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

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Abstract:	Biodelignification is a complex and misunderstood mechanism. To study it, a new analytical approach was proposed using <i>Pseudomonas putida</i> KT2440. The strategy originality lies in: 1) the concomitant bioconversion of two lignins (Kraft lignin and synthetic dehydrogenopolymer (DHP)) separately highlighting substrate specific mechanisms; 2) the kinetics study (over 7d.) spotlighting the dynamic; 3) the use of a non-delignifying bacterium (<i>Escherichia coli</i>) as control (in addition to conventional no-cell control); 4) the analyses of lignin contained in bacterial pellets after the development of an adequate strategy.
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Highlights

- Incubation with *P. putida* KT2440 prevented self-polymerization of Kraft lignin
- Apparent DHP depolymerization took place during incubation with *P. putida* KT2440
- *P. Putida* KT2440 metabolized phenolic monomers during the first 24 h incubation
- Dihydroferulic acid was transitory formed by lignin treatment with *P. putida* KT244
- Lignin was found associated to non-delignifying *E. coli* bacterium after incubation

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

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Abstract

Biodelignification is a complex phenomenon whose mechanisms are misunderstood. In order to help decipher bacterial activity on lignins, a new analytical approach was proposed using *Pseudomonas putida* KT2440 as delignifying bacterium. The strategy originality lies in: 1) the concomitant bioconversion of two lignins types (Kraft lignin and synthetic dehydrogenopolymer (DHP)) highlighting substrate specific mechanisms; 2) the kinetics study (over 7 days) spotlighting transitory conversions and long-term physico-chemical effects; 3) the use of a non-delignifying bacterium (*Escherichia coli*) as control (in addition to conventional no-cell control); 4) the original analyses of lignin contained in bacterial pellets.

In contrast to *E. coli* control, lignin losses in *P. putida* pellets could not account for the changes observed in the supernatant upon incubation. These changes were specific of *P. putida* and brought insight into its ligninolytic activity: phenolics metabolism, prevention of spontaneous repolymerization for Kraft lignin, and apparent depolymerization followed by repolymerization for both lignins.

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

1. Introduction

The world is currently facing concerns of global warming, population rising, 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.

Lignins, the second most abundant biopolymer on Earth, are recalcitrant polyphenolic polymers (Linger et al. 2014) with huge diversity and very diverse properties (Monlau et al. 2013). Lignin valorization into high-added value molecules, 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 industrial lignins such as Kraft lignin, which are currently low-value byproducts from pulp and paper industry. Thus, the biological conversion of industrial 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). In nature, delignifying microorganisms are composed of fungi and bacteria. 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 (Salvachúa et al. 2015) 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 (Odier et al. 1981; 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: C α -C β 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 ¹⁴C-labeled synthetic lignins (dehydrogenopolymers (DHP)) whereas only a small ¹⁴CO₂ release was obtained with corn stalk (Haider et al. 1978). Polymeric part of other lignified substrates after bacterial

fermentation were studied (Odier et al. 1981; Taylor et al. 2012; Salvachúa et al. 2015) but impacting parameters (i. e. molar mass (MM), solubility, etc.) on delignification and pathways by which polymeric lignin is broken down are still not clearly determined, so. In particular, the size and mechanism of uptake of the intermediates into the cell remains to be clarified (Bugg et al. 2016).

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 kinetics study upon bacterial treatment is provided, covering both short terms (0-24 h) and long terms (7 days) incubation times.

Two distinct substrates were tested, i.e. a commercial Kraft lignin and a synthetic DHP lignin obtained by polymerization of coniferyl alcohol. *Pseudomonas putida* KT2440 was chosen as biocatalyst since it is a non-pathogenic bacterium considered as an experimental model for many biotechnological applications, and it has a known ability to degrade a wide variety of aromatic compounds (Jiménez et al. 2002; Salvachúa et al. 2015). The bioconversion of Kraft lignin and DHP by *P. putida* is discussed in order to validate the developed experimental strategy (including the analysis of lignin in bacterial pellets).

2. Materials and methods

2.1. Bacterial culture

2.1.1. Bacterial strains

P. putida KT2440 (ATCC 47054) is a Gram-negative bacterium, restriction-deficient derivative of *P. putida* mt-2 (Franklin et al. 1981). *E. coli* W(ATCC 9637) was used as a control strain 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).

A second substrate consisted in a synthetic DHP lignin 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; $(\text{NH}_4)_2\text{SO}_4$, 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 molar mass distribution changes (see supplementary data). 250 mL-erlenmeyers containing 100 mL of culture medium were inoculated with the corresponding bacterial strains to an OD₆₀₀ of 0.1, then incubated at 30°C with continuous stirring at 200 rpm. Cell growth was monitored by measuring the absorbance at 600 nm (OD₆₀₀). 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), and the supernatants freeze-dried for further analysis. Cell pellets were stored at - 80°C.

2.2.2. APPL preparation and extraction of acid-soluble compounds from the supernatants

One mass unit of the freeze-dried supernatant was dissolved in 35 mass unit of acidified water (HCl 2M) to obtain a pH lower than 3. After 30 min at 4°C, samples were centrifuged (15 min, 4000 g, 4°C). The pellet contained acid-precipitable polymeric lignin (APPL) (Bugg et al. 2011). Precipitations were carried out in duplicates.

To isolate soluble lignin from acid-soluble part, 6.3 mL of the acid-soluble fraction were extracted three times with 3 mL of CH₂Cl₂/AcOEt (50/50, v/v) 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 µg.µL⁻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, 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 in a mixture of pyridine (200 μ L) and acetic anhydride (400 μ L) in the dark at room temperature for 48 h. Then, acetylation reagents were eliminated by methanol addition (200 μ L) and evaporation at 40°C under a stream of nitrogen gas, followed by three washing steps with toluene (200 μ L) evaporating the samples to dryness, and a final washing step with methanol (200 μ L). Samples were freeze-dried before dissolution in stabilized tetrahydrofuran (THF) and filtration (0.45 μ m PTFE membrane syringe filters) for HPSEC analysis. The acetylated APPL sample was dissolved in 1 mL of THF and acetylated bacterial pellets samples were dissolved in THF at a concentration of 20 mg.mL⁻¹. Acid-soluble extracts in CH₂Cl₂/AcOEt (50/50, v/v) were analyzed without acetylation, after evaporation of the solvent and dissolution in a twice lower volume of THF to get a twice higher final concentration.

2.3.1.2. HPSEC-UV analysis

HPSEC of the acetylated samples was performed at room temperature using a PL-gel mixed-C pore type column (Polymer Laboratories, 5 μ m, 600 x 7.5 mm) with detection at 280 nm, and stabilized THF (1 mL.min⁻¹) as eluent as in Baumberger et al. (2007). Polystyrene standards (Polymer Labs EasyCal PS2A and B; mass range 580-364000 g.mol⁻¹) were used for calibration. Chromatograms were converted into log M-distribution using the calibration equation $\log M = 10.93 - 0.4403 \times \text{Retention Time (RT)}$. Non-acetylated samples (acid-soluble compounds) were analyzed with a 100 Å PL-gel column (Polymer Laboratories, 5 μ m, 600 mm x 7.5 mm). Calibration of this column was carried out with polyethylene oxide

standards (Igepal, Sigma–Aldrich, Saint-Louis, USA) and purified lignin model compounds (Demont-Caulet et al., 2010).

2.3.2. Analysis of the CH₂Cl₂/EtOAc soluble phenolic compounds by GC-MS

CH₂Cl₂/EtOAc extracts recovered from the acid-soluble fraction of the supernatants were 10-fold concentrated before Gas Chromatography-Mass Spectrometry (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), gas chromatography 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

3.1. Overall experimental strategy

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. Interestingly, in the current study, the effect of the treatments on the whole lignin samples is determined following the experimental strategy outlined in Fig. 1.

After bacterial incubation in the presence of lignin, the culture medium was centrifuged. The bacterial pellets and supernatants were freeze-dried and both samples were submitted to sequential extraction before analysis. The supernatant was composed of lignin soluble polymers and oligomers as well as phenolic monomers, initially present or released during the incubation. In order to investigate changes in polymeric lignin, an APPL fraction was isolated from the supernatant using acid precipitation (Ball et al. 1990; Arias et al. 2016). Phenolic compounds were recovered from the acid soluble fraction by extraction with a $\text{CH}_2\text{Cl}_2/\text{AcOEt}$ mixture in order to get rid of the sugars and other possible contaminants coming from the culture medium or from bacteria. On the other hand, to investigate lignin present 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).

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.

3.2. Main structural features of the selected lignin substrates

Two distinct substrates were used to study the effect of *P. putida* in delignification: 1) a commercial industrial softwood Kraft lignin sample, Indulin, and 2) a synthetic lignin obtained by the laccase-initiated polymerization of coniferyl alcohol, DHP. Indulin was previously subjected to a purification step by extraction in a CH₂Cl₂/AcOEt mixture known to eliminate some sugars, free phenolic monomers and dimers (Baumberger et al. 1998). DHP was completely deprived from carbohydrate contaminants due to their synthesis protocol involving only monolignols, buffer and acetone (Jaufurally et al., 2016). Thioacidolysis analysis confirmed the majority of lignin G-unit type (at least 98, 5% for Indulin and 100% for DHP), which facilitated their analysis and investigation of structural changes. The main differences between these two lignin substrates consisted in their intersubunit bonding pattern and molecular mass (MM) distribution. Indulin was previously shown to be mainly composed of condensed linkages (C-C bonds) as indicated by a low proportion of β -O-4 linked units (200-300 $\mu\text{mol/g}$) (Baumberger et al., 1998). In contrast, the DHP β -O-4 linked units content ($1129 \pm 36 \mu\text{mol.g}^{-1}$) was closer to that of native wood lignin (Rolando et al. 1992) and accounted for 20% of the total G-units in the polymer. In addition to its lower proportion in β -O-4 bonds (1 000 $\mu\text{mol/g}$ native lignin for G(C₆C₃), extracted Indulin exhibited specific functional groups, such as catechols and enols, which are diagnostic of structural degradation during the Kraft process (Baumberger et al. 1998).

The apparent MM distributions of samples (supplementary data) indicated that DHP contained a higher proportion of oligomers and polymers ($2.7 < \log M < 3.5$) than the extracted Indulin. However, both samples were almost deprived from free monomers, in agreement with the purification of Indulin and the polymerization process of coniferyl alcohol for DHP. Extractable phenolic monomers in the initial (0 h) culture supernatant were in low proportion even after a substrate solubilization step. In extracted Indulin at 0 h, monomers measured by

GC-MS accounted for around 0.02% of dry supernatant (Table 1). For DHP, only traces of vanillin and vanillic acid were detected (less than 10 µg of total phenolic content per gram of dry supernatant).

In conclusion, extracted Indulin and DHP turned out to be suitable samples to investigate the effect of bacterial conversion on lignin polymerized fractions representative of industrial and native lignins, respectively.

3.3. Effect of *P. putida* on the polymeric Indulin and DHP lignins

3.3.1. Evolution of APPL amount in *P. putida* cultures

Whereas no clear tendency (data not shown) appeared for the proportion of DHP APPL (1.5-2% of the initial sample), evolution of the APPL recovered from extracted Indulin samples (Fig. 2) indicated lower content (2.5% of the dry weight) than in the no cell control (3% of the dry weight). Moreover, *P. putida* 7d recovered APPL is around 1% inferior to *P. putida* 0h samples. Thus, *P. putida* delignifying action is suspected. To assess whether this decrease was due to a conversion into lower-MM soluble compounds, HPSEC was applied both to the soluble and insoluble lignin fractions.

3.3.2. Change in the MM distribution of the APPL and acid-soluble fractions

For each sample, main changes concerning lignin polymeric fraction can be observed through the APPL MM distribution (HPSEC). This fraction was analyzed in parallel to the acid-soluble extract fraction to get insights into the depolymerization process. To better visualize changes in the APPL MM distributions, the absorbance was normalized (Fig. 3a and 4a) with respect to the maximum polymer peak intensity.

3.3.2.1. Indulin

Incubation of Indulin with *P. putida* induced only slight changes in the normalized apparent MM distribution of the polymeric APPL lignin fraction, whereas 24 h control exhibited a shift of the distribution towards higher MM (Fig. 3a). This shift suggested that lignin self-

264 polymerization or aggregation took place in the absence of bacteria and that *P. putida*
 265 prevented (or delayed) this apparent lignin self-assembling phenomenon. Since no significant
 266 changes were observed in the soluble fraction of the no-cell control after 24 h incubation
 267 (overlapping of the *P. putida* 0 h and No cell 24 h profiles in Fig. 3b), it was concluded that
 268 low-molar-mass compounds were not involved in this phenomenon.

269 A closer look at the MM distribution indicated a slight shift towards lower MM during *P.*
 270 *putida* growing phase (0-24 h; Fig. 3a). A slight increase of the acid-soluble oligomers
 271 proportion at 10 h followed by a decrease at 24 h was observed (RT = 14-17.5 min; Fig. 3b).
 272 This observation was consistent with the release of soluble compounds by lignin
 273 depolymerization followed by their degradation and subsequent absorption by *P. putida*. On
 274 the other hand, monomers appeared to be converted by *P. putida* as soon as 10 h incubation
 275 (Fig. 3b, peak at 18 min), which was confirmed by GC-MS analysis of the consumption of
 276 phenolic monomers initially present in Indulin (Table 1). After 7 days, a slight shift towards
 277 higher MM was observed (Fig. 3a, 7 d- line higher and righter at than 0 h) together with a
 278 decrease in the proportion of all soluble compounds (Fig. 3b, 7 d-line is the lowest one).
 279 Taken together, these results suggest that the polymer APPL fraction of Indulin was subjected
 280 to two antagonist processes, depolymerization during *P. putida* growing phase followed by
 281 repolymerization that appeared to be a spontaneous phenomenon in the no cell control.

282

283 The analysis of the phenolic monomers by GC-MS (Table 1) indicated that vanillin, vanillic
 284 acid, and dihydroconiferyl alcohol (DHCA) were converted by *P. putida*, with transitory
 285 formation of dihydroferulic acid (DHFA) between 10 and 24 h. In agreement with the initial
 286 molar content of DHCA ($0.3 \mu\text{mol g}^{-1}$), DHFA (max $0.1 \mu\text{mol g}^{-1}$ at 10 h) could be formed
 287 from DHCA in a way analogous to the conversion of coniferyl alcohol into ferulic acid
 288 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, *P. putida* specific increase of acetovanillone at the end of the bacterial 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 whether not metabolized or maybe consumed at the same time that it was formed.

3.3.2.2. DHP

In the case of DHP, slightly increased proportion of higher masses APPL compounds was observed for the control but in contrast to Indulin no shift of the MM distribution took place (Fig. 4a), suggesting that DHP was less inclined to self-polymerization.

This increase in the proportion of higher MM compounds was enhanced by the incubation with *P. putida* after 24 h and was maximum at 7 days among measured samples. As for Indulin, a slight decrease was observed at short incubation time (10 h). Changes in the lower MM portion of the curves (M range 400-900 g.mol⁻¹) took place as soon as 10 h incubation, with the progressive formation of a new peak corresponding to an apparent MM of 520 g.mol⁻¹ and the disappearance of the shoulder corresponding to an apparent MM of 630 g.mol⁻¹ (equivalent to tetramers). Consistently, the analysis of the soluble fraction (Fig. 4b) showed also a decrease with time of tetramers and oligomers of higher MM (chromatogram portion at RT = 14-15.5 min), with subsequent enrichment in dimers (peak at 15.8 min). The broadening of the dimer peak at 7 d (Fig. 4b) indicated that new dimers were formed. DHP oligomers of polymerization degree >3 seem to be converted by *P. putida* into lower MM compounds.

Compared to Indulin, DHP exhibited higher apparent reactivity of its oligomer fractions with a clearer depolymerization effect when incubated with *P. putida*. The concomitant increased of oligomers and higher polymeric fraction in APPL would explain the lack of clear tendency in the evolution of APPL amount studied earlier (3.3.1).

Differences between DHP and Indulin were also observed through the comparison of the non-normalized APPL chromatograms (figure dedicated to *E. coli* in supplementary data and Fig. 5). Indeed, polymer total area ($2.8 < \log M < 4.5$) increased at 7 d for DHP but decreased with Indulin (Fig. 5). The increase in the absorbance of DHP APPL at long time is due to the action of *P. putida*. It may reflect an increased solubility in THF and/or extinction coefficient changes due to phenols oxidation by some secreted bacterial oxidases. Indeed, Munk et al. (2015) suggested modifications of functional groups caused by laccases. Those lignin oxidases necessitate copper as cofactor and a multicopper lignin oxidase (Bugg et al. 2016) was reported with *P. putida* GB-1 (Brouwers et al., 1999; Geszvain et al., 2016).

3.4. Non-specific lignin interaction with non-delignifying bacteria

3.4.1. Effect of *E. coli* on APPL and polymeric soluble fractions

Incubation of the lignin samples with non-delignifying *E. coli* bacteria showed also some 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.

3.4.2. 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 days pellets (Fig. 6). Indeed, the MM distribution and UV spectra were found similar to that of lignin in the

supernatant. Despite the low amount of extract recovered (less than 1.5 mg) led to quantitative variations, 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 part of them 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 in pellets was also observed in *P. putida* (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 important in the case of *E. coli*. This difference is probably explained by the highest specific membrane surface area of *E. coli* due to higher size (Bronk et al., 1992; Mulakhudair et al., 2017) or number of cells (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.

4. Discussion

The present study brought two main results of importance from a methodological point of view. The first one is that some lignin fraction is lost in the bacterial pellets through centrifugation of the culture media. This loss can lead to an apparent decrease in the proportion of high-molar compounds in the supernatant and erroneous subsequent conclusion that depolymerization occurs. The second important result is that the depolymerizing effect of bacterial incubation on lignin can be hidden by lignin self-polymerization as for Kraft lignin

or by recondensation of oxidized lignin products, as observed for DHP. In light of these conclusions, our experimental analyzes and literature results are discussed below.

4.1. 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 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 would be due to a higher concentration of bacterial cells at 7 d for independent experiment of campaign 2 (higher O.D. Table 2) leading possibly to a higher depolymerization activity but also to a slight increase in lignin bacterial pellets losses.

4.2. Reconsideration of previous evidence of lignin bacterial depolymerization and metabolizing

In a previous study, Salvachúa et al. (2015) assessed the ability of different bacteria to depolymerize and/or metabolize lignin from maize alkali-pretreated liquor containing 30% lignin associated to hemicelluloses. This assessment was made based on the HPSEC profile of the whole acetylated culture supernatants recovered from a given mass of substrate incubated during 7 days. As mentioned by the author, the consumption of hemicelluloses and sugars can lead to an apparent increase in lignin content. Among the bacteria tested *P. putida* KT2440 was concluded to convert the phenolic monomers and to convert up to 30% lignin, according

to the Klason lignin determination in the substrate. In the light of our results, a contribution of lignin losses with bacterial pellets to this apparent conversion cannot be ruled out. In the same line, Chai et al.(2014) concluded that the bacteria *Comamonas* sp. B-9 was able to decolorize and degrade Kraft lignin, based on the spectrophotometry analysis of the supernatant after 7 days of incubation. It is possible that lignin selective loss and self-polymerization contributed to the change in UV spectra in addition to lignin conversion by the bacteria. Due to these possible artefacts, experimental strategies based on the use of ^{14}C -labeled lignin substrates and determination of released $^{14}\text{CO}_2$ allows to unambiguously demonstrate lignin metabolizing by bacteria, as applied by Kern and Kirk (1987) to *Xanthomonas* sp. Using this criteria, these authors showed that *Xanthomonas* could degrade lignin compounds only up to a mass of 600-1000 g.mol⁻¹, which corresponds to oligomers ($\leq$ 5 units). Moreover, by analyzing aqueous dioxan lignin soluble fractions recovered from the culture medium after 14 days they observed formation of low MM compounds diagnostic of depolymerization. However, the amount of cell-associated ^{14}C in each case was equal to about half of the evolved $^{14}\text{CO}_2$. It confirmed that some lignin remained associated to the cell. Moreover, the absence of kinetics analysis involving short times hampered the full comprehension of the conversion mechanisms.

A valid strategy for HPSEC analysis of lignins contained in bacterial pellets was developed here; as our results showed the necessity to take it into account when assessing lignin bacterial conversion. This strategy combined several advantages: 1) it is easier to carry out due to the absence of radioactive labelled compounds, 2) it allows complete analysis of all the polymer and oligomer lignin fractions present in the medium, 3) it provides a way to investigate specifically depolymerization mechanisms.

4.3. Interest of a treatment with *P. putida* for lignin valorization

Though specific effects of *P. putida* on Kraft lignin and DHP were observed, its use as depolymerizing agent for bioconversion was found limited notably by simultaneous polymerization phenomenon. The competition between depolymerization and repolymerization is a widespread phenomenon observed during different processes, including chemical processes (Ball et al., 1990; Li et al., 2007a). This phenomenon is due to the reactivity of lignin products released by cleavage of interunit bonds, in particular species carrying carbonium ions formed in acidic medium (Li et al., 2007b). For example, a polymerization of Kraft lignin occurred after 72h of incubation with soil bacteria followed by a depolymerization at 192h (Taylor et al., 2012). Using steam explosion as depolymerizing process, Li et al. (2007b) suggested the use of a scavenger for carbonium ions as phenol, 2-naphthol to avoid this phenomenon. In a white biotechnological approach, some bacterial enzymes, such as dehydrolipoamide dehydrogenase, would also be advantageously used to avoid spontaneous repolymerization (Rahmanpour et al., 2017). Such approaches would be helpful whether for understanding purpose or for future industrial application.

In this paper, we used the substrate fraction that was soluble in the culture medium using pH variations and got rid of the non-soluble highly polymerized fraction by filtration prior to incubation to obtain sterile substrate. In contrast, Tian et al. (2016) incubated the non-water extractable polymerized Kraft lignin fraction for the strain screening of soils and sediments bacteria and showed the production of soluble phenolic compounds. Brown and Chang (2014) reported several papers dealing with the release of water soluble APPL metabolites, from non-water soluble lignins with soil bacteria. This suggests that bacterial treatment with *P. putida* might have greatest interest to solubilize non-soluble recalcitrant fractions of lignin rather than the water soluble fraction.

439

440 In this work *P. putida* exhibited specific activity on both Indulin and DHP. The original
441 kinetic approach proposed was found efficient to better understand involved lignin modifying
442 mechanisms. *P. putida* limited the self-repolymerization of Indulin after 7d incubation and a
443 depolymerization occurred during growing phase (monomers metabolizing and decrease in
444 polymer apparent MM during the first 24 h). Moreover, evolution of phenolics and the
445 transitory formation of DHFA during Indulin incubation is helpful to further investigate *P.*
446 *putida* metabolic pathways. Finally, evidences of DHP oligomers progressive conversion with
447 formation of dimers were obtained.

448

449 **Electronic supplementary material**

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

452

453 **Conflict of Interest**

454 All authors declare that they have no conflict of interest.

455

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Figure captions

Fig.1 Scheme of the experimental strategy settled to analyze lignin fractions

Fig. 2 Indulin APPL amount (% dry weight of the supernatant) for (▲; ◆) *P. putida* and (△; ◇) no cell control of (◆; ◇) campaign 1 and (▲; △) campaign 2, respectively

Fig. 3 HPSEC analysis of Indulin in culture supernatants: (a) Apparent MM distribution of the acetylated APPL (polymer peak normalized with respect to the maximum absorbance and the sample acetylated amount; mixed C type PL-gel column) (b) HPSEC profiles of the acid-soluble AcOEt/CH₂Cl₂ extracts (100 Å PL-gel column). Numbers indicate the oligomerization degree corresponding to (1) monomers of the C₆C₁ and C₆C₃ types, (2) dimers, (3) trimers, (4) tetramers

Fig. 4 HPSEC analysis of DHP in culture supernatants: (a) Apparent MM distribution of the acetylated APPL (polymer peak normalized with respect to the maximum absorbance and the sample acetylated amount; mixed C type PL-gel column) (b) HPSEC profiles of the acid-soluble AcOEt/CH₂Cl₂ extracts (100 Å PL-gel column). Numbers indicate the oligomerization degree corresponding to (1) monomers of the C₆C₁ and C₆C₃ types, (2) dimers, (3) trimers, (4) tetramers, (6) hexamers; the arrow indicates the broadening of the dimers peak

Fig. 5 Apparent MM distribution of the acetylated APPL recovered from (a) Indulin and (b) DHP culture supernatant (non-normalized absorbance; mixed C type PL-gel column).

Fig. 6 MM distribution of the lignin dioxan/water extract of the bacterial pellets recovered from (a) Indulin and (b) DHP.

Figure 1

Figure 2

Figure 4

Figure 5

Figure 6

Table 1. Phenolic monomers determined by GC-MS in the Indulin acid soluble fraction ($\mu\text{g}\cdot\text{g}^{-1}$ freeze-dried culture supernatant). nd: not detected; tr: traces (<4)

	<i>P. putida</i>				No cell			<i>E. coli</i>			
Incubation time	0h	10h	24h	7d	0h	10h	24h	0h	10h	24h	7d
Vanillin	79.8	tr	tr	tr	89.9	93.8	75.3	45.1	74.9	tr	tr
Acetovanillone	22.8	28.5	28.7	45.1	26.1	24.0	21.0	11.5	27.4	21.8	tr
Vanillic acid	55.7	tr	tr	tr	45.2	47.9	47.2	11.5	41.7	5.9	tr
Dihydroconiferyl alcohol (DHCA)	47.4	32.7	tr	tr	55.1	53.9	47.6	40.4	54.8	30.9	tr
Dihydroferulic acid (DHFA)	nd	22.2	7.5	nd	nd	nd	nd	nd	nd	nd	nd

Table 2. Summary of taken samples and their corresponding optical density at 600 nm for the DHP and Indulin (Ind 1) 1st and (Ind 2) 2nd campaigns. Data between parentheses indicate a measurement of optical density without fermentation stop.

<i>P. putida</i> KT2440						<i>E. coli</i>				No cell			
Time	0h	6h	10h	24h	7d	0h	6h	24h	7d	0h	10h	24h	7d
DHP	0.1		0.1	1	0.7	(1.6)		1		0			
Ind 1	0.1		1	1.1	0.8	0.1	1.6	1.4	1.1	0	0	0	
Ind 2	0.1	0.7		1.1	0.9					0			

Declaration of interests

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

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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