). Samples of initial substrates and SMS were also analyzed for comparison.

Additionally, ash was produced by combusting ground samples (30 g) of each substrate and leftover in a laboratory-scale muffle furnace at 1000 °C. The elemental composition of each ash sample was analyzed using a Carl Zeiss EVO LS15 scanning electron microscope combined with an Oxford

Instruments Xmax-80 energy dispersive X-ray spectrometer (SEM-EDS). As a complementary analysis, the ash fusion process was also analyzed at a certified laboratory (EUROFINS, Sweden) following the ISO 21404:2020 standard method. The shrinkage, deformation, hemispherical, and liquid temperatures were determined.

2.8 Modelling

D-optimal experiment with combined formula (sawdust species: birch, alder and aspen) and quantitative factors (nitrogen addition), and central composite face (CCF) design with two independent factors (bark and nitrogen addition to substrate) and was designed using software MODDE 11.0 (Umetrics AB, Sweden), respectively, in Paper I and Paper IV. The models were evaluated using the coefficients of determination (R^2 and Q^2), which explained the goodness of fit and the predictive ability of the model; R^2 and Q^2 values close to 1 indicate that the model fits the data very well. For each response, a model including all the independent factors and their interactions was created. Interaction terms showing no significant effect on the target response variable ($p > 0.05$) were excluded from the model to obtain optimized R^2 and Q^2 values.

3 Results and discussion

3.1 Effects of nitrogen supplementation and hardwood species on the production of shiitake and lignocellulose degradation of substrates

3.1.1 Shiitake mushroom production

The evaluation of the mushroom production includes both the time for complete colonization in the substrate of the mycelium (full colonization) and mushroom fruiting bodies harvest, together with the mushroom yield, in order to do the assessment of the cultivation process. In paper I, hyphal growth from added spawn started to be observed in most of the experimental runs with formulated substrates of either single species ingredient or two- or three-species mixtures. The full colonization, which is defined as all six surfaces of substrate block are 100% covered by white hypha though observation, took 18–28 days for the different experimental runs (Fig. 4a-c). A significantly faster ($p < 0.001$) colonization was observed in the substrates with lower whey additions. Low fraction of aspen was correlated with a short time of colonization, in opposite to that of alder, while birch had a milder effect compared with alder and aspen.

The entire time to harvest varied from 74 to 100 days for the different experimental runs, and no significant effect ($p > 0.05$) of either whey addition or tree species was detected (data not shown).

The yield of the first flush of fresh fruiting bodies (normalized to moisture content of 90%) ranged between 520 and 741 g per kg of dry substrate (Fig. 4d-f). The yields were in general comparable to results of previous shiitake studies (650.8–675 g/kg hardwood) (Lin et al., 2015; Xiong et al., 2019). Whey additions had a significant ($p < 0.05$) quadratic correlation with mushroom yield; a higher yield was found with 1% whey, followed by 2% and 0% whey addition (Fig. 4d-f). For all whey

supplementations, high ratio of alder in the substrate can be associated with high yields of fruiting bodies. On the other hand, inclusion of aspen generally resulted in low mushroom yield. However, the Q^2 value was marginally significant for the mushroom yield, and the significance was mostly due to nitrogen supplementation rather than hardwood species.

In Paper III where the birch-based substrate was used, similar results were achieved regarding the negative correlation between mycelial colonization speed and nitrogen addition/loading, the time to harvest (66–85 days), and the yield of shiitake fruit bodies (651 g per kg DM, on average).

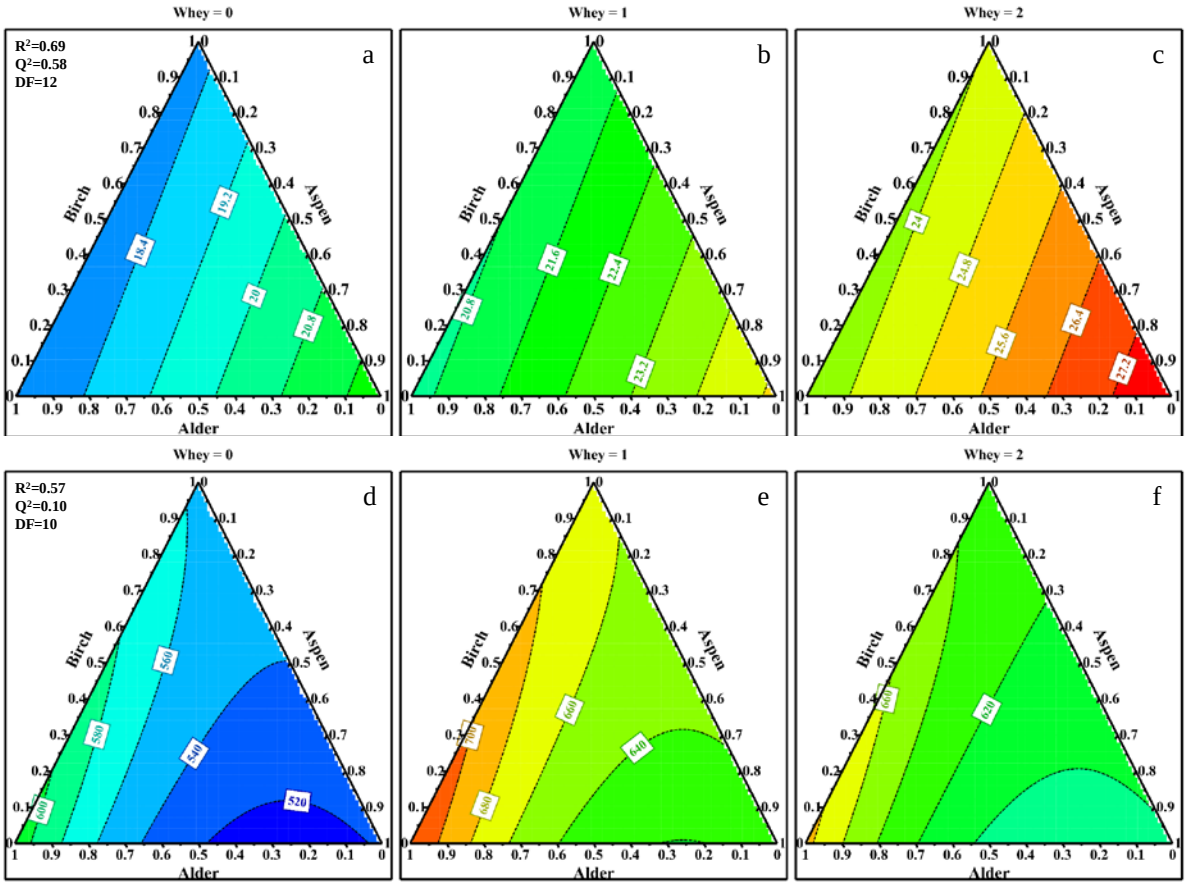

Figure 4. Response contour plots predicted with PLS regression models for the colonization time (a-c) and fresh mushroom yield (d-f) in relation to whey addition and tree species. The units are days for the colonization time and g/kg DM of initial substrate for the mushroom yield (Paper I).

3.1.2 Major changes of substrate lignocellulose after shiitake cultivation

3.1.2.1 Lignocellulose mass degradation

In the work presented in Paper I, the average mass degradation of lignin (the sum of Klason and acid-soluble lignin), xylan and glucan in the substrates after fungal treatment using shiitake was 60.4, 61.3 and 26.0%, respectively, of the initial values. Glucan had a much lower rate of degradation than

lignin and xylan, and that is consistent with the results of previous study (Xiong et al., 2019). The relative mass reductions of lignin and glucan are well described by PLS models with high R^2 and Q^2 values (Fig. 5), which were significantly ($p < 0.05$) affected by the level of whey additions, while the effect of the wood species used in the substrate was less important.

Lignin degradation was linearly and negatively correlated with whey addition ($p < 0.01$), and showed maximal value of 67.6% with no whey addition (Fig. 5a-c), where the only nitrogen source was that intrinsic of the wood substrates, which corresponded to less than 0.58% (w/w). In contrast to lignin, glucan mass degradation was positively correlated with the whey addition ($p < 0.05$) (Fig. 5d-f). Low losses of glucan (16–24%) were observed for the experimental runs without whey addition, while larger losses (24–32%) were found for those with 1% and 2% whey. The contour plot in Fig. 5 reveals that lignin was degraded slightly more in birch than in aspen, in contrast to glucan, which was degraded more in alder than in birch substrate. That points to the importance of the specific type of hardwood species in the degradation pattern of lignocellulose components. Compared with those of lignin and glucan, mass reduction of xylan was not significantly ($p > 0.05$; data not shown) affected by either whey addition or tree species.

The facts that the lowest glucan degradation and maximal lignin degradation at the 0% of whey addition regardless substrate composition of wood species (Paper I) are confirmed by the results from Paper III, in which the regulatory role of nitrogen in lignocellulose degradation by shiitake was the major study topic. In Paper III, only birch-based substrate was used but substrate composition varied with different fractions of nitrogen and birch bark. Consistently with Paper I, the minimal glugan consumption (< 26%) and maximal lignin degradation (64%) were associated with the lowest nitrogen loading in initial substrate (0.64%). With the experiment settings and substrate compositions adopted in my studies, the nitrogen is shown to be a significant regulatory factor regarding efficiency of the fungal pretreatment.

The high degree of degradation of lignin and xylan, together with the good preservation of glucan, are positive features of the fungal cultivation using shiitake investigated in this work as a potential pretreatment for enzymatic saccharification of cellulose. The results of the mass balances revealed that glucan recovery as high as 80% can be achieved, which is within the typical range for other hydrothermal pretreatment approaches (Ilanidis et al., 2021a; Ilanidis et al., 2021b; Martín et al., 2019; Tang et al., 2021).

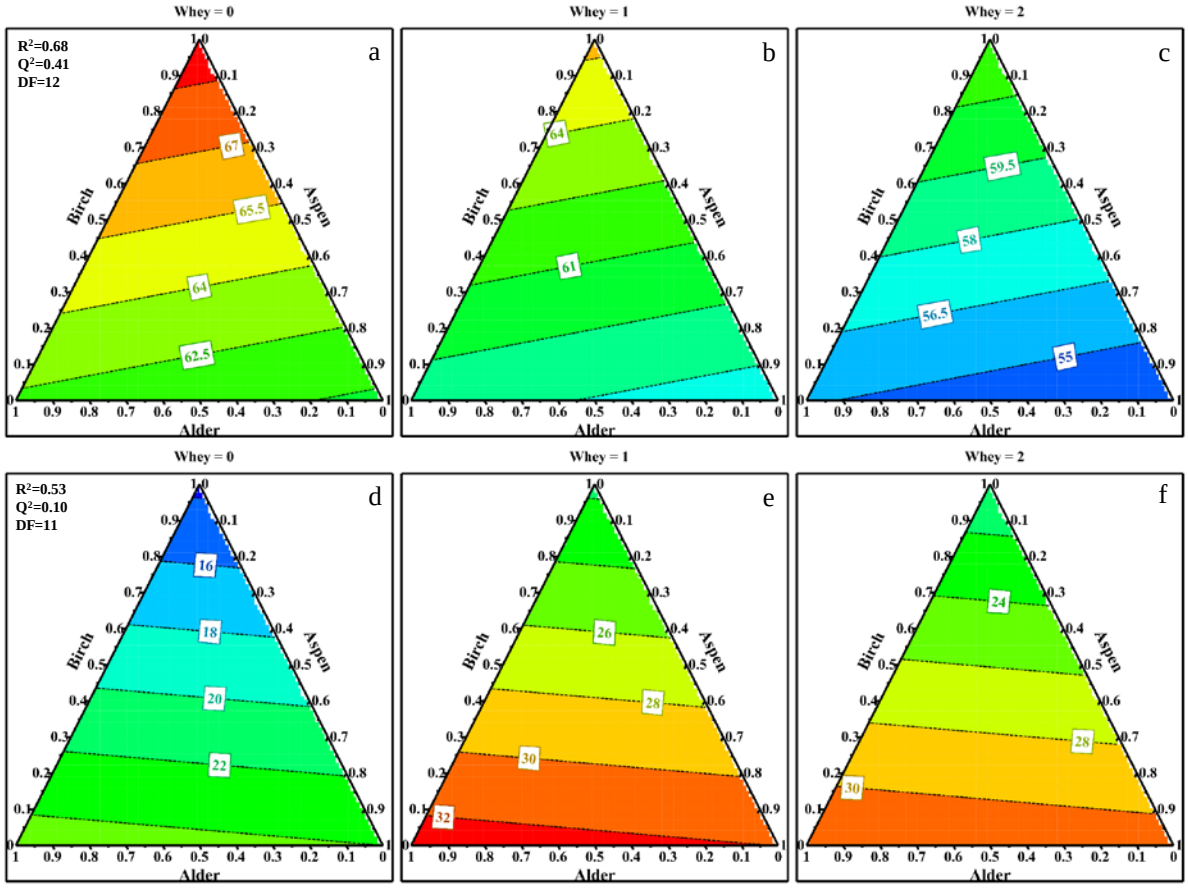

Figure 5. Effects of whey addition and tree species on lignin (a-c) and glucan (d-f) mass degradation (Paper I).

3.1.2.2 Lignin composition

The initial substrates, based on birch, alder, aspen had S:G ratio from 2.2 to 2.9. Shiitake cultivation, in addition to removing part of lignin, changed the ratio of lignin phenylpropane units, and the S:G ratio decreased to 1.2–1.7 in the SMS (Table 3 in Paper I). The reduction of S:G ratio was 41.5% on average (Fig. 6), which was much higher than the previously reported reduction observed after acid pretreatment of aspen (20.8%) and birch (26.2%) (Wang et al., 2018a). The reduction of S:G ratio suggests that S-units were more degraded than G-units. These findings were however in contrast to that from alkaline pretreatment by (Santos et al., 2012), who reported a general increase in S:G ratio for four hardwood species, which was attributed to a larger proportion of G lignin degradation.

As shown in Fig. 6, the whey additions had a significant ($p < 0.001$) effect on the relative change (%) of S:G ratio. High whey addition resulted in less change of the S:G ratio. The decreased S:G ratio during fungal pretreatment could be attributed to a higher lignin-degrading enzymatic reactivity of S-units in the substrates of this study. It had been reported that both manganese peroxidase (MnP) and laccase are the major lignin-degrading enzymes secreted by shiitake (Janusz et al., 2013). Those

enzymes have different preferences regarding degradation of different lignin units. For example, MnP is capable of oxidizing nonphenolic compounds and minor phenolic moieties of lignin, while laccase oxidase that oxidizes numerous phenolic compounds (Janusz et al., 2013; Wan and Li, 2012). Wood lignin rich in S-units often has low content of free phenolic groups due to their involvement in formation of methoxy groups (Camarero et al., 1999). Plausibly, the high reactivity of S-lignin in this study can be attributed to high MnP activity, compared to laccase activity, involved in lignin degradation. High nitrogen content might have caused the repression in MnP activities, and that resulted in even higher ratio of S:G in the SMS and low mass degradation of total lignin (Fig. 6, Fig. 5a-c). A description of the mechanism is beyond the scope of this study, but it deserves being investigated in future studies including assessment of enzymatic activities.

Research on different materials has shown that the effectiveness of fungal pretreatment is dependent on lignocellulose species and assortment (Kuijk, 2016; Shirkavand et al., 2016). For example, lignin structure is as important as content, the G-lignin unit is more difficult to degrade than the S one was evidenced in this study. This might also be the part of potential explanation that softwood consisting almost exclusively of G-lignin are difficult to colonize for most white-rot fungi therefore are rarely used for fruiting body production. Differences in chemical and structural features between lignocellulosic biomass, such as hardwood, softwood and crops, are observed (Zhu and Pan, 2010), but within hardwoods the variation among tree species might not significant. Overall, in this study, compared with the effects by substrate nitrogen loading, the choice of hardwood species had the minor influence in shiitake “production” and lignocellulose degradation.

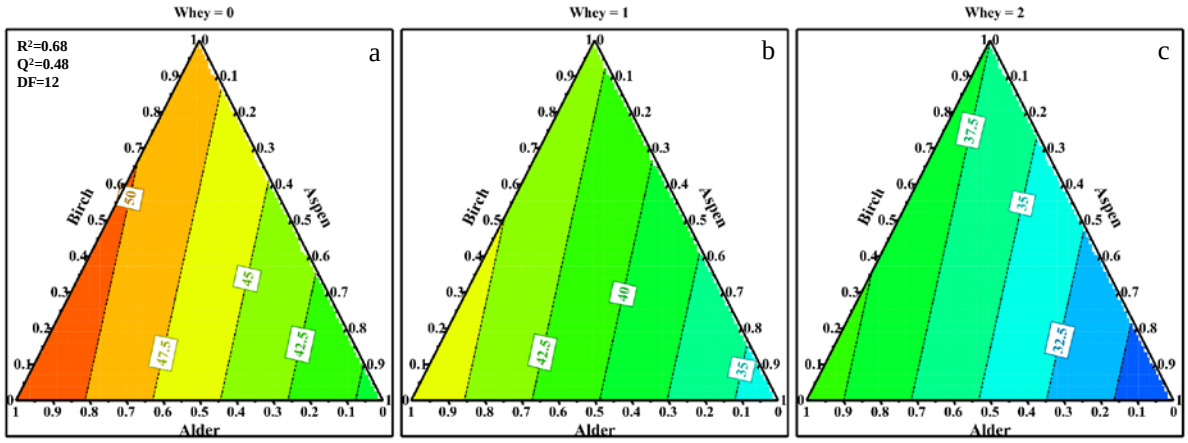

Figure 6. The change of the S:G lignin ratio in substrates (Paper I).

3.1.2.3 Substrate crystallinity

All initial substrates regardless of tree species had comparable crystallinity indices, and they ranged from 66.6 to 72.4% (Paper I). The crystallinity in the substrate is mainly due to cellulose, which

is a highly crystalline polymer with a compact supramolecular structure stabilized by hydrogen bonds (Fengel and Wegener, 1989). The fungal cultivation did not cause statistically significant changes in the crystallinity (Table 3 Paper I). This is in contrast with the expectations of a possible increase of the crystallinity after fungal pretreatment due to retention of crystalline sectors and more degradation of amorphous sectors of cellulose (Shirkavand et al., 2016). However, this agrees with a report showing that losses of crystalline and non-crystalline regions of cellulose occurred in parallel during growth of shiitake on oak bark during a 101-month cultivation (Vane et al., 2006). No significant correlations ($p > 0.05$) were found between lignocellulose degradation and the relative change of crystallinity during fungal pretreatment.

3.2 Chemical and structural factors influencing enzymatic saccharification and fermentation of shiitake pretreated SMSs

Investigating the enzymatic saccharification of the SMS, and the ethanolic fermentation of the resulting hydrolysates is important for assessing the potential of using shiitake cultivation as a pretreatment for production of cellulosic ethanol. The study on downstream biochemical processes were performed in Paper II.

3.2.1 Factors affecting the enzymatic saccharification of SMS

As indicated by the results of the enzymatic saccharification, the glucan digestibility in the initial substrates was rather low for the different tree species. Around 16–27% of initial glucan was hydrolyzed for the different materials (Fig. 7a). Fungal pretreatment using shiitake resulted in an enhancement of the susceptibility of the substrate to enzymatic saccharification. Glucan digestibility of the SMSs averaged 80.3%, which corresponds to an increase by 3.5 times compared with the values of initial substrates.

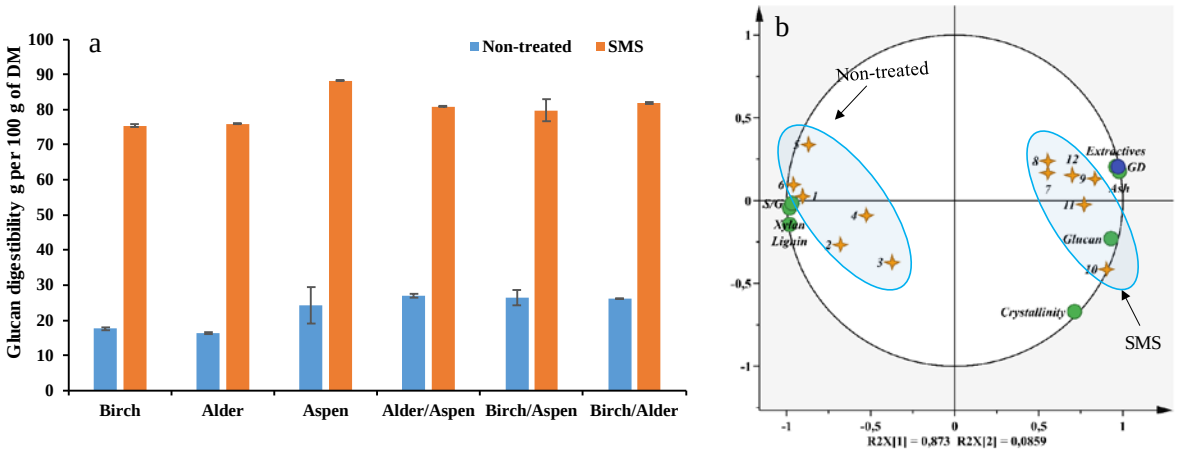

Figure 7. Enzymatic digestibility of glucan contained in the non-pretreated woody substrates (initial substrates) and SMSs, (a); PCA biplot showing major chemical components of substrates and glucan digestibility (GD) (b). Each star represents an observation. (Paper II).Based on a 12×7 data matrix

including twelve analytical enzymatic hydrolysis experimental runs and seven characteristic variables of substrates, a PCA was performed to identify multivariable relations (Fig. 7b). The biplot, composed of the first two PCA components and explaining 95.9% of the total variation, shows that the observations were visually clustered into two groups that were separated from left to right of PC1 axis by the initial substrates and SMSs. The negative effect of lignin and xylan on enzymatic digestibility was anticipated (Jönsson and Martín, 2016; Shirkavand et al., 2016), whereas, the negative effect of S:G ratio and the positive effect of the crystallinity index were less expected, since they have often been assumed to affect enzymatic saccharification in a different way to what our results show (Guo et al., 2014; Hall et al., 2010).

It has previously been reported that low S:G ratio is deleterious for enzymatic digestibility. That has been associated with lignin guaiacyl units having higher adsorption capacity than syringyl ones, thus causing non-productive adsorption of cellulases onto lignin (Guo et al., 2014), which is a key problem affecting enzymatic hydrolysis of cellulose (Oliva-Taravilla et al., 2020). However, in the current study, an opposite trend, e.g., a good correlation between low S:G ratios and enzymatic digestibility, was observed. As a possible explanation, one might hypothesize that the reduced amount of syringyl units, which are more voluminous than guaiacyl ones, can weaken steric impediment limiting lignin role as barrier blocking the access of enzymes to cellulose. It has been shown that in some pretreated biomass, lignin inhibits the enzymatic hydrolysis by acting as a physical barrier rather than by inducing non-productive adsorption of cellulases (Djajadi et al., 2018).

Cellulose crystallinity has traditionally been considered one of the main factors behind the poor enzymatic convertibility of cellulose (Hall et al., 2010). However, although the role of crystallinity is unambiguous for pure cellulose, it is not that straightforward for pretreated lignocellulose. There are many examples, where improved enzymatic convertibility of pretreated biomass and its crystallinity index are directly correlated, or even when no relationship at all is found between digestibility and crystallinity (Karimi and Taherzadeh, 2016). Acid pretreatment of de-starched cassava stems led to enhancement of the enzymatic hydrolysis of cellulose in spite of the observed increase of the crystallinity index (Martín et al., 2017). Other authors have also stressed that ultrastructural and compositional changes caused during pretreatment are more important than cellulose crystallinity for explaining different enzymatic hydrolysis of pretreated materials (Agarwal et al., 2013; Pardo et al., 2019).

In summary, although the increase of lignin guaiacyl units and substrate crystallinity was observed, that did not affect the enzymatic saccharification of the SMSs. The higher glucan digestibility in the SMS compared to initial substrate was mostly related to the removal of lignin and hemicelluloses during fungal cultivation.

3.2.2 Assessment of inhibitory compounds in the SMS hydrolysates

A drawback of hydrothermal pretreatment is the occurrence of side reactions resulting in formation of lignocellulose-derived by-products, which are potential inhibitors of cellulolytic enzymes and fermenting organisms. In the studies presented in Papers I and II, the formation of those compounds was investigated.

3.2.2.1 Furfural, HMF, levulinic acid and formic acid

The toxic effects of furan aldehydes, formic acid and levulinic acid on ethanolic fermentation has been investigated for different hydrothermal pretreatment approaches (Bolado-Rodriguez et al., 2016; Martín et al., 2018). The results presented in Paper I and II revealed that no detectable signals of furfural, HMF, levulinic acid and formic acid were found in either any of the samples after shiitake cultivation, or in the SMS hydrolysates. Furan aldehydes are typically formed by sugar degradation during hydrothermal treatments in acidic media, and if the temperature is high or the reaction time is long, they can get degraded further to formic acid and levulinic acid (Fengel and Wegener, 1989). The absence of furan aldehydes, levulinic acid and formic acid, indicates that hot-air pasteurization is unlikely to produce these inhibitors for either enzymatic saccharification and microbial fermentation.

3.2.2.2 Acetic acid

Acetic acid, a known inhibitor of ethanolic fermentation, was found in the SMS hydrolysates in a range between 1.8 and 2.0 g/L (Table 4 in Paper II). The formation of acetic acid was not related to fungal pretreatment, but rather to enzymatic hydrolysis process. As a results of Paper I, acetic acid was hardly detectable in the SMS. Acetic acid is typically formed from splitting of acetyl groups during xylan hydrolysis. Its absence indicates that no hydrolysis of hemicelluloses occurred during pasteurization and shiitake cultivation. On the other hand, hydrolysis of hemicelluloses did occur during the enzymatic saccharification, as shown by the high concentrations of xylose found in the hydrolysates (Table 4 in Paper II), which is explained by the presence of hemicellulases in the used enzyme preparation (Cellic CTec 2). The acetic acid observed in the SMS hydrolysate was, as xylose, a product of the hydrolysis of remaining hemicelluloses during enzymatic saccharification,

3.2.2.3 Phenolic compounds

The SMS hydrolysates contained phenolic compounds with concentration of 1.8–2.0 g/L (Table 4 in Paper II), which were formed by lignin degradation during shiitake cultivation. In the work described in Paper I, the content of soluble phenolics in the SMS ranged from 1.8 to 2.4% (w/w), which corresponds to 1.4–7.2-fold increases compared with that in the initial substrates. Phenols ended up in the liquid fraction when the SMS was suspended in the buffer solution.

3.2.3 Nitrogen, an important nutrient in the SMS hydrolysates

Differently from other lignocellulose-based media, SMS hydrolysates might provide not only carbon sources, but also nutrients such as nitrogen required for fermentation. The results show that the content of solubilized nitrogen in the SMS hydrolysates ranged between 810 and 890 mg/L (Table 4 in Paper II). This soluble nitrogen comes from the fungal mycelium retained in SMS and from the nitrogen remaining in the partly degraded substrate. During the shiitake cultivation, accumulations of nitrogen in the substrates was observed, the contents increased from 0.50–0.64% in raw substrates to 0.67–0.76% in the SMSs. The nitrogen from the lignocellulosic biomass is partly incorporated into fungal proteins, which also increased the solubility. As can be seen in the nitrogen solubilization test (Table 3 Paper II), the solubilized nitrogen in initial substrates was only 10–20% of the total nitrogen in the solid sample, while in the SMSs it was 48–55%. Meanwhile, inorganic nitrogen (NH_4^+, NO_3^- and NO_2^-) forms represent a minor proportion (on average 1.8%) of the total solubilized nitrogen in SMSs (Table 3 in Paper II), suggesting that the major part of the solubilized nitrogen was in organic forms, likely as protein existing in remaining fungal mycelia.

3.2.4 Potential of SMS hydrolysates for cellulosic ethanol production

It is hypothesized that the high nitrogen content is a strength of SMS hydrolysates if they are going to be used as substrates for ethanolic fermentation. In order to assess that feature of SMS hydrolysates, a fermentation experiment was included in Paper II. A nutrient mixture, typically used for fermentation using baker's yeast, was added in four different dosages (2%, 1%, 0.5% and 0% (v/v)) where the 2% dosage corresponded to the full nutrient supplementation reported previously in fermentation of lignocellulosic hydrolysates (Martín et al., 2018).

The results showed that the yeast cell growth in the fermentations with higher nutrient dosage was significantly higher than in those with no nutrient addition (Fig. 8). Thus, some nutrient deficiency likely impaired growth in the hydrolysate of the SMS. Meanwhile, with the reduction of the nutrient amount, there was significant decrease of the glucose consumption and volumetric productivity of ethanol during the fermentation process (Fig. 9 and Fig. 10). The ethanol concentrations, however, became comparable between nutrition loadings of the hydrolysates at the end of the fermentation (12 h), and reached values corresponding to 84–87% of the theoretical yield.

The good fermentation behavior of hydrolysates supplemented with low nutrient dosages or not supplemented at all can be attributed to the presence of nutritional substances produced during the fungal pretreatment. The glucose-to-nitrogen ratios in SMS hydrolysates seems to be enough for ensuring the carbon-nitrogen balance that is required for ensuring an efficient fermentation by yeast. Apparently, the nitrogen sources contained in fungal biomass remnants in the SMS are also suitable to be used by yeast as nutrients. Among other nitrogen forms, ammonia, glutamine, and asparagine have long been considered important sources of yeast assimilable nitrogen (YAN) (Gobert et al., 2019). In the current work, all soluble inorganic nitrogen accounted for < 0.3% DM of the total nitrogen contained

in SMS hydrolysates (Table 3 in Paper II). That points towards involvement of other unidentified nitrogen forms, probably organic components, such as amino acids, originated from fungal mycelium. This might be associated with the increase of pH of the SMS hydrolysates (from 5.5 to 6.2) during fermentation, and which was not observed in the reference media, whose final pH was below 4.1 (Fig 6 in Paper II). The increase of the pH during the fermentation of the SMS hydrolysates might be linked with the dissociation of amino acids in slightly acidic conditions, which results in the protonation of the amino group with formation of an alkylammonium cation (Russo and Casazza, 2012). The pH increase was inversely proportional to the amount of added nutrients. An explanation might be that high nutrient supplementation provided more direct nitrogen source, and thus reduced use of amino acid, resulting a significant lower pH ($p < 0.05$). In general, although increased addition of nutrients resulted in some increase of glucose consumption rate and volumetric productivity of ethanol for the SMS hydrolysates, it did not affect the final ethanol yields.

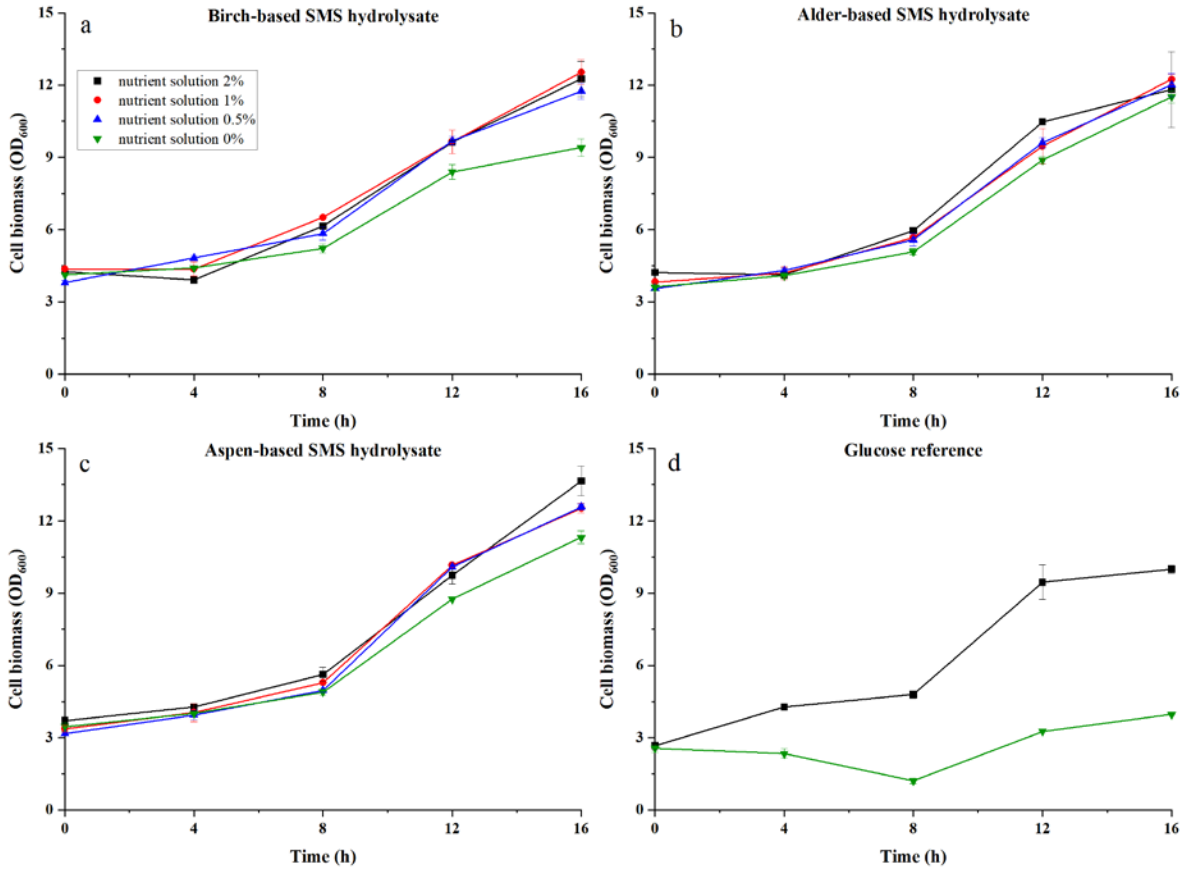

Figure 8. Cell growth of *S. cerevisiae* during fermentation of SMS hydrolysates (a-c) and reference media (d) with different nutrient loadings. The data refer to mean values of duplicate experiments, and the error bars represent the standard errors. (Paper II).

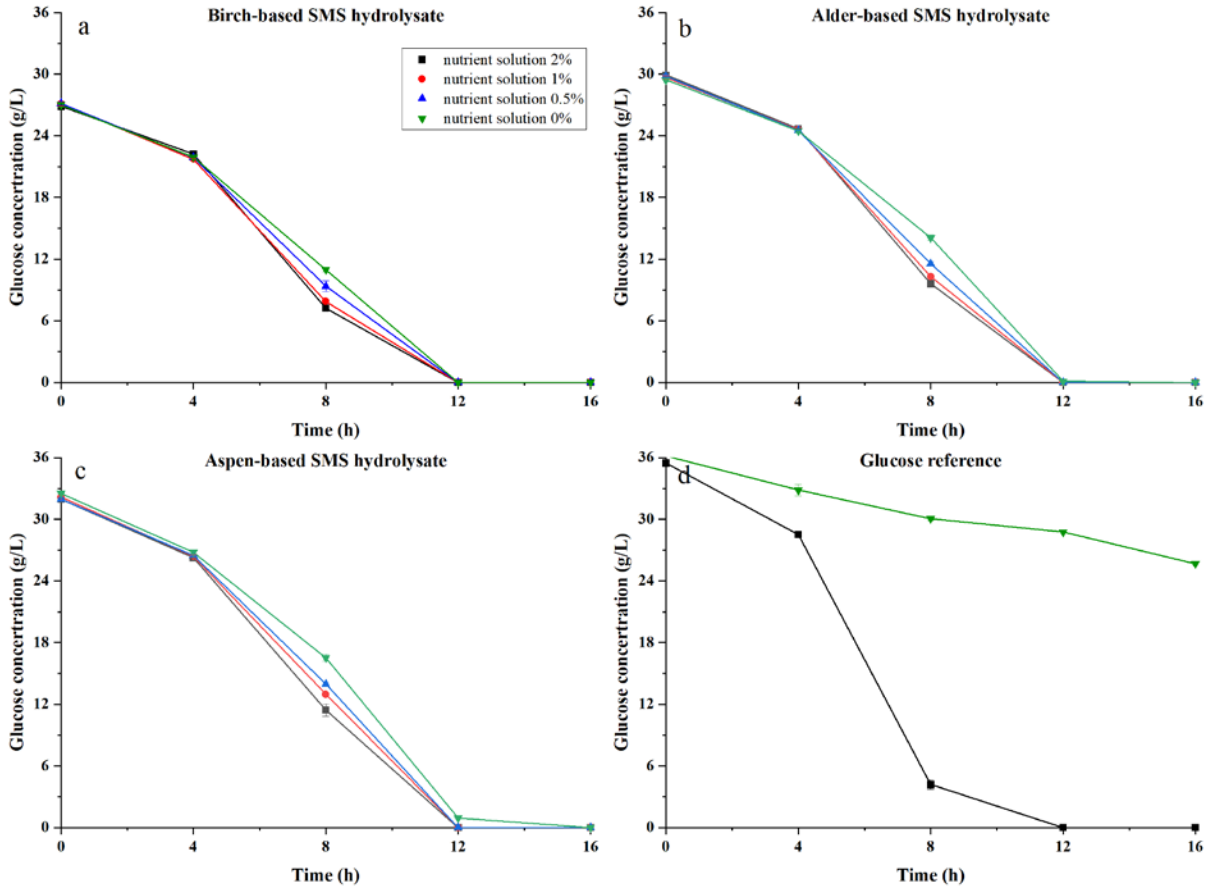

Figure 9. Glucose consumption during *S. cerevisiae* fermentation of SMS hydrolysates (a-c) and reference media (d) with different nutrient loadings. The data refer to mean values of duplicate experiments and the error bars represent the standard errors. (Paper II).

When comparing ethanol production in the SMS hydrolysates and in the glucose reference fermentation, some inhibition was observed for the volumetric productivity, while the final yield was not affected (Paper II). The inhibition of the productivity might have been caused by phenolic compounds and acetic acid in the hydrolysates. However, since the concentration of those inhibitors was low, their effect on fermentation was weak and did not last long, which is a different phenomenon compared with the stronger inhibition reported in fermentation of hydrolysates resulting from other pretreatment methods leading to high formation of inhibitors. Acetic acid concentration in the hardwood-based SMS hydrolysates used in this work was up to 2 g/L, while in hydrolysates of acid-pretreated hardwood and agricultural residues it is typically above 4 g/L (Du et al., 2020; Ilanidis et al., 2021b; Ko et al., 2016). In addition, it has been reported that inhibition by acetic acid is pH dependent, i.e., high toxicity towards *S. cerevisiae* is observed when the extracellular pH is below the pKa of acetic acid (4.7), but at pH above that value, no severe inhibitory effect occurs (Ko et al., 2016; Taherzadeh et al., 1997; Wei et al., 2013). During the fermentation, the pH of the media was always above acetic acid pKa, since it was 5.5 at the beginning, and then it increased to up to 6.2, especially at low nutrient supplementation. That might have further weakened the inhibition effect of acetic acid. Furthermore,

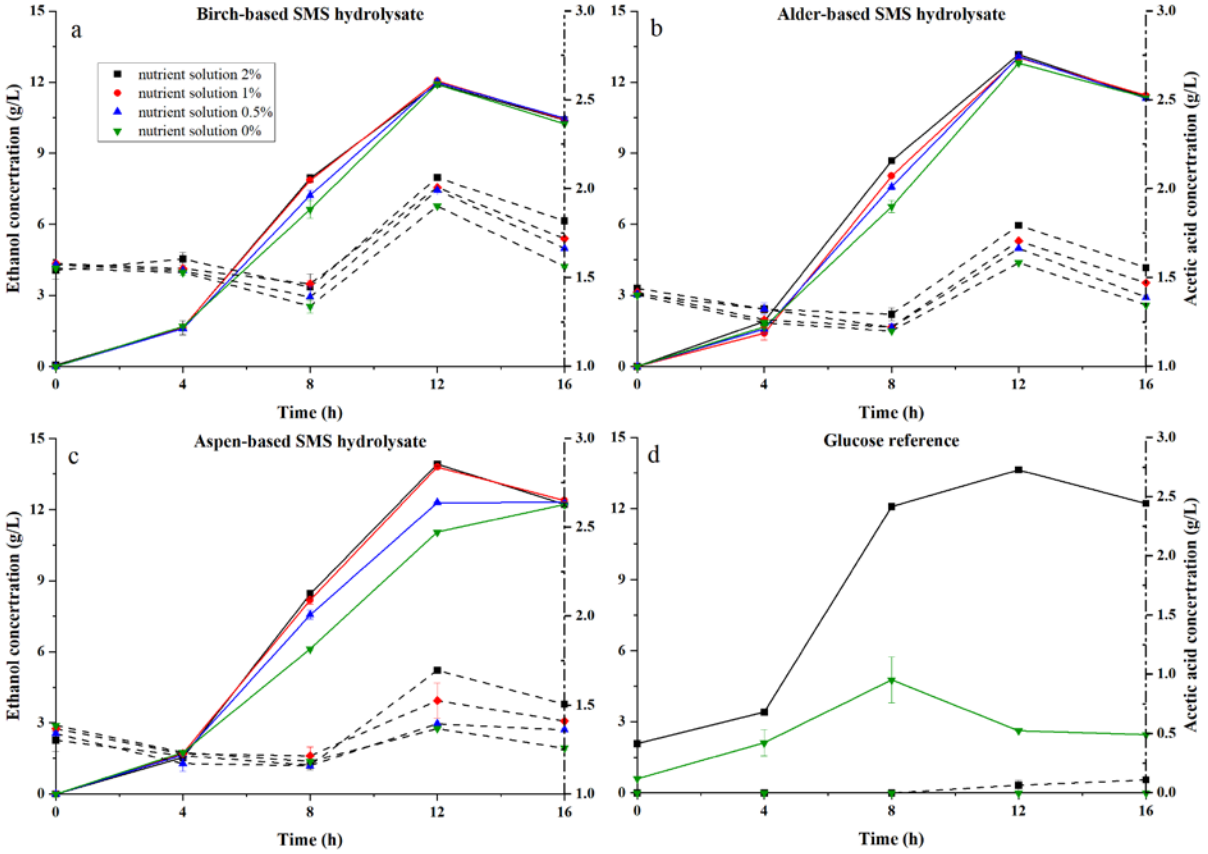

Figure 10. Ethanol (solid line) and acetic acid (dotted line) production during S. cerevisiae fermentation of SMS hydrolysates (a-c) and reference media (d) with different nutrient loadings. The data refer to mean values of duplicate experiments, and the error bars represent the standard errors. (Paper II).

differently from hydrolysates produced by hydrothermal methods (Bolado-Rodriguez et al., 2016; Martín et al., 2018), SMS hydrolysates do not contain other inhibitors, apart from phenolic compounds, which in this study were detected in comparable amounts as in previous reports on acid-pretreated sugarcane bagasse and spruce hydrolysates (Ilanidis et al., 2021b).

3.3 Mass balance analysis on combined production of shiitake mushroom and biofuel

Table 4 summarizes the mass balance of converting initial birch, alder and aspen-based substrates (1000 g) to fresh shiitake fruit bodies and SMS ethanol. About 62–67% of the initial substrate remained as SMS after the harvest of edible mushroom (Table. 4). After enzymatic saccharification, about 40–50% of SMS was solubilized and recovered as monosaccharides in the hydrolysates. The obtained glucose, around 216.6–274.3 g of 1000 g initial substrate (or 349.2–412.3 g of 1000 SMS), was then fermented to ethanol yielding 93.2–109.5 g (i.e. 124–147 mL of 95% ethanol fuel). This reveals the high potential of fungal pretreatment using shiitake as a biorefinery approach producing ethanol and edible mushrooms with high value as food and source of nutraceuticals and pharmaceuticals.

Table 4. Mass balance during shiitake mushroom cultivation, enzymatic sacchararification and fermentation. (Paper I and II)

	Units	Substrate species		
		Birch	Alder	Aspen
Initial substrate	g	1000	1000	1000
		After harvest		
Fruiting body[1]	g	596.4	621.4	375.0
SMS	g	620.4	667.5	665.4
		Enzymatic saccharification of recovered SMS[2]		
Enzyme	g	84.7	91.1	90.8
Buffer chemicals	g	145.2	156.2	155.7
		After enzymatic saccharification		
Hydrolysates				
Glucose	g	216.6	251.1	274.3
Xylose[3]	g	85.8	83.3	78.4
Solid leftover				
Glucan	g	45.2	55.2	52.2
Xylan	g	19.5	21.4	15.9
Lignin		67.3	82.5	82.9
Extractives	g	118.1	154.4	108.9
Ash etc.[4]	g	58.8	67.7	68.8
		After fermentation of hydrolysates[5]		
Ethanol	g	95.5	109.5	93.2
CO_2 loss	g	91.4	104.7	89.1
Non-used sugars And other residues				
Glucose	g	0	0	1.2
Xylose	g	76.9	64.1	60.8
Others[6]	g	267.5	302.0	302.5

[1] with moisture content of 90%, [2] With addition of 200 CMCase units/g biomass; [3] Includes other hemicellulosic sugars; [4] Includes unidentified components; [5] Without nutrient supplementation; [6] Estimated by differences.

3.4 Using bark as substrate ingredients for shiitake cultivation

Lignocellulosic biomass from different anatomic parts of a tree has not always the same composition. Differences in chemical features between stemwood and bark from the same tree species is sometimes larger than divergences between different stemwoods of different species. Still, the possibility of including these different parts in the substrate would be beneficial from a sustainability perspective. The effect of birch bark addition in the substrate on fungal pretreatment using shiitake was revealed in Paper III. Bark addition played a positive and significant ($p < 0.05$) role in lignin content but negatively correlated with the amount of carbohydrates in the raw substrate. Although bark has been reported containing antioxidant and antimicrobial properties, the addition of 0–20% did not affect

shiitake mycelium growth in the beginning stage (Paper III). Marginally significant (p = 0.04) and quadratic effect of bark addition on mushroom production and harvest time was observed. At ~10% bark fraction of initial substrate, shiitake mushroom achieved lower yields (610.1 g/kg) with shortest cultivation period (66 days), compared with the substrates with 0 and 20% bark (Paper III). Bark addition was significantly correlated with the degradation of lignin, xylan and glucan. The highest degradation of lignin was about 63.5% with the bark addition close to 20% (Paper III). Bark also had a significant and strong quadratic correlation ($p < 0.05$) with xylan degradation. The MLR model contour plots (Fig. 3c-d in Paper III) indicate that the region with highest xylan degradation was found at bark addition ranging between 4.1 and 9.1%. Although glucan degradation could not be well described by the MLR predictive models, it could be found that the mass reduction tended to be slightly higher at 10% bark than at 0 and 20% (Fig. 4 in Paper III).

3.5 Potential use of other edible fungi for combined production of food and biofuel

Over last two decades, the global consumption of cultivated edible mushrooms was doubled and reached about 40 million tons in year of 2013 (Royse et al., 2017). Shiitake, oyster mushroom and wood ear are the top three species, contributing 22%, 19% and 18% global edible mushroom production, respectively (Royse et al., 2017). Therefore, an investigation on the potential use of oyster and wood ear mushrooms also for combined production of food and fuel is of a significant interest. To do this, the thesis studied the adaptation of these two species to novel cultivation process, namely hot-air pasteurization, and their efficiency regarding fungal pretreatment as well as enzymatic saccharification (Table 5). The hot-air pasteurization has been set as a basis for a sustainable and future production of edible mushroom and biofuels (Wei et al., 2020; Xiong et al., 2019).

Table 5. Wood ear and summer oyster mushroom fruiting bodies produced after different heat treatments, mass change of lignocellulose after the cultivation and glucan digestibility of SMS (Paper IV and V). Data refer to mean ± SE.

Mushroom Species	Pasteurisation Methods	Mushroom yields g/kg substrate	Degradation (%) of Lignin	Hemicellulose	Cellulose	Glucan digestibility of SMS (%)
Wood ear[1]	121 °C	190.2±18	75.3±0.5	80.3±1.4	70±2.5	45.9±2.8
	100 °C	139.2±20.5	78.8±0.7	88.2±1.3	68.7±3.1	NA
	85 °C	217.1±46.7	73.2±3.5	83.4±3.0	74.5±2.2	46±3.2
	75 °C	171.4±14.5	79.7±0.2	91±0.9	76.8±0.9	NA
Summer oyster[2]	121 °C	140±27.7	39.3±5.2	41.6±3.3	36.6±3.8	NA
	65 °C	174±40.4	39.9±5.4	45.2±2.8	37.2±2.6	32.5±3.6

1, cultivated on birch based substrate; 2, cultivated on alder based substrate; NA, data not available.

Substrate pasteurization is a key process to remove/deactivate competitive microorganisms before inoculation of fungal spawn. As shown in Table 5, this study demonstrated that hot-air pasteurization even at temperatures as low as 75 °C and 65 °C, is as effective as conventional autoclaving at 121 °C in allowing wood ear mushroom and summer oyster mycelium, respectively, to complete the whole biological cycle (Paper IV and V). Regardless pasteurization regimes, comparable production of fruiting bodies was achieved for each studied mushroom, and the impact of pasteurization methods on the lignocellulose degradation and enzymatic saccharification of the generated SMS was minor. The effectivity of hot air pasteurization were in agreement with a previous study on shiitake mushroom that served as background to this thesis (Xiong et al., 2019). Compared to conventional autoclaving method, hot air pasteurization at relatively lower temperature might allow the survival of certain microbial species, but if that happened, the hypothetical survivors would not cause severe competition with the growing fungi. The fact that both autoclaving and hot-air pasteurization resulted in comparable biological cycle makes it possible to save up to 60% of the energy (2240 vs 814 kJ/kg dry substrate) required for mushroom cultivation today (Wei et al., 2020).

Overall, compared with wood ear and summer oyster mushroom, shiitake has a much higher selective degradation ability on lignin and hemicelluloses fraction rather than cellulose. Wood ear mushroom cultivation resulted in degradation of lignin (73%) and hemicelluloses (83%) of birch substrate, but it caused 75% cellulose losses; for summer oyster mushroom, there were 40, 45 and 37% mass reduction on lignin, hemicellulose and cellulose, respectively, in alder substrate. As indicated by the results of the analytical enzymatic saccharification (Paper IV and Paper V), between 84 g and 153 g glucose was yielded per kg of dry SMS, which corresponds to cellulose conversion ratio ranging from only 30 to 46% for wood ear and summer oyster. Thus, before applying these two species for the food and bioethanol concept, further studies are needed to investigate the factors that may regulate the degradation of lignocellulose and the possibilities to maximize lignin degradation with minimal cellulose consumption while obtaining acceptable quantity mushroom fruit bodies with good quality.

3.6 Utilization of side streams

Although a large part of the shiitake SMS entering the enzymatic saccharification was solubilized during the process, between 49 and 57% of the initial weight of shitake-based SMS remained as a solid residue (Table 4). This part is the largest solid side stream along entirely chain from the initial shiitake mushroom substrate to the end-product, cellulosic ethanol.

The large amount of solid residues was not exclusive for shiitake. Enzymatic saccharification of SMS from wood ear mushroom cultivation generated an even larger amount of solid leftover than that resulting from shiitake SMS (Table 2 in Paper IV vs. Table 4). Such a remarkable amount of side stream requires being utilized in an economic way in order to moving closer to a circular economy concept. A way of dealing with such a large residue might be good to use it as solid biofuel, since it contains rather much lignin (or fragments) with high calorific value. In order to elucidate the potential of such a use,

fuel characteristics of the leftovers were assessed. A good example comes from the wood ear mushroom cultivation (Paper IV).

Due to the degradation of lignocellulose caused by the fungal growth, the SMS had lower C content and higher contents of ash-forming elements, such as Ca, K, P, Si, and Mg, (Table 3 in Paper IV). For all substrates, the relatively high fraction of Ca could be explained by the addition of $CaCO_3$ that was used to adjust the pH of the substrate. The most notable difference between the SMS and the solid leftover from enzymatic saccharification was a considerable increase in Na content, in contrast to the decreases in contents of most ash-forming elements. The high Na content of the leftover from enzymatic saccharification was due to the sodium citrate buffer that was used during the process. The calorific value of the substrates decreased slightly during mushroom cultivation and enzymatic saccharification, but the ash-free net calorific values were comparable (18.0–18.5 MJ kg^{-1}).

It can be seen that Na, Ca, Si, and K accounted for approximately 90% of mass of the ash resulting from calcination of the solid leftover. On the other hand, the ash resulting from calcination of the raw substrate and SMS were dominated by Ca, P, and K (Fig. 3 in Paper IV). An attempt to predict the ash behavior of the studied samples during combustion was further carried out by plotting the elemental compositions of the produced ashes determined by SEM-EDS analysis. The elemental compositions of each ash were normalized to the contents of Na, K, Ca, Mg, Si, and P, recalculated to the oxide form, and then plotted in the ternary phase diagram for the $(Na_2O + K_2O)-(CaO + MgO)-SiO_2/P_2O_5$ system proposed by (Böstrom et al., 2012) (Fig. 11). Liquidus isotherms indicate compositional areas where strong slagging tendencies can be anticipated, as well as the directions where these trends are declining. As can be seen, the positions of the SMSs were similar to the raw substrate. The high relative amounts of (CaO + MgO) of the two is a common feature that determines ash behavior. The upper part of the triangle suggests that SMSs have high ash-fusion temperatures, which means a low slagging tendency during combustion. The positions of the ash from the solid leftovers were grouped together toward to the $(Na_2O + K_2O)$ corner of the ternary diagrams. They were located at the low-risk zone of slagging. Theoretically, as the concentration of Na increases, the ash melting temperature may decrease. Ash fusion analysis indicated that a decrease in shrinkage temperature from 1460–1490 °C of SMS to 755–785 °C of leftover did occur (Table 4 in Paper IV). However, the liquid temperatures of the ash from these leftovers were still above 1500 °C, probably due to the $CaCO_3$ addition from the mushroom cultivation. This indicates a low tendency for slag formation at combustion temperatures in fixed- and fluidized bed technologies. It should be noticed that, due to the introduce of sodium citrate buffer during enzymatic hydrolysis, the leftover ash had a high molar ratio of (K + Na + Ca + Mg)/(P + Si) (6.2–7.7) (Table 3 in Paper IV). According to a previous study (Díaz-Ramírez et al., 2012), ratios above 3 indicate that the risk of fly ash particle emissions during combustion should be considered. But the formation of fine particulate emissions can be easily controlled using existing technologies, for instance, the soot blowing techniques have been widely used to reduce the deposition.

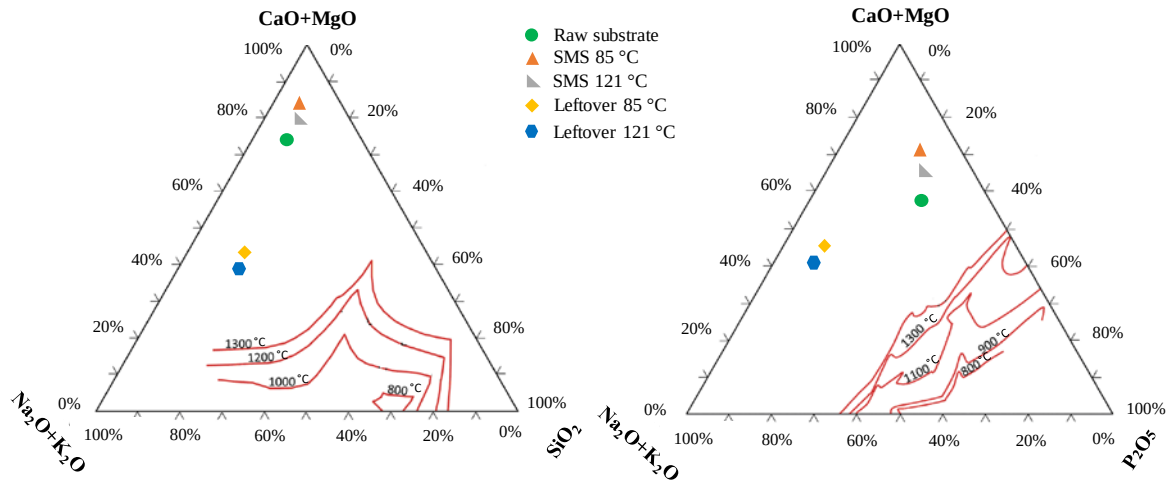

Figure 11. Compositional triangle for the system ($Na_2O + K_2O$)−(CaO + MgO)−SiO_2/P_2O_5. Contents of the three axes are given in weight fractions and normalized to 100%. Liquidus isotherms for the ternary phase diagram are adopted from (Böstrom et al., 2012). (Paper IV).

4 Conclusion

- Shiitake was shown to be a promising model species to develop the food-cellulosic ethanol concept. Shiitake cultivation on substrates based on three different hardwood species resulted in selective degradation of lignin and hemicelluloses with good preservation of cellulose, whose susceptibility to enzymatic saccharification was considerably enhanced as result of the fungal action on the substrate.
- Increased nitrogen supplementation of shiitake substrate inhibited the colonization speed and increased the production of fruiting bodies, while low supplementation enhanced selective delignification.
- Delignification by shiitake was correlated with degradation of syringyl units of lignin, and it did not cause statistically significant changes in the substrate crystallinity.
- Phenolic compounds and acetic acid were detected in SMS hydrolysates, but their concentrations were not high enough as to cause a strong inhibition of the fermentation with *S. cerevisiae*. The high nitrogen content of SMS hydrolysates made it possible their fermentation without nutrient supplementation.
- The solid leftover resulting from SMS saccharification revealed promising features for being used as solid fuel for satisfying the energy requirements of the fungal pretreatment process.
- Hot-air treatment at temperatures below 85 °C and atmospheric pressure is as effective as autoclaving at 121 °C and 2 bar for ensuring substrate pasteurization for cultivation of shiitake, wood ear and summer oyster mushrooms.

5 Future study

5.1 Mushroom cultivation on softwood

As for many other white-rot fungi, softwood are difficult to colonize and is rarely used for fruiting body production. From the perspective of the northern hemisphere, where most forests and wood resources are dominantly composed of coniferous tree species such as spruce and pine, it is desirable to extend the mushroom substrate range from hardwood to softwood in industrial scale from the view of both edible mushroom and biofuel production. Our study (Paper V) showed an example that summer oyster mushroom could utilize spruce for production of food and cellulosic ethanol (but this part is not included in this thesis), but the mechanism is not known. Although the higher reactivity of S-units by lignin degrading enzyme is pointed out as the possible reason of the strong recalcitrance of the softwood for mushroom (e.g. shiitake) cultivation, assessment of enzymatic activities should be investigated in future studies.

In comparison to the structural components, extractives represent a minor fraction comprising < 10% in wood, but the compounds are more complex. It has been reported that extractives protect trees against microbial attack in the nature. The recalcitrance of softwood to shiitake mushroom cultivation might also be attributed to the presence of extractives. This opens up the possibilities of pre-heating and pre-extracting to reduce the content of extractives and their inhibition of softwood colonization and lignin degradation by shiitake.

5.2 Valorization of lignin in the solid leftover

Although the energetic potential of the solid leftover of enzymatic saccharification of SMS was shown, the combustion of biomass wastes is typically regarded as a less resource efficient application. Conversion of it to high-value products is a route to impulse the development of the circular economy. After removing extractive compounds, the lignin and polysaccharides in the remaining extractive-free solid leftover were rough 50–50 distribution (Table 4), can still further be processed by biorefinery approaches for upgrading of the components. For instance, post-pretreatment could be performed to use the carbohydrates still present in the hydrolysis leftover. Lignin then be extracted for production of carbon fibers, engineered plastics, polymeric foams, or commodity chemicals, which will enhance overall biorefinery competitiveness (Ragauskas et al., 2014). Assessment of the technical feasibility and systems analysis studies on those expectations have to be carried out in future studies.

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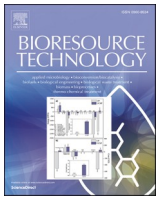

Shiitake cultivation as biological preprocessing of lignocellulosic feedstocks – Substrate changes in crystallinity, syringyl/guaiacyl lignin and degradation-derived by-products

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HIGHLIGHTS

- Three wood species combined with whey additions were designed for shiitake substrate.
- Whey affected more fungal production and lignocellulose degradation than wood species.
- Low nitrogen maximise delignification but minimise glucan degradation.
- Delignification was correlated with reduction of syringyl-to-guaiacyl ratio.
- Slight changes in substrate crystallinity but phenolics increased up to seven-fold.

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ABSTRACT

Formulation of substrates based on three hardwood species combined with modulation of nitrogen content by whey addition (0–2%) was investigated in an experiment designed in D-optimal model for their effects on biological preproceesing of lignocellulosic feedstock by shiitake mushroom (*Lentinula edodes*) cultivation. Nitrogen loading was shown a more significant role than wood species for both mushroom production and lignocellulose degradation. The fastest mycelial colonisation occurred with no nitrogen supplementation, but the highest mushroom yields were achieved when 1% whey was added. Low nitrogen content resulted in increased delignification and minimal glucan consumption. Delignification was correlated with degradation of syringyl lignin unit, as indicated by a significant reduction (41.5%) of the syringyl-to-guaiacyl ratio after cultivation. No significant changes in substrate crystallinity were observed. The formation of furan aldehydes and aliphatic acids was negligible during the pasteurisation and fungal cultivation, while the content of soluble phenolics increased up to seven-fold.

1. Introduction

Forest residues, wood-processing by-products and other lignocellulosic materials are promising renewable resources for bioconversion to advanced biofuels and platform chemicals. That would allow reducing environmental problems caused by the use of fossil resources and supporting bioeconomy development. Enzymatic saccharification of cellulose is crucial in lignocellulose bioconversion, since it produces sugars that can then be converted to bio-based products. However, due to feedstock recalcitrance, enzymatic saccharification of raw lignocellulose results in low rates and yield (Shirkavand et al., 2016; Wang et al., 2018). Pretreatment is required to reduce biomass recalcitrance and to facilitate enzymatic saccharification (Jönsson and Martín, 2016; Zhu and Pan, 2010). By removal lignin or hemicelluloses, or other actions on biomass chemistry and structure, pretreatment reduces feedstock recalcitrance (Zhao et al., 2012).

Many pretreatment approaches have been investigated (Galbe and Wallberg, 2019), and the research on different materials has shown that the effectiveness of a given method is feedstock-dependent (Martín, 2021). Biological pretreatment, consisting in lignin removal by lignin-

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degrading microorganisms, has attracted researchers' interest because it has low input of energy and chemical use (Wan and Li, 2012). Drawbacks of conventional biological pretreatment are its slow rate and high rate of cellulose consumption. Recent studies, showing the possibility of combining biological pretreatment with production of edible fungi bring back the attention to this method (Xiong et al., 2019).

The example of shiitake (*Lentinula edodes*), a white-rot fungus, which is the most cultivated edible mushroom in the world (Royse et al., 2017), is rather explicit. After growing shiitake on wood, the spent mushroom substrate (SMS) is delignified to a large extent (Wei et al., 2020) as a result of the fungal activity during cultivation. Due to its low lignin content, SMS is less recalcitrant to a bioconversion to fermentable sugar for ethanol production than the initial substrate. Such a concept of integrated production of bioenergy (ethanol) and food (edible mushroom) suits the bio-based circular economy concept. In order to develop this concept towards industrial implementation, basic understanding on the fundamental mechanisms behind the reduction of the recalcitrance is necessary.

Biomass properties vary considerably between species (Kan et al., 2016), which may result in different degrees of fungal degradation. Lignin, in close association with polysaccharides in lignin-carbohydrate complexes, and its composition and structure play a key role in blocking enzyme access to cellulose (Jönsson and Martín, 2016; Shirkavand et al., 2016). Syringyl (S) and guaiacyl (G), are the main units of lignin macromolecule, and their relative fractions varied with tree species (Wang et al., 2018). A low S:G ratio is believed to cause higher non-productive adsorption of cellulases to lignin, and thus a poorer enzymatic hydrolysis, than a high S:G ratio (Guo et al., 2014). Thus, whether biological pretreatment of woody biomass using shiitake affects lignin S:G ratio, and how it does for different tree species is an important question to be explored. Wang et al. (2018) reported that sulfuric-acid-pretreatment decreased the S:G ratio of lignin, and that was more remarkable for aspen and birch than for spruce. Santos et al. (2012), on the other hand, found that kraft pretreatment removed relatively more G-lignin in several hardwood materials, including *Alnus rubra*, *Eucalyptus urograndis*, *Quercus rubra* and *Acacia mangium*. However, to the best of our knowledge, no experimental research on changes of lignin S:G ratio following wood treatment with shiitake has been reported.

A drawback of several conventional thermochemical pretreatments is the occurrence of side reactions resulting in formation of by-products, such as furan aldehydes, aliphatic acids and phenolic compounds, which are potential inhibitors of cellulolytic enzymes and fermenting organisms (Jönsson and Martín, 2016; Kim et al., 2013). Since biological pretreatment is performed under mild conditions, the formation of most inhibitors that are typical of thermochemical pretreatments might not be expected. Anyway, formation of phenolics and acetic acid might result from the fungal degradation of lignin and hemicelluloses, respectively. Furthermore, as heating is used for pasteurisation, which is essential for substrate preparation, the formation of inhibitors might be possible. Another concern is how different the formation of inhibitors could be for substrates based on diverse tree species.

Wood cellulose has a high degree of crystallinity (Fengel and Wegener, 1989), which is a factor contributing to substrate recalcitrance, and often associated with low reactivity to enzymatic saccharification (Shirkavand et al., 2016; Zhu et al., 2008). Crystallinity changes, attributed to the selective degradation of lignin and hemicelluloses, have been reported after biological pretreatment of lignocellulose. In a comparison of 12 studies on pretreatment with white-rot fungi, the crystallinity incresed in six reports, decreased in three, while in other three almost no change was observed (Shirkavand et al., 2016). Since those reports covered non-edible fungal species grown on and different substrates, it is difficult to conclude if the divergence was caused by differences in fungal species or substrate difference.

Fungal pretreatment is performed by lignin-degrading enzymes secreted by the fungi that colonize lignocellulose. Depending on cultivation conditions, including the nutritional status, fungi can secrete different types and quantities of enzymes and thus results in different outcomes in terms of lignocellulose degradation. Nitrogen, which is often added as nutritional supplement, is important not only for production of edible mushroom bodies (Koutrotsios et al., 2014), but also for the lignocellulose degradation. In previous studies (Chen et al., 2020a; Xiong et al., 2019), birch-based substrates with as low as 0.5–0.6% DM of nitrogen content led to a significantly higher lignin degradation (60–70% of initial mass) and glucan recovery (~70%) than that achieved with a higher nitrogen content (≥0.8%). Nitrogen-limited conditions enhanced lignin-degrading enzymes, and that resulted in more delignification. However, which of S-type or G-type lignin would be more degraded by white-rot fungi, and how this is affected by interaction between substrate species and nitrogen loadings is a question that remains to be investigated. It is also interesting to explore how initial substrate composition and different nitrogen contents would affect the consequent change in substrate crystallinity and formation of degradation by-products by edible white-rot fungi.

In this work, an experiment designed in D-optimal model with combined formula and quantitative factors was performed. Three hardwood substrate species were treated as formula factor and three levels of whey addition as quantitative factor. The parameters (yield and cultivation duration) of shiitake mushroom production and substrate properties (composition of carbohydrates, lignin, substrate crystallinity and lignocellulose-derived by-products) were response variables. The effects of the factors on the response variables were revealed using partial least squares regression (PLS) models, which was expected to be applied for an optimisation of fungal treatment.

2. Materials and methods

2.1. Shiitake cultivation

2.1.1. Substrate materials

Small trees of white birch (*Betula pubescens* Ehrh.), alder (*Alnus incana* (L.) Moench) and aspen (*Populus tremula* L.) with diameter of 4–12 cm at 1.3 m height removed during thinning of a natural forest in Vännäs, Sweden, were used in the experiment. The stems without branches and top materials were debarked, chipped, dried and finally ground. Wheat (*Triticum aestivum* L.) bran was purchased from the Swedish agricultural cooperative (Lantmännen, Sweden). Whey powder (Whey-100, SHNG AB, Sweden), a by-product of the manufacture of cheese, was used as nitrogen additive. Some features of the substrate ingredients are provided in Table 1.

2.1.2. Experimental design

An experiment composed of 17 runs ("treatments") was designed in D-optimal model with combined formula and quantitative factors using the software MODDE 11.0 (Umetrics AB, Sweden) (Table 2). Firstly, a formulation factor 'sawdust species' referring to the mass fraction of birch, alder and aspen sawdust was created. Secondly, additions of 0, 1 and 2% (w/w) whey as quantitative factor were used to adjust the C/N ratio in the substrates. The whey, being a by-product of cheese industry, was proved as effective nitrogen sources for shiitake growth in the previous study (Chen et al., 2020a). The design incorporated five replicated center points using blends containing equal proportions of all

Table 1
Substrate ingredients and chemical composition.

Parameters	pH	Ash %DM	C %DM	H %DM	N %DM	S:G	Particle size mm
Birch	5.0	0.28	49.8	6.1	0.11	3.8	1.4–2.8
Alder	4.9	0.46	50.2	6.2	0.25	2.4	1.4–2.8
Aspen	4.8	0.42	49.5	6.1	0.11	2.4	1.4–2.8
Wheat bran	5.9	5.71	46.5	6.1	2.6	0.5	≤ 3
Whey	6.3	–	50	–	13.2	–	≤ 0.2

DW, dry weight; S:G, syringyl-to-guaiacyl ratio in lignin.

Table 2
Experimental design and fractions of ingredients in initial substrate.

Treatments	Substrate ingredients % DM			Sawdust species × 100% DM			Nitrogen %
	Wheat bran	Sawdust	Whey	Birch	Alder	Aspen	
N1	20	80	0	1	0	0	0.53
N2	20	80	0	0	1	0	0.64
N3	20	80	0	0	0	1	0.50
N4	20	80	0	0	0.5	0.5	0.64
N5	20	80	0	0.5	0	0.5	0.55
N6	20	80	0	0.5	0.5	0	0.58
N7	19.6	78.4	2	1	0	0	0.91
N8	19.6	78.4	2	0	1	0	1.07
N9	19.6	78.4	2	0	0	1	0.68
N10	19.6	78.4	2	0	0.5	0.5	0.84
N11	19.6	78.4	2	0.5	0	0.5	0.80
N12	19.6	78.4	2	0.5	0.5	0	0.88
N13	19.8	79.2	1	0.333	0.333	0.333	0.72
N14	19.8	79.2	1	0.333	0.333	0.333	0.68
N15	19.8	79.2	1	0.333	0.333	0.333	0.70
N16	19.8	79.2	1	0.333	0.333	0.333	0.71
N17	19.8	79.2	1	0.333	0.333	0.333	0.74

three tree species, with 1% (w/w) whey.

The substrates were prepared by mixing all ingredients, and then adding water up to a moisture content of 65% (wet based). The pH of the substrates was adjusted to approximately 6.3–6.5 by adding 1% (w/w) $CaCO_3$ of substrate DM. Four replicates were used for each run.

2.1.3. Mushroom cultivation and sampling

Substrate pasteurisation (85 °C for 4 h), incubation and mushroom harvest (first flush only), as well as fresh mushroom yield determination, was performed as previously described (Chen et al., 2020a). When the entire block was fully covered with white mycelia, judged by a visual observation, the colonisation period was considered complete. Samples for chemical analyses were collected from (1) initial substrates right after pasteurisation, and (2) SMSs (all material remaining in each container) right after the harvest of fruit bodies. Initial substrates and SMSs were dried at 45 °C, then milled to ≤ 0.5 mm, and stored in air-tighten plastic bags at room temperature. Before the chemical analysis, the replicated samples of each treatment were proportionally pooled (20% of every replicate by weight) into one mixed sample.

2.2. Characterisation of substrates

2.2.1. Analysis of nitrogen and pH in the substrate

Total nitrogen and total carbon contents were determined using an elemental analyzer-isotope ratio mass spectrometer (DeltaV, Thermo Fisher Scientific, Germany). A SensION PH31 pH meter was used to determine pH values.

2.2.2. Compositional analysis

Determination of extractives (water and ethanol) and structural components in the substrates was performed using NREL methods (Sluiter et al., 2008; Sluiter et al., 2005). Analytical acid hydrolysis (AAH) was applied for determination of lignin and carbohydrates. Klason lignin was determined gravimetrically as the AAH residue, and acid-soluble lignin in the hydrolysates was determined spectrophotometrically at 240 nm (Shimadzu, Kyoto, Japan). Glucose and xylose in the hydrolysates were analyzed with HPLC (High-Performance Liquid Chromatography), using an Aminex HPX-87H column and an RI detector. Elution was performed with isocratic flow of a 5 mM aqueous solution of sulfuric acid. The flow rate was 0.6 mL/min and the column temperature was set to 55 °C.

The relative mass change of each component was determined using mass balances

$$\text{Relative mass degradation}\% = \frac{1-(M_{SMS}\times C_{SMS})}{M_{INI}\times C_{INI}}\times 100$$

where M and C refer, respectively, to mass of substrate and content of component (extractives, glucan, xylan, lignin or soluble phenolics) of SMS and initial (INI) substrates, respectively (Equation. 1).

2.2.3. Pyrolysis-gas chromatography/mass spectrometry (Py-GC/MS) analysis

Py-GC/MS was used to determine the relative ratio of dimethoxylated (syringyl, S) and monomethoxylated (guaiacyl, G) lignin units in raw substrate ingredients, initial substrates and SMSs. The analysis was performed at Umeå Plant Science Centre (Umeå, Sweden) according to the method described by Gerber et al. (2012).

2.2.4. Determination of degradation-derived by-products

For determining the possible formation of degradation products, liquid media were prepared by suspending aliquots of the initial substrates and SMSs in 50 mM sodium citrate buffer (pH 5.2) at 10% solids content in the same way as used for enzymatic hydrolysis (Gandla et al., 2018). The suspensions were vortexed and then incubated for 2 h. After that, the liquid phase was separated by centrifugation, acetic acid, formic acid and levulinic acid were determined by HPAEC-PAD (Dionex ICS-5000, Sunnyvale, CA, USA) using a 4 × 50 mm AG15 guard column and a 4 × 250 mm AS15 separation column (Dionex) and a conductivity detector set at 35 °C. A 10 mM aqueous solution of sodium hydroxide at a flow rate of 1.2 mL/min was used as eluent, as previously indicated (Martín et al., 2019). Possible presence of the furan aldehydes furfural and 5-hydroxymethylfurfural (HMF) was evaluated with an HPLC system (Dionex UltiMate 300; ThermoFisher, Waltham, MA, USA) with a diode-array detector and a 3 × 50 mm, 1.8-μm Zorbax RRHT SB-C 18 column. The temperature was set to 40 °C.

For determination of soluble phenolics, boiling water extraction was performed for 3 h. Soluble phenolic compounds in the extracts were determined by Folin Ciocalteu's method (Singleton et al., 1999) using vanillin as calibration standard.

2.2.5. X-ray diffraction (XRD) analysis

To determine the crystallinity index (CrI) of cellulose in the studied samples, powder X-ray diffraction (XRD) analyses were performed using a Malvern Panalytical X'Pert^3 Powder diffractometer equipped with an Empyrean Cu LFF HR X-ray generator and a X'Celerator detector. The patterns were acquired by exposing the samples to Cu Kα1 radiation (1.54056 Å). The instrument was operated at 1.8 kW and the samples were scanned in the 2 θ range between 5 and 45°. The crystallinity index

was calculated by Kumar et al. (2009), based on the height ratio between the intensity of the crystalline peak and the total intensity after the background signal (non-crystalline) using the expression

$$\mathrm{CrI} = \frac{I_{200} - I_{non-cr.}}{I_{200}} \times 100$$

where I_{200} is the maximum intensity of the peak corresponding to the plane in the sample with the Miller indices 200 at a 2 θ between 22 and 24°; $I_{non\text{-}cr}$ is the intensity of the non-crystalline material, which is taken at an angle of aproximatelly 18° 2 θ in the valley between the peaks (Equation 2).

2.3. Statistical analysis

The influence of whey additions and tree species was studied in an experiment with a D-optimal factorial design and its response variables were evaluated by partial least squares (PLS) regression using the software MODDE 11.0 (Umetrics, Sartorius Stedim Biotech, Umea, Sweden). R^2 and Q^2 values were used to indicate the goodness of fit and predictive ability of the model, respectively; values close to 1 indicated that the model fits the data completely. Q^2 is considered good when it is above 0.5, and the difference between R^2 and Q^2 is lower than 0.2–0.3. A Q^2 value above 0.1 is considered as significant (Eriksson et al., 2008). The number of factors used in the models was determined by optimization of Q^2. The interaction terms showing no significant effect on the target response variable ($p > 0.05$) were excluded. Principal component analysis (PCA) was performed to gain an overview of the data using SIMCA 14.0 (Umetrics, Sartorius Stedim Biotech, Umea, Sweden). Bivariate correlation followed by Pearson test was conducted to analyse the correlation between data using SPSS statistical analysis software (IBM SPSS version 26.0).

3. Results and discussion

3.1. Fungal colonisation and fresh mushroom production

After some days of inoculation of shiitake mycelium, colonisation signals started to be evident in most of the experimental runs with formulated substrates of either single species ingredient or two- or three-species mixtures. The colonisation pattern was variable for different substrates, and it was sensitive to the tree species used and to the whey addition. It should be noted that one of the experimental runs (N7, birch based substrates with 2% whey, corresponding to 0.91% N) suffered from unexpected termination of the colonisation around two–three weeks after inoculation. Repeated trials (5 × 4) ended up the same results. Based on the experimental results, PLS regression models were developed. The observations of the experimental run N7 were excluded as missing data in the modelling, which was confirmed as valid in terms of the statistical program used. The PLS models revealed the effect of both wood species and whey addition on the colonisation time by shiitake mycelia (Fig. 1a-c) and on the fresh mushroom yield (Fig. 1d-f). The colonisation time exerted significant effect according to Q^2 values, and the whey addition was more influential than the tree species used as substrate. The full colonisation took 18.3–27.8 days for the different experimental runs. A significantly faster ($p < 0.001$) colonisation was observed in the substrates with lower whey additions, which is in

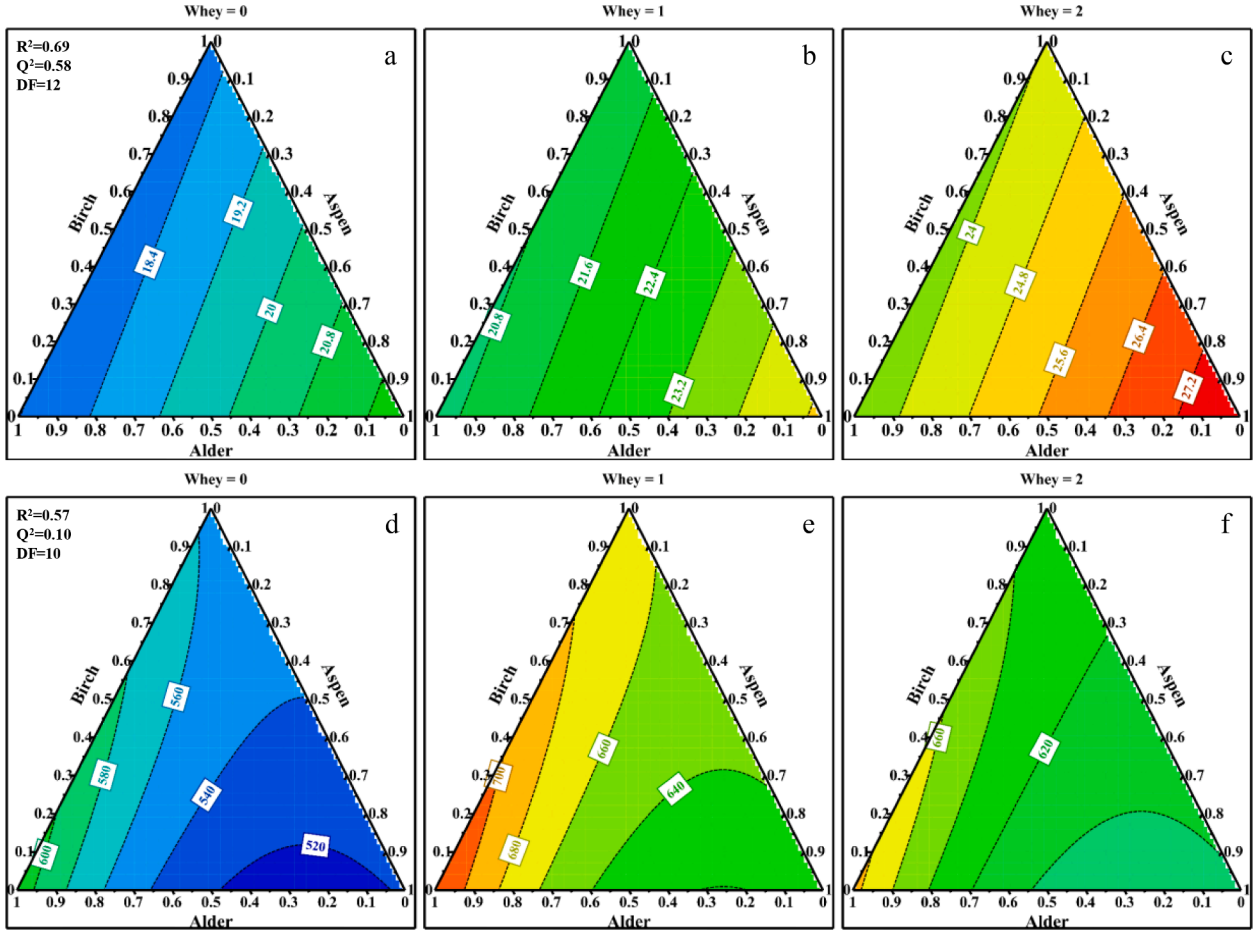

Fig. 1. Response contour plots predicted with PLS regression models for the colonisation time (a-c) and fresh mushroom yield (d-f) in relation to whey addition and tree species. The units are days for the colonisation time and g/kg dry substrate for the mushroom yield.

agreement with a previous study (Chen et al., 2020a). Low fraction of aspen was correlated with a short time of colonisation, in opposite to that of alder, while birch had a milder effect compared with alder and aspen.

Although the entire shiitake cultivation time varied from 74 to 100 days for different experimental runs, no significant effect ($p > 0.05$) of either whey addition or tree species was detected (data not shown).

The yield of the first flush of fresh fruit bodies (with moisture content of 90%) ranged between 520 and 741 g per kg of dry substrate (Fig. 1d-f). The yields were in general comparable to results of previous shiitake studies (650.8–675 g/kg hardwood) (Chen et al., 2020a; Lin et al., 2015; Xiong et al., 2019). Whey additions had a significant ($p < 0.05$) quadratic correlation with mushroom yield; a higher yield was found with 1% whey, followed by 2% and 0% whey addition (Fig. 1d-f). For all whey supplementations, high ratio of alder in the substrate can be associated with high yields of fruit bodies. On the other hand, inclusion of aspen generally resulted in low mushroom yield. However, the Q^2 value was marginally significant for the mushroom yield, and the significance was mostly due to nitrogen loading rather than substrate species.

3.2. Major changes of substrate lignocellulose

3.2.1. Lignocellulose composition

Table 3 shows the contents of major components in the initial substrates and SMSs. The major changes can be generalized as follows: after fungal pretreatment, the contents of lignin (sum of Klason lignin and acid-soluble lignin) and xylan in the SMS were on average 14.4% and 7.4%, respectively. That corresponds to a reduction of 39.6% and 42.8%, respectively, compared with the initial values. On the other hand, average glucan content increased from 31.8% in the initial substrates to 36.9% in the SMSs, which corresponds to around 16% increase.

The compositional changes are evidently associated with the whey additions but not with the tree species (Table 3). The recovered SMS without whey addition had significant ($p < 0.001$) lower lignin content than the ones with 1% and 2 % whey additions. The glucan content in SMS significantly increased with decreasing of whey addition ($p < 0.01$). For the experiments with no whey addition, the nitrogen content in SMS ranged between 0.67 and 0.76%, while for those with whey supplementation of 1 and 2% (w/w), the determined nitrogen content was 0.85% and 0.88–1.13%, respectively. These nitrogen values in the SMSs were higher than those in the initial substrates (Table 2), and they positively correlated ($p < 0.001$) with whey additions.

3.2.2. Lignocellulose mass degradation

Although the previously mentioned changes in the composition of SMSs (Table 3) with respect to the initial substrate point at apparent decreases of the contents of lignin and hemicelluloses, and at increase of glucan content, a better representation is provided by mass balances, which allow calculating the actually recovered and degraded amounts of each biomass constituent. Based on the contents of each component (Table 3), and substrate mass recovery (averaging 64.4% of the starting amount, data not shown) after mushroom cultivation, the relative mass reduction/degradation of major components were calculated (Fig. 2) using the equation 1.

The average mass degradation of lignin, xylan and glucan in the substrates after fungal treatment was 60.4, 61.3 and 26.0%, respectively, in proportion to initial values. Glucan had a much lower rate of degradation than lignin and xylan, and that is consistent with previous studies on birch treatment with shiitake (Chen et al., 2020a; Xiong et al., 2019). The relative mass reductions of lignin and glucan are well described by PLS models with high R^2 and Q^2 values (Fig. 2), which were significantly ($p < 0.05$) affected by the level of whey additions, while the

Table 3
Content of major components in initial substrates and SMSs. Syringil-to-guaiacyl (S:G) ratios of lignin, and crystallinity index are also included. The data refer to mean of duplicate experiments, and the standard errors are included.

Whey	Sawdust species	Glucan		Xylan		Lignin		S:G		CrI		Extractives		S-phenolics		Nitrogen
		Initial	SMS	Initial	SMS	Initial	SMS	Initial	SMS	Initial	SMS	Initial	SMS	Initial	SMS	SMS
%	Bir/Ald/Asp	%	%	%	%	%	%			%	%	%	%	*	*	%
0	1/0/0	28.3	37.0	14.1	8.9	24.1	12.8	2.9	1.6	70	72	10.9	31.2	0.34	2.4	0.74
	0/1/0	30.6	36.7	14.4	8.1	24.7	14.8	2.3	1.2	72.4	71	12.1	29.8	1.56	2.4	0.76
	0/0/1	37.8	39.3	12.1	5.5	22.0	12.5	2.4	1.3	72.3	72.3	9.6	28.8	0.47	2.0	0.74
	0/0.5/0.5	32.7	41.0	12.0	6.6	23.2	13.0	2.1	1.2	70.4	76.8	9.8	29.3	0.80	2.2	0.70
	0.5/0/0.5	30.2	39.7	13.8	7.7	23.1	12.3	2.6	1.4	66.6	73.6	10.6	30.8	0.33	2.2	0.67
	0.5/0.5/0	29.2	38.3	14.7	7.3	24.9	12.8	2.9	1.3	68.9	72	11.0	30.9	0.73	2.1	0.73
1	0.3/0.3/0.3	30.2 ± 0.6	33.5 ± 0.7	12.2 ± 0.2	8.0 ± 0.5	23.7 ± 0.1	15.0 ± 0.4	2.5 ± 0.1	1.5 ± 0.1	69.9 ± 1.3	70.9 ± 1.1	12.0 ± 0.3	29.0 ± 0.6	0.69 ± 0	2 ± 0.1	0.85 ± 0
2	1/0/0	na	na	na	na	na	na	2.8	na	68.3	na	na	na	0.34	na	na
	0/1/0	30.5	29.1	12.3	7.6	24.7	17.6	2.5	1.3	70.9	70.6	13.4	29.4	1.32	2.2	1.13
	0/0/1	36.6	38.9	11.2	7.7	22.5	16.3	2.0	1.6	67.5	73.5	13.0	25.8	0.40	1.9	0.88
	0/0.5/0.5	33.5	31.9	12.4	6.8	24.6	16.2	2.2	1.4	70.8	68	11.6	29.1	0.78	2.0	1.08
	0.5/0/0.5	30.9	38.0	14.2	7.3	23.5	14.6	2.5	1.7	70.9	70.9	11.7	29.6	0.30	1.9	0.89
	0.5/0.5/0	29.6	36.4	11.9	8.5	24.4	15.1	2.6	1.5	69.5	78.5	12.7	30.0	0.65	2.1	1.11
Effects of																
Whey		ns	b	ns	ns	ns	a	ns	b	ns	ns	b	ns	ns	ns	a
Birch		ns	ns	ns	ns	ns	ns	c	ns	ns	ns	ns	ns	ns	ns	ns
Alder		ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	a	ns	ns
Aspen		b	ns	ns	ns	ns	ns	c	ns	ns	ns	ns	ns	ns	ns	ns
Whey × Whey		–	c	–	–	–	–	–	–	–	–	–	–	–	–	–
Aspen × Aspen		b	–	–	–	–	–	–	–	–	–	–	–	–	–	–

Bir/Ald/Asp, Birch/Alder/Aspen; Lignin amount corresponds to the sum of the acid-insoluble (Klason) and acid-soluble fractions; CrI, Crystallinity index; S-phenolics, Soluble phenolics; * vanillin equivalent units; na, data not available; a, significant at $p < 0.001$, b, significant at $p < 0.01$, c, significant at $p < 0.05$, ns, non-significant ($p > 0.05$), -, excluded interaction terms ($p > 0.05$; details in section 2.3).

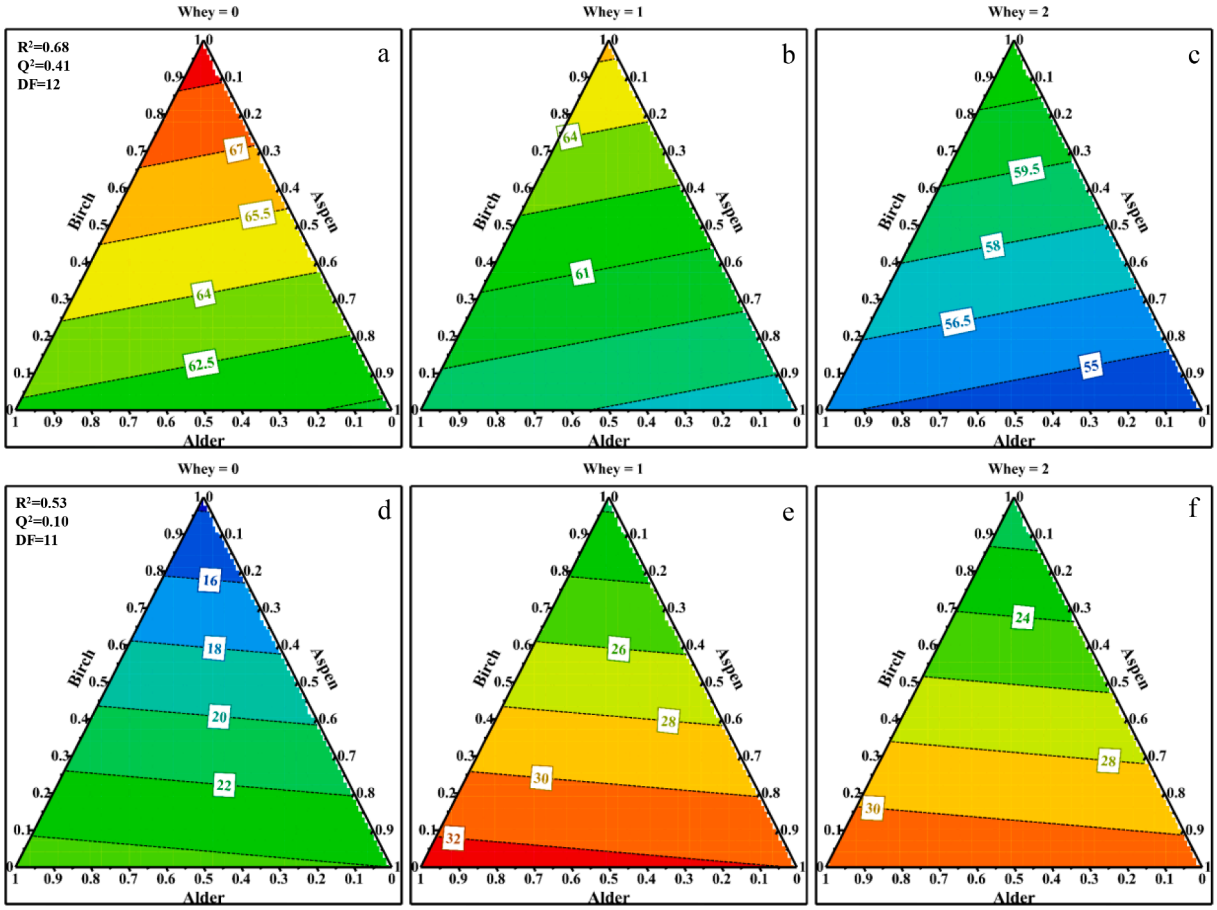

Fig. 2. Response contour plots predicted with PLS regression models for the relative mass reduction (%) of lignin (a-c) and glucan (d-f) in SMSs with respect to the initial substrates.

effect of the wood species used in the substrate was less important.

Lignin degradation was linearly and negatively correlated with whey addition ($p < 0.01$), and showed maximal value of 67.6% with no whey addition (Fig. 2a-c), where the only nitrogen source was that intrinsic of the wood substrates, which corresponded to<0.58% (w/w) (Table 2). Differently, glucan mass degradation was positively correlated with the whey addition ($p < 0.05$) (Fig. 2d-f). Low losses of glucan (16–24%) were observed for the experimental runs without whey addition, while larger losses (24–32%) were found for those with 1% and 2% whey. Although the exact reason for this could not be determined by this study, the nitrogen-rich condition might have increased the growth of competing microbes that consume carbohydrates. Whatever, the fact that maximal degradation of lignin but minimal glucan loss was associated with no addition of whey agrees with what was observed at nitrogen loading of < 0.6% in a previous study (Chen et al., 2020a). Substrate species seemed to be important also, as indicated by contour in Fig. 2: lignin was degraded slightly more in birch than in aspen, in contrast to glucan, which was degraded more in alder than in birch substrate. Compared with those of lignin and glucan, mass reduction of xylan was not significantly ($p > 0.05$; data not shown) affected by either whey addition or tree species.

The high degree of degradation of lignin and xylan, together with the good preservation of glucan, are positive features of the fungal cultivation investigated in this work as a potential pretreatment for enzymatic saccharification of cellulose. The results of the mass balances revealed that glucan recovery as high as 80% can be achieved, which is comparable with the typical range (70–90%) achieved in different lignocellulose thermochemical pretreatment approaches (Ilanidis et al., 2021a; Ilanidis et al., 2021b; Martín et al., 2019; Tang et al., 2021).

3.2.3. *Relative change of syringyl-to-guaiacyl ratio in lignin*

The substrates used for fungal cultivation in this work were based on three hardwood species, namely birch, alder and aspen. Hardwood lignin is composed of a mixture of syringyl and guaiacyl units, but their relative ratios can vary for different species. The raw birch, alder and aspen used for formulating the substrates had syringyl-to-guaiacyl (S:G) ratios of 3.8, 2.4 and 2.4 respectively (Table 1). The higher S:G ratio of birch compared to the other species is in agreement with a previous investigation reporting S:G ratios of 3.3 and 1.7, respectively for birch and aspen (Wang et al., 2018). Another major constituent of the substrates was wheat bran, a byproduct of wheat milling. Wheat bran is composed mostly of carbohydrates and a small lignin fraction. The lignin of the wheat bran used in this work contained 55.6% G-units.

As consequence of the addition of wheat bran, the S:G ratio of the initial substrates was 2.0–2.9, which is lower than the values for the raw wood (Table 3). After the shiitake cultivation, the S:G ratio decreased to 1.2–1.7 in the SMS. The reduction of S:G ratio was 41.5% on average (Fig. 3), which was much higher than the results reported after acid pretreatment of aspen (20.8%) and birch (26.2%) (Wang et al., 2018). The findings in this study were, however, in contrast with reports on alkaline pretreatment showing a general increase in S:G ratio of four hardwood species, which was attributed to a larger proportion of G-lignin been degraded (Santos et al., 2012).

As shown in Fig. 3, whey addition had a significant ($p < 0.001$) effect on the relative change (%) of S:G ratio; high addition resulted in less change of S:G ratio. The effect of the different wood species was, however, only marginally significant (Table 3). Large changes were associated with higher fraction of alder, and lower changes were associated with more aspen in the substrate, while the influence of birch was minor

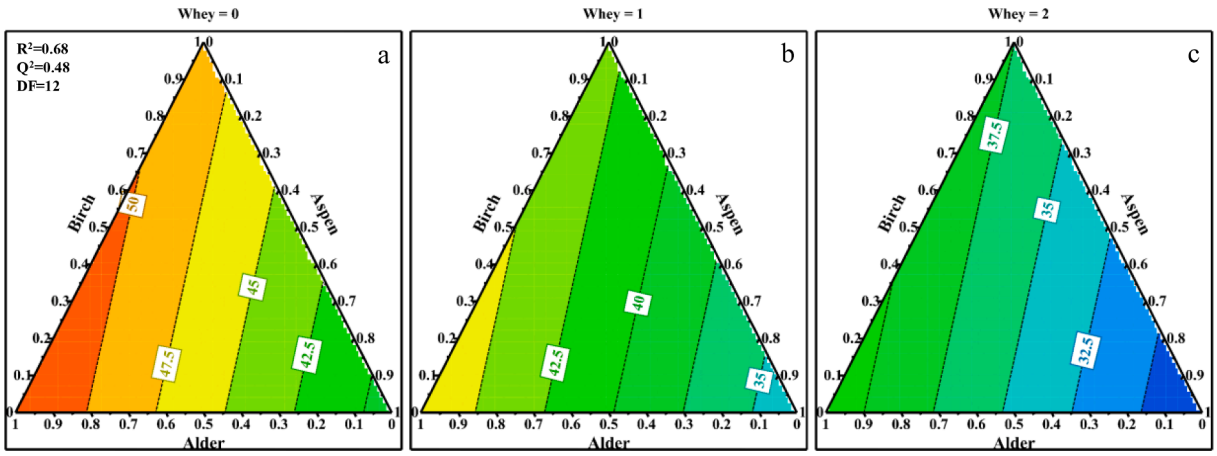

Fig. 3. Response contour plots predicted with PLS regression models for the relative change (%) of the S:G ratio of lignin in SMSs with respect to the initial substrates.

(Fig. 3).

The decrease of the S:G ratio during fungal cultivation might be attributed to a higher enzymatic reactivity of S units in the substrates of this study. It has been reported that both manganese peroxidase (MnP) and laccase, the major lignin-degrading enzymes secreted by shiitake (Janusz et al., 2013), have different preferences regarding degradation of lignin units. For example, MnP generates Mn^{3+} and is capable of oxidizing nonphenolic compounds and minor phenolic moieties of lignin, while laccase is a copper-containing oxidase that oxidizes numerous phenolic compounds (Janusz et al., 2013; Wan and Li, 2012). Wood lignins rich in S units often have low content of free phenolic groups due to their involvement in formation of methoxy groups (Camarero et al., 1999). Plausibly, the high reactivity of S lignin in this study can be attributed to more MnP activity, compared to laccase activity, involved in lignin degradation. For unknown reasons, high nitrogen loading might have caused the repression in MnP activities, and that resulted in even higher ratio of S:G in the SMS and low mass degradation of total lignin. A description of the mechanism is beyond the scope of this study, but it deserves being investigated in future studies including assessment of enzymatic activities.

3.2.4. *Formation of lignocellulose degradation by-products*

Furfural, HMF, levulinic acid and formic acid were not found in any of the samples, whereas acetic acid was not detected in the initial substrates, but it was contained in low amounts in the SMSs (Below 0.1% (w/w), data not shown). Furan aldehydes are typically formed by sugar degradation during hydrothermal treatments in acidic media, and if the temperature is high or the reaction time is long, they can get degraded further to formic acid and levulinic acid (Fengel and Wegener, 1989). The absence of furan aldehydes, levulinic acid and formic acid, indicates that, as expected, hot-air pasteurisation at 85 °C did not cause any major carbohydrate degradation. The absence of acetic acid, which is typically formed from splitting of acetyl groups during xylan hydrolysis (Jönsson and Martín, 2016), in the initial substrates indicates that no hydrolysis of hemicelluloses occurred during pasteurisation. Furan aldehydes and aliphatic acids are known inhibitors of enzymatic saccharification and microbial processes (Jönsson and Martín, 2016). The results presented in this study show that hot-air pasteurisation at 85 °C, and the following fungal cultivation are unlikely to produce these inhibitors if shiitake cultivation would be used as a fungal pretreatment of lignocellulose for bioconversion by enzymatic saccharification and microbial fermentation.

Soluble phenolic compounds were determined in water extracts of the initial substrates and SMSs. As shown in Table 3, the content of soluble phenolics in initial substrates ranged between 0.3 and 1.6% (w/w). Soluble phenolics in the initial substrates are mostly wood extractives (Chen et al., 2020c), such as lignans, isoflavones and other polyphenols. The amount and composition of the phenolics compounds of the wood extractives varies widely from species to species (Valette et al., 2017). In alder-based substrates, a significantly ($p < 0.001$) higher content of soluble phenolics, compared to the other two wood species, was found. A considerable higher content of phenolics was detected in the water extracts of spent substrates after the cultivation. The content of soluble phenolics in the SMS ranged from 1.8 to 2.4% (w/w), which corresponds to 1.4–7.2-fold increases. No significant differences regarding the content of soluble phenolics were found between SMSs resulting from experimental runs with different nitrogen loadings or different tree species. The increased content of soluble phenolics in the SMSs compared with those of initial substrates might be a consequence of formation of relatively small phenolic compounds as result of lignin degradation during fungal cultivation. However, in this work, in the experimental runs with low nitrogen loading, which led to large mass reduction of lignin, the accumulation of soluble phenolics did not seem to be affected significantly (Table 4). The lack of significant correlation between lignin degradation and soluble phenolics might be attributed to the formation of lignin-degradation products that are not water-soluble. Some phenolic compounds are regarded as more toxic compounds towards fermenting microorganisms compared to furan aldehydes and aliphatic acids (Chen et al., 2020c; Jönsson and Martín, 2016). However, since enzymatic hydrolysis is generally performed at 45–50 °C (Gandla et al., 2018), the expected concentrations of phenolics in the hydrolysates are lower than the amounts released by water extraction at boiling temperature. Such a low release of phenolics should probably result in low inhibitory effects in the fermentation.

3.2.5. *Substrate crystallinity*

As presented in Table 3, all initial substrates regardless of tree species had comparable crystallinity indices, and they ranged from 66.6 to 72.4%. The crystallinity in the substrate is mainly due to cellulose, which is a highly crystalline polymer with a compact supramolecular structure stabilized by hydrogen bonds (Fengel and Wegener, 1989). The fungal cultivation did not cause statistically significant changes in the crystallinity. This is in contrast to the proposal that the crystallinity was increased by fungal pretreatment due to a possible retention of crystalline form and more degradation of amorphous forms of cellulose (Shirkavand et al., 2016). However, this agrees with results by Vane et al. (2006), who found that loss of crystalline and non-crystalline regions of cellulose occurred in parallel during growth of shiitake on oak bark during a 101-month cultivation. No significant correlations ($p > 0.05$) were found between lignocellulose degradation and the relative change of crystallinity during fungal pretreatment (Table 4).

Table 4
Correlation analysis of the mass change of substrate major components and relative changes of crystallinity index and S:G ratio.

	Mass change of						Relative change of	
	Block mass	Extractives	Lignin	Glucan	Xylan	S-phenolics	CrI	S:G
Block mass	1	−0.18	0.73b	−0.07	0.44	−0.15	0.00	0.50c
Extractives		1	−0.72b	0.31	−0.66b	0.27	0.10	−0.45
Lignin			1	−0.47	0.64b	−0.29	−0.16	0.68b
Glucan				1	−0.05	0.40	0.44	−0.16
Xylan					1	−0.15	0.31	0.48
S-phenolics						1	0.13	0.27
CrI							1	0.13
S:G								1

b, significant at $p < 0.01$, c, significant at $p < 0.05$.

3.3. Principle components analysis (PCA) and overview of fungal pretreatment using shiitake

An overview of chemical and physical characteristics from all initial substrates and SMSs was performed by a PCA based on data matrix of 10 substrate variables and 32 observations (experimental runs, observations of N7 and NS7 were excluded as missing data). As generalized by the biplot of PC1 × PC2 in Fig. 4, which explains 87.5% of the total variations, the cultivation resulted in distinct divergence of properties from initial substrate cluster (N1-N17) to SMSs (NS1-NS17). The initial substrates revealed an obviously high content of lignin and hemicellulose (see also Table 3), while highly delignified SMS was positively correlated to glucan-rich SMS. The cluster of NS1-NS17 seemed to be more dispersed along PC2 (vertical) axis than that of N1-N17, which was clearly driven by nitrogen and glucan in opposite directions. However, there was no clear pattern that either clustering or dispersion was associated with the tree species. All these are consistent to the direct results from previous sections that nitrogen is more important than the options of studied wood species when the effect of fungal pretreatment is concerned.

In this study, cultivation of shiitake mushroom on hardwood was demonstrated to be a successful model for food production with potential of using the spent substrate for bioconversion to cellulosic ethanol. The achieved yield of fresh fruit bodies (Fig. 1d-f) ensures a good market value. During the cultivation, >60% (w/w) of initial lignin and xylan was degraded (Fig. 2), while as much as 80% of the initial glucan mass remained in the SMS. The high glucan recovery after cultivation, together with the low content of lignin and hemicelluloses of the SMS are indications of the potential of a shiitake-based biological pretreatment for facilitating enzymatic saccharification in a bioconversion process to produce ethanol or other sugar-platform products. Compared with other white-rot fungal species, such as summer oyster mushroom (*Pleurotus pulmonarius*) (Chen et al., 2020b) and wood ear mushroom (*Auricularia auricular-judae*) (Chen et al., 2021), shiitake has

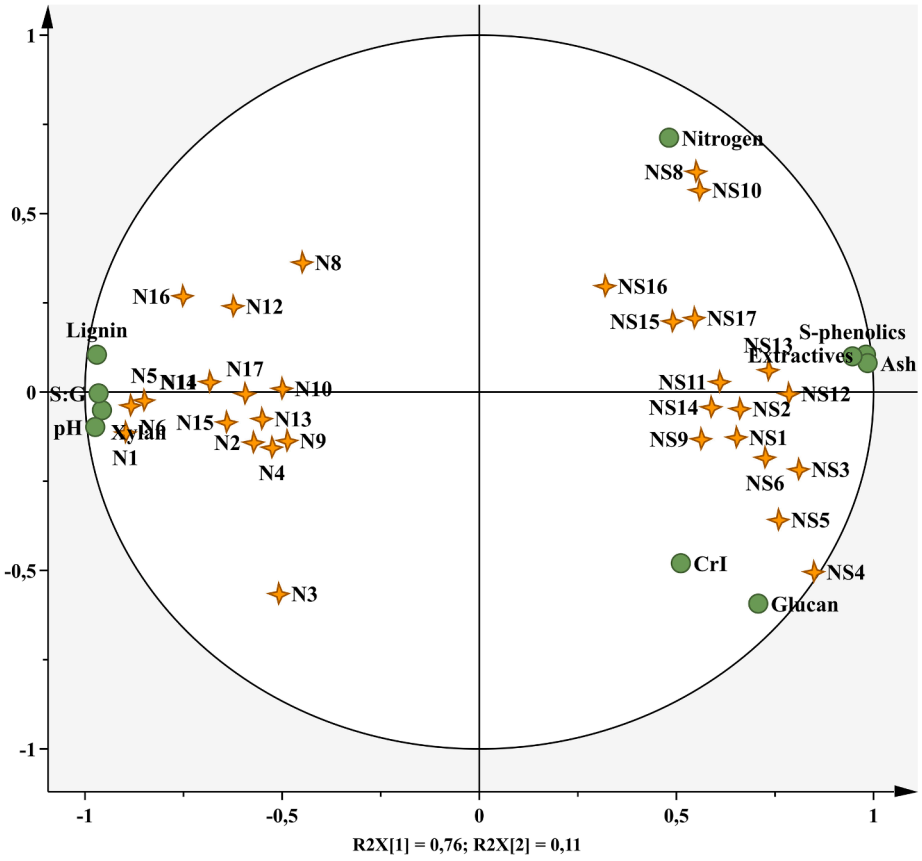

Fig. 4. PCA biplot showing major chemical components of substrates before and after shiitake mushroom cultivation. PCA was based on 32 × 10 matrix of wet chemical data.

a higher ability to selectively degrade lignin and hemicelluloses. It is worth mentioning that in a previous study (Chen et al., 2020a), it was found that glucan losses could be minimized by lowering nitrogen loading in birch-based substrates. In this study, where alder and aspen were included, that finding is proven to be true also for other hardwood species. Furthermore, the negligible formation of inhibitors found in this study reveals an advantage of shiitake cultivation as potential biological pretreatment for lignocellulose bioconversion.

Anyway, a deeper understanding on the mechanisms of lignocellulose pretratment using shiitake is still required. Among other issues, the different susceptibility of S- and G-lignin to degradation and the kinetics of lignin-degrading enzymes remain to be explored. The availabilities of different forms of nitrogen (NH_4^+, NO_3^-, NO_2^-) in the substrate during the fungal cultivation and their effects on the pretreatment should also be investigated. A comprehensive characterisation of the fraction of extractives, which represents as much as 30% of SMS dry mass, including the screening of its composition and the elucidation of the utilisation potential of its components, is required. Although fungal pretreatment resulted in an efficient removal of lignin and hemicelluloses from wood, which is clearly positive for enzymatic hydrolysis, other changes, such as an increase of the share of G-lignin and formation of soluble phenolics were also detected. Elucidating whether and to what extent those phenomena affect the enzymatic hydrolysis are relevant questions to be answered.

4. Conclusion

Shitake cultivation on birch-, alder- and aspen-based substrates resulted in selective degradation of lignin and hemicelluloses, together with good preservation of cellulose. Nitrogen supplementation played a significant regulatory role, and the low levels of nitrogen resulted in fast mycelial colonisation, increased delignification and relatively low glucan consumption. No statistically significant changes in substrate crystallinity were caused by shiitake cultivation. Some formation of phenolic compounds was detected independently on the substrate composition. Favourable features of shiitake cultivation as potential pretreatment for bioconversion of lignocellulose were identified.

CRediT authorship contribution statement

Feng Chen: Investigation, Writing – original draft, Writing – review & editing. **Carlos Martín:** Writing – original draft, Writing – review & editing. **Torbjörn A. Lestander:** Investigation. **Alejandro Grimm:** Investigation. **Shaojun Xiong:** Conceptualization, Project administration, Funding acquisition, Data curation, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Spent mushroom substrates for ethanol production – Effect of chemical and structural factors on enzymatic saccharification and ethanolic fermentation of *Lentinula edodes*-pretreated hardwood

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HIGHLIGHTS

- Spent shiitake mushroom substrate (SMS) was studied for potential ethanol production.
- Enzymatic saccharification resulted in 80–90% glucan digestibility in SMS.
- The relative ratio of guaiacyl units and SMS crystallinity did not affect hydrolysis.
- The high nitrogen content in SMS hydrolysates ensured efficient fermentation by yeast.
- Phenolics and acetic acid in the hydrolysates had only a minor inhibitory effect.

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ABSTRACT

Spent mushroom substrates (SMS) from cultivation of shiitake (*Lentinula edodes*) on three hardwood species were investigated regarding their potential for cellulose saccharification and for ethanolic fermentation of the produced hydrolysates. High glucan digestibility was achieved during enzymatic saccharification of the SMSs, which was related to the low mass fractions of lignin and xylan, and it was neither affected by the relative content of lignin guaiacyl units nor the substrate crystallinity. The high nitrogen content in SMS hydrolysates, which was a consequence of the fungal pretreatment, was positive for the fermentation, and it ensured ethanol yields corresponding to 84–87% of the theoretical value in fermentations without nutrient supplementation. Phenolic compounds and acetic acid were detected in the SMS hydrolysates, but due to their low concentrations, the inhibitory effect was limited. The solid leftovers resulting from SMS hydrolysis and the fermentation residues were quantified and characterized for further valorisation.

1. Introduction

With the increasing concerns on environmental issues, cellulosic ethanol, which is produced by enzymatic saccharification and fermentation of lignocellulosic biomass, has received considerable attention as an alternative to fossil-based transportation fuels. Since lignocellulose is recalcitrant to the action of enzymes, pretreatment is needed for improving susceptibility to enzymatic saccharification of cellulose (Jönsson and Martín, 2016; Shirkavand et al., 2016). Pretreatment effect is related to removal of lignin and hemicelluloses, among other factors, and high temperature and expensive materials are typically required (Jönsson and Martín, 2016). To be relevant, pretreatment methods should be environmentally friendly, resource efficient and cost effective. Biological pretreatment with lignin-degrading fungi is a low-cost alternative, but its slow rate and the preferential consumption of carbohydrates of many fungi have so far limited its viability as a stand-alone option (Shirkavand et al., 2016; Wan and Li, 2012). Using edible fungi can open new perspectives for biological pretreatment of lignocellulose. During the last 15 years, the global consumption of edible mushrooms is doubled and has reached near 40 million tons per year (Royse et al., 2017). The rapid growth of the mushroom market results in generation of large quantities of spent mushroom substrate (SMS). For example,

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production of shiitake (*Lentinula edodes*), the most cultivated edible mushroom (Royse et al., 2017), results in up to 12.5 million tons SMS dry mass worldwide per year (Wei et al., 2020).

Recent studies have shown that the SMS resulting from cultivation of shiitake on birch is a potential feedstock for production of fermentable sugars through enzymatic saccharification (Chen et al., 2020; Xiong et al., 2019). The SMS resulting from shiitake cultivation is delignified to a high degree, which implies a potential reduction of the substrate recalcitrance towards the action of cellulolytic enzymes. In a recent study in parallel with this paper (Chen et al., 2022), a considerable degradation of lignin and hemicelluloses, up to 67.6% and 61.3% respectively, together with a preservation of around 80% of the initial cellulose, were observed in SMSs, where shiitake grew in different combinations of three hardwood species, namely white birch (*Betula pubescens* Ehrh.), alder (*Alnus incana* (L.) Moench), and aspen (*Populus tremula* L.). Although previous studies have shown that shiitake cultivation can be an effective biological pretreatment for facilitating enzymatic saccharification of cellulose (Chen et al., 2020; Xiong et al., 2019), the issue regarding the suitability of different SMSs for downstream biochemical processing to ethanol have not been well investigated (but see Asada et al., 2011 for oak based SMS; Hiyama et al., 2016 for salix based SMS). Investigating the enzymatic saccharification of the SMSs from the above mentioned cultivations, and the ethanolic fermentation of the resulting hydrolysates is important for assessing the potential of using shiitake cultivation as a pretreatment for production of cellulosic ethanol.

An issue to be understood is how SMS chemical and structural differences, either caused by the fungal growth or intrinsic to the nature of the used wood species, influence the enzymatic saccharification. For example, the share of guaiacyl (G) lignin units and the substrate crystallinity in SMS differed from those of the initial substrates due to removal of lignin and xylan during fungal pretreatment (Chen et al., 2022). Changes of lignin content, composition and structure, and crystallinity have often been associated with the improvement of susceptibility to enzymatic saccharification observed after many pretreatment methods. For example, Santos et al. (2012) reported that kraft pretreatment increased the S/G ratio of lignin in wood chips, which increased enzyme adsorption and resulted in higher enzymatic hydrolysis efficiency. While the changes of S/G ratio and cellulose crystallinity did not affect the hydrolysis of sulfuric-acid pretreated birch, spruce and aspen (Wang et al., 2018a). It would be of interest to know whether the fungal-pretreated materials behave differently.

In fermentation of cellulosic hydrolysates, nutrient supplementation is required for ensuring the metabolic activity of the ethanologenic microorganism, usually baker's yeast (*Saccharomyces cerevisiae*), and avoiding sluggish fermentations. Nitrogen is required, and urea, ammonium sulphate and peptone are some of the sources typically used as nitrogen-rich nutrient supplements (Liu et al., 2014; Martín et al., 2002). The situation might be different if SMS hydrolysates are used, since they can already contain nitrogen due to nutrient supplements, such as wheat bran and straw, which are added initially during substrate preparation. In previous study, it was found that more than 70% of initial nitrogen remained in the SMS after shiitake mushroom harvesting (Chen et al., 2022). Nitrogen is used in fungal metabolic reactions and it is likely incorporated in the mycelia remaining in the SMS (Koutrotsios et al., 2014; Parchami et al., 2021). The SMS nitrogen might be released to the liquid phase during hydrolysis, and serve as nutrient in the fermentation of the hydrolysate. That would minimize external nutrient supplementation, thus resulting in decreasing the fermentation cost and environmental impact. However, differently from nitrogen contained in the nutrient supplements, which is effectively solubilised in fermentation media, the solubilisation of SMS nitrogen and the forms of solubilised nitrogen, as well as its use during fermentation process is still an open question worth being explored.

The potential inhibition of cellulolytic enzymes and fermenting microorganisms by lignocellulose degradation by-products is another issue of interest. By-products, such as furan aldehydes, formic acid and levulinic acid, exerting toxic effects on ethanol fermentation have been investigated for hydrothermal pretreatment methods (Bolado-Rodríguez et al., 2016; Martín et al., 2018), but they are not relevant for biological pretreatment (Chen et al., 2022). On the other hand, acetic acid and phenolic compounds can be relevant inhibitors in fungal pretreatment. Acetic acid resulting from splitting of xylan acetyl groups, and phenolic compounds formed as a consequence of lignin degradation, and both of them are known to be inhibitory (Jönsson and Martín, 2016). Previous study revealed the presence of phenolics in water extracts of spent substrates after shiitake mushroom cultivation (Chen et al., 2022). Meanwhile, even if acetic acid was not detected in the SMS extracts (Chen et al., 2022), there is concern about its formation during enzymatic saccharification due the splitting of acetyl groups in the remaining hemicelluloses. Since the potential toxicity of by-products of fungal pretreatment is poorly understood, investigating whether and to what extent by-products affect bioconversion is a relevant question to be answered, especially considering that SMSs from different tree species might have divergent characteristics regarding inhibitory compounds.

In this work, the enzymatic digestibility of SMSs resulting from growing shiitake on three hardwood species (birch, alder and aspen) was assessed by small-scale analytical enzymatic saccharification trials. Additionally, preparative enzymatic hydrolysis in shake-flask experiments was performed, and the fermentability of the resulting hydrolysates with *S. cerevisiae*, both with and without nutrient supplementation, was evaluated. Furthermore, a preliminary characterization of the hydrolysis leftovers and fermentation residues was performed in order to evaluate their suitability for further valorisation using biorefinery processes.

2. Materials and methods

2.1. Materials

Six initial substrates (non-treated woody substrates) and their SMSs after shiitake cultivation were collected from a parallel research (Chen et al., 2022). Each initial substrate contained 80% hardwood sawdust (in dry mass, DM) and 20% wheat bran (*Triticum aestivum* L.). White birch, alder, and aspen, as well as their combinations (50–50%), were the sources of hardwood sawdust. Four replicates were used for each substrate treatment. Before the chemical analysis, the replicated samples of each treatment were proportionally pooled (20% of every replicate by weight) into one mixed sample (Chen et al., 2022). The characteristics of the initial substrates and SMSs were cited from Chen et al. (2022) and shown in Table 1 of this study.

2.2. Enzymatic saccharification

2.2.1. Analytical enzymatic saccharification

The susceptibility of the mushroom substrates to enzymatic hydrolysis was determined by analytical enzymatic saccharification (Gandla et al., 2018). The commercial enzyme preparation Cellic CTec2, which is a blend of cellulases, β-glucosidases and hemicellulases acquired from Sigma-Aldrich Chemie GmbH (Steinheim, Germany), was added at a load of 100 CMCase units/g biomass. Details of the protocol were described in a previous study (Xiong et al., 2019).

2.2.2. Preparative enzymatic saccharification

Preparative enzymatic saccharification of the SMSs was performed in order to produce hydrolysates to be used in the fermentation experiment. Fifty g (DM) of SMS were suspended in sodium citrate buffer (50 mM, pH 5.2) at 10% solids content in 2 L Erlenmeyer flasks. The enzyme preparation Cellic CTec2 was added at a load of 200 CMCase units/g biomass. The reaction mixtures were incubated for 72 h, at 50 °C and 130 rpm in an Infors Ecotron orbital incubator (Infors AG, Bottmingen, Switzerland). At the end of hydrolysis, the slurry was vacuum filtered for

Table 1
Characteristics of non-treated and biologically-treated (SMS) woody substrates (data are reproduced from Chen et al. (2022)).

		Tree species					
		Birch	Alder	Aspen	Alder/Aspen	Birch/Aspen	Birch/Alder
Glucan, %	Non-treated	28.3	30.6	37.8	32.7	30.2	29.2
	SMS	37.0	36.7	39.3	41.0	39.7	38.3
Xylan, %	Non-treated	14.1	14.4	12.1	12.0	13.8	14.7
	SMS	8.9	8.1	5.5	6.6	7.7	7.3
KLL, %	Non-treated	16.0	17.9	15.6	17.0	16.1	17.2
	SMS	8.4	10.6	8.5	9.1	8.3	8.8
ASL, %	Non-treated	8.1	6.8	6.4	6.2	7.0	7.7
	SMS	4.4	4.2	4.0	4.0	4.1	4.0
S/G	Non-treated	2.9	2.3	2.4	2.1	2.6	2.9
	SMS	1.6	1.2	1.3	1.2	1.4	1.3
Extractive, %	Non-treated	10.9	12.1	9.6	9.8	10.6	11.0
	SMS	31.2	29.8	28.8	29.3	30.8	30.9
Nitrogen, %	Non-treated	0.53	0.64	0.50	0.64	0.55	0.58
	SMS	0.74	0.76	0.74	0.70	0.67	0.73
CrI, %	Non-treated	70.0	72.4	72.3	70.4	66.6	68.9
	SMS	72.0	71.0	72.3	76.8	73.6	72.0

KLL, Klason lignin; ASL, acid-soluble lignin; S/G, Ratio of lignin syringyl and guaiacyl units; CrI, Crystallinity index.

separating the hydrolysate from the hydrolysis residue. The hydrolysate was centrifuged at 10 000 rpm (~12 000 g-force) for 15 min for removing small particles, adjusted to pH 5.5, and filter-sterilized through 0.22 μm sterile filtration unit under vacuum (Corning, Darmstadt, Germany). The solid leftover of enzymatic saccharification was air-dried until 90% DM content, and stored for compositional analysis.

2.3. Nitrogen solubilisation test

The solubilisation of nitrogen contained in the initial substrates and SMSs was examined. Aliquotes of 5 g (DM) of the substrates were suspended in sodium citrate buffer (50 mM, pH 5.2), at 10% solids content, for 72 h, mimicking the environment of the preparative enzymatic saccharification, but without adding enzymes. After that, the liquid phase was separated by centrifugation. Samples of the supernatants, in parallel with samples of SMS preparative enzymatic hydrolysates, were analysed by an accredited laboratory (EUROFINS, Sweden) for the contents of ammonium nitrogen (NH_4^+-N), nitrate nitrogen (NO_3^--N), nitrite nitrogen (NO_2^--N) and total nitrogen.

$$\text{Nitrogen solubilisation}\% = \frac{\text{Total nitrogen of liquid phase (g)}}{\text{Total nitrogen of substrate (g)}} \times 100$$

2.4. Fermentation of SMS hydrolysates and reference media

2.4.1. Inoculum and media

For preparing the inoculum, freeze-dried yeast (*S. cerevisiae* Ethanol Red, Fermentis Ltd., Marcq-en-Baroeul, France) was suspended in sterile deionized water at 35 °C and for 30 min. The cell concentration in each fermentation flask was 1 g/L. The fresh yeast suspension was prepared right before inoculation.

The fermentation media consisted of 92.4% (v/v) filter-sterile SMS hydrolysate, 5.6% (v/v) yeast inoculum and nutrient solution in four different doses (2%, 1%, 0.5% and 0%; Table 2). The raw nutrient solution, corresponding to total nitrogen content of 30.9 g/L, contained 150 g/L yeast extract, 75 g/L $(NH_4)_2HPO_4$, 3.75 g/L $MgSO_4{\cdot}7\ H_2O$ and 238.2 g/L $NaH_2PO_4{\cdot}H_2O$ as previously described (Ilanidis et al., 2021; Martín et al., 2018). The 2% dosage corresponded to the full nutrient supplementation reported previously in fermentation of lignocellulosic hydrolysates (Ilanidis et al., 2021; Martín et al., 2018), while the 1% and 0.5% corresponded to half and one quarter of the full nutrient charge. Two reference fermentations, one with full nutrient charge (Reference 1) and one with no nutrients (Reference 2), of a glucose medium with concentration ~ 35 g/L were also included. The pH of all the media was adjusted to 5.5.

Table 2
Overview of fermentation experimental set-up.

Medium	Fermentation media × 100% (v/v)				
	Glucose	Hydrolysate	Yeast inoculum	Nutrient solution	Water
Birch-based SMS hydrolysate	–	92.4	5.6	2.0	0
				1.0	1.0
				0.5	1.5
				0	2.0
Alder-based SMS hydrolysate	–	92.4	5.6	2.0	0
				1.0	1.0
				0.5	1.5
				0	2.0
Aspen-based SMS hydrolysate	–	92.4	5.6	2.0	0
				1.0	1.0
				0.5	1.5
	–			0	2
Reference 1	92.4	–	5.6	2	0
Reference 2	92.4	–	5.6	0	2

2.4.2. Fermentation

Filter-sterile SMS hydrolysates and reference media were mixed under aseptic conditions with the required amount of nutrient solution (Table 2) in 25-mL bottles with a working volume of 18 mL. One mL of the yeast inoculum was added, and the flasks were sealed with cotton plugs to allow the release of CO_2 formed during fermentation. The fermentation media were incubated in an Ecotron orbital incubator at 35 °C and 180 rpm under oxygen-limited conditions. Samples were taken at the beginning of the fermentation, and after 4, 8, 12 and 16 h. The fermentations were monitored by regular OD (optical density) measurements at 600 nm using a spectrophotometer (Shimadzu, Kyoto, Japan), and by glucose determination using an Accu-Chek Aviva glucometer (Roche Diagnostics, Basel, Switzerland). Ethanol and glucose concentrations were also determined by HPLC and used for calculating the ethanol volumetric productivity and yield. The volumetric productivity (g/L h) was based on grams of ethanol produced per litre of culture medium per hour during the first 8 h of the fermentation because the maximum volumetric productivity in the reference was obtained after 8 h as previously reported (Martín et al., 2002). The ethanol yield (g/100 g) was calculated as the maximum amount of ethanol formed per 100 g of initial glucose. Duplicate experiments were run for each condition.

2.5. Analytical methods

Total nitrogen was determined in solid biomass using an elemental analyzer-isotope ratio mass spectrometer (DeltaV, Thermo Fisher Scientific, Germany). The substrates crystallinity and the relative ratios of guaiacyl and syringyl units of lignin were determined by X-ray diffraction (XRD) and Pyrolysis-gas chromatography/mass spectrometry (Py-GC/MS), respectively, according to the methods described previously (Chen et al., 2022). Determination of the extractives in the substrates, as well as solid leftover after preparative enzymatic saccharification, was performed by extraction according to an NREL protocol (Sluiter et al., 2005). Extractive-free materials were air-dried and used for the analysis of structural carbohydrates and lignin by analytical acid hydrolysis combined with high performance liquid chromatography (HPLC) (Sluiter et al., 2008).

The concentrations of glucose, xylose, levulinic acid, formic acid and acetic acid in the enzymatic hydrolysates and in fermentation samples, as well as ethanol in fermentation samples, were determined by HPLC, using an Aminex HPX-87H column and an RI detector. Elution was performed with isocratic flow of a 5 mM aqueous solution of sulfuric acid. The flow rate was 0.6 mL/min and the column temperature was set to 55 °C. Total phenolics compounds were determined using Folin Ciocalteu's method (Singleton et al., 1999) using vanillin as calibration standard. Residues dry weight in the remained media after fermentation was determined by drying 1 mL aliquots of the broth at 105 °C.

2.6. Data analysis

Principal component analysis (PCA) was performed using SIMCA 14.0 (Umetrics, Sartorius Stedim Biotech, Umea, Sweden) to gain an overview of the data and multivariate relations. Each experiment treatment was carried out in duplicate (triplicates if too much variation) and mean values and standard error (SE) are reported. One-way ANOVA followed by Post Hoc Multiple Comparisons (Tukey) was conducted using SPSS software (IBM SPSS version 26.0) to analyse statistical significance of differences of examined variable between samples.

3. Results and discussion

3.1. Characterisation of substrates

3.1.1. Characteristics of the SMSs

Glucan, with a content ranging between 36.7 and 41% (w/w), was the main component of the SMSs, followed by lignin (12.4–14.8%), which was calculated as the sum of the Klason and acid-soluble fractions, and xylan (5.5–8.9%) (Table 1). In general, the SMSs had higher glucan content and lower content of lignin and xylan than the non-treated woody substrate, which is a result of selective degradation on lignocellulose during fungal pretreatment (Chen et al., 2020; Xiong et al., 2019). Among the wood species, aspen contained a higher glucan but lower xylan and lignin than birch and alder in both non-treated woody substrate and SMS (Table 1). The ratio of syringyl to guaiacyl units of lignin (S/G ratio) was lower in the SMSs than in the initial woody material, while the crystallinity was generally higher in SMSs than in wood. The nitrogen content in the SMSs ranged from 0.67% to 0.76%, and it was remarkably higher than that of the initial woody substrates (0.50–0.64%). The content of extractive compounds was around 30% of the SMS.

3.1.2. Nitrogen solubilisation in non-treated wood substrates and SMSs

Solubilised nitrogen in the supernatants of the suspensions of SMSs in sodium citrate buffer (without enzyme preparation) is presented in Table 3. Woody substrate not subjected to fungal treatment was also included. The solubilised nitrogen in initial substrates was only 10–20% of the total nitrogen in the solid sample, while in the SMSs it was 48–55%. The highest value of solubilised nitrogen was achieved for alder SMS. Remarkably, the solubilisation of SMS nitrogen was higher than that previously reported for nitrogen recovered from brewer's spent grain after a hydrothermal pretreatment (Parchami et al., 2021). Shiitake mushroom cultivation not only resulted in nitrogen accumulation in the woody substrate (Table 1), but it also increased the solubility of nitrogen sources contained in the spent substrate. As consequence, the availability of nitrogen for downstream processes increased considerably. Meanwhile, inorganic nitrogen (NH_4^+, NO_3^- and NO_2^-) forms represent a minor proportion (on average 1.8%) of the total solubilised nitrogen in SMSs (Table 3), suggesting that the major part of the solubilised nitrogen was organic, likely as protein existing in remaining fungal mycelia.

Table 3
Solubilised nitrogen forms in non-treated woody substrates and SMS.

Parameters	Unit		Tree species		
			Birch	Alder	Aspen
NH_4^+-N	mg/L	Non-treated	0.03	0.04	0.09
	mg/L	SMS	5.3	5.8	7.1
NO_3^--N	mg/L	Non-treated	< 0.10	< 0.10	< 0.10
	mg/L	SMS	< 0.10	< 0.10	< 0.10
NO_2^--N	mg/L	Non-treated	< 0.002	< 0.002	< 0.002
	mg/L	SMS	0.3	0.41	0.33
Total N	mg/L	Non-treated	98.3	60.5	94.2
	mg/L	SMS	350	390	330
Nitrogen solubilisation	% of substrate	Non-treated	20.0	10.2	20.3
	% of substrate	SMS	49.3	55.4	47.8

3.2. Enzymatic hydrolysis of the SMSs

3.2.1. Analytical enzymatic saccharification

As indicated by the results of the analytical enzymatic saccharification (Fig. 1a), the enzymatic digestibility of glucan in the non-treated woody was rather low for the different tree species. Around 16–27% of initial glucan was hydrolysed for the different materials, and it was slightly higher for aspen, either alone or mixed, than for the other wood species.

Fungal pretreatment resulted in an enhancement of the susceptibility of the substrate to enzymatic saccharification (Fig. 1a). Glucan digestibility of the studied SMSs reached values averaging 80.3%, which corresponds to an increase of 3.5 times compared with the non-treated materials. For the SMSs based on a single wood species, the aspen-based one displayed a significant higher ($p < 0.05$) glucan digestibility (88%) than the birch- and alder-based ones (75–76%). For the SMSs based on wood mixtures, no remarkable differences were observed between the enzymatic digestibility of different samples. These results confirm previous reports on shiitake cultivation as pretreatment method for enhancing the enzymatic convertibility of birch cellulose (Chen et al., 2020; Xiong et al., 2019). Furthermore, the current results show that the method is suitable also for other hardwood species, and that for some species, for example, aspen, it can even be more effective than for birch.

Based on a 12 × 7 data matrix including the twelve analytical enzymatic hydrolysis experimental runs and seven characteristic variables of substrates, a PCA was performed (Fig. 1b) to identify multivariable relations. The biplot, composed of the first two PCA components and explaining 95.9% of the total variation, shows that the observations were visually clustered into two groups that were separated from left to right by the non-treated woody substrates and SMSs. The negative effect of lignin and xylan on enzymatic digestibility is anticipated (Jönsson and Martín, 2016; Shirkavand et al., 2016), which is in line with the highest enzymatic digestibility of the aspen-based SMS, which had the lowest content of both lignin and xylan (Table 1). The negative effect of S/G ratio and the positive effect of the crystallinity index were less expected, since they have often been assumed to affect the enzymatic saccharification in a different way to what our results

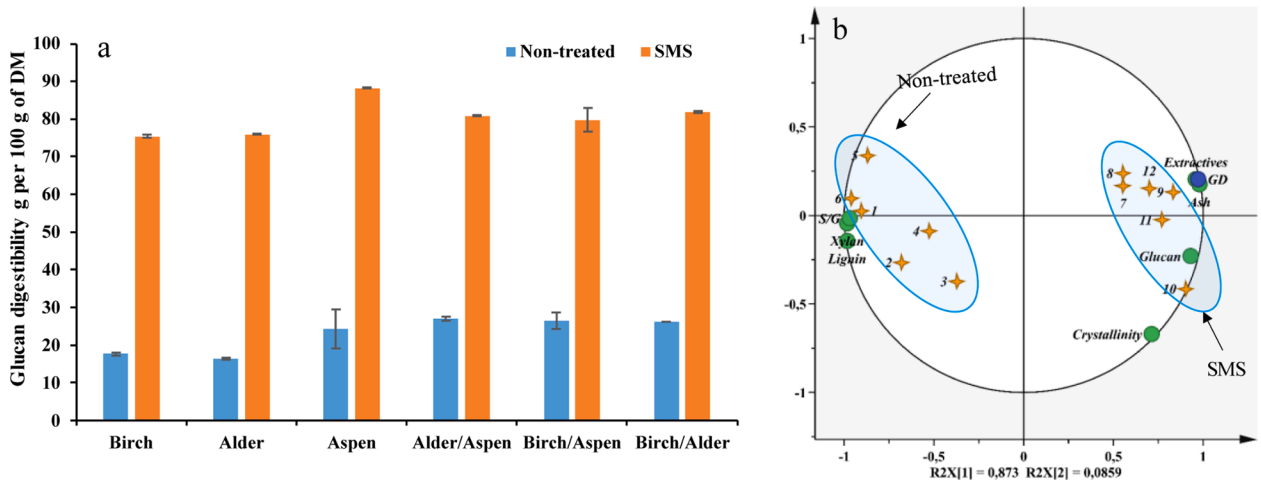

Fig. 1. Enzymatic digestibility of glucan contained in the non-treated woody substrates and SMSs (a); PCA biplot showing major chemical components of substrates and glucan digestibility (GD) (b). Each star represents an observation.

show (Guo et al., 2014; Hall et al., 2010).

It has previously been reported that low S/G ratio is deleterious for enzymatic digestibility. That has been associated with lignin guaiacyl units having higher adsorption capacity than syringyl ones, thus causing non-productive adsorption of cellulases onto lignin (Guo et al., 2014), which is a key problem affecting enzymatic hydrolysis of cellulose (Oliva-Taravilla et al., 2020). However, in the current study, an opposite trend, *e.g.*, a good correlation between low S/G ratios and enzymatic digestibility, was observed. As a possible explanation, one might hypothesize that the reduced amount of syringyl units, which are more voluminous than guaiacyl ones, can weaken steric impediment limiting lignin role as barrier blocking the access of enzymes to cellulose. It has been shown that in some pretreated biomass, lignin inhibits the enzymatic hydrolysis by acting as a physical barrier rather than by inducing non-productive adsorption of cellulases (Djajadi et al., 2018).

Cellulose crystallinity has traditionally been considered one of the main factors behind the poor enzymatic convertibility of cellulose (Hall et al., 2010). However, although the role of crystallinity is unambiguous for pure cellulose, it is not that straightforward for pretreated lignocellulose. There are many examples, where improved enzymatic convertibility of pretreated biomass and its crystallinity index are directly correlated, or even when no relationship at all is found between digestibility and crystallinity (Karimi and Taherzadeh, 2016). We have previously found that acid pretreatment of de-starched cassava stems led to enhancement of the enzymatic hydrolysis of cellulose in spite of the observed increase of the crystallinity index (Martín et al., 2017). Other authors have also stressed that ultrastructural and compositional changes caused during pretreatment are more important than cellulose crystallinity for explaining different enzymatic hydrolysis of pretreated materials (Agarwal et al., 2013; Pardo et al., 2019).

3.2.2. *Preparative enzymatic saccharification*

Preparative enzymatic saccharification was performed for generating hydrolysates to be used in fermentation experiments. Larger volumes, and higher biomass load and enzyme dosage were used compared to those of the analytical enzymatic saccharification. The experiment was performed with only three SMS samples, namely, those based on wood from individual tree species. The resulting hydrolysates contained glucose concentrations roughly between 30 and 35 g/L (Table 4), which are equivalent to glucose yields of 350–412 g/kg SMS. Aspen hydrolysate had the highest glucose content, which is consistent with the results of the analytical enzymatic saccharification (Fig. 1a). It is noteworthy that the glucose yield upon preparative hydrolysis was higher than previously reported values for acid pretreated aspen and birch (274–312 g/kg) (Wang et al., 2018a). The glucan digestibility in the preparative enzymatic hydrolysis (80.3–90.2%) was, in general, higher than the range of values achieved in the analytical enzymatic saccharification (75–88%) (Fig. 1a). That can be attributed to the higher enzyme dosage used in the preparative enzymatic hydrolysis (200 CMCase units/g biomass) compared with analytical enzymatic saccharification (100 CMCase units/g biomass).

Table 4
Composition and pH of the SMS hydrolysates.

Parameters	Unit	SMS hydrylysates		
		Birch	Alder	Aspen
Glucose	g/L	29.6	31.4	34.6
Xylose	g/L	11.7	10.4	9.9
Acetic acid	g/L	2	1.8	1.8
Formic acid	g/L	ND	ND	ND
Levulinic acid	g/L	ND	ND	ND
Phenolic compounds	g/L	2	1.8	1.8
NH_4^+-N	mg/L	0.37	0.42	0.36
NO_3^--N	mg/L	< 0.10	< 0.10	< 0.10
NO_2^--N	mg/L	1.5	1.5	1.5
Total N	mg/L	840	890	810
Glucose/Total N		35.2	35.3	42.7
pH		4.2	4.2	4.2

ND, Not detected.

The hydrolysis resulted in xylose concentrations between 10 and 12 g/L (Table 4). The high formation of xylose was due to the hydrolysis of SMS hemicelluloses. In the used enzyme preparation (Cellic CTec 2), in addition to cellulases, contains also different hemicellulases. The xylanase activity of that enzymatic preparation has been reported before (Yang et al., 2017). Acetic acid was another product of the hydrolysis of hemicelluloses. Although in a previous study, acetic acid was hardly detectable in SMSs (Chen et al., 2022), it was found in the hydrolysates in a range between 1.8 and 2.0 g/L (Table 4) and the concentration trend correlated well with that of xylose, which can be explained because both of them are products of the hydrolysis of hardwood hemicelluloses (Fengel and Wegener, 1989). Acetic acid, as well as formic and levulinic acids, is a known inhibitor of ethanolic fermentation (Jönsson and Martín, 2016). However, formic acid and levulinic acid are not a problem in SMS hydrolysates because they cannot be formed under the low temperatures typical of biological pretreatment process (Chen et al., 2022).

The hydrolysates contained also phenolic compounds with concentration of 1.8–2.0 g/L (Table 4), which were formed by lignin degradation during fungal cultivation (Chen et al., 2022). Phenols ended up in the liquid fraction when the SMS was suspended in the buffer solution. A

part of the phenolics was released right away before the hydrolysis (Table 4), while some additional amount was released during the incubation (Data not shown). The concentration of phenolic compounds, which are inhibitors of cellulolytic enzymes and fermenting microorganisms (Jönsson and Martín, 2016), was the highest in birch-based SMS hydrolysate. Their higher concentration in the initial reaction mixture of the birch-based SMS used in the analytical enzymatic saccharification might have been another reason behind the lower digestibility of that SMS compared with those of the aspen and alder.

The total content of solubilised nitrogen in the SMS hydrolysates ranged between 810 and 890 mg/L (Table 4). The highest value was detected in the hydrolysate of alder-based SMS, while the lowest one was found in that of aspen-based SMS, which is in agreement with the amounts released in the solubilisation test (Table 3). Nitrogen comes mostly from the fungal mycelium retained in SMS, but some contribution by the enzyme preparation is also possible since the amount detected in the hydrolysates was higher than the values released in the solubilisation test. The inorganic nitrogen in forms of ammonium (0.36–0.42 mg/L) and nitrate (1.5 mg/L) represented a minor proportion. To facilitate the understanding of the fermentation of the SMS hydrolysates, a ratio of glucose to total nitrogen in hydrolysates was calculated. For the hydrolysates of birch- and alder-based SMSs, the glucose-to-nitrogen ratio was the same, 35.2, while for the hydrolysate of aspen-based SMS, that value was higher (42.7).

3.3. Fermentation of hydrolysates to ethanol

It is hypothesized that the high nitrogen content and the possible presence of other substances originated from fungal biomass remnants in SMS hydrolysates could contribute to the nutrient requirements of fermenting organisms grown on them. That is a strength of SMS hydrolysates if they are going to be used as substrates for ethanolic fermentation. In order to assess that feature of SMS hydrolysates, a fermentation experiment was included in this study.

The fermentation experiment (Table 2) was first directed to assess the effect of addition of different amounts of nutrients on yeast growth and ethanol formation. A nutrient mixture, typically used for yeast fermentation, was added in three different dosages based on the supplementation reported previously for lignocellulosic hydrolysates (Ilanidis et al., 2021; Martín et al., 2018). Fermentations with non-supplemented hydrolysates, and with glucose reference media, either with no nutrients or fully-supplemented were also run. Based on the data presented in Tables 2 and 3, at the highest nutrient dosage, the expected nitrogen contents, including the nitrogen contained in the hydrolysate and that from the nutrient solution, were ~ 1394, 1440 and 1367 mg/L, respectively, for the hydrolysates of birch-, alder- and aspen-based SMSs. For the non– supplemented SMS hydrolysate, the corresponding total nitrogen values were ~ 776, 822 and 748 mg/L, respectively. That were higher than that in the glucose reference fermentation medium with full nutrient charge (2% dosage), which had nitrogen content of 618 mg/L.

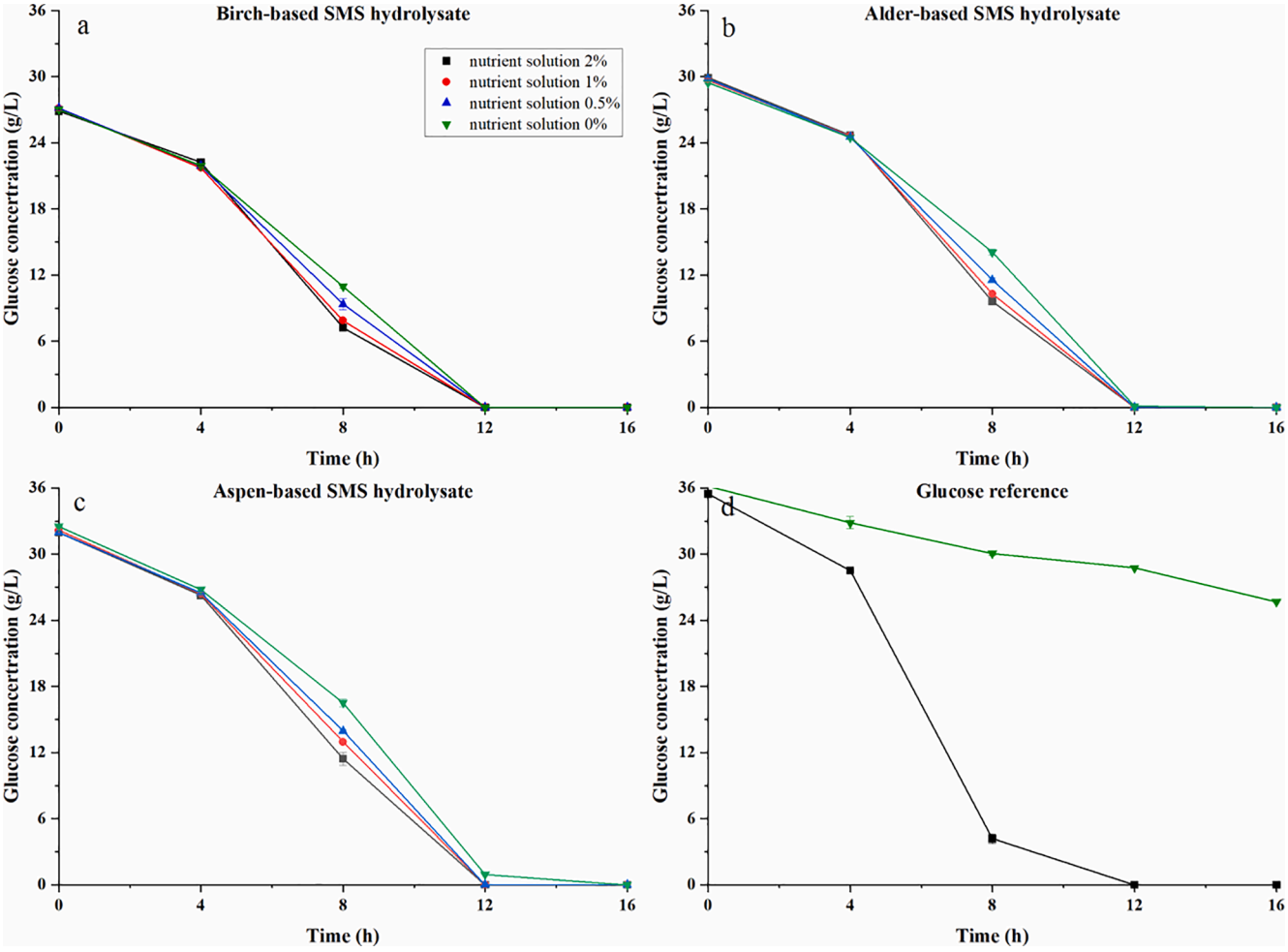

Fig. 2. Glucose consumption during *S. cerevisiae* fermentation of SMS hydrolysates (a-c) and reference media (d) with different nutrient loadings. The data refer to mean values of duplicate experiments, and the error bars represent the standard errors.

3.3.1. Glucose consumption

During the first 4 h of fermentation, around 19, 17 and 18% of the initial glucose was consumed by yeast in the hydrolysates of birch-, alder- and aspen-based SMS, respectively, and the values were comparable between media with different nutrient loadings (Fig. 2a, b and c). The consumed glucose in the hydrolysates after 8 h ranged between 50 and 73% of the initial amount, and a significant slower consumption was observed in non-supplemented hydrolysates than in those containing externally-added nutrients ($p < 0.05$) and in the fully-supplemented reference (88%) (Fig. 2d). After 12 h, glucose was depleted as in that reference as in nearly all the hydrolysates independently of the nutrient supplementation, and no clear differences were observed. The only exception was the fermentation of non-supplemented hydrolysate of aspen-based SMS, where 2.9% of the initial glucose was still unconsumed after 12 h (Fig. 2c). Compared with the birch- and alder-, aspen-based SMS hydrolysate had a higher glucose-to-nitrogen ratio (42.7; Table 4), and it is reasonable that the large amount of glucose required longer time to be consumed by yeast, especially considering a lower amount of nitrogen available for fermentation. Anyway, the fermentation behaviour of all the non-supplemented hydrolysates, including that from aspen-based SMS, was visibly better than that of the non-supplemented glucose reference. In the fermentation of the non-supplemented reference, glucose consumption was clearly restricted by the nutrient deficiency, and only 29% of the initial glucose was consumed after 16 h (Fig. 2d).

3.3.2. Yeast growth

The effect of nutrient limitation was more visible for yeast growth than for glucose consumption as can be seen from the faster OD increase in the fermentations with higher nutrient dosage than in that with no nutrient addition (Fig. 3). The OD measurements of the fermentation samples indicated that yeast growth started to be affected by the nutrition deficiency after 4 h, and the effect was more visible for the rest of the fermentation. Cell growth was more affected in the hydrolysates of birch-based and aspen-based (Fig. 3a and c) SMSs than in that of alder-based SMS (Fig. 3b). Anyway, the cell growth pattern for all the hydrolysates was comparable with that of the fully-supplemented reference medium, and it was significantly higher than that of the non-supplemented reference (Fig. 3d). Evidently, nutrient deficiency caused the inhibition of yeast metabolic activity and sluggish fermentation.

3.3.3. Formation of ethanol and acetic acid

For the fermentations of SMS hydrolysates, ethanol formation was comparable for the different loadings of the nutrient solution during the first 4 h, but it was different after 8 h (Fig. 4a, b and c). With the reduction of the nutrient amount, there was significant decrease ($p < 0.05$) of the volumetric productivity of ethanol, calculated with concentration values corresponding to the first eight hours of fermentation (Fig. 5a). The fully supplemented SMS hydrolysates resulted in ethanol productivities ranging between 0.99 and 1.09 g/L h, while the corresponding values were 0.77–0.84 g/L h in non-supplemented fermentation. The poor performance of the hydrolysate of aspen-based SMS (productivity 0.77 g/L h) can be attributed to its lower nutrient richness, since it had the lowest nitrogen content (810 mg/L) among the different non-supplemented hydrolysates (Table 4). Compared with the volumetric productivity of ethanol in the fermentation of the fully-supplemented reference medium (1.51 g/L h), the values in the

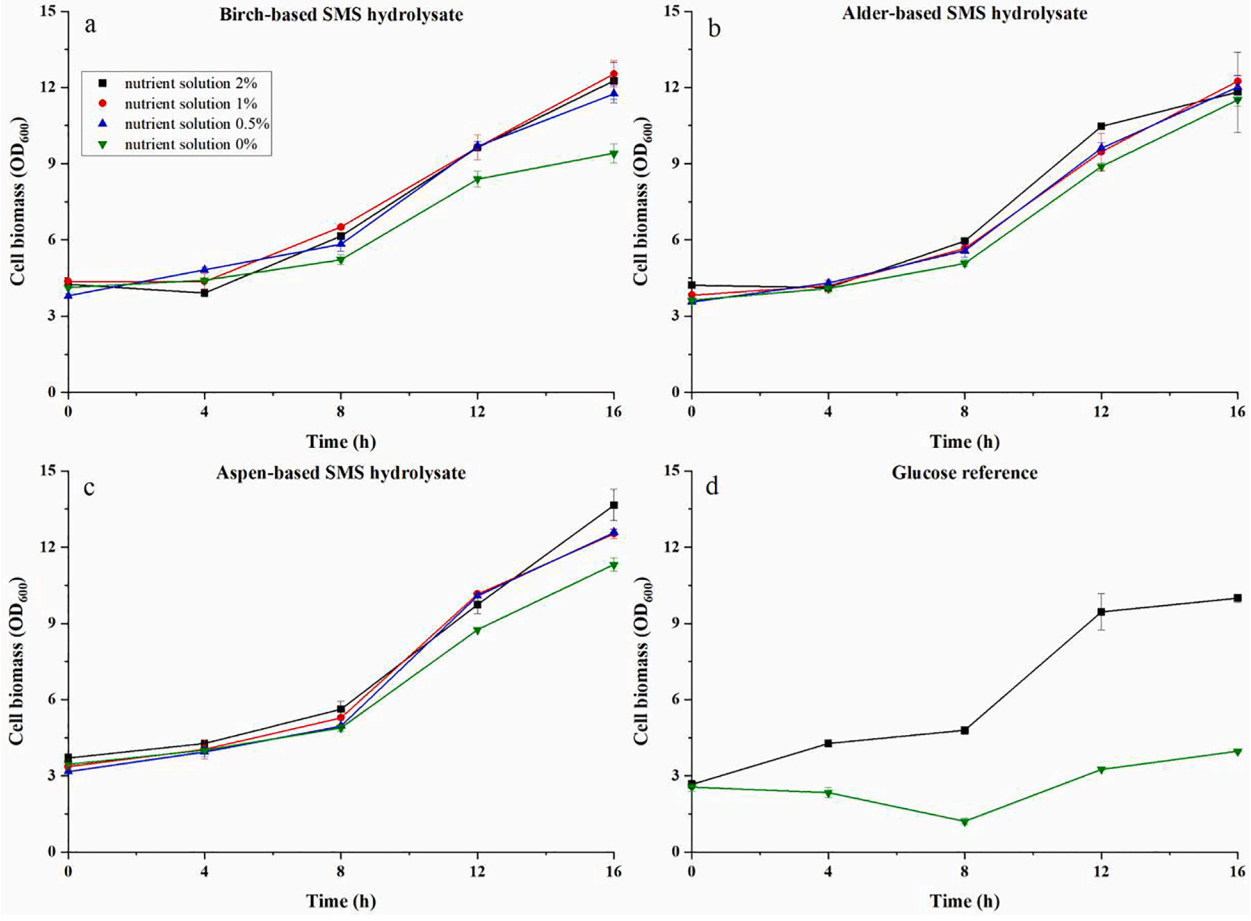

Fig. 3. Cell growth of *S. cerevisiae* during fermentation of SMS hydrolysates (a-c) and reference media (d) with different nutrient loadings. The data refer to mean values of duplicate experiments, and the error bars represent the standard errors.

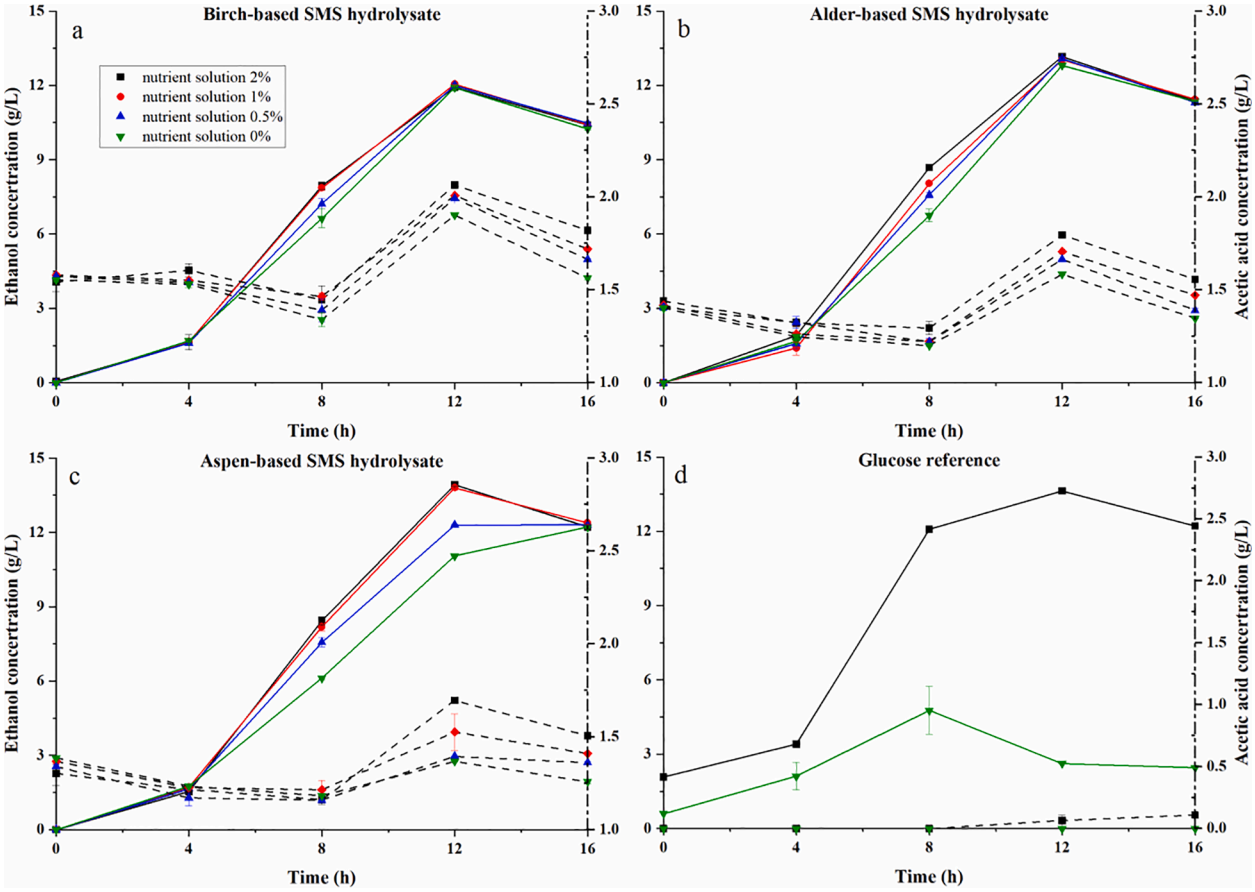

Fig. 4. Ethanol (solid line) and acetic acid (dotted line) production during *S. cerevisiae* fermentation of SMS hydrolysates (a-c) and reference media (d) with different nutrient loadings. The data refer to mean values of duplicate experiments, and the error bars represent the standard errors.

fermentation of hydrolysates was significantly lower, but they were obviously higher than that of the non-supplemented reference (0.6 g/L h).

Although ethanol concentrations were slightly different in fermented hydrolysates of birch- and alder-based SMS between nutrition loadings during the first 8 h, the values became comparable at the end of the fermentation (Fig. 4a and b). At 12 h, the concentrations of ethanol in the hydrolysate of birch-based SMS reached the maximum (11.9–12.1 g/L) (Fig. 4a), which corresponds to yields of 44.1–44.4 g/100 g glucose (Fig. 5b), and then decreased due to evaporation. For the hydrolysate of alder-based SMS, 12.6–13.2 g/L ethanol (Fig. 4b), corresponding to yields of 43.5–44.0 g/100 g glucose (Fig. 5b), was achieved. In the fermentation of the hydrolysate of aspen-based SMS, ethanol formation was clearly affected ($p < 0.05$) by differences in nutrient dosages. Supplementation with the full nutrient loading (2%) and half loading (1%) resulted in comparably high ethanol formation after 12 h (13.3–13.9 g/L) (Fig. 4c), corresponding to yields of 42.9–43.6 g/100 g glucose (Fig. 5b), whereas for the fermentation with 0.5% nutrient supplement and for the non-supplemented one, ethanol formation was remarkably lower, and it decreased proportionally with the decrease of the nutrient dosage. The higher glucose-to-nitrogen ratio in the aspen-based SMS hydrolysis (42.7) compared with the other two hydrolysates (35.2) might be behind reason for the difference. Anyway, aspen-based hydrolysate performed better than the non-supplemented glucose reference, which displayed and ethanol yield of only 7.3 g/100 g glucose (Fig. 5b).

For most hydrolysates, independently on the nutrient supplementation, the ethanol yield was high, and corresponded to 84–87% of the theoretical maximum yield (51.1) (Krishnan et al., 1999). Only the hydrolysates of aspen-based SMS with the lowest nutrient supplementation or with no supplementation at all behaved differently. Ethanol yields were higher in the hydrolysates than in the fully-supplemented reference (38.5 g/100 g glucose) (Fig. 5b). The explanation might be that in fully-supplemented reference, glucose was consumed faster and the fermentation was probably completed soon after 8 h, and no ethanol concentration value was measured at that point. In general, although increased addition of nutrients resulted in some minor increase of glucose consumption rate and volumetric productivity of ethanol for the hydrolysates, it did not affect the final ethanol yields.

The dynamics of acetic acid during the fermentation is shown in Fig. 4. There was a clear increase of acetic acid in the hydrolysate media after 12 h of fermentation. The increases of acetic acid above its initial amount can be attributed to its transient accumulation in the medium as it typically happens during glucose fermentation by yeasts. That is generally associated with the presence of contaminant microbes, other environmental issues, and it is species- and strain-dependent (Dzialo et al., 2017). Acetic acid formation increased proportionally with the increase of loading of nutrients, and the increase was more significant ($p < 0.05$) in the media with the full nutrient charge than in those with less or no nutrients (Fig. 4a, b and c). Acetic acid concentration decreased by the end of the fermentation. That can be attributed to its consumption by yeast when no more sugars were available, since acetate use as carbon source in *S. cerevisiae* is repressed by glucose (Palma et al., 2018). Although formation of acetic acid was also observed in the fermentation of fully-supplemented glucose reference (Fig. 4d), it was negligible (0–0.1%) compared with those in SMS hydrolysates.

3.3.4. SMS hydrolysates as nutrient-rich fermentation media

The good fermentation behaviour of hydrolysates supplemented with low nutrient dosages or not supplemented at all can be attributed to

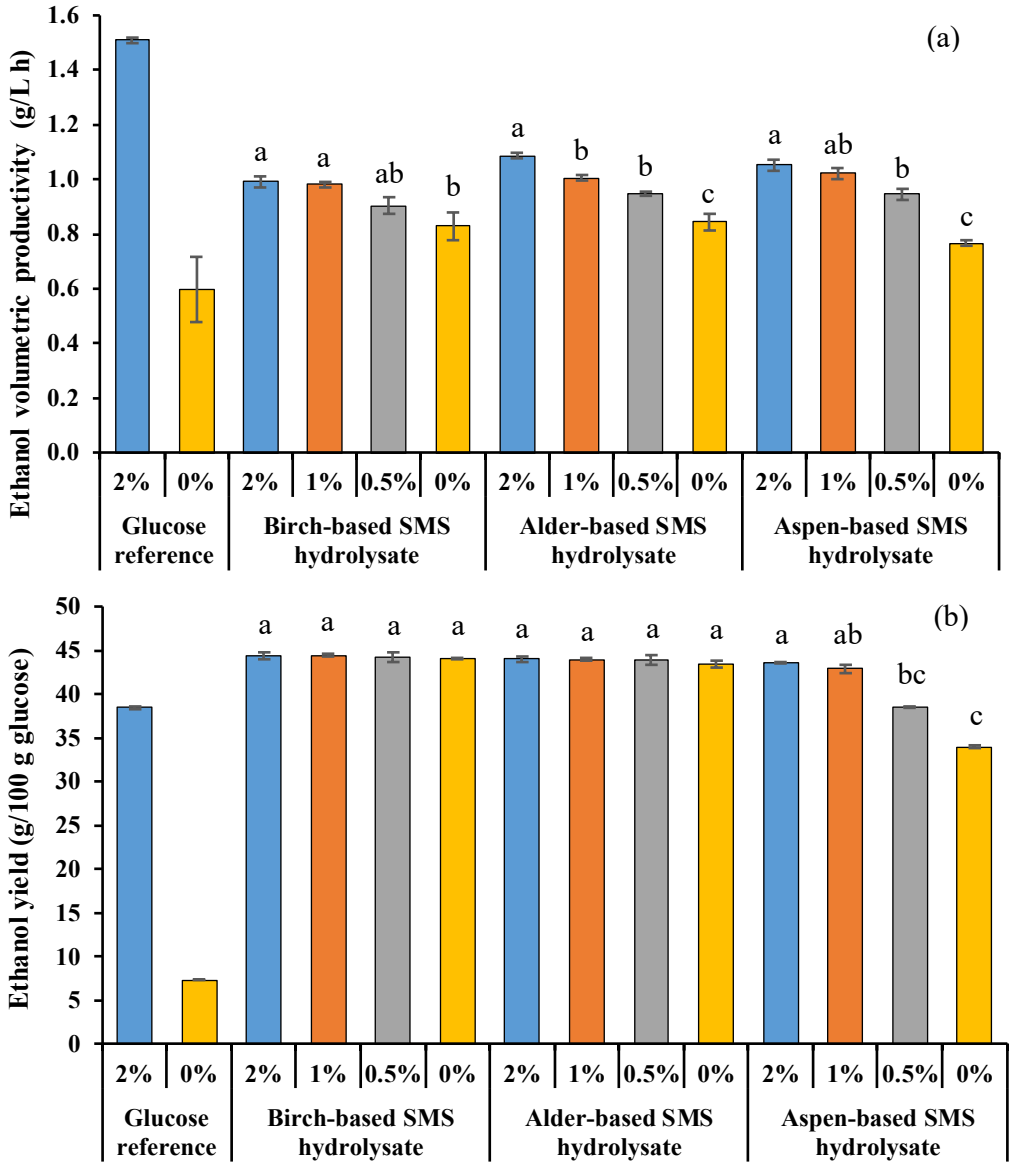

Fig. 5. Ethanol volumetric productivity (a) and yield (b) in *S. cerevisiae* fermentation of SMS hydrolysates and reference media with different nutrient loadings. The data refer to mean values of duplicate experiments, and the error bars represent the standard errors. Values within one fermentation type followed by different letters (a, b and c) indicate significant differences ($p < 0.05$) between nutrient loadings.

the presence of nutritional substances of fungal origin. Components of fungal biomass remnants contained in the SMS might have been solubilised during the enzymatic saccharification, and might be suitable as nutrient sources for yeast. Therefore, differently to other lignocellulose-based media, SMS hydrolysates can provide not only carbon sources, but also nutrients required for fermentation. The glucose-to-nitrogen ratios in SMS hydrolysates seems to be enough for ensuring the carbon–nitrogen balance that is required for ensuring an efficient fermentation by yeast.

Apparently, the nitrogen sources contained in fungal biomass remnants in the SMS (Table 3) are suitable to be used by yeast as nutrients. Among other nitrogen forms, ammonia, glutamine, and asparagine have long been considered the main sources of yeast assimilable nitrogen (YAN) (Gobert et al., 2019). In the current work, all soluble inorganic nitrogen accounted for < 0.3% DM of the total nitrogen contained in SMS hydrolysates (Table 4). In spite of that, fermentation of non-supplemented birch- and alder-based SMS hydrolysates resulted in excellent fermentation performance. That points towards involvement of other unidentified nitrogen forms, probably organic components, such as amino acids, originated from fungal mycelium. For example, glutamic and aspartic acids, which make up 4–10% of the fruit bodies, are important component of edible mushrooms (Bach et al., 2017). They might be also expected to account for a high proportion in the fungal mycelium. This might be associated with the increase of pH of the SMS hydrolysates (from 5.5 to 6.2) during fermentation (Fig. 6a), and which was not observed in the reference media, whose final pH was below 4.1. The increase of the pH during the fermentation of the SMS hydrolysates might be linked with the dissociation of amino acids in slightly acidic conditions, which results in the protonation of the amino group with formation of an alkylammonium cation (Russo and Casazza, 2012). The pH increase was inversely proportional to the amount of added nutrients. An explanation might be that high nutrient supplementation provided more direct nitrogen source, and thus reduced use of amino acid, resulting a significant lower pH ($p < 0.05$).

3.3.5. Potential effect of inhibitors on fermentation performance

Regardless of nutrient supplementation, *S. cerevisiae* growth during fermentations of SMS hydrolysates was inhibited in comparison with

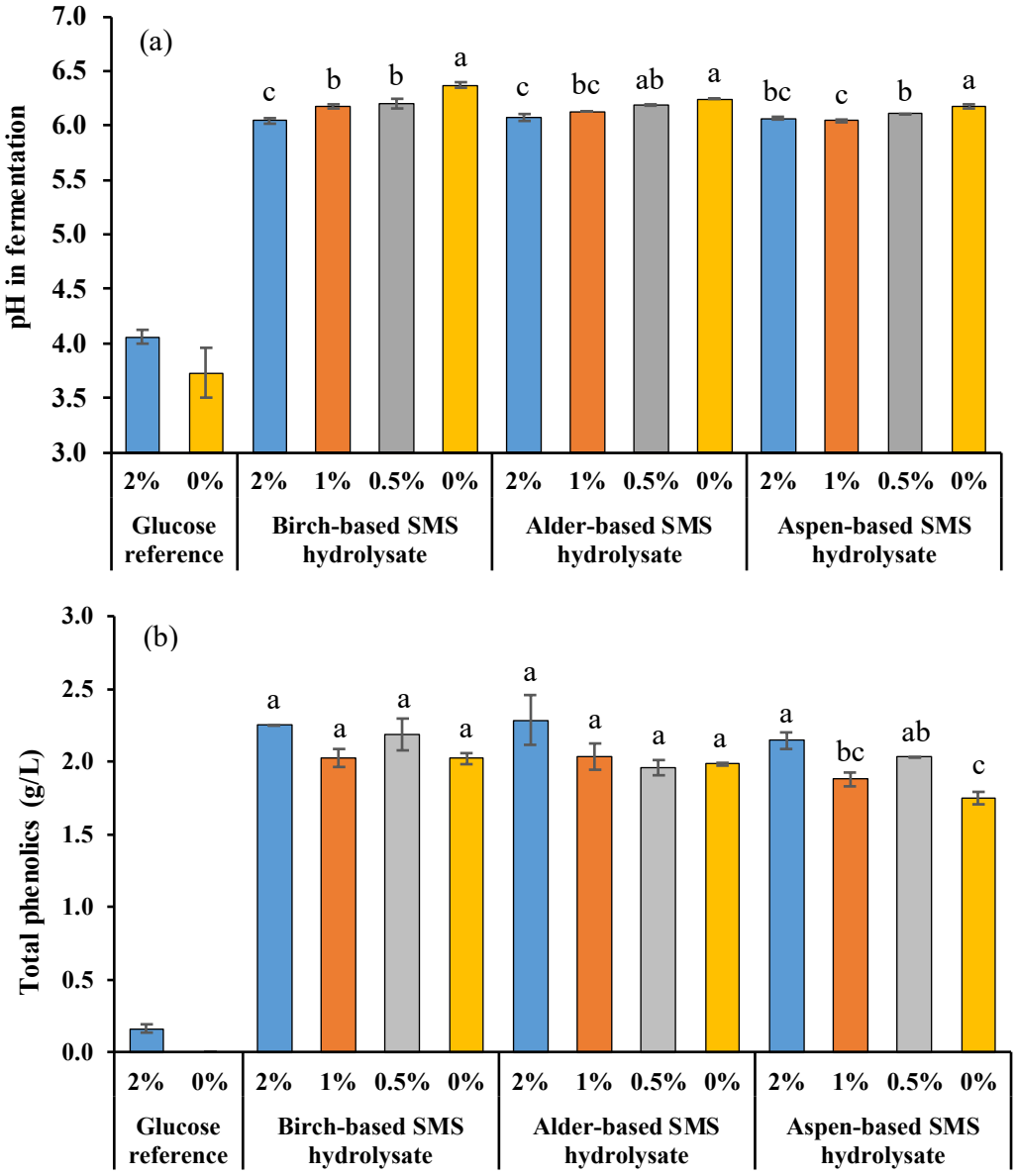

Fig. 6. Final pH (a) and concentrations of total phenolic compounds (b) in *S. cerevisiae* fermentation of SMS hydrolysates and reference media with different nutrient loadings. The data refer to mean values of duplicate experiments, and the error bars represent the standard errors. Values within one hydrolysate type followed by different letters (a, b and c) indicate significant differences ($p < 0.05$) between different nutrient loadings.

that of the reference medium with full nutrient charge. In the reference fermentation, where the initial nitrogen concentration was 618 mg/L, the cell growth, measured as optical density, increased by 60, 80 and 254% of the initial value after 4, 8 and 12 h, respectively (Fig. 3d). In contrast, the non-supplemented SMS hydrolysates (Fig. 3a, b and c), which had higher nitrogen concentration than the reference (greater than748 mg/L), resulted in lower increases of cell growth, namely up to 12, 36 and 134% after 4, 8 and 12 h, respectively. The lower cell growth in SMS hydrolysates can be attributed to inhibition caused by toxic compounds, such as acetic acid and phenolic compounds, which are known to inhibit microbial processes (Jönsson and Martín, 2016), and were contained in all the hydrolysates (Table 4).

When comparing ethanol production in the SMS hydrolysates and in the reference fermentation, some inhibition was observed for the volumetric productivity (Fig. 5a), while the yield was not affected (Fig. 5b). The inhibition of the productivity might has been caused by phenolic compounds and acetic acid in the hydrolysates. However, the concentration of those inhibitors was low; their effect on fermentation was weak and did not last long. The situation is different from the stronger inhibition reported in fermentation of hydrolysates produced by methods resulting in high formation of inhibitors. Acetic acid concentration in SMS hydrolysates was up to 2 g/L (Table 4), while in hydrolysates of acid pretreated corn cob, spruce and oak it is typically above 4 g/L (Du et al., 2020; Ilanidis et al., 2021; Ko et al., 2016). In addition, it had been reported that inhibition by acetic acid is pH dependent, i.e., high toxicity towards *S. cerevisiae* is observed when the extracellular pH is below the pKa of acetic acid (4.7), but at pH above that value, no severe inhibitory effect occurs (Ko et al., 2016; Taherzadeh et al., 1997; Wei et al., 2013). During the fermentation, the pH of the media was always above acetic acid pKa, since it was 5.5 at the beginning, and then it increased to up to 6.2, especially at low nutrient supplementation (Fig. 6a). That might have further weakened the inhibition effect of acetic acid. Furthermore, differently from hydrolysates produced by conventional pretreatment methods (Bolado-Rodríguez et al., 2016; Martín et al., 2018), SMS hydrolysates do not contain other inhibitors, such as furan aldehydes, formic acid and levulinic acid. Phenolic

compounds are the only type of inhibitors that was detected in comparable amounts in this study and in acid pretreated sugarcane bagasse and spruce hydrolysates (Ilanidis et al., 2021). The dynamics of acetic acid during fermentation is shown in Fig. 4, and it is discussed in 3.3.3, while the concentration of phenolic compounds in SMS hydrolysates remained rather similar before (Table 4) and after the fermentation (Fig. 6b).

3.4. Implication of using shiitake SMS for biorefinery

Table 5 summarizes the mass balance of converting shiitake SMSs (100 g) to ethanol, with an emphasis of lignocellulose fractions. After enzymatic saccharification, about 40–50% of SMS was solubilised and recovered as monosaccharides in the hydrolysates. The obtained glucose, around 35–41 g of 100 g SMS, was then fermented to ethanol yielding 14–17 g. The amount of produced ethanol from SMSs is comparable or higher to what is produced from pretreated solids from other pretreatment methods (Du et al., 2020; Qureshi et al., 2015; Wang et al., 2018b). This reveals the high potential of fungal pretreatment using shiitake as a biorefinery approach producing ethanol and edible mushrooms with high value as food and source of nutraceuticals and pharmaceuticals.

During the fermentation without nutrient supplementation, except for the hydrolysate of aspen-based SMS, glucose was depleted after 12 h. There was some apparent xylose consumption, which is unexpected since *S. cerevisiae* cannot ferment xylose. That might be attributed to consumption of mannose, resulting from mannan contained in SMS, for example, it represented around 2% of the weight of birch-based SMS in a previous study (Wei et al., 2020). Mannose and xylose elute as a single peak with the used HPLC method. Anyway, it was found that per each 100 g of SMS, 9–12.4 g of xylose, corresponding to 77–90% of the initial amount, remained unconsumed in the fermentation broth. Such a remarkable amount should be considered for conversion to other products in future research.

Approximately 49–57 g out of 100 g SMS remained as a solid residue after enzymatic saccharification (Table 5). That residue was composed of 33–40% extractives, 21–25% lignin and 20% carbohydrates. It is noticeable that Klason lignin was completely recovered after the hydrolysis, while acid-soluble lignin was only partially (50%) retained in the solid leftovers (Table 1 vs. Table 5). The explanation of that phenomenon is beyond the scope of the current investigation, and a deeper understanding remains to be studied. Anyway, the solid leftover, a major side stream of enzymatic hydrolysis, implies an important potential resource for producing additional bio-based products, probably alongside the same biorefinery chain. Although a previous study showed its potential for thermal energy production (Chen et al., 2021), alternative valorisation routes, for example, can be direct its lignin or polysaccharide components to advanced biofuels or other bio-based products. Assesment of the technical feasibility and systems analysis studies on those expectations have to be carried out in future studies.

4. Conclusions

It was found that using shiitake cultivation as pretreatment for facilitating lignocellulose bioconversion results in a good enhancement of the enzymatic saccharification of cellulose and provides easily-accessible nitrogen sources for supporting ethanolic fermentation. The initial nitrogen contained in the SMS hydrolysates ensured good ethanol production even without supplementing additional nutrients. The presence of phenolic compounds and acetic acid was detected in the enzymatic hydrolysates, but their concentrations were low and caused only some limited inhibitory effect on ethanol production. Mass balance analysis of the presented fermentation system revealed important amounts of xylose and hydrolysis leftover that require being valorised.

Table 5
Mass balance during enzymatic hydrolysis and fermentation.

	Units	Substrate species		
		Birch	Alder	Aspen
SMS	g	100	100	100
Enzyme preparation	g	13.6	13.6	13.6
Buffer chemicals	g	23.4	23.4	23.4
After preparative enzymatic saccharification [1]				
Hydrolysates				
Glucose	g	34.9	37.6	41.2
Xylose [2]	g	13.8	12.5	11.8
Solid leftover				
Glucan	g	7.3	8.3	7.8
Xylan	g	3.1	3.2	2.4
KLL	g	8.5	10.3	10.3
ASL	g	2.3	2.1	2.2
Extractives	g	19.0	23.1	16.4
Ash and other [3]	g	9.5	10.1	10.3
After fermentation of hydrolysates [4]				
Ethanol	g	15.4	16.4	14.0
CO_2 loss	g	14.7	15.7	13.4
Residuals				
Glucose	g	0	0	1.2
Xylose [2]	g	12.4	9.6	9.1
Others [5]	g	43.1	45.2	45.5

[1]With addition of 200 CMCase units/g biomass; [2] Includes other hemicellulosic sugars; [3] Includes unidentified components; [4] Without nutrient supplementation; [5] Estimated by differences.

CRediT authorship contribution statement

Feng Chen: Investigation, Data curation, Writing – original draft, Writing – review & editing. **Shaojun Xiong:** Conceptualization, Project administration, Funding acquisition, Writing – original draft, Writing – review & editing. **Madhavi Latha Gandla:** Investigation. **Stefan Stagge:** Investigation. **Carlos Martín:** Conceptualization, Data curation, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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III

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ORIGINAL ARTICLE

Enabling efficient bioconversion of birch biomass by *Lentinula edodes*: regulatory roles of nitrogen and bark additions on mushroom production and cellulose saccharification

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Abstract
Pretreatment with edible white-rot fungi has advantages in low inputs of energy and chemicals for reducing the recalcitrance of woody biomass for bioethanol production while harvesting protein-rich food. The effectiveness of fungal pretreatment may vary with substrate composition. In this study, birch with or without bark and nitrogen additives were experimentally studied for their effects on shiitake production, substrate lignocellulosic degradation and enzymatic convertibility with cellulolytic enzymes. Whey was added as protein nitrogen and led to successful outcomes, while non-protein nitrogen urea and ammonium-nitrate resulted in mortality of fungal mycelia. The mushroom yields of one harvest were generally comparable between the treatments, averaging 651 g fresh weight per kilogram dry substrate, and high enough as to be profitable. Nitrogen loading (0.5–0.8%, dry mass) negatively affected lignin degradation and enzymatic convertibility and prolonged cultivation/pretreatment time. The added bark (0–20%) showed quadratic correlation with degradation of lignin, xylan and glucan as well as enzymatic digestibility of glucan. Nitrogen loading of < 0.6% led to maximal mass degradation of xylan and lignin at bark ratios of 4–9% and 14–19%, respectively, peak saccharification of glucan at 6–12% and the shortest pretreatment time at 8–13% bark. The designed substrates resulted in 19–35% of glucan mass loss after fungal pretreatment, less than half of the previously reported values. Nitrogen and bark additions can regulate lignocellulose degradation and saccharification of birch-based substrates. The designed substrate composition could considerably reduce cellulose consumption during fungal pretreatment, thus improving bioconversion efficiency.

Keywords White-rot fungi · Biological pretreatment · Birch · Delignification · Enzymatic hydrolysis · Multiple-linear-regression (MLR) model

1 Introduction

Global climatic changes and a growing population call for the increased production of renewable energy. It is estimated that by 2050, around 10–40% of the world's primary energy consumption could be covered by woody biomass [1, 2]. Wood lignocellulose, such as in forest residues, is a potential source of advanced biofuels such as second-generation ethanol. However, the high lignin content in wood (about 20–35% of dry mass) limits enzymatic hydrolysis of cellulose for the production of ethanol [3]. The development of pretreatment technologies, including physical, chemical and biological methods, aimed at reducing biomass recalcitrance to improve enzymatic saccharification of cellulose, for an eventual industrial production of bioethanol, has been a focus of intense academic research in the last two decades [4].

Biological pretreatment using lignin-degrading microorganisms, mainly white-rot fungi, has received research attention due to its low energy input, reduced formation of inhibitors and environmental friendliness [5, 6]. During white-rot fungus growth on woody substrates, lignin is degraded by the oxidoreductases, such as laccases and peroxidases, and used for mycelium formation. This results in a significant decrease of the lignin content and in changes in some physical and biochemical characteristics of the substrate, which improves the efficiency of enzymatic hydrolysis of cellulose [7].

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In spite of the interest within academic research, unless it is combined with other methods [8], biological pretreatment is not yet viable for industrial implementation, due to its slow rate [9] and the partial consumption of carbohydrates by pretreatment microorganisms [10]. A novel concept for biological pretreatment that deserves attention is based on the cultivation of edible white-rot fungi on woody substrates, and shiitake (*Lentinula edodes* (Berk.) Pegler) has been revealed as a promising model species [11]. Although this novel biological pretreatment strategy is still time-consuming and associated with carbohydrate losses, the drawback can be compensated by the co-production of high value-added edible mushrooms. Shiitake is an edible white-rot mushroom that is becoming increasingly popular on the global market because of its flavour, high nutritional content and health-promoting and medicinal properties [12]. Our previous research has revealed that the spent mushroom substrate (SMS) resulting from harvesting shiitake fruiting bodies on woody biomass is a cellulose-enriched material with a low lignin content and enhanced enzymatic digestibility [11]. Pretreatment of woody biomass by shiitake cultivation is also effective in improving anaerobic digestion for biomethane production [13]. However, it is still unclear whether and how cellulose consumption can be reduced while obtaining a high degradation of lignin and a good mushroom yield.

Global shiitake production amounts to 7.5 million tons per year, which corresponds to 22% of the global mushroom market [14]. It is estimated that around 4–5 kg SMS are generated per kilogram of mushroom harvested [15]. This suggests a great potential to integrate shiitake and biofuel production, provided that the cellulose that remained in the SMS is efficiently saccharified and converted to ethanol. Different mushroom producers may use different biomass mixtures for the substrates [16], which may result in differences in lignocellulose degradation and enzymatic digestibility of the spent substrates. An understanding of how the degradation of lignocellulose components during cultivation and the susceptibility of the spent substrate to enzymatic hydrolysis are affected by initial substrate composition is a prerequisite for the further development of a process combining mushroom production and biological pretreatment for lignocellulose bioconversion to ethanol.

Bark constitutes around 10–25% of the dry mass (DM) of a tree stem depending on the species, growing conditions and anatomical part [17]; for birch, it can be 13–20% in correlation with stems/shoot diameters. It is often a major by-product of sawmills where stemwood is processed. Bark typically has lower carbohydrate contents but higher contents of lignin, extractives and ash than stemwood [18]. Although the antioxidant and antimicrobial properties detected in bark extractives may inhibit mycelium growth [19, 20], bark still contains nutrients (minerals) and carbohydrates [21, 22]. However, due to its higher lignin content (33% DM) compared with stemwood (23% DM), based on our pilot analysis, it might be included as an additive to substrates only within a certain percentage range. However, the inclusion of bark in the substrate formulation for shiitake production based on the nutrient-supplemented woodchip method and the effect of initial lignocellulose fractions on changes in substrate composition and the enzymatic convertibility of SMS cellulose have rarely been addressed in the literature.

Nitrogen supplementation is considered a key factor in substrate formulation for industrial mushroom cultivation. Supplying nitrogen of good quantity and bioavailability has been an important topic for both research and industrial practice for mushroom production [23], and the effects may vary with the ratio and source (protein and non-protein nitrogen). The activity of the enzymes involved in lignin degradation may be affected differently [24, 25], probably depending on the bioavailable nitrogen and carbon concentrations. Since the mechanism behind that phenomenon is not yet well understood, additional research is needed to clarify whether the differences in initial substrate composition may have an effect.

This study aimed at a further development of our novel fungal pretreatment process to delignify lignocellulose by cultivation of edible mushrooms on forest residues [11]. The focus of this study was on how substrate formulation could affect the efficiency of substrate delignification and mushroom production, and our hypothesis was that the nitrogen and bark additions in the substrate were important factors for process optimisation. A factorial experiment was designed to investigate the effects of bark and nitrogen addition on (i) mushroom production on a birch-based substrate, (ii) degradation of lignocellulosic components, and (iii) enzymatic digestibility of cellulose contained in the SMS. The potential use of non-protein versus protein nitrogen resources was also evaluated.

2 Materials and methods

2.1 Substrate materials

White birch (*Betula pubescens*) was used as a model species for major substrate material based on our previous study [11]. Birch is frequently found among early forest thinning residues and remains underutilised in the northern hemisphere [26]. Birch trees with a breast diameter of 4–12 cm were freshly harvested from a natural forest area in Vännäs, Sweden, in October 2017. The main stems were split into stemwood and bark by manual debarking. Stemwood and bark were then chipped with an Edsbyhuggen chipper (*Edsbyhuggen* AB, Sweden) to a particle size of 15–20 mm, dried at 45 °C and ground to < 4 and < 5 mm, respectively (Table 1). Wheat (*Triticum aestivum*) bran and barley (*Hordeum vulgare*) grain were supplied by a Swedish food and fodder company

Table 1 Substrate ingredients and chemical composition

Parameter	pH	Ash %DM	Carbon %DM	Hydrogen %DM	Nitrogen %DM	Bulk density kg m^{-3}	Glucan %DM	Xylan %DM	Lignin %DM	Extractives %DM
Birch stemwood (< 4 mm)	5.0	0.3	49.8	6.1	0.1	243.5	37.2	19.9	22.8	4.46
Birch bark (< 5 mm)	4.8	2.2	56.0	6.7	0.5	152.5	16.2	12.3	33.0	22.1
Barley grain (< 8 mm)	5.9	2.1	45.7	6.0	1.6	668.6	55.3	4.4	6.7	20.4
Wheat bran (< 3 mm)	5.9	5.7	46.5	6.1	2.6	238.5	18.0	14.9	12.5	32.6
Whey (< 0.2 mm)	4.6	–	–	–	13.9	–				

(Lantmännen). Whey powder (Whey-100, HSNG AB, Sweden), urea and ammonium nitrate (Sigma-Aldrich) were used as nitrogen additives.

2.2 Experimental design and treatments

A central composite face (CCF) design with two independent factors (bark and nitrogen addition to substrate) was used, each of them at three levels. Three replicated centre points were included, resulting in a total of 11 treatments (Table 2). Each treatment was replicated four times.

To examine the effects of nitrogen on mushroom growth and lignocellulose degradation in different substrates, the treatments were arranged so that the nitrogen content was expected to range from 0.51 to 0.87%, but the total carbon content was around 50% of the DM. Three types of nitrogen additive were studied in three separate experiments: whey (0, 1, 2%), urea (0, 0.5, 1%) and ammonium-nitrate (1, 2, 3%). The doses of nitrogen additive were chosen based on the nitrogen contents of all substrate ingredients; thus, each of the three doses of nitrogen addition should have a similar C/N ratio, regardless of the type of nitrogen source. The bark added had a higher lignin content than stemwood, and thus, the designed addition of bark from 0 to 20% should form a gradient from a low to a high ratio of lignin to total carbohydrates in the substrates. It is understood that the range of bark doses also represents different assortments from stemwood (0% bark) to whole trees or branches with bark [17].

Table 2 Experimental design and fractions of substrate ingredients (% of DM). Bark and whey fractions are the design factors. Total carbon (C) and total nitrogen (N) were used to calculate the C/N ratio

Treatment	Ingredients					Expected content	
	Bark	Whey	Stemwood	Grain	Bran	N	C/N
N 1	0	0	80	10	10	0.51	97.0
N 2	0	1	79	10	10	0.64	75.4
N 3	0	2	78	10	10	0.78	61.5
N 4	10	0	70	10	10	0.55	90.5
N 5-1	10	1	69	10	10	0.69	71.6
N 5-2	10	1	69	10	10	0.69	71.6
N 5-3	10	1	69	10	10	0.69	71.6
N 6	10	2	68	10	10	0.83	59.0
N 7	20	0	60	10	10	0.59	85.0
N 8	20	1	59	10	10	0.73	68.2
N 9	20	2	58	10	10	0.87	56.8

2.3 Substrate preparation and shiitake cultivation

The substrates were prepared by mixing all ingredients according to Table 2; subsequently, water was added to adjust the moisture content of the substrate to 65%, which is a usual industrial practice. The pH was adjusted to around 6.5 by adding 1% $CaCO_3$ based on substrate DM.

The moisturised substrate was packed into transparent polypropylene microcontainers (125 × 65 × 80 mm) which were sealed by a lid equipped with microporous filters for gas exchange and biofiltration (Microsac, http://saco2.com/). Each container was filled with 200 g wet substrate (70 g on DM) and then pasteurised immediately in an oven at 85 °C for 4 h in the same way as in a previous study [11]. After that, the containers were left overnight in the oven to cool down to room temperature before inoculation.

Inoculation was done manually under a sterile hood. Each substrate container was inoculated with 5 g of shiitake spawn M3790 (2.5% of wet mass) (Mycelia BVBA http://www.mycelia.be/). After that, the containers were incubated under controlled conditions at around 22 °C and 70% relative humidity in the dark in a climate chamber. When the entire block was fully covered with mycelia, the colonisation period was considered complete. When the mushroom fruit bodies emerged, the plastic lid was removed, the temperature was lowered to 18 °C, humidity was increased to 90% and some light (about 500 lx) was induced in the climate chamber until the harvest was completed.

2.4 Mushroom harvest and yield

According to the current standard practice in most European mushroom industries, only one harvest (first flush) of fruit

Table 3 Substrate mass and content of major components in initial and spent substrates. All data are based on dry mass (DM)

Model components		Substrate mass		KLL		ASL		Glucan		Xylan		Extractives	
Bark %	Whey	Initial g	SMS	Initial %	SMS	Initial %	SMS	Initial %	SMS	Initial %	SMS	Initial %	SMS
0	0	70	47.5	15.3	9.2	8.3	4.7	28.8	30.9	15.2	9.7	11.8	27.3
0	1	70	48.6	15.8	9.3	8.6	5.3	28.2	33.1	14.3	8.5	11.5	24.9
0	2	70	46.5	15.2	9.6	8.5	5.1	28.7	31.5	14.9	8.5	12.6	28.3
10	0	70	47.9	18.4	10.4	8.1	4.7	27.1	29.9	16.5	8.6	11.1	28.3
10	1	70	45.2	18.2	10.6	7.4	4.5	26.0	28.8	15.2	8.8	14.3	32.3
10	2	70	46.2	17.7	10.8	7.9	4.2	25.4	25.1	14.4	8.7	13.4	34.2
20	0	70	45.3	21.0	11.6	7.3	4.2	24.4	28.0	14.7	11.4	13.6	32.5
20	1	70	47.5	21.2	12.4	7.1	5.0	24.1	27.5	13.1	11.8	12.6	28.6
20	2	70	47.2	20.9	12.7	7.4	5.0	23.6	25.7	13.5	11.2	13.5	29.1
Mean value		70	46.6	18.2	10.7	7.7	4.7	26.2	28.9	14.7	9.5	13.0	30.0
Standard deviation		–	0.8	0.1	0.1	0.2	0.2	0.2	0.6	0.2	0.2	0.6	1.3
Effects of													
Bark		–	ns	2.7a	1.5a	− 0.6b	ns	− 2.3a	− 2.4b	− 0.5c	1.3a	ns	ns
Whey		–	ns	ns	0.3c	ns	ns	− 0.4c	ns	− 0.6c	ns	ns	ns
Bark × bark		–	ns	ns	ns	ns	ns	ns	ns	− 1.2b	1.5b	ns	− 3.4a
Whey × whey		–	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Bark × whey		–	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

KLL Klason lignin, *ASL* acid-soluble lignin, *ns* non-significant ($p > 0.05$), *a* significant at $p < 0.001$, *b* significant at $p < 0.01$, *c* significant at $p < 0.05$

bodies was conducted. The fruit bodies were harvested manually and then dried at 45 °C for 96 h to determine the DM. The date of harvesting was registered for each individual container. Yield was calculated as the weight of fresh fruit bodies (90% water) divided by the DM of the initial substrate for each container and expressed as grams of fresh fruit body per kilogram of dry substrate.

2.5 Substrate sampling

Sampling was performed for initial (day 1, before pasteurisation) and spent substrates (day 65–80, immediately after mushroom harvest). Whenever sampling was carried out, the entire substrate block from each container was manually collected as one sample. The substrate samples were dried at 45 °C for 96 h, milled to ≤ 0.5 mm and stored in airtight plastic bags at room temperature prior to further analyses.

2.6 Chemical analysis

Prior to chemical analysis, the replicated samples of each treatment were pooled into a single grand sample containing equal proportions (20% by weight) of each replicate. Initial substrates and SMS were used for wet chemical analysis with two replicates.

The chemical composition of initial substrates and SMS was determined using standard procedures for wood analysis. The extractive content was determined by successive extraction with water and ethanol according to an NREL protocol [27]. The structural components were determined by analytical acid hydrolysis followed by quantitation of the sugars and lignin [28]. Glucose and xylose in the hydrolysates were analysed via HPLC (Shimadzu, Kyoto, Japan), using a Shodex NH_2P-50 4E column and an RI detector operating at 50 °C. As the mobile phase, we used HPLC-grade acetonitrile, supplied at a flow rate of 1.0 mL/min. Klason lignin was determined gravimetrically as the solid residue remaining after analytical acid hydrolysis, while for acid-soluble lignin contained in the analytical acid hydrolysate, spectrophotometric determination at 240 nm (Shimadzu, Kyoto, Japan) was used. Total nitrogen and total carbon contents were determined using an elemental analyzer-isotope ratio mass spectrometer (DeltaV, Thermo Fisher Scientific, Germany). A SensION PH31 pH meter was used to determine pH values, following the method described in [11].

2.7 Mass degradation of components

The mass degradation of major lignocellulose components from the initial mass could then be calculated using the following equation:

Relative mass degradation (%)

$$= [1-(MSMS*CSMS/MINI*CINI)]*100,$$

where M and C refer to mass and content of component (cellulose, hemicellulose or lignin) of SMS and initial (INI) substrates, respectively. All data are based on dry mass.

2.8 Enzymatic hydrolysis

Analytical enzymatic saccharification [29] was used for determining the enzymatic susceptibility of cellulose contained in the initial substrates and in the SMS. For each sample, 50 mg DM was suspended in 900 μL of 50 mM sodium citrate buffer (pH 5.2) in 2.0-mL Eppendorf tubes. The tubes containing the reaction mixture were placed in an Ecotron orbital incubator (INFORS HT, Bottmingen, Switzerland) at 45 °C and 170 rpm for 1 h for mixing and attemperation. After that, 6 μL of Cellic CTec2, an enzyme blend containing cellulases, β-glucosidases and hemicellulases, acquired from Sigma-Aldrich Chemie GmbH (Steinheim, Germany), was added. The final enzyme activity in the reaction mixture was 100 CMCase units per gram of biomass. After adding the enzyme blend, the tubes containing the reaction mixture were incubated for 72 h under the above-stated conditions. At the end of the hydrolysis, the tubes were centrifuged; the supernatant was stored frozen at − 18 °C until further analyses, and the precipitate was discarded. Glucose in the supernatants was analysed by HPLC and used for calculating the enzymatic convertibility of cellulose.

2.9 Statistical analysis

Multiple linear regression (MLR) was used to model the relationship between the response variables and the independent factors as well as the factor-to-factor interactions. Modelling and statistical evaluation were performed using the MODDE 11.0 software (Umetrics AB, Umeå, Sweden). The MLR models were evaluated using the coefficients of determination (R^2 and Q^2), which explained the goodness of fit and the predictive ability of the model; R^2 and Q^2 values close to 1 indicate that the model fits the data very well. For each response, a model including all the independent factors and their interactions was created. Terms showing no significant effect on the target response variable ($p > 0.05$) were excluded from the model to obtain optimised R^2 and Q^2 values, and the model was considered reliable. Principal components analysis (PCA) was used to examine the relations and relative importance of the compositional variables of SMS. The data matrix of compositional variables (11 × 7) was analysed by PCA using SIMCA 14.0 (Umetrics AB, Umeå, Sweden) after mean-centring.

3 Results and discussion

3.1 Effect of nitrogen additive sources

Although in the urea-containing substrates, the start of shiitake mycelia colonisation was evident, growth was terminated after 1–2 weeks of incubation. Survival was longer in the substrate with 0.5% addition of urea than in that with 1% addition. In all substrates with added NH_4NO_3, mycelia died within the first week. The two non-protein nitrogen sources at the given doses were apparently not preferred by the shiitake. However, shiitake mycelia grew and fructified well in the substrates supplemented with whey powder that contained 87% of protein, < 1% of carbohydrates, 1% of fat and 0.5% of fibre (data from producer). The major results reported below are therefore from the experiments with whey as a nitrogen source.

3.2 Composition of the initial substrates

The initial substrates based on the design (Table 2) contained varying concentrations of nitrogen, lignin and carbohydrates, depending on the different additions of whey and bark. Actual nitrogen content of the dry substrates ranged between 0.54 and 0.84%, while total lignin, including Klason lignin (KLL) and acid-soluble lignin (ASL), amounted to 23.6–28.3%, and carbohydrates, including glucans (GLU) and xylans (XYL), fluctuated between 37.1 and 44.0% (Table 3). Figure 1 shows the MLR models for the two most important compositional characteristics, namely the total nitrogen content and the total lignin/carbohydrates ratio, of the initial substrate. The models had very good predictive capacity for the initial substrate composition for both the nitrogen content ($R^2 = 0.98$ and $Q^2 = 0.94$) and the total lignin/carbohydrate ratio ($R^2 = 0.98$ and $Q^2 = 0.93$). The condition numbers of the models were 1.7 and 2.7, suggesting a valid experimental design [30].

As expected and indicated in Table 2 and Fig. 1, the added whey played a positive and significant ($p < 0.05$) role in actual nitrogen loading of initial substrate. Bark addition was significantly ($p < 0.05$) and positively correlated with lignin content but negatively correlated with the amount of carbohydrates. The contents of both glucan and xylan in the substrate were diluted by the addition of bark. The added whey contributed almost no carbohydrate (< 0.001% DM) to the substrate, but it had a negative influence on the content of carbohydrates due to a dilution effect.

3.3 Effects of bark and whey addition on shiitake mushroom production

The number of days from inoculation to the first harvest in this experiment, i.e. cultivation time, varied from 66 to 85 days and was significantly ($p < 0.01$) affected by both whey and bark ratio (Fig. 2a). The effect of whey was positively and

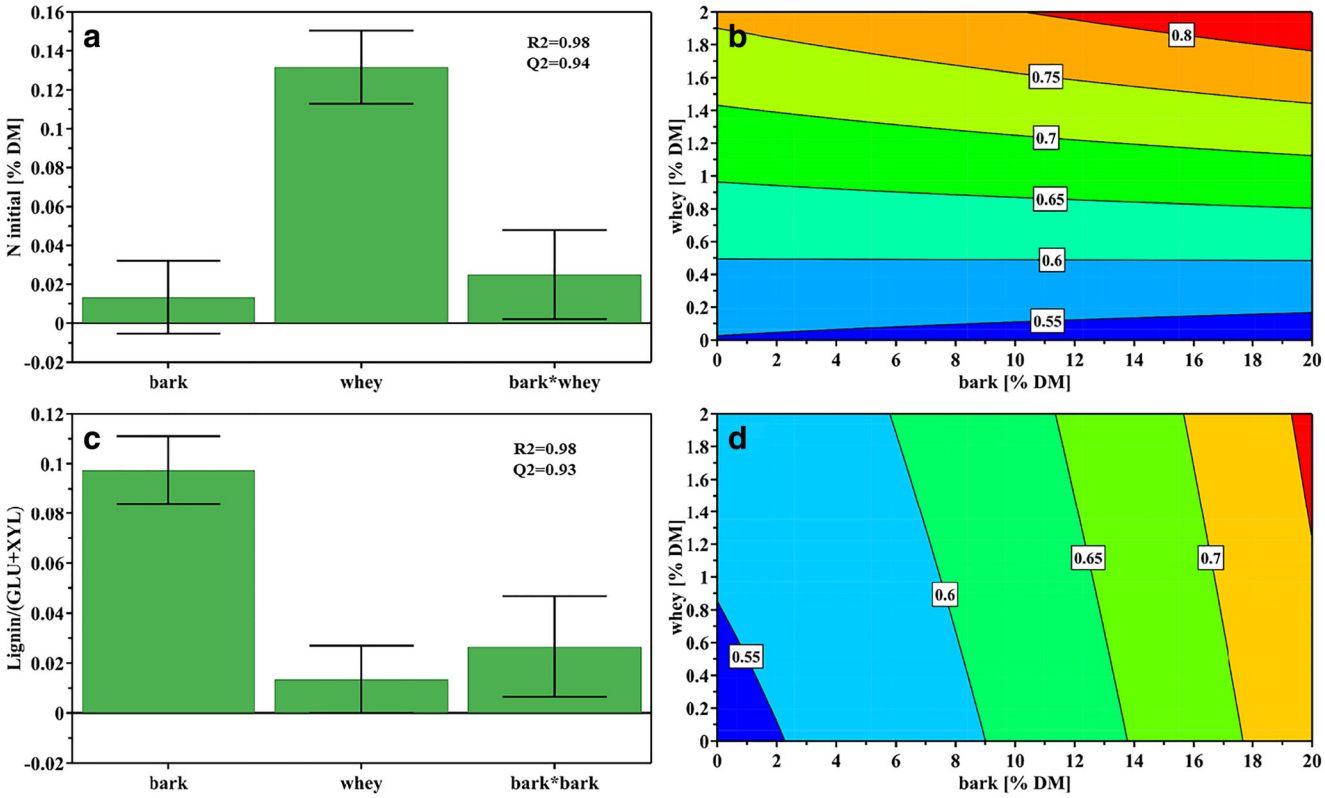

Fig. 1 Actual total nitrogen content (**a** and **b**) and ratio of lignin to carbohydrate (**c** and **d**) in initial substrate in relation to added whey and bark. **a** and **c** Main effect plots for scaled and centralised factors, the bars referring to 95% confidence level. **b** and **d** Response contour plots predicted with the MLR model

linearly correlated with the length of cultivation time, while the effect of bark showed a quadratic function with cultivation time. As indicated by the response contour plot (Fig. 2b) based on the MLR model ($R^2 = 0.78$, $Q^2 = 0.46$), the shortest cultivation period (66 days) was found for the initial substrate composed of $10.5 \pm 2.5\%$ bark, corresponding to a lignin/carbohydrate ratio of 0.60–0.65, together with $< 0.2\%$ whey addition, corresponding to a nitrogen content of 0.54–0.55% DM. Ratios higher or lower than the above-mentioned values for bark and N would slow mycelium growth and fructification resulting in longer cultivation time.

Mean shiitake mushroom yield (wet mass with 90% moisture content) reached up to 650.8 g kg^{-1} dry substrate, ranging from 472.3 to 773.6 g kg^{-1} (Fig. 2c). These values are comparable with the yield achieved in our previous report using a substrate that contained the same ingredients as in this study but without whey nor bark [11]. The MLR model for yield was not acceptable because of the low Q^2 value. There was only a marginally significant and quadratic relation with bark addition ($p = 0.04$), showing that the average fresh mushroom yield from the substrates with 10% bark (610.1 g kg^{-1}) was lower than that from the substrates with 0 and 20% bark (671.2 g kg^{-1} for both). The fast mycelial spreading with 10% bark addition shortened the cultivation time, but was not necessarily related to yields, which is in accordance with previous reports [31]. The exact underlying reasons could not be given directly by this study, but hypothetic explanations might be attributed to the physical and chemical characteristics of the bark.

It is noticeable that the shiitake yields from this study compare favourably with the values reported in other works using higher nitrogen loadings. For instance, our yields were comparable to those reported by Lin et al. [13] (yield = 650.8 g kg^{-1} dry hardwood substrate), despite higher N loadings in their study (0.8–1.4%), and even higher than those reported by Philippoussis et al. [32] (yield = 251 g kg^{-1} dry oak substrate, $N = 1.2\%$) and Ozcelik and Peksen [33] (yield = 437 g kg^{-1} dry hazelnut-husk substrate, $N = 0.8\%$). Our results are even more remarkable considering that they were obtained from only one harvest, whereas in the above-referred reports, the yields were mostly obtained from two to three harvests. Based on this, it can be concluded that the designed N levels used in this study are good enough to reach a profitable mushroom yield, when whey is used as the N-additive to birch-based substrate.

3.4 Compositional changes of substrates during shiitake cultivation

Compositional changes can be typically characterised by the changes in mass of the major lignocellulosic components. Based on the substrate mass remaining and the component

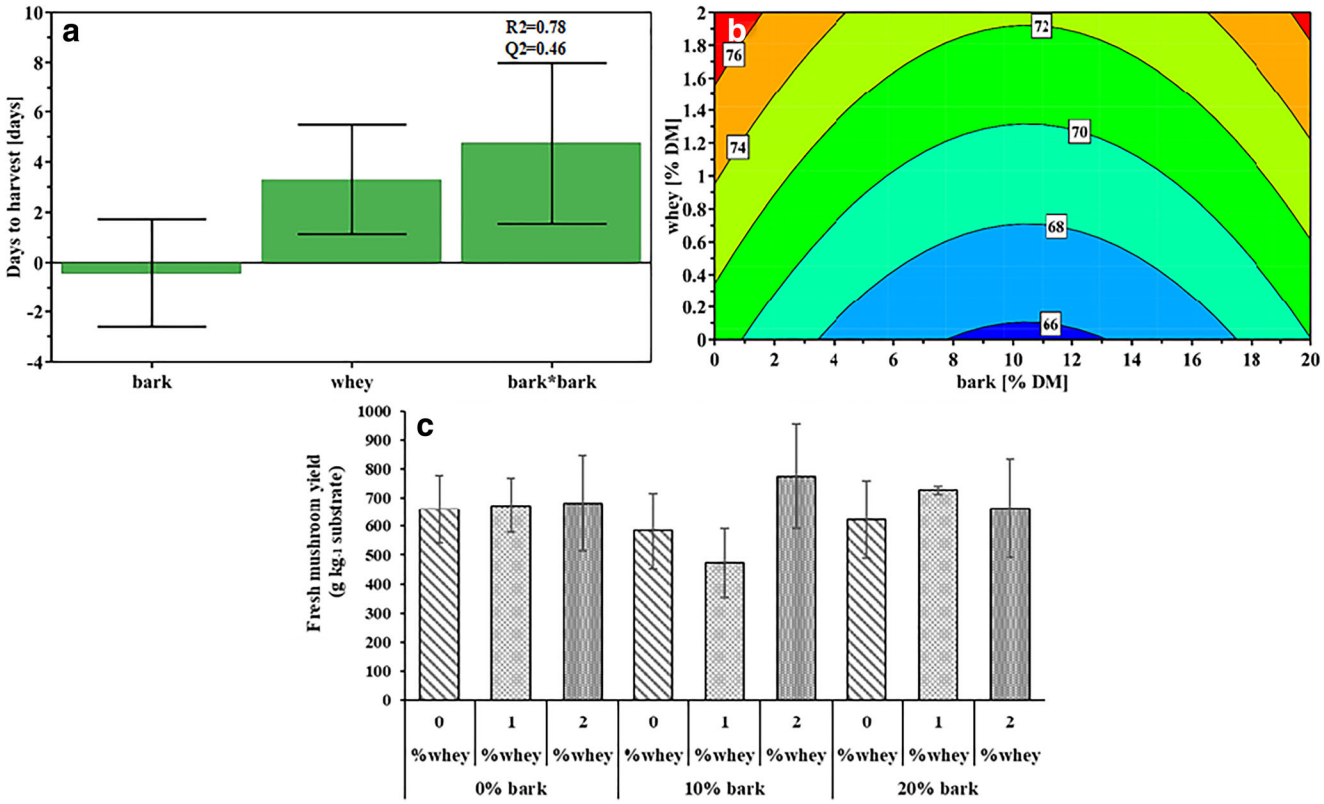

Fig. 2 Cultivation time (days from inoculation to harvest) (**a** and **b**) and fresh mushroom yield (**c**) in relation to additions of bark and whey. **a** Main effect plot for scaled and centralised factors, the bars referring to 95% confidence level. **b** Response contour plot predicted with the MLR model

content in SMS (Table 3), the relative change in mass of each component was calculated using the equation described in Section 2.7.

The average relative reduction in mass of the major components from the initial levels showed the following order: KLL (60.8%) > ASL (59.5%) > XYL (56.7%) > GLU (26%). The reductions in KLL and XYL mass were closely correlated with the amount of added whey and bark and could be well described by the MLR predictive models ($R^2 = 0.83$ and $Q^2 = 0.40$ for KLL; $R^2 = 0.89$ and $Q^2 = 0.65$ for XYL), as illustrated in Fig. 3. The MLR models for ASL and GLU were not acceptable ($Q^2 < 0$), and their mass degradation was considered comparable between treatments. No models were achieved for the relative changes in mass of extractives either; there were no significant differences in extractives between treatments.

As shown in Fig. 3a, b, the degradation ratio, i.e. relative change in mass, of KLL increased ($p < 0.05$) with an increase of bark but decreased with increasing whey fraction. The lowest degradation was 58.3% when adding 0–1.1% bark and 0.8–2% whey and when adding 19.3–20% bark and 1.9–2% whey, where initial nitrogen levels corresponded to > 0.62% and a lignin/carbohydrate ratio of 0.54–0.56% and 0.74–0.75%. The highest ratio was about 63.5% with the addition of 0–0.07% whey and 14–19% bark, corresponding to N loading at 0.53–0.54% and a lignin/carbohydrate ratio of around 0.66–0.71.

Mass degradation of xylan was marginally affected by whey addition, but had a significant and strong quadratic correlation ($p < 0.05$) with bark ratio (Fig. 3c). The MLR model contour plots (Fig. 3d) indicate that the lowest xylan degradation region was found (44%) for the addition of about 20% bark and 1.5–2% whey, where nitrogen levels corresponded to > 0.75% and the lignin/carbohydrate ratio was around 0.75. The highest degradation was about 64%, when additions of whey and bark were 0–0.85% and 4.1–9.1%, respectively, which corresponded to an N content of 0.55–0.64% and a lignin/carbohydrate ratio of 0.57–0.62.

Compared with those of Klason lignin and xylan, mass reduction of glucan was remarkably lower: around 18.5–34.9% of glucan in the initial substrate was consumed during cultivation. Glucan mass degradation was significantly affected by bark only ($p = 0.04$) and tended to be slightly higher at 10% bark than at 0 and 20% (Fig. 4a, b).

Bark addition was significantly correlated with the mass reduction patterns of all three components, namely lignin, xylan and glucan (Figs. 3 and 4), while whey played a significant role in Klason lignin degradation only. This fact suggests that, within the conditions investigated in this study, the addition of bark was more important for lignocellulose degradation than that of whey. The amount of added bark resulted in a gradient of lignin/carbohydrate ratios (Fig. 1), and it was

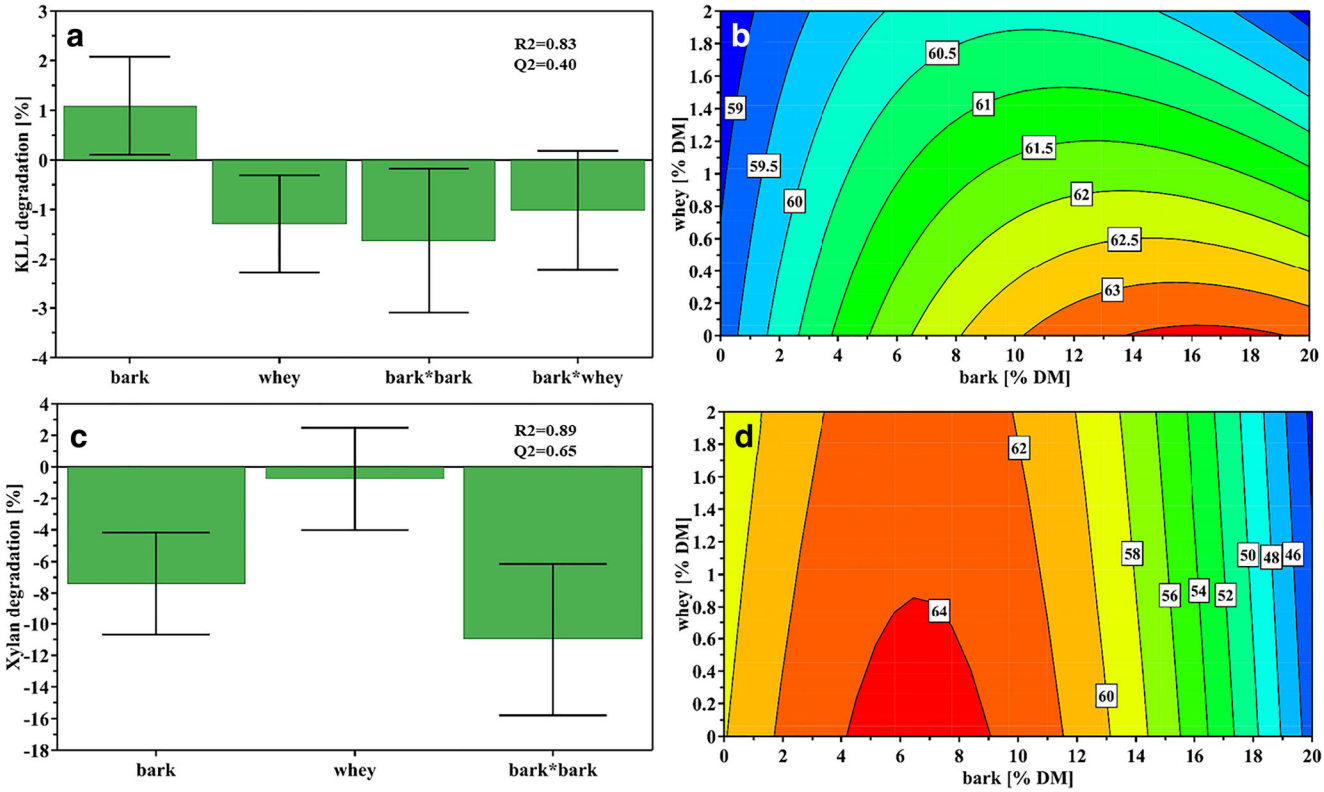

Fig. 3 Effects of whey and bark on the mass degradation of Klason lignin (**a** and **b**) and xylan (**c** and **d**). **a** and **c** Main effect plots for scaled and centralised factors, the bars referring to 95% confidence level. **b** and **d** Response contour plots predicted with the MLR model

possible to identify a threshold (around 0.65) distinguishing favourable ratios for lignin and xylan degradation. Lignin/carbohydrate ratios above 0.65 were favourable for lignin degradation, while values below 0.65 favoured xylan degradation. It is interesting that with the increase of the bark fraction and the lignin/carbohydrate ratio, the shiitake mycelia shifted their preference from degrading xylan to degrading lignin. In this aspect, the addition of bark functioned as a regulatory factor.

The added bark might also have changed the physical structure of the substrate. Since birch bark was fluffier and had a lower bulk density than the stem sawdust (Table 1), its addition could have increased the porosity and aerobic microenvironment inside the substrate. Elevated oxygen levels would increase the rate of lignin degradation through the promotion of peroxidase enzyme secretion [34, 35]. However, the resistance of bark lignin as compared to stem lignin might be attributed in part to the inhibiting effect of tannins and suberin on fungal growth [19], which could be why fructification was delayed when the bark ratio was close to 20% (Fig. 2b), resulting in impairment of xylan degradation with high bark loadings (Fig. 3d). It is remarkable that lignin (Fig. 3b) and

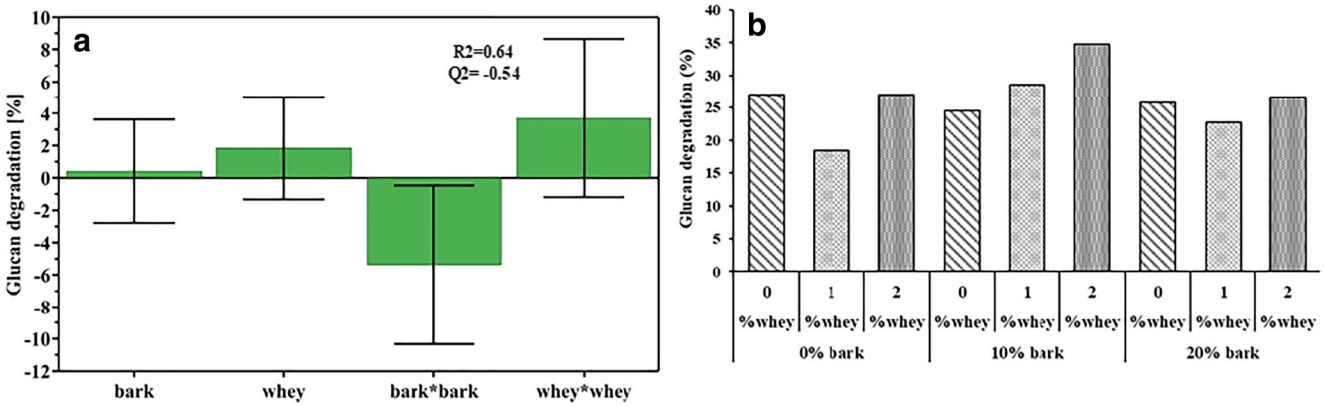

Fig. 4 Effects of whey and bark on glucan mass degradation. **a** Main effect plot for scaled and centralised factors, the bars referring to 95% confidence level. **b** Mass change ratio of components in the initial substrate to those in spent substrate

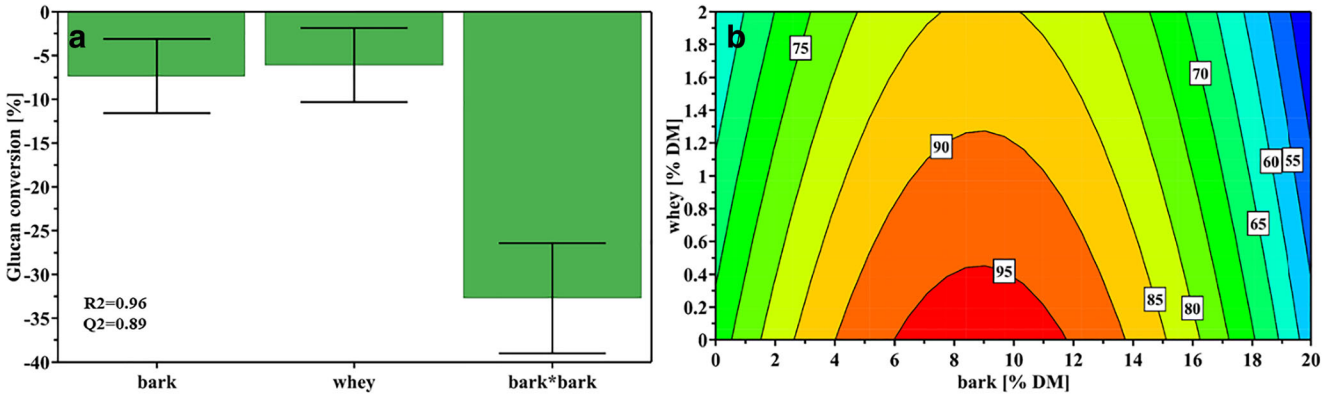

Fig. 5 Glucan conversion (**a** and **b**) during enzymatic hydrolysis of SMS. **a** Main effect plot for scaled and centralised factors, the bars referring to 95% confidence level. **b** Response contour plot predicted with the MLR model

glucan (Fig. 4) degradation was not affected by high doses of bark as much as xylan degradation was.

From contour plots paired with responses (Figs. 2 and 3), a nitrogen content of around 0.55%, at which the highest degradation rate of Klason lignin and the shortest cultivation time were found, appeared to be optimal. The fact that Klason lignin degradation was correlated with the amount of nitrogen in the substrate is in accordance with the finding that nitrogen-limited conditions enhance the production of lignin-degrading enzymes (LiP and MnP) and thus induce the depolymerisation of lignin, facilitating colonisation [24, 25]. In this study, the resulting lignin mass degradation (59–64%) was higher but the cellulose mass degradation was lower (19–35%) than those reported for shiitake cultivation on other hardwood materials [13, 36]. Lin et al. [13] reported an average lignin mass degradation of 54% and a cellulose mass degradation of 69% after shiitake cultivation, with no significant differences between nitrogen loadings of 0.8–1.4%. Based on data of Atila's [36] study, a calculation was performed and showed that up to 45% of lignin mass degradation and 46% of cellulose mass degradation resulted from oak substrate with 0.34% nitrogen content. Interestingly, the N loadings of 0.51–0.87% in the initial substrate in this study were moderate, but seemed to have resulted in a more selective degradation of lignin and cellulose, which could be a positive feature for a biological pretreatment of birch-based substrate. However, future studies are needed to examine whether N loadings may interact with substrate species to affect the biological pretreatment process during shiitake cultivation.

3.5 Enzymatic digestibility of substrates

As indicated in Fig. 5, the enzymatic digestibility of glucan contained in the SMS ranged from 41.3 to 92.8%, compared with 20–22% for raw/initial substrate prior to cultivation [11], showing a considerably positive effect of fungal pretreatment. Enzymatic digestibility showed a negative linear correlation with whey ($p < 0.05$) and a strong quadratic correlation with bark addition ($p < 0.001$). Only around 50% of glucan could be converted to glucose when bark addition was closer to 20% and whey addition was > 1.3%, which could be explained by less degradation of lignin and xylan (Fig. 3) and probably also by the high ratio of intermediate products from lignin degradation, such as organic acids, which can inhibit hydrolytic enzymes [37]. The relatively lower digestibility at a low bark content might be a consequence of the low degradation of

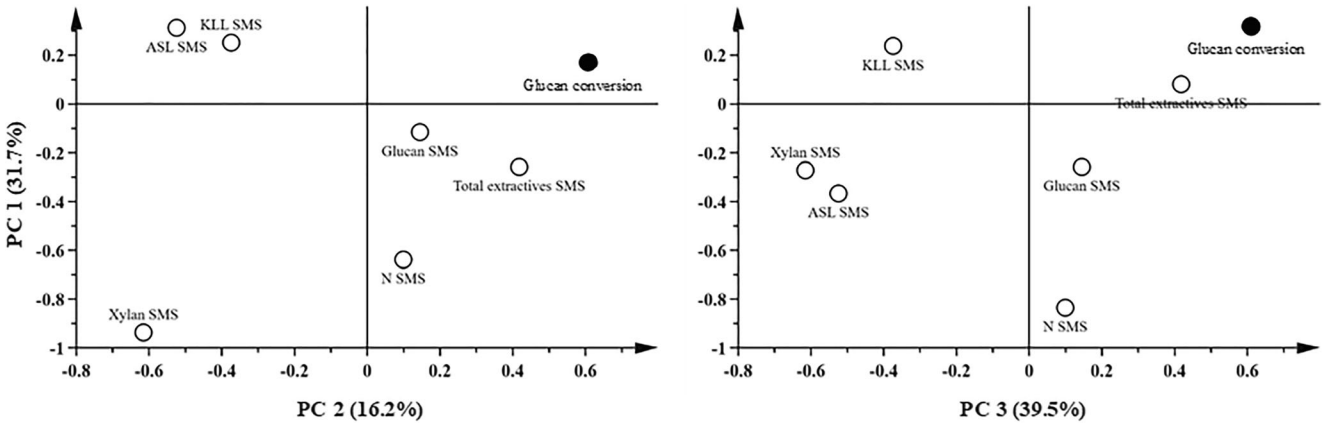

Fig. 6 PCA loading plot showing major chemical components of SMS (open circles) and glucan conversion (filled circles) following enzymatic hydrolysis

lignin during fungal growth under those conditions (Fig. 3b). Initial nitrogen loading below 0.59% (i.e. 0.45% whey) and a lignin/carbohydrate ratio from 0.59 to 0.64 (i.e. 8.9 ± 2.9% bark) resulted in the highest hydrolytical conversion (95%) of glucan contained in the SMS, which remarkably coincided with the most favourable conditions, in terms of nitrogen content and lignin/carbohydrate ratio in the substrate, for the degradation of both lignin and xylan (Fig. 3).

Based on the data matrix (11 × 7) of major chemical composition and enzymatic digestibility of glucan in SMS, a PCA was performed. The three first PCA components explained 87.4% of the total variation. The loading plots, for both PC1 × PC2 and PC1 × PC3 (Fig. 6), show that lignin and xylan are on the opposite side to glucan conversion, which is grouped together with total extractives and glucan content, confirming a negative effect of lignin and xylan on enzymatic hydrolysis [4] and a positive relation between glucan conversion and glucan concentration in SMS. It is understandable that, because of the lower lignin and xylan contents (Table 3), the spent substrate remaining after shiitake cultivation is less recalcitrant than the initial substrate, and therefore, glucan is more digestible by the enzymes (see also [11]). The increase in extractives was an effect of major biochemical processes. Theoretically, the incomplete degradation of cellulose might result in short-chained polysaccharides and in oligosaccharides remaining in the SMS [38]. In the presence of cellulases, those oligo- and short-chained polysaccharides undergo hydrolysis, which could explain why the high content of extractives in the SMS is positively related to glucan conversion.

4 Conclusions

This study showed that by regulating the addition of whey/nitrogen and bark in the initial substrate, it is possible to minimise glucan degradation and maximise the degradation of lignin and hemicellulose during shiitake cultivation, thereby enhancing enzymatic saccharification of cellulose in the spent substrate. Keeping in mind that whey and non-debarked birch wood are underused bioresources, our results shall have a considerable implication to promote a cost-effective, energy-efficient and environmentally friendly combined production of food and renewable energy by using forest residues, which would finally benefit a biobased circular economy.

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Authors' contributions SJX initiated the project and designed the experiment. FC co-designed the experiment, performed fungal cultivation and chemical analyses and drafted the manuscript. CM provided the methods for chemical analysis. SJX, CM and MF provided key comments for manuscript writing. All authors read and approved the final manuscript.

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Code availability Not applicable.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Ethics approval and consent to participate Not applicable.

Consent for publication All authors agree to the publishing of the paper.

Abbreviations SMS, spent mushroom substrate; DM, dry mass; KLL, Klason lignin; ASL, acid-soluble lignin; GLU, glucan; XYL, xylan; MLR, multiple linear regression; CCF, central composite face; PCA, principal component analysis

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IV

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Integrated production of edible mushroom (*Auricularia auricular-judae*), fermentable sugar and solid biofuel

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Solid biofuel

ABSTRACT

This study aimed to develop an energy- and resource-efficient process for the coproduction of edible mushroom, fermentable sugar and solid biofuel from wood residues. A promising potential was revealed for wood ear fungus (*Auricularia auricular-judae*), which yielded about 200 g mushroom per kg dry birch-based substrate, with concomitant degradation of 76.8 and 85.7% of lignin and xylan, respectively, in the substrate. Substrate pasteurisation by hot-air (85–100 °C) was as effective as by energy intensive autoclaving (121 °C), resulting comparable mushroom growth and degradation of lignocellulose. The spent mushroom substrate (SMS) contained 28–33% glucan, which upon analytical enzymatic saccharification released around 46% of the potentially-achievable glucose, corresponding to a 2.3–fold enzymatic digestibility compared with that of the raw substrate. The solid leftover generated after enzymatic hydrolysis revealed high thermal energy value and promising combustion characteristics, showing a plausibility to be recycled as solid fuel for self-supporting energy system and space heating.

1. Introduction

According to the Food and Agriculture Organization of the United Nations, the world's population is expected to reach near 10 billion people in 2050 [1]. At the same time, the emission of greenhouse gases caused by human activities is expected to increase, which in the long run may aggravate problems caused by climate change and global warming. The development of resource- and energy-efficient biotechnology for the sustainable production of food and feedstock for the production of CO_2-neutral fuels could be an important strategy to reduce the above-mentioned problems. For reducing further over-exploitation of the natural ecosystem, it is necessary to promote biological conversion approaches for producing food and biofuels from lignocellulosic biomass residues, which are resources largely underused today.

Lignocellulosic biomass, including forest residues, is an abundant bioresource suitable for the production of both edible fungi and second-generation biofuels. Forestry activity and wood-processing industries generates large amounts of by-products such as logging residues and sawdust, which are so far underutilized. Only in Sweden, around 15 Mt forest residues are generated yearly, and only a quarter of that amount is utilized, mostly for energy purposes through direct combustion [2]. Enzymatic hydrolysis of cellulose contained in lignocellulosic materials is a way of generating glucose, which could then be converted by microbial fermentation to biofuels, for instance ethanol. The cellulose-containing materials, however, are typically recalcitrant and low-reactive towards enzymes [3]. Therefore, in bioconversion of lignocellulosic materials, a pretreatment step for removing lignin and/or hemicelluloses has to be implemented prior to enzymatic hydrolysis in order to improve the susceptibility of cellulose to cellulases [4]. The history of growing edible fungi on wood substrates can be traced back thousands of years [5]. Recent research has shown that the cultivation of white-rot edible fungi on lignocellulosic substrates is an efficient way to degrade lignin and hemicelluloses, and therefore the resulting spent mushroom substrate (SMS) has a better digestibility by cellulolytic enzymes, which allows production of fermentable sugar from cellulose [6,7]. Thus, edible fungi cultivation on lignocellulose, followed by enzymatic hydrolysis of the SMS, is a bioconversion alternative of

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interest. Compared with conventional pretreatment methods, biological pretreatment with white-rot fungi allows reducing the energy demand and usage of chemicals for increasing cellulose reactivity towards cellulolytic enzymes.

We consider that there are three scientific issues of major relevance regarding biological pretreatment with white-rot fungi as a platform for production of food and energy carriers. The first one is the assessment of fungal species suitable to be included in the integrated production of edible fungi and biofuel. Although studies have shown the potential of using *Lentinula edodes* [8,9], *Pleurotus ostreatus* [10], and *Pleurotus pulmonnarius* [11], the capacity of *Auricularia auricular-judae*, especially in the degradation of lignocellulose, has so far not been well documented. *A. auricular-judae,* known as wood ear or Juda's ear, a white-rot fungus [12], is one of the five most cultivated edible fungi in the world and is a valuable food product due to its high nutritional value and health improvement functions [13]. The global annual production of wood ear accounts for 6.1 million tons, approximately 18% of total cultivated edible mushrooms [13]. It has been reported that in a well-managed mushroom industry for every ton of mushroom produced, about 5 tons of SMS is generated [14,15], suggesting proximate 30 million tons of SMS are from global wood ear production every year.

The second issue is how to improve the energy and cost-effectiveness for processing wood biomass into food and fuels. Substrate pasteurisation is a key process to remove/deactivate competitive microorganisms before inoculation of fungal spawn. However, it is also one of the most energy-consuming steps throughout the whole cultivation chain. Steam sterilisation at 121 °C with overpressure is a conventional method in the mushroom industry, but it has high energy demand and equipment cost. Recent studies [9,11] have demonstrated that hot-air pasteurisation, with up to 60% energy savings, at temperatures as low as 75–85 °C for shiitake (*L. edodes*) and 65 °C for summer oyster (*P. pulmonarius*), resulted in comparable mushroom yield and substrate lignocellulose degradation as those achieved by steam sterilisation at 121 °C and 2 bar. It is expected that hot air pasteurisation will also work for wood ear, but biological processes and the preferable temperature may be different.

The third issue involves the utilisation of side streams. In one of our pilot studies, it was found that approximately 60% of SMS on dry mass remained as solid leftover after enzymatic hydrolysis. Theoretically, the leftover contains a relatively high ratio of lignin, with a higher energy value than other components [16], which could be used as fuel for the production of heat. Heat is needed for mushroom substrate pasteurisation and space heating for the rest cultivation processes. It would be ideal to recycle the hydrolysis solid leftovers for a self-supporting energy system and thereby, reduce the costs associated with the mushroom production process. However, a prerequisite is to understand ash composition and ash behaviour during combustion. The ash content of the substrate increased after fungal cultivation [17] and ash composition might be altered due to the elementary translocation to fungal fruit bodies during the cultivation and addition of buffer chemicals when SMS is hydrolysed. The ash forming elements (such as Ca, Mg, K, Na, Si, P, Al, and Cl) contained in the biofuels have a crucial role in the combustion in terms whether slagging, corrosion, and particle emissions could be induced. There is little, if any, published research regarding the fuel characteristic of the solid leftover from the enzymatic hydrolysis of SMS.

This paper shows the results of our experimental studies on (1) the potential of using birch sawdust for the integrated production of wood ear mushroom and fermentable sugar; (2) the possibility of using hot-air pasteurisation to improve energy efficiency for mushroom cultivation; and (3) the characterisation of the solid leftover after enzymatic saccharification, in a consideration of using the leftovers for combustion.

2. Materials and methods

2.1. Substrate preparation

Substrate ingredients included birch (*Betula* ssp.) sawdust (0.5–2 mm), which was collected from a local sawmill in the province of Västerbotten, Sweden, and wheat (*Triticum aestivum*) bran (≤ 2 mm) and grain (3–5 mm), which were purchased from a Swedish agriculture cooperative (Lantmännen). The substrate ingredients were mixed according to the proportions indicated in Table 1. Water was added to obtain mixtures with a moisture content of approximately 65% wet mass. The pH of the substrates was adjusted to approximately 6.4 by adding $CaCO_3$. After blending all ingredients, the substrates were packed into polypropylene bags with inbuilt micro-porous filters (Microsac) for gas exchange that allowed the mycelia to breathe. Each bag was filled with 1 kg substrate (wet mass), which resulted in "synthetic logs" of approximately 11 cm in diameter and 15 cm in height.

The bagged substrates were pasteurized using four heating treatments (Table 1) based on autoclave using pressurized steam (121 °C and 2 bar) and hot-air oven (100-75 °C, under atmospheric pressure). For each pasteurisation treatment, the temperature and time were measured by using a 'control' substrate bag filled with the same substrate. A thermocouple was placed at the core of the substrate cylinder, and the process data was recorded using a logger. When the desired temperature and the required treatment time was reached, pasteurisation was terminated. The bags were then left overnight in the autoclave or oven to cool to room temperature.

2.2. Mushroom cultivation

Grain spawns of commercial wood ear mushroom strain M9610 (Mycelia BVBA http://www.mycelia.be/) was used in this study. The inoculation process was performed in a laminar-flow cabinet. Each substrate bag was inoculated with 25 g of the inoculum (2.5% of wet mass), after which the bags were placed in an incubation room. During the first four weeks of incubation, the bags were kept in darkness at a temperature of about 22–24 °C. Once the entire substrate cylinder was covered with mycelia, the maturation phase was induced using light (<500 lx) and an air temperature of around 19–21 °C. Six columns of small holes (1–2 mm), with ten holes per each column, were punctured through the plastic bag all around the lateral area of each synthetic log of the substrate, allowing for mycelium penetrating and fruiting.

2.3. Substrate sampling and mushroom fruit bodies harvest

The substrate samples were collected as follows: (1) raw substrate samples were collected right after ingredients blending but before pasteurisation; (2) initial substrate samples were taken from one of the replicates immediately after inoculation (Day 1); (3) one of the replicated bags was collected when the substrate was fully covered by fungal mycelia (colonisation period); (4) the remaining replicated bags were used for harvesting and estimating the yield of fruit bodies, after which the SMS were collected. Every substrate sample was from the entire substrate bag and dried at 45 °C until constant weight and then milled to ≤0.5 mm before further

Table 1
Substrate ingredients and pasteurisation treatments.

Treatment	Substrate ingredients (g per kg wet mass)					Pasteurisation		
ID	Birch sawdust	Wheat bran	Wheat grain	Water	$CaCO_3$	Medium	Temperature (°C)	Duration (h)
1	280	35	35	650	3.5	Steam	121	3
2						Hot air	100	3
3						Hot air	85	3
4						Hot air	75	3

Particle size: birch sawdust 0.5–2 mm, wheat bran ≤ 2 mm, wheat grain 3–5 mm.

analysis. The mushroom yield was defined as the fresh weight (g) per kg dry raw substrate, where the fresh weight was normalised to 90% moisture content.

2.4. Compositional analysis of the substrates

The determination of extractive contents was performed by successive extraction with water and ethanol according to an NREL protocol [18]. Extractive-free materials were then air-dried and stored for the determination of structural components by analytical acid hydrolysis [19]. Klason lignin was determined gravimetrically, and acid-soluble lignin was determined spectrophotometrically at 240 nm (Shimadzu, Kyoto, Japan). The amount of carbohydrates was determined by gas chromatography in combination with mass spectrometry (GC-MS instruments Agilent Technologies 6890N GC connected to MSD 5973) based on a previously described protocol [20].

2.5. Enzymatic hydrolysis of SMS

The enzymatic saccharification of the raw substrate and SMSs from the 121 °C and 85 °C pasteurisation treatments were assayed according to a method described by Gandla et al. [21]. The used protocol, including the enzyme dosage, was based in the method that we have used in previous studies with SMS [11,22]. The substrate was suspended in 50 mM sodium citrate buffer (pH 5.2) at a 10% consistency. After that, the commercial enzyme preparation Cellic CTec2 (acquired from Sigma-Aldrich Chemie GmbH, Steinheim, Germany), a blend of cellulases, β-glucosidases and hemicellulases, was added at a load of 200 CMCase units/g biomass. After adding the enzyme blend, the flasks containing the reaction mixture was incubated for 72 h in an Ecotron orbital incubator (INFORS HT, Bottmingen, Switzerland) at 45 °C and 170 rpm. At the end of hydrolysis, (1) the hydrolysate was separated by centrifugation, and a sample was diluted, filtered, and subjected to HPLC (Shimadzu, Kyoto, Japan) analysis for quantitation of glucose, the enzymatic digestibility was calculated as the percentage of hydrolysed glucan out of the amount contained in the substrate; (2) the solid leftover was air-dried until 90% DM content and stored for further analysis. All measurements were performed in duplicates, and the average values are reported.

2.6. Estimate of the theoretical yield of ethanol

The theoretical yield of ethanol was calculated according to the following equation [9]:

Y_{EtOH} (mL) = [mass of SMS (g)] * [glucan content (%)] * [enzymatic digestibility (%)] * 1.11 * 0.511/0.789.where 1.11 is a factor considering the addition of water molecule during hydrolysis, 0.511 (g g^{-1}) is the theoretical yield of ethanol from glucose by fermentation [23,24], and 0.789 is the density of ethanol (g mL^{-1}).

2.7. Fuel characterisation of the solid leftover from enzymatic hydrolysis

To investigate whether the solid leftovers after enzymatic hydrolysis can be used as fuel for heat production, samples of the hydrolysis residues corresponding to processing after both pasteurisation treatments were analysed for their fuel characteristics. The samples were analysed by a certified laboratory (EUROFINS, Sweden) for the contents of ash, calorific values, main energy elements (C, H, O, N, S), and main ash forming elements. Samples of raw substrates, initial substrates and SMS from the same treatments were also analysed for comparison.

To be able to understand the fuel quality of the leftover after enzymatic hydrolysis, it is important to explore how elements in the fuel may react during combustion. As the first step, three molar ratios were adopted to predict ash transformation behaviors: Cl/(K + Na), Na/(K + Na), and (K + Na + Ca + Mg)/(P + Si). The ratio Cl/(K + Na) has been shown to correlate with the proportion of alkali that could easily be vaporised as volatile chloride [25]. When this ratio is > 0.3, there is a risk for the formation of corrosive chlorine-rich deposits. The ratio Na/(K + Na) has been used to indicate whether there is a risk of forming a eutectic phase [25], i.e. an unusual reduction in the melting temperature of the ash that occurs when the contents of K and Na are similar. The hazardous range for this ratio is suggested to be between 0.2 and 0.8. The index (K + Na + Ca + Mg)/(Si + P) was introduced to indicate the probability of the leftover ash for deposits and formation of fine particulate emissions [25,26].

Additionally, the ash composition was further investigated. Ash was produced by combusting ground samples (30 g) of each substrate and leftover in a laboratory-scale muffle furnace at 1000 °C. The produced ashes were thoroughly homogenised by grinding them in a mixer mill (MM 400 Retsch GmbH, Germany). The elemental composition of each ash sample was analysed using a Carl Zeiss EVO LS15 scanning electron microscope combined with an Oxford Instruments Xmax-80 energy dispersive X-ray spectrometer (SEM-EDS). Six area analyses on each sample were performed, and the average values were reported. As a complementary analysis, the ash fusion process was also analysed at a certified laboratory (EUROFINS, Sweden) following the ISO 21404:2020 standard method. The shrinkage, deformation, hemispherical, and liquid temperatures were determined. In parallel, the ternary phase diagrams suggested by Böstrom et al. [27] were adopted to make general predictions of ash behaviour, especially for slagging tendencies during combustion.

2.8. Data analysis

One-way ANOVA followed by Post Hoc Multiple Comparisons (Duncan) was conducted to analyse the difference ($p < 0.05$) between data using SPSS statistical analysis software (IBM SPSS version 26.0).

3. Results and discussion

3.1. Wood ear mushroom production

There were no significant differences between the pasteurisation treatments in fungal colonisation rate (data not shown) and fructification time. The wood ear fruit bodies were harvested between 151 and 156 days after inoculation. Only one flush was harvested, and the average moisture contents was comparable (around 90%) for all the collected batches.

The average yields of fresh (90% moisture) fruit bodies ranged from 139.2 ± 21 to 217.1 ± 47 g per kg dry raw substrate. There was no statistically significant difference in the yield between pasteurisation treatments ($P > 0.5$). Compared with our previous estimation in a global context [17], in which the between 300 and 700 g fresh wood ears per kg dry substrate were obtained in total, the yields achieved in this study are relatively low. The harvest of only the first flush might be a possible explanation. A pilot study by us indicated that the first flush could contribute 39–54% to total production from 4 harvests/flushes. Nevertheless, the present study demonstrated that hot air pasteurisation as low as 75 °C is as effective as saturated steam at 121 °C and 2 bar in allowing wood ear mycelium to complete the whole biological cycle and yield comparable production of fruit bodies.

3.2. Compositional changes of the substrate over time

Regardless of the pasteurisation method, the content of the major components of the initial substrates (Day 1) was comparable to that of the raw substrates (Fig. 1). This is in agreement with a previous report by Xiong et al. [9], showing that hot air pasteurisation did not significantly differ from autoclaving in affecting in the way they affect lignocellulose composition.

The comparison of the composition of the raw substrate and the spent mushroom substrate (SMS) reveals that wood ear cultivation led to a general reduction in the content of lignin (Klason lignin and acid-soluble lignin), xylan, and glucan, and to an increase in extractives content in the substrates over time (Fig. 1). There was an increase of 8.4 and 3.2% in glucan content for substrates treated at 121 °C and 100 °C from day 1 to full fungal colonisation. The slight increase was consistent with results reported previously for shiitake [9]. They might be attributed to possible changes in the wood particle structure (e.g. cracks) caused by pasteurisation under high temperature, and affecting lignocellulose degradation by fungi. That phenomenon was not observed for the substrates pasteurized at 75 °C and 85 °C, which displayed decreased glucan contents in all the cultivation phases.

After cultivation, a stronger reduction in the content of xylan and lignin than that of glucan could be detected (Fig. 1). The pasteurisation treatment had a significant effect on the contents of lignin, xylan, and glucan in the SMS and hot air with 75 °C resulted in the largest decrease in all of them ($P < 0.05$). The largest reduction of the relative amount of the main lignocellulose components with the 75 °C heat treatment was somehow associated with the highest content of extractives in SMS. It could be hypothesised that the lower pasteurisation temperature (75 °C) allowed survival of other microorganisms that also degraded the lignocellulose in the substrate [9]. Nevertheless, the compositional changes over time are much larger than what can be attributed to heat treatments, which also agrees with our previous study on shiitake [17].

3.3. Mass reduction of substrate

After mushroom harvest, 36.7–39.1% of the raw substrate mass was recovered as SMS (Table 2), suggesting > 60% mass degradation

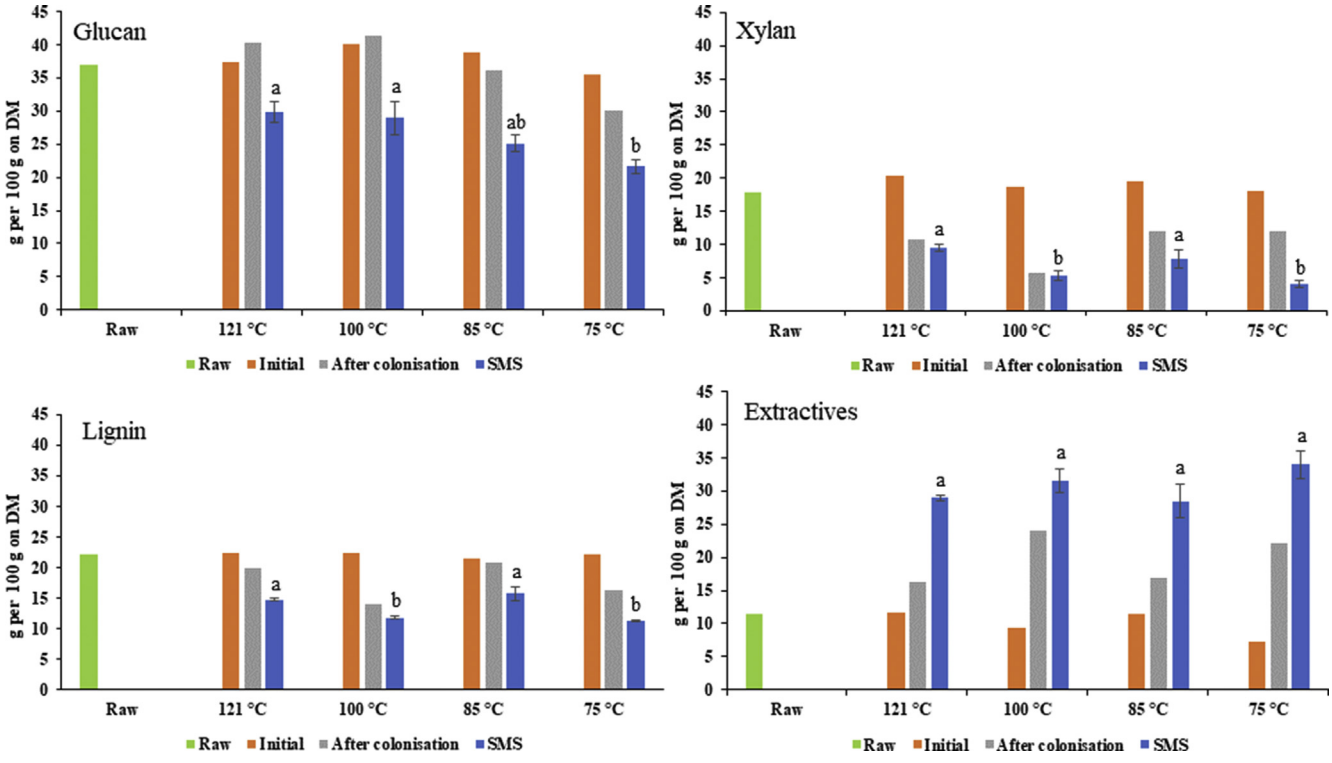

Fig. 1. Content of the major components in the substrate before pasteurisation (Raw), at the beginning of incubation (Initial), after full colonisation, and after harvest (SMS). Data refer to mean values ± standard error (SE). Different letters (a, b) indicate significant differences between the treatments ($P < 0.05$).

Table 2
Mass change of substrate during wood ear cultivation and enzymatic hydrolysis, and theoretical yield of ethanol. Data refer to mean ± SE.

	Unit	Pasteurisation treatments			
		121 °C	100 °C	85 °C	75 °C
Raw substrate	per 100 g	100	100	100	100
SMS		37.1 ± 2.2a	39.1 ± 2.3a	36.7 ± 3.1a	38.7 ± 0.1a
Ratio of remaining component mass in the SMS to the content in raw substrate					
Lignin	% (w/w)	24.7 ± 0.5ab	21.2 ± 0.7ab	26.8 ± 3.5a	20.3 ± 0.2b
Glucan	% (w/w)	30 ± 2.5ab	31.3 ± 3.1a	25.5 ± 2.2ab	23.2 ± 0.9b
Xylan	% (w/w)	19.7 ± 1.4a	11.8 ± 1.3bc	16.6 ± 3.0ab	9 ± 0.9c
Ratio of recovered leftover after enzymatic hydrolysis to initial SMS mass					
Leftover	% (w/w) SMS	75.1 ± 3.3	NA	86.7 ± 0.6	NA
Potential ethanol yield from fermentation of SMS hydrolysates					
	mL/kg SMS	98.7 ± 8.2	NA	83.2 ± 4.5	NA

Values within a row followed by different letters (a, b and c) indicate significant differences between the treatments ($p < 0.05$). NA = data not available.

during cultivation. No remarkable effect of pasteurisation treatments on SMS recovery was observed ($P > 0.5$). There was 20.3–26.8% of lignin and 23.2–31.3% of glucan mass recovered, with significant differences ($P < 0.05$) between the heat treatments (Table 2). Meanwhile, significant differences ($P < 0.01$) were observed for xylan recovery, which ranged between 9% (75 °C) and 19.7% (121 °C). Remarkably, the mass degradation of the total substrate and carbohydrates (glucan and xylan) in this study was much more than those found in shiitake cultivation on the same substrates for the same running time (ca. 150 days) as this study [9], while the mass degradation of lignin was comparable. The high mass loss of total substrate can be attributed mainly to a high degradation of glucan and xylan by wood ear. Wood ear seems to produce a non-selective degradation pattern in terms of the preferential removal of lignin or glucan but it requires higher amount of hemicelluloses to grow.

3.4. Enzymatic hydrolysis

For assessing how the fungal growth affected the susceptibility of the substrate to cellulolytic enzymes, parallel enzymatic hydrolysis assays were performed for the raw substrate and the SMSs resulting from the cultivations including 121 °C and 85 °C pasteurisation treatments. The hydrolysis of the SMSs resulted in a glucan digestibility corresponding to 46% of the theoretically based on the content of the SMS sample in the assay (Fig. 2). Even if the hydrolytic conversion was rather modest, it was 2.3 times higher than the value achieved in the hydrolysis of the raw substrate (20% of the theoretical value). This indicates that wood ear mushroom cultivation led to certain enhancement of the enzymatic hydrolysis of cellulose. No significant differences in the glucan digestibility during enzymatic hydrolysis were observed between the SMS samples from autoclaving (121 °C) or hot air (85 °C) pasteurisation. This result is consisting with previous study, where the choice of the pasteurisation method did not affect the digestibility of the cellulose retained in the substrate after shiitake cultivation [9]. An implication of this result is that both pasteurisation treatments contributed to a similar extent to reduction of the recalcitrance of the substrate. Anyway, the hydrolysis of the SMS corresponding to the pasteurisation by autoclaving resulted in a higher glucose yield (152.3 g kg^{-1}) than that of the SMS from the hot air pasteurisation (128.5 g kg^{-1}) (Fig. 3). However, that difference can be attributed to the higher glucan content in the SMS from the pasteurisation by autoclaving (29.8%) compared with that of the hot air treatment (25.1%) (Fig. 1).

Assuming that the theoretical yield is achieved in the fermentation of the produced hydrolysates with baker's yeast (*Saccharomyces cerevisiae*), ethanol production of 99 mL per kg of SMS can be expected from the process including autoclaving as pasteurisation method and 83 mL per kg for hot-air pasteurized process. Based on a mass balance over the whole process, the potential ethanol yields would be 37 and 31 mL per kg of raw substrate, respectively, for autoclaving and hot air pasteurisation (Table 2).

It should be noticed that in spite of the relative enhancement of

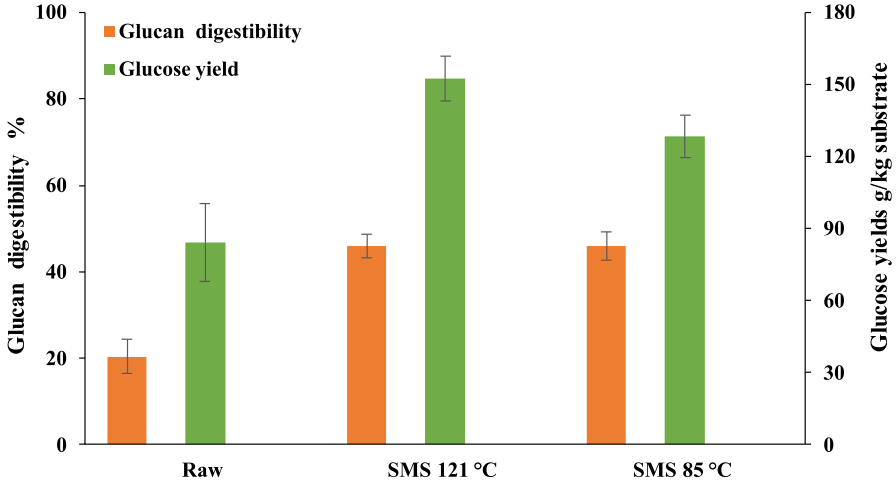

Fig. 2. Enzymatic digestibility of glucan contained in the raw substrates and in the SMSs. The glucose yield is the amount of glucose resulting per each kg of material subjected to enzymatic hydrolysis. Data refer to mean values ± SE.

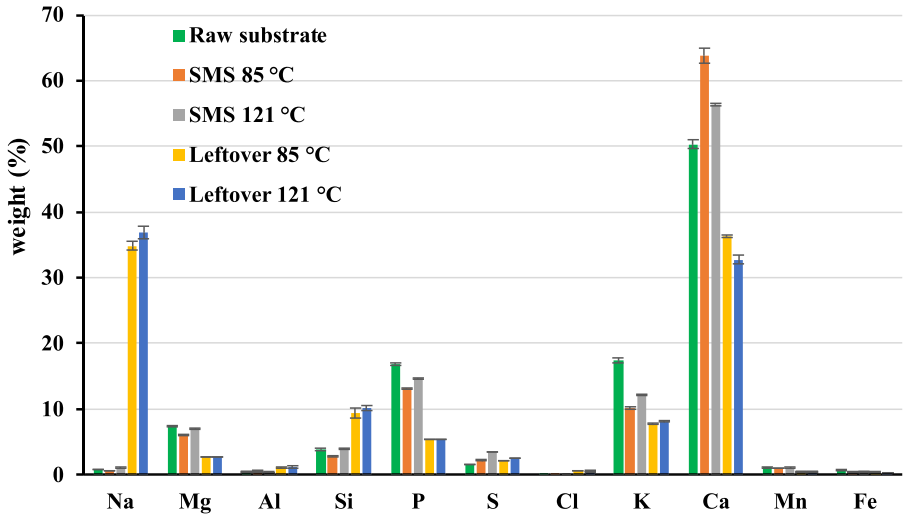

Fig. 3. Elemental composition (SEM-EDS) of the ash produced by calcination at 1000 °C of the raw substrate, SMS, and solid leftover from the enzymatic hydrolysis of SMS, % (w/w). The values are normalised to 100% for each element. Data refer to mean ± SE.

the enzymatic hydrolysis of glucan by cultivation of wood ear mushroom on the lignocellulosic substrate, the effectiveness of such a system as pretreatment method for improving cellulose bioconversion is lower than as previously reported for shiitake. The observed glucan digestibility in this study with wood ear mushroom (46%) was far from the enzymatic digestibility reported for shiitake in a previous study by this group (87%) [8,9], and lower than the value (62%) reported by Lin et al. [8], and than those reported for the white-rot fungus *Irpex lacteus* on agricultural residues, such as corn stover (59%) and wheat straw (54%) [28]. Anyway, the results of the current study are comparable with the values achieved in some of the initial studies with shiitake (40%) [29], and higher than the values (23–38.9%) achieved after oyster mushroom cultivation on different substrates [11,30,31], and after *I. lacteus* cultivation on corn cobs (30%) [28].

3.5. Fuel characterisation of substrates and solid leftover after enzymatic hydrolysis

Since more than 50% of the cellulose in the assays was not hydrolysed, a large part of the SMSs used in the enzymatic hydrolysis assay remained as a solid leftover, which, in addition to cellulose, contains also the whole lignin fraction and unhydrolysed hemicelluloses. The yield of solid leftover was 75% for the SMS from the autoclaving-based process and above 80% for the one using hot air pasteurisation (Table 2). The lower mass recovery of the hydrolysis residue corresponding to the pasteurisation by autoclaving was consistent with the higher glucan digestibility in the hydrolysis of that SMS (Section 3.4).

A way of economically dealing with such a large residue might be to use it as solid biofuel. In order to elucidate the fuel potential of the enzymatic hydrolysis residue its fuel characteristics were evaluated. The fuel characterisation included not only the solid leftover after enzymatic hydrolysis, but also the raw and initial substrates, as well as the SMSs corresponding to both pasteurisation treatments (Table 3).

Regardless of the pasteurisation method and temperature (even for 100 °C and 75 °C, though data are not shown), the elemental composition of the raw and the initial substrates were similar. Due to the degradation of the lignocellulose caused by the fungi during the growing period, the SMS has a lower C content and higher contents of main ash forming elements. For all substrates, the relatively high fraction of Ca could be explained by the addition of $CaCO_3$ that was used to adjust the pH of the substrate (Table 1). The most notable difference between the SMS and the solid leftover from enzymatic hydrolysis was a considerable increase in Na content, in contrast to the decreases in contents of most ash-forming elements (Table 3). The high Na content of the leftover from enzymatic hydrolysis was due to the sodium citrate buffer that was used during the process (See section 2.5). The content of the major ash forming elements, such as Ca, K, P, Si, and Mg, decreased due to the enzymatic hydrolysis. Part of these elements was probably dissolved in the hydrolysates. Although the calorific value of the substrates decreased slightly during mushroom cultivation and enzymatic hydrolysis, the ash-free net calorific values were comparable (18.0–18.5 MJ kg^{-1}).

Remarkably, the molar ratios indicated that the use of hydrolysis leftover as fuel had a rather low risk of either corrosion in conjunction with alkali chlorine [25] or formation of eutectic phases during the combustion due to an external addition of Na during hydrolysis. The Cl/(K + Na) of the leftover (0.01, Table 3) was not only lower than those in the SMS but was also far below the critical values of 0.3. The value of Na/(K + Na) of the leftover (0.91, Table 3) was much higher than the initial and spent substrates, but outside the hazardous range of 0.2–0.8. However, the leftover ash had a high ratio of (K + Na + Ca + Mg)/(P + Si) (6.2–7.7). According to a previous study [26], ratios above 3 indicate that the risk of fly ash particle emissions during combustion should be considered.

The elemental composition (SEM-EDS) of the ash produced at 1000 °C is shown in Fig. 3. It can be seen that the composition of the ash produced at 1000 °C resembles the composition of the fuel (Table 3), and Na, Ca, Si, and K accounts for approximately 90% of the ash mass for the solid leftover, compared to the raw substrate and SMS that were dominated by Ca, P, and K. The ash formed at 1000 °C is probably composed of different oxides and silicates so that each of them contributes with different quantities of vitrifying (Si, Al), melting (Na, K), and stabilising (Ca, Mg) agents in the final ash and so that different chemical features can be obtained [27].

An attempt to predict the ash behaviour of the studied samples during combustion was further carried out by plotting the elemental compositions of the produced ashes determined by SEM-EDS analysis, Fig. 3. The elemental compositions of each ash were

Table 3
Fuel characteristics of raw substrate, initial substrate, SMS, and solid leftover from enzymatic hydrolysis of SMS. All data are based on dry mass.

		Pasteurisation treatments						
			Autoclaving (121 °C)			Hot air (85 °C)		
Analysis	Unit	Raw	Initial	SMS	Leftover	Initial	SMS	Leftover
GCV	MJ kg^{-1}	19.5	19.5	18.4	17.9	19.1	18.2	17.9
NCV	MJ kg^{-1}	18.2	18.1	17.1	16.7	17.8	17.0	16.6
NCV ash-free	MJ kg^{-1}	18.5	18.5	18.1	18.4	18.1	18.0	18.0
Carbon (C)	w/w%	48.6	48.4	47.2	47.2	48.5	46.7	47.6
Hydrogen (H)	w/w%	6.1	6.2	5.8	5.7	6.2	5.6	5.8
Oxygen (O)	w/w%	42.8	42.9	40.1	36.0	42.9	40.0	37.3
Nitrogen (N)	w/w%	0.5	0.5	1.5	1.6	0.5	1.5	1.1
Sulphur (S)	w/w%	0.04	0.04	0.17	0.12	0.04	0.13	0.10
Chlorine (Cl)	w/w%	0.02	0.01	0.06	0.03	0.01	0.05	0.02
Aluminium (Al)	mg/kg	22	17	25	75	13	27	55
Phosphorus (P)	mg/kg	2100	2100	4400	2200	1800	4500	1900
Iron (Fe)	mg/kg	42	45	97	79	30	110	55
Calcium (Ca)	mg/kg	5200	5400	16000	11000	4900	20000	13000
Potassium (K)	mg/kg	2700	2200	6500	3400	2200	6600	2800
Magnesium (Mg)	mg/kg	870	850	2100	1000	790	2200	740
Manganese (Mn)	mg/kg	99	100	240	120	100	250	93
Sodium (Na)	mg/kg	<53	<52	250	21000	<52	220	17000
Silicium (Si)	mg/kg	560	550	3300	2800	1300	3700	3600
Ash (550 °C)	w/w%	1.9	1.9	5.1	9.4	1.9	6.1	8.0
molar ratios of								
Cl/(K + Na)		≈0.08	≈0.05	0.09	0.01	≈0.05	0.08	0.01
Na/(K + Na)		≈0.03	≈0.04	0.06	0.91	≈0.04	0.05	0.91
(K + Na + Ca + Mg)/(P + Si)		≈2.70	≈2.61	2.55	7.71	≈2.04	2.77	6.15

GCV, Gross calorific value; NCV, Net calorific value.

normalised to the contents of Na, K, Ca, Mg, Si, and P, recalculated to the oxide form, and then plotted in the ternary phase diagram (Fig. 4) for the (Na_2O + K_2O)–(CaO + MgO)–SiO_2/P_2O_5 system proposed by Böstrom et al. [27]. Liquidus isotherms indicate compositional areas where strong slagging tendencies can be anticipated, as well as the directions where these trends are declining. As can be seen, the positions of the SMSs were similar to the raw substrate. The high relative amounts of (CaO + MgO) of the two is a common feature that determines ash behaviour. The upper part of the triangle suggests that SMSs have high ash-fusion temperatures, which means a low slagging tendency during combustion.

The positions of the ash from the solid leftovers (from both the 121 °C and 85 °C pasteurized substrate) were grouped together toward to the (Na_2O + K_2O) corner of the ternary diagrams (Fig. 4). They were located at the low-risk zone of slagging. Theoretically, as the concentration of Na increases, the ash melting temperature may decrease. Ash fusion analysis (Table 4) indicated that a decrease in shrinkage temperature from 1460 to 1490 °C of SMS to 755–785 °C of leftover did occur. However, the liquid temperatures of the ash from these leftovers were still above 1500 °C (Table 4), probably due to the $CaCO_3$ addition from the mushroom cultivation. This, together with the fact that the ratio of Na/(K + Na) was not plausible to lead a eutectic phase formation, indicates a low

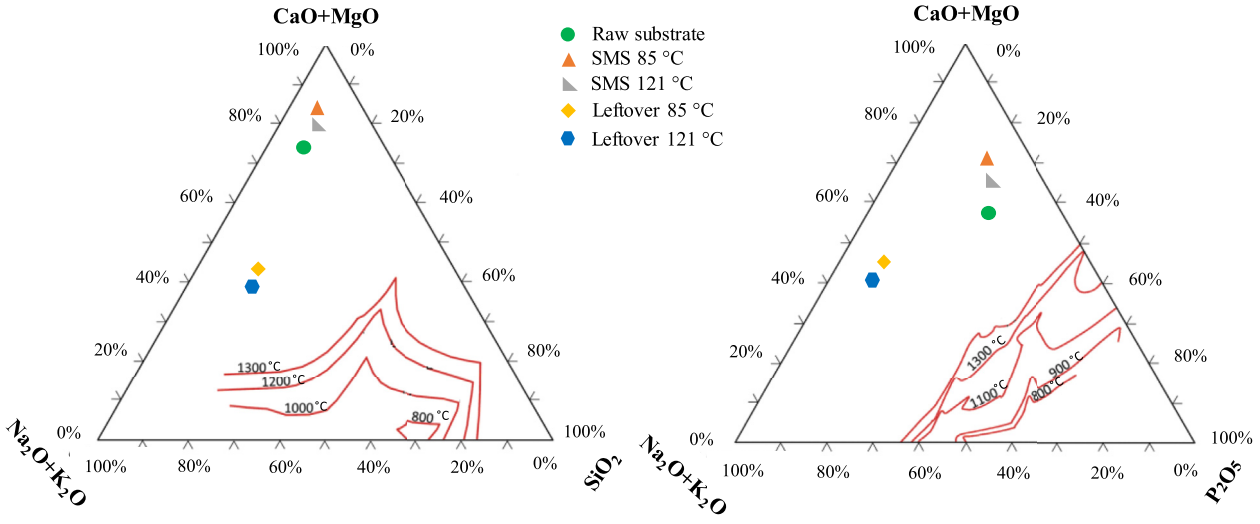

Fig. 4. Compositional triangle (weight per cent) for the system (Na_2O + K_2O)–(CaO + MgO)–SiO_2/P_2O_5. Contents of the three axes are given in weight fractions and normalised to 100%. Liquidus isotherms for the ternary phase diagram are adopted from Böstrom et al. [27].

Table 4
Ash melting behaviour of raw substrate, SMS, and solid leftover from the enzymatic hydrolysis of SMS.

		Pasteurisation treatments				
		Autoclaving (121 °C)			Hot air (85 °C)	
Ash melting process	Unit	Raw	SMS	leftover	SMS	leftover
Shrinkage temperature	°C	1330	1490	755	1460	785
Deformation temperature	°C	>1500	>1500	>1500	>1500	>1500
Hemispherical temperature	°C	>1500	>1500	>1500	>1500	>1500
Liquid temperature	°C	>1500	>1500	>1500	>1500	>1500

tendency for slag formation at combustion temperatures in fixed- and fluidised bed technologies.

3.6. Potential of combined production of food and bioenergy

This study has demonstrated a new approach for the integrated production of food and biofuel from wood residues, in which three products were produced one after the other: edible mushroom as food from the cultivation, fermentable sugars from the enzymatic hydrolysis of cellulose contained in the SMS, and fuels from the solid leftovers after enzymatic hydrolysis. These results suggest that, with the experimental settings and materials of this study, every kg of dry raw substrate may potentially produce about 200 g of wood ear fruit bodies, 31–37 mL ethanol, and 280–320 g DM combustible fuels of 4639–5295 kJ thermal energy. The fact that both autoclaving and hot air pasteurisation resulted in comparable yields of wood-ear fruit bodies suggest a possibility of saving up to 60% of the energy (2240 vs 814 kJ kg^{-1} dry substrate) used for mushroom cultivation today [17]. Compared with the raw substrate, the SMS generated after cultivation was more susceptible to be hydrolysed, and it yielded 2.3 times more glucose, which can converted to ethanol by fermentation with yeast. That suggests that fungal growth, due to the concomitant removal of lignin and hemicelluloses, is a pretreatment method that contributes to enhancing the enzymatic hydrolysis of cellulose contained in the lignocellulosic substrate. The fuel characterisation of the recovered leftovers after enzymatic hydrolysis provides clear indications of useable biofuel for combustion. In consideration of bioeconomic development, this scenario has a considerable potential, especially in the aspect of energy-and-resource efficiency and near-zero-waste mode for production from lignocellulose feedstock.

However, several challenges have to be tackled in future studies. One of them is that the substrate mass loss (up to 60% DM of the raw substrate) during the cultivation in this study was higher than that after shiitake and oyster cultivation (up to 30–40%) [11,22], which was due to a high consumption of cellulose and hemicelluloses. A consequence is the low yield of ethanol (Table 2). An optimisation of cultivation substrate and environment (temperature, light, and humidity) should be studied. The high content of Na in the ash of the solid leftover after enzymatic hydrolysis may cause particle emissions during combustion. However, the formation of fine particulate emissions can be easily controlled using existing technologies, for instance, the soot blowing techniques [32] have been widely used to reduce the deposition.

This study has an implication on an alternative scenario for combined mushroom and combustible fuel only. That is to say, SMS may be directed to solid fuels for heat production as a part of self-energy support system for a mushroom producer [33]. As shown in this study (Table 3), every kg of DM raw substrate may end up as 370 g DM SMS that could be recovered as combustible fuels providing 6240 kJ thermal energy. However, both economic and environmental systematic analyses are needed before an industrial application.

The proposed approach of this study could have also an implication in bioconversion systems based on other white-rot fungi species than wood ear. For example, shiitake could be good model in a consideration of more sugar production, since it displayed a relative stronger effect on enhancing cellulose susceptibility to enzymatic hydrolysis [22]. Analyses on compositions of the leftover and mass balance regarding to product options would be necessary for a substantial evaluation.

4. Conclusions

This investigation revealed that wood ear mushroom has good potential for integrated production of food, fermentable sugar, and solid fuels using forest industry residues. Hot-air pasteurisation of substrates (100-85 °C) and autoclaving with 121 °C pressurized steam resulted in comparable mushroom yields and lignocellulose degradation. The spent substrate generated after fungal cultivation displayed enhanced enzymatic digestibility in cellulose saccharification compared with that of the raw substrate, as shown by the 2.3 times higher glucan digestibility in hydrolysis. After enzymatic hydrolysis, 75–87% of SMS was left as a solid leftover that had energy value as high as the raw substrate and revealed promising combustion characteristics. It would be ideal to recycle leftover for self-supporting system and extra space heating.

CRediT authorship contribution statement

Feng Chen: Investigation, Writing - original draft, Writing - review & editing. **Alejandro Grimm:** Investigation, Writing - original draft. **Lill Eilertsen:** Investigation. **Carlos Martín:** Writing - original draft, Writing - review & editing. **Mehrdad Arshadi:** Investigation. **Shaojun Xiong:** Conceptualization, Project administration, Funding acquisition, Data curation, Writing - original draft, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Potential for combined production of food and biofuel: Cultivation of *Pleurotus pulmonarius* on soft- and hardwood sawdusts

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ABSTRACT

This study aimed at developing an integrated process of production of edible summer oyster mushroom (*Pleurotus pulmonarius*) and preprocessing of the substrate lignocellulose for producing 2nd-generation biofuels based on softwood. Sawdust-based mushroom substrates of softwood spruce (*Picea abies*) versus hardwood alder (*Alnus glutinosa*) as a reference were used for production of summer oyster mushrooms. The substrates had been either hot-air pasteurised or steam sterilised before growing the mushroom. The potential of using spent substrate (SMS) after harvest for biofuel production was evaluated by examining the lignocellulosic composition and enzymatic convertibility. The biological efficiency of the substrates ranged 14.0–33.8% and no significant difference was observed between the treatments. The fruiting bodies had similar total protein concentrations ranging between 26.0 and 28.5% regardless of differences in treatments. The average mass degradation of Klason lignin and acid soluble lignin in the substrates after mushroom production were 35.0 and 22.6%, respectively. Glucan, the major carbohydrate component, was initially present in concentrations ranging from 24 to 29% of total dry matter and with similar concentrations observed in both alder-based and spruce-based substrates. After mushroom production, a significant difference was observed between the substrates with the lowest consumption of glucan, 3.9% of the initial mass, in the spruce-based substrate. The selective degradation ability of *P. pulmonarius* on the lignin fraction, rather than the cellulose component of softwood, is suggested in the present study. Between 84 and 126 g glucose was yielded per kg of dry SMS, spruce based substrates resulted a higher yield than alder substrate from enzymatic saccharification of the spent substrates. The heat treatment of the mushroom's substrate had in general a minor impact on the mushroom production and fungal pretreatment of the substrates; hot-air pasteurisation is apparently more energy efficient method than steam sterilisation.

1. Introduction

The growing world population demands increased food and energy production and there is a need for new production systems (Specht et al., 2014) and biobased-circular-economy strategy to increase resource and energy efficiency while maintaining ecological sustainability. This calls for a broader understanding and innovative ways to recycle nutrients from biomass resources back into food production as well as for energy production. Recent studies have suggested that a combined production of protein-rich edible fungi and bioethanol/biogas fuels could be an interesting strategy (Lin et al., 2015; Xiong et al., 2019), in which the cultivation of white-rot edible mushrooms, using indigenous biomass resources as substrate material, is functioning as pretreatment to delignify the substrate during cultivation and enabling enzymatic conversion of the spent substrate to 2nd generation biofuels. However, the effectiveness of such a system, in terms of both produced mushroom (quantity and quality) and substrate lignocellulose degradation and saccharification, may vary with fungal species, substrate ingredients (e.g. soft-vs. hardwood) and cultivation processes, which emphasizes a necessity of researches.

Edible white-rot fungi, such as most oyster mushrooms, are usually cultivated on lignocellulosic plant material provided it has a suitable composition (e.g., carbon/nitrogen ratio) and a physical

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structure that allows gas exchange during fungal growth (Sanchez, 2009). A commonly used substrate for commercial mushroom production is sawdust from hardwood amended with a nitrogen source such as bran (Stamets, 2000). Sawdust from softwood, such as spruce and pine, are difficult to colonise for most white-rot fungi and is rarely used for fruiting body production. The reasons for the low colonisation are poorly understood but the presence of volatiles and extractives are pointed out as inhibitory factors (Croan, 2004). However, from the perspective of the northern hemisphere, where most forests and wood resources are dominantly composed of coniferous tree species such as spruce and pine, it is desirable to extend the substrate range from hardwood to softwood. In this context, it is of interest that oyster mushrooms are aggressive colonisers of a wide array of substrates (Fernandes et al., 2015). There are also reports on cultivation of summer oyster mushroom *Pleurotus pulmonarius* on logs of softwood (Stamets, 2018). However, few studies, if any, have been quantifying and qualifying the effects of softwood substrates on potential industrial production of *P. pulmonarius*. To be able to develop combine production of mushroom and biofuel, it is important to understand the compositional changes of coniferous substrate during the mushroom cultivation, which is lack today.

Biofuel production through cellulosic process is one of the most promising technologies to replace fossil fuel and support bioeconomy development (Formas, 2012). Bioconversion of lignocellulosic feedstocks to advanced biofuels such as ethanol is based on a sugar platform process, in which hemicelluloses and cellulose are converted to monosaccharide sugars for further processing. Efficient bioconversion is however hampered by the recalcitrance of the feedstock, which to a large extent can be explained by the negative effect of lignin on convertibility. Thus, pretreatment for delignification (Jönsson and Martin, 2016; Singh et al., 2015) is a prerequisite to overcome recalcitrance and allow enzyme accessibility to cellulose and maximise product recovery for improved economics of second-generation lignocellulosic biorefineries. Today, physiochemical pretreatments are dominant methods, but they end up in high economic cost in addition to environmental consequences. Since white-rot fungi have a high capacity for extracellular release of powerful lignin-degrading enzymes such as laccases and peroxidases, biological pretreatment of lignocellulosic feedstocks can be an alternative method to physiochemical pretreatment (Singh, 2006; Wan and Li, 2012). Compared with established methods, fungal pretreatment has advantages of lower capital cost, fewer chemicals and less environmental impact (Shirkavand et al., 2016; Wan and Li, 2012). Furthermore, the use of edible and protein-rich white-rot fungi, such as shiitake and oyster mushrooms with a high market value, for example Swedish price of them is today approximately 15 € per kg fresh weight, may further improve the economy of the process due to parallel production of fruiting bodies. Oyster mushroom such as *P. pulmonarius* may have advantage to be applied in this context, since it often have a shorter cultivation cycle (about 40 days Wu et al., 2019) than our previously studied shiitake (>130 days Xiong et al., 2019). This may reduce the concerns about the drawback of slow rate of edible fungal pretreatment compared with physiochemical methods.

An important step in fruiting body production is heat treatment of the substrate, by pasteurisation or autoclavation, in order to deactivate competitive microorganisms. Conventional methods such as saturated steam are often used (Sanchez, 2009) with the disadvantage of high energy demand. Our recent study (Xiong et al., 2019) has demonstrated that hot-air pasteurisation, with temperatures as low as 75 °C, is a promising low-cost alternative to steam sterilisation of shiitake substrates. The production of hot air consumes 40% less energy and is much cheaper and simpler than that of saturated steam for sterilisation (Xiong et al., 2019). While saturated steam has to be produced using costly pressurised equipment, leading to even more expenses for maintenance, the hot air can be obtained from recovered or waste heat or produced using a small-medium hot water boiler. Our study showed also that hot-air pasteurisation resulted in improved growth and yield of shiitake parallel with a slight change in composition of the used lignocellulosic feedstock. Thus, it is worth to examine whether the hot air pasteurisation may also have an impact on quantity and quality of *P. pulmonarius* production and degradation of lignocellulose in the substrates.

The objective of present study was to investigate the potential of using softwood (spruce) for co-production of oyster mushrooms *P. pulmonarius* and bioethanol fuel, using hardwood (alder) as a reference. The study will examine the mushroom yield and quality, lignocellulose degradation in substrate, and glucose conversion from spent mushroom substrate (SMS). Also, the impact of two different heat treatment regimes, namely hot-air pasteurisation and steam sterilisation, were studied in order to determine whether it is possible to use hot air pasteurisation to further level up cost- and energy-effectiveness of the co-production system.

2. Materials and methods

2.1. Mushroom production

The summer oyster mushroom strain *P. pulmonarius* (Fr.) Quél. (phoenix oyster) was used in the experiments. The strain was obtained from Fungi Perfecti LLC, Olympia, USA as an agar culture. Spawn was produced by propagating the strain for 12 days on sterile rye grain amended with 4% $CaCO_3$ and 2% $CaSO_4$ (w/w of dry mass) at 25 °C.

For preparation of the mushroom substrates, fresh sawdust from spruce (*Picea abies*) was obtained from the wood producer Setra, Sweden. Sawdust from alder (*Alnus glutinosa)* was obtained from a pet store (VetZoo AB, Kista, Sweden). The sawdust was dried at 45 °C to constant weight and milled to a size of 2–4 mm. All substrates were based on sawdust (73% dry weight), wheat bran (24.6% dry weight) and calcium sulfate (2.4% dry weight). Three substrates were prepared based on either alder sawdust only, mixed (1:1 dry weight/dry weight) alder and spruce sawdust (alder/spruce) and spruce sawdust only, respectively. All three substrates were rewetted to a moisture content of 65% using distilled water. In aliquots of 0.5 kg substrate (wet weight), the substrates were packed into gas-permeable bags suitable for mushroom production (Sac O_2, Nevele, Belgium). The bags were either autoclaved by steam (121 °C for 15 min) or pasteurised by hot air at 65 °C inside an air dryer for 8 h. Every treatment was replicated three times.

After heat treatments and cooling down, spawn was added to each bag in a concentration of 10% (dry weight/dry weight). The inoculated substrate bags were cultivated at 24 °C and 75% humidity in a climate chamber. On day 16, the substrate was densely colonised by mycelium and fruiting body formation was induced by opening the bags, lowering the temperature to 17 °C and raising humidity to 90%. When fruiting body formation was clearly visible (day 22) the temperature was increased to 24 °C while keeping humidity at 90%.

2.2. Analysis

2.2.1. Mushroom production and quality

Both fresh and dry weight, after lyophilisation, of harvested mushrooms were determined. Mushroom yield (fresh weight) was related to the amount of substrate (dry matter), in order to determine the biological efficiency (BE) of the substrate, according to the formula:

BE = (fresh weight of mushroom/dry mass of substrate) x 100.

The total amount of protein in the fruiting bodies was analysed by the Dumas method (Bellomonte et al., 1987) using a Thermo Scientific™ FLASH 2000 CHNS/O Analyzer and a conversion factor of 4.38 for total nitrogen (Barros et al., 2008). Amino acid composition, including alanine, arginine, aspartic acid, cysteine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tyrosine and valine, was determined at a certified laboratory (Eurofins Food & Agro Testing Sweden AB, Linköping, Sweden) by ion exchange chromatography according to the method used by Llames and Fontaine (1994). To determine the concentration of the heavy metal cadmium, the lyophilised biomass was wet-combusted in HNO_3 (65%) using a microwave technique (CEN Mars 5) (Huang et al., 2004). The metal was then analysed by atomic absorption spectrometer (AAS) (Agilent Technologies 200 AA) amended with a graphite tube atomizer (GTA 120). A reference standard (CRM, BCR-679) was also analysed. For determination of amino acid composition and cadmium content samples from pretreatments, hot-air pasteurisation and steam-sterilisation, were pooled in a 1:1 ratio (dry weight/dry weight).

2.2.2. Evaluation of the spent mushroom substrate (SMS) for biofuel production

Initial substrate samples were collected after heat pretreatments but before inoculation of the fungi. The SMS samples were collected after the harvest of fruiting bodies. All substrate samples were dried at 45 °C for 72 h, then milled to a size of ≤0.5 mm and stored in air-tight plastic bags at room temperature before further analyses. The samples were analysed for extractives (water and ethanol), lignin (Klason and acid soluble), glucan and xylan using the NREL (USA National Renewable Energy Laboratory) method (Sluiter et al., 2008). After a two-step acid hydrolysis for extractive-free samples, glucose and xylose in the hydrolysates were analysed with a high-performance liquid chromatography (HPLC) (Shimadzu Kyoto, Japan) fitted with a Shodex NH_2P-50 4 E column and RI detector, using HPLC-grade acetonitrile as the mobile phase at a flow rate of 1.0 mL/min operating at 50 °C. Klason lignin was determined gravimetrically, while a spectrophotometer (Shimadzu, Kyoto, Japan) was used for acid soluble lignin spectrophotometric determination at 240 nm. Total nitrogen and total carbon content of the substrates were determined by Elemental Analyzer-Isotope Ratio Mass Spectrometer (EA-IRMS) at the Stable Isotope Laboratory at SLU in Umeå.

The mass of degradation of major components from the initial mass could then be calculated using the following equation:

$$\text{Mass reduction of component (\%)} = [1 - (\text{C-mass}_{sms}/\text{C-mass}_{Initial})]* 100$$

where C-mass refers to the mass of the component of either cellulose, hemicellulose or lignin, in the SMS and initial substrate, respectively.

The SMS samples with hot air treatment were used for enzymatic analysis. For each sample, 50 mg DM was suspended in 900 μL of 50 mM sodium citrate buffer (pH 5.2) in 2.0 mL Eppendorf tubes. The tubes containing the reaction mixture were placed in an Ecotron orbital incubator (INFORS HT, Bottmingen, Switzerland) at 45 °C and 170 rpm for 1 h for mixing and attemperation. After that, 6 μL of Cellic CTec2 enzyme blend, acquired from Sigma-Aldrich Chemie GmbH (Steinheim, Germany), was added so that the final activity was 100 CMCase units per gram of biomass, and the tubes containing the reaction mixture were incubated for 72 h under the above-stated conditions. At the end of the hydrolysis, the tubes were centrifuged; the supernatant was stored frozen until further analyses, and the precipitate was discarded. Glucose in the supernatants was analysed by HPLC and used for calculating the enzymatic convertibility of cellulose.

The following formula was used to calculate the theoretical yield of ethanol:

$$y\ (mL) = [\text{mass of SMS (g)}]*[\text{glucan content (\%)}]*[\text{enzymatic digestibility (\%)}]* 1.11*0.511/0.789$$

where 1.11 is a factor related to the addition of water during hydrolysis, 0.511 (g g^{-1}) is the theoretical yield of ethanol from glucose by fermentation (Krishnan et al., 1999) and 0.789 is the density of ethanol (g mL^{-1}).

2.3. Statistical analysis

Each experiment treatment was carried out in triplicate and mean values and standard error (SE) are reported. The data were analysed by analysis of variance followed by Tukey's multiple comparison test. Differences were considered significant at $p < 0.05$ (SPSS, version 21).

3. Results and discussion

3.1. Mushroom production and quality of the fruiting bodies

There was no visible difference between the mushroom substrates in the mycelial colonisation time and speed, and the fruiting bodies were harvested on day 26. No significant differences in the amount of produced fruiting bodies or BE were observed between the hot-air (65 °C) and steam-sterilised (121 °C) treatments for any of the mushroom substrates (Table 1). The BE values (14.0–33.8%), presented in Table 1, were comparable to reported BE values of 33.5% (Koutrotsios et al., 2014), who used beech sawdust as the main substrate. However, the BE values can still be considered low as oyster mushrooms may have BE values reaching above 100% (Koutrotsios et al., 2014). Part of an explanation for this low BE might be that only the first flush of fruiting bodies was harvested in the present study. Also, among mushroom producers, it is generally considered that the amount of mushroom substrate (wet weight) should not be below 1 kg in order to sustain high production of fruiting bodies. This, larger harvest of fruiting bodies when using a substrate amount larger than 1 kg, have been confirmed experimentally by Owaid et al. (2015). In the present study, the experiment was performed using a lower amount of substrates (0.5 kg wet weight). Thus, it is most likely that higher fruiting body production can be obtained in commercial production.

The fruiting bodies produced on the different substrates had similar total protein concentrations of 28.4–28.5% on the alder-based substrates, 27.5–27.6% on alder/spruce and 26.0–26.6% on spruce (Table 1). These values are in the range of previously reported figures for mushrooms (Kalac, 2013). It should be pointed out that variations in total protein concentration of fruiting bodies, dependent on the substrates used for cultivation, are commonly reported (Koutrotsios et al., 2014). Thus, the finding of similar total protein content suggests a similarity between the substrates. When the amino acid composition of the fruiting bodies was studied on pooled samples from both heat treatments, minor differences were observed in the fruiting bodies produced on the alder-based substrate demonstrating higher levels of three amino acids: glutamic acid, histidine and serine, as compared to the spruce-based substrate (Table 2). Of these amino acids, histidine is essential and the decrease in the fruiting bodies produced in the spruce-based substrate corresponds to a reduction of 13.4% compared to the alder-

Table 1
Three mushroom substrates based on sawdust of alder and/or spruce (with C/N-ratio) were pretreated by either hot air pasteurisation or steam sterilisation and used for cultivation of *P. pulmonarius*. The amount of fruiting bodies produced, the BE of the substrates and the content of protein in the fruiting bodies produced from the different mushroom substrates are presented below (mean ± SE).

Substrate	C/N-ratio	Pretreatment	Fruiting body (g, FW)	BE (%)	Protein (g/100 g dry weight)
Alder	74.5	Hot-air	31.6 ± 12.6a	17.4 ± 7.0a	28.5 ± 0.9a
		Steam	25.8 ± 8.8a	14.0 ± 4.8a	28.4 ± 0.2a
Spruce	97.2	Hot-air	48.2 ± 12.8a	26.5 ± 7.1a	26.6 ± 0.4a
		Steam	38.0 ± 20.2a	20.3 ± 10.8a	26.0 ± 0.4a
Alder/Spruce	85.9	Hot-air	61.3 ± 2.9a	33.8 ± 1.6a	27.6 ± 1.0a
		Steam	46.1 ± 14.7a	24.7 ± 7.8a	27.5 ± 0.6a

*Values within a column followed by different letters indicate significant differences between the rows ($p \leq 0.05$).

Table 2
Amino acid composition (g 100 g^{-1} dry weight) of fruiting bodies produced in the different substrates (mean ± SE).

Amino acid	Content in fruiting bodies		
	Alder	Spruce	Alder/Spruce
Alanine	2.08 ± 0.11a*	1.99 ± 0.02a	2.00 ± 0.10a
Arginine	1.59 ± 0.04a	1.52 ± 0.04a	1.43 ± 0.13a
Aspartic acid	2.78 ± 0.03a	2.62 ± 0.04a	2.63 ± 0.11a
Cysteine	0.33 ± 0.01a	0.32 ± 0.01a	0.32 ± 0.01a
Glutamic acid	7.07 ± 0.45a	5.25 ± 0.49 b	5.98 ± 0.21 ab
Glycine	1.37 ± 0.03a	1.31 ± 0.02a	1.30 ± 0.07a
Histidine	0.67 ± 0.01a	0.58 ± 0.02 b	0.63 ± 0.03 ab
Isoleucine	1.03 ± 0.03a	1.03 ± 0.01a	1.00 ± 0.11a
Leucine	1.74 ± 0.06a	1.69 ± 0.01a	1.68 ± 0.10a
Lysine	1.53 ± 0.05a	1.50 ± 0.02a	1.45 ± 0.11a
Methionine	0.48 ± 0.02a	0.48 ± 0.01a	0.45 ± 0.03a
Phenylalanine	1.20 ± 0.05a	1.16 ± 0.01a	1.15 ± 0.06a
Proline	1.84 ± 0.09a	1.67 ± 0.03a	1.75 ± 0.02a
Serine	1.53 ± 0.03a	1.30 ± 0.04 b	1.32 ± 0.07 ab
Threonine	1.48 ± 0.01a	1.31 ± 0.03a	1.33 ± 0.07a
Tyrosine	1.15 ± 0.05a	1.10 ± 0.01a	1.12 ± 0.05a
Valine	1.30 ± 0.04a	1.28 ± 0.01a	1.26 ± 0.07a

*Values within a row followed by different letters indicate significant differences between the columns ($p \leq 0.05$).

based substrate.

The cadmium content, also studied on pooled samples from both heat treatments, varied significantly between alder and spruce sawdust, ranging from 0.015 ± 0.003 μg kg^{-1} of dry weight in alder to 0.073 ± 0.007 μg kg^{-1} of dry weight for spruce. This difference was reflected in the amount of cadmium accumulated in the fruiting bodies, which was significantly higher in the spruce-based substrate, 0.79 ± 0.10 μg kg^{-1}, compared to the alder-based substrate, 0.49 ± 0.01 μg kg^{-1}, while the alder/spruce-based substrate had an intermediate concentration of 0.62 ± 0.08 μg kg^{-1}. For the most frequently traded mushrooms, including oyster mushrooms, the limit allowed have been set at 0.2 mg Cd/kg fresh weight (EU, 2006). The highest concentration of cadmium in fruiting bodies cultivated on spruce, related to the fresh weight, would have been approximately 0.14 μg kg^{-1} of fresh weight. Thus, the concentration of cadmium in the fruiting bodies was considerably below (by a factor of 1000) the statutory limit for traded mushrooms even in the replicate with the highest concentration.

The present study clearly demonstrates that fruiting body production is possible on softwood using the species *P. pulmonarius*. It can also be concluded that no significant effect was seen in total protein concentration depending on the different tree species used in the substrates. For amino acid composition and cadmium content, minor variation was observed between the substrates. However, no major disadvantage in using softwood compared to hardwood was observed.

3.2. Impact of treatments on substrate composition

3.2.1. Composition of initial substrate

When the content of water extractive, ethanol extractive, Klason lignin, acid soluble lignin, glucan and xylan were compared for the initial substrates (Table 3), lower content of acid soluble lignin and xylan was observed in the spruce-based substrate. Contents of Klason lignin and glucan are comparable regardless of soft- or hardwood substrate. The low content of xylan is in agreement with previous reports (Taherzadeh et al., 1997; Wang et al., 2018), which showed a considerably lower mass fraction of xylan in spruce than in hardwood such as alder, birch and aspen.

The effects of the heat treatments were generally minor, however, higher content of ethanol extractive in alder and acid soluble lignin in spruce after steam sterilisation were observed (Table 3). These observations may be explained by that saturated and pressurised steam is commonly used for pretreatment in biorefinery industries to alter the structure of the lignocellulosic biomass (Zimbardi et al., 2009).

3.2.2. Compositional and mass change during cultivation

As expected, due to degradation of organic carbon during fungal growth, oyster mushroom cultivation led to a reduction in substrate mass. This decrease ranged from 22.0 to 32.0% of the initial substrate dry mass (Fig. 1), and no differences were observed between the alder-based, alder/spruce-based or spruce-based substrates in this regard. However, there was a significant difference related to the heat treatments, and the hot-air pasteurisation resulted in lower mass reduction compared with steam sterilisation (Fig. 1). Taken into account that less effect was observed on the initial composition by the treatments (Table 3), this finding was unexpected. Still, this could be explained by changes in the physical structure of the woody biomass and release of volatile inhibitors (Kim et al., 2013; Wan and Li, 2012) due to the high temperature and pressure provided during steam sterilisation resulting in increased fungal metabolisation.

Parallel with the weight reduction, the chemical mass of substrates changed after mushroom production (Fig. 2). This is reflected in the finding of major changes in both the total amount and composition of the extractives after mushroom cultivation (Fig. 2A). The significant decrease in ethanol extractives parallel with an increase in water extractives after mushroom production suggests that the biochemical processes taking place during the mycelial growth results in an increase in more polar extractives, such as free sugars and fatty acids, during the lignocellulosic degradation (Lin et al., 2014).

The average mass degradation of Klason lignin in the substrates after mushroom production were 35.0% (Fig. 2B). No statistically significant difference ($p > 0.05$) was observed between the

Table 3
Contents of water extractive (W-EXT), ethanol extractives (E-EXT), Klason lignin (KLL), acid soluble lignin (ASL), glucan and xylan, in initial vs spent substrates of the alder-based and spruce-based materials (g 100 g^{-1} dry weight, mean ± SE).

Substrates		W-EXT		E-EXT		KLL		ASL		Glucan		Xylan	
		Initial	SMS	Initial	SMS	Initial	SMS	Initial	SMS	Initial	SMS	Initial	SMS
Alder	Hot-air	12.0 ± 1.6a	21.6 ± 0.3a	2.0 ± 0.1 b	1.0 ± 0.1a	20.6 ± 1.3a	17.0 ± 0.5a	5.3 ± 0.1a	5.2 ± 0.1a	29.3 ± 0.9a	26.3 ± 0.4 b	11.2 ± 0.3a	8.8 ± 0.1a
	Steam	10.7 ± 0.8a	21.1 ± 1.1a	2.4 ± 0.1a	1.1 ± 0.1a	20.5 ± 1.7a	18.0 ± 0.6a	5.4±0a	5.0 ± 0.1 ab	29.3 ± 1.2a	27.3 ± 1.4 b	10.2 ± 0.5a	8.8 ± 0.5a
Spruce	Hot-air	13.5 ± 0.4a	22.6 ± 0.9a	1.8 ± 0.1 b	1.0 ± 0.1a	22.9 ± 0.3a	18.9 ± 0.3a	3.8 ± 0.1c	4.4 ± 0.1c	28.7 ± 1.8a	35.3 ± 1.4a	4.3 ± 0.2c	1.6±0c
	Steam	10.6 ± 0.4a	23.6 ± 0.3a	2.0 ± 0.1 b	0.9 ± 0.2a	21.6 ± 0.7a	18.9 ± 0.6a	4.2 ± 0.1 b	4.6 ± 0.1bc	24.8 ± 1.4a	33.3 ± 0.5a	3.5 ± 0.6c	2.9 ± 0.1c
Alder/Spruce	Hot-air	14.5 ± 2.2a	22.6 ± 0.2a	1.9 ± 0.1 b	1.1 ± 0.1a	19.6 ± 1.7a	18.3 ± 1.0a	3.9 ± 0.1bc	4.4 ± 0.1c	25.0 ± 1.5a	29.5 ± 1.1 ab	7.2 ± 0.2 b	5.6 ± 0.5 b
	Steam	11.3 ± 0.4a	22.9 ± 1.0a	1.9 ± 0.1 b	1.2 ± 0.1a	20.0 ± 0.2a	19.9 ± 1.0a	4.1 ± 0.1bc	4.5 ± 0.1c	24.0 ± 1.0a	31.3 ± 1.8 ab	7.8 ± 0.5 b	5.7 ± 0.7 b

*Values within a column followed by different letters indicate significant differences between the rows ($p \leq 0.05$).

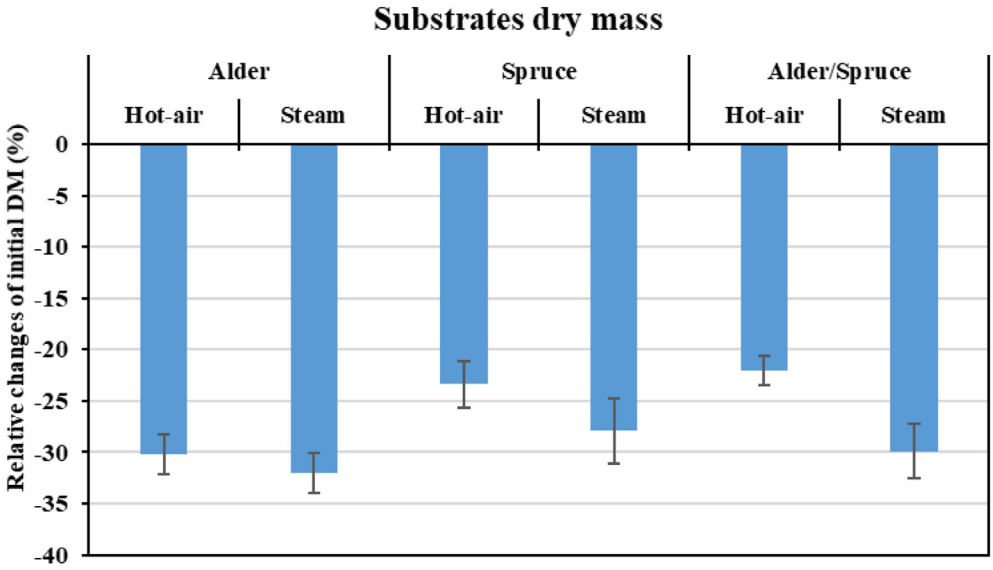

Fig. 1. Relative mass reduction in the substrates after harvest (mean ± SE).

substrates related to degradation of Klason lignin (Fig. 2B). The fact that degradation of Klason lignin was comparable between spruce and alder substrate and between hot-air and steam heat treatments was probably correlated to the observations that no differences in time and speed of mycelial colonisation. The fungus seemed to attack Klason lignin in cell wall, for building its mycelium, equally between different substrates, without discriminating which species and how it was pretreated.

However, the mass degradation of acid soluble lignin was significantly lower in the spruce-based and spruce/alder-based substrate compared to the alder-based substrate (15.9% and 17.4% vs 34.3%; $p < 0.001$). There was also a statistically significant difference in acid soluble lignin mass degradation related to the heat pretreatments, and the steam sterilisation resulted in a larger degradation. This result could suggest that acid soluble lignin was probably more sensitive than Klason lignin to the treatment of high-temperature and pressurised steam and thereafter became easier accessible to fungal degradation, although the mechanism behind could not be given by this study. Potentially, the increased degradation of the acid soluble lignin may partly explain the larger weight loss observed in the steam-sterilised substrate after mushroom production (Fig. 1).

Glucan, the major carbohydrate component, was initially present in concentrations ranging from 24 to 29% of total dry matter, with similar values observed in both alder-based and spruce-based substrates (Table 3). The glucan content was in general lower than those reports using pure wood materials (Taherzadeh et al., 1997; Wang et al., 2018), which could be largely due to the dilution effect by adding wheat bran (24.6%) in the substrates. Wheat bran contains 13–16% cellulose but 5–8% lignin, according to our pilot analyses. Nevertheless, after mushroom production, a significant difference was observed between the substrates with a very low mass consumption of glucan (approximately 3.9%) in the spruce-based in contrast to 36.9% in the alder-based substrate. (Fig. 2C). The reason why *P. pulmonarius* behaved differently between the softwood and hardwood, when cellulose degradation is concerned, remains mystery in this study but shall be included in future studies. Xylan mass reduction was rather comparable among treatments except for the hot-air pasteurised spruce-based substrate, where it reached as high as 70% of that in the initial substrate (Fig. 2C).

Lignin and xylan are considered main barriers limiting the enzymatic hydrolysis for biofuel production (Jönsson and Martin, 2016). After mushroom cultivation, the sum of the remaining lignin and xylan fractions amounted to 28.1% on average of all hot-air treatments for alder-based and spruce-based SMS (Table 3), compared with 29.4% for all steam treatments. This suggests that both heat treatments lead to a generally comparable condition for enzymatic hydrolysis that could be negatively affected by either lignin or xylan. It is already known that both heat pretreatment methods did not differ from each other regarding the digestibility of glucan in SMS (Xiong et al., 2019). Furthermore, when the thermal efficiency is considered during the pasteurisation process, hot air should be considered as a substitute for steam sterilisation.

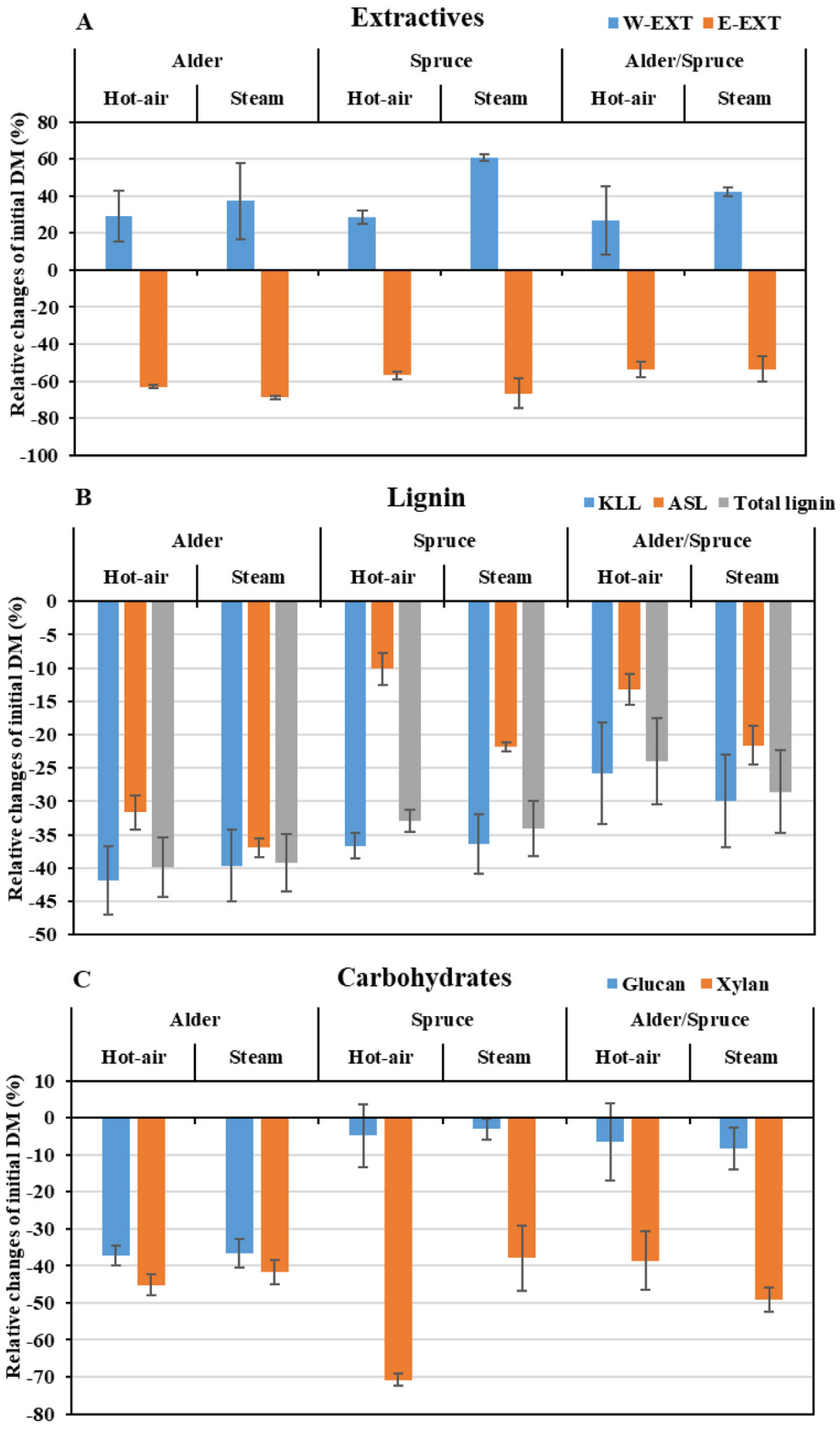

Fig. 2. Relative changes of substrate composition for water extractives (W-EXT), ethanol extractives (E-EXT), Klason lignin (KLL), acid soluble lignin (ASL), Total lignin, glucan and xylan compared to initial substrate (mean ± SE).

3.2.3. P. pulmonarius vs. non-edible white-rot fungi

Overall, a greater lignin degrading ability of *P. pulmonarius* on alder, irrespective of the heat treatments, was observed in the present study (Fig. 2B). This difference might be attributed to the fact that softwood is more recalcitrant to degradation by most white-rot fungi compared with hardwood (Zhu and Pan, 2010). In comparison with published data on degradation of hardwood by other fungal species, our results of 39.6 and 43.4% reduction of total lignin and hemicellulose mass (Fig. 2B and C), respectively, in the alder-based substrate were slightly lower than other reported

values (Table 4). The cellulose reduction on alder was 36.9%, the value was comparable to that reported within 30 days of fungal cultivation.

For softwood (spruce-based substrate), a degradation of 33.5% of total lignin was observed in the present study (Fig. 2B). This value is comparable to the previous reported degradation of 39.8% of total lignin (Yu et al., 2009), using the white-rot fungal species *Echinodontium taxodii*, which was high compared to several other studies (Table 4). It should be pointed out that in our present study, the degradation was obtained after only 26 days of cultivation, while a much longer time period of 120 days was used in the study by Yu et al. (2009). Furthermore, it should be pointed out that, in our study, only 3.9% of the glucan content was consumed, parallel to the degradation of 33.5% of total lignin.

The performance of lignocellulose degradation demonstrated in the present study suggests that *P. pulmonarius* has a greater selective degradation ability on the lignin fraction rather than the cellulose component of softwood, whereas the performance of degradation on hardwood was not obviously different from other white-rot fungi (Table 4). After the cultivation of *P. pulmonarius*, a comparable lignin content was found in both spruce-based and alder-based substrate (Table 3), suggesting that the lignin-induced recalcitrance to enzymatic conversion was non-discriminately reduced by the mushroom.

3.3. Enzymatic saccharification of SMS for biofuel production

As indicated by the results of the analytical enzymatic saccharification (Fig. 3), between 84 and 126 g glucose was yielded per kg of dry SMS, which corresponds to cellulose conversion ratio ranging from 29 to 33%. The glucose yields are higher than what has been reported in the literature for raw wood, and are in the same order as values obtained for acid pretreated softwood and hardwood (Wang et al., 2018). That is a good indication that the cultivation of *P. pulmonarius* has the complementary bonus of pretreating biomass for bioconversion to sugar-platform products, such as bioethanol and other biocommodities. It was also interesting that the effectiveness of cultivation of *P. pulmonarius* as wood pretreatment was higher for softwood than for hardwood, in terms of glucose yield. The best glucose yield (126 g/kg) was achieved for spent spruce substrate, and that value corresponds to 74% of the yield achieved in sulfuric acid pretreatment of spruce (Wang et al., 2018). That, together with the lower glucan degradation occurred during cultivation on spruce compared with the alder substrates (Fig. 2C), reveals the potential of *P. pulmonarius* as an appropriate organism for biological pretreatment of softwood biomass.

The sugar yields achieved in this work in the enzymatic hydrolysis of spent alder substrate are lower than those achieved for spruce, and lower than those reported previously by this group for birch (Xiong et al., 2019). However, in our opinion that does not demerit the potential of oyster mushroom cultivation for hardwood. It should be taken into consideration that in the current study different hardwood substrate and different fungal species were used than in our previous investigation, where birch and shiitake were model species. Another major issue is that in that study, mushroom was cultivated for 150 days, while in the results discussed in the current work correspond to a considerably shorter cultivation time (26 days).

Apparently, use of *P. pulmonarius* may have advantages over other non-edible white rot fungi for pretreating specially softwood for biofuel production. About 68–78% of the raw substrate were recovered as SMS after the harvest; the theoretical yields of ethanol converted from glucan in the SMS was estimated (using the formula of Section 2.2.2) to be 4.3–7.0 mL per 100 g dry mass of raw substrate (or 6.1–9.2 mL per 100 g SMS) (Table 5), using the data of enzymatic

Table 4
Mass degradation of sawdust-based substrates after fungal growth in published reports.

Data source	White rot fungi	Major ingredient of substrates	Cultivation days	Relative mass reduction of (g per 100 g of initial DM)		
				Cellulose	Hemicellulose	Lignin [a]
This study	*Pleurotus pulmonarius*	Hardwood	26	36.9	43.4	39.6
		Softwood	26	3.9	54.3	33.5
Lin et al. (2015)	*Lentinula edodes*	Hardwood	110	69.1	61.6	54.3
Yu et al. (2009)	*Echinodontium taxodii*	Hardwood	120	26.7	50.8	45.6
Rudakiya and Gupte (2017)	*Pseudolagarobasidium acaciicola*	Hardwood	30	36.1	41.8	43.6
	Pseudolagarobasidium acaciicola	Hardwood	30	40.6	50.1	53.7
Yu et al. (2009)	*Echinodontium taxodii*	Softwood	120	12.6	31.4	39.8
	Ceriporia lacerata	Softwood	56	8 (Holocellulose)		13.1
Lee et al. (2007)	*Polyporus brumalis*	Softwood	56	10.6 (Holocellulose)		11.6
	Stereum hirsutum	Softwood	56	7.8 (Holocellulose)		14.5
Ferraz et al. (2001)	*Poria medula-panis*	Softwood	90	10	15	18

[a] Klason lignin and acid soluble lignin.

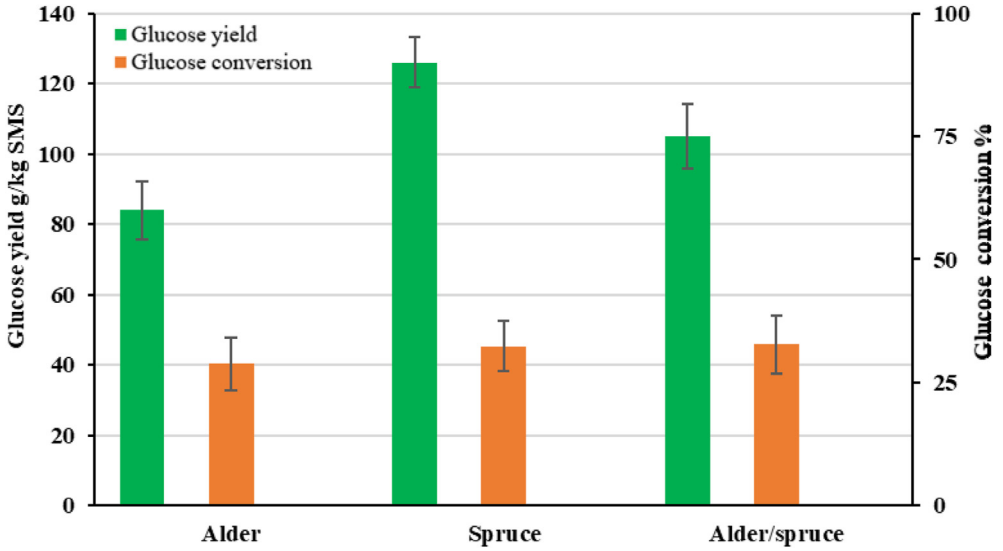

Fig. 3. Results of analytical enzymatic saccharification (mean ± SE). The glucose conversion is calculated as the percentage of hydrolysed cellulose out of the amount contained in the test SMS. The yield is here expressed as the amount of the glucose in analytical enzymatic hydrolysis of SMS. Data are based on dry mass.

Table 5
Mass balance during mushroom cultivation and theoretical yield of ethanol.

	-	Alder		Spruce		Alder/Spruce	
		Hot-air	Steam	Hot-air	Steam	Hot-air	Steam
Raw substrate	per 100 g	100	100	100	100	100	100
SMS		69.8 ± 2.0	68 ± 2.0	76.6 ± 2.3	72.1 ± 3.3	78.0 ± 1.5	70.1 ± 2.8
		Theoretical ethanol from hydrolysed glucan in the SMS					
Ethanol	ml (raw)	4.3 ± 0.5	/	7.0 ± 0.3	/	6.8 ± 1.0	/
	ml (SMS)	6.1 ± 0.6	/	9.2 ± 0.5	/	8.6 ± 1.0	/

digestibility (Fig. 3). Apart from co-producing quality and edible mushrooms, the short pretreatment time, 26 days in this study, could considerably reduce the common concerns for time consuming of fungal pretreatment (Wan and Li, 2012), compared with physiochemical treatment. All these may have important implications for developing a cost-effective, energy-efficient and environmental friendly process for combined production of edible mushroom and biofuels. However, future studies are needed to investigate the factors that may regulate the degradation of lignocellulose and thus the possibilities to increase degradation of lignin with low consumption of cellulose while producing acceptable quantity mushroom fruit bodies with good quality. From elevation of cleaner production and circular economy point of view, an exploration of utilizing the side streams, e.g. the solid byproducts after enzymatic hydrolysis of the spent substrate should be also included in the future development.

4. Conclusion

The present study clearly demonstrates that fruiting body production is possible on softwood using the oyster species *P. pulmonarium*, although the biological efficiency (14.0–33.8%) was low but comparable to some published studies. Compared to fruiting bodies produced on substrates based on hardwood, no difference was observed in mushroom quality in terms of total protein concentration (26.0–28.5%). After mushroom harvest, all remaining substrates had improved properties for producing bioethanol due to fungal degradation of the lignin and xylan components. A significant difference was observed between the substrates with a very low mass consumption of glucan (3.9%) in the spruce-based in contrast to 36.9% in the alder-based substrate. The selective degradation ability of *P. pulmonarius* on the lignin fraction, rather than the cellulose component of softwood, is suggested in the present study. Between 84 and 126 g glucose was yielded per kg of dry SMS, spruce based substrates resulted a higher yield than alder substrate from enzymatic saccharification of the spent substrates. From these released glucose, a production about 61–92 mL ethanol per kg SMS may be theoretically estimated based on current experiment settings. Furthermore, concerning pretreatment of the mushroom's substrate, hot-air pasteurisation can be considered to substitute for the less thermal efficient use of steam sterilisation. To be able to scale up the concept to an industrial usage, an increase of mushroom production shall be one of the focuses for future development, which will include both optimization of substrate composition and improvement of the processes. At the same time, mechanisms and possibilities of maximizing lignin degradation while obtaining acceptable mushroom yield at minimal cellulose lose shall be important research tasks.

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Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Feng Chen: Writing - original draft, Writing - review & editing, Validation, Software. **Shaojun Xiong:** Writing - original draft, Writing - review & editing, Supervision. **Jonatan Sundelin:** Writing - original draft, Methodology, Resources. **Carlos Martín:** Writing - review & editing, Validation. **Malin Hultberg:** Methodology, Resources, Writing - original draft, Writing - review & editing, Validation, Supervision.

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Increased production of biofuels from lignocellulosic material would allow reduced environmental problems caused by the use of fossil resources while still supporting development of bioeconomy. In this thesis, the possibility of pretreating the lignocellulosic biomass with edible white-rot fungi for integrated production of edible mushroom, cellulosic bioethanol and solid fuels were investigated.

Feng Chen received his doctoral education at the Department of Forest Biomaterials and Technology, Swedish University of Agricultural Sciences, Umeå. He received the Master degree from China Agricultural University.

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