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, Gulfrat, & 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,
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, Ajajarapu, Choi, & Jarboe, 2011; Lian et al., 2010; Lian, Garcia-Perez, & Chen, 2013; Lian, Garcia-Perez, Wu, & Chen, 2012; Linger, Hobday, 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.
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; Li et al., 2013; 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, & Haidour, 1995; Weber, Ooijkaas, Schemen, Hartmans, & Bont, 1993), a potential biorefinery route for microbial valorization of bio-oil is shown.

### 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.

### 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-butane| 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.
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 unpecific 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 aqueous condensate (AC) used in this study were prepared 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 OD600 0.1. The main medium was adapted to Wilm's KP medium (Wilms et al., 2001): 6.58 g/L K2HPO4, 1.64 g/L KH2PO4, 5 g/L (NH4)2SO4, 0.5 g/L NH4Cl, 2 g/L Na2SO4, 0.5 g/L MgSO4·7H2O, 0.05 g/L Thiamin HCl, 3 mL/L trace element solution, pH 7.4; trace element solution: 0.18 g/L ZnSO4·7H2O, 0.16 g/L CuSO4·5H2O, 0.1 g/L MnSO4·H2O, 13.9 g/L FeCl3·6H2O, 10.05 g/L EDTA Titriplex III, 0.18 g/L CoCl2·6 H2O, 0.662 g/L CaCl2·2H2O. 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 plates, 1 g OC or 1 ml AC, respectively, was added to 100 ml Wilm's KP 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 (OD600).

The remaining sample was centrifuged (12,500xg, 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 ± 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 \( \mu \) (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](image2.png)

**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](image3.png)

**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.
OCFANC for 45 hr. Cell-free medium with OCSPE or ACSPE (Figure 2b) only changed slightly. Noticeable is the comparably high standard deviation of high concentrations of ACFANC 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 OD600 (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 OD600 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 KP1 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 OCFANC or ACFANC 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 OCFANC, 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 OCFANC, which again resulted in a slight dilution of the inhibitors.

The time-course of the OD600 of cultivations with 1 g/L acetate content in OCFANC or ACFANC as sole carbon source is depicted in Figure 4c,d. Shown values for OD600 are mean OD600 values of the medium inoculated with

**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 KP1 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: OCFANC (c) and ACFANC (d) cultivations.
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 OCFANC up to a ΔOD₆₀₀ of 1 in 45 hr (Figure 4c). At the maximal ΔOD₆₀₀, acetate was completely depleted. For higher concentrations of about 5 g/L acetate in OCFANC growth was not detectable. OD₆₀₀ values of the medium with 5 g/L acetate content in OCFANC 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 ACFANC, there is no dilution and the concentration of inhibitors within ACFANC is much higher compared to ACFANC. In addition, no adjustment to a higher acetate concentration has to be performed, because the acetate concentration in ACFANC is ~42 g/L. Therefore, it is obvious that growth on ACFANC poses a significantly higher challenge than on OCFANC. Cultivations with 1 g/L acetate content in ACFANC as sole carbon source showed no increase of ΔOD₆₀₀ values and no decrease in acetate concentration over time (Figure 4d). Also, OD₆₀₀ values of medium containing 5 g/L acetate in ACFANC inoculated with microorganisms and medium without inoculation increased equally (Supporting information Figure S1), while the acetate concentration remained unchanged during cultivation (data not shown).

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

|acetate (g/L) growth | µ_max (hr⁻¹) | Y_XS (g/g) | maxOD₆₀₀ (–) |
|---------------------|-------------|------------|-------------|
| 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. |             |            |             |
| OCFANC 1 ✓          | 0.1–0.2     | 0.32       | 1.0         |
| 3 × n.a. n.a. n.a.  |             |            |             |
| 5 × n.a. n.a. n.a.  |             |            |             |
| ACFANC 1 × 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.  |             |            |             |
| ACSPE 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.

### 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 OD600 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 OD600 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 YXS 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 acetal 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.

ACKNOWLEDGEMENTS

This work was supported by a grant (Az.:33–7533-10-5/75A) from the Ministry of Science, Research and the Arts of Baden-Württemberg, Germany as part of Bioeconomy Research Program Baden-Württemberg.

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