Evaluation of small organic acids present in fast pyrolysis bio-oil from lignocellulose as feedstocks for bacterial bioconversion

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Abstract
Small organic acids derived from fast pyrolysis of lignocellulosic biomass represent a significant proportion of microbially accessible carbon in bio-oil. However, using bio-oil for microbial cultivation is a highly challenging task due to its strong adverse effects on microbial growth as well as its complex composition. In this study, the main small organic acids present in bio-oil as acetate, formate and propionate were evaluated with respect to their suitability as feedstocks for bacterial growth. For this purpose, the growth behavior of four biotechnological production hosts—Escherichia coli, Pseudomonas putida, Bacillus subtilis, and Corynebacterium glutamicum—was quantified and compared. The bacteria were cultivated on single acids and mixtures of acids in different concentrations and evaluated using common biotechnological efficiency parameters. In addition, cultivation experiments on pretreated fast pyrolysis-derived bio-oil fractions were performed with respect to the suitability of the bacterial strains to tolerate inhibitory substances. Results suggest that both P. putida and C. glutamicum metabolize acetate—the major small organic acid generated during fast pyrolysis of lignocellulosic biomass—as sole carbon source over a wide concentration range, are able to grow on mixtures of small organic acids present in bio-oil and can, to a limited extent, tolerate the highly toxic inhibitory substances within bio-oil. This work provides an important step in search of suitable bacterial strains for bioconversion of lignocellulosic-based feedstocks and thus contributes to establishing efficient bioprocesses within a future bioeconomy.

KEYWORDS
Bacillus, bacteria, bioeconomy, biomass, bio-oil, Corynebacterium, E. coli, fast pyrolysis, lignocellulose, Pseudomonas

Abbreviations: AC_{SPF}, aqueous condensate after solid phase extraction; OC_{SPF}, organic condensate after solid phase extraction.
INTRODUCTION

Today, most value-added products of industrial biotechnology are produced by bioconversion of glucose as carbon source (Wendisch et al., 2016). However, within the frame of a biobased economy, the search for alternative sustainable carbon sources is driven by a competing application of sugars in human and animal nutrition.

As an abundant, renewable and no direct food and feed competing resource, lignocellulosic biomass is being considered as a potential carbon source for biotechnological production of sustainable value-added compounds. Lignocellulose is the major structural component of plants and is primarily composed of cellulose, hemicellulose, and lignin, which are strongly connected with each other. Therefore, the conversion of lignocellulosic biomass into value-added compounds requires a prior effective degradation of this complex structure. For this, a different separation and degradation methods such as mechanical, chemical, biological, physicochemical, thermochemical, and/or biochemical methods (e.g., reviewed in Anwar, Gulfraz, & Irshad, 2014; Barakat, Vries, & Rouau, 2013; Kumar & Sharma, 2017) are normally used. Fast pyrolysis, which is a thermochemical degradation method, converts lignocellulosic biomass into liquid bio-oil. This complex mixture is principally composed of water and many organic components including pyrolytic sugars, small organic acids, phenolic compounds, alcohols, furans, aldehydes, and ketones (Arnold, Moss, Henkel, & Hausmann, 2017; Mohan, Pittman, & Steele, 2006; Piskorz, Scott, & Radlein, 1988).

Of special interest for biotechnological processes are fast pyrolysis-derived sugars and small organic acids, as they constitute a significant proportion of microbially accessible carbon in bio-oil. To date, the main focus of biotechnological application of bio-oil has been microbial utilization of pyrolytic sugars as carbon source (Chi et al., 2013; Kim, Um, Bott, & Woo, 2015; Layton, Ajjarapu, Choi, & Jarboe, 2011; Lian et al., 2010; Lian, Garcia-Perez, & Chen, 2013; Linger, Hobday, Franden, Fulk, & Beckham, 2016; Prosen, Radlein, Piskorz, Scott, & Legge, 1993; Wang et al., 2012). Less is known about bioconversion of small organic acids derived from fast pyrolysis of lignocellulosic biomass (Dang et al., 2014; Lian, Garcia-Perez, Coates, Wu, & Chen, 2012; Liang et al., 2013), especially regarding their bacterial valorization.

Acetic acid, primarily generated during deacetylation of hemicellulose, is with about 10 wt% the most common organic acid in bio-oil (Table 1) and has gained attention as a low-cost alternative carbon source for microbial cultivation with no direct competition with food supplies. The ability of using C2 compounds as carbon sources and converting them to anaplerotic compounds is found in some plants and microorganisms. Kornberg and Krebs (1957) identified two enzymes, isocitrate lyase (ICL; gene: aceA) and malate synthase (MS; gene: aceB), which, together with reactions of the tricarboxylic acid (TCA) cycle, enable Escherichia coli to grow on acetate as sole carbon source. This modified TCA cycle is named glyoxylate cycle. While glucose is taken up in bacteria by the phosphotransferase system resulting in intracellular glucose-6-phosphate, which enters the glycolytic pathway and TCA cycle to generate NADH + H+, FADH2, GTP and biosynthetic precursors, acetate is directly transported into the cell, where it is activated to acetyl coenzyme A (acetyl-CoA) by either the ACK-PTA (acetate kinase, encoded by ack and phosphotransacetylase, encoded by pta) or acetyl-CoA synthase (ACS, encoded by acs) pathway, both being ATP-dependent reactions (Figure 1). Different studies showed that ICL and MS are active and their genes aceA and aceB are highly upregulated when acetate is present.

| Biomass raw material | Small organic acids (wt%) | Acetic acid | Formic acid | Propionic acid |
|----------------------|---------------------------|------------|-------------|---------------|
| Beech wood (Demirbas, 2007) | 12.6 | 0.72 | 0.49 |
| Spruce wood (Demirbas, 2007) | 12.2 | 0.92 | 0.57 |
| Olive husk (Demirbas, 2007) | 10.2 | 0.87 | 0.59 |
| Hazelnut shell (Demirbas, 2007) | 11.4 | 0.99 | 0.64 |
| Pine sawdust (Bertero, Puente, & Sedran, 2012) | 5.58 | 0.95 | 0.30 |
| Mesquite sawdust (Bertero et al., 2012) | 4.33 | 1.07 | 0.19 |
| Wheat shell (Bertero et al., 2012) | 6.18 | 1.86 | 0.97 |
| Rice husk (Guo, Wang, Wang, Guo, & Luo, 2011) | 13.49 | n.a. | 1.65 |
| Pine (Guo et al., 2010) | 17.1 | n.a. | 0.88 |
| Average | 10.34 | 1.05 | 0.70 |

TABLE 1 Proportion of small organic acids in bio-oil obtained from different biomass raw materials
as the sole carbon source for microbial growth (Collins & Kornberg, 1960; Gerstmeir, Cramer, Dangel, Schaffer, & Eikmanns, 2004; Hayashi et al., 2002; Kornberg, 1966; Kornberg & Madsen, 1958; Reinscheid, Eikmanns, & Sahm, 1994a, 1994b; Wendisch, Graaf, Sahm, & Eikmanns, 2000; Wendisch et al., 1997). However, many bacteria lack the key glyoxylate cycle enzyme ICL. It has been reported that these bacteria utilize alternate pathways for acetate assimilation (Alber, Spanheimer, Ebenau-Jehle, & Fuchs, 2006; Ensign, 2006; Ivanovsky, Krasilnikova, & Berg, 1997; Khomyakova, Bukmez, Thomas, Erb, & Berg, 2011). Besides acetic acid, other small organic acids found in bio-oil serve as additional carbon sources for microbial growth.

Formic acid, one of the simplest organic compounds, is typically present in bio-oil with about 1 wt% (Table 1). In the past, bioconversion of formic acid was mainly investigated in methylotrophs and lithoautotrophs (Li et al., 2012; Lidstrom & Stirling, 1990; Schauer & Ferry, 1980; Ensing, 2006; Ivanovsky, Krasilnikova, & Berg, 1997; Khomyakova, Bukmez, Thomas, Erb, & Berg, 2011). Besides acetic acid, other small organic acids found in bio-oil serve as additional carbon sources for microbial growth.

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acid found in bio-oil with about 0.7 wt% (Table 1). Based on various studies, it is assumed that in most bacteria, yeast, and filamentous fungi, the 2-methylcitrate cycle is the main pathway used to oxidize propionate to pyruvate (Brock, Maerker, Schutz, Volker, & Buckel, 2002; Claes, Pühler, & Kalinowski, 2002; Horswill & Escalante-Semerena, 1999; London, Allen, Gabel, & DeRose, 1999; Miyakoshi, Uchiyama, Someya, Satoh, & Tabuchi, 1987; Pronk, Linden-Beuman, Verduyn, Scheffers, & Dijken, 1994; Tabuchi, Serizawa, & Uchiyama, 1974; Textor et al., 1997). As for acetate, propionate is first activated to propionyl-CoA by either ACS (encoded by acs) and propionyl-CoA synthase (encoded by prpE) or a sequential reaction of phosphorylation of propionate catalyzed by ACK and PTA (Figure 1). The first step of the 2-methylcitrate cycle involves the condensation of propionyl-CoA with oxaloacetate to 2-methylcitrate by methylcitrate synthase (encoded by prpC) followed by an isomerization reaction that converts 2-methylcitrate to 2-methylisocitrate via a 2-methyl-cis-aconitate intermediate. This step is accomplished by a methylcitrate dehydratase (encoded by prpD) followed by subsequent hydration catalyzed by an aconitase (encoded by acn). 2-Methylisocitrate is finally cleaved by 2-methylisocitrate lyase (encoded by prpB) or by ISL into pyruvate and succinate.

For the utilization of fast pyrolysis-derived small organic acids as feedstocks for bacterial growth, it is important that the applied bacterial strain is able to use acetate, formate, and propionate as carbon and/or energy source, can handle different concentrations thereof, as well as tolerates inhibitory substances that are present in bio-oils. This work aims to assess four major bacterial production systems of industrial biotechnology—E. coli, Pseudomonas putida, Bacillus subtilis, and Corynebacterium glutamicum—for their suitability of utilizing fast pyrolysis-derived small organic acids as carbon sources.

2 MATERIALS AND METHODS

2.1 Chemicals

All chemicals used in the current study were either purchased from Carl Roth GmbH (Karlsruhe, Germany) or Sigma-Aldrich (Munich, Germany) unless stated otherwise.

2.2 Strains

The four applied industrial bacterial strains E. coli K12 DSM498, P. putida KT2440 DSM6125, B. subtilis DSM10^7, and C. glutamicum DSM20300 were obtained from the DSMZ strain collection (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) and were conserved as glycerol stocks at −80°C.

2.3 Culture media and conditions

All precultures were performed in a 250 ml baffled shake flask containing 25 ml LB medium (5 g/L yeast extract, 10 g/L tryptone, 10 g/L NaCl; pH 7.0) and 100 µl of the respective glycerol stock. The shake flasks were incubated at 30°C, respectively 37°C for E. coli, and 120 rpm in a shaking incubator (Newbrunswick™/Innova® 44, Eppendorf AG, Hamburg, Germany) for 18 hr. To obtain cell solutions free of medium components, the precultures were centrifuged for 4 min at 4,550 g and 20°C (Heraeus X3R, Thermo Fisher Scientific GmbH, Braunschweig, Germany) and the cell pellets were washed with 1 vol. sterile 9 g/L NaCl solution, centrifuged again and resuspended in fresh sterile 9 g/L NaCl solution. The cell-NaCl solutions were then diluted at 0.1 OD_{600} in 50 ml of the respective main culture medium in 500 ml baffled shake flasks and incubated in a shaking incubator at 120 rpm and 30°C (37°C for E. coli). Each strain was cultivated in its suitable culture medium.

Escherichia coli was cultivated in a minimal medium (Henkel et al., 2015) consisting of the following components: 5 g/L NH4Cl, 14.6 g/L K2HPO4, 3.6 g/L Na2HPO4 · 2H2O, 5.6 g/L Na2SO4, 0.54 g/L MgCl2 · 6H2O complexed with 1 g/L sodium citrate · 2H2O, 0.26 g/L CaCl2 · 2H2O, 0.01 g/L thiamin, 3 mL/L trace element solution (trace element solution: 25.8 g/L sodium citrate · 2H2O, 0.18 g/L MnSO4 · 7H2O, 0.1 g/L MnSO4 · H2O, 8.35 g/L FeCl3 · 6H2O, 0.16 g/L CuSO4 · 5H2O, 0.18 g/L CoCl2 · 6H2O, 0.01 g/L NiCl2 · 6H2O, 0.016 g/L NaMoO4 · 2H2O, 0.001 g/L Na2SeO3 · 5H2O). The pH value was adjusted to 7.0.

The employed main culture medium for cultivation of *P. putida* was adapted Wilm's KP1 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 NaCl, 3 mL/L trace element solution (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 NaCl, 10.05 g/L EDTA Titrplex III, 0.18 g/L CoCl2 · 6H2O, 0.062 g/L CaCl2 · 2H2O). The pH value of the buffer solution was adjusted to 7.4.

*Bacillus subtilis* was cultivated in a modified mineral salt medium based on culture medium of Cooper, Macdonald, Duff, and Kosaric (1981) (Willenbacher et al., 2014): 0.1 M NH4Cl, 0.03 M KH2PO4, 0.04 M Na2HPO4 · 2H2O, 8.0 × 10⁻⁴ M MgSO4 · 7H2O, 1 mL/L trace element solution (4.0 × 10⁻³ M Na2 EDTA · 2H2O, 7.0 × 10⁻³ M CaCl2, 4.0 × 10⁻³ M FeSO4 · 7H2O, 1.0 × 10⁻³ M MnSO4 · H2O). The pH value of the buffer solution was adjusted to 7.0.

Modified CGXII minimal medium (Eikmanns, Metzger, Reinscheid, Kircher, & Sahm, 1991; Keilhauer, Eggeling, & Sahm, 1993; Lange et al., 2017) was used for cultivation of *C. glutamicum* containing the following components: 5 g/L (NH4)2SO4, 5 g/L urea, 21 g/L 3-(N-morpholino)
propanesulphonic acid, 1 g/L K$_2$HPO$_4$, 1 g/L KH$_2$PO$_4$, 0.25 g/L MgSO$_4$ · 7H$_2$O, 10 mg/L CaCl$_2$ · 2H$_2$O, 10 mg/L MnSO$_4$ · H$_2$O, 16.4 g/L FeSO$_4$ · 7H$_2$O, 1 mg/L ZnSO$_4$ · 7H$_2$O, 0.2 mg/L CuSO$_4$ · 5H$_2$O, 0.02 mg/L NiCl$_2$ · 6H$_2$O, 0.2 mg/L biotin. The pH value was adjusted to 7.2.

2.4 Carbon sources

The effect of small organic acids on bacterial growth was investigated by adding them aseptically to the respective culture medium. The following stock solutions of carbon sources were prepared and sterilized by autoclaving (glucose), respectively, by sterile filtration: 500 g/L glucose stock solution, 200 g/L acetate stock solution, 200 g/L propionate stock solution, and 200 g/L formate stock solution. Cultivations on glucose were performed with a final glucose concentration of 10 g/L. Up to seven concentrations of acetate (1, 5, 10, 15, 20, 25, and 30 g/L) were investigated. Propionate and formate were added to the culture medium at final concentrations of 1, 5, and 10 g/L. Cultivations with mixtures of organic acids were performed with 10 g/L acetate supplemented with either 1 g/L formate or 1 g/L propionate or with 1 g/L formate and 1 g/L propionate.

Cultivation experiments with lignocellulosic degradation products as sole carbon sources were accomplished by using pretreated bio-oil fractions: organic condensate after solid phase extraction (OCSPE) and aqueous condensate after solid phase extraction (AC SPE) as described previously (Arnold, Moss, Dahmen, Henkel, & Hausmann, 2018). Briefly, bio-oil fractions were added to the medium and adjusted to final concentrations of 1, 3, and 5 g/L acetate.

2.5 Analytical methods

During cultivation, samples of 1 ml culture were taken to monitor cell growth and consumption of carbon source. The optical density at a = 600 nm (OD$_{600}$) was determined using a spectrophotometer (UV-3100 PC; VWR GmbH, Darmstadt, Germany). After measuring the OD$_{600}$, the samples were centrifuged for 15 min at 15,500 g and 4°C (Centrifuge 5430 R; Eppendorf AG) to obtain the cell-free supernatant for glucose and acetate determination. Consumption of glucose and acetate as carbon sources was measured from the supernatant samples using enzymatic assay kits (Enztech yellow line; R-Biopharm AG, Darmstadt, Germany) following the manufacturer’s instructions. The photometric measurements were performed using a spectrophotometer (WPA CO8000 Cell density meter; Biochrom Ltd., Cambridge, UK).

A correlation between OD$_{600}$ and biomass concentration $c_{BM}$ (g/L) ($c_{BM}$ (g/L) = OD$_{600}$ (g/L) / (a (g/L))) was determined for each bacterial strain by measuring the cell dry mass in shake flask cultivations of the respective culture medium containing 10 g/L glucose. The correlation factor a is 3.3 g/L for E. coli, 2.6 g/L for P. putida, 4.2 g/L for B. subtilis, and 3.7 g/L for C. glutamicum.

2.6 Data analysis

Experimental data were obtained as duplicates from at least two individual biological experiments, resulting in at least four individual measurements for all datasets. Data were fitted applying a logistic four-parameter equation in a scientific data analysis and graphing software (Sigma Plot 13.0; Systat, San Jose, CA, USA). Obtained data as well as the resulting fits were used to calculate the specific growth rate $\mu$ (per hour) and maximum substrate-to-biomass yields $Y_{ac,max}$ (g/g).

3 RESULTS

3.1 Characterization of growth on acetate

For comparison of growth behavior on acetate to growth on glucose as a sole source of carbon, growth experiments with culture media containing 10 g/L glucose or 10 g/L acetate, respectively, were performed exemplarily and depicted in Figure 2. As expected, addition of 10 g/L acetate promoted growth to a lower cell density, resulted in lower specific growth rates, as well as longer lag-phases than the same concentration of glucose. While E. coli achieved a maximal biomass concentration of 4.84 g/L after 18.5 hr on glucose, the maximal biomass yield on 10 g/L acetate was reached after 53 hr and is about 10 times less (0.52 g/L; Figure 2a). The acetate concentration decreased slightly during cultivation and at a concentration of about 8 g/L left the growth stagnated until the cultivation was finally stopped after 80 hr. On glucose as sole carbon source, the biomass production of P. putida reached a maximum of 6.92 g/L after 16 hr cultivation and a maximal growth rate of 0.77 per hour (Figure 2b). The biomass yield of P. putida on acetate is approximately half of that achieved on glucose while simultaneously displaying longer lag times. The growth of C. glutamicum on 10 g/L glucose or 10 g/L acetate, respectively, is shown in Figure 2d. The addition of 10 g/L acetate to the culture medium of C. glutamicum led to a maximal growth rate of 0.55 per hour and generated biomass of 3.24 g/L within 44 hr, whereas cultivations on glucose reached maximal growth rates of 0.65 per hour and biomass yields of 5.1 g/L. Bacillus subtilis yielded a biomass of 2.2 g/L and a specific growth rate of 0.7 per hour on glucose (Figure 2c). After the addition of acetate, neither an increase in biomass nor a decrease in acetate was observed.

The effect of acetate on bacterial growth was investigated by using up to seven different acetate concentrations (1, 5, 10, 15, 20, 25, and 30 g/L; Table 2). In the presence of acetate as sole carbon source in the culture medium, all
applied bacterial strains were able to grow, except *B. subtilis*. Cultivation experiments with *E. coli* on different acetate concentrations showed that the concentration of acetate is not completely depleted but rather decreases around 12% during cultivation. The overall maximal biomass concentration of 0.52 g/L was achieved in cultivation with 10 g/L acetate. The growth behavior of *E. coli* on different acetate concentrations indicates that *E. coli* is inhibited by acetate. The biomass production of *P. putida* increased with increasing acetate concentrations from 0.35 g/L for 1 g/L acetate to 6.54 g/L for 15 g/L acetate in the culture medium. For all four concentrations (1, 5, 10, and 15 g/L), acetate was completely depleted during cultivation. For higher acetate concentration, growth was not detectable. In contrast, *Corynebacterium glutamicum* was able to grow on all seven acetate concentrations by complete depletion of acetate. The biomass concentration increased from 0.27 to 10 g/L. In the presence of 30 g/L acetate, *C. glutamicum* was not able to grow in each cultivation experiment. While *C. glutamicum* showed a long stationary phase for all acetate concentrations in which the number of cells remained the same (Figure 2d right), the cells of *P. putida* started decreasing immediately after reaching the maximal biomass concentration resulting in a peak in the cell growth curve (Figure 2b right).

3.2 | Characterization of growth on further pyrolytic small organic acids

Further growth experiments were performed by using three different concentrations (1, 5, and 10 g/L) of other small organic acids such as formate and propionate, which can be present in bio-oil.

Cultivations on formate revealed that all applied bacterial strains are unable to grow on formate as sole carbon source. There was no increase in the biomass concentration.

Growth experiments with propionate as sole carbon source showed that only *P. putida* is able to grow on all three applied propionate concentrations. The biomass concentration increased from 0.58 to 4.62 g/L with increased propionate concentrations. *Escherichia coli* showed growth on 1 g/L propionate; however, only at higher concentrations of propionate, biomass growth could be measured. During the entire cultivation, a maximal biomass yield (1.12 g/L) was achieved on 5 g/L propionate in the culture medium. With higher propionate concentrations of 10 g/L, *E. coli* reached a maximal biomass concentration of 0.61 g/L, which afterward decreased until the cultivation was finally stopped after 200 hr. *Corynebacterium glutamicum* was only able to grow on 1 g/L propionate reaching a maximal biomass concentration of 10.4 g/L.
| Strain: | E. coli K12 DSM498 | P. putida KT2440 DSM6125 | B. subtilis DSM10^T | C. glutamicum DSM20300 |
|--------|-------------------|-------------------|-------------------|-------------------|
| Acetate (A) | | | | |
| 1 | ✓ | n.a. | n.a. | n.a. | ✓ | 0.35 | 0.60 | 0.31 | ✓ | 0 | 0 | 0 | ✓ | 0.27 | 0.32 | 0.26 |
| 5 | ✓ | 0.33 | 0.40 | 0.17 | ✓ | 1.62 | 0.77 | 0.28 | ✓ | 0 | 0 | 0 | ✓ | 1.89 | 0.61 | 0.37 |
| 10 | ✓ | 0.52 | 0.33 | 0.37 | ✓ | 3.85 | 0.68 | 0.31 | ✓ | 0 | 0 | 0 | ✓ | 3.24 | 0.55 | 0.32 |
| 15 | ✓ | 0.48 | 0.15 | 0.39 | ✓ | 6.54 | 0.46 | 0.44 | ✓ | 0 | 0 | 0 | ✓ | 6.22 | 0.48 | 0.39 |
| 20 | ✓ | 0.30 | 0.08 | 0.16 | ✓ | 0 | 0 | 0 | ✓ | 0 | 0 | 0 | ✓ | 7.84 | 0.39 | 0.37 |
| 25 | n.d. | n.a. | n.a. | n.a. | n.d. | n.a. | n.a. | n.a. | n.d. | n.a. | n.a. | n.a. | ✓ | 9.91 | 0.22 | 0.36 |
| 30 | n.d. | n.a. | n.a. | n.a. | n.d. | n.a. | n.a. | n.a. | n.d. | n.a. | n.a. | n.a. | ✓ | 10.0 | 0.16 | 0.33 |
| Formate (F) | | | | |
| 1 | × | 0 | 0 | n.d. | × | 0 | 0 | n.d. | × | 0 | 0 | n.d. | × | 0 | 0 | n.d. |
| 5 | × | 0 | 0 | n.d. | × | 0 | 0 | n.d. | × | 0 | 0 | n.d. | × | 0 | 0 | n.d. |
| Propionate (P) | | | | |
| 1 | ✓ | n.a. | n.a. | n.d. | ✓ | 0.58 | 0.64 | n.d. | × | 0 | 0 | n.d. | ✓ | 0.24 | 0.15 | n.d. |
| 5 | ✓ | 1.12 | 0.12 | n.d. | ✓ | 2.12 | 0.54 | n.d. | × | 0 | 0 | n.d. | × | 0 | 0 | n.d. |
| 10 | ✓ | 0.58 | 0.07 | n.d. | ✓ | 4.62 | 0.39 | n.d. | × | 0 | 0 | n.d. | ✓ | 0 | 0 | n.d. |
| AP | 10 + 1 | ✓ | 0.28 | 0.17 | 0.13 | ✓ | 2.69 | 0.36 | 0.24 | × | 0 | 0 | 0 | ✓ | 2.70 | 0.23 | 0.27 |
| AF | 10 + 1 | ✓ | 0.42 | 0.34 | 0.27 | ✓ | 3.85 | 0.55 | 0.32 | × | 0 | 0 | 0 | ✓ | 3.51 | 0.34 | 0.35 |
| AFP | 10 + 1 + 1 | ✓ | 0.32 | 0.21 | 0.36 | ✓ | 3.85 | 0.59 | 0.30 | × | 0 | 0 | 0 | ✓ | 3.51 | 0.39 | 0.26 |
concentration of 0.24 g/L, whereas *B. subtilis* did not grow on any of the applied propionate concentrations.

### 3.3 | Characterization of growth on mixtures of small organic acids

The effect of mixtures of small organic acids on the growth of the applied bacterial strains was investigated by adding different combinations of small organic acids to the culture medium. According to typical proportions of small organic acids in bio-oil (Table 1), acetate was used as the major carbon source with a concentration of 10 g/L in the culture medium, whereas formate and propionate were applied as supplementary carbon sources with a respective concentration of 1 g/L. Figure 3 shows the cell growth of the four bacterial strains on three different mixtures of small organic acids: (a) AF = 10 g/L acetate + 1 g/L formate, (b) AP = 10 g/L acetate + 1 g/L propionate, and (c) AFP = 10 g/L acetate + 1 g/L formate + 1 g/L propionate. As for cultivations on 10 g/L acetate as sole carbon source, the growth behavior on mixtures of small organic acids is mostly similar. While *E. coli*, *P. putida*, and *C. glutamicum* are able to grow on all three mixtures, *B. subtilis* shows no growth on any small organic acid mixtures (Table 2). Similar to cultivations with acetate, *P. putida* and *C. glutamicum* completely depleted acetate during the cultivations, whereas the acetate concentration only decreased by around 22% during cultivation of *E. coli* on small organic mixtures.

The results also confirmed that formate has an inhibitory effect on bacterial growth. While maximal biomass concentrations of 0.52 g/L for *E. coli*, 3.85 g/L for *P. putida*, and 3.24 g/L for *C. glutamicum* on 10 g/L acetate were achieved, maximal biomass production on AF was about 30% less. Interestingly, when cultivations were performed on AFP mixtures, formate has only an adverse effect on *E. coli*.

Cultivations of *P. putida* on AP and AFP reached the same values for the process parameters as on 10 g/L acetate. *Corynebacterium glutamicum* achieved slightly higher

![Figure 3](image-url)
growth parameters on AP and AFP than on 10 g/L acetate. The lag-phase of both bacterial strains is briefer on small organic mixtures compared to 10 g/L acetate (Figures 2 and 3).

### 3.4 Characterization of growth on pretreated bio-oil fractions

In order to evaluate the suitability of the bacterial strains for their growth on bio-oil and tolerance toward inhibitory substances within this complex mixture, cultivation experiments on pretreated bio-oil fractions were performed. Figure 4 shows the maximal biomass concentrations of the four bacterial strains achieved by cultivation on the two pretreated bio-oil fractions OC$_{SPE}$ and AC$_{SPE}$ applied in three different concentrations. The concentrations of the fractions were adjusted based on acetate as described previously (Arnold et al., 2018). According to the other growth experiments, no growth was detectable for _B. subtilis_ (Figure 4c). Although _E. coli_ was able to grow on OC$_{SPE}$ with 1 g/L acetate with complete depletion of acetate, the maximal biomass concentration of 0.27 g/L was only reached after 216 hr (Figure 4a). Both _P. putida_ and _C. glutamicum_ showed growth on all adjusted concentrations of OC$_{SPE}$ with a simultaneous decrease in acetate until it was completely depleted (Figure 4b,d). The comparison of the two strains revealed that _C. glutamicum_ achieves higher biomass yields than _P. putida_. However, _P. putida_ requires less cultivation time for reaching maximal biomass concentration compared to _C. glutamicum_, e.g., cultivations on OC$_{SPE}$ with 5 g/L acetate resulted in a maximal biomass production of 1.62 g/L for _C. glutamicum_ after 169 hr and 0.85 g/L for _P. putida_ within only 70 hr. Additionally, _P. putida_ is the only bacterial strain investigated that is able to grow on the pretreated aqueous bio-oil fraction AC$_{SPE}$ (Figure 4b). Cultivations of _P. putida_ on AC$_{SPE}$ with 1 g/L acetate resulted in a maximal biomass production of 0.5 g/L, which is even higher than the biomass yield on OC$_{SPE}$ with 1 g/L acetate amounting to a maximum of 0.33 g/L. However, the cultivation time for achieving maximal biomass concentration on AC$_{SPE}$ is 15 times longer than on OC$_{SPE}$ containing 1 g/L acetate.

**FIGURE 4** Cultivation of (a) _Escherichia coli_ K12, (b) _Pseudomonas putida_ KT2440, (c) _Bacillus subtilis_ DSM 10$^{T}$, and (d) _Corynebacterium glutamicum_ DSM 20300 on pretreated bio-oil fractions OC$_{SPE}$ and AC$_{SPE}$ containing 1, 3, or 5 g/L acetate
The ability to use small organic acids derived from fast pyrolysis of lignocellulosic biomass is an interesting and non-conventional possibility to establish efficient bioprocesses within a future bioeconomy. However, knowledge of the effect and valorization of small organic acids on bacterial growth is scarce. Therefore, this study evaluated four bacterial strains for their suitability using different concentrations of acetate, mixtures of small organic acids, as well as pretreated bio-oil fractions as carbon source for their growth. Results show that \textit{E. coli}, \textit{P. putida}, and \textit{C. glutamicum} are able to grow on acetate as a sole source of carbon, whereas growth of \textit{B. subtilis} on acetate was not detectable. This behavior can potentially be attributed to the glyoxylate cycle (Berg, Tymoczko, & Stryer, 2002). While \textit{E. coli}, \textit{P. putida}, and \textit{C. glutamicum} possess the key enzymes of the glyoxylate cycle and thus are able to grow on acetate as sole carbon source (Cortay et al., 1989; Gerstmeir et al., 2003; Kornberg & Krebs, 1957; Reinscheid, Eikmanns, & Sahm, 1994a; Sudarsan, Dethlefsen, Blank, Siemann-Herzberg, & Schmid, 2014; Wendisch et al., 2000), \textit{B. subtilis} lacks enzymes required for the glyoxylate cycle (Freese & Fortnagel, 1969). To enable growth of \textit{B. subtilis} on acetate, Kabisch et al. (2013) transferred an operon encoding the two key enzymes of the glyoxylate cycle, ISL and MS, from \textit{B. licheniformis}.

Although \textit{E. coli} has the ability to grow on acetate as sole carbon source, it is obvious that its growth is affected by acetate in the growth medium. Previous reports suggest that this effect is potentially caused by an interfering effect of acetate on methionine biosynthesis leading to the accumulation of homocysteine, which inhibits the growth of \textit{E. coli} (Roe, O’Byrne, McLaggan, & Booth, 2002). Roe et al. (2002) demonstrated that even 0.5 g/L acetate in the culture medium reduced the specific growth rate of \textit{E. coli} by 50% compared to an uninhibited control. This inhibition can be relieved by adding methionine to the culture medium (Han, Hong, & Lim, 1993).

With regard to the effect of acetate on \textit{P. putida}, results suggest that \textit{P. putida} can efficiently grow on acetate up to a concentration of 15 g/L. Higher acetate concentrations, however, have an adverse effect on cell growth. The phenomenon of subsequent decrease in biomass after reaching maximal biomass concentration was also observed by Arias-Barrau, Olivera, Sandoval, Naharro, and Luengo (2006).

In agreement with previous studies (Claes et al., 2002; Gerstmeir et al., 2003; Jolkver et al., 2009; Wendisch et al., 2000), the findings of this study show that \textit{C. glutamicum} can grow on acetate. The specific growth rates achieved in our cultivations were, within typical margin of error, in the same range compared to those of Wendisch et al. (2000). In contrast to the previous studies, higher acetate concentrations were tested and it was shown that \textit{C. glutamicum} can grow on up to 30 g/L acetate. The results reveal that the growth rate decreases with increasing acetate concentrations, which was in the past attributed to its effects on the transmembrane pH gradient (Baronofsky, Schreurs, & Kashket, 1984). Growth inhibition of \textit{C. glutamicum} by acetate was initially observed at a concentration of 30 g/L acetate. Although \textit{C. glutamicum} was able to grow on 30 g/L acetate reaching maximal biomass concentrations of 10 g/L, growth was not detectable for every cultivation experiment with 30 g/L acetate.

Furthermore, the effect of formate and propionate on cell growth was investigated. It was found that none of the tested bacterial strains was able to grow on formate as sole carbon source, whereas propionate was used for cell growth by some bacteria. The fact that the strains cannot use formate as a carbon source might be attributed to the lack of metabolic enzymes for C1 metabolism in the genome of these strains (Roca, Rodriguez-Herva, Duque, & Ramos, 2008).

Propionate utilization was observed for \textit{E. coli}, \textit{P. putida}, and \textit{C. glutamicum}. The results are in agreement with other studies, which showed that the methylcitrate cycle is present in these bacterial strains (Claes et al., 2002; Textor et al., 1997).

Cultivations on mixtures of small organic acids revealed that formate has an adverse effect on cell growth. Formate is known as both an energy source and a compound potentially toxic to growth. Under certain conditions, formate can be used as an auxiliary energy substrate and thus has a beneficial effect on cell growth and product formation (Bruinenberg et al., 1985; Harris et al., 2007; Lian et al., 2012). However, depending on cell density, pH, activity of FDH, formate concentration, and physiological state of the cells, formate was also found to have inhibitory effects.

On the basis of growth analysis in the presence of pretreated bio-oil fractions, \textit{P. putida} and \textit{C. glutamicum} were found to be suitable for bioconversion of lignocellulosic-based feedstocks.

Both \textit{P. putida} and \textit{C. glutamicum} were able to grow on all three \textit{OC}_{SPE} concentrations. However, \textit{C. glutamicum} reached higher maximal biomass concentrations than \textit{P. putida}. This could be attributed to the ability of \textit{C. glutamicum} to grow on a wide variety of carbohydrates and organic acids as single or combined sources of carbon and energy (Arndt, Auchter, Ishige, Wendisch, & Eikmanns, 2008; Claes et al., 2002; Cocaing, Monnet, & Lindley, 1993; Wendisch et al., 2000). In comparison to cultivations on acetate, the results suggest that low concentrations of \textit{OC}_{SPE} have no significant effect on cell growth, but the higher the \textit{OC}_{SPE} concentration, the higher the adverse effect on cell growth, especially for \textit{P. putida}. The results suggest that \textit{P. putida} is not able to use all carbon sources present in \textit{OC}_{SPE} just as efficiently as \textit{C. glutamicum} does. Nevertheless, cultivations on \textit{AC}_{SPE} revealed that \textit{P. putida} is the only strain able to grow on this bio-oil fraction, even with a higher biomass yield than achieved on \textit{OC}_{SPE} and 1 g/L acetate.
*Pseudomonas putida* is known as an organic solvent–tolerant strain, able to degrade and adapt to high concentrations of organic solvents (Inoue & Horikoshi, 1989; Kim & Park, 2014; Nikel & de Lorenzo, 2014; Rojas et al., 2004; Rühl, Schmid, & Blank, 2009; Weber, Ooijkaas, Schemen, Hartmans, & Bont, 1993). It is assumed that ACSPE contains compounds, which are less inhibitory to *P. putida* as well as additional carbon sources for *P. putida*, whereas these substances have an adverse effect on the growth of *C. glutamicum*.

Bio-oil derived from fast pyrolysis of lignocellulosic biomass provides a sustainable resource for biotechnological production of value-added compounds with no direct competition to food and feed. The small organic acid fraction of bio-oil represents a significant proportion of microbially accessible carbon, which is being considered as one potential carbon source for an envisioned future industrial biotechnology. However, due to inhibitory substances as well complex composition, accessing these organic acids as substrates for growth remains a challenging task. The results of this study suggest that both *P. putida* and *C. glutamicum* are able to metabolize acetate as sole carbon source over a wide concentration range and are able to grow on mixtures of the main small organic acids present in bio-oil. Both *P. putida* and *C. glutamicum* show a distinct potential to tolerate inhibitory substances within bio-oil, which provides an important step in search of suitable bacterial strains for bioconversion of lignocellulosic-based feedstocks.

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