Assessment strategy for bacterial lignin depolymerization: Kraft lignin and synthetic lignin bioconversion with *Pseudomonas putida*

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Abstract

In order to better understand bacterial depolymerization of lignin, a new analytical approach was proposed using *Pseudomonas putida* KT2440 as delignifying bacterium and *Escherichia coli* as non-delignifying control. Two different types of lignins, technical Kraft lignin and synthetic dehydrogenopolymer (DHP), were submitted to a bioconversion kinetic study over 7 days at pH 7.5 in the presence of glucose. The concomitant analysis of the supernatant acid-precipitable lignin fraction and water-soluble extractives by HPSEC and GC-MS highlighted the specific action of *P. putida* towards these substrates, with the transitory formation of phenolic metabolites (dihydroferulic acid for Kraft lignins and dimers for DHP) and the

prevention of Kraft lignin self-assemblying. In both cases lignin apparent depolymerization followed by repolymerization was observed. The analysis of the bacterial pellets indicated the time-increasing content of lignins associated to bacterial cells, which could account for the apparent structural changes observed with *E. coli* in the supernatant.

Keywords: *bacterial conversion, depolymerization, HPSEC, lignin, phenolic metabolites*

1. Introduction

The world is currently facing concerns of global warming, rising population, and resource depletion, which enhance the need for sustainable industries based on renewable resources and low waste generation. However, the transition towards biomass-based industry is largely dependent on financial benefits.

Lignin, the second most abundant biopolymer on Earth, is a recalcitrant polyphenolic polymer with huge diversity and very diverse properties (Longe et al. 2020). Lignin valorization into molecules with high added value, such as bioplastics building-blocks, commodity chemicals, pharmaceutical products, etc., could help the alliance of the economic, societal and environmental requirements associated to sustainable industries. The environmental benefit would be particularly high with a biological transformation process, including a biotransformation by microorganisms (Rouches et al. 2016). It would also be enhanced by the use of technical lignins such as Kraft lignin, which are currently low-value byproducts from pulp and paper industry. Thus, the biological conversion of technical lignins into valuable functional biomolecules has become a technical and industrial challenge, and recent studies addressed the possibility to use technical lignins or their derivatives as sole carbon source for bioconversions (Ravi et al. 2019; Jusselme et al. 2020; Daou et al. 2021). Lignin conversion by fungi, white-rot fungi in particular, has been much more studied than bacterial conversion. Bacterial enzymatic systems show less oxidative power than fungal ones

(Brown and Chang 2014). However, bacterial delignification efficiency is far from being fully characterized. Bacterial enzymes may be adapted to a wider range of physico-chemical conditions (Díaz-García et al. 2020) that would be advantageous for industrial application. The relative ease of bacterial genetic manipulation may also be a powerful advantage to improve products yields and use a one-step process (depolymerization and useful monomer production in the same reactor) (Salvachúa et al. 2015). Moreover, due to their higher growth rate, bacteria might be quicker lignin converters than fungi.

As for fungal delignification (Rouches et al. 2016), bacterial delignification efficiency is dependent on culture conditions (Shi et al. 2013) and substrates (Ahmad et al. 2010), and catabolism is partly strain dependent (Vicuña 1988). Different biological modifications of lignin have been described: Cα-Cβ cleavage, alkyl-aryl cleavage, cross linking, demethoxylation, and ring-cleavage of aromatic compounds (Salvachúa et al. 2015). Delignifying bacteria are able to metabolize monomeric phenols from lignins but their activity on polymeric lignin substrates is still poorly understood (Asina et al., 2017). However, some conversion pathways of lignin and related oligomers have been described in some bacteria (Bugg et al., 2011; Johnson and Beckham, 2015; Masai et al., 2007). Diverse existing lignin linkages in dimer model compounds were shown to be cleaved by bacteria (Vicuña 1988). Bacterial cleavage of aromatic rings and side chains was shown on ^{14}C-labeled synthetic lignins (dehydrogenopolymers (DHP)) whereas only a small $^{14}CO_2$ release was obtained with corn stalk (Haider et al. 1978). The polymeric part of lignin substrates after bacterial fermentation were studied (Taylor et al. 2012; Salvachúa et al. 2015), but important parameters (i.e. molar mass (MM), solubility, etc.) and pathways by which polymeric lignin is broken down are still not clearly determined. In particular, the size and mechanism of uptake of the intermediates into the cell remains to be clarified (Bugg et al. 2016).

Pseudomonas putida KT2440, a non-pathogenic bacterium, is considered as an experimental model for many biotechnological applications since it has a known ability to degrade a wide variety of aromatic compounds (Jiménez et al. 2002) and has been reported to metabolise polymeric lignin (Salvachúa et al. 2015). Involved enzymes of biodelignification with *P. putida* KT2440 would be laccases and Mn^{2+}-oxidising and/or Mn^{2+}-independent peroxidases as the three enzymatic activities were measured in the culture medium of *P. putida* KT2440 when growing in the presence of a lignin-enriched stream, i.e., alkaline pretreated liquor (APL). Those activity were considerably higher in stationary phase cultures growing in nutrient-rich conditions (glucose used as additional carbon source) (Salvachúa et al., 2015). Biological depolymerization of lignin mainly relaying in redox attack by the above-mentioned kind of enzymes and some accessory enzymes such as H_2O_2-generating oxidases (Becker J. and Wittmann C., 2019). A more recent work also points out to the potential involvement of two azoreductases, a xenobiotic reductase, and a 2,3 quercetin dioxygenase in the modification of lignin, given that production of such enzymes was only detected or enriched in the extracellular medium when *P. putida* was cultured with oligomeric lignin as the major carbon source (Salvachúa et al., 2020). Thus, *P. putida* KT2440 was chosen as biocatalyst in the current study.

High-performance size-exclusion chromatography (HPSEC) is the main technique employed to investigate structural changes within the lignin polymer fraction. It provides a way to compare molar-mass distributions and thus assess changes in composition and polymerization degrees. Thus, it is often used to study bacterial lignin depolymerization, generally after a removal step of bacterial pellets (Kern and Kirk 1987; Chandra et al. 2007; Salvachúa et al. 2015). However, lignin in bacterial pellets might impact the interpretation of analyses and is often not studied nor discussed. This study aims to propose a valid analytical strategy to investigate bacterial lignin bioconversion. Several bottlenecks and artefacts that should be taken into consideration when analyzing bacterial lignin depolymerization are highlighted.

For the first time, a kinetic study of bacterial treatment is provided, covering both short term (0-24 h) and long term (7 days) incubation times.

Two distinct substrates were tested, i.e. a commercial pine Kraft lignin and a synthetic lignin (dehydrogenopolymer (DHP)) obtained by enzymatic polymerization of coniferyl alcohol (Jaufurally et al., 2016). Those lignin samples of guaïacyl-unit type were selected as they are representative of industrial technical lignins and native lignins, respectively. Moreover, the DHP lignin was considered as model since it did not contain any contaminating carbohydrates nor lignin degradation products generated by pulping processes. In bacterial delignification studies, a control without bacterial inoculation (no cell control) is generally used at the final incubation time. This allows detecting possible lignin structural changes due to the physico-chemical conditions. In the current study, an additional control based on the incubation of lignin with a non-delignifying bacteria (*E. coli*) (Ahmad et al. 2010) was implemented to facilitate the identification of the specific effects of the lignin-metabolizing *P. putida* KT2440 cells.

The bioconversion of Kraft lignin and DHP by *P. putida* and *E. coli* is discussed in order to validate the developed experimental strategy.

2. Materials and methods

2.1. Bacterial culture

2.1.1. Bacterial strains

P. putida KT2440 (ATCC 47054) is a Gram-negative bacterium derivative of *P. putida* mt-2 (Franklin et al. 1981). *E. coli* W(ATCC 9637) was used as a control strain as it is not able to metabolize lignin compounds (Archer et al. 2011).

2.1.2. Lignin substrates

A sample of commercial pine Kraft lignin Indulin® AT (MeadWestvaco; Richmond, USA) was extracted with methylene chloride (Baumberger et al. 1998) before its use as substrate

for bacterial culture ("Indulin" substrate). The second substrate consisted of a synthetic DHP lignin. It was obtained by laccase-initiated polymerization of coniferyl alcohol ("DHP" substrate) using *Trametes versicolor* laccase and a 20/80 wt. acetone/pH 4.5 buffer medium (Jaufurally et al., 2016).

2.1.3. Culture conditions and bacterial growth analysis

M63 minimal medium (KH_2PO_4, 13.6 g/L; $(NH_4)_2SO_4$, 2 $g.L^{-1}$) supplemented with $MgSO_4$ (0.12 $g.L^{-1}$) and microelements was used (Jiménez et al. 2002). Glucose was used as co-substrate at 1 $g.L^{-1}$as the addition of an easily degradable carbon source was already shown to improve lignin degradation with *P. putida*. (Asina et *al.*, 2017). Lignin substrates (1 $g.L^{-1}$) were dissolved in M63 medium. Lignin was dissolved by increasing the pH to 12 using 10 M NaOH. Then, to ensure bacterial growth, pH was decreased to 7.5 by adding 10 M HCl. This step did not lead to the formation of precipitate. In addition, medium was sterilized by filtration at 0.2 µm. It was ensured that medium preparation did not lead to significant MM distribution changes (see supplementary data). 250 mL-Erlenmeyer flasks containing 100 mL of culture medium were inoculated with the corresponding bacterial strains to an OD_{600} of 0.1, then incubated at 30ºC with continuous stirring at 200 rpm. Cell growth was monitored by measuring the absorbance at 600 nm (OD_{600}). In order to assess reproducibility, an independent experiment with Indulin and *P. putida* were repeated (excepted for bacterial pellets extraction), using the same substrate and bacterial strain but with newly prepared culture media and no cell control (independent experiment of campaign 2).

2.2. Culture sample recovery and purification

2.2.1. Collection of supernatants

To study the kinetics of delignification, samples were taken along the growth curve. Samples were collected by centrifugation of the whole culture medium (15 min, 4000 rpm, 4°C). The 100 mL of culture medium was divided into two falcon tubes, and the supernatants

freeze-dried during 48 h for further analysis. After freeze-drying samples from a same culture condition were pooled again. Cell pellets were stored at - 80°C.

2.2.2. Acid-precipitable polymeric lignin (APPL) preparation and extraction of acid-soluble compounds from the supernatants

The freeze-dried supernatant was dissolved in 35 equivalents (w/w) of water, then acidified with 2M HCl to pH < 3. After 30 min at 4°C, samples were centrifuged (15 min, 4000 g, 4°C). The pellet contained APPL (Bugg et al. 2011). Precipitations were carried out in duplicate.

To isolate soluble lignin from acid-soluble part, 6.3 mL of the acid-soluble fraction was extracted three times with 3 mL of a CH_2Cl_2/AcOEt (50/50, v/v) mixture and concentrated under reduced pressure (rotary evaporation) to a final volume of 1800 µL. Before extraction, 15 µL of a heneicosane (C21) solution at 0.245 $\mu g.\mu L^{-1}$ were added in order to constitute the internal standard for gas-chromatography.

2.2.3. Lignin extraction from the bacterial pellets

Sequential extractions with excess of solvent were applied on the freeze-dried bacterial pellets. After a first extraction step with hexane (lipids extraction), the remaining pellet was suspended in a dioxan/water mixture (90/10, v/v), vortexed and bath-sonicated for 20 min before centrifugation (15 min, 4000 g, 4°C) steps. The dioxan/water extracts and the remaining pellets were freeze-dried separately and acetylated for further analysis.

Samples without bacterial growth (less than 1 mg weight) were analyzed once and other samples (around 15 mg weight) were analyzed in duplicates.

2.3. Sample analysis

2.3.1. Apparent molar-mass (MM) distribution assessment by HPSEC)

2.3.1.1. Sample preparation

Initial lignins, APPL and bacterial pellets samples (10 mg) were acetylated at room temperature for 48 h using a mixture of pyridine (200 µL) and acetic anhydride (400 µL).

175 Acetylation was performed in absence of light. Then, acetylation reagents were eliminated by
176 methanol addition (200 µL) and evaporation at 40ºC under a stream of nitrogen gas, followed
177 by three washing steps with toluene (200 µL) evaporating the samples to dryness, and a final
178 washing step with methanol (200 µL). Samples were freeze-dried before dissolution in
179 stabilized tetrahydrofuran (THF) and filtration (0.45 µm PTFE membrane syringe filters) for
180 HPSEC analysis.
181 The acetylated APPL sample was dissolved in 1 mL of THF and acetylated bacterial pellets
182 samples were dissolved in THF at a concentration of 20 $mg.mL^{-1}$.
183 Acid-soluble extracts in CH_2Cl_2/AcOEt (50/50, v/v) were analyzed without acetylation, after
184 evaporation of the solvent and dissolution in a 2-fold lower volume of THF.
185 ***2.3.1.2. HPSEC-UV analysis***
186 HPSEC analysis of the acetylated samples was performed at room temperature on a system
187 equipped with a PL-gel mixed-C pore type column (Polymer Laboratories, 5 µm, 600 x 7.5
188 mm), with UV detection at 280 nm and stabilized THF (1 $mL.min^{-1}$) as eluent (Baumberger et
189 al., 2007). Polystyrene standards (Polymer Labs EasyCal PS2A and B; mass range 580-
190 364000 $g.mol^{-1}$) were used for calibration. Chromatograms were converted into log M-
191 distribution using the calibration equation log M=10.93-0.4403xRetention Time (RT). Non-
192 acetylated samples (acid-soluble compounds) were analyzed with a 100 Å PL-gel column
193 (Polymer Laboratories, 5 µm, 600 mm × 7.5 mm). Calibration of this column was carried out
194 with polyethylene oxide standards (Igepal, Sigma–Aldrich, Saint-Louis, USA) and purified
195 lignin model compounds (Demont-Caulet et al., 2010).
196 ***2.3.2. Analysis of the CH_2Cl_2/EtOAc soluble phenolic compounds by Gas Chromatography-***
197 ***Mass Spectrometry (GC-MS)***
198 CH_2Cl_2/EtOAc extracts recovered from the acid-soluble fraction of the supernatants were 10-
199 fold concentrated before GC-MS analysis. Aliquots (20 µL) of the concentrated solutions

were silylated with bistrimethylsilyltrifluoroacetamide (BSTFA, 50 µL) and pyridine (20 µL) prior to GC-MS analysis. According to Baumberger et al. (1998), GC was run on a SPB5 poly(5% diphenyl-95% dimethylsiloxane) capillary column (Supelco, 30 m x 0.2 mm i.d., 0.25 µm) using helium as carrier gas (0.5 bar inlet pressure) and increasing 2°C per min the temperature from 180°C up to 260°C. Using heneicosane C21 as internal standard, phenolic compounds were quantified using pure commercial compounds (vanillin, vanillic acid and acetovanillone) and synthesized models (dihydroferulic acid and dihydroconiferyl alcohol, see S1 for NMR synthesis protocols and characterization).

2.3.3. Thioacidolysis of the initial DHP sample

Thioacidolysis was carried out in duplicate as in Sipponen et al. (2013) with 20 mg of DHP (considering 100% of Klason content) and with 0.5 mg heneicosane C21 (Fluka) as internal standard. Trimethylsilyl derivatives of guaicyl (G) thioacidolysis monomers derived from DHP were quantified by GC–MS.

3. Results

The effect of the bacterial treatments on the whole Kraft lignin Indulin and DHP samples was investigated according to the experimental strategy summarized in Fig. 1. After each incubation, the culture medium was centrifugated in order to separate the bacterial cells contained in the pellet from the lignin soluble compounds present in the supernatant. These compounds were expected to consists of polymers and oligomers as well as phenolic monomers, initially present or released during incubation. In order to investigate changes in polymeric lignin, an APPL fraction was isolated from the supernatant using acid precipitation, as previously experimented (Ball et al., 1990; Arias et al., 2016). In parallel, phenolic compounds were recovered from the acid soluble fraction by extraction with a CH_2Cl_2/AcOEt mixture in order to get rid of the sugars and other possible contaminants coming from the

culture medium or from bacteria. To investigate lignin possibly associate to the cells in the bacterial pellets, an extraction method in dioxan/water solution (90/10, v/v) was developed. This lignin-selective solvent is generally used at laboratory scale for lignin isolation (Kern and Kirk 1987). Proteins and DNA coming from the bacterial biomass were not expected to be solubilized in such an apolar organic solvent (Chin et al., 1994).

This section reports on the changes observed concomitantly in the APPL and acid-soluble fractions of each sample upon incubation with *P. putida*, then on the non-bacterial specific effects evidenced with *E. coli* and its impact on reproducibility.

3.1. Effect of P. putida on Indulin and DHP lignins

3.1.1. Conversion of APPL

Incubation of Indulin with *P. putida* induced a 30% decrease in the APPL yield within the first 6 hours (Fig. 2). Similar decrease was observed in the no-cell control. However, after 7 days, the APPL yield of the incubated samples was 17% lower than that of the no cell control and 28% lower than the initial APPL yield. This result suggested a delignifying effet of *P. putida* on Indulin. In the case DHP the APPL yield was too low to detect any changes with precision (data not shown). To assess whether the APPL yield decrease of Indulin was due to a conversion of non-soluble polymer into lower-MM soluble compounds, HPSEC was applied both to the APPL and acid-soluble lignin fractions.

3.1.2. Changes in the MM distribution of the APPL and acid-soluble fractions

The apparent MM distributions of the Indulin and DHP APPL fractions were obtained from the HPSEC profiles and normalized with respect to the maximum absorbance (Fig. 3a and 4a). These distributions confirmed that APPL was mainly composed of polymers (log M up to 5). In contrast, the HPSEC profiles of the acid-soluble fractions indicated the presence of monomers, dimers and oligomers (polymerization degree (PD) inferior to 10 according to

their retention time). For both samples, extractable phenolic monomers in the initial (0 h) culture supernatant were present only in low proportion (0.02% of dry supernatant for Indulin and only traces of vanillin and vanillic acid for DHP (Table 1)). This was consistent with the pre-extraction of Indulin with methylene chloride and with the polymerization process of coniferyl alcohol for DHP. The initial composition of the Indulin monomer fraction was in agreement with literature and reflects depolymerization processes occurring during Kraft pulping (Baumberger et al., 1998).

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3.3.2.1 Changes observed for Indulin

Incubation of Indulin with *P. putida* induced only slight changes in the normalized apparent MM distribution of the polymeric APPL lignin fraction, whereas the 24 h control sample exhibited a shift of the distribution towards higher MM (Fig. 3a). This shift suggested that lignin self-polymerization or aggregation took place in the absence of bacteria and that *P. putida* prevented (or delayed) this apparent lignin self-assembling phenomenon. Since no significant changes were observed in the soluble fraction of the no-cell control after 24 h incubation (overlapping of the *P. putida* 0 h and no cell 24 h profiles in Fig. 3b), it was concluded that low-MM compounds were not involved in this phenomenon.

A closer look at the MM distribution indicated a slight shift towards lower MM during the *P. putida* growth phase (0-24 h; Fig. 3a). A slight increase in the proportion of acid-soluble oligomers at 10 h was observed, followed by a decrease at 24 h (RT = 14-17.5 min; Fig. 3b). This observation was consistent with the release of soluble compounds by lignin depolymerization followed by their degradation and subsequent absorption by *P. putida*. On the other hand, monomers appeared to be converted by *P. putida* as soon as 10 h incubation (Fig. 3b, peak at 18 min), which was confirmed by GC-MS analysis of the consumption of phenolic monomers initially present in Indulin (Table 1). After 7 days, a slight shift towards higher MM was observed (Fig. 3a, 7 d- line higher and righter at than 0 h) together with a

decrease in the proportion of all soluble compounds (Fig. 3b, 7 d-line is the lowest one). Taken together, these results suggest that the polymer APPL fraction of Indulin was subjected to two antagonist processes, depolymerization during the *P. putida* growth phase, followed by repolymerization that appeared to be a spontaneous phenomenon in the no cell control.

The analysis of the phenolic monomers by GC-MS (Table 1) indicated that vanillin, vanillic acid, and dihydroconiferyl alcohol (DHCA) were converted by *P. putida*, with transitory formation of dihydroferulic acid (DHFA) between 10 and 24 h. In agreement with the initial molar content of DHCA (0.3 µmol g^{-1}), DHFA (max 0.1 µmol g^{-1} at 10 h) could be formed from DHCA in a way analogous to the conversion of coniferyl alcohol into ferulic acid reported for white rot fungi lignin degradation (Priefert et al., 2001). DHFA could then be converted into vanillic acid as reported for *P. fluorescens* (Andreoni et al. 1995). In parallel, a specific increase in concentration of acetovanillone at the end of the *P. putida* cultivation (Table 1) suggested that this compound was formed after 7d. Indeed, this compound was shown to be released from lignin by β-O-4 linkage bacterial cleavage (Bugg et al. 2011). Before 7d, acetovanillone was either not metabolized or maybe consumed at the same time that it was formed.

3.3.2.2. Changes observed for DHP

In the case of DHP, a slightly increased proportion of higher mass APPL was observed for the control, but in contrast to the results observed with Indulin, no shift of the MM distribution took place (Fig. 4a), suggesting that DHP was less inclined to self-assembly.
This increase in the proportion of higher MM compounds was enhanced by the incubation with *P. putida* after 24 h and reached a maximum at 7 days. As for Indulin, a slight decrease was observed at short incubation time (10 h). Changes in the lower MM portion of the curves (400-900 $g.mol^{-1}$) took place as soon as 10 h, with the progressive formation of a new peak

300 corresponding to an apparent MM of 520 g.mol^{-1} and the disappearance of the shoulder
301 corresponding to an apparent MM of 630 g.mol^{-1} (equivalent to tetramers). Consistently, the
302 analysis of the soluble fraction (Fig. 4b) showed also a decrease with time of tetramers and
303 oligomers of higher MM (chromatogram portion at RT = 14-15.5 min), with subsequent
304 enrichment in dimers (peak at 15.8 min). The broadening of the dimer peak at 7 d (Fig. 4b)
305 indicated that new dimers were formed. DHP oligomers of polymerization degree >3 seem to
306 be converted by *P. putida* into lower MM compounds. Compared to Indulin, DHP exhibited
307 higher apparent reactivity of its oligomer fractions with a clearer depolymerization effect
308 when incubated with *P. putida*. The concomitant increase in oligomers and higher polymeric
309 fraction in APPL would explain the lack of clear tendency in the amount of APPL noted
310 earlier (3.3.1).
311 Differences between the DHP and Indulin experimental data were also observed through the
312 comparison of the non-normalized APPL chromatograms (figure dedicated to *E. coli* in
313 supplementary data and Fig. 5). Polymer total area (2.8< log M < 4.5) of APPL increased at 7
314 d for DHP treated with *P. putida*, but decreased in the case of Indulin (Fig. 5). The increase in
315 absorbance of APPL formed from DHP at t = 7 d is due to the action of *P. putida*. It may
316 reflect an increased solubility in THF and/or extinction coefficient changes due to phenol
317 oxidation by some secreted bacterial oxidases. Indeed, Munk et al. (2015) suggested possible
318 modification of lignin functional groups caused by laccases. These lignin oxidases require
319 copper as cofactor, and a multicopper lignin oxidase (Bugg et al. 2016) was reported for *P.*
320 *putida* GB-1 (Brouwers et al., 1999; Geszvain et al., 2016).
321 ***3.2. Non-specific lignin interaction with non-delignifying bacteria***
322 ***3.2.1. Effect of E. coli on APPL and polymeric soluble fractions***
323 Incubation of the lignin samples with non-delignifying *E. coli* bacteria showed also some
324 changes in the lignin MM distribution pattern, but the effects were clearly distinct from those

observed with *P. putida* cells (Fig. 5). The main difference consisted in a lower proportion of high MM polymers in the APPL fraction of both Indulin and DHP when incubated with *E. coli* (Fig. 5). In the case of DHP an additional difference was the lowest overall concentration of lignin polymer in the APPL fraction (Fig. 5b). Such effects, apparently diagnostic of the consumption of lignin polymer by bacteria, were not expected from a non-delignifying bacterium such as *E. coli*. Thus, loss of lignin compounds in the bacterial pellets was suspected, as lignin association to bacterial cell was previously reported (Kern and Kirk 1987). Further experiments were carried out on bacterial pellets to check this assumption.

3.2.2. Analysis of lignin in the bacterial pellets

HPSEC analysis of the dioxan-extracted pellet after acetylation and dissolution in THF of Indulin and DHP confirmed the presence of lignin in the *E. coli* 7 day pellets (Fig. 6). Indeed, the MM distribution and UV spectra were found to be similar to that of lignin in the supernatant. Despite the low amount of extract recovered (less than 1.5 mg), good repeatability in terms of chromatogram profile between two extractions of the same sample was obtained. Analysis of the pellet extraction residue indicated that extraction of the polymer and oligomers was exhaustive, since only traces remained (data not shown). However, low repeatability was obtained regarding phenolic monomers, as some often remained in the pellet after extraction. Thus, the dioxan extract was considered representative of the lignin oligomer and polymer fraction (log M>2.7) present in the pellet.

The presence of Indulin and DHP was also observed in *P. putida* cell pellets (Fig. 6). However, the proportion of polymer and oligomers, compared to monomers, was lower than in *E. coli* extracts, suggesting that non-specific interactions with bacteria were more significant in the case of *E. coli*. This difference is probably explained by the highest specific membrane surface area of *E. coli*, due to larger cell size (Bronk et al., 1992; Mulakhudair et al., 2017), or higher cell density (higher OD for *E. coli*, Table 2). Interestingly, the lignin losses in *E. coli* pellets

could account for the apparent depolymerization observed in APPL (Fig. 5). In contrast, polymeric lignin losses with *P. putida* pellets (Fig. 6) had a different pattern to that observed for APPL (Fig. 5). Consequently, the specific delignifying action of *P. putida* mainly accounted for the structural changes observed in APPL.

3.2.3. Possible impact of lignin loss in bacterial pellets on technical reproducibility

In order to assess the qualitative reproducibility of bacterial conversion, the analytical results were compared to those obtained from the second incubation series of Indulin with *P. putida*. The analysis of the soluble fraction provided similar results, with the specific conversion of phenolic monomers at short time (≤ 10 h), and an increase followed by a decrease (from 24 h) in the proportion of all soluble compounds. Moreover, slight lignin polymerization in the no cell control APPL was also observed, as well as with *P. putida* at 24 h. However, at longer time (7 d), an apparent APPL depolymerization was suggested by a lower proportion of higher MM compounds (see supplementary data), which was not the case for the other series. In view of the present study, this discrepancy could be due to a higher density of bacterial cells at 7 d for campaign 2 (higher O.D. Table 2) leading possibly to a higher depolymerization activity (Salvachúa et al., 2015) but also to a slight increase in lignin bacterial pellets losses.

4. Discussion

4.1. Relevance of the experimental approach

The novel features of the strategy developed in this study were: 1) the concomitant bioconversion of two types of lignin (a technical lignin and a DHP) highlighting substrate specific mechanisms; 2) a kinetic study (over 7 days) highlighting transitory conversions and long-term physico-chemical effects; 3) the use of a non-delignifying bacterium (*E. coli*) as

374 control (in addition to conventional no-cell control); 4) the analyses of lignin contained in
375 bacterial pellets.

376 This strategy brought two main results of importance for lignin analysis, from a
377 methodological point of view. The first one is that some lignin is lost in the bacterial pellets
378 through centrifugation of the culture media. This loss can lead to an apparent decrease in the
379 proportion of high-molar compounds in the supernatant and erroneous subsequent conclusion
380 that depolymerization occurs (e.g. by non-degrader *E. coli*). The second important result is
381 that the depolymerizing effect of bacterial incubation on lignin can be hidden by lignin self-
382 polymerization, as observed for Kraft lignin, or by recondensation of oxidized lignin products,
383 as observed for DHP. In light of these conclusions, previous evidence of lignin bacterial
384 depolymerization and metabolizing are discussed below.
385
386 In a previous study, Salvachúa et al. (2015) assessed the ability of different bacteria to
387 depolymerize and/or metabolize lignin from maize alkali-pretreated liquor containing 30%
388 lignin associated to hemicelluloses. This assessment was made based on the HPSEC profile of
389 the whole acetylated culture supernatants recovered from a given mass of substrate incubated
390 during 7 days. As mentioned by the author, the consumption of hemicelluloses and sugars can
391 lead to an apparent increase in lignin content. Among the bacteria tested *P. putida* KT2440
392 was concluded to convert the phenolic monomers and to convert up to 30% lignin, according
393 to the Klason lignin determination in the substrate. In the light of the present results, a
394 contribution of lignin losses with bacterial pellets to this apparent conversion cannot be ruled
395 out. In the same line, Chai et al. (2014) concluded that the bacteria *Comamonas* sp. B-9 was
396 able to decolorize and degrade Kraft lignin, based on the spectrophotometry analysis of the
397 supernatant after 7 days of incubation. It is possible that lignin selective loss and self-
398 polymerization contributed to the change in UV spectra in addition to lignin conversion by the

399 bacteria. Due to these possible artefacts, experimental strategies based on the use of ^{14}C-
400 labeled lignin substrates and determination of released $^{14}CO_2$ allows to unambiguously
401 demonstrate lignin metabolizing by bacteria, as applied by Kern and Kirk (1987) to
402 *Xanthomonas* sp. Using this criteria, these authors showed that *Xanthomonas* could degrade
403 lignin compounds only up to a mass of 600-1000 $g.mol^{-1}$, which corresponds to oligomers (≤
404 5 units). Moreover, by analyzing aqueous dioxan lignin soluble fractions recovered from the
405 culture medium after 14 days they observed formation of low MM compounds diagnostic of
406 depolymerization. However, the amount of cell-associated ^{14}C in each case was equal to about
407 half of the evolved $^{14}CO_2$. It confirmed that some lignin remained associated to the cell.
408 Moreover, the absence of kinetic analysis involving short times hampered the full
409 comprehension of the conversion mechanisms.
410 In conclusion, compared to previous approaches previously implemented, the strategy
411 proposed herein combined several advantages: 1) it is easy to carry out due to the absence of
412 radioactive labelled compounds, 2) it allows complete analysis of all the polymer and
413 oligomer lignin fractions present in the medium, 3) it provides a way to investigate
414 specifically depolymerization mechanisms.
415 ***4.2. Insights into lignin reactivity towards treatment with P. putida***
416 In the present study, comparison between the two substrates, Indulin and DHP, indicated
417 distinct reactivity towards *P. putida* treatment, which can be tentatively related to the
418 structural specificities of these substrates. The main differences between these two substrates
419 consisted in their MM distribution and inter-unit bonding pattern. According to MM
420 distribution (supplementary data), DHP contained a higher proportion of oligomers and
421 polymers ($2.7<\log M<3.5$) than the extracted Indulin. Concerning inter-unit bonding pattern,
422 Indulin was previously shown to be mainly composed of condensed linkages (C-C bonds) as
423 indicated by a low proportion of β-O-4 linked units (200-300 µmol/g) (Baumberger et al.,

1998). In contrast, the DHP β-O-4 linked units content (1129 ± 36 µmol.g^{-1}) was closer to that of native wood lignin (Rolando et al., 1992) and accounted for 20% of the total G-units in the polymer. As a consequence of its lower proportion in β-O-4 bonds, lower MM and the presence of demethylated units (catechol structures) (Baumberger et al., 1998), Indulin is expected to contain a higher proportion of phenolic OH groups than DHP. This last characteristic could explain its higher propention to aggregration, a phenomenon known to depend on lignin structure and to involve intermolecular interactions through hydrogen bonds (Ratnaweera et al., 2015; Mishra and Ekielski, 2019). The fact that incubation with *P. putida* apprently prevents such phenomenon suggests that MM decrease through depolymerization compensates the MM increase due to aggregation but could also result from the precipitation of Indulin aggregates with bacterial cells. Thus, no proof of Indulin depolymerization by *P. putida* is provided. However, it capacity to metabolize some lignin phenolic compounds was demonstrated by the transitory formation DHFA. In the case of DHP, no DHFA was formed but changes in the composition of soluble oligomers and monomers occurred with the formation of new dimers. Thanks to the absence of phenolic monomers in DHP, it can be concluded that DHFA is formed by conversion of phenolic monomers in Indulin, and that the formation of new dimers is DHP result from a depolymerization and not dimerization process. Formation of new dimers can be explained by the action of lignolytic enzymes excreted by the bacteria in the culture medium, in particular laccases and Mn2+-oxidising and/or Mn2+-independent peroxidases previously shown to be secreted by *P. putida KT2440* (Salvachúa et al., 2015). Indeed these types of enzymes are likely to catalyze the oxidative cleavage of different intra- and inter-unit lignin bonds and the higher proportion of β-O-4 linkages in DHP is in favour of higher reactivity towards depolymerization (Bugg et al., 2020). Though polymerization was in contrast previously observed when these enzymes were implemented

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448 on DHP *in vitro* (Kondo et al., 1990) synergy between enzymes might explain the apparent
449 depolymerization observed herein *in vivo*.
450
451 ***4.3. Interest of a treatment with P. putida for lignin valorization***
452 Though specific effects of *P. putida* on Kraft lignin and DHP were observed, its use as
453 depolymerizing agent for bioconversion was found limited notably by simultaneous
454 polymerization phenomenon. The competition between depolymerization and
455 repolymerization is a widespread phenomenon observed during different processes, including
456 chemical processes (Ball et al., 1990; Li et al., 2007a). This phenomenon is due to the
457 reactivity of lignin products released by cleavage of interunit bonds, in particular species
458 carrying carbonium ions formed in acidic medium (Li et al., 2007b). For example, a
459 polymerization of Kraft lignin occurred after 72h of incubation with soil bacteria, followed by
460 a depolymerization at 192h (Taylor et al., 2012). Using steam explosion as depolymerizing
461 process, Li et al. (2007b) suggested the use of phenol or 2-naphthol as a scavenger for
462 carbonium ions to avoid this phenomenon. In a biotechnological approach, some bacterial
463 enzymes such as dehydrolipoamide dehydrogenase, could also be advantageously used to
464 avoid spontaneous repolymerization (Rahmanpour et al., 2017). Such approaches would be
465 helpful for future industrial application.
466
467 In this paper, we used the substrate fraction that was soluble in the culture medium using pH
468 variation, removing the non-soluble highly polymerized fraction by filtration prior to
469 incubation, to obtain a sterile substrate. In contrast, Tian et al. (2016) incubated the non-water
470 extractable polymerized Kraft lignin fraction for the strain screening of soils and sediments
471 bacteria, and showed the production of soluble phenolic compounds. Brown and Chang
472 (2014) reported several papers dealing with the release of water soluble APPL metabolites

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from insoluble lignin with soil bacteria. This suggests that bacterial treatment with *P. putida* could be used to solubilize non-soluble recalcitrant fractions of lignin, rather than the water-soluble fraction.

5.Conclusion

In this work *P. putida* exhibited specific depolymerization activity on both Indulin and DHP. The original kinetic approach proposed was found efficient to better understand the lignin modifying mechanisms. With DHP, 4 and 5 units oligomers decreased after 7d with *P. putida* whereas new dimers were obtained. *P. putida* limited the self-repolymerization of Indulin after 7d incubation, and a depolymerization occurred during the growth phase (monomers metabolizing and decrease in polymer apparent MM during the first 24 h). Moreover, evolution of phenolics and the transitory formation of DHFA during Indulin incubation is helpful to further investigate *P. putida* metabolic pathways.

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Electronic supplementary material

The online version of this article contains supplementary material which is available to authorized users.

Conflict of Interest

All authors declare that they have no conflict of interest.

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