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Research article

The use of spent mushroom substrate as biologically pretreated wood and its fibrillation

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ABSTRACT

Utilization of biomass and reuse of industrial by-products and their sustainable and resource-efficient development into products that are inherently non-toxic is important to reduce the use of hazardous substances in the design, manufacture and application of biomaterials. The hypothesis in this study is that spent mushroom substrate (SMS), a by-product from mushroom production, has already undergone a biological pretreatment and thus, can be used directly as a starting material for fibrillation into value-added and functional biomaterial, without the use of toxic substances. The study show that SMS can be effectively fibrillated at a very high concentration of 6.5 wt % into fibrils using an energy demand of only 1.7 kWh kg^{-1}, compared to commercial and chemically pretreated wood pulp at 8 kWh kg^{-1}, under same processing conditions. SMS is a promising resource for fibrillation with natural antioxidant activity and network formation ability, which are of interest to explore further in applications such as packaging. The study shows that biological pretreatment can offer lower environmental impact related to toxic substances emitted to the environment and thus contribute to reduced impacts on categories such as water organisms, human health, terrestrial organisms, and terrestrial plants compared to chemical pretreatments.

1. Introduction

Efficient use of our bioresources and their sustainable processing are key aspects for sustainable development that requires an integrated approach that takes into consideration environmental impact along with economic development. For sustainable development, making use of biomass and reuse of industrial by-products such as residues from agriculture needs to be combined with the development of alternative green and sustainable technologies that reduces or eliminates hazardous substances in the design, manufacture and application of materials (Anastas and Warner, 1998).

The mushroom production in Europe reached well over a million tons of cultivated mushrooms in 2022 and is expected to increase in the coming years (Eurostat, 2022). For every ton of mushrooms, up to five times the amount of spent mushroom substrate (SMS) is generated as a by-product, which to date has little commercial use (Beckers et al., 2019). The substrate can be composed of various lignocellulosic materials, such as sawdust, sugar cane bagasse, or wheat straw and bran. After harvest, the vast amount of SMS results in significant logistical problems as well as disposal costs for farmers (Beckers et al., 2019). There are however many different uses that are being expanded and explored, including mushroom recultivation (Beckers et al., 2019), recycling as fertilizer (Zied et al., 2021; Li et al., 2023; Ré et al., 2024), animal feed (Martín et al., 2023), energy (Williams et al., 2001; Finney

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et al., 2008), water purification (Hultberg et al., 2020), source of bioactive compounds (Klausen et al., 2023), or to produce biofuels (Xiong et al., 2018; Chen et al., 2022). It has been previously demonstrated that during cultivation of edible mushrooms, the woody substrate undergoes a selective degradation of lignin and hemicellulose (Xiong et al., 2018; Chen et al., 2020). After harvest, the SMS materials are rich in fungal mycelium, fungal metabolites (Li et al., 2022), that are not present in conventional biomass, and a known source for bioactive components (Muiruri et al., 2023).

Separation, or fibrillation of lignocellulosic biomass and by-products into fibrils is one approach to develop value-added materials from, for example industrial side streams (Berglund et al., 2016; Squinca et al., 2021). For the fibrillation process to achieve fibrils at nanoscale (cellulose nanofibers), the production generally involves a combination of chemical and mechanical treatments and for industrial use the starting material is typically wood pulp. Altogether, the production process is associated with several processing steps that includes the use of chemicals, a high energy consumption, low production efficiencies (low solid content) being the largest contributors to the environmental impact (Berglund et al., 2020; Ai et al., 2022). Many researchers have investigated and reported on the use of "eco-friendly or greener" alternatives for fibrillation. For example, the use of oxidative enzymes to promote an energy-efficient fibrillation and reduce the use of toxic chemicals (Koskela et al., 2019), or the patented process of high consistency enzymatic fibrillation at a consistency of 25 wt % and an energy demand of only 0.6 kWh kg^{-1} (Pere et al., 2020). However, both studies started their processes from already pretreated or delignified softwood fibers and the environmental impacts of the processes were not assessed. Previously, we have reported on the fibrillation of industrial bio-residues after various chemical pretreatment with a high yield and efficiency (Hassan et al., 2018; Berglund et al., 2020). We have also shown that by making use of the natural composition that the biomass offers, in turn reducing fractionation, or pretreatment steps, not only promote energy efficient conversion, but also contribute to an overall more eco-efficient process. An environmental impact reduction of more than 75% for carbon footprint, freshwater ecotoxicity, and human toxicity, along with a cost reduction of more than 50% was shown going from bio residue to nanofibers (Berglund et al., 2020).

Very few researchers have investigated the use of SMS as a starting material for fibrillation into fibrils (Konno et al., 2016). separated cellulose nanofibers from SMS using TEMPO-mediated oxidation and in a recent study (Li et al., 2022) reported the successful fibrillation of SMS into nanofiber. These studies demonstrate the potential of the SMS as a raw material for fibrillation, however, in both studies, SMS was subjected to one or up to six chemical pretreatment steps prior to fibrillation, which is not only reducing the yield, but it also adds to the environmental impact.

From an eco- and resource-efficiency perspective, we hypothesize that SMS has undergone a selective degradation of lignin during mushroom cultivation and thus is already biologically pretreated, and therefore could be fibrillated without applying any chemical pretreatments. It is, however, an open question if fibrillation of SMS can be made efficiently by making use of the natural composition of the SMS material, and what functional properties that can be attained.

The purpose of this study is to evaluate the fibrillation potential of SMS according to consistency, energy efficiency, and network formation ability. The aim is to effectively fibrillate functional fibrils from SMS without any chemical pretreatments and with a low environmental footprint for increased resource-efficiency combined with the development of fibrils with specific functionality. The fibrillation of biologically pretreated SMS is compared to a commercially, chemically pretreated wood pulp in terms of life cycle analysis, network formation and bioactivity.

2. Material and methods

Materials. Wood particles (birch (*Betula pubescens)* particles) was used as substrates for bench-scale cultivation of edible fungi, shiitake (*Lentinula edodes*) mushroom at SLU Svamplabb. The spent mushroom substrates (SMS) were collected after harvesting and evaluated for fibrillation. A commercial Kraft pulp kindly supplied by SCA (Munksund, SE) was used as a reference material composed of 70 ± 0.7 wt % cellulose, 21 ± 0.2 wt % hemicellulose and 5 ± 0.1 wt % lignin (Berglund et al., 2017). The SMS was frozen (−18 °C), and before use, left to thaw for 16 h at room temperature (23 °C), and subsequently disintegrated by hand prior to fibrillation methods applied in next steps. The SMS material was prepared at concentrations of 2.5, 4.5, 6.5 and 8.5 wt % and the reference material at a concentration of 6.5 wt %. Distilled water was used for all experiments. Prior to fibrillation the material was soaked for 48 h at the prepared concentration and pre-dispersed using a mixer (Ystral GmbH, Ballrechten-Dottingen, Deutschland) for 10 min.

Fibrillation. A super mass colloider (MKZA6-3, Masuko Sangyo Co., Ltd., Kawaguchi, Japan) with coarse silica carbide (SiC) grinding stones was used for the grinding (fibrillation) process, with the aim to separate the cellulosic biomass into smaller fibrils. The fibrillation was conducted in contact mode, with the gap of the two discs set to contact and then gradually adjusted to −90 μm, at 1500 rpm (Fig. S1a). The energy consumption of the fibrillation process was established by the direct measurement of the power with an energy analyzer, EM24 DIN, Carlo Gavazzi (Belluno, Italy) and from the monitored processing time. The cumulative energy demand integrated over the complete fibrillation time was calculated from the following equation:

$$\text{Energy} = \text{power (W)} \times \text{time (h)} \quad (1)$$

The total energy consumption for the process was expressed as kilowatt hours per kilogram of dry weight of fibers. Samples were collected at regular intervals to evaluate the degree of fibrillation. The process was finalized when a plateau was reached in the viscosity and no larger intact structures could be visualized by optical microscope.

Film formation. Films were prepared from the fibrillated materials using a vacuum filtration setup followed by drying processes to obtain a dry fiber network with a grammage of 150 g m^{-2}. The suspension was diluted to 0.3 wt % and then stirred with a magnetic for about 30 min prior to vacuum filtration using a Büchner funnel and flask with a filter membrane (Durapore PVDF from Millipore, Darmstadt, Germany, diameter: 90 mm, filter-Type: 0.1 μm). After the filter cake was formed, the filter membrane was removed followed by drying in a vacuum oven (NSV 9000, LABEX, Helsingborg, Sweden) between aluminum plates (300 mm × 300 mm x 1.8 mm, 0.36 kg), paper, two thin-meshed metal nets (Pore size: 50 μm) closest to the filter cake on each side. A weight of 5.3 kg was then placed on top, and the setup was dried at 80 °C for 30 min. The film was subsequently removed and hot-pressed using a hot press (LabEcon 300, Fontijne Press, Vlaardingen, Netherlands), between metal plates (300 mm × 300 mm x 1.8 mm, 0,36 kg) and Mylar films (100 MICRON MYLAR, Lohmann Technologies, Milton Keynes, UK) at 100 °C and 2.5 MPa for 10 min before being cooled down to 20 °C to obtain the finished film.

Chemical composition. The chemical composition of the substrate was determined before and after mushroom cultivation. The determination of the content of extractive compounds was based on a standard protocol of the National Renewable Energy Laboratory (NREL) (Sluiter et al., 2005).

The content of structural carbohydrates and lignin was determined by analytical acid hydrolysis followed by chromatographic determination of sugars, spectrophotometric determination of acid-soluble lignin, and gravimetric quantification of acid-insoluble lignin. The procedure was based on a NREL standard protocol (Sluiter et al., 2012), except for the determination of monosaccharides, which was carried out by High-Performance Anion-Exchange Chromatography (HPAEC). An

ICS-5000 (Dionex, Sunnyvale, CA, USA) instrument was used for the HPAEC determination of monosaccharides. Free fatty acids in the non-polar extracts were determined following a standard method (Grieve and Lau, 2018). Esterified fatty acids were determined by alkali-catalyzed transesterification by a modification of the protocol described by (Glass et al., 1965). In detail, sequential extractions with hexane (for non-polar extractives) and ethanol (for polar extractives) using an accelerated solvent extraction system (Dionex ASE 350, Sunnyvale, CA) were performed. Hexane extraction was performed at 100 °C and 1500 psi for three 8-min cycles. After the run, liquid portions were weighed to determine the extractives mass and the solid residues were air-dried overnight, and submitted to ethanol extraction, which was carried at 80 °C with three 8-min extraction cycles. The solid residues recovered from the ethanol extraction was used for determination of structural carbohydrates and total lignin. Free fatty acids in the non-polar extracts were determined of consisting in methylation followed by chromatographic quantification. Esterified fatty acids were determined by alkali-catalyzed transesterification. The method included dissolving the dried extracts in heptane. The resulting fatty acid methyl esters (FAME) were analyzed with an Agilent 7890A gas chromatograph equipped with a multimode inlet (MMI) and with a Zebron ZB-FAME 20 m × 0.18 mm i.d. fused silica capillary column with a chemically bonded 0.15 μm stationary phase (Phenomenex, Torrance, USA). The injector temperature was set to 250 °C, the carrier gas flow rate was 1 mL min $^{-1}$.

Viscosity. The viscosity was measured during fibrillation process of SMS and reference material using a Vibro Viscometer SV-10 from A&D Company, Ltd. (Tokyo, Japan) at a constant shear rate and periodical circulation of the sensor plates from zero to peak (sine-wave vibration) at a frequency of 30 Hz. Because the temperature increased during the fibrillation owing to the compression and abrasive shearing forces, the viscosity measurements were repeated at a stabilized temperature of 22.3 ± 1.0 °C to confirm that a viscosity plateau had been reached during the process.

Optical microscopy (OM). The optical microscope used was a Nikon Eclipse LV100N POL (BergmanLabora AB, Danderyd, Sweden), equipped with imaging software NIS-Elements D 4.30. OM images were captured before and during fibrillation for each batch and used as an indicator to assess the degree of fibrillation. In addition, all samples were concentrated to 0.25 wt % after fibrillation to compare the viscosity between the different batches.

Scanning electron microscopy (SEM). The material morphology of the SMS and reference material before and after fibrillation was studied using a JCM-6000 (JEOL, Tokyo, Japan) at an acceleration voltage of 15 kV. Prior to SEM the samples were diluted to 0.1 wt % and dropped onto a carbon tape placed on the sample holder using a pipette and subsequently submerged in liquid nitrogen and freeze-dried using Martin Christ Alpha 1–4 LSC plus freeze dryer (Svenska LABEX AB, Helsingborg, Sweden) at 10 °C and at a pressure of 1 mbar for 40 h. The samples were sputtered with platinum 13 nm layer using the LEICA EM ACE200 (Leica Microsystems GmbH, Wetzlar, Germany). High-resolution SEM (FEI Magellan 4000 XHR-SEM, Thermo Fisher Scientific, OR, USA) with an accelerated voltage of 15 kV, was used to visualize SMS after fibrillation. The 6.5 wt % batch was diluted to 0.1 wt % and magnetically stirred for 30 min. A drop was placed onto a carbon tape and dried in a vacuum oven (NSV 9000, LABEX, Helsingborg, Sweden) at 90 °C for 30 min. The sample surface was sputtered with platinum using the LEICA EM ACE200 with a 10 nm thick layer.

Mechanical properties. The SMS films were tested in tensile mode using a tensile testing system (AG X; Shimadzu, Tokyo, Japan) equipped with a 1 kN load cell was used at an extension rate of 2 mm min^{-1} and with a gauge length of 20 mm. All samples were cut into 5.9 mm wide and 50 mm long strips with the thickness of the strips measured in triplicate at different points using an ID-C112XBS thickness measuring instrument. The elastic modulus was measured from the linear region without an extensometer. Prior to testing, the samples were conditioned at 50 ± 2.0% relative humidity at 22 ± 1.0 °C for at least 48 h. The results are averages of at least 10 sets of measurements for each material.

Antioxidant activity. The radicals scavenging activity of the SMS and reference material after fibrillation and freeze-drying were assayed according to (Xu et al., 2021). The final concentration of freeze-dried material varied between 0.0625 and 12.5 mg mL^{-1} and DPPH ethanol solution was added with a final concentration of DPPH to 0.1 mM. A series of blank samples containing the materials without DPPH adding 0.4 mL distilled water and 0.4 mL ethanol were prepared, followed by shaking at 1000 rpm and room temperature under dark conditions. An homogeneous aliquot was withdrawn, at 30 min and 20 h, and the supernatant was pipetted into a 96-well plate and its absorbance was measured at 517 nm via a microplate reader. The radical scavenging activity was calculated as following:

$$\text{DPPH radicals scavenging activity } (\%) = [1 - (\text{As} - \text{A1}) / \text{A0}] \times 100 \quad (2)$$

where As is the absorbance of the sample and DPPH, A1 is the absorbance of the sample (gel in water: ethanol 50:50 v/v), and A0 is the absorbance of the control (DPPH 0.1 mM in water: ethanol 50: 50 v/v).

2.1. Fourier-transform infrared (FTIR)

To evaluate the unique composition of SMS, scans of SMS, raw birch wood particles, and chitin-glucan extract from shiitake mushrooms were carried out using FTIR spectrometer (Nicolet™ Summit, Thermo Fisher Scientific, Karlsruhe, Germany) equipped with an Everest™ Diamond ATR, with a range of 4000–800 cm^{-1} and a 4 cm^{-1} scan resolution was used. To identify the peak positions, the curves were analyzed using SpectraGryph software (Spectragryph, version 1.2.16.1, Oberstdorf, Germany).

The extraction of chitin-glucan complex from shiitake mushrooms was done according to (Fazli Wan Nawawi et al., 2019). Briefly, mushrooms were rinsed with water, blended, and heated to 85 °C under magnetic stirring for 30 min. The excess water was removed using a centrifuge (Avanti J 251, Beckman Coulter Life Sciences, Beckman Coulter AB, Sweden) at 6000 rpm for 15 min and diluted with 1M NaOH and heated to 65 °C for 3h under stirring. The slurry was neutralized, and the final suspension was diluted to 3 wt %.

Life cycle analysis (LCA). The life cycle analysis followed the four phases of the ***ISO 14040 standard (2006)***. The goal was to find out if there are significant differences between the processes of obtaining a fibrillated material from the biologically pretreated biomass (SMS) and obtaining a chemically pretreated commercial Kraft pulp. The input data inventory for modelling used lab-scale values for SMS and used industrial-scale values for obtaining the commercial pulp, accessed from the ***Ecoinvent database v.3.8 (2023).*** The method used for assessing the life cycle impact was ReCiPe 2016 v1.1 midpoint (Huijbregts et al., 2017), in the software SimaPro. The following assumptions were made for the modelling of the material from the biologically pretreated biomass (SMS): a distance of 50 km was assigned between the SMS production site and the fibrillation site; the materials included in the modelling were: wheat bran, tap water and diodes; the amount of electricity covered the modelling of all the processes that are usually included in the indoors mushroom cultivation flow, electricity, medium voltage {SE}| electricity, medium voltage, residual mix | Cut-off, S. The reference used for comparison in the modelling is a bleached sulfate hardwood pulp production.

3. Results and discussion

3.1. SMS material and its fibrillation

Composition. The mushroom cultivation using wood residues as a substrate and subsequent harvesting of the protein-rich shitake

mushrooms leaving SMS as a by-product is presented in Fig. 1.

Previous studies that have evaluated potential uses of SMS have reported that the composition can vary depending on factors such as type of substrate and cultivation time (Xiong et al., 2018; Klausen et al., 2023). Furthermore, addition of other components is typical for cultivation but are assumed to be largely consumed upon growing of the mushrooms. The chemical composition was studied before and after cultivation/harvesting (Table 1), for a better understanding of the biological pretreatment that the wood residues undergo during cultivation and its effect on the composition of the biomass.

Birch wood contained around 37% (w/w) glucan, 24% (w/w) total lignin, and 23% (w/w) hemicelluloses (Table 1). Xylan was the main hemicellulosic component. Total lignin was determined as the sum of Klason lignin and acid-soluble lignin. The mass fraction of total extractives was 2.1% (w/w), and polar components were predominant. Birch-based SMS also had a typical lignocellulosic composition. The predominant compositional difference after cultivation and harvest of mushrooms was observed in the lignin content of the spent substrate (10.6% (w/w)), which was reduced by almost 60% (Table 1), and highlights that the material undergo a selective degradation of lignin during the biological pretreatment. SMS was enriched in glucan (52.1% (w/w)), meanwhile the content of xylan (16% (w/w)) was slightly lower after the biological pretreatment, compared to raw birch wood. However, the mass fraction of total extractives remained comparable for both materials, indicating that the extractives are preserved after biological pretreatment. The results are in agreement with previously reported compositions of SMS material where wood have been used as a substrate (Xiong et al., 2018).

The fatty acid profile is provided in Table S1 before and after biological pretreatment. The lipophilic extractives of the wood sample consisted mainly of esterified saturated fatty acids. Except for palmitic acid, the content of most of the esterified fatty acids decreased in SMS compared with raw birch wood, while the content of esterified linoleic acid increased. On the other hand, SMS contained also esterified capric acid and pentadecylic acid, which were not detected in wood. The content of most of the free fatty acids, except behenic acid, linoleic acid, and lignoceric acid, decreased in SMS compared to wood (Table S1). On the other hand, tricosylic and pentadecylic acid, which were not contained in the free fatty acids fraction of wood, were present in SMS after the biological pretreatment.

Fibrillation efficiency. The SMS material was manually disintegrated by hand and distilled water was added to prepare SMS suspensions at different concentrations before mixing and subsequent mechanical fibrillation as presented in the overview in Fig. 2. The separation process at different concentrations was evaluated during fibrillation according to the viscosity as a function of the energy consumption and their size reduction in optical microscope (Fig. 2c), as well as SEM (Fig. 2d, Fig. S1b). In general, chemically pretreated biomass is fibrillated using an ultrafine grinder at concentrations between 2 and 3 wt % (Adu et al., 2018). However, higher concentrations enable more material to be processed and could contribute to a more effective separation process. Since biologically pretreated biomass, without any chemical pretreatment, have not been evaluated for fibrillation previously, the range of concentrations for fibrillation were studied.

Fig. 2a shows how the visual appearance of the material changed with the fibrillation process where a clear difference in gel formation between the four batches can be seen. While the 2.5 wt % sample displayed a liquid-like appearance, it appeared more gel-like with increased concentration. In previous studies, we have shown that the viscosity is an indirect measurement of the degree of fibrillation signifying an increased network formation with separated fibrils (Berglund et al., 2016, 2017). Viscosity measurements were thus performed continuously during process with the intent to provide an assessment of the fibrillation. For successful fibrillation into high aspect ratio fibrils, the process was continued while an increase in viscosity was observed and subsequently terminated when a plateau was reached. Additional processing, leading to a decrease of viscosity would indicate that the fibers are being cut (i.e shorter), rather than separated, which has a negative effect on the network formation as well as the energy consumed. From Fig. 2b, it can be observed that the viscosity indeed increases for all batches upon fibrillation. For the 4.5 wt % and 6.5 wt % batch, a clear plateau of the viscosity was observed, however the 2.5 wt % batch did not display a large increase of the viscosity, nor was a plateau reached upon fibrillation. For the 8.5 wt % batch the fibrillation process was terminated before a viscosity plateau was reached due to too high temperature increase originating from the high shear friction during fibrillation. A concentration range between 2.5 and 8.5 wt % was thus considered as under and upper limit for feasible fibrillation of the SMS material at the applied processing conditions. The 6.5 wt % sample displayed a direct and sharp viscosity increase and reached the viscosity plateau at 1520 m Pa s consuming the lowest energy of 1.7 kWh kg^{-1} (Fig. 2b). Thus, no further fibrillation than up to 1.7 kWh kg^{-1} is required for optimal fibrillation of the 6.5 batch to avoid affecting the length of the fibrils.

For microscopy study, all batches were diluted to same concentration for comparison. From Fig. 2c, larger intact structures were observed with OM for all batches prior to fibrillation and were measured in the range of 10–400 μm in width and 40–1500 μm in length (Fig. S1c). After fibrillation, a size reduction was observed for all batches and no visible intact structures were detected with OM. The SEM images are in agreement with the OM study, displaying a size reduction of all batches after fibrillation (Fig. 2d), regardless of if the samples were oven dried or freeze-dried (Fig. S1b), although agglomeration owing to drying of the samples was also apparent.

3.2. Comparison to chemically pretreated pulp

Network formation and bioactivity. The fibrillation process and material of biologically pretreated biomass were further studied and compared to that of chemically pretreated commercial Kraft pulp that

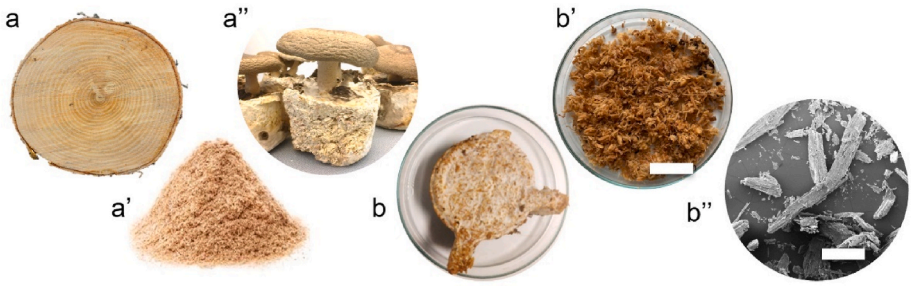

Fig. 1. Overview of photographs from birch wood to SMS material (a) cross section of birch, (a') wood particles, (a'') Shiitake mushroom cultivated in a wood substrate, and SMS (b) directly after the mushroom have been harvested (b') after manual disintegration, scalebar: 2.5 cm and (b'') SEM after manual disintegration, scalebar: 1 mm (SEM).

Table 1
Chemical composition of birch wood particles used as substrate and birch-based spent mushroom substrate. The compositions are given in % (w/w), and standard deviations of the different fractions are shown in brackets.

	Arabinan	Galactan	Glucan	Mannan	Xylan	Total lignin	Hexane extractives	Ethanol extractives
Wood	0.3 (0.0)	0.7 (0.0)	36.8 (0.1)	2.2 (0.1)	19.9 (0.2)	24.4 (0.3)	0.4 (0.1)	1.7 (<0.1)
SMS	0.3 (0.0)	0.4 (0.0)	52.1 (0.4)	2.5 (0.1)	16.0 (0.4)	10.6 (0.1)	0.1 (<0.1)	1.7 (0.2)

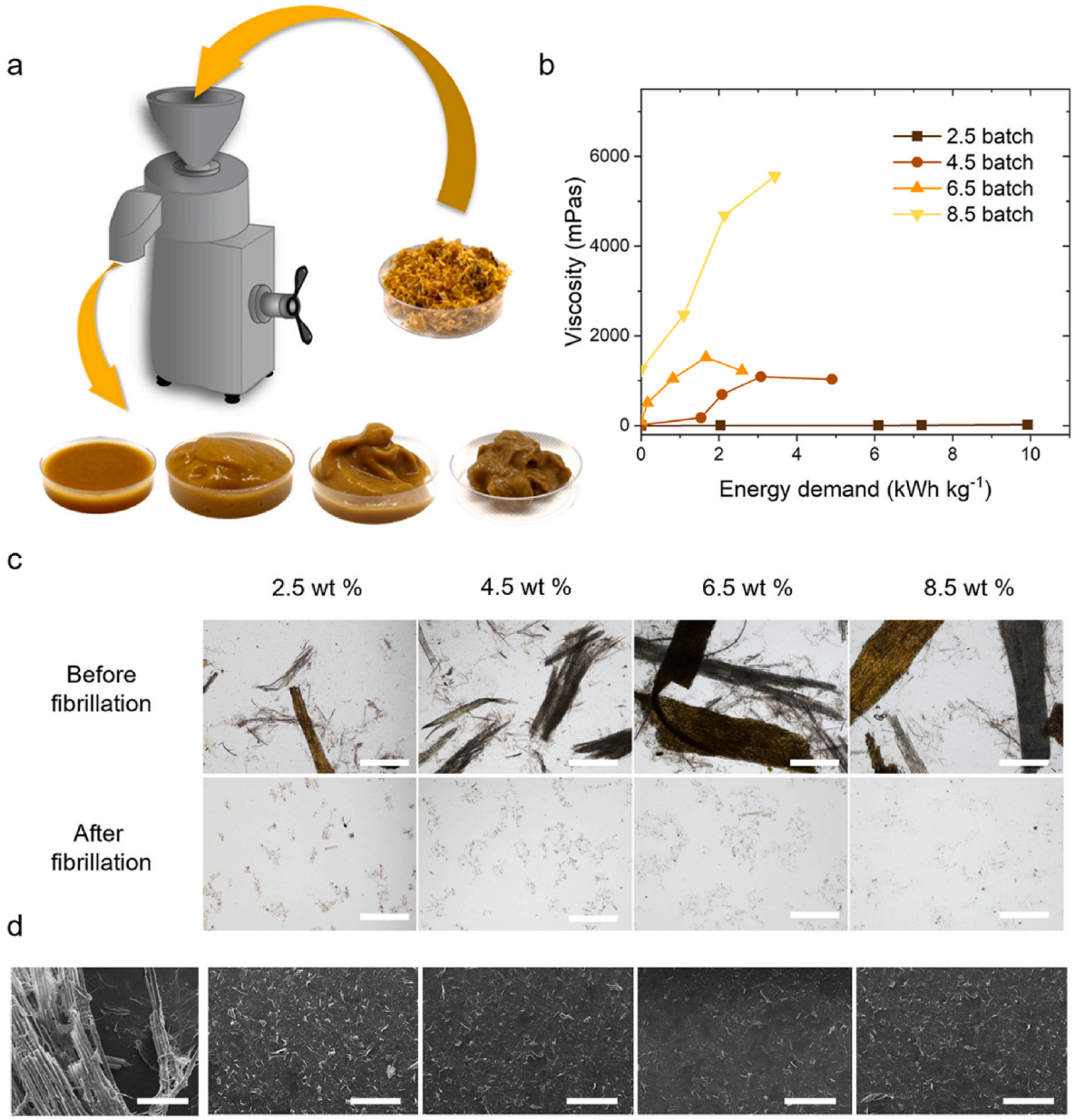

Fig. 2. (a) Photographs of SMS after manual disintegration by hand, the ultrafine friction grinder used for the mechanical fibrillation, and SMS after fibrillation at concentrations from left to right 2.5 wt %, 4.5 wt %, 6.5 wt %, and 8.5 wt %. (b) Viscosity as a function of the energy demand measured during the fibrillation process. (c) OM and (d) SEM images captured at a concentration of 0.3 wt % before and after the fibrillation process of the different batches, OM scale bar: 500 μm, SEM scale bar: 200 μm.

was used as a reference material in this study. To put SMS, in the context of biological pretreatment, and in comparison to that of a chemical pretreatment, the materials were fibrillated under the same conditions. The network formation of dried films, bioactivity assessed by antioxidant activity, and FTIR spectra are presented together with results from relative environmental impact assessment of the reference material and SMS (both fibrillated at 6.5 wt %) in Fig. 3.

The network formation was further evaluated for all fibrillated batches by preparing dry films using vacuum-assisted filtration, drying and hot-pressing (Fig. S1d) for further tensile testing. The SMS materials could successfully be formed into films after fibrillation, appearing as brown-colored and bendable dried networks, compared to chemically pretreated films that appeared having a whitish color (inset, Fig. 3a). The different batches displayed comparable mechanical properties, yet

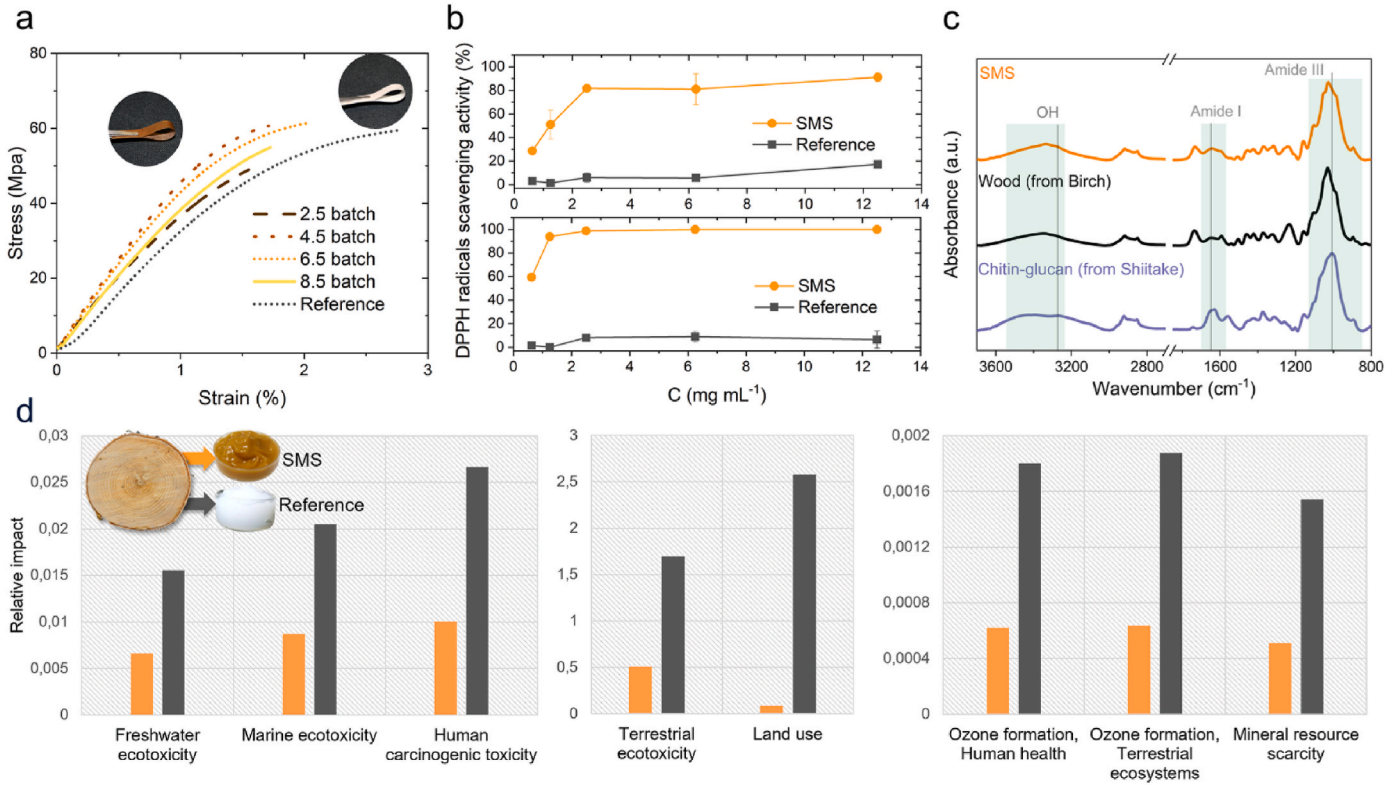

Fig. 3. (a) Representative stress and strain curves from all batches and the reference material, inset photographs of films (b) Antioxidant activity of fibrillated and freeze-dried SMS and reference material as a function of the free radical after 30 min (above) and 20 h (below) of incubation, where the reference material was assessed as a negative control (c) FTIR spectra of SMS, raw birch wood, and chitin-glucan extract from Shiitake mushroom (d) Relative impact from LCA per 1 kg gel material (6.5 wt%) for selected impact categories and their corresponding contributions in absolute values of SMS batch 6.5 and reference from raw material to fibrillated gel, cradle to gate.

6.5 wt % showed overall slightly higher average mechanical properties (Fig. 3a–Table S2), further signifying fibrillation into fibrils. In order to compare biological pretreatment to that of chemical pretreatment and their fibrillation process and corresponding network formation, the reference material was fibrillated at a concentration of 6.5 wt % (which was the most energy-efficient batch of SMS) using comparable fibrillation settings, at an energy demand of 8 kWh kg^{-1}. From Fig. 3a, it can be seen that at a concentration of 6.5 wt %, the mechanical properties of the SMS film and the reference are comparable, although SMS films display slightly higher elastic modulus, meanwhile the reference material display slightly higher strain at break (Fig. 3a–Table S2). However, it should be noted that nanofibrillation of Kraft pulp under other conditions i.e. lower concentrations and with a higher energy input (13 kWh kg^{-1}) can result in films with overall much higher mechanical properties of up to 9.9 GPa in elastic modulus, 174 MPa in strength, and 6% strain (Berglund et al., 2016).

From Fig. 3b, for the fibrillated SMS material, at the highest tested concentration of 12.5 mg mL^{-1}, 91.3% scavenging activity was observed after 30 min of incubation, while the reference material had negligible antioxidant activity even at the highest loading. Furthermore, for SMS, a 100% scavenging activity was observed at only 2.5 mg mL^{-1}, which signifies that biological pretreatment could be beneficial to preserve functional properties such as antioxidant activity compared to chemically pretreated and fibrillated materials. The unique composition of SMS was further studied and compared to that of birch wood particles (before mushroom cultivation), and chitin-glucan extracts from Shiitake mushrooms. The wood particles present a very broad spectrum, with most of the peaks overlapping the chitin-glucan complex. SMS presents a higher intensity peak with a small shoulder at the region 3600-3200 cm^{-1}, which is related to the OH stretching vibration group present in both birch (3342 cm^{-1}) and chitin-glucan (3432 and 3267 cm^{-1}). The Amide I group at 1647 cm^{-1}, found in chitin, also shows a higher intensity peak in SMS when compared with birch, and the peak around 1000 cm^{-1} is related to Amide III in chitin (Fazli Wan Nawawi et al., 2019).

Environmental impact. Making use of residual biomass as a resource for high-value applications, instead of a residue to be disposed of is considered a core of the circular bioeconomy model (Leppänen et al., 2020; Gaffey et al., 2024). However, environmental performance quantifications of industrial symbiosis networks are complex (Martin et al., 2013). For a better holistic understanding of the fibrillation of SMS as a biologically pretreated biomass, in comparison to chemical pretreatments, the environmental impacts of the value chains from wood to fibrillated material were assessed using LCA, with system boundaries visualized in Fig. S2. For SMS this included the production of woody substrate, the mushroom production, and subsequent reuse of SMS for fibrillation into a gel, while for the reference material, the pulp production from wood and its subsequent fibrillation were accounted for. The studied processes involve the production of editable mushrooms on one hand, and the production of pulp on the other and for interpretation of the environmental burden the categories with the largest differences (more than 100%) between the materials were selected: ozone formation (human health); ozone formation (terrestrial ecosystems; terrestrial-, freshwater-, and marine ecotoxicity; human carcinogenic toxicity; land use and mineral resource scarcity as seen in Fig. 3d. The relative environmental contributions are shown in Fig. S2, and comparative impact assessment (absolute values) are summarized in Table S3 for all impact categories. From Fig. 3, all selected impact categories indicated a lower environmental impact for the SMS material, compared to the reference material. The avoidance of chemicals, by biological pretreatment, compared to a chemical process is reflected in the lower relative impacts observed in Fig. 3d for all ecotoxicology categories, including

impact on water organisms, humans, terrestrial organisms and terrestrial plants of toxic substances emitted to the environment. This is in agreement with a previous study on the environmental assessments of fibrillation from chemically pretreated residual biomass from juice industry that also showed a reduction of ecotoxicology impact categories in relation to reduced chemical input. Synergistic effects were also observed for environmental and economic assessments, where life cycle costing was also reduced by 50% connected to that the main contribution was the cost associated with chemicals (Berglund et al., 2020).

The impact from land use, quantified as amount of land that is occupied for a certain period to produce a product showed the largest difference between the processes, however, the implications of using different assumptions and choices of system boundaries, and land-use baseline should be considered further, since it has shown to have a large influence on the results (Peñaloza et al., 2019). Large differences were also observed for ozone formation, divided in human health and terrestrial eco systems categories and are indicators of emissions to air, partly connected to use of chemicals, that causes the destruction of the stratospheric ozone layer. The biological pretreatment was also promising from a mineral resource scarcity perspective which is an indicator of the depletion of natural non-fossil resources.

3.3. Outlook and limitatins of SMS materials

The use of spent mushroom substrate as biologically pretreated wood and its fibrillation is in this section evaluated in the context of a future bioresource and compared to existing technologies, wherein advantages and limitations, as well as potential for further optimization, is discussed. The SMS prepared from the 6.5 batch was further evaluated and presented according to film formation as a function of energy demand during fibrillation in Fig. 4.

To further study the fibrillated material, HR-SEM was used, which revealed very fine fibrils, however it was difficult to identify and study structures at a nanoscale since the fibrils appear to be mainly embedded in what is hypothesized to be mycelium, or plausibly other components presents (Fig. 4a). It has previously been reported that nanofibrils from SMS after TEMPO-oxidation and sonication with a yield of 18% displayed nanofibers with 2–3 nm in width, as measured using transmission electron microscopy, and cellulose as the main component (Konno et al., 2016). Another study reported that fibrils from SMS, after multiple chemical pretreatments resulted in a yield of about 25%, and that the fibrillation was carried out at a concentration of 1 wt %. Cellulose was the main component after the pretreatments, but chitin from mycelium was also detected using Fourier-transform infrared spectroscopy. The same study showed a wide size distribution of fibrils from SMS and that the width of the smallest structures were measured to about 10 nm from SEM images (Li et al., 2022). The structure of SMS in relation to its fibrillation into fibrils and different components is of interest to study further using different microscopy techniques to learn more about the role of mycelium.

From Fig. 4b, it is shown that the films from fibrillated SMS are bendable and slightly translucent, and possible to form into for example a straw-shape, yet the films are not foldable without breaking and it is of interest to further study and optimize the film properties for potential use in for example packaging applications. In previous studies, we have shown that the viscosity plateau upon fibrillation corresponds to a strong network formation and the highest mechanical properties of dried films (Berglund et al., 2017; Adu et al., 2018). To confirm this for the fibrillated 6.5 wt % SMS material, films were prepared from the samples collected during processing and tested in tensile mode. It was observed that the reached viscosity plateau (Fig. 3b) appears to correspond to the highest mechanical properties of the films (Fig. 4c), suggesting that further fibrillation i.e. longer processing time and higher energy input, would not further improve the mechanical properties of the network at the applied processing conditions. Hence, further optimization of the film properties could explore alternative technologies or

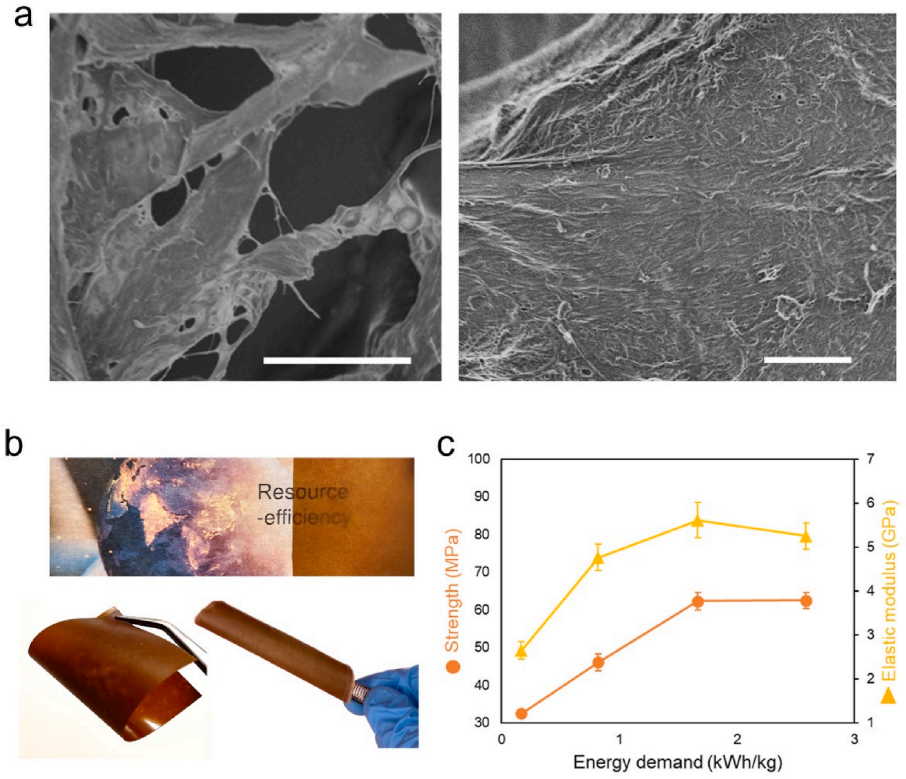

Fig. 4. (a) HR-SEM images after fibrillation of batch 6.5 wt %, after freeze-drying and sputter coating, scale bar: 10 μm and 1 μm, respectively. Film prepared from 6.5 batch **(**b) Photograph of film flat on a background and folded and rolled with a tweezer. (c) Tensile strength and elastic modulus of SMS films as a function of the energy demand of the fibrillation process.

material combinations.

The European Green Deal defined a zero pollution/toxic-free environment as a key policy goal (European Commission, 2019). To support such ambition, the EU Chemicals Strategy for Sustainability includes actions for safe and sustainable by design materials by integrating safety, circularity and functionality, to minimize impacts on human health and the environment associated with chemicals, materials, and products (European Commission, 2020). SMS is a very promising resource from an environmental perspective being a renewable by-product that is becoming more abundant, possible to fibrillate at a yield of 100% without the use of toxic chemicals and could make out an alternative material that further could contribute to minimize the environmental impacts of plastic pollution (De Gisi et al., 2022). Furthermore, SMS have several natural characteristics which are of interest to explore further and can be effectively fibrillated at a high concentration into fibrils with an overall lower environmental impact in the majority of the assessed impact categories, compared to chemically pretreated wood pulp. It should be noted that water consumption, marine eutrophication and stratospheric ozone depletion impact categories were higher for SMS compared to the reference material as shown in Table S4. Further evaluation, beyond the scope of this study is needed to describe the separate contributions, however the results is plausibly connected to the different scales (lab and industrial) of the processes. The total global warming potential (GWP) was 0.24 kg CO_2 eq and 0.32 kg CO_2 eq for SMS and reference material, respectively (Table S3). For the SMS scenario, the aim was to develop an integrated production, where the by-product can be fibrillated for added value, and high value edible mushrooms is produced in parallel. Meanwhile, wood pulping (Kraft or sulfate pulp) is an industrial process, and the reference scenario include data on wood production, chemical pulping, and bleaching, drying, energy production on-site, recovery cycles of chemicals, and internal wastewater treatment. A previous study reported that the pulp production had a relatively small contribution on the environmental impact considering the additional processing steps typically applied for production of cellulose nanofibrils (Arvidsson et al., 2015).

In a life cycle assessment scale-up framework, the environmental impact per kg of produced nanofiber yarn from carrot reside was shown to be lowered by a factor of up to 6.5 compared to the laboratory production (Piccinno et al., 2018). The same study also highlighted the synergetic effects of lower environmental impacts connected to lower costs. In view of a larger or industrial scale of mushroom cultivation and thus, an industrial generation of SMS including recovery to a larger extent, it is expected that the overall CO_2 footprint would be even lower. The environmental impact for SMS material is recommended to be further investigated from an up-scalable perspective and including different scenarios and sensitivity analysis for the assumptions made in the present LCA study. Further investigation, including both environmental and economic perspective, would also be of interest at the early development stage of SMS materials to promote its potential use for high-value applications.

4. Conclusions

In this study, we have demonstrated that SMS, a mushroom production by-product can be considered biologically pretreated lignocellulose, that furthermore can be processed into fibrils without the additions of toxic chemicals. SMS provides interesting benefits for inherently non-toxic and resource-efficient fibrillation with added value and function compared to existing fibrillation processes. SMS was successfully fibrillated at a high concentration of 6.5 wt % using an energy demand of only 1.7 kWh kg^{-1}, compared to commercial and chemically pretreated wood pulp at 8 kWh kg^{-1}, under same processing conditions. The use of SMS as biologically pretreated wood and its fibrillation offers an integrated production with overall lower environmental impact, compared to chemically pretreated wood pulp, meanwhile producing edible mushrooms in parallel. The unique composition of SMS, which is rich in bioactive components, provides natural antioxidant activity. The fibrillation process resulted in fibrils with strong network formation and thus enabled the preparation of SMS into films that has potential for further development into packaging materials. It is essential to explore the SMS material structure and its valorization further for a better understanding of its material properties at all scales and alternative processing approaches and subsequent environmental impact to make real use of it as a resource for applications such as bio-based and bio-active packaging.

CRediT authorship contribution statement

Linn Berglund: Writing – review & editing, Writing – original draft, Visualization, Supervision, Project administration, Funding acquisition, Conceptualization. **Luisa Rosenstock Völtz:** Writing – review & editing, Visualization, Investigation, Formal analysis, Data curation. **Timon Gehrmann:** Investigation, Formal analysis. **Io Antonopoulou:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Carmen Cristescu:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Shaojun Xiong:** Writing – review & editing, Writing – original draft, Resources, Conceptualization. **Pooja Dixit:** Investigation, Formal analysis. **Carlos Martín:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Ola Sundman:** Writing – review & editing, Methodology, Formal analysis. **Kristiina Oksman:** Writing – review & editing, Resources.

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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jenvman.2024.123338.

Data availability

Data will be made available on request.

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