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ORIGINAL RESEARCH

The impact of xylan on the biosynthesis and structure of extracellular lignin produced by a Norway spruce tissue culture

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Abstract

In order to develop more economic uses of lignin, greater knowledge regarding its native structure is required. This can inform the development of optimized extraction methods that preserve desired structural properties. Current extraction methods alter the polymeric structure of lignin, leading to a loss of valuable structural groups or the formation of new non-native ones. In this study, Norway spruce (*Picea abies*) tissue-cultured cells that produce lignin extracellularly in a suspension medium were employed. This system enables the investigation of unaltered native lignin, as no physicochemical extraction steps are required. For the first time, this culture was used to investigate the interactions between lignin and xylan, a secondary cell wall hemicellulose, and to study the importance of lignin–carbohydrate complexes (LCCs) on the polymerization and final structure of extracellular lignin (ECL). This has enabled us to study the impact of xylan on monolignol composition and structure of the final lignin polymer. We find that the addition of xylan to the solid culture medium accelerates cell growth and impacts the ratio of monolignols in the lignin. However, the presence of xylan in the lignin polymerization environment does not significantly alter the structural properties of lignin as analyzed by two-dimensional nuclear magnetic resonance (NMR) spectroscopy and size exclusion chromatography (SEC). Nevertheless, our data indicate that xylan can act as a nucleation point, leading to more rapid lignin polymerization, an important insight into biopolymer interactions during cell wall synthesis in wood. Lignin structure and interactions with a secondary cell wall hemicellulose were investigated in a model cell culture: we found that the polymerization and final structure of lignin are altered when the hemicellulose is present during cell growth and monolignol production. The physicochemical interactions between lignin and xylan partly define the extractability and utility of native lignin in high value applications, so this work has implications for lignin extraction as well as fundamental plant biology.

Abbreviations: DHP, Dehydrogenation polymer; ECL, Extracellular lignin; G-units, Guaiacyl monomeric units/lignin subunit; HPAEC-PAD, High-performance anion-exchange chromatography with pulsed amperometric detection; HSQC, Heteronuclear single coherence spectroscopy; H-units, *p*-Hydroxyphenyl monomeric units/lignin subunit; LCC, Lignin–carbohydrate complex; MWL, Milled wood lignin; NMR, Nuclear magnetic resonance; SEC, Size exclusion chromatography.

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KEYWORDS
biosynthesis, LCC, lignin, NMR, Norway spruce, *Picea abies*, Xylan

1 | INTRODUCTION

Lignin is an abundant biopolymer comprising 15–40% of the dry weight of woody biomass (del Río et al., 2020). Lignification in higher plants takes place in the cell walls of water conducting cells and in dead supportive cells (sclerenchyma) (Boerjan et al., 2003). The building blocks of lignin, so-called monolignols, are transported across the plasma membrane into the cell wall where they polymerize (Boerjan et al., 2003; Perkins et al., 2019, 2022; Ralph et al., 2004; Väisänen et al., 2020). Oxidative enzymes called peroxidases and laccases introduce a radical onto the monolignols, which then couple together to form the lignin polymer via an end-wise polymerization mechanism, with new radicals being added to one end of a growing chain (Blaschek et al., 2023; Grabber et al., 2003; Hoffmann et al., 2022; Koutaniemi et al., 2015; Laitinen et al., 2017; Ralph et al., 2019; Sarkanen & Ludwig, 1971; Schuetz et al., 2014). For softwoods such as Norway spruce, the most common monolignols come from the canonical lignin biosynthesis pathway and derive from coniferyl alcohol (forming so-called G-units), and to a lesser extent from *p*-coumaryl alcohol (H-units) (Ralph et al., 2019). The lignification process is thought to continue after programmed cell death in lignifying tissue, by incorporation of monolignols produced by non-dying neighboring cells, in a cell-autonomous and non-cell autonomous way, respectively (Pesquet et al., 2013).

Lignin has been under investigation for many decades and yet, fundamental properties of the native polymer, such as its linearity, are still keenly debated (Balakshin et al., 2020; Ralph et al., 2019). Several issues hinder the analysis of true native lignin. First, lignin is natively deposited into a dense cell wall matrix comprising complex carbohydrates that confound extraction and necessitate physical and chemical treatment. Interactions between lignin and carbohydrates are thought to underlie lignin recalcitrance. The formation of covalent linkages in so-called lignin–carbohydrate complexes (LCCs) is well established in the cell walls of grasses, and these seem to be important for the localization of lignin in specific cell wall domains, acting as anchors or nucleation sites for lignin polymerization (Hatfield et al., 2017; Ralph et al., 1995; Zhang et al., 2019). This mechanism is thought to be more prominent in grasses because of the presence of ferulic acid substitutions on some grass hemicelluloses, which are lacking in species like Norway spruce. However, similar mechanisms could be in place for woody tissue that would facilitate lignin polymerization. Second, the polymer consists of a large aromatic network with radical scavenging properties, making it susceptible to alterations during conventional extraction methods. Approaches with organic solvents such as the milled wood lignin (MWL) extraction technique (Björkman, 1956), and enzymatic treatments that selectively remove carbohydrates from the cell wall, go some way to overcoming these issues (Guerra et al., 2006; Wu & Argyropoulos, 2003). However, low extraction yields raise the question of whether fully representative structures are obtained, or whether important structural moieties are under-represented in such analyses. In addition, the limited accessibility of enzymes to the wood structure and the need to increase the surface area available to solvent require the use of mechanical pre-treatments such as ball milling. These have themselves been shown to alter the lignin structure because of the high mechanical and thermal energies introduced (Balakshin et al., 2020; Sapouna & Lawoko, 2021; Zinovyev et al., 2018). To avoid the extraction problem, whole cell wall analysis has been performed with both solid and solution state nuclear magnetic resonance (NMR) spectroscopy (Crestini et al., 2011; Kim & Ralph, 2010; Yue et al., 2016). However, the need for a ^{13}C-enriched substrate in solid state NMR is limiting and, in the case of solution NMR, mechanical pre-treatments are still required for efficient cell wall dissolution. Alternative approaches that do not require extensive, damaging pre-treatments or extraction methods are needed for us to get closer to a full picture of the true native structure.

An alternative route is to study dehydrogenation polymer (DHP), a form of biomimetic synthetic lignin (Terashima et al., 1995). It can be produced in cell wall-mimicking in vitro conditions in the presence of polysaccharides and is considered a good model to understand the structure and biosynthesis of lignin, as well as its interactions with cell wall polysaccharides during polymerization. Many insights have been gained through DHP analysis regarding the polymerization mechanism and the parameters that affect the final structure of the polymer, such as the composition of the monolignols, the pH, and the addition rate (Harman-Ware et al., 2017; Terashima et al., 1996; Wang et al., 2021, 2022). However, there is poor control of the monolignol concentration during DHP synthesis, which results in a polymer more branched and of smaller molecular weight than cell wall lignin (Brunow et al., 1993), and these experiments fail to accurately reflect the complexity of the living plant.

As a third approach to studying lignin synthesis and structure, plants and plant cell cultures can be used to directly investigate lignin biosynthesis and cellular control over lignification (Pesquet et al., 2013, 2019). However, there are limitations with these systems too. Plants need to be provided with ^{13}C-rich substrates and atmosphere to allow lignin investigation by NMR and in most cases, extraction steps are still needed even with young plant tissue. Specially developed plant cell cultures that produce lignin in a way that allows it to be collected by simple filtration of culture medium, avoiding all chemical and mechanical extraction techniques, are a useful alternative that can overcome these issues.

In this study, a Norway spruce (*P. abies* [L.] Karst.) tissue culture was used to investigate the biosynthesis and structure of lignin, as well as the impact of a secondary cell wall polysaccharide, xylan, on lignin formation. This cell culture comprises mostly non-differentiated cells with a primary cell wall and well-developed chloroplasts

(Simola & Santanen, 1990). Approximately 3% of cells are differentiated to tracheids with secondary cell wall (Karkonen & Koutaniemi, 2010). The cells are maintained as calli on agar plates. When transferred into liquid conditions, the cells secrete monolignols into the culture medium that mimics the apoplastic fluid, and lignin forms. The laccases and peroxidases related to lignin polymerization in this culture have been studied (Kärkönen et al., 2002), and the extracellular lignin (ECL) has been structurally characterized (Brunow et al., 1990; Giummarella et al., 2019; Warinowski et al., 2016). These studies concluded that the polymer has an intermediate structure between DHP and cell wall lignin extracted as MWL (Brunow et al., 1993). Although important for understanding lignin structure and biosynthesis, the interactions between lignin and carbohydrates in this system have not yet been addressed, to our knowledge. ECL has generally been considered a response to stress in the culture, since it is not deposited to the cell wall and it contains higher than normal levels of H-units (Brunow et al., 1993; Giummarella et al., 2019; Viljamaa et al., 2018).

In the present investigation, we made use of all the aforementioned advantages of the ECL system to study native lignin structure and interactions by incorporating xylan into culture treatments. We investigated both the impact of xylan in the growing environment to explore changes in cell behavior and the impact of xylan in the lignin-producing environment to uncover any changes in the ECL structure. We found that the presence of xylan in the solid culture medium used to grow calli led to an increased mass of collected cells and a higher ECL yield upon transfer into the suspension medium. Interestingly, a higher level of peroxidase activity was detected in xylan-containing suspensions although there were only minor structural changes in the lignin, identified via size exclusion chromatography (SEC) and 2D NMR. Our analyses show that the presence of xylan at different stages can be correlated with changes in both cell mass and lignin polymerization. Both physical and chemical interactions between lignin and hemicelluloses were demonstrated for the first time in this model system, an important aspect that partly defines the extractability of these abundant biopolymers, influencing their use in high value applications.

2 | RESULTS AND DISCUSSION

To study the impact of a secondary cell wall hemicellulose on monolignol production and the structure of ECL, beechwood xylan was added into the solid medium on which Norway spruce cells grow as a callus culture, and into the liquid medium into which monolignols are secreted. Xylan solubility in aqueous conditions was the limiting factor for the range of concentrations that could be tested. Our hypothesis that insoluble xylan can act as a nucleation point for lignin polymerization was investigated by the addition of 1 g/l xylan into the suspension culture medium. Lower amounts of xylan were also tested, to check for any effect from dissolved hemicellulose. Potential changes in cell growth rate, monolignol production, or relevant enzyme activities were investigated after adding xylan into the solid culture medium used for cell growth. The combinations of xylan concentrations in the solid and suspension culture media resulted in the development of 12 treatments that were studied in terms of ECL production and structural properties (Table 1). The concentration of .1 g/l was considered too low as no significant changes were observed in the investigated properties, as can be seen for example from the sugar composition analysis in Table S1, suggesting that the xylan needs to be at least partially insoluble to have any impact.

2.1 | Xylan addition to the solid and suspension culture media affects cell growth rate

During in planta lignification of cell walls, lignin is deposited on a polysaccharide environment consisting of cellulose, pectins, and hemicelluloses (Barros et al., 2015). Addition of xylan into the solid medium might therefore give information on the impact of secondary cell wall hemicelluloses being present before monolignol production begins. We hypothesized that when monolignol production is induced in cells in the presence of a secondary cell wall hemicellulose, they may produce lignin with an altered monolignol composition, to be deposited in possible neighboring lignifying cells, without inducing cell death, as has been observed for non-tracheary-element-differentiating cells in other culture systems (Pesquet et al., 2013).

For all cultures, the ECL-containing product was collected once it had reached a certain visual density, allowing sufficient product to be collected for structural analysis of the lignin moiety, but before cells had begun to die. The impact of adding xylan into the solid growth medium was examined and showed clearly that xylan induced faster cell growth on plates, as presented in Figure S1. Cells to be used for the production of ECL in the following experiments were sub-cultured from reference plates (no xylan) to xylan-containing plates and sub-cultured twice more before monolignol production was induced by transferring cells to the suspension culture medium.

TABLE 1 Treatments showing the combination of solid and liquid media with different xylan concentrations.

		Xylan in suspension medium (g/L)			
		0	.1	.5	1
Xylan in solid medium (g/L)	0	0 → 0	0 → .1	0 → .5	0 → 1
	.1	.1 → 0	-	-	.1 → 1
	.5	.5 → 0	-	-	.5 → 1
	1	1 → 0	1 → .1	1 → .5	1 → 1

The effect of adding xylan into the suspension culture medium was examined next. This experiment approximates the lignification environment in wood. Our hypothesis was that xylan, especially when insoluble, could act as a nucleation point for lignin polymerization, and that the ECL produced would accordingly have a higher molecular weight. In addition, physicochemical interactions between xylan and the ECL might result in a polymer structurally closer to cell wall lignin. We first observed an effect of xylan on the mass of cells that could be collected from plates, as presented in Table S2. When the increase in cell dry mass for the treatments 0 → 0 and 1 → 0 was compared in representative culture batches, it was clear that the cells appeared to multiply faster when there was a high amount of insoluble xylan in the solid culture medium.

2.2 | ECL yield increases with increasing xylan concentration in the solid medium

Monolignol production was induced via a change in culture conditions while cells were actively multiplying. This is induced by a decrease in auxin (2,4-D), a change in the NH_4^+/NO_3^- ratio, and the use of agitated cultivation conditions (Simola et al., 1992). Lignin formation then happens spontaneously in the suspension medium.

Because of its intrinsic insolubility in water, ECL is collected from the suspension medium via centrifugation. As a result, insoluble exogenous xylan is pelleted together with ECL, contributing to the sample's apparent yield. It is likely that the cells secrete hydrolytic enzymes that can reduce the molecular weight of xylan, hence increasing its solubility. The extent of xylan depolymerization by such enzymes is not known, but this could explain why the observed product mass from some treatments prepared with a high concentration of insoluble xylan are lower than expected (Table S2, ECL mass). For example, the product collected from 1 L of suspension culture medium weighs less than 1 g although it should in theory contain 1 g of xylan in addition to the ECL and any cell-secreted polysaccharides. In order to calculate the ECL yields from these treatments more accurately, samples produced by cells that were exposed to xylan in the suspension medium were treated with a xylanase enzyme. However, because of practical issues with re-suspension or possible enzyme inhibition caused by lignin, this step was not efficient, as can be observed in the sugar composition data in Table S1 (discussed below). It was therefore only possible to reliably determine ECL yields collected from suspension cultures to which no xylan was added. The higher ECL yields observed in the treatment 1 → 0 compared with 0 → 0 (Figure 2a) are attributed to the presence of more cells in these flasks (Tables S2, S3).

2.3 | The sugar composition of the ECL-containing product is affected by xylan addition into the solid culture medium

Sugar composition analysis was performed on the ECL-containing material collected from suspension medium to allow a complete chemical characterization, and to investigate possible effects from the exogenously added xylan. The inefficiency of xylanase treatment is apparent when comparing the highly similar xylose contents of samples analyzed both before and after xylanase treatment (Table S1). As a result of this, any potential xylan or xylose deriving from the cells themselves is masked in these analyses by the exogenous xylan provided. The comparison of xylose content in the ECL-containing product is therefore only discussed here using samples collected from xylan-free suspension medium (Figure 1), although the sugar composition of all samples is presented as supplementary information in Table S1. A correlation in the detected xylose and the xylan concentration in the solid medium is visible in Figure 1. The xylose detected in the reference samples is presumed to originate from the endogenous xylan and xyloglucan secreted by the cells as no xylan was added to the suspension medium in the compared treatments. Qualitatively, the sugar composition observed in the product is characteristic of a primary cell wall, comprising mainly pectins and xyloglucan (Roger et al., 2012; Stevanic & Salmén, 2007). The findings are in accordance with previous work, where pectin-type sugars were detected in spruce ECL (Warinowski et al., 2016).

Fucose, rhamnose, glucose, and mannose concentrations were highest in the ECL-containing products of cells grown on .5 g/l xylan plates. In particular, glucose concentration in the sample was elevated when xylan was present in the solid medium. This monosaccharide is present in xyloglucan, the major hemicellulose in primary cell walls, but can also be attributed to callose, a β-1,3-glucan that is known to be produced during cell division and as a response to stress (Chen & Kim, 2009). Since the cells pre-grown on xylan-containing solid medium seemed to grow more than on the reference solid medium (Figure S1, Table S2), an increased callose production is logical. As previously mentioned, xylose concentration in the product increased in line with the increasing xylan concentration in the solid medium. The concentrations of the uronic acids, arabinose, galactose, and mannose seemed to be independent of the xylan concentration in the solid medium.

2.4 | Elevated peroxidase activity correlates with a higher degree of lignin polymerization in the presence of insoluble xylan

The peroxidase activity in the suspension medium was monitored during lignin polymerization as an indication of the effects of xylan addition on enzyme activity and polymerization efficiency. Starting from the first day the cells were transferred into the suspension medium (denoted as day 0 in Table S3), and every 2 days until ECL was collected, samples were taken from the medium for quantification of peroxidase activity. Note that ECL was not collected on the same day for all treatments, because of differences in cell growth rate and to ensure that ECL was collected while the cells were still alive. Peroxidase activity increased over the course of ECL production (Figure 2b-d). In most treatments, the activity was reduced before the collection day, and in some cases was below the detection limit of the assay (Figure 2b-d, Table S3).

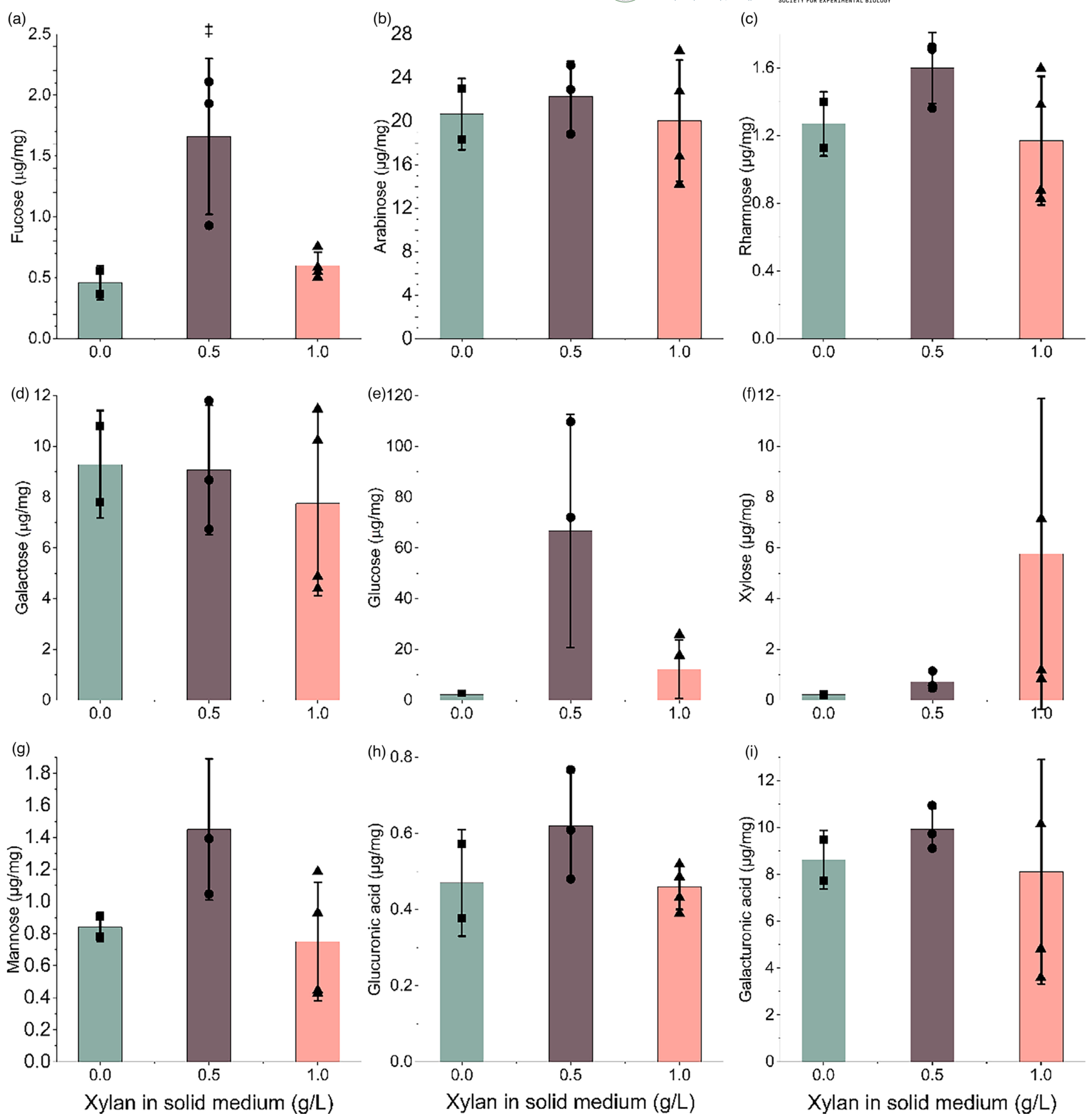

FIGURE 1 Sugar composition of the ECL-containing product of cultured Norway spruce cells in reference liquid medium of the 0 → 0, .5 → 0, and 1 → 0 treatments. The polysaccharide portion of the product was hydrolyzed with TFA and analyzed by HPAEC to quantify neutral sugars and uronic acids. The bars represent the average values and the error is the standard deviation. Student t-test was performed to identify significantly different samples, compared with the 0 → 0 treatment (control). There are no significant differences between the treatments. Raw data are presented in Table S1. Only the concentration of Fucose in panel a was statistically different from the control at $p = .1$; this is marked with a double dagger (‡) and shows that the different culture treatments did not lead to an altered carbohydrate content in the ECL-containing product. The data points for 0 → 0 (squares), .5 → 0 (circles), and 1 → 0 (triangles) are depicted in the respective bar.

Although there was no general trend connecting enzyme activity and the xylan concentration in the suspension medium, in the case of reference (xylan-free) and 1 g/l xylan-containing solid medium, the activity seemed to increase with increasing provision of insoluble xylan in suspension. In addition, in the case of the reference liquid medium, the peroxidase activity seemed to be higher when xylan had been present during pre-growth on the solid medium. The same trend connecting ECL yield and cell mass can be seen in Figure 2a and Table S2. As a result, peroxidase activity can be linked to the higher cell mass in these treatments. However, a greater abundance of cells cannot explain the increased peroxidase activity observed for the treatments 1 → 0, 1 → .5 and 1 → 1 (Table S3). Here, the activity was

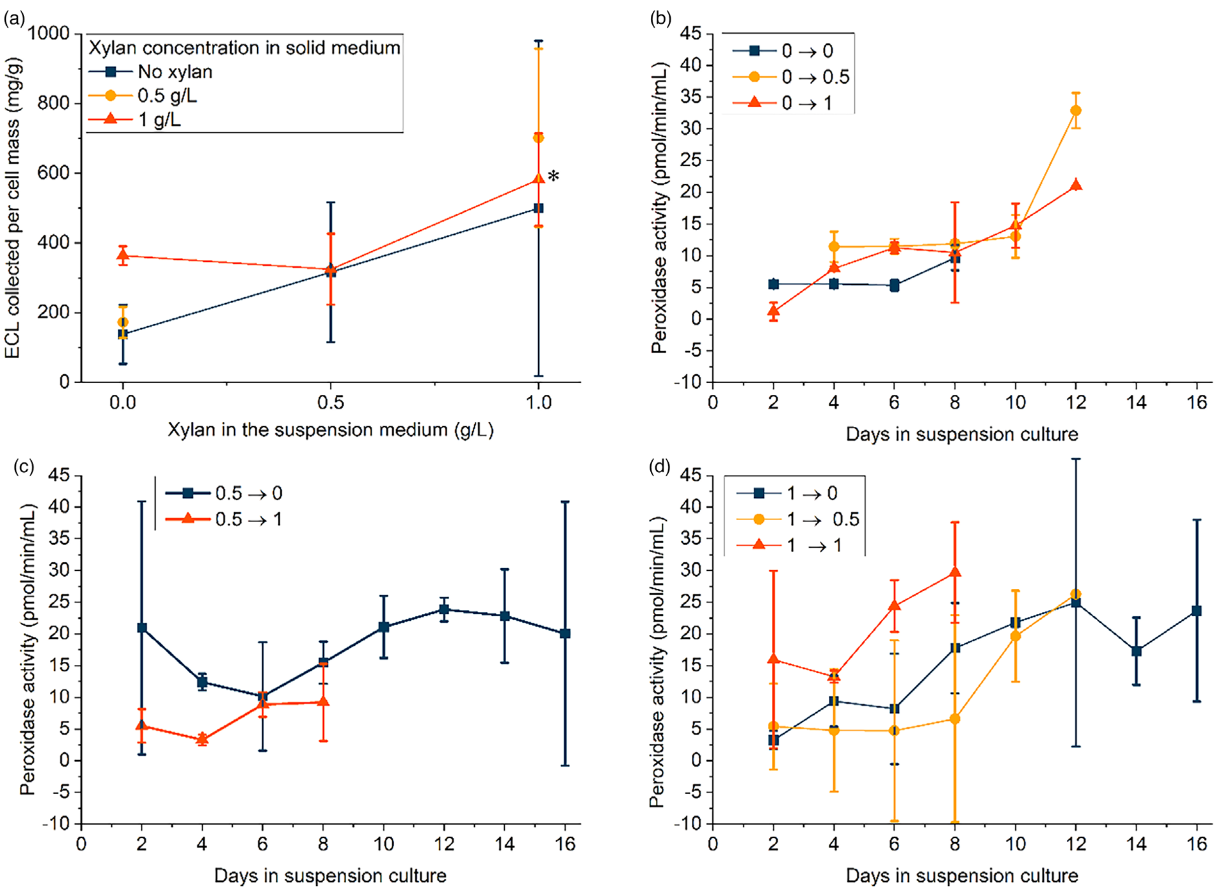

FIGURE 2 (a) ECL yield reported as mg of product per g of initial dried cell mass. The data point marked with star (*) are significantly different with p = .05. (b)-(d) peroxidase activity measured in the liquid suspension medium supplemented with different concentrations of xylan. All measurements are averages of two technical replicates. The last measurement for each culture was taken when ECL was collected. The raw data for the ECL mass and peroxidase activity is presented in Tables S2 and S3 respectively.

increasing, despite the apparently slower cell growth. As a result, the presence of xylan in the suspension medium seemed to correlate with increased peroxidase activity, either through an increased enzyme production by the cells or via a larger monolignol amount produced in the 1 → 1 treatment, compared with the 1 → 0 and 1 → .5.

After ECL removal but prior to disposal of experimental waste, culture medium was stored at 4 °C. By chance, we observed that ECL production continued in the culture medium even after cells had been removed by filtration. This ECL likely results from unreacted monolignols that had not yet been incorporated into the lignin polymer at the time of ECL collection. Because our data show that peroxidase activity was low at that time, laccases are presumed to be active during this post-filtration period. The role of these enzymes in lignin polymerization has been studied before and some activity has been detected in the medium of this cell culture, although the in vitro activity levels were much lower than that of peroxidase (Kärkönen et al., 2002). We therefore propose that laccases are likely actively radicalizing monolignols for many days post cell death or removal, since radical stability for several days under these conditions is highly improbable (Pesquet et al., 2013).

2.5 | Addition of xylan in the culture media impacts early stages of lignin polymerization

We hypothesized that insoluble xylan in the suspension medium would act as a nucleation point for lignin polymerization, allowing larger polymers to be produced. This is generally supported by the results from SEC analysis. The average molecular weight values presented in Table S4 were rather similar, so no conclusions can be drawn from this presentation of the data regarding the polymerization mechanism. In addition, SEC methods are sensitive to polymer-solvent interactions, and as a result, these data should not be considered as absolute values. The chromatograms can however provide information regarding the relative molecular size of the samples. Indeed, the overlaid SEC data in Figure 3 confirm the nucleation hypothesis.

Comparing the chromatogram overlays of ECL produced by cells pre-grown on reference solid medium, the molecular weight of the largest population eluting at approximately 28 min was almost the same for all three treatments, suggesting that no significant change in the molecular weight of the overall, final polymer was induced by the presence of xylan in the suspension medium (Figure 3a). However,

there was a small lignin population of high molecular weight in the 0 → 1 treatment (dotted line) that was absent in the reference treatment (0 → 0). This likely represents the small amount of ECL that started to grow at the early stages of monolignol secretion, for which xylan acted as nucleation point, resulting in the observed higher molecular weight in the final product. In ECL produced by cells pre-grown on 1 g/l xylan plates, this high molecular weight population eluting at approximately 23 min gave a much more pronounced peak for all samples (Figure 3b). The size of the peak varied depending on the xylan concentration in the suspension culture, but there was no clear trend, suggesting there may be multiple, perhaps conflicting impacts of having soluble or insoluble xylan present in the ECL polymerization environment. In this case, this higher heterogeneity of the product could be attributed to a combination of faster monolignol production (1 → 0, Table S2) and the addition of xylan in the suspension medium (1 → 1). In all cases, the peak at 23 min was larger than the corresponding shoulder in Figure 3a, confirming the impact of xylan in the solid medium.

Xylan was partly soluble in the suspension medium at .5 g/l but formed a particulate dispersion at the highest tested concentration of 1 g/l. At the early stages of monolignol secretion, their concentration is low and the probability of dimerization and subsequent chain growth is rather small. The insoluble xylan in the medium may act as a nucleation point, interacting with the monolignols in a physicochemical way, either through the formation of LCCs, the presence of which was confirmed by NMR analysis (discussed below), or by spatially restricting the monolignols, increasing the probability that two radicals will couple, starting the formation of a new lignin polymer, but without the formation of an LCC (Figure 4a). After several days of monolignol secretion into the suspension medium, their high concentration ensures a greater probability of radical coupling for the formation of new ECL chains or their incorporation into an already formed ECL

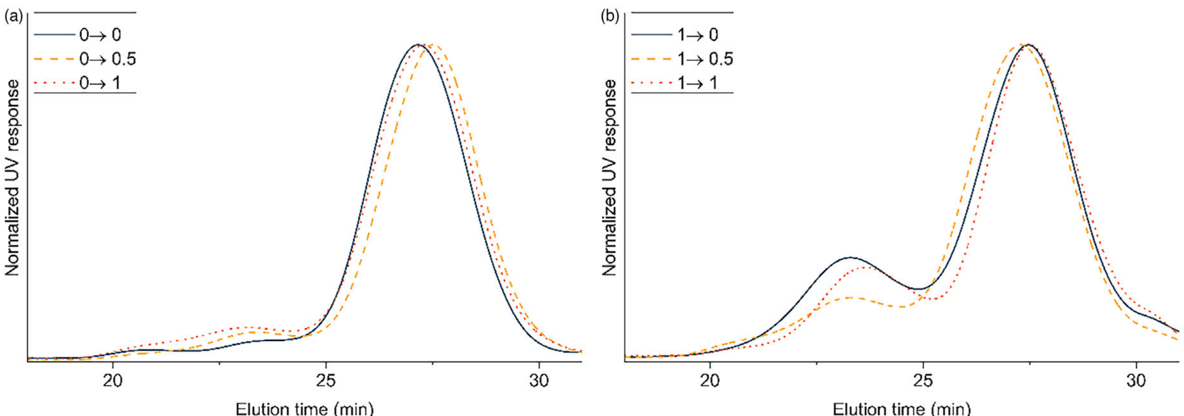

FIGURE 3 THF-SEC chromatogram at 254 nm of the ECL collected from different treatments (see Table 1). The samples were acetylated before the analysis and compared with polystyrene standards.

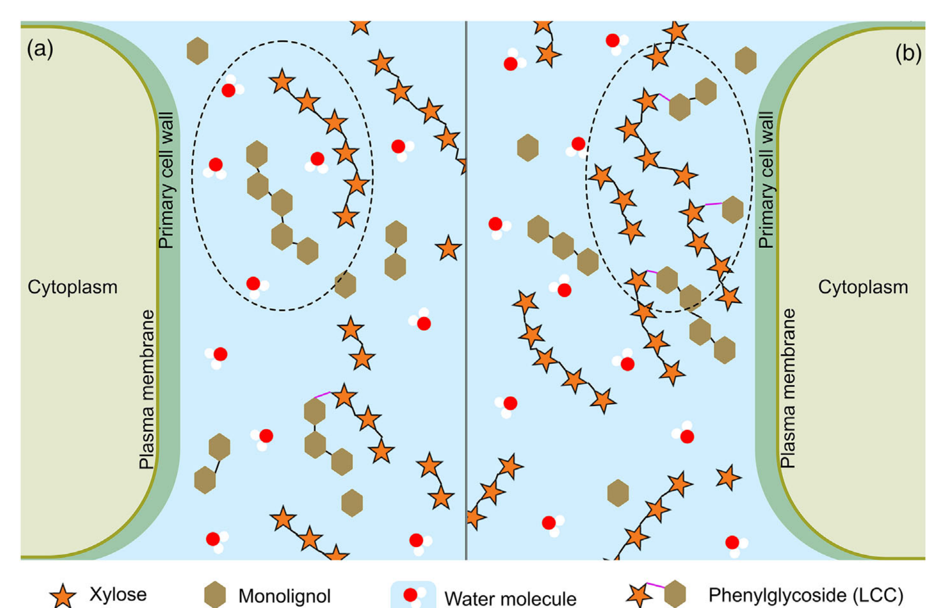

FIGURE 4 Proposed mechanism of lignin polymerization in the suspension medium. (a) Xylan chains spatially restrict monolignols, increasing the probability of two radicals to meet and couple. The addition of water molecules to the radicalized monolignol/polymer is more favorable (marked area), hence the formation of LCCs is not high (b) the formation of LCCs by nucleophilic addition of hydroxyl groups of xylan to the radicalized monolignol/propagating lignin polymer becomes favored through water exclusion (marked area), when insoluble xylan occupies areas close to the cell surface. This "anchoring" enables lignin polymerization by locally increasing the monolignol concentration.

chain, regardless of the surrounding insoluble xylan. We therefore speculate that the ECL chains anchored to particulate xylan originate from the interactions between monolignols and the hemicellulose while the concentration of the former is still low, i.e. in the early days of their secretion. In order for LCC formation to be favored over the addition of water, it is probable that this is happening close to the surface of the cell, where xylan can create areas from which water is excluded (Figure 4b). As a result, these chains reach a higher molecular weight over the course of lignin polymerization compared with the LCC-free ECL, for which polymerization started at a later point in time. In Figure 4, we present a theoretical model for lignin polymerization in our system based on these findings.

In previous structural characterizations of ECL produced by this spruce cell culture, a comparison with MWL showed that ECL exhibits characteristics of a "bulk" polymer, i.e., it is highly branched, with high amounts of C-C bonds such as resinol structures, and has a high proportion of cinnamyl alcohol end groups (Brunow et al., 1993; Lu & Ralph, 1999). The bulk polymerization mechanism is supported by a locally higher concentration of monolignol radicals that dimerize quickly (Grabber et al., 2003; Lu & Ralph, 1999). On the other hand, more recent studies on ECL suggest that peroxidases that are bound to ECL of this cell culture can produce a polymer with a structure closer to native lignin (Warinowski et al., 2016). We compared molecular weight dispersity (Đ) values for ECL produced in this investigation and with those from MWL extracted from spruce wood from the literature (Sapouna & Lawoko, 2021), and found no significant differences between the two polymers (Table S4). ECL had a slightly lower molecular weight than MWL and lower Đ. The extent of polymer branching in the ECL is rather inconclusive based on the SEC data, as coiling of the polymer due to interactions with the solvent can be misleading, so NMR studies were pursued to clarify this.

2.6 | 2D NMR analysis shows correlation between LCC formation and the concentration of xylan in the ECL polymerizing environment

Characterization of the chemical structure of lignin was performed using semi-quantitative heteronuclear single coherence spectroscopy (HSQC) experiments, wherein the proportion of functional groups

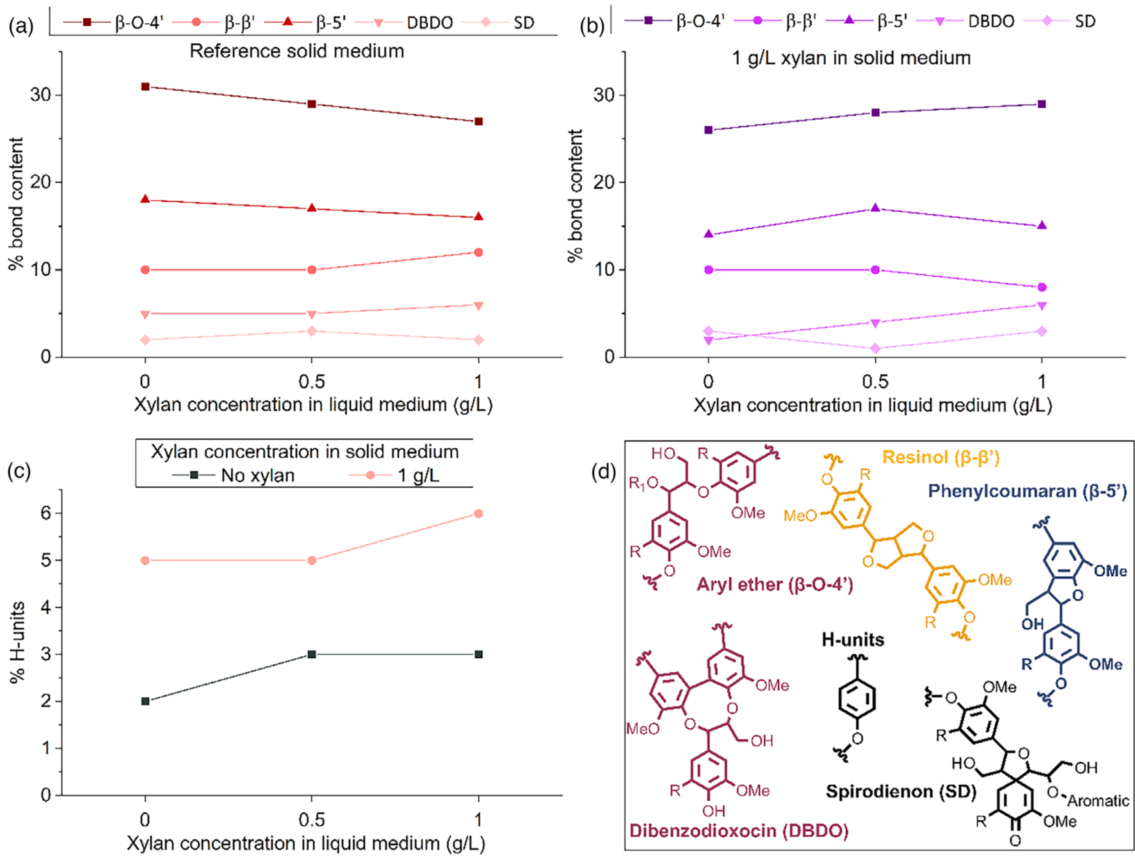

FIGURE 5 ECL composition as calculated by semi-quantitative 2D NMR (HSQC). The bond content is calculated relative to the C2/H2 signal of G-units within the same spectrum, and reported for the reference solid medium (a) and the solid medium with 1 g/l xylan (b). The proportion of H-units quantified from HSQC for both reference and the solid medium with 1 g/l xylan is shown in (c). The structures of the lignin inter-unit linkages plotted in a-c are presented in (d). Since the semi-quantification of the different bonds presented is not based on the same carbon because of overlapping signals and the bonds reported are only the most representative ones, the final bond content does not reach 100%.

visible as peaks in the spectra can be relatively quantified, by comparison to the integral of the C2-H2 peak of G-units, within the same spectrum. ECL produced from the spruce tissue cultures comprised mainly G-units, as is expected in softwood lignin (Ralph et al., 2019). Hence, the C2 of the aromatic ring in G-units was used as an internal standard for the relative quantification of the characteristic lignin bonds β-O-4′ β-β', and β-5′ (Figure 5 and Table S5). Overall, there was no general trend connecting bond composition with xylan concentration in the solid or suspension media. Some variations were observed in the different treatments, with β-O-4′ content ranging between 26–32% (Figure 5, Table S5), but the proportions of each bond are within the expected range (Ralph et al., 2019). Interestingly, the secretion of H-units appeared to increase when xylan concentration in the solid medium increased. These units represent 1–5% of softwood lignin (Ralph et al., 2019). The increased amount could be due to stress (Pesquet et al., 2019), although this was not indicated by the healthy growth of cells in the respective treatments (Figure S1, Table S2). This observation may indicate that the cells responded to the presence of xylan in the growth medium by changing the type of monolignol produced.

The secreted product had low solubility in the deuterated solvent DMSO-d_6 and for some treatments it was not possible to obtain a clear NMR spectrum. This low sample solubility was attributed to the presence of pectins, confirmed by sugar composition and NMR experiments (Figure 1, Figure 6). An enriched ECL fraction was obtained by extracting the product with 80% ethanol overnight at room temperature. This procedure was applied to representative samples collected from treatments with high xylan concentration to ensure the accuracy of the integral values of the major lignin bonds that possibly overlap with carbohydrate signals. In Figure 6, the HSQC spectra before and after ethanol extraction are presented and confirm that most of the carbohydrates were effectively removed, and that there was no significant broadening of the peaks because of gelling of the samples (Figure 6a). The signals remaining after ethanol extraction may be attributable to glycans that are covalently linked to lignin via LCCs. Alternatively, there may be some low molecular weight

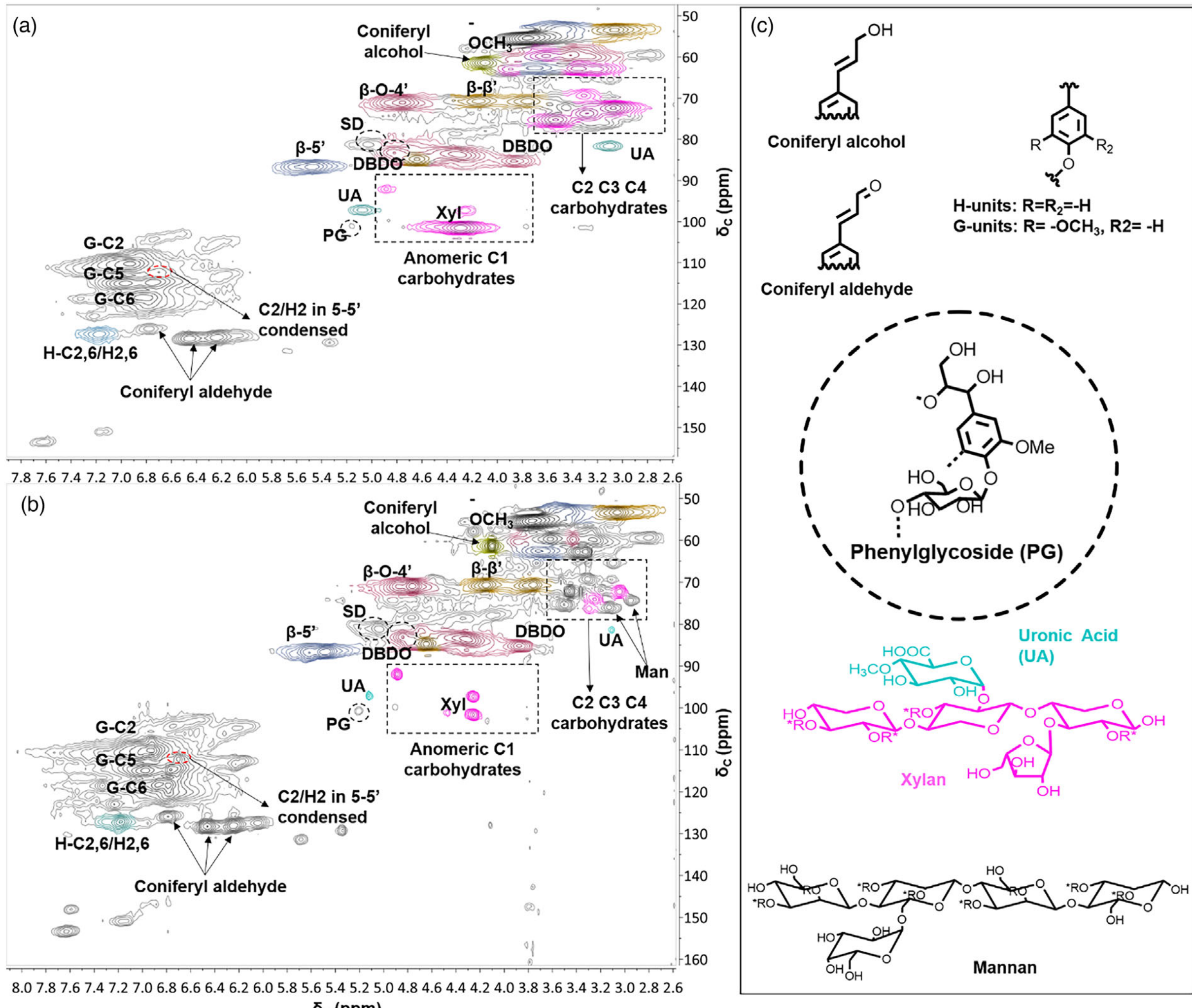

FIGURE 6 HSQC spectra of ECL (a) before and (b) after ethanol extraction of the product from the .5 → 1 treatment. The interunit linkages in lignin as well as the carbohydrates identified are presented in (c) with color coding as in Figure 5d. NMR solvent was DMSO-d_6.

oligosaccharides remaining in the sample, but not covalently linked to the lignin moiety, which adhere to the ECL sample in some way that prevented their removal.

In Figures 6a-b, signals at 100/4.9 ppm were assigned to phenylglycoside LCC (the structure is shown in Figure 6c), in accordance with previous studies (Giummarella et al., 2019; Sapouna & Lawoko, 2021). An earlier study that aimed to confirm the nativity of such phenylglycoside structures detected the linkage in both ECL and DHP (Giummarella et al., 2019), which suggested that the linkage does not solely derive from changes during the wood extraction process, as had been theorized previously. In our work, we see a distinct correlation between the abundance of the phenylgycoside linkage and the amount of xylan present in the polymerization environment. The integral of the phenylglycoside peak observed in the anomeric carbon region in NMR spectra seems to increase with increasing concentration of xylan in the suspension medium. From .2% in the 0 → 0 treatment, the value increased to .6% in the 0 → .5, and reached 1.2% in the 0 → 1 treatment.

However, it does not appear that all xylan took part in LCC formation, as the signal of the peak is lower than would be expected otherwise. It was concluded that the xylan signals are due to both ethanol-soluble, low molecular weight oligosaccharides, as well as these LCC structures that we find in ECL that has not been subjected to any extraction processes. This observation confirms the hypothesis that xylan in the extracellular polymerization environment interacts with the secreted monolignols and spontaneously forms covalent LCC bonds (Figure 4).

Interestingly, sugar composition analysis of the ECL from the reference treatment (0 → 0) showed a small amount of xylose in the sample (Figure 1, Table S1). As observed in Figure S2, the anomeric region showed no carbohydrate peaks. However, there was a peak attributed to the phenylglycoside structure in that region with a relative amount of .2%. A xylan peak at 3.27/69.7 ppm assigned to C2/H2 in α-D-xylopyranoside was also detected in that spectrum, supporting the presence of the LCC as a native structure the cells are producing from intracellularly generated components.

A comparison between ECL samples and spruce MWL showed a significant difference in the amount of 5–5′ condensed bonds (Table S5). These structures are known branching points in lignin and have been identified in wood extracts in previous studies (Balakshin et al., 2020; Sapouna & Lawoko, 2021). Their presence in these ECL samples may indicate that the ECL is a branched polymer. In addition, the amount of cinnamyl alcohol units, an end group in lignin (Ralph et al., 2019), was higher in the ECL samples compared with a MWL sample (Table S5), suggesting a higher degree of branching in ECL. Cinnamyl aldehyde units, another end group in spruce lignin (Ralph et al., 2019), had similar abundance in spruce MWL and reference ECL (0 → 0) but appeared at lower concentrations in ECL from the xylan treatments (Table S5). Although this could support the end-wise mechanism of ECL polymerization (Lu & Ralph, 1999) in the presence of xylan, the total amount of cinnamyl end groups (both alcohol and aldehyde) is higher in ECL compared with MWL, suggesting that ECL is a “bulk” polymer.

3 | CONCLUSIONS AND OUTLOOK

Structural properties of ECL derived from a Norway spruce tissue culture were here investigated using NMR, SEC, and sugar composition analysis, as well as peroxidase activity of the environment where ECL was produced via monolignol polymerization. The presence of xylan in the solid and suspension culture media resulted in greater amounts of cell mass and a higher ECL yield, which may derive from an observed increase in peroxidase activity. Future studies examining changes in the expression of relevant genes in cultured cells with different xylan provision would be useful to reveal the biochemical mechanisms driving the variations observed here in ECL production and cell growth. There were only minor changes in the structure of the ECL as shown by NMR, which indicates that the xylan did not noticeably influence the manner in which the monolignols connected to each other. Our data suggest that xylan in the polymerization environment can act as a nucleation point for lignin formation, depending on the amount and solubility of the xylan provided. Our work supports the theory that polymerization of lignin is altered in the presence of xylan, not only through the formation of covalent bonds but also because of physical interactions, which can be influenced by extraction steps, but are often overlooked in the design of extraction protocols. Future work on lignin extraction would benefit from considering these interactions for optimization of extracting polymer with targeted structural properties and high yields.

4 | EXPERIMENTAL PROCEDURES

4.1 | Chemicals and reagents utilized

All chemicals, materials and the peroxidase activity assay kit (product MAK092) were purchased from Sigma-Aldrich, Germany. Absolute ethanol was purchased from VWR, Sweden. The chemicals were used without further purification, unless stated otherwise. Purified beechwood xylan was purchased from Megazyme, Ireland. Xylanase 10A (*Ruminococcus champanellensis)* was purchased from NZYTech, Portugal.

4.2 | Culture media and treatments

The composition of both solid and suspension nutrient media is described in Simola et al. (1992) and Kärkönen et al. (2002). For experiments where xylan was added into the solid culture medium or the liquid suspension medium, it was added at concentrations of .1 g/l, .5 g/l, or 1 g/l before pH adjustment and autoclaving. As an example of the denotation used, *0 → .5* describes the treatment in which the cells growing on reference (no xylan) solid medium are transferred into suspension medium that contains .5 g/l xylan. To confirm the absence of acetyl groups in the xylan used, NMR analysis was performed (Figure S3). All treatments are presented in Table 1.

4.3 | Maintaining the cell culture and ECL production experiments

Norway spruce cells (*P. abies* [L.] Karst., line A3/85) were maintained as a callus culture on solid medium and triggered to produce ECL by transferring the cells into suspension culture as described by Simola et al. (1992). The cells were sub-cultured to fresh solid medium every three weeks and transferred to suspension culture for the production of ECL two weeks after the subculture. Approximately 3 g cells (fresh weight) were transferred into 100 ml of liquid medium, in 500 ml flasks. Prior to the experimental work with xylan supplementation, we performed a simple habituation study to ensure that the cells are able to survive when xylan is added to media. For one year, the cells were acclimatized to the presence of xylan in the solid culture medium up to a concentration of 2 g/l, to ensure that cells were healthy in the presence of the polysaccharide. In suspension culture, even at 1 g/l, when the xylan is largely insoluble, there were no detrimental health effects. No reduction in growth rate or biomass production, and no changes in the coloration of cells or calli, were observed during the acclimatization period.

4.4 | ECL collection

Once the white-beige ECL was visibly produced in the flasks, but while the cells were still alive (evidenced by a bright green color), the cells were filtered out with Miracloth (Merck Millipore). Several flasks were used per treatment to ensure a sufficient ECL amount for chemical analyses. The filtrate of all collected flasks of the same treatment was combined and centrifuged at 17,000 *g* for 40 min, using a JA10 rotor in an Avanti J-265 XP centrifuge (Beckman Coulter). The pelleted ECL was washed twice with Milli-Q water and freeze-dried before further analysis.

4.5 | Xylanase treatment of secreted ECL-containing product

To remove the exogenous xylan added to the suspension cultures, a xylanase treatment was performed, to degrade it into soluble oligosaccharides. The freeze-dried products were suspended in 50 mM citrate buffer, pH 6, at 37 °C under agitation. Based on the manufacturer's information, an enzyme concentration of .5 μM was used. After overnight incubation, 40% more enzyme was added to ensure maximum hydrolysis and the samples were incubated for additional 24 h. Afterwards, the samples were washed twice with Milli-Q water before freeze-drying.

4.6 | Sugar composition analysis

The sugar composition of the pelleted product was characterized using high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) (Dionex ICS-3000; Sunnyvale, CA, USA). Before analysis, the samples were hydrolyzed with trifluoroacetic acid (TFA) as follows. One mg of the sample was dissolved in 1 ml of 2 M TFA solution containing .01 g/l *myo*-inositol as an internal standard. The solution was heated at 120 °C for 3 h to hydrolyze all non-crystalline polysaccharides, and then centrifuged. The supernatant was dried overnight under nitrogen flow and re-suspended in 1 ml Milli-Q water. The solution was finally filtered through a .2 μm Nylon syringe filter, and analyzed using an HPAEC-PAD Dionex ICS-3000 equipped with CarboPac PA-1 4 × 250 mm column (Thermo Fisher Scientific, USA). For the separation of the neutral and acidic monosaccharides, different gradients of the eluents were used (300 mM sodium hydroxide, 200 mM sodium hydroxide with 170 mM sodium acetate, 1 M sodium acetate and MilliQ water) with a P1 column set to 22 °C as previously reported (McKee et al., 2016).

4.7 | Peroxidase activity assay

Peroxidase activity was measured as an indication of the ECL polymerization rate in the suspension medium. One mL sample of the culture medium was collected every two days, starting from the first day the cells were transferred into liquid suspension culture, and until the ECL collection day. All samples were kept frozen at −20 °C until the measurement. The peroxidase activity assay was performed in duplicates, as recommended in the manufacturer's instructions. Briefly, the enzyme activity was calculated by a colorimetric reaction that enabled monitoring the change in absorbance at 570 nm. A master reaction mix (50 μl) consisting of the assay buffer, a fluorescent peroxidase substrate, and H_2O_2 were incubated with 50 μl of each sample in a 96 well flat-bottom plate at 37 °C for 3 min before the initial measurement. Subsequent measurements were taken every 10 min until no change in absorbance was observed. The activity was calculated with the use of a standard curve where the absorbance at 570 nm is plotted against H_2O_2 solutions of known concentrations.

4.8 | Size exclusion chromatography

The molecular weight and dispersity (Đ) of ECL-containing samples were studied with SEC. Experiments were performed in a gel permeation chromatography (GPC) system from Waters (Waters Sverige AB, Sollentuna, Sweden) consisting of a Waters-515 high pressure liquid chromatography (HPLC) pump, a 2,707 autosampler, a 2,998 photodiode array detector operated at 254 nm and 280 nm and a 2,414 refractive index (RI) detector. Waters Ultrastyragel HR4, HR2 and HR.5 (4.6 × 300 mm) solvent efficient columns were used, connected in series with a Styragel guard column, set to 35 °C. Standard calibration was performed using polystyrene standards with molecular weights from 176 kDa to 370 Da, using the data from the 254 nm channel of the photodiode array detector. A

flow rate of .3 ml/min was used. The ECL samples were acetylated before analysis to increase their solubility in tetrahydrofuran (THF). The protocol is described in previous work (Sapouna & Lawoko, 2021). Briefly, 2 mg of sample was mixed with 100 µl pyridine and 100 µl acetic anhydride, at room temperature, overnight. Pyridine was then removed by addition of ice cold toluene: methanol (1:1) solution, and evaporating several times under nitrogen flow.

4.9 | NMR spectroscopy

2D NMR experiments were performed for the structural characterization and semi-quantification of the bond composition in the ECL samples. A Bruker 400 DMX instrument was used (Bruker Corporation, Billerica, MA, USA), equipped with a multinuclear inverse Z-grad probe. The pulse sequence used for the HSQC experiments was hsqcetgpsi. The pulse length was optimized at 9.2 sec with 1.49 sec relaxation delay and 176 scans per sample. ECL samples (70 mg) were dissolved in DMSO-d_6 (550 µl) for all the experiments.

AUTHOR CONTRIBUTIONS

IS performed all experimental work, data analysis, and data presentation, and drafted the manuscript. LSM contributed to experimental design and data interpretation. AK supplied the cell line for experiments and offered guidance on the maintenance of the cultures. LSM and AK made substantial contributions in revising the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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