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Pretreatment strategies for microbial valorization of bio-oil fractions produced by fast pyrolysis of ash-rich lignocellulosic biomass

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Abstract

This work evaluates a biorefinery approach for microbial valorization of bio-oil fractions produced by fast pyrolysis of ash-rich lignocellulosic biomass. Different methods are presented for the pretreatment of the low-sugar complex bio-oil consisting of organic condensate (OC) and aqueous condensate (AC) to overcome their strong inhibitory effects and unsuitability for common analytical methods. Growth of *Pseudomonas putida* KT2440, which was chosen as a reference system, on untreated bio-oil fractions was only detectable using solid medium with OC as sole carbon source. Utilization of a pretreated OC which was filtered, autoclaved, neutralized and centrifuged enabled growth in liquid medium with significant remaining optical instability. By subjecting the pretreated fractions to solid phase extraction, more stable and less inhibitory bio-oil fractions could be obtained enabling the appliance of common analytical methods. Furthermore, this pretreatment facilitated growth of the applied reference organism *Pseudomonas putida* KT2440. As there is currently no convincing strategy for reliable application of bio-oil as a sole source of carbon in industrial biotechnology, the presented work depicts a first step toward establishing bio-oil as a future sustainable feedstock for a bio-based economy.

KEYWORDS
bioeconomy, bio-oil, lignocellulosic biomass, pretreatment, *Pseudomonas putida* KT2440, pyrolysis

1 | INTRODUCTION

The most abundant nonfood renewable raw material is lignocellulosic biomass, which may present a sustainable alternative platform to petrochemicals (Anwar, Gulfraz, & Irshad, 2014; Iqbal, Kyazze, & Keshavarz, 2013; Menon & Rao, 2012). A significant challenge, however, lies in its conversion because lignocellulose is a plant-based, sturdy and compact biocomposite material consisting of lignin,

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Abbreviations: AC, aqueous condensate; AC_{FANC}, aqueous condensate after filtration, autoclaving, neutralization and centrifugation; AC_{SPE}, aqueous condensate after solid phase extraction; OC, organic condensate; OC_{FANC}, organic condensate after filtration, autoclaving, neutralization and centrifugation; OC_{SPE}, organic condensate after solid phase extraction; SPE, solid phase extraction.

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cellulose and hemicellulose. In the last decades, a number of conversion methods have been developed mainly focusing on the production of second-generation biofuels (Brethauer & Studer, 2015; Dahmen et al., 2016; Maurya, Singla, & Negi, 2015; Seidl & Goulart, 2016). One of such a conversion method is fast pyrolysis—a thermochemical method which converts lignocellulosic biomass in the absence of oxygen mainly into an energy-rich liquid referred to as bio-oil, also referred as to bio-oil (Bridgwater, Meier, & Radlein, 1999).

One example for a fast pyrolysis process with fractional condensation, which addresses pyrolysis of nonwoody biomass such as herbaceous, ash-rich biomass like wheat straw, was shown by Pfitzer et al. (2016) (bioliq® process, Karlsruhe Institute of Technology (KIT), Germany). The applied two-stage condensation allows a controlled formation of two condensates: a viscous condensate rich in organic substances with <20 wt% water (OC) obtained at condensation temperatures between 60 and 90°C and an aqueous condensate (AC) consisting of up to 85 wt% water and water-soluble organic compounds formed at around 30°C (Figure 1; Pfitzer et al., 2016). An excerpt of their composition is shown in Table 1. These condensates are mixed with pyrolysis char in different proportions forming energy-rich and free-flowing bioslurries, which are further used for gasification to generate synthetic fuels (Dahmen, Henrich, Dinjus, & Weirich, 2012). Depending on the applied biomass and the mixing ratio, unexploited side streams can accrue. As a future bioeconomy relies on the efficient and sustainable utilization of renewable resources, it is important that for all products and side streams, which accrue during an overall conversion process of lignocellulosic biomass an adequate valorization is found. Furthermore, it should be taken into account that the use of biomass as a feedstock for energy and fuel production is restricted by its relatively low volumetric energy content, seasonality, and discrete geographic availability (FitzPatrick, Champagne, Cunningham, & Whitney, 2010; Lipinsky, 1981).

An alternative application of lignocellulosic-based bio-oil and its fractions is its use as a carbon source for microbial cultivation (Arnold, Moss, Henkel, & Hausmann, 2017; Islam, Zhisheng, Hassan, Dongdong, & Hongxun, 2015; Jarboe, Wen, Choi, & Brown, 2011). Bio-oil is mainly composed of water, pyrolytic lignin, and a wide variety of organic components such as organic acids, sugars, alcohols, aldehydes, ketones, and phenolic components (Piskorz, Scott, & Radlein, 1988). Especially, pyrolytic sugars and organic acids are of particular interest to be used as carbon sources by microorganisms (Bennett, Helle, & Duff, 2009; Chi et al., 2013; Kim, Um, Bott, & Woo, 2015; Layton, Ajjarapu, Choi, & Jarboe, 2011; Lian et al., 2010; Lian, Garcia-Perez, & Chen, 2013; Lian, Garcia-
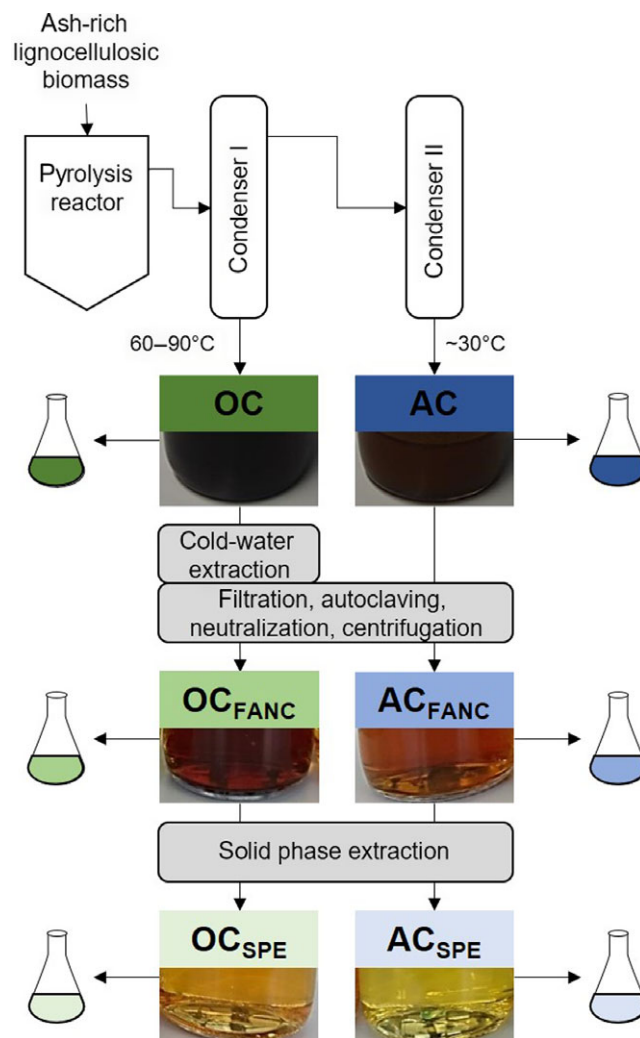

FIGURE 1 Schematic diagram of the different pretreatment steps and its products originating from condensable vapor of fast pyrolysis process with a two-stage condensation. The resulting organic condensate (OC) and aqueous condensate (AC) are furthermore subjected to filtration, autoclaving, neutralization, and centrifugation (FANC). Subsequently, OC_{FANC} and AC_{FANC} are subjected to solid phase extraction (SPE)

Perez, Coates, Wu, & Chen, 2012; Linger, Hobdey, Franden, Fulk, & Beckham, 2016; Prosen, Radlein, Piskorz, Scott, & Legge, 1993). But bio-oil also comprises many unidentified substances, as well as components which are inhibitory to microbial growth such as furans, phenolic compounds, and ketones (Chi et al., 2013; Jarboe et al., 2011; Lian et al., 2010; Prosen et al., 1993). Furthermore, due to the rapid quenching in pyrolysis process, bio-oil is not at equilibrium and changes chemically and physically with time if no measures for stabilization are taken (Diebold, 2000). Especially, the presence of reactive aldehydes and ketones, organic acids, but also the presence of char cause reactions within various bio-oil functionalities and contribute toward the instability of bio-oil.

TABLE 1 Composition of the two bio-oil phases determined by GC-MS and typical ranges extracted from the literature (Islam et al., 2015)

Compound (wt%)	OC	AC	Typical range
Formic acid	n.a.	n.a.	0.3–9.1
Acetic acid	5.004	4.492	0.5–17.0
Propionic acid	1.302	0.404	0.1–2.0
Methanol	n.d.	1.689	0.4–8.2
Ethylene glycol	1.258	0.437	0.7–2.0
Levoglucosan	0.965	n.d.	0.1–30.5
Acetol	4.631	3.484	0.2–7.4
1-Hydroxy-2-butanone	0.844	0.49	0.3–1.3
2-Cyclopenten-1-one	0.308	0.262	0.3–1.5
Furfural	0.265	0.281	1.5–3.0
Phenol	0.384	0.041	0.1–3.8
Cresol (o-,p-,m-)	0.455	0.058	1.0–2.5
Guaiacol	0.469	0.104	2.8–2.8
Syringol	0.556	0.011	0.7–4.8
Isoeugenol	0.524	n.d.	0.1–7.2

Note. AC: aqueous condensate; OC: organic condensate; n.a.: data not available, n.d.: not detectable.

Different strategies have been developed to prevent inhibition including bio-oil fractionation (Pollard, Rover, & Brown, 2012; Westerhof et al., 2011), detoxification (Chi et al., 2013; Li et al., 2013; Lian et al., 2010, 2012 ; Prosen et al., 1993; Vitasari, Meindersma, & Haan, 2012), and improvement of the tolerance of biocatalysts (Chan & Duff, 2010; Liang et al., 2013; Wang et al., 2012; Yu & Zhang, 2003).

Regarding bio-oil fractions produced from wheat straw by fast pyrolysis in the bioliq® plant at Karlsruhe Institute of Technology (KIT, Karlsruhe, Germany), two studies have already been reported investigating their potential as carbon source for microbial cultivation (Dörsam et al., 2016; Lange et al., 2017). Noticeable is that the sugar content of these fractions is very low in the OC fraction or even nonexistent in AC. The major component present in these fractions is acetic acid (Table 1), which can be a potential carbon source for some microorganisms (Berg, Tymoczko, & Stryer, 2002; Gerstmeir et al., 2003; Li et al., 2016). In 2016, Dörsam et al. determined the bio-oil tolerance limits of fungi, as well as investigated the reason for their low tolerance level by assessing 11 substances from bio-oil in different concentrations regarding the production of malic acid. The authors used the organic-rich bio-oil fraction of the so-called organic condensate (OC). For tolerance experiments, cultivations were performed on solid medium. Among the 22 screened fungi, 15 showed tolerance toward OC in minimal medium agar plates while growing on glucose. Cultivations with OC as sole carbon source suggest that only *Aspergillus oryzae* is able to grow on up to 1% of OC; however, quantitative data are not provided. Beside OC also an aqueous bio-oil phase is formed during the two-stage condensation in the KIT fast pyrolysis plant. In 2017, Lange et al. evaluated the suitability of *Corynebacterium glutamicum* to grow on this so-called aqueous condensate (AC). The authors reported that after supplementation of minimal medium with AC, turbidity analyses were no longer possible. Therefore, they established a protocol for pretreatment of AC consisting of four steps: pH adjustment, centrifugation, filtration, and heat treatment, aiming for a fermentable substrate by removing residual oils and solids as well as diminishing volatile substances such as methanol. However, also with the addition of pretreated AC to the medium growth was not detectable. Only supplementation of yeast extract to pretreated AC enabled growth of an engineered *Corynebacterium glutamicum* and the production of 1,2-propanediol.

Even though the bioconversion of bio-oil was investigated in the past, studies are mainly based on cultivations with sugar-rich, hydrolyzed, and detoxified bio-oil fractions added to complex media, which mostly include yeast extract and peptone (Bennett et al., 2009; Chan & Duff, 2010; Lian et al., 2010; Luque et al., 2014; Prosen et al., 1993; Sukhbaatar et al., 2014; Wang et al., 2012; Yang et al., 2011; Yu & Zhang, 2003). Furthermore, their focus generally lies on product formation and less on the biomass generation. As of today, the effect of reactivity of bio-oil during cultivation by quantitative means such as suitable investigation of supplemented culture medium over time as negative control has not been reported in detail.

In this article, the challenges of using sugar-poor bio-oil fractions as sole carbon sources for bacterial cultivation are described. Different pretreatment methods are presented to address problems such as reactive and inhibitory substances in bio-oil fractions produced by fast pyrolysis of ash-rich lignocellulosic biomass. Using the example of *Pseudomonas putida* KT2440, which is generally regarded as an organic solvent tolerant bacterial strain capable of degrading aromatic hydrocarbons and using a wide range of carbon sources (Cruden, Wolfram, Rogers, & Gibson, 1992; Inoue & Horikoshi, 1989; Ramos, Duque, Huertas, & Haïdour, 1995; Weber, Ooijkaas, Schemen, Hartmans, & Bont, 1993), a potential biorefinery route for microbial valorization of bio-oil is shown.

2 | MATERIALS AND METHODS

2.1 | Chemicals

All chemicals used in this study were either purchased from Carl Roth GmbH (Karlsruhe, Germany) or Sigma-Aldrich (Munich, Germany) if not stated otherwise.

2.2 | Bio-oil fractions and their pretreatments

The two bio-oil fractions organic condensate (OC) and aqueous condensate (AC) used in this study were prepared from wheat straw by fast pyrolysis in the bioliq® plant at KIT in Karlsruhe, Germany (Pfitzer et al., 2016). The substances within the two condensates were analyzed by Thünen Institute Hamburg by GC-MS. As a result of unspecific pyrolytic reactions, OC and AC contain hundreds of different molecules, many of which have not been identified. This is most prominent in OC, where pyrolytic lignin and unidentified components constitute a major part of dry weight. An excerpt is shown in Table 1 comprising the most abundant substances in either OC or AC (above 0.1 wt%) and compared with typical ranges in the literature (Islam et al., 2015). OC is low in sugar (in sum 1.63 wt% and thereof 0.965 wt% levoglucosan), and AC even contains no monomeric sugars. Solids in OC are usually <10 wt% and the water content is adjusted between 12 and 18 wt% by varying the condensation temperature (Pfitzer et al., 2016). AC consists of up to 85 wt% water including water-soluble organic compounds and practically no solids. Its pH is ~2.5. To maintain integrity to favor stability, OC samples were stored at 4°C and AC at −21°C until further use. To obtain stable and fermentable bio-oil fractions, various pretreatment methods were applied. One of the first purification steps of OC was an extraction. The aim was to extract water-soluble substances such as organic acids and sugars from bio-oil and get an aqueous extract rich in these substances. For this, OC was slowly added to ice-cooled water at the ratio of 1:6 while mixing with a stirrer. A filtration with a bottle top filter with PES membrane (pore size: 0.22 μm; membrane Ø: 75 mm; volume: 500 ml) obtained from Carl Roth GmbH (Karlsruhe, Germany) was performed for both OC extract and AC to remove pyrolytic lignin (water-insoluble fraction) and other solid substances. This modified approach is described by Scholze and Meier (2001), who used this procedure for the characterization of pyrolytic lignin. The resulting filtrates were autoclaved, neutralized, and centrifuged at 4,700 rpm for 15 min, whereby the middle phase represents the final aqueous fractions OC_{FANC}, respectively, AC_{FANC} (Figure 1), which were sterile-filtered at the end. Neutral and aromatic compounds within OC_{FANC} and AC_{FANC} were removed using solid phase extraction (SPE) cartridges (Strata-X 33 μm Polymeric Reversed Phase, 200 mg stationary phase, specific surface of 800 m²/g, Phenomenex Ltd., Aschaffenburg, Germany). Extraction of each sample was performed with a fresh column, by employing 20 ml of OC_{FANC} and AC_{FANC}, which were passed through the column in a single run according to the manufacturers' recommendations. The neutrals and aromatics bind at the column and the sterile-filtered flow-through was used as a more purified fraction called OC_{SPE}, respectively, AC_{SPE} (Figure 1). All pretreated bio-oil fractions were stored at 4°C.

2.3 | Microorganism, culture medium, and conditions

The used bacteria strain *P. putida* KT2440 (DSM 6125) was obtained from the DSMZ strain collection (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany). *P. putida* was grown at 30°C and 120 rpm. For the preculture LB medium (5 g/L yeast extract, 10 g/L tryptone, 10 g/L NaCl; pH 7.0) was used. After ~18 hr of incubation, the cells were centrifuged (4,700 rpm, 4 min, 20°C) and the cell pellet was washed with 1:1 (v/v) 9 g/L NaCl solution, centrifuged again and resuspended in fresh 9 g/L NaCl solution. The main medium was inoculated with OD_{600} 0.1. The main medium was adapted to Wilm's KP_i medium (Wilms et al., 2001): 6.58 g/L K_2HPO_4, 1.64 g/L KH_2PO_4, 5 g/L $(NH_4)_2SO_4$, 0.5 g/L NH_4Cl, 2 g/L Na_2SO_4, 0.5 g/L $MgSO_4·7H_2O$, 0.05 g/L Thiamin HCl, 3 ml/L trace element solution, pH 7.4; trace element solution: 0.18 g/L $ZnSO_4·7H_2O$, 0.16 g/L $CuSO_4·5H_2O$, 0.1 g/L $MnSO_4·H_2O$, 13.9 g/L $FeCl_3·6H_2O$, 10.05 g/L EDTA Titriplex III, 0.18 g/L $CoCl_2·6H_2O$, 0.662 g/L $CaCl_2·2H_2O$. As carbon source either OC/AC, OC_{FANC}/AC_{FANC} or OC_{SPE}/AC_{SPE} was used. Acetate was used as reference carbon source. For OC or AC Wilm's KP_i agar plates, 1 g OC or 1 ml AC, respectively, was added to 100 ml Wilm's KP_i medium containing 1.5% agar. To enable the comparison of different cultivations with OC_{FANC}/OC_{SPE}, acetate concentrations were adjusted using 200 g/L sodium acetate solution, which was required due to initial dilution by cold-water extraction.

2.4 | Sampling, sample processing, and analytics

All photometric measurements were taken using a spectrophotometer (WPA CO8000 Cell density meter, Biochrom Ltd., Cambridge, UK) by measuring the optical density at $\lambda = 600$ nm (OD_{600}).

The remaining sample was centrifuged ($12,500 \times g$, 15 min) to obtain cell-free supernatant for acetate detection. The concentration of acetate was determined from the supernatant samples using acetate assay kit (Enztech yellow line, R-Biopharm AG, Darmstadt, Germany) according to the manufacturers' instructions.

2.5 | Data analysis

All experimental data were obtained at least as duplicates from at least two individual biological experimental setups

and shown as average values $\pm$ standard deviation. Data were fitted using a logistic equation with four parameters in a scientific data analysis and graphing software (Sigma Plot 13.0, Systat, San Jose, USA). Obtained data and the resulting fits were used to calculate the specific growth rate μ (hr). Substrate-to-biomass yields $Y_{X/S}$ (g/g) were calculated using the contained amount of acetate.

3 | RESULTS AND DISCUSSION

3.1 | Pretreatment of bio-oil fractions

The major component of the two bio-oil fractions produced in the bioliq® process is acetic acid (Table 1), which can be a potential carbon source for some microorganisms (Berg et al., 2002; Gerstmeir et al., 2003; Li et al., 2016; Lian et al., 2012). However, solids and pyrolytic lignin in OC, and residual oil in AC, as well as reactive and inhibitory

substances make their application as microbial substrates very challenging. By the first pretreatment step, water-soluble substances such as organic acids and sugars within the viscous OC were extracted by cold-water extraction to be separated from residual bio-oil, pyrolytic lignin, and solids. The resulting fractions were sterile-filtered and referred to as OC_{FANC}, respectively, AC_{FANC} (Figure 1). The acetate concentration of OC_{FANC} and AC_{FANC} was about 6 g/L, respectively, 43 g/L. The relatively low acetate content in OC_{FANC} originates from its cold-water extraction resulting in a dilution of water-soluble substances.

A more purified fraction was achieved by solid phase extraction. Here, neutral and aromatic compounds within OC_{FANC} and AC_{FANC} were reduced. These fractions are called OC_{SPE}, respectively, AC_{SPE} (Figure 1) and exhibit acetate concentrations of about 5 g/L and 42 g/L. There is a small decrease in acetate content when treated with solid phase extraction.

To see the effect of pretreatments on the reactivity of bio-oil fractions, incubations of cell-free medium supplemented with bio-oil fractions were performed and the drift of optical density was measured during incubation and depicted in Figure 2. Measuring the OD_{600}, no utilizable results could be obtained due to strong drift and variability between individual measurements as well as control measurements. Incubation of cell-free medium with OC_{FANC} or AC_{FANC} (Figure 2a) revealed significant increase in OD_{600} reaching values up to 2 when incubated on 5 g/L acetate in
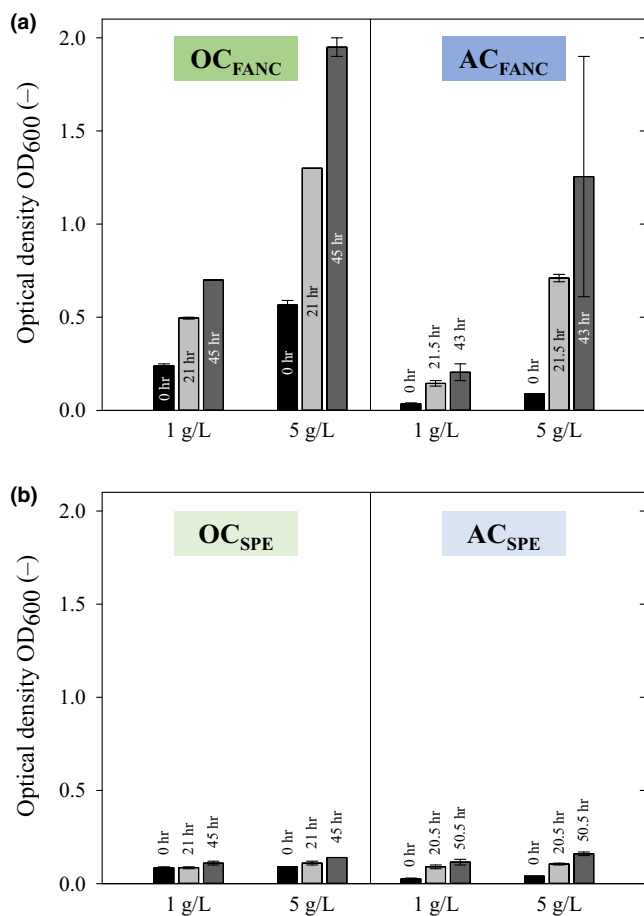

FIGURE 2 Time-course of optical density OD_{600} in cell-free medium supplemented with bio-oil fractions. (a) organic condensate and aqueous condensate after filtration, autoclaving, neutralization, and centrifugation (OC_{FANC} (left), AC_{FANC} (right)), (b) organic condensate and aqueous condensate after solid phase extraction (OC_{SPE} (left), AC_{SPE} (right))

FIGURE 3 Photograph of organic condensate (OC). High viscosity of OC and the formation of elastic-type particles interferes the measurement of OD_{600}, dry weight, and cell counting with microscopy

(a)

OC

(b)

AC
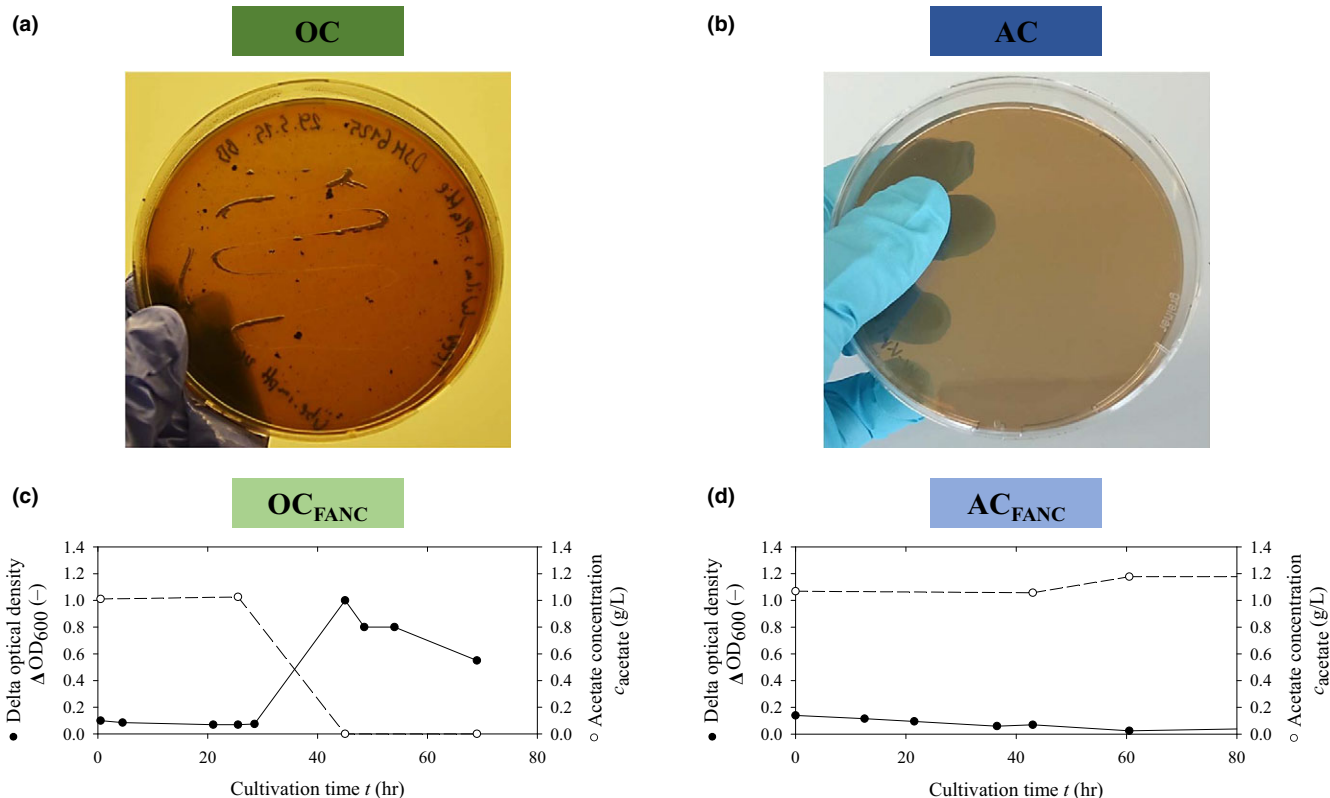
(c)

OC$_{FANC}$

(d)

AC$_{FANC}$

FIGURE 4 Colony formation and growth of *P. putida* KT2440 on organic condensate (OC) and aqueous condensate (AC). Growth on agar plates: Untreated OC (a) and AC (b) in Wilm's KP$_i$ agar plates. Growth in liquid medium in shake flasks: OC and AC subjected to filtration, autoclaving, neutralization, and centrifugation (FANC) and adjusted to 1 g/L acetate: OC$_{FANC}$ (c) and AC$_{FANC}$ (d) cultivations

OC$_{FANC}$ for 45 hr. Cell-free medium with OC$_{SPE}$ or AC$_{SPE}$ (Figure 2b) only changed slightly. Noticeable is the comparably high-standard deviation of high concentrations of AC$_{FANC}$ and long incubation period (Figure 2a).

3.2 | Cultivations with OC or AC as sole carbon source

Experiments with untreated bio-oil fractions in culture suspensions severely hinder analytical procedure and the determination of the optical density of the culture. Especially, the high viscosity of OC and the formation of elastic-type particles interferes with the measurement of the OD$_{600}$ (Figure 3) as well as the dry weight. Also, cell counting with microscopy is difficult, because it is not feasible to differentiate between small OC particles and *P. putida* cells (Figure 3). But the major problem is the reactivity of bio-oil fractions. During cultivation, the culture suspension containing bio-oil fractions is getting darker and particles precipitate. This reactivity leads to strong variations within an OD$_{600}$ measurement as well as dry weight determination making it impossible to verify biomass formation. Growth of *P. putida* on untreated OC was only detected using Wilm's KP$_i$ agar plates containing OC as carbon source (Figure 4a). The bacteria seem to form biofilm to overcome

the inhibitors within OC making growth possible (Khiyami, Pometto, & Brown, 2005). Growth of *P. putida* is neither detectable on liquid nor on solid medium containing AC as sole carbon source (Figure 4b). This may be explained by the increased bioavailability of growth inhibitory water-soluble compounds in AC such as furans and methanol (Table 2).

3.3 | Cultivation with OC$_{FANC}$ or AC$_{FANC}$ as sole carbon source

By mixing OC with water and removing the pyrolytic lignin, first enables the detection of growth in liquid medium (Figure 4c). This could be explained by the fact that the mixing with water caused a dilution, which leads to a lower concentration of inhibitors within OC$_{FANC}$, but also to a lower concentration of microbial accessible components such as acetate (only 5 g/L). This low concentration of acetate was adjusted to a final acetate concentration of 20 g/L acetate in OC$_{FANC}$, which again resulted in a slight dilution of the inhibitors.

The time-course of the OD$_{600}$ of cultivations with 1 g/L acetate content in OC$_{FANC}$ or AC$_{FANC}$ as sole carbon source is depicted in Figure 4c,d. Shown values for OD$_{600}$ are mean OD$_{600}$ values of the medium inoculated with

TABLE 2 Summary of process parameters of different cultivations using untreated and pretreated bio-oil fractions

	$c_{acetate}$ (g/L)	growth	μ_{max} (hr^{-1})	$Y_{X/S}$ (g/g)	$^{max}OD_{600}$ (–)
Acetate	1	✓	0.6–0.9	0.33	0.9
	3	✓			2.7
	5	✓			4.2
OC		only on agar plate	n.a.	n.a.	n.a.
AC		✗	n.a.	n.a.	n.a.
OC$_{FANC}$	1	✓	0.1–0.2	0.32	1.0
	3	✗	n.a.	n.a.	n.a.
	5	✗	n.a.	n.a.	n.a.
AC$_{FANC}$	1	✗	n.a.	n.a.	n.a.
	3	✗	n.a.	n.a.	n.a.
	5	✗	n.a.	n.a.	n.a.
OC$_{SPE}$	1	✓	0.1–0.3	0.26	0.9
	3	✓			1.8
	5	✗	n.a.	n.a.	n.a.
AC$_{SPE}$	1	✓	0.1–0.2	0.46	1.3
	3	✗	n.a.	n.a.	n.a.
	5	✗	n.a.	n.a.	n.a.

Note. Cultivation on acetate is given as reference. n.a.: not available.

microorganisms subtracted by values obtained from medium without inoculation (Supporting information Figure S1). *P. putida* was able to grow on a final concentration of 1 g/L acetate content in OC$_{FANC}$ up to a ΔOD_{600} of 1 in 45 hr (Figure 4c). At the maximal ΔOD_{600}, acetate was completely depleted. For higher concentrations of about 5 g/L acetate in OC$_{FANC}$ growth was not detectable. OD$_{600}$ values of the medium with 5 g/L acetate content in OC$_{FANC}$ inoculated with microorganisms and medium without inoculation increased equally (Supporting information Figure S1) and the acetate concentration remained unchanged during cultivation (data not shown).

As AC was not mixed with water to obtain AC$_{FANC}$, there is no dilution and the concentration of inhibitors within AC$_{FANC}$ is much higher compared to AC$_{FANC}$. In addition, no adjustment to a higher acetate concentration has to be performed, because the acetate concentration in AC$_{FANC}$ is ~42 g/L. Therefore, it is obvious that growth on AC$_{FANC}$ poses a significantly higher challenge than on OC$_{FANC}$. Cultivations with 1 g/L acetate content in AC$_{FANC}$ as sole carbon source showed no increase of ΔOD_{600} values and no decrease in acetate concentration over time (Figure 4d). Also, OD$_{600}$ values of medium containing 5 g/L acetate in AC$_{FANC}$ inoculated with microorganisms and medium without inoculation increased equally (Supporting information Figure S1), while the acetate concentration remained unchanged during cultivation (data not shown).
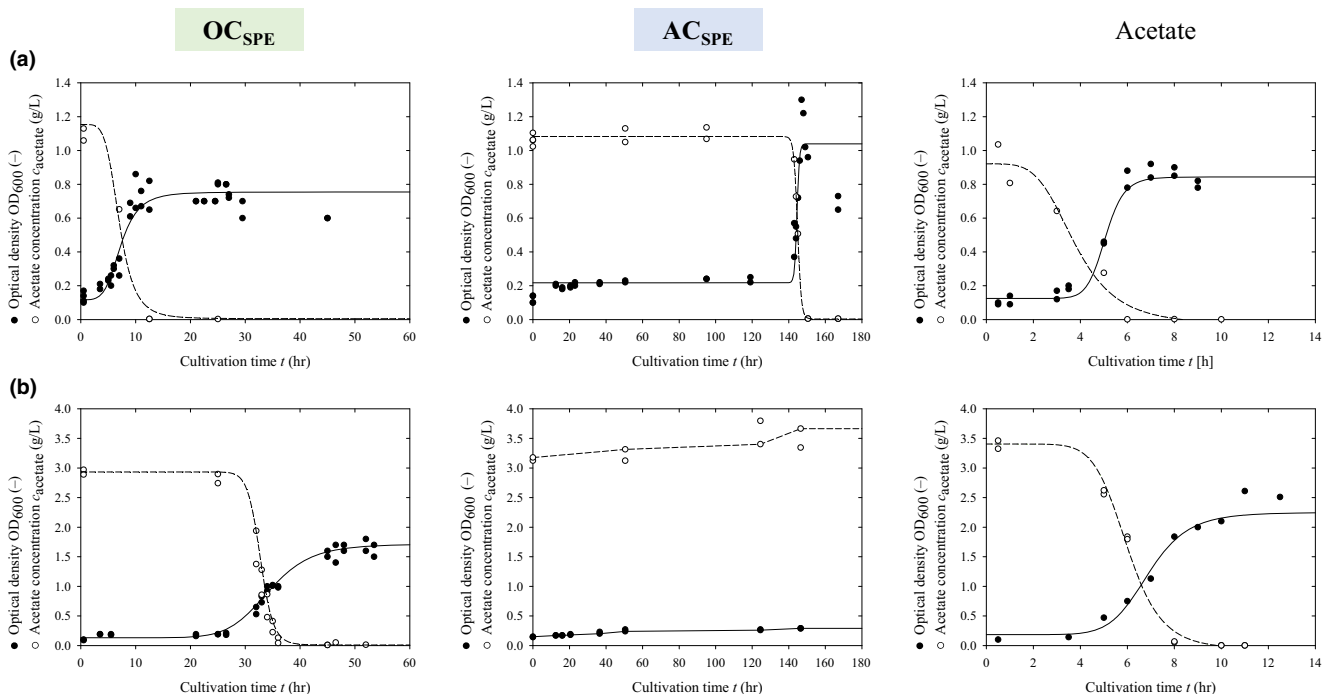

FIGURE 5 Cultivations of *P. putida* KT2440 on solid phase extracted bio-oil fractions. Cultivations were performed with OC$_{SPE}$ or AC$_{SPE}$ containing medium equivalent to concentrations of 1 g/L acetate (a) and 3 g/L acetate (b) as well as reference cultures using pure acetate, respectively

As shown in Figure 2a, there is still a strong reactivity of the pretreated bio-oil fractions OC_{FANC} and AC_{FANC} during incubations, which results in a significant drift in optical density and hinders common analytical methods.

3.4 | Cultivation with OC_{SPE} or AC_{SPE} as sole carbon source

SPE facilitates more stable bio-oil fractions (Figure 2b). Using bio-oil fractions after SPE as sole carbon source, *P. putida* was able to grow on higher concentrations of OC (up to 3 g/L acetate content in OC_{SPE}), as well as the first time on AC (1 g/L acetate content in AC_{SPE}) (Figure 5). This is attributed to the employed SPE, which retains aromatic and hydrophobic compounds that have been reported to interfere with microbial growth (Chi et al., 2013; Jarboe et al., 2011; Lian et al., 2010; Prosen et al., 1993).

Considering the different concentrations of $OC_{SPE,}$ it is obvious that the higher the concentration of OC_{SPE} the longer the duration of the lag-phase is (Figure 5). Cultivations with higher acetate concentrations (5 g/L) in OC_{SPE} showed no increase in OD_{600} and no decrease in acetate during cultivation after incubation time of up to 7 days (data not shown). Using AC_{SPE} as sole carbon source, *P. putida* grew on 1 g/L acetate content in AC_{SPE} up to a maximal OD_{600} of 1.3 within 147 hr (Figure 5a). While a total of 1 g/L acetate in AC_{SPE} cultivations was depleted completely, acetate concentrations of AC_{SPE} cultivations with higher AC_{SPE} concentrations remain constant during cultivation supposing that *P. putida* is not able to grow on higher AC_{SPE} concentrations (Figure 5b). Reference cultivations on different acetate concentrations without any addition of bio-oil fractions were performed for comparison (Figure 5). Growth of *P. putida* was detected up to an acetate concentration of 5 g/L.

3.5 | Comparison of cultivation parameters

As a first assessment of potential suitability of pretreated bio-oil fractions as carbon sources for biotechnology, process parameters of different cultivations using untreated and pretreated bio-oil fractions are compared (Table 2). While maximum growth rates on pretreated bio-oil fractions are in a similar range (0.1–0.3 hr^{-1}), in contrast, the maximal growth rate of cultivations on pure acetate is significantly higher (0.6–0.9 hr^{-1}). This may mainly be attributed to the fact that many inhibitory substances (Table 1) are not or only partially removed by the applied pretreatment methods. Substrate-to-biomass conversion yields $Y_{X|S}$ are in a similar range for cultivation on pure acetate, OC_{FANC} and OC_{SPE} of ~0.3 g/g. Cultivation on AC_{SPE}, however, shows significantly higher yields of 0.46 g/g. This can mainly be attributed to the fact that AC_{SPE} as the aqueous fraction of pyrolysis contains additional potential

carbon sources such as propionate, formate, and acetol compared to OC_{SPE}.

Using bio-oil as an alternative carbon source for microbial cultivation is very challenging due to its instability and highly complex composition along with strong adverse effects on microbial growth and existing analytical procedures. In this study, it was shown that SPE may be a suitable tool to obtain stable bio-oil fractions with less inhibitory substances which facilitates growth of *P. putida* KT2440 on low-sugar bio-oil fractions.

This provides a first step toward establishing bio-oil as a feedstock for microbial valorization. Future work should address direct bioconversion and investigate appropriate fractionating condensation designs and conditions.

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REFERENCES

Anwar, Z., Gulfraz, M., & Irshad, M. (2014). Agro-industrial lignocellulosic biomass a key to unlock the future bio-energy: A brief review. *Journal of Radiation Research and Applied Sciences*, *7*, 163–173. https://doi.org/10.1016/j.jrras.2014.02.003

Arnold, S., Moss, K., Henkel, M., & Hausmann, R. (2017). Biotechnological perspectives of pyrolysis oil for a bio-based economy. *Trends in Biotechnology*, *35*, 925–936. https://doi.org/10.1016/j.tibtech.2017.06.003

Bennett, N. M., Helle, S. S., & Duff, S. J. B. (2009). Extraction and hydrolysis of levoglucosan from pyrolysis oil. *Bioresource Technology*, *100*, 6059–6063. https://doi.org/10.1016/j.biortech.2009.06.067

Berg, J. M., Tymoczko, J. L., & Stryer, L. (2002). The glyoxylate cycle enables plants and bacteria to grow on acetate. *Biochemistry*, Section, 17.4.

Brethauer, S., & Studer, M. H. (2015). Biochemical conversion processes of lignocellulosic biomass to fuels and chemicals - a review. *Chimia International Journal for Chemistry*, *69*, 572–581. https://doi.org/10.2533/chimia.2015.572

Bridgwater, A. V., Meier, D., & Radlein, D. (1999). An overview of fast pyrolysis of biomass. *Organic Geochemistry*, *30*, 1479–1493. https://doi.org/10.1016/S0146-6380(99)00120-5

Chan, J. K. S., & Duff, S. J. B. (2010). Methods for mitigation of bio-oil extract toxicity. *Bioresource Technology*, *101*, 3755–3759. https://doi.org/10.1016/j.biortech.2009.12.054

Chi, Z., Rover, M., Jun, E., Deaton, M., Johnston, P., Brown, R. C., … Jarboe, L. R. (2013). Overliming detoxification of pyrolytic sugar syrup for direct fermentation of levoglucosan to ethanol. *Bioresource Technology*, *150*, 220–227. https://doi.org/10.1016/j.biortech.2013.09.138

Cruden, D. L., Wolfram, J. H., Rogers, R. D., & Gibson, D. T. (1992). Physiological properties of a *Pseudomonas* strain which grows with p-xylene in a two-phase (organic-aqueous) medium. *Applied and Environmental Microbiology*, *58*, 2723–2729.

Dahmen, N., Abeln, J., Eberhard, M., Kolb, T., Leibold, H., Sauer, J., … Zimmerlin, B. (2016). The bioliq process for producing synthetic transportation fuels. *Wiley Interdisciplinary Reviews: Energy and Environment*, *6*, e236.

Dahmen, N., Henrich, E., Dinjus, E., & Weirich, F. (2012). The bioliq® bioslurry gasification process for the production of biosynfuels, organic chemicals, and energy. *Energy, Sustainability and Society*, *2*, 1–44. https://doi.org/10.1186/2192-0567-2-3

Diebold, J. (2000) *A review of the chemical and physical mechanisms of the storage stability of fast pyrolysis bio-oils*. Report No. NREL/SR-570-27613. Golden, CO: National Renewable Energy Laboratory.

Dörsam, S., Kirchhoff, J., Bigalke, M., Dahmen, N., Syldatk, C., & Ochsenreither, K. (2016). Evaluation of pyrolysis oil as carbon source for fungal fermentation. *Frontiers in Microbiology*, *7*, 2059. https://doi.org/10.3389/fmicb.2016.02059

FitzPatrick, M., Champagne, P., Cunningham, M. F., & Whitney, R. A. (2010). A biorefinery processing perspective: Treatment of lignocellulosic materials for the production of value-added products. *Bioresource Technology*, *101*, 8915–8922. https://doi.org/10.1016/j.biortech.2010.06.125

Gerstmeir, R., Wendisch, V. F., Schnicke, S., Ruan, H., Farwick, M., Reinscheid, D. J., & Eikmanns, B. J. (2003). Acetate metabolism and its regulation in *Corynebacterium glutamicum*. *Journal of Biotechnology*, *104*, 99–122. https://doi.org/10.1016/S0168-1656(03)00167-6

Inoue, A., & Horikoshi, K. (1989). A *Pseudomonas* thrives in high concentrations of toluene. *Nature*, *338*, 264. https://doi.org/10.1038/338264a0

Iqbal, H. M. N., Kyazze, G., & Keshavarz, T. (2013). Advances in the valorization of lignocellulosic materials by biotechnology: An overview. *BioResources*, *8*, 3157–3176. https://doi.org/10.15376/biores.8.2.3157-3176

Islam, Z. U., Zhisheng, Y., Hassan, E. B., Dongdong, C., & Hongxun, Z. (2015). Microbial conversion of pyrolytic products to biofuels: A novel and sustainable approach toward second-generation biofuels. *Journal of Industrial Microbiology & Biotechnology*, *42*, 1557–1579. https://doi.org/10.1007/s10295-015-1687-5

Jarboe, L. R., Wen, Z., Choi, D., & Brown, R. C. (2011). Hybrid thermochemical processing: Fermentation of pyrolysis-derived bio-oil. *Applied Microbiology and Biotechnology*, *91*, 1519–1523. https://doi.org/10.1007/s00253-011-3495-9

Khiyami, M. A., Pometto, A. L., & Brown, R. C. (2005). Detoxification of corn stover and corn starch pyrolysis liquors by *Pseudomonas putida* and *Streptomyces setonii* suspended cells and plastic compost support biofilms. *Journal of Agricultural and Food Chemistry*, *53*, 2978–2987.

Kim, E. M., Um, Y., Bott, M., & Woo, H. M. (2015). Engineering of *Corynebacterium glutamicum* for growth and succinate production from levoglucosan, a pyrolytic sugar substrate. *FEMS Microbiology Letters*, *362*, pii: fnv161.

Lange, J., Müller, F., Bernecker, K., Dahmen, N., Takors, R., & Blombach, B. (2017). Valorization of pyrolysis water: A biorefinery side stream, for 1,2-propanediol production with engineered *Corynebacterium glutamicum*. *Biotechnology for Biofuels*, *10*, 277. https://doi.org/10.1186/s13068-017-0969-8

Layton, D. S., Ajjarapu, A., Choi, D. W., & Jarboe, L. R. (2011). Engineering ethanologenic *Escherichia coli* for levoglucosan utilization. *Bioresource Technology*, *102*, 8318–8322. https://doi.org/10.1016/j.biortech.2011.06.011

Li, Y., Huang, B., Wu, H., Li, Z., Ye, Q., & Zhang, Y. H. P. (2016). Production of succinate from acetate by metabolically engineered *Escherichia coli*. *ACS Synthethic Biology*, *5*, 1299–1307.

Li, Y., Shao, J., Wang, X., Yang, H., Chen, Y., Deng, Y., … Chen, H. (2013). Upgrading of bio-oil: Removal of the fermentation inhibitor (furfural) from the model compounds of bio-oil using pyrolytic char. *Energy & Fuels*, *27*, 5975–5981. https://doi.org/10.1021/ef401375q

Lian, J., Chen, S., Zhou, S., Wang, Z., O'Fallon, J., Li, C. Z., & Garcia-Perez, M. (2010). Separation, hydrolysis and fermentation of pyrolytic sugars to produce ethanol and lipids. *Bioresource Technology*, *101*, 9688–9699. https://doi.org/10.1016/j.biortech.2010.07.071

Lian, J., Garcia-Perez, M., & Chen, S. (2013). Fermentation of levoglucosan with oleaginous yeasts for lipid production. *Bioresource Technology*, *133*, 183–189. https://doi.org/10.1016/j.biortech.2013.01.031

Lian, J., Garcia-Perez, M., Coates, R., Wu, H., & Chen, S. (2012). Yeast fermentation of carboxylic acids obtained from pyrolytic aqueous phases for lipid production. *Bioresource Technology*, *118*, 177–186. https://doi.org/10.1016/j.biortech.2012.05.010

Liang, Y., Zhao, X., Chi, Z., Rover, M., Johnston, P., Brown, R., … Wen, Z. (2013). Utilization of acetic acid-rich pyrolytic bio-oil by microalga *chlamydomonas reinhardtii*: Reducing bio-oil toxicity and enhancing algal toxicity tolerance. *Bioresource Technology*, *133*, 500–506. https://doi.org/10.1016/j.biortech.2013.01.134

Linger, J. G., Hobdey, S. E., Franden, M. A., Fulk, E. M., & Beckham, G. T. (2016). Conversion of levoglucosan and cellobiosan by *Pseudomonas putida* KT2440. *Metabolic Engineering Communications*, *3*, 24–29. https://doi.org/10.1016/j.meteno.2016.01.005

Lipinsky, E. S. (1981). Chemicals from biomass: Petrochemical substitution options. *Science*, *212*, 1465. https://doi.org/10.1126/science.212.4502.1465

Luque, L., Westerhof, R., Van Rossum, G., Oudenhoven, S., Kersten, S., Berruti, F., & Rehmann, L. (2014). Pyrolysis based bio-refinery for the production of bioethanol from demineralized ligno-cellulosic biomass. *Bioresource Technology*, *161*, 20–28. https://doi.org/10.1016/j.biortech.2014.03.009

Maurya, D. P., Singla, A., & Negi, S. (2015). An overview of key pretreatment processes for biological conversion of lignocellulosic biomass to bioethanol. *Biotech*, *5*, 597–609.

Menon, V., & Rao, M. (2012). Trends in bioconversion of lignocellulose: Biofuels, platform chemicals & biorefinery concept. *Progress in Energy and Combustion Science*, *38*, 522–550.

Pfitzer, C., Dahmen, N., Tröger, N., Weirich, F., Sauer, J., Günther, A., & Müller-Hagedorn, M. (2016). Fast pyrolysis of wheat straw in the bioliq pilot plant. *Energy & Fuels*, *30*, 8047–8054. https://doi.org/10.1021/acs.energyfuels.6b01412

Piskorz, J., Scott, D. S., & Radlein, D. (1988). Composition of oils obtained by fast pyrolysis of different woods. *Pyrolysis Oils from Biomass (ACS Symposium Series)*, Vol. *376* (pp. 167–178). Washington, DC: American Chemical Society.

Pollard, A. S., Rover, M. R., & Brown, R. C. (2012). Characterization of bio-oil recovered as stage fractions with unique chemical and physical properties. *Journal of Analytical and Applied Pyrolysis*, *93*, 129–138. https://doi.org/10.1016/j.jaap.2011.10.007

Prosen, E. M., Radlein, D., Piskorz, J., Scott, D. S., & Legge, R. L. (1993). Microbial utilization of levoglucosan in wood pyrolysate as a carbon and energy source. *Biotechnology and Bioengineering*, *42*, 538–541. https://doi.org/10.1002/bit.260420419

Ramos, J. L., Duque, E., Huertas, M. J., & Haïdour, A. (1995). Isolation and expansion of the catabolic potential of a *Pseudomonas putida* strain able to grow in the presence of high concentrations of aromatic hydrocarbons. *Journal of Bacteriology*, *177*, 3911–3916. https://doi.org/10.1128/jb.177.14.3911-3916.1995

Scholze, B., & Meier, D. (2001). Characterization of the water-insoluble fraction from pyrolysis oil (pyrolytic lignin). Part I. PY–GC/MS, FTIR, and functional groups. *Journal of Analytical and Applied Pyrolysis*, *60*, 41–54. https://doi.org/10.1016/S0165-2370(00)00110-8

Seidl, P. R., & Goulart, A. K. (2016). Pretreatment processes for lignocellulosic biomass conversion to biofuels and bioproducts. *Current Opinion in Green and Sustainable Chemistry*, *2*, 48–53. https://doi.org/10.1016/j.cogsc.2016.09.003

Sukhbaatar, B., Li, Q., Wan, C., Yu, F., Hassan, E. B., & Steele, P. (2014). Inhibitors removal from bio-oil aqueous fraction for increased ethanol production. *Bioresource Technology*, *161*, 379–384. https://doi.org/10.1016/j.biortech.2014.03.051

Vitasari, C. R., Meindersma, G. W., & de Haan, A. B. (2012). Renewable glycolaldehyde isolation from pyrolysis oil-derived aqueous solution by reactive extraction with primary amines. *Separation and Purification Technology*, *95*, 103–108. https://doi.org/10.1016/j.seppur.2012.04.027

Wang, H., Livingston, D., Srinivasan, R., Li, Q., Steele, P., & Yu, F. (2012). Detoxification and fermentation of pyrolytic sugar for ethanol production. *Applied Biochemistry and Biotechnology*, *168*, 1568–1583. https://doi.org/10.1007/s12010-012-9879-1

Weber, F. J., Ooijkaas, L. P., Schemen, R. M., Hartmans, S., & de Bont, J. A. (1993). Adaptation of *Pseudomonas putida* S12 to high concentrations of styrene and other organic solvents. *Applied and Environmental Microbiology*, *59*, 3502–3504.

Westerhof, R. J. M., Brilman, D. W. F., Garcia-Perez, M., Wang, Z., Oudenhoven, S. R. G., van Swaaij, W. P. M., & Kersten, S. R. A. (2011). Fractional condensation of biomass pyrolysis vapors. *Energy & Fuels*, *25*, 1817–1829. https://doi.org/10.1021/ef2000322

Wilms, B., Hauck, A., Reuss, M., Syldatk, C., Mattes, R., Siemann, M., & Altenbuchner, J. (2001). High-cell-density fermentation for production of L-N-carbamoylase using an expression system based on the *Escherichia coli* rhaBAD promoter. *Biotechnology and Bioengineering*, *73*, 95–103. https://doi.org/10.1002/bit.1041

Yang, Z., Kang, H., He, X., Zhuang, G., Zhang, H., & Bai, Z. (2011) Microbial degradation and utilization of lignocellulosic biomass pyrolysis oil. In *Proceedings of the 2nd International Conference on Mechanic Automation and Control Engineering*: July 15–17 1996; Hohhot. IEEE; 2011: 7457–7459.

Yu, Z., & Zhang, H. (2003). Ethanol fermentation of acid-hydrolyzed cellulosic pyrolysate with *Saccharomyces cerevisiae*. *Bioresource Technology*, *90*, 95–100. https://doi.org/10.1016/S0960-8524(03)00093-2

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