

# From Compost to Colloids—Valorization of Spent Mushroom Substrate

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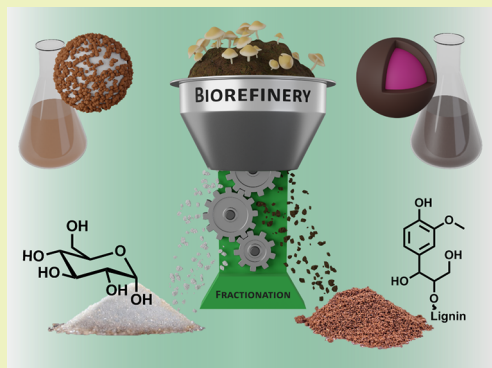
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## S Supporting Information

**ABSTRACT:** Yearly the mushroom industry generates several million tons of spent mushroom substrate (SMS), a mixture of composted soil and fungal mycelium, left after the mushroom harvest. Although containing valuable ingredients like carbohydrates, lignin, and diverse enzymes, the substrate is unutilized and causes immense disposal costs. In order to valorize SMS, it is essential to fractionate the complex mixture into its valuable components, which is a challenge for current biorefineries and has only been partly achieved. We have developed a novel biorefinery strategy in order to separate carbohydrates and soluble lignin from SMS. Therefore, SMS was subjected to two different extraction methods in order to break the insoluble biopolymer residues: A, a thermochemical treatment (water/basic or acidic catalyst) yielding a carbohydrate-enriched liquid fraction; B, an organosolv extraction (with ethanol/water) solubilizing mainly lignin. The carbohydrate fraction possesses surface-active properties and was investigated as a potential biobased surfactant. The soluble lignin fraction was used for the formation of nanocarriers via an inverse miniemulsion polymerization. The lignin-based nanocarriers were biodegradable by laccases, which renders them of high interest for drug delivery systems for advanced plant protection. This novel biorefinery is a powerful strategy for the upcycling of SMS into various high-value products.

**KEYWORDS:** Lignin, Carbohydrates, Biorefinery, Nanocarrier, Surfactant



## INTRODUCTION

The fresh mushroom production in Europe amounted to 1 million tonnes in 2013 with a value of 1.8 billion euro.<sup>1–3</sup> For every ton of mushrooms, approximately three times the amount of spent mushroom substrate (SMS) is generated, which to date has no commercial use.<sup>4</sup> This means such as for Ireland in an annual SMS production of 254 000 t or a production volume of 800 000 t SMS for The Netherlands.<sup>5</sup>

The SMS remains from the mushroom cultivating process and is a complex mixture of composted organics and fungal mycelium left after the mushroom harvest.<sup>5</sup> The substrate is based on various agricultural waste materials, such as sawdust, sugar cane bagasse, oil palm empty fruit bunch, wheat straw-bedded horse manure, hay, poultry manure, ground corncobs, cottonseed meal, cocoa shells, and gypsum.<sup>6</sup> A typical mushroom cultivation cycle lasts for 1 month; farmers usually store the SMS temporarily before it is landfilled, spread on land, or sold as a soil amendment or conditioner product.<sup>5</sup> Due to the vast amounts of SMS generated during the harvest, farmers are confronted with significant logistical problems.<sup>4</sup> Additionally, current costs for SMS disposal amounts range from 10 to 50 €/t in Europe, leading to an immense financial

burden of up to 150 000 000 €/year for the mushroom industry.

Valorization of the unutilized SMS feedstock is demanding, as the substrate composition varies locally, seasonally, and over the period of the harvest. Nevertheless, few studies had succeeded in using SMS directly or as feedstock for valuable biobased materials.<sup>7</sup> For example, extracellular fungal enzymes like laccases and peroxidases were extracted from SMS after the mushroom harvest.<sup>8</sup> Especially due to its lignases, which enzymatically catalyze the metabolism of lignin and lignin-related compounds, SMS is a potential candidate for bioremediation (e.g., for polycyclic aromatic carbons or agrochemicals like pentachlorophenol, carbendazim, or mancozeb).<sup>5,9</sup> Besides the enzymes contained in SMS, various natural ingredients such as lignin, proteins, or diverse carbohydrates have been detected in the used compost.<sup>5</sup> To date, only crude carbohydrate fractions containing up to 25% of SMS's sugars were separated by hot water alkali extractions.<sup>10,11</sup> Carbohydrates from SMS were also used for

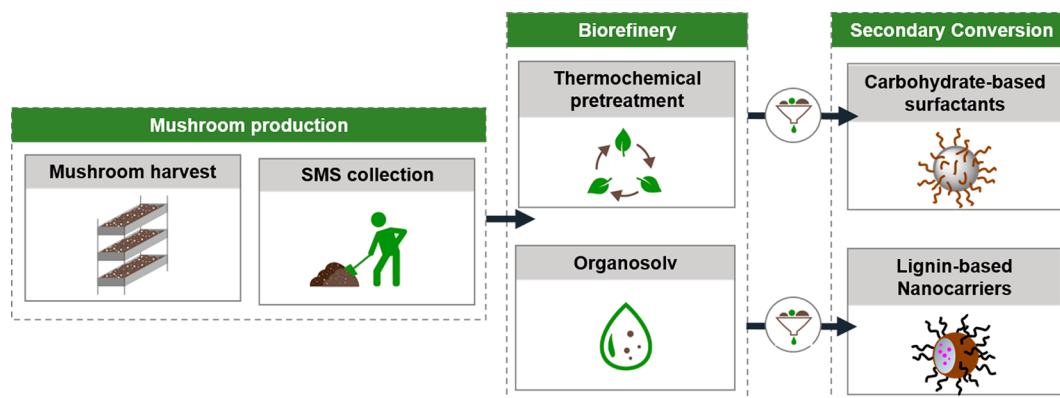
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Scheme 1. Valorization of the Spent Mushroom Substrate to Ecofriendly Surfactants and Biodegradable Nanocarriers



biofuel production,<sup>12</sup> after extracting the majority of the lignin from the crude SMS by an organosolv protocol.<sup>13</sup> An efficient fractionation of the complex mixture is still missing. Particularly, as SMS is not competing with food production, the exploitation of its components as a resource is of great interest.

If an efficient fractionation is established, the SMS-derived chemicals might be useful as biodegradable building blocks for nanomaterials. Especially, the development of biobased nanocarriers as interesting nanomaterials for biomedicine or agriculture is a focus of recent studies.<sup>14</sup> As SMS is composed of carbohydrates and lignin, the development of biobased surfactants or degradable nanocarriers might be attractive. We have recently used carbohydrates<sup>15,16</sup> or lignin<sup>17,18</sup> to prepare biodegradable nanocarriers. The use of SMS derived materials for nanocarrier production would be an attractive pathway of an “upcycling” of a waste product.

Herein, we present an innovative biorefinery concept for fractionation of SMS (and SMS supplemented with wheat straw) into carbohydrate- or lignin-enriched extracts. The soluble carbohydrates or lignins act as starting materials for valuable biobased products; here we used them as biobased surfactants or building block for biodegradable nanocarriers.

The SMS was either thermochemically pretreated (at elevated temperatures in water containing an acidic or basic catalyst) in order to solubilize carbohydrates or extracted with ethanol–water mixtures (“organosolv” treatment) to obtain soluble lignin fractions (Scheme 1). The carbohydrate fraction was used as a biobased surfactant, whereas the lignin-enriched SMS extracts could be applied to prepare biodegradable lignin nanocarriers by a miniemulsion polymerization. With this concept, a step toward “upcycling” of SMS into biobased and biodegradable materials is made, which might be used in agricultural applications.

## EXPERIMENTAL SECTION

**Materials.** 2,4-Toluene diisocyanate (TDI), sodium dodecyl sulfate (SDS), sulforhodamine-B (SR-B), hexadecane, styrene, 2,2'-azobis(2-methylbutyronitrile), 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane, *N*-hydroxy-5-norbornene-2,3-dicarboxylic acid imide, and solvents were purchased from Sigma-Aldrich. Polyglycerol polyricinoleate (PGPR) was obtained from Danisco. The laccase cocktail MetZyme PURECO 020 (L371) was kindly provided by MetGen (Finland). All chemicals were used without further purification.

Spent mushroom compost was generated at Tyholland Farm (Monaghan, Ireland) and kindly supplied by Monaghan Mushrooms. Wheat straw was harvested in Spain and supplied by local farmers.

The SMS was grounded with a mesh of 6 mm and stored at 4 °C. The wheat straw was milled to a mesh of 4 mm and stored at room temperature. After drying, the moisture content was adjusted to 50%, and the SMS was thermochemically pretreated in combination with wheat straw or extracted without additives with ethanol–water mixtures (Scheme 1).

**Thermochemical Pretreatment.** The thermochemical pretreatment assays were carried out in a steam pressurized plug-flow horizontal reactor (Advancebio Systems LLC, USA) that allows a high solid load (50% w/w) and operation up to 192 °C. In order to avoid clogging, SMS was mixed with wheat straw in a wet weight ratio of 4 to 6 (2 to 8 in dry basis). The operating temperature was adjusted to 160, 175, or 190 °C and pressures of 6.5, 8.5, and 15.5 bar, respectively, using a residence time of 10 min. The catalyst (NaOH, Na<sub>2</sub>CO<sub>3</sub>, or H<sub>2</sub>SO<sub>4</sub>) was added in a relation of 4% by dry weight to the total solid content. For homogenization, the mixture was blended manually first and left overnight at room temperature before feeding the reactor afterward. Finally, the pretreated samples were filtered with a filter press, and subsequent filter cake, as well as extract, was analyzed separately. In total, from 1 kg of SMS and 1.65 kg of wheat straw, 1.4 kg of extract with a solid content of 50–60 mg/mL was generated. In average, a carbohydrate yield of 20% relative to the number of carbohydrates contained in the initial SMS:wheat straw mixture (ratio 4:6) was reached (Figure S7b).

**Preparation of Oil-in-Water Miniemulsion Stabilized by SMS-Derived Biobased Surfactant.** After thermochemical pretreatment (190 °C, NaOH catalyzed), the slurry was filtered, and the resulting extract was dried under reduced pressure. From the solid obtained, 50 mg was dissolved in 5 mL of Milli-Q water to yield a 10 mg/mL solution. Two hundred microliters of hexadecane was added, and the mixture was sonicated (Branson Digital Sonifier W450-D, 1/2" tip, 70% amplitude, 3 min, 20 s ultrasound followed by 10 s pauses, 0 °C) to form a miniemulsion. Afterward, the dispersion was stirred gently for at least 7 days. To investigate the impact of pH value or salt, the biobased surfactant was dissolved in the respective aqueous NaOH (pH 11), HCl (pH 2), or NaCl (10 mg/mL, 100 mg/mL) solution instead of pure Milli-Q water. Furthermore, the stability of the aqueous dispersions (at pH 7, without the addition of salt) at 50 or 70 °C was investigated.

**Formation of Polystyrene Nanoparticles Stabilized by an SMS-Derived Biobased Surfactant.** Carbohydrate-enriched fractions generated by the thermochemical pretreatment were dried under reduced pressure. Two hundred forty milligrams of the obtained biobased surfactant was dissolved in 24 mL of Milli-Q water. Afterward, 6 g of styrene, 152 mg of hexadecane, and 102 mg of 2,2'-azobis(2-methylbutyronitrile) were added, and the mixture was stirred vigorously for 1 h. After sonication (Branson Digital Sonifier W450-D, 1/2" tip, 90% amplitude, 2 min, 20 s ultrasound followed by 10 s pauses, 0 °C) the reaction was allowed to proceed at 70 °C overnight. After the polymerization, the reaction mixture was filtered through a Kimtech wipe to remove any aggregates (typically no macroscopic aggregates were detected). For further purification, the dispersion was

centrifuged for 45 min at 12k rpm and redispersed in Milli-Q water (the solid content was adjusted to ca. 8 mg/mL).

**Organosolv Treatment of SMS.** As starting material pristine SMS was used. The organosolv treatment was performed in a stirred batch reactor (working volume 70 mL) at 250 °C for 60 min using either 10 wt % substrate–solvent mixture. Water and ethanol were applied in a ratio of 1:1, 6:4, or 7:3, respectively. After extraction, the mixture was filtered, producing 50 mL of lignin-enriched extract with a solid content of ca. 10 mg/mL.

**Formation of Nanocarriers from Lignin-Enriched Organosolv Fractions.** Lignin-enriched extracts obtained from previous organosolv treatment (EtOH–water 50:50) were dried under reduced pressure. The residue was dissolved in acetone and precipitated in water afterward. The solid was isolated by centrifugation and was dried at 50 °C at reduced pressure. Forty milligrams of the fine brown powder was dissolved in 520  $\mu$ L of DMSO adding optionally 5 mg of sulforhodamine B. The solution was then mixed vigorously for 1 min at 20k rpm with a 1 wt % cyclohexane–PGPR solution using an IKA Ultraturrax disperser in order to form a pre-emulsion. Subsequently, the emulsion was treated with ultrasound for 3 min (Branson Digital Sonifier W450-D, 1/2" tip, 70% amplitude, 20 s ultrasound followed by 10 s pauses) cooled by water. A solution consisting of 2 g of cyclohexane, 6 mg of PGPR, and 60 mg of TDI was added then dropwise, and the reaction was allowed to proceed overnight at RT under stirring.

For solvent exchange, 400  $\mu$ L of the dispersion was added slowly under vigorous shaking to 5 mL of a 1 mg/mL aqueous sodium dodecyl sulfate (SDS) solution. The mixture was sonicated for a further 3 min in a sonication bath (25 kHz) and finally stirred for 24 h with an open cap in order to guarantee evaporation of the cyclohexane. For purification, the dispersions were centrifuged (30 min, 4k rpm), followed by solvent exchange and redispersion in aqueous SDS solution (0.1 wt %) afterward.

In order to determine the encapsulation efficiency, the nanocarriers were removed by centrifugation (30 min, 14k rpm) directly after evaporation of the cyclohexane, and free SR-B in the supernatant was quantified by UV/vis spectroscopy.

**Enzymatic Degradation of SR-B Loaded Nanocarriers.** First 0.3 mL of the laccase cocktail L371 was mixed with 1.7 mL of glycine/sodium hydroxide buffer (pH 10.5) and 0.5 mL of a nanocarriers dispersion (solid content: 1.5 mg/mL). The degradation reaction proceeded for 24 h at room temperature under stirring while being kept open to enrich the solution with oxygen. In order to determine the dye release, the nanocarriers were removed by centrifugation, and the amount of SR-B in the supernatant was quantified by UV/vis spectroscopy.

**Moisture Content.** Moisture content in SMS was calculated by drying the samples at 105 °C up to constant weight.

**Ash Content.** Ash content was determined after air combustion for 2 h at 550  $\pm$  10 °C in a muffle oven.

**Dynamic Light Scattering.** The hydrodynamic diameters of the particles were measured by DLS with NICOMP 380 submicron particle sizer (Nicom Particle Sizing systems, USA) at a fixed angle of 90° and a laser diode running at 635 nm. The sample was diluted to a concentration of 0.01 wt % with water or cyclohexane before measurement.

**Surface Tension Measurements.** The surface tension at the water–air interface was determined with the ring tensiometer DCAT 21 from DataPhysics. An aqueous surfactant solution of 0.15 g/mL was added dropwise into the water. After each addition, the mixture was stirred for 2 min, and afterward the surface tension was measured.

**The Interfacial Tension.** The interfacial tension between chloroform and water was determined with a spinning drop tensiometer (SVT 20N from DataPhysics). A glass capillary was filled with chloroform and a small droplet of Milli-Q water (as a reference) or aqueous extract mixture (10 mg/mL). Then the capillary was placed horizontally and equilibrated at 20 °C for 10 min under rotation at 8000 rpm until a cylindrical droplet at the axis of rotation was obtained. The interfacial tension based on the theory of Cayias, Schechter, and Wade was used for data analysis.

**Extract Analysis.** HPLC analysis was performed with an Agilent Technologies system (model 1200). The content of monosaccharides (D-glucose, D-xylose, L-arabinose) was quantified at an ICSEP ION 300 column (7.8  $\times$  300 mm, Transgenomics, Glasgow, U.K.) at 72 °C with a refractive index detection. As an eluent, an aqueous 8.5 mM H<sub>2</sub>SO<sub>4</sub> solution was used.

Carbohydrate degradation compounds such as furans (furfural and 5-hydroxymethylfurfural) and phenolic compounds (benzaldehyde, ferulic acid, vanillin-syringaldehyde, vanillin/syringic, coumaric, benzoic, 4-hydroxybenzoic, phenol, syringol, guaiacol) were measured using a Zorbax column (250  $\times$  4.6 mm, Agilent Technologies) at 50 °C with a diode array detector. The mobile phase consisted of water and acetonitrile in a ratio of 8:2.

**Filter Cake Analysis.** Quantification of carbohydrates and lignin content in the insoluble fraction were obtained following the NREL procedures no. 42618 and no. 42627, respectively.

**Size Exclusion Chromatography.** SEC analyses of the extracts obtained from the thermochemical pretreatments were performed using an Agilent Technologies 1260 instrument consisting of a pump, autosampler, and column oven. A column set consisting of two columns: MCX10<sup>3</sup> Å and MCX10<sup>5</sup> Å (PSS Standards Service GmbH, Mainz, Germany), both of 300  $\times$  8 mm and 10  $\mu$ m average particle size, were used at a flow rate of 1.0 mL/min and a column temperature of 30 °C. As eluent, 80% 0.1 M NaOH and 20% acetonitrile were used. The injection volume was 10  $\mu$ L. Detection was accomplished with an RI detector (RI-101, ERC) and a UV detector (UV/vis-2487, Waters) at 270 nm. Data acquisition and evaluation were performed using PSS WINGPC UniChrom (PSS Polymer Standards Service GmbH, Mainz, Germany). Calibration was carried out by using poly(styrenesulfonate) sodium salt provided by PSS Polymer Standards Service GmbH (Mainz, Germany). High-performance size exclusion chromatography (HPSEC) was used for analyses of the organosolv extracts. As eluent, a solution of 0.1% lithium bromide in *N,N'*-dimethylformamide was used under isocratic conditions. The column was a Polargel-M, and detection was accomplished with an RI detector. Polystyrene was used as a reference material.

**NMR Spectroscopy.** <sup>1</sup>H and <sup>31</sup>P nuclear magnetic resonance (NMR) spectroscopy was performed at a Bruker AVANCE (USA) system at 300 MHz. For <sup>1</sup>H NMR spectroscopy, 5 mg of the sample was dissolved in 600  $\mu$ L of DMSO-*d*<sub>6</sub>. To determine the number of OH groups in the sample, <sup>31</sup>P NMR spectroscopy was used after derivatization according to the literature for other lignin derivatives.<sup>19</sup> 20 mg of dried sample was dissolved in 550  $\mu$ L of a CDCl<sub>3</sub>–pyridine-*d*<sub>5</sub> (4/6 v/v ratio) mixture and was then treated with 100  $\mu$ L of 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane in the presence of the internal standard endo-*N*-hydroxy-5-norbornene-2,3-dicarboximide and the relaxation agent Cr(III)acetylacetonate in an NMR tube, and <sup>31</sup>P NMR spectra were recorded.

## RESULTS AND DISCUSSION

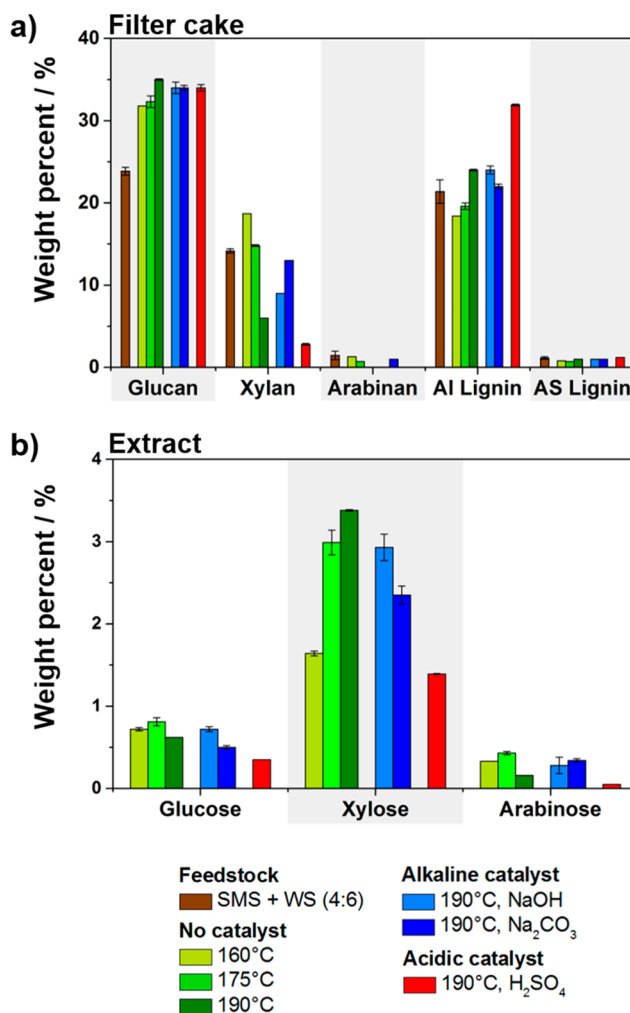
**Compositional Analysis of SMS.** The substrate prepared specifically for growing mushrooms is a blend of natural products with high organic content. The major ingredients in the compost investigated in this study were wheat straw (62 wt %), gypsum (6 wt %), and poultry manure (30 wt %) as well as ammonium sulfate (0.6 wt %) as a nitrogen source. During the growth, the mycelium penetrated continuously the compost consuming carbohydrates, lignin, and nutrients. After the harvest, the spent mushroom substrate consisted mainly of water (>60%), lignin, ash (25–35%), residual carbohydrates, and fungal mycelium. The majority of the SMS dry matter (82–85%) was insoluble in water and was composed of ca. 30% lignin. Additionally, saccharides like glucan (8%) and xylan (4%), which are related to cellulose and hemicellulose respectively, were identified. Hence, SMS contains higher lignin amounts than agricultural residues like wheat straw (9–17%), corn stover (7–20%), or sugar cane bagasse (10–20%)



but also than lignocellulosic feedstocks like oak, birch, and poplar (with each ca. 25%) and is therefore considered as a particularly useful lignin source.<sup>20</sup>

**Thermochemical Treatment of SMS.** The dried SMS was grounded with a mesh size of 6 mm, and the moisture content was adjusted to improve processing and reproducibility. In spite of the presence of fungal enzymes (e.g., lignases, cellulases) and the mycelium penetration during the mushroom cultivation, which might lose the dense lignocellulose structure in the compost,<sup>20</sup> the spent mushroom substrate was found to be recalcitrant and demanding to process. In order to prevent clogging of the plug-flow reactor, the SMS was supplemented by wheat straw in a ratio of 4:6, which is another seasonal underutilized lignocellulosic feedstock.<sup>21</sup> The SMS (or the mixture with wheat straw) was treated at elevated temperatures in a pressure reactor with the addition of acid or base. Due to the recalcitrant structure of SMS and its buffering capacity, fractionation needed to be performed at high temperatures and with high catalyst concentrations. After the thermochemical treatment, the slurry was filtered, and the resulting filter cake was investigated separately from the liquid extract. In order to find optimal process conditions, the reaction temperature was varied from 160 to 190 °C. SEC proved the extraction of a polydisperse mixture of oligomers, with molar masses slightly decreasing when the temperature of the process was increased, which indicates a more efficient fractionation of SMS under harsher conditions (Figure S5). Independent of the temperature during the process, the monosaccharide xylose was the major extracted compound, while glucose was solubilized always in lower amounts (Figure 1b, green). Respectively, the insoluble filter cake contained a large fraction of glucan but significantly less xylan (Figure 1a, green). With increasing temperature, the concentration of xylose increased by the factor of 3 in the extract, and a reduced amount of xylan in the filter cake was detected. Additionally, arabinan was extracted, while in the filter cake it was only detected in small amounts. Interestingly, the amount of lignin which was left in the solid slightly increased comparing the processes at 160 and 190 °C, indicating more effective lignin solubilization at lower temperatures. However, from the <sup>1</sup>H NMR spectra of these extracts, mainly aliphatic species, i.e., carbohydrates, were identified (Figure S1). Lignin and other aromatic compounds were extracted only in trace amounts. Phosphorylation of the extracted compounds allowed determination of aliphatic and aromatic hydroxyl groups, respectively, according to a literature protocol.<sup>19</sup> The extracts were reacted with 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane, and <sup>31</sup>P NMR spectra of the reaction products were recorded. The spectra indicated mainly signals of phosphorylated aliphatic alcohols, whereas almost no resonances belonging to phenolic compounds were detected (Figure 3b, black).

Alkaline catalysts promote the cleavages of ester linkages and glycosidic bonds in the cell walls, resulting in cellulose swelling and partial solubilization.<sup>22</sup> Particularly Na<sub>2</sub>CO<sub>3</sub><sup>23</sup> and NaOH,<sup>24</sup> two inexpensive and abundant bases, showed high effectivity regarding the degradation of lignocellulosic biomass in previous studies.<sup>20,25,26</sup> Therefore, the influence of the catalysts on the thermochemical treatment of an SMS–wheat straw mixture was studied. Interestingly, the basic reaction conditions only reduced the amount of xylose in the extract, correlating with increasing xylan content in the respective filter



**Figure 1.** Compositional analysis of (a) filter cake (weight percent refers to the dry mass of filter cake; AI = acid insoluble, AS = acid soluble; Table S2) and (b) carbohydrate-enriched extract after thermochemical pretreatment and filter press (weight percent of liquid extract; Table S1).

cakes, but did not affect the amount of solubilized lignin or glucan (Figure 1, blue).

Moreover, H<sub>2</sub>SO<sub>4</sub> is a commonly employed reagent to pretreat diverse biomass, as it effectively hydrolyzes hemicellulose to xylose, which then can be converted to furfural and derivatives.<sup>27</sup> In contrast to Na<sub>2</sub>CO<sub>3</sub> and NaOH, the addition of the acid led to decreased yields of xylan in the filter cake but also to a reduction of xylose in the extract (Figure 1, red). Simultaneously, we found significantly increased concentrations of 5-HMF (745 mg L<sup>-1</sup>, 250%) and furfural (1412 mg L<sup>-1</sup>, 413%) in the extract, suggesting acidic degradation reactions of xylose (Table S1).<sup>27–29</sup>

Thus, all thermochemical treatments resulted in extracts enriched in a mixture of carbohydrates and carbohydrate derivatives. Independent of the catalyst, xylose was solubilized as the major compound, whereas lignin remained almost completely in the filter cake. In total, a carbohydrate yield of ca. 20% (Figure S7b) was reached, which is comparable to results already reported from SMS extractions in literature.<sup>10,11</sup> In comparison, higher yields of carbohydrates were documented when fractionating lignocellulosic feedstocks like wheat straw, soybean straw,<sup>30</sup> spruce,<sup>31</sup> and pine<sup>32</sup> under

similar conditions (e.g., liquid hot water, acid, or alkaline treatments) underlining the recalcitrancy of SMS.<sup>20,26</sup> To further increase the carbohydrate yield, the recalcitrant filter cake could be treated additionally by enzymatic hydrolysis (e.g., cellulases) followed by an additional extraction step.<sup>33</sup>

**Biobased Surfactants from Carbohydrate-Enriched SMS.** Due to their versatile applicability such as in bioremediation, food, cosmetics, pharmaceuticals, or nanotechnology, biobased surfactants are expected to reach a market value of up to 2 billion USD by 2020.<sup>34</sup> Especially, regarding waste management and exploitation of renewable sources, surfactants produced from inexpensive waste feedstock are of growing interest.<sup>34,35</sup>

The carbohydrate-enriched liquid fractions obtained after the thermochemical treatment of SMS were tested on their capacity to decrease the surface tension. The extracts, which had pH values of 3.47 (for the H<sub>2</sub>SO<sub>4</sub>-treated SMS) and ca. 4.8 for all others, were lyophilized and added at different concentrations to water, and the surface tension at the air–water interface was measured by a ring tensiometer. All extracts reduced the surface tension of pure water (with 72 mN m<sup>−1</sup>); at concentrations of 3 g L<sup>−1</sup>, extracts obtained by treatments using no or alkaline catalysts decreased the surface tension to ca. 57 mN m<sup>−1</sup> proving their capability to be used as surfactants (Figure 2b). At the same weight concentration, the

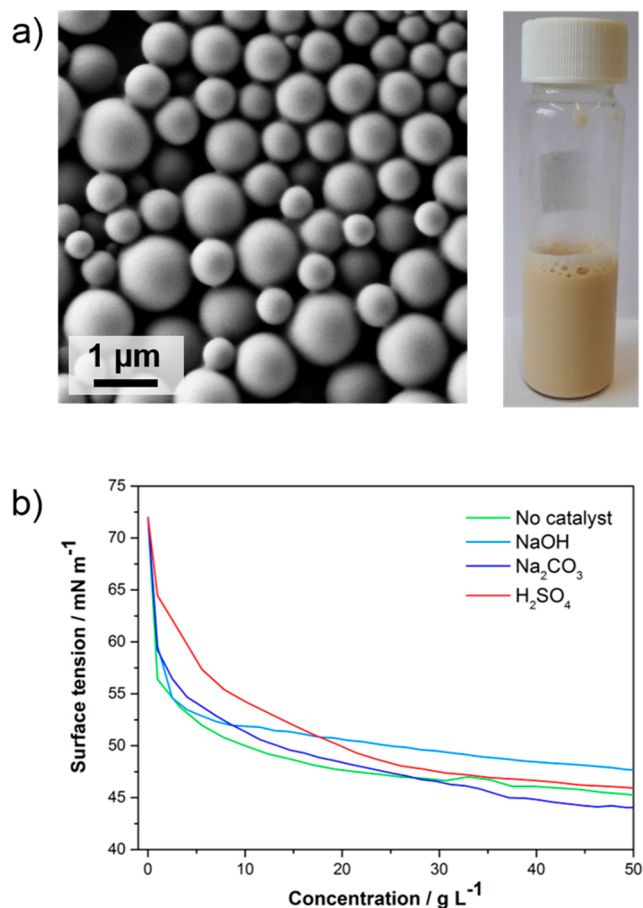
acidic extract only reduced the interfacial tension to ca. 62 mN m<sup>−1</sup>, probably due to partial protonation of the compounds in the mixture resulting in lower interfacial activity. However, a further increase of the extracts (up to 50 g L<sup>−1</sup>) still resulted in a steady decrease of the surface tension to values between 45 and 50 mN m<sup>−1</sup>, indicating an incomplete surface coverage by surfactant molecules. A constant value for the surface tension was not reached, probably due to aggregates, which were detected by dynamic light scattering, contained in the SMS extract, which could not be separated by centrifugation or filtration. By further increasing the concentration of the extract, these aggregates were enriched steadily, leading to an additional surface on which the surfactant molecules can arrange, which led to a slight further decreased surface tension.<sup>36</sup>

Additionally, we determined the interfacial tension between chloroform and the aqueous SMS extracts (solid content of 10 mg mL<sup>−1</sup>) with a spinning drop tensiometer. In accordance to our previous results, the interfacial tension between water and CHCl<sub>3</sub> was significantly reduced from 26.6 to 7.3 mN m<sup>−1</sup> after the addition of the extract obtained from SMS treatments using no catalyst. Similar values of ca. 11 mN m<sup>−1</sup> were measured for the other extracts (cf. Table S4).

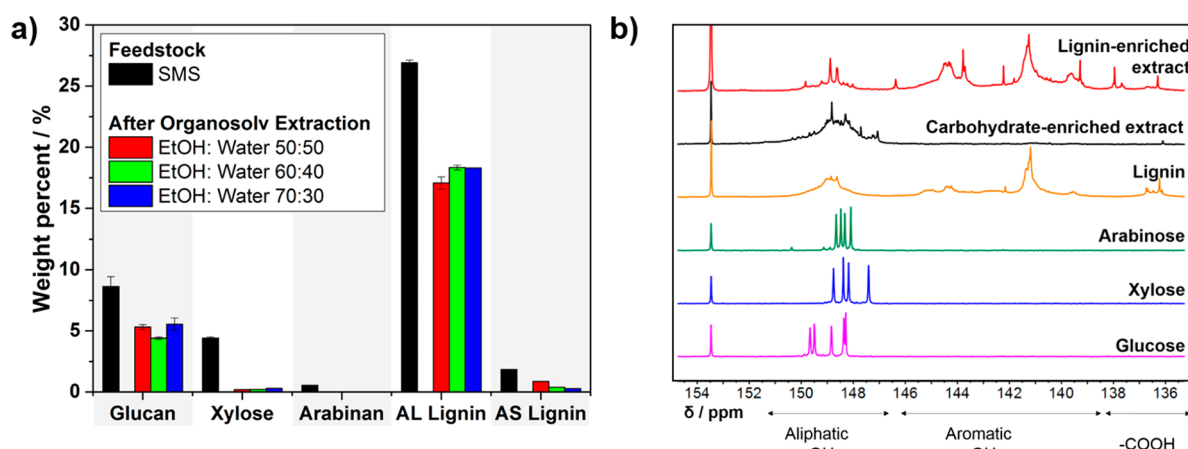
The ability to decrease the interfacial tension suggests an extraction of amphiphilic hydrolysates from cellulose, hemicellulose, or lignin generated during the thermochemical treatment. As <sup>1</sup>H and <sup>31</sup>P NMR spectra (the latter after derivatization, Figure 3b) proved the solubilization of aromatic compounds only in minor extent, mainly UV-active oligomers were detected by SEC though (Figure S6) the extracts contained probably phenolic oligoglycosides. This correlates with slightly negative log *D* values (distribution coefficient determined between water and octanol, Table S5) determined for all extracts and underlines the hydrophilic character of the amphiphilic oligosaccharides. Due to their amphiphilicity, such phenolic glycosides are interfacially active and can stabilize oil-in-water emulsions.

The extracts were successfully used to stabilize oil-in-water miniemulsions–heterophase systems in which stable nanodroplets of one phase are dispersed in a second continuous phase.<sup>37</sup> In an oil-in-water miniemulsion, each nanodroplets of the oil can be considered as a nanoscopic, individual batch reactor that enables a variety of reactions, e.g., polymerizations, and can be used to prepare various nanomaterials.<sup>38</sup> As an initial test for emulsion stabilization, 1 mL of hexadecane (as dispersed phase) was dispersed into 25 mL of an aqueous SMS extract (catalyst: NaOH, 190 °C) with a solid content of 1 wt %. After sonication, yellowish/light-brown turbid dispersions were obtained which remained macroscopically stable for at least 7 days at room temperature. Phase separation was not observed when the dispersion was heated up to 70 °C or after the addition of NaCl (100 mg/mL). Moreover, the dispersion did not undergo phase separation when the pH of the continuous phase was changed from 2 to 12, underlining the versatile applicability of the SMS-based surfactant.

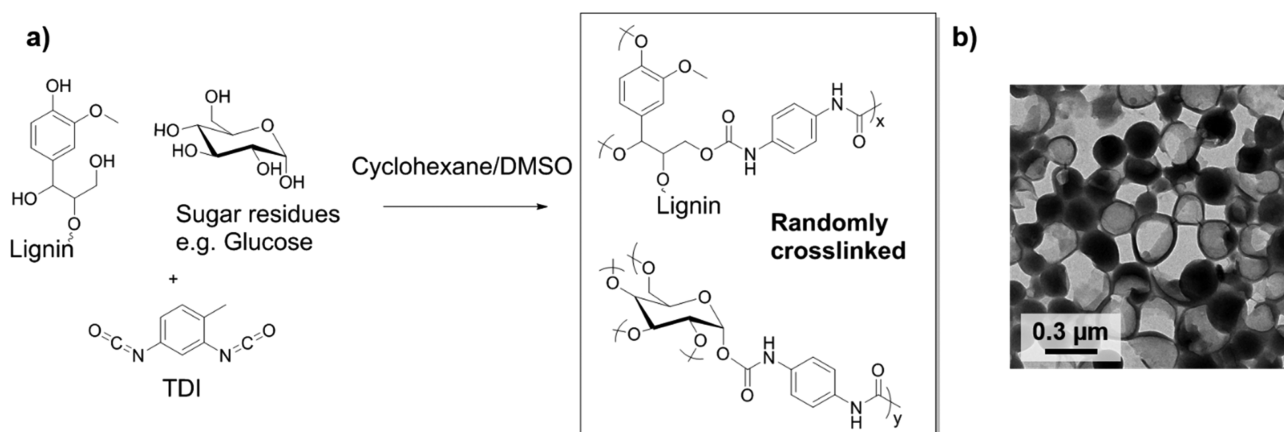
As the results suggest that the carbohydrate-enriched fractions can stabilize oil-in-water droplets, we applied the extracts as stabilizers during a free radical miniemulsion polymerization of styrene to prepare polystyrene nanoparticles. A mixture of styrene, hexadecane (as costabilizer), and the initiator 2,2'-azobis(2-methylbutyronitrile) was emulsified in an aqueous SMS extract solution by vigorous stirring. After ultrasonication, a stable miniemulsion was obtained. The



**Figure 2.** (a) SEM image (left) and photo (right) of the polystyrene nanocarrier dispersion stabilized with the SMS-derived biobased surfactant and (b) surface tension measurements of SMS-derived biobased surfactants produced by thermochemical treatments at 190 °C using different catalysts measured by ring tensiometry.



**Figure 3.** (a) Compositional analysis of the filter cake left after extracting SMS with water–ethanol mixtures (organosolv treatment). (b) Comparison of the  $^{31}\text{P}$  NMR spectra obtained from SMS-derived extracts and commercially available lignin as well as carbohydrates after phosphorylation.



**Figure 4.** (a) Possible reaction of organosolv–SMS extract with TDI at the droplet interface of DMSO nanodroplets dispersed in cyclohexane.<sup>18</sup> (b) TEM image of cross-linked organosolv–SMS nanocarriers.

polymerization of styrene was carried out at 70 °C for 15 h. After the reaction, a colloidal stable dispersion of PS particles was obtained, independent of the extracts which was used for the stabilization. Dynamic light scattering (DLS) and electron microscopy proved the successful formation of spherical polystyrene particles (Figure 2a) with diameters between 500 and 1000 nm, which did not show any signs of aggregation for at least 5 months of storage at room temperature, proving the potential of SMS-derived extracts as biobased surfactants.

**Organosolv Treatment of SMS.** Organosolv is a pulping technique applied in paper industry that uses an organic solvent to solubilize lignin and hemicellulose.<sup>39</sup> As pure SMS is composed of ca. 30% lignin, it would be an ideal lignin feedstock. Therefore, we extracted SMS with different ethanol–water mixtures at 250 °C in a batch reactor. Using a 50:50 ethanol–water mixture, the lignin amount in the solid residue was reduced by almost 40% (Figure 3a). By increasing the ethanol percentage from 50% to 70%, the extracted lignin amount remained unchanged. Besides lignin, also glucan and hemicelluloses were extracted partially. However,  $^1\text{H}$  NMR and  $^{31}\text{P}$  NMR (after derivatization) spectroscopy of the respective extracts showed mainly the lignin typical signals (e.g.,  $^1\text{H}$ : 6.0–8.0 ppm aromatic protons;  $^{31}\text{P}$ : 151–147 ppm aliphatic –OH, 146–139 ppm phenolic –OH), and hence lignin can be assumed as the major compound solubilized

(Figure 3b, red; Figure S1). Fully in line with these results, FT-IR spectra proved the presence of aromatic compounds by intense bands at 1609 and 1457  $\text{cm}^{-1}$  belonging to the vibration of the aromatic skeleton (Figure S2). Additionally, SEC analyses confirmed the presence of oligomeric and polymeric compounds in the extracts with relatively broad molar mass dispersity (with apparent  $M_n$ : ca. 800 g/mol,  $M_w$ : 3300 g/mol;  $D = 4.2$ ; Figure S8).

**Preparation of Biodegradable Nanocarriers from Lignin-Enriched Fractions.** In recent years, the exploitation of lignin was heavily researched particularly as a renewable feedstock for chemical syntheses and as fuel alternatives. However, the valorization to nanomaterials gained increasing interest recently, too.<sup>20,26,29</sup> Until now, solid or hollow particles with core–shell structures were prepared mainly by nanoprecipitation, whereas only a few studies investigated the preparation of chemically cross-linked lignin nanoparticles.<sup>40</sup> Herein, dye-loaded, biodegradable lignin nanocarriers were synthesized from lignin-enriched extracts obtained after the organosolv treatment. An interfacial polyaddition in inverse miniemulsion, similar to previously reported for commercial lignin, was adapted for the extracts.<sup>18</sup> For that, the solid was dissolved in DMSO and the dark-brown solution was dispersed in cyclohexane, containing the biocompatible and -degradable surfactant PGPR.<sup>41</sup> To further increase the sustainability of the



process, biobased solvents, e.g., alcohols, could be used as a dispersed phase. In order to prove that the nanocarriers might be used to deliver drugs, sulforhodamine-B was encapsulated as a model cargo. The emulsion was sonicated to generate stable nanodroplets (DLS: 200–300 nm), which act as a template for the nanocarrier production. We used toluene–diisocyanate (TDI) as a cross-linker, as its isocyanate groups react with lignin's hydroxyl groups as well as with hydroxyl groups of carbohydrate residues at the droplet interface forming a cross-linked shell (Figure 4a). After the reaction, the solvent was exchanged by dispersing the product mixture into an aqueous 0.1 wt % sodium dodecyl sulfate solution and subsequent evaporation of cyclohexane. The resulting dispersion was centrifuged and resuspended in fresh water. A stable aqueous dispersion of the SMS-based nanocarriers was obtained with diameters of ca. 290 nm (PDI 0.15) as determined by dynamic light scattering. After storage of 6 months at room temperature, no change in the particle size distribution was detected by DLS, proving the colloidal stability of the prepared nanocarrier dispersions. The morphology of the nanocarriers was a mixture of spherical core–shell and full particles. Previous studies with commercial lignin produced only core–shell structures under the same reaction conditions. The mixture of both core–shell and solid nanoparticles from the SMS-based lignin might be attributed to the diverse mixture of the organosolv SMS-extract containing molecules of different hydrophilicity and reactivity so that the cross-linking with TDI occurred both at the interface and the inside of the DMSO droplets (Figure 4b). In spite of the different morphologies of the nanocarriers, ca. 80% of the SR-B was determined inside of the nanocarriers after the transfer into an aqueous environment, proving that a DMSO-soluble cargo was effectively loaded into the SMS-based nanocarriers. The remaining 20% of SR-B was either not encapsulated or removed during the redispersion process. The aqueous dispersion could be stored at room temperature without any signs of degradation or leakage of the cargo for at least 4 weeks.

As reported previously, lignin-based nanocarriers can be degraded enzymatically by laccases, which occur naturally in diverse fungi and catalyze the oxidation of phenolic compounds by simultaneous oxygen reduction.<sup>42</sup> In order to prove that also SMS-derived nanocarriers undergo enzyme-triggered release of the cargo, we quantified the amount of free SR-B before and after addition of a laccase cocktail. After the addition of laccase to the dispersion, a 40% release of SR-B was determined after 24 h. As several pathogenic fungi segregate laccases,<sup>43</sup> the lignin-based nanocarriers might be applied as an enzyme-triggered drug delivery system, which could be of high interest to develop advanced fungal plant protection.

## SUMMARY

This work presents the valorization of the spent mushroom substrate (SMS) by two biorefinery processes allowing the isolation of either carbohydrates (by a thermochemical treatment) or lignin (by an organosolv extraction).

The obtained carbohydrate-enriched fractions contained xylose as the major compound whose concentration increased with rising temperature but decreased when using the alkaline catalysts  $\text{Na}_2\text{CO}_3$  or  $\text{NaOH}$ .  $\text{H}_2\text{SO}_4$  was found effective to solubilize xylan but resulted in low xylose concentrations due to following carbohydrate decomposition reactions. As the carbohydrate-enriched fractions significantly decrease the surface tension at a water–air interface, they are promising

biobased surfactants and were successfully used for mini-emulsion polymerization.

Soluble lignin was obtained after extracting SMS with ethanol–water mixtures. The organosolv–SMS (i.e., lignin-enriched fractions) were used as a raw material to generate loaded nanocarriers by an inverse miniemulsion approach. Due to their degradability by laccases, the nanocarriers are considered to be of high interest as biobased drug delivery systems useful such as for sustainable plant protection.

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssuschemeng.8b06710.

Tables with compositional analysis of extracts and filter cakes; interfacial tensions; log *D* values; NMR and FT-IR spectra; SEM and TEM images; SEC elugrams (PDF)

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### Notes

The authors declare no competing financial interest.

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