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Ustilago Rabenhorstiana—An Alternative Natural Itaconic Acid Producer

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Received: 22 November 2019; Accepted: 24 December 2019; Published: 2 January 2020

Abstract: Itaconic acid is an industrial produced chemical by the sensitive filamentous fungus *Aspergillus terreus* and can replace petrochemical-based monomers for polymer industry. To produce itaconic acid with alternative renewable substrates, such as lignocellulosic based hydrolysates, a robust microorganism is needed due to varying compositions and impurities. Itaconic acid producing basidiomycetous yeasts of the family *Ustilaginaceae* provide this required characteristic and the species *Ustilago rabenhorstiana* was examined in this study. By an optimization of media components, process parameters, and a fed-batch mode with glucose the final titer increased from maximum 33.3 g·L^{-1} in shake flasks to 50.3 g·L^{-1} in a bioreactor. Moreover, itaconic acid was produced from different sugar monomers based on renewable feedstocks by *U. rabenhorstiana* and the robustness against weak acids as sugar degradation products was confirmed. Based on these findings, *U. rabenhorstiana* has a high potential as alternative natural itaconic acid producer besides the well-known *U. maydis* and *A. terreus*.

Keywords: *Ustilago*; itaconic acid; process improvement; lignocellulosic feedstock

1. Introduction

Itaconic acid is an interesting chemical for the polymer industry, which is produced in a biotechnological process based on renewable substrates [1]. Petrochemical-based substances, like methacrylic or acrylic acid, can be replaced by this single unsaturated dicarbonic acid and its derivatives. Therefore, the field of products and applications is widespread, e.g., synthetic latex, styrene-butadiene rubber, superabsorbent polymers, or unsaturated polyester resins [2–6].

Since the 1960s, the filamentous fungus *Aspergillus terreus* is industrially used with a titer of 85–100 g·L^{-1}, whereas in laboratory scale, final titers of 160 g·L^{-1} itaconic acid are described [4,7–9]. *A. terreus* achieves a productivity up to 1.15 g (L·h)$^{-1}$ and a yield of 0.64 (*w*/*w*), whereby the theoretical yield with glucose is 0.72 (*w*/*w*) [7,8]. Besides pure glucose, itaconic acid was successfully produced by *A. terreus* with glycerol, starch hydrolysates, molasses, and different monosaccharides, like xylose, arabinose, galactose, and rhamnose [10]. A great cultivation challenge is caused by sugar degradation products or other impurities in lignocellulosic hydrolysates, which influence the morphology and itaconic acid production of the fungus. Due to the sensitivity of the fungus, complex purification processes are used for such hydrolysates or more resistant strains are generated by mutagenesis [11–13]. Another alternative is itaconic acid producing yeasts of the species *Candida*, *Pseudozyma*, or *Ustilago*, which are more robust and not as sensitive to metal ions as *A. terreus* [14–18]. For wildtype strains of the species *Ustilago*, low final titers of 44.5 g·L^{-1} itaconic acid, low yields up to 0.24 (*w*/*w*), and a low productivity of maximum 0.31 g (L·h)$^{-1}$ are disadvantageous [19]. This is due to a variety of by-products like other organic acids, glycolipids,

and intracellular triacylglycerols, which are produced in parallel to itaconic acid [16,18,20,21]. Nevertheless, in addition to the robustness of the yeasts, the formation of haploid yeast-like cells is an advantage compared to the filamentous growth or formation of pellets of *A. terreus* with a decreased oxygen supply or increased viscosity [5,22].

In recent years, the research interest in itaconic acid production with the phytopathogenic basidiomycete *Ustilago maydis* increased. It was found, that an ammonium limitation triggers the itaconic acid overproduction in *U. maydis* [5,19] and itaconic acid is synthesized in the cytosol via the intermediate cis-aconitate and trans-aconitate and can be further converted to 2-hydroxyparaconic acid [23–25]. The itaconic acid gene cluster was also characterized and relevant enzymes, transporters, and promoters were found [23,25,26], whereby a summary of metabolic aspects is given by Wierckx et al. [27]. Based on these findings, metabolic engineering strategies and process optimization of *U. maydis* resulted in a reduction of by-product concentrations of malic acid and 2-hydroxyparaconic acid with a significant increased itaconic acid titer of 63.2 $g \cdot L^{-1}$ and a yield of 0.48 (*w/w)* [23]. All in all, detailed examinations are available for itaconic acid production of *U. maydis*, but also other wildtype strains of the family *Ustilaginaceae* could offer advantages of less sensibility or a yeast-like morphology for using second-generation feedstocks. This family is well-known for organic acid production [16,18,28,29], but the level of knowledge about alternative itaconic acid producer, like *U. cynodontis* or *U. rabenhorstiana,* are low.

This study considers the cultivation of *Ustilago rabenhorstiana* for itaconic acid production and its potential as alternative natural producer. Although the used strain is known as natural itaconic acid producer [18,30], the microorganism was not examined in literature more precisely. Only the growth of the organism with glycerol as substrate was described, whereby non-formation of organic acids was detected [28]. Concerning itaconic acid production based on renewable resources, usability of different sugar monomers and robustness towards influence of sugar degradation products were examined in this study. Moreover, the influences of media and fermentation parameters on the production of itaconic acid and by-products as well as the morphology of the yeast were investigated.

2. Materials and Methods

2.1. Microorganism

The basidiomycete *Ustilago rabenhorstiana* NBRC 8995 was purchased from the National Institute of Technology and Evaluation (Tokyo, Japan) and was stored at −80 °C as 50% (*v/v*) glycerol stock culture.

2.2. Media Compositions

YEPS-medium was used for the preparation of agarplates and preculture (20 $g \cdot L^{-1}$ sucrose, 10 $g \cdot L^{-1}$ yeast extract, 20 $g \cdot L^{-1}$ peptone, optional 20 $g \cdot L^{-1}$ agar-agar).

If not mentioned otherwise, the production media was a Tabuchi-medium [18] containing 120 $g \cdot L^{-1}$ glucose, 0.5 $g \cdot L^{-1}$ KH_2PO_4, 1.6 $g \cdot L^{-1}$ NH_4Cl, 0.2 $g \cdot L^{-1}$ $MgSO_4 \cdot 7\ H_2O$, 10 $mg \cdot L^{-1}$ $FeSO_4 \cdot 7\ H_2O$, 1 $g \cdot L^{-1}$ yeast extract, and 30 $g \cdot L^{-1}$ $CaCO_3$. All components were prepared separately in stock solutions; the pH-value was adjusted to pH 6.0 for all solutions with 0.5 M H_2SO_4 or 1 M NaOH and autoclaved. The pH-value of the iron-solution was not corrected, and the solution was sterile filtered. $CaCO_3$ was weighed in the glassware and autoclaved.

All media components were p.a. quality and purchased from Merck (Darmstadt, Germany), Sigma-Aldrich (St. Louis, MO, USA) or Roth (Karlsruhe, Germany). In fed-batch cultivation the glucose concentration was monitored during the cultivation. If necessary, glucose was added in solid form without previous sterilization to prevent a glucose limitation.

2.3. Cultivation

Precultures were conducted in a 250 mL shake flask with three baffles, a filling volume of 50 mL, and inoculated with a single colony from a YEPS-agar plate (30 °C, 3 days). The preculture was

cultivated at 30 °C and 120 rpm (50 mm shaking diameter) for 24 h until an optical density of 10 at 605 nm was achieved. All experiments were inoculated with 1% (*v*/*v*) of the preculture.

The main cultures in shake flasks were carried out at 30 °C and 120 rpm in 250 mL shake flasks with three baffles and a filling volume of 100 mL Tabuchi-medium.

To identify potential impurities and the utilization of monosaccharides based on lignocellulosic feedstock, test tubes with Kapsenberg caps were used and a working volume of 2 mL (ø 16 mm × 100 mm). The test tubes were incubated for 4 days at 30 °C and 120 rpm in an inclined test tube holder with an inclination angle of 30°. Shake flasks and test tubes were continuously rotated by hand while sampling avoiding inhomogeneity.

The cultivation in bioreactors were conducted in four parallel 1 L-bioreactors, equipped with a Rushton impeller and an L-sparger (model SR0700ODLS, DASGIP GmbH, Jülich, Germany). DASGIP Control software (DASGIP GmbH, Jülich,Germany) was used for the regulation of gassing, temperature, pH-value, and stirring rate, as well as recording the data of dissolved oxygen (DO) and pH. The pH regulation to pH 6.0 was carried out with 4 M NaOH, if not otherwise mentioned. At the beginning of the cultivation, 0.5 mL antifoam solution (Ucolup N-115, Brenntag, Mühlheim/Ruhr, Germany) was added to the broth. The experiments were carried out at 30 °C, 500 rpm, a filling volume of 500 mL, and an aeration of 0.1 vvm, unless otherwise mentioned. All cultivations were carried out in minimum duplicates, whereby the deviation from the mean value was < 5%. All results are presented as mean values without error bars on account of readability.

2.4. Analytical Methods

The samples were centrifuged at 21,000 g for 20 min at 20 °C and the supernatant was used for further analysis. A Shimadzu HPLC (Shimadzu Corp., Kyoto, Japan) with a HPX-87H column (BioRad, Munich, Germany) with a refractive index detector (RI) and UV detector at 210 nm was used to analyze the concentrations of sugars and organic acids. The column was tempered at 40 °C and as mobile phase a 5 mM H_2SO_4 solution at a flow rate of 0.6 mL·min^{-1} was used. The concentration of an unknown product was estimated by the peak area of the RI-signal compared to a calibration of succinic acid. For samples of bioreactor experiments, the pellet was washed twice with deionized water and dried to a constant weight at 105 °C for at least 48 h to determine the cell dry weight (CDW).

The composition of fatty acids was analyzed by transesterification of the fatty acids as described by Lewis et al. [31]. The biomass of reactor cultivation was separated from the broth by centrifugation (21,000 g for 20 min at 20 °C). The supernatant was discarded, and the pellet was washed twice with 0.9% (*v*/*v*) NaCl-solution and suspended in 0.9% (*v*/*v*) NaCl-solution. The cells were disrupted by an ultrasonic-homogenisator on ice (4 cycles: 15 s at 65%, break 30 s; Sonopuls HD2200 with sonotrodetype UW2200, Bandelin electronic, Berlin, Germany). The suspension was stored at −80 °C and freeze-dried (Alpha 1-2 LD, Christ, Osterode, Germany). The fatty acids were derivatized to fatty acid methyl esters (FAME) [31] and analyzed by GC-MS on a GC-17A (Shimadzu Corp., Kyoto, Japan) with a Zebron™ ZB-WAX plus column (60 m × 0.25 mm × 0.25 µm), using 1.4 mL·min^{-1} helium as carrier gas. The temperature gradient of 60 °C was increased to 150 °C at a rate of 30 °C·min^{-1}, and afterwards increased up to 240 °C at a rate of 13 °C·min^{-1}. The temperature of 240 °C was kept for 30 min and raised to 255 °C for 5 min. The FAMEs were identified with the software LabSolutions (Shimadzu Corp., Kyoto, Japan) and the mass spectral data were compared with the database of the National Institute of Standards and Technology (Gaithersburg, MD, USA).

2.5. Microscopy

The cells were examined using a phase-contrast microscope (Axioplan, Carl Zeiss AG, Jena, Germany) with the software analysis pro (Analysis 5.1, Olympus Soft Imaging Solutions GmbH, Münster, Germany). Intracellular lipids were visualized after coloring with nil-red by fluorescence microscopy [32].

3. Results

3.1. Standard Cultivation in Shake Flasks

A standard cultivation of *U. rabenhorstiana* with pure glucose as substrate was performed in shake flasks (Figure 1). After one day, the itaconic acid production started, and additionally, succinic acid and malic acid were produced. Furthermore, an unknown product accumulated after 48 h approximately in a concentration range of <1 g·L^{-1}. Malic acid was consumed in further course of cultivation, and after four days, α-ketoglutaric acid was formed and increased parallel with the itaconic acid concentration. Glucose was completely consumed after 9.7 days, resulting in 31.3 g·L^{-1} itaconic acid, 13.6 g·L^{-1} α-ketoglutaric acid, 2.3 g·L^{-1} malic acid, and traces of an unknown metabolite, followed by a further production of α-ketoglutaric acid. The overall productivity was 0.13 g (L·h)$^{-1}$ with a yield of 0.26 (*w*/*w*) after 9.8 days. Despite the use of $CaCO_3$ as buffer, the pH-value constantly decreased from pH 6.8 to 4.9 throughout the cultivation. The morphology of *U. rabenhorstiana* changed from yeast-like single cells (0–2 days; Figure 1A) via a development of pseudomycel (2–7 days, Figure 1B) to filamentous growth like long branched mycel (7–11 days, Figure 1C). Moreover, intracellular lipids deposits were visible under the microscope, which became smaller in size after the glucose limitation at day 9.7.

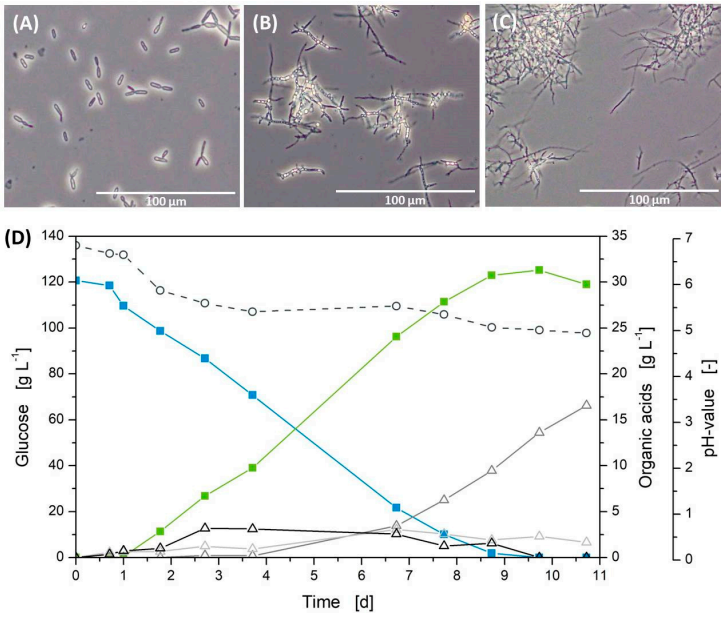

Figure 1. Cultivation of *U. rabenhorstiana* in 250 mL shake flasks in standard Tabuchi-medium (**D**) and its corresponding morphology (**A**): 0–2 days; (**B**): 2–7 days; (**C**): 7–11 days. Glucose (blue square), itaconic acid (green square), α-ketoglutaric acid (grey triangle), succinic acid (black triangle), malic acid (light grey triangle), pH (black circle), 120 rpm, 30 °C, 1% (*v*/*v*) inoculum.

3.2. Influence of Media Components

To determine the influence of media components, titer, yield, and productivity of a cultivation with 120 g·L^{-1} initial glucose, performed in shake flasks after 7.8 days, are shown in Figure 2. In the case of the variating initial glucose concentrations (Figure 2A), the point in time of glucose limitation was analyzed. 50 g·L^{-1} glucose were consumed in 3.7 days, 100 g·L^{-1} in 6.7 days, 120 g·L^{-1} in 8.7 days, and 150 g·L^{-1} in 10.7 days. Further, 200 g·L^{-1} glucose was not completely consumed by *U. rabenhorstiana* and 35 g·L^{-1}, and remained while the concentration of itaconic acid was constant after 15 days. With increasing initial glucose concentration, the titer of itaconic acid increased, but the productivity and yield decreased slightly from 0.16 g (L·h)$^{-1}$ to 0.09 g (L·h)$^{-1}$ and from 0.27 (*w*/*w*) to 0.20 (*w*/*w*) for glucose concentrations larger than 100 g·L^{-1}. The amount of α-ketoglutaric, succinic, and malic acid of the total organic acid concentration was raised from 15.5% (50 g·L^{-1} glucose) over 33.7% (120 g·L^{-1} glucose) to 50.1% (200 g·L^{-1} glucose). In case of different ammonia chloride

concentrations, the highest titer of 31.8 g·L^{-1} itaconic acid, a productivity of 0.17 g (L·h)$^{-1}$, and a yield of 0.26 (*w*/*w*) was achieved using 1.6 g·L^{-1} NH_4Cl, which corresponded to the used concentration in standard Tabuchi-medium (Figure 2B). With a lower concentration of 1 g·L^{-1} NH_4Cl and higher concentrations between 3 and 7 g·L^{-1} NH_4Cl the titer, productivity and yield decreased up to 35%. Also, the chosen concentration of 0.2 g·L^{-1} $MgSO_4 \cdot 7\ H_2O$ in Tabuchi-medium was optimal for itaconic acid production with *U. rabenhorstiana*, and a titer of 28.9 g·L^{-1} with a productivity of 0.16 g (L·h)$^{-1}$ was reached (Figure 2C). Lower or higher levels of magnesium resulted in a decrease of all target values. In the concentration range of 0.1–1 g·L^{-1} KH_2PO_4, there were no significant differences between the titer, yield, and productivity. All cultivations yielded in titers of 29.3 g·L^{-1} ± 1.2 g·L^{-1} with a productivity between 0.15–0.16 g (L·h)$^{-1}$ and a yield of 0.24–0.25 (*w*/*w*) (Figure 2D). In the range of 0.5–25 mg·L^{-1} $FeSO_4 \cdot 7\ H_2O$, the itaconic acid decreased from 32.4 g·L^{-1} to 24.2 g·L^{-1} (Figure 2E). The productivity of 0.17 g (L·h)$^{-1}$ was reduced by 17% and the yield of 0.3 (*w*/*w*) itaconic acid by 25%. With increasing yeast extract concentration (0.25–1.5 g·L^{-1}) the titer increased to 27.2 g·L^{-1} with a productivity of 0.15 g (L·h)$^{-1}$ at a concentration of 1.5 g·L^{-1} yeast extract (Figure 2F). A further increase in the yeast extract concentration up to 2 g·L^{-1} resulted in a decreased titer of 24 g·L^{-1} and a lowered productivity of 0.13 g (L·h)$^{-1}$. None of the media components had an influence on the filamentous growth.

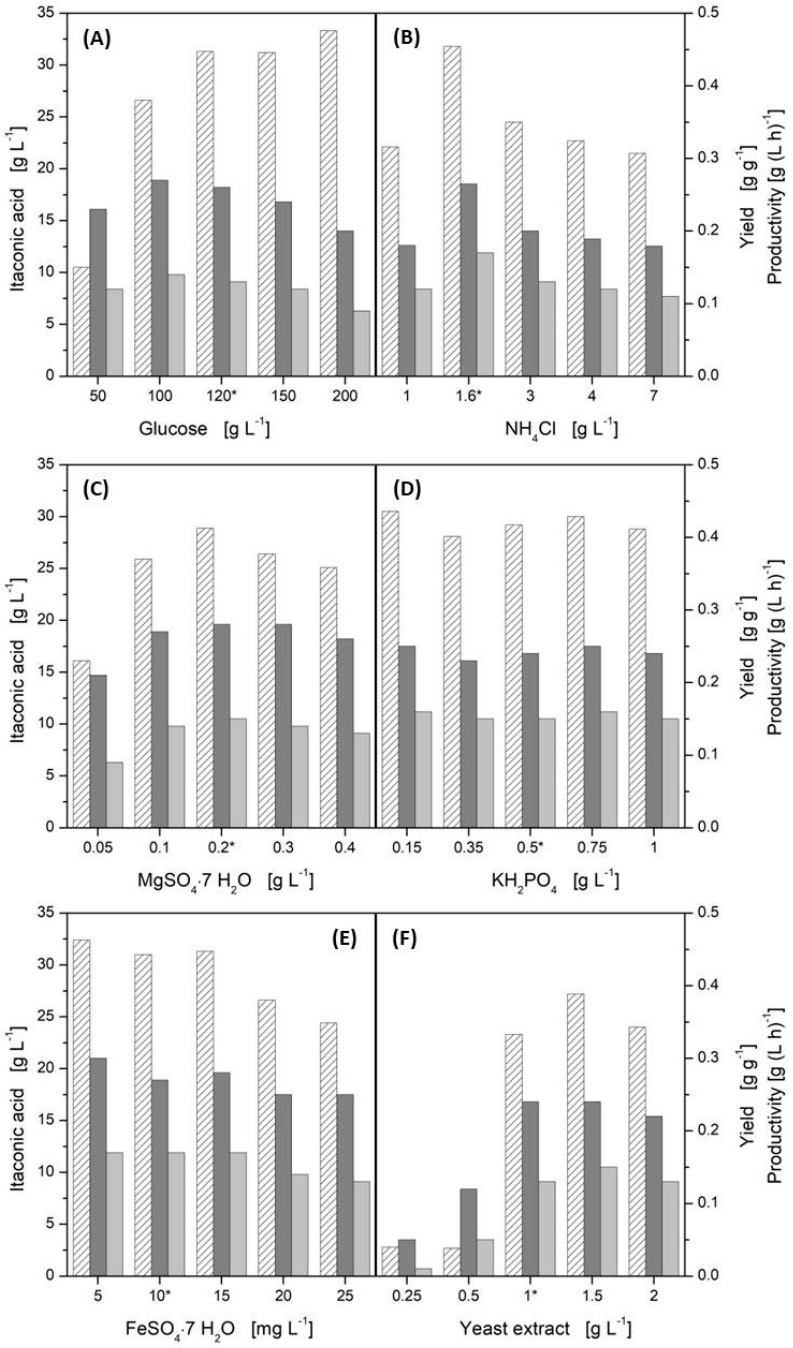

Figure 2. Influence of Tabuchi-medium components in 250 mL shake flasks on the final titer (dashed bar), yield (black bar), and productivity (grey bar) of *U. rabenhorstiana* at 120 rpm and 30 °C after 7.8 days (**B**–**F**). Cultivation time for different initial glucose concentrations depended on the point of glucose limitation (**A**). Asterisks highlight the standard media composition.

3.3. Monosaccharide Utilization

It is intended to produce itaconic acid based on renewable feedstocks, e.g., lignocellulosic biomass, biomass with a high starch content, or molasses. The usability of the monosaccharides based on those feedstocks (arabinose, fructose, galactose, glucose, mannose, rhamnose, and xylose) were investigated with 100 g·L^{-1} of each sugar in test tubes (Table 1). For the precultivation, sucrose was used. Filamentous growth occurred on glucose, fructose, mannose, and xylose. An accumulation of long hyphae, a buildup of pellets with a diameter of 50 µm grew with arabinose as substrate. The yield and productivity were very different depending on the substrate. For the reference cultivation with glucose, the productivity was 0.16 g (L·h)$^{-1}$ with a yield of 0.24 (*w*/*w*). The

productivity of 0.09 g (L h)$^{-1}$ of itaconic acid with mannose was 44% lower, while the yield was in the same range with 0.22 (*w*/*w*). Using fructose, the same productivity compared to mannose was achieved, but with a lower yield of 0.17 (*w*/*w*). *U. rabenhorstiana* was able to use both pentoses for itaconic acid production, whereby the productivity with arabinose with 0.04 g (L·h)$^{-1}$ was twice as high as with xylose. In the cultivation with glactose, only traces of itaconic acid were detected. The yeast was not able to produce itaconic acid or even grow with rhamnose as single substrate.

Table 1. Cultivation of *U. rabenhorstiana* in test tubes with different monosaccharides as substrate (four days, 30 °C, 120 rpm, inclination angle of 30°, and 1% (*v*/*v*) inoculum).

Monosaccharide	Productivity [g (L·h)$^{-1}$]	$Y_{P/S}$ [*w*/*w*]
Glucose	0.16	0.24
Mannose	0.09	0.22
Fructose	0.09	0.17
Arabinose	0.04	0.06
Xylose	0.02	0.04
Galactose	<0.01	<0.01
Rhamnose	-	-

3.4. Influence of Sugar Degradation Products

In case of lignocellulosic feedstocks, different sugar degradation products are formed due to the harsh conditions in the pretreatment. To test the inhibitory effect of sugar degradation products like weak acids or furan derivates, the components were added by the lowest expected concentration levels to the media. The effect of 0–2 g·L^{-1} acetic acid, formic acid, furfural, or hydroxymethylfurfural (HMF) was carried out in test tubes (Figure 3). A productivity of 0.15 g (L h)$^{-1}$ with standard Tabuchi-medium without the addition of inhibitory components was reached. Up to a concentration of 0.5 g·L^{-1} formic acid, the productivity did not differ. The addition of 1 g·L^{-1} resulted in an increased productivity of approximately 1.4 times and decreased to 0.13 g (L h)$^{-1}$ with 2 g·L^{-1} formic acid. The result was very similar with the addition of acetic acid. The standard productivity increased up to 0.19 g (L h)$^{-1}$ by adding 0.5 g·L^{-1} acetic acid and was reduced to 0.13 g (L h)$^{-1}$ by increasing the acetic acid concentration. Both furan derivates influenced the microorganism very strongly, amounts of 0.1 g·L^{-1} of HMF or 0.5 g·L^{-1} furfural already resulted in a growth inhibition. If the growth was not inhibited, the yeast grew filamentous and stored intracellular lipid droplets comparable with the cultivation without addition of inhibitors.

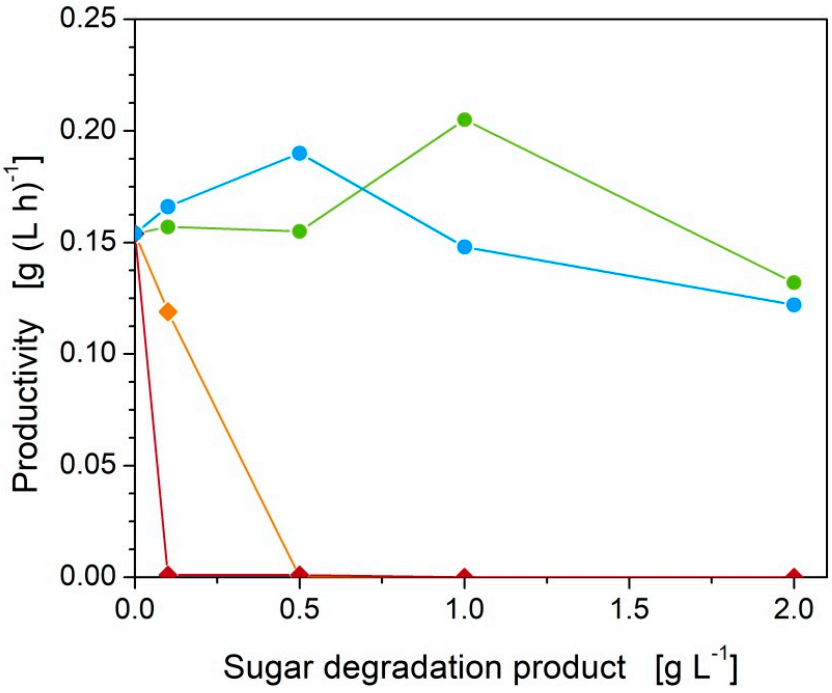

Figure 3. Inhibition effects of sugar degradation products on the itaconic acid productivity with *U. rabenhorstiana* in test tubes with Tabuchi-medium after four days, 30 °C, 120 rpm, inclination angle of 30°, pH > 5.5, and 1% (*v*/*v*) inoculum. Acetic acid (blue circle), formic acid (green circle), HMF (red diamond), furfural (orange diamond).

3.5. Influence of the pH-Value in 1 L-Bioreactor

The pH-value dropped in shake flask cultivations from pH 6.7 to pH 4.9 with $CaCO_3$ as buffer. To estimate the influence of the pH-value, the cultivation was transferred in 1 L-bioreactors with pH-control using 4 M NaOH (Table 2). Moreover, the modified Tabuchi-medium with 100 g·L^{-1} glucose and 1.5 g·L^{-1} yeast extract was used, based on the findings regarding the tested media components. The highest titer of 31.7 g·L^{-1} itaconic acid with a productivity of 0.23 g (L h)$^{-1}$ and a yield of 0.34 (*w*/*w*) was reached with a controlled pH of 6.0. Beside itaconic acid, 0.4 g·L^{-1} α-ketoglutaric acid, 2 g·L^{-1} malic acid, 2.9 g·L^{-1} succinic acid, and the unknown product (< 1 g·L^{-1}) were produced. The rate of the byproducts did not differ among the tested pH-values; also, the pH-value did not have any influence on the filamentous growth of the yeast and formation of intracellular lipids.

Table 2. Cultivation results of *U. rabenhorstiana* with pH-control in modified Tabuchi-medium with 100 g·L^{-1} glucose and 1.5 g·L^{-1} yeast extract in 1 L-bioreactor (30 °C, 500 rpm, 0.1 vvm, 4 M NaOH).

pH-Value [-]	Itaconic Acid [g·L^{-1}]	Productivity [g (L·h)$^{-1}$]	$Y_{P/S}$ [*w*/*w*]
5.5	23.7	0.15	0.25
6.0	31.7	0.23	0.34
6.5	18.6	0.12	0.19
7.0	15.9	0.10	0.16

3.6. Influence of Aeration in 1 L-Bioreactor

The effect of aeration was tested in 1 L-bioreactors by different aeration rates between 0.1–1 vvm, the stirring rate was kept constant, and the pH-value was regulated to pH 6.0 (Table 3). An increasing aeration rate from 0.1 to 1 vvm resulted in a decreased yield and titer of 20%, as well as in a 45% lower productivity. In contrast, the formed biomass increased from 15.7 g·L^{-1} at 0.1 vvm to 21.3 g·L^{-1} at 1 vvm. There were no significant differences between the by-product concentrations depending on the aeration rate, which corresponds to the concentrations described in Section 3.5.

Table 3. Influence of aeration on the cultivation of *U. rabenhorstiana* in modified Tabuchi-medium with 100 g·L^{-1} glucose and 1.5 g·L^{-1} yeast extract in 1 L-bioreactor (30 °C, 500 rpm, pH 6.0).

Aeration [vvm]	Itaconic Acid [g·L^{-1}]	Productivity [g·(L·h)$^{-1}$]	$Y_{P/S}$ [*w*/*w*]	CDW [g·L^{-1}]
0.1	29.8	0.22	0.30	15.7
0.5	26.1	0.16	0.26	17.5
1.0	23.6	0.12	0.24	21.3

3.7. Fed-Batch Mode in 1 L-Bioreactor with Glucose

Glucose concentrations larger than 150 g·L^{-1} resulted in a decreased yield and productivity in shake flasks (Figure 2A). For this reason, a fed batch with glucose was realized at a constant pH of pH 6.0 in a 1 L-bioreactor (Figure 4). An initial glucose concentration of 100 g·L^{-1} was chosen. After five days, 73 g·L^{-1} and after 10 days, 25 g·L^{-1} glucose were added into the cultivation broth, in which the average glucose consumption rate was 0.73 g (L h)$^{-1}$ for the first batch (0–5 days), 0.53 g (L h)$^{-1}$ for the second batch (5–10 days), and 0.28 for the third batch (10–15 days). The yield amounted to 0.31 (*w*/*w*) in the first batch and was constant with 0.26 (*w*/*w*) in the second and third batches. The DO decreased to 2% within the first day and varied between 2%–20% during the further cultivation. After one day, the itaconic acid production started and rose to a final titer of 50.3 g·L^{-1} within 15 days. Beside itaconic acid, 3.6 g·L^{-1} malic acid, 13.6 g·L^{-1} succinic acid, 2.5 g·L^{-1} α-ketoglutaric acid, and the unknown product (< 10 g·L^{-1}) were formed by 17.2 g·L^{-1} filamentous biomass (Appendix A, Figure A2). This cultivation resulted in a productivity of 0.14 g (L·h)$^{-1}$ with an overall yield of 0.27 (*w*/*w*) after 15 days. Further, 175 mL of a 4 M NaOH was used to keep the pH-value constant at pH 6.0. After 15 days, the cell dry weight was analyzed regarding the fatty acids (Appendix A, Table A1); C16:0, C18:0, and C18:2 were the main elements.

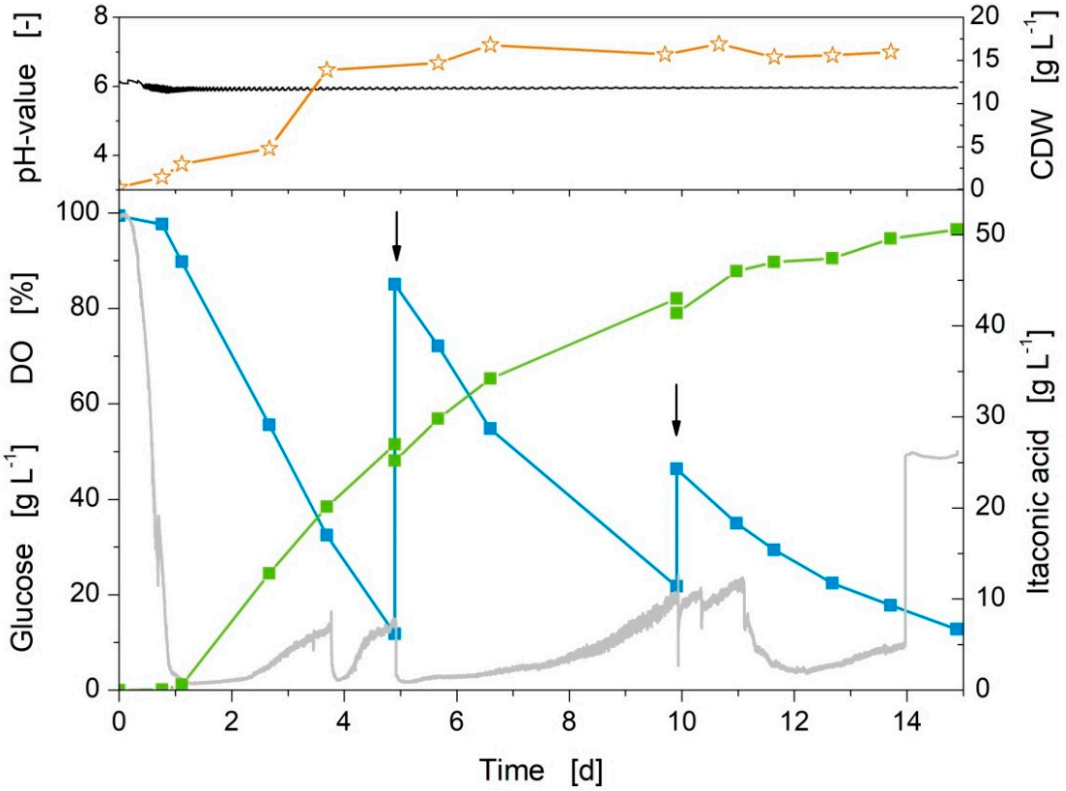

Figure 4. Fed batch with glucose in 1 L-bioreactor with modified Tabuchi-medium (initial glucose concentration 100 g·L^{-1} and 1.5 g·L^{-1} yeast extract) at 30 °C, 500 rpm, 0.1 vvm, pH 6.0 with 4 M NaOH as base and 1% (*v*/*v*) inoculum. Glucose (blue square), itaconic acid (green square), pO_2 (grey line), pH (black line), cell dry weight (CDW) (orange asterisk), arrows symbolize the addition of glucose.

4. Discussion

In a standard cultivation in shake flasks, a final titer of 31.3 g·L^{-1} itaconic acid was achieved after 9.7 days without media and process optimization of *U. rabenhorstiana*. Additionally, succinic acid, malic acid, α-ketoglutaric acid, an unknown product, and intracellular lipids were formed. Guevarra and Tabuchi reached a titer of about 16 g·L^{-1} itaconic acid in the same media after seven days and verified 2-hydroxyparaconic acid, itatartaric acid, and erythritol as byproducts with a total concentration of 30 g·L^{-1} [18]. Furthermore, they reduced the byproduct concentration to 19 g·L^{-1} with a constant itaconic acid titer by using an unbuffered media, whereby the final pH-value was pH 2.8 [30]. Erythritol as the unknown byproduct could be excluded in this work. In comparison with the literature, the unknown product (<1 g·L^{-1}) could either be 2-hydroxyparaconic acid or itatartaric acid (Appendix A, Figure A1) [18,23,30] and must be characterized precisely in a further study. Moreover, the formation of different organic acids and intracellular lipid bodies by *Ustilaginaceae* besides itaconic acid production are very well known [5,19], as well as the production of cellobiose lipids and mannosylerythritol lipids [20,29], which were not proved in this study.

A point-by-point analysis of each media component indicated that the chosen concentrations of each component in the Tabuchi-medium were nearly optimal for itaconic acid production with *U. rabenhorstiana*. Only the glucose and yeast extract concentrations were adjusted from 120 g·L^{-1} to 100 g·L^{-1} for glucose and from 1 g·L^{-1} to 1.5 g·L^{-1} for yeast extract in the modified Tabuchi-medium. Increased glucose concentration resulted in an increased final titer, but the yield and productivity decreased with glucose concentrations > 100 g·L^{-1}. The decreased yield and productivity can be explained by a higher osmotic stress and a higher number of byproducts. A very similar result was obtained by the production of itaconic acid with *U. maydis* [19]. This microorganism also showed lower yields at higher initial glucose concentrations, explained by the formation of other organic acids, polyols, and glycolipids. The increase of yeast extract by 0.5 g·L^{-1} raised the titer, productivity, and yield. A further increase of yeast extract did not result in an improved titer, suggesting that a limitation of vitamins, amino acids, salts, trace elements or nucleic acids [33] is prevented with a concentration > 1.5 g·L^{-1} yeast extract. The itaconic acid overproduction by *U. maydis* or *P. antarctica* are mainly triggered by an ammonium limitation, higher concentrations of NH_4Cl > 4 g·L^{-1} resulted in lower itaconic acid yields and an increase in biomass for *U. maydis* [5,14,19]. What is more, in this study, the ammonium concentration had the strongest influence on the fermentation performance of *U. rabenhorstiana.* A concentration of 1.6 g·L^{-1} NH_4Cl was optimal for itaconic acid production, and higher concentrations resulted in reduced yields and productivities as described already for *U. maydis*. For this reason, it can be assumed that an ammonium limitation caused the itaconic acid overproduction of *U. rabenhorstiana*. Further, the nitrogen limitation caused the accumulation of intracellular lipid droplets, which are mostly triacylglycerols [21,34]. A secretion of cellobiose lipids and mannosylerythritol lipids in the form of needle-like crystals or oily droplets of *U. maydis* [20], could not be verified for *U. rabenhorstiana*.

To use alternative, low-cost, or lignocellulosic feedstocks for itaconic acid production, it is important to use a microorganism, which is able to consume different monosaccharides and is robust towards varying impurities. In this study, it was worked out that *U. rabenhorstiana* is able to grow and produce itaconic acid from different monosaccharides like glucose, fructose, mannose, xylose, arabinose, and galactose. The highest productivity was reached with glucose, followed by fructose and mannose. Because the precultivation was based on sucrose, composed of glucose and fructose, it can be assumed that the cultivation with fructose was therefore such successful. An adaption to the used monosaccharide of the microorganisms in the preculture or a mixture of several monosaccharides with glucose would probably lead to higher productivity and yield using that single monosaccharide in the main culture. Furthermore, the plant pathogen *U. rabenhorstiana* is supposed to degrade a range of biomass-based polymers [35–37]. For industrial itaconic acid production, the filamentous fungus *A. terreus* is used, which is very sensitive to weak acids, furan derivates, metal ions, and other impurities, which are contained in such substrates [11,12,38]. *U. maydis* is described as a very robust microorganism [5,22,39]. *U. rabenhorstiana* was also not influenced by the addition of weak acid concentrations up to 2 g·L^{-1} in the main culture. A major

advantage of the cultivation of *Ustilaginaceae* is the pH range of 5.0–6.5, whereby the dissociated weak acids cannot cross the plasma membrane into the cytosol and affect the intracellular pH-value [40,41]. A positive effect on the itaconic acid productivity was even achieved with the addition of 0.5 $g \cdot L^{-1}$ acetic acid or 1 $g \cdot L^{-1}$ formic acid. This positive influence of low weak acid concentrations in cultivation media is already known from itaconic acid production with *A. terreus*, ethanol production with *S. cerevisiae*, or enzyme production with *T. reesei* [12,42–44]. In contrast, low concentrations of 0.1 $g \cdot L^{-1}$ HMF or 0.5 $g \cdot L^{-1}$ furfural are growth limiting factors; both furan derivates reduce the activity of a number of important intracellular enzymes of the maintenance metabolism, e.g., pyruvate dehydrogenase [45]. In particular, the activity of the pyruvate dehydrogenase is essential for cells, because this enzyme links glycolysis and citric acid cycle, which supplies the cell with energy intermediates. When using lignocellulosic biomass as feedstock for *U. rabenhorstiana*, it should be taken into account, that some robustness in relation to weak acids exists, but furan derivates influence the microorganisms mostly up to growth inhibition.

For further characterization of *U. rabenhorstiana*, the fermentation was transferred in 1 L-bioreactors to investigate the influence of pH-value and aeration. A constant pH of 6.0 and modified Tabuchi-medium yielded in the highest itaconic acid titer of 31.7 $g \cdot L^{-1}$, which is comparable with the titer in standard shake flask cultivation with Tabuchi-medium. However, the overall productivity was 1.7 times higher at a constant pH-value than in shake flask experiments with $CaCO_3$, whereby the pH-value continuously decreased to pH 4.9. Moreover, the total concentration of by-products decreased by 66%, which resulted in an increased itaconic acid yield. These suggested that the buffer capacity in shake flasks is insufficient. Buffer systems like $CaCO_3$ or MES and its buffer capacity have a significant impact on the organic acid production of *Ustilaginaceae* not only on titer, but also on the ratio of products [16,24,30]. The higher the buffer capacity, the better is the itaconic acid titer in small-scale experiments, but *U. maydis* achieved the highest itaconic acid titer of 45.5–63.2 $g \cdot L^{-1}$ in cultivations with a constant pH of 6.0–6.5 in bioreactors [19,23]. Consequently, *U. maydis* and *U. rabenhorstiana* have nearly the same requirements in pH and productivity. Titer and yield are positively influenced by a constant pH-value.

In the literature, no detailed studies regarding influence of oxygen levels on the organic acid production with *Ustilaginaceae* exist. Only cultivation parameters like high shaking frequencies or stirrer speeds led to the conclusion that a high input of oxygen is necessary [10]. Contrary results were achieved for itaconic acid production with *U. rabenhorstiana*; the lowest aeration rate of 0.1 vvm and a constant stirring rate of 500 rpm yielded the best result regarding titer, productivity, and yield. Presumably, the increase in these values is related to the formation of 36% more biomass at higher aeration rates, because of a better supply of oxygen. In batch experiments, a maximum itaconic acid concentration of 33.3 $g \cdot L^{-1}$ with an initial glucose concentration of 200 $g \cdot L^{-1}$ was achieved, proving that initial glucose concentration ≥ 100 $g \cdot L^{-1}$ and a constant pH-value of pH 6.0 have a significant impact on the itaconic acid production with *U. rabenhorstiana*. Therefore, a cultivation in fed-batch mode with glucose was realized at pH 6.0 and resulted in 50.3 $g \cdot L^{-1}$ itaconic acid. Comparing the batch cultivation with 200 $g \cdot L^{-1}$ glucose with the fed-batch cultivation in a bioreactor, the productivity and yield were 1.4 times and the final titer 1.5 times higher. Also, the formation of organic acid as byproducts was reduced. The number of byproducts of the overall organic acid concentration decreased from 50% in batch cultivation to 28% in fed-batch mode. Thereby, the amount of organic acids shifted from α-ketoglutaric acid as the main byproduct in batch mode to succinic acid in fed-batch mode, due to the pH-value of each cultivation [16]. The final titer and yield of this study are slightly increased compared to a wildtype strain of *U. maydis*, which reached 44.5 $g \cdot L^{-1}$ [19]. A higher final titer up to 63.2 $g \cdot L^{-1}$ or yield of 0.48 (*w*/*w*) was only obtained by a genetical modification of *U. maydis* [23]. All in all, the fermentation broth was diluted by addition of 175 mL NaOH as base. Considering the dilution, the wildtype of *U. rabenhorstiana* demonstrates the potential to produce up to 68 $g \cdot L^{-1}$ itaconic acid. However, in all main cultures, the unicellular growth of *U. rabenhorstiana* shifted to filamentous cells with depots of intracellular lipids. Neither the variation of media components nor the investigation of process parameters influenced the filamentous growth. The typical unicellular yeast-like growth of *U. maydis* for itaconic acid could not be achieved for *U.*

rabenhorstiana in this study. This morphology would be a great advantage compared to the filamentous *A. terreus* regarding oxygen supply or viscosity, especially in large-scale fermentations [5,22], but was not focused in this study. In general, it is possible to generate a stable unicellular growth by deleting several genes [24]. The filamentous growth involved the accumulation of intracellular lipid droplets, which is initiated by nitrogen limitation, which in turn is needed for itaconic acid overproduction [5,19,34]. Furthermore, 89% of all fatty acids in *U. rabenhorstiana* were long-chain fatty acids C16:0, C18:0, and C18:2 and suggested the accumulation of triacylglycerols in the cells. The lipid bodies in *U. maydis* mainly contain triacylglycerols consisting of palmitic, linoleic, and oleic acids [21].

5. Conclusions

This study describes a known, but so far unspecified, itaconic acid producer—*U. rabenhorstiana*. The cultivation in shake flasks with a maximal final titer of 33.3 $g \cdot L^{-1}$ itaconic acid was transferred in a bioreactor. With a controlled pH-value, a low initial glucose concentration, and fed-batch mode, a final titer of 50.3 $g \cdot L^{-1}$ was achieved, which is comparable with titer of other wildtype strains of *Ustilago* described in literature. However, the productivity and yield are rather low compared to *U. maydis*, which was studied very precisely in the last years regarding cultivation and process parameters as well as metabolic engineering strategies for further improvements in itaconic acid production. Transferring this knowledge from *U. maydis* to *U. rabenhorstiana* could result in a further increased final titer and improved yield and productivity. Particular attention should be paid to the morphology of the yeast and minimization of byproducts, mainly the formation of intracellular lipid droplets. Moreover, the wildtype strain *U. rabenhorstiana* turned out to be a robust and promising alternative itaconic acid producer based on renewable resources. All in all, this study serves a basis for further promising research regarding lignocellulosic hydrolysates.

Author Contributions: S.K. and M.L. designed and performed the experiments. U.P. and A.K. supervised the experiments. S.K. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This work was carried out in the framework of the European Research Area Network for Industrial Biotechnology (ERA-IB project "Production of Organic Acids for Polyester Synthesis (POAP)") and was funded by the German Federal Ministry of Food and Agriculture, following a decision of the German Bundestag, via the Agency of Renewable Resources (Grant No. 22029312).

Conflicts of Interest: The authors declare no conflict of interest. The funder had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Appendix A

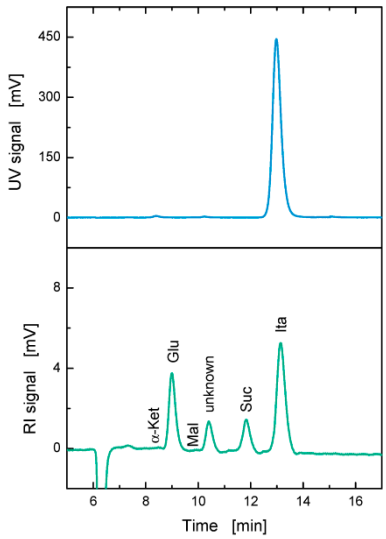

Figure A1. Representative HPLC analysis of a final sample (15 days) of a fed-batch cultivation in 1 L-bioreactor (Section 3.7.). α-ketoglutaric acid (α-Ket), glucose (Glu), malic acid (Mal), unknown product, succinic acid (Suc), itaconic acid (Ita).

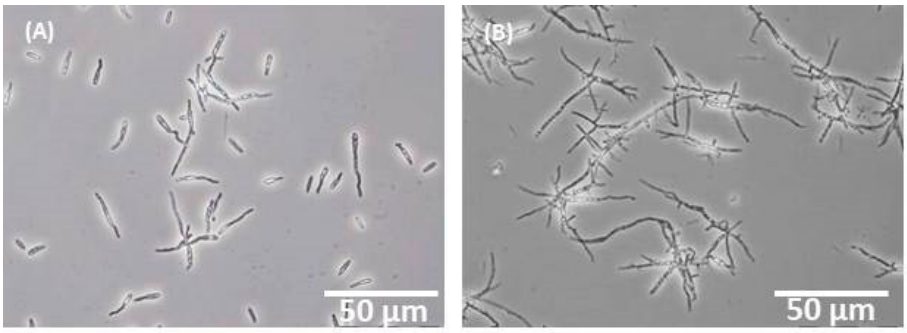

Figure A2. Corresponding morphology of *U. rabenhorstiana* in a fed-batch cultivation in 1 L-bioreactor (Section 3.7.) after 1.1 days (**A**) and 6.6 days (**B**).

Table A1. Analysis of fatty acids in intracellular lipid droplets in *U. rabenhorstiana* of a fed-batch cultivation in 1 L-bioreactor (Section 3.7.).

Fatty Acid	Concentration [mg·g^{-1}$_{CDW}$]
C14:0	1.46
C16:0	30.04
C16:1	1.55
C18:0	16.94
C18:1	1.83
C18:2	48.38
C22:0	4.42
C24:0	2.45

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