Rhodotorula kratochvilovae outperforms Cutaneotrichosporon oleaginosum in the valorisation of lignocellulosic wastewater to microbial oil

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Abstract

Rhodotorula kratochvilovae has shown to be a promising species for microbial oil production from lignin-derived compounds. Yet, information on *R. kratochvilovae's* detoxification and microbial oil production is scarce. This study investigated the growth and microbial oil production on the phenolic-containing effluent from poplar steam explosion and its detoxification with five *R. kratochvilovae* strains (EXF11626, EXF9590, EXF7516, EXF3697, EXF3471) and compared them with *Cutaneotrichosporon oleaginosum*. The *R. kratochvilovae* strains reached a maximum growth rate up to four times higher than *C. oleaginosum*. Furthermore, all *R. kratochvilovae* strains generally degraded phenolics more rapidly and to a larger extent than *C. oleaginosum*. However, the diluted substrate limited the lipid production by all strains as the maximum lipid content and titre were 10.5% CDW and 0.40 g/L, respectively.

Keywords

Wastewater; Lignocellulose; Oleaginous yeast; Phenolics; Lipids; Phenol

1 Introduction

Lignocellulosic materials are one of the most promising sustainable alternatives to fossil resources for the production of chemicals [1]. Lignocellulose is mainly composed of lignin, cellulose, and hemicellulose. These three components are interwoven to form a natural biocomposite that is resistant to degradation [2].

The recalcitrance of lignocellulosic materials is a significant barrier to enzymatic hydrolysis to release their fermentable sugars. Several pre-treatment methods have been

proposed to overcome the recalcitrance: bio-based (e.g., white rot fungi [3], physical (e.g., milling), chemical (e.g., organosolv), and physicochemical pre-treatment methods (e.g., steam explosion) [2].

All these technologies aim to increase sugar recovery by facilitating the enzymes' access to the carbohydrate polymers [4]. However, most of these processes release compounds that inhibit subsequent hydrolysis and bioconversion [5], i.e., organic acids, furans, phenols, and lignin fragments [6].

Phenolic compounds derived from lignocellulose are generally the most toxic, with growth and ethanol production inhibition observed at concentrations as low as 0.1 g/L [6]. Furthermore, these compounds often end up in the wastewater, posing a risk to the environment [7]. As a result, detoxification processes have been developed to remove these compounds from pre-treated lignocellulose and its waste-streams. They can be broadly classified into four groups: physical, chemical, enzymatic, and microbial. Although generally slower, microbial processes are environmentally friendly because they are operated under mild conditions, and produce little waste [2].

Several moulds, yeasts, and bacteria have been proposed as catalysts for the detoxification of lignocellulosic hydrolysates, e.g., *Trichoderma reesei*, and *Coniochaeta ligniaria,* among others [8]. However, these processes mainly focus on the removal of pollutants. In the light of resource efficiency, valorisation of the inhibitors is necessary. Especially, lipid production from lignocellulosic wastewaters has been researched as a valorisation strategy [9]. Several well-known oleaginous yeasts, i.e., *Rhodotorula toruloides*, *Lipomyces starkeyi, Rhodotorula glutinis, Yarrowia lipolytica*

48 [10-12] and *Cutaneotrichosporon oleaginosum*, have been used for lipid production on
49 lignocellulosic effluents, most often supplemented with sugars or glycerol [13].

50

51 A less studied yeast, *Rhodotorula kratochvilovae*, was recently discovered by Patel et
52 al. as a promising species for the detoxification and valorisation of lignocellulosic
53 effluents [14]. Patel et al. showed that *R. kratochvilovae* HIMPA01 could remove up to
54 99.6% of lignin and 94.27% of phenolics from glucose-supplemented lignocellulosic
55 effluent [15] . Furthermore, they showed that the yeast could use phenol as a sole
56 carbon source by degrading phenol through the meta-cleavage pathway [16]. Broos et
57 al. used *R. kratochvilovae* EXF7516 for the lipid production of steam explosion effluent
58 in a repeated batch process without additional sugar. From the diluted waste stream, the
59 yeast removed up to 77% of the monomeric substrates, i.e., sugars, aromatics, and
60 organic acids, and up to 21% of non-monomeric substrates, i.e., partially degraded
61 xylan, lignin, cellulose, and accumulated 5.4 g/L (23% cell dry weight) of lipids
62 intracellularly [9].

63 The literature on *R. kratochvilovae* for detoxification and valorisation of lignocellulosic
64 effluent is scarce, with only a hand full of published research articles and two strains of
65 *R. kratochvilovae*, i.e., HIMPA01, and EXF7516, studied in this context. The lack of
66 information makes it hard to predict whether *R. kratochvilovae* is suited for detoxifying
67 or valorising a particular lignocellulosic effluent. Furthermore, the diversity within *R.*
68 *kratochvilovae* has been little studied.

69 Therefore, this work investigates the performance of six strains of *Rhodotorula*
70 *kratochvilovae*, including *R. kratochvilovae* EXF7516, for the detoxification and

valorisation of poplar steam-explosion effluent. *C. oleaginosum* was used as a baseline for comparison, as *Cutaneotrichosporon* yeasts are considered most capable of metabolising a broad spectrum of lignocellulose-derived aromatics [17]. First, the six yeast strains were screened for growth on the steam-explosion effluent and ligninolytic enzyme production. The growth inhibition of individual phenolics was evaluated for the fastest growing strain. Finally, the simultaneous detoxification of steam-explosion effluent and lipid production were studied in shake flasks.

2 Materials and methods

2.1 Phenolic waste stream

The phenolic waste stream (PWS) used in this study was steam explosion effluent from poplar wood. The PWS was from the same batch used in the work of Broos et al. [9]. Briefly, one kilogram of poplar sawdust (48±1% moisture) was added to one litre of water. The mixture was steam exploded at a severity of 3.9±0.05 in a 50 L-pilot scale steam exploder [18]. The PWS was collected by filter-centrifugation and stored at -20 °C. Before use, the PWS was centrifuged at 3894 g for 30 min to remove particles.

2.2 Strains

The *R. kratochvilovae* strains (EXF11626, EXF9590, EXF7516, EXF3697, EXF3471) were obtained from the Ex Culture Collection of the Infrastructural Centre Mycosmo, MRIC UL, Slovenia: http://www.ex-genebank.com/, at the Department of Biology, Biotechnical Faculty, University of Ljubljana. *C. oleaginosum* ATCC20509 was obtained from the American Type Culture Collection.

2.2 Growth screening

The strains were screened for their tolerance to PWS, based on the maximum growth rate, duration of the lag phase, and maximum optical density reached during growth on PWS media.

2.2.1 Inoculum preparation

100 µL of *R. kratochvilovae* was inoculated in 100 mL yeast peptone dextrose medium (YPD) consisting of 20 g/L glucose (VWR), 20 g/L soy-peptone (Merck), 10 g/L yeast extract (Merck) in a 500 mL Erlenmeyer flask. The Erlenmeyer flask was incubated for 48 hours, until the late exponential phase, in a Sanyo Orbishake incubator at 30 °C and 150 rpm. The cells were centrifuged at 3500 g for 10 min. and washed twice with sterile YPD. The inoculum was diluted to an OD of 1.4.

2.2.2 Fermentation

The fermentation was performed in a microplate (Greiner, 655090). Each well contained 200 µL medium and 20 µL washed cell suspension. The overall volume percentage of PWS in each well was varied between 22 v/v% and 91 v/v% by dilution with nutrient medium. The PWS and nutrient medium contained the same nutrients and were prepared according to Yarrow [19] with minor modifications, and contained the following: 5 g/L NH_4Cl (Chem-Lab), 0.15 g/L K_2HPO_4 (Merck), 8.5 g/L KH_2PO_4 (Merck), 0.5 g/L $MgSO_4$ (Merck), 0.1 g/L NaCl (Fisher), 0.1 g/L $CaCl_2$ (Chem-Lab), 10 mg/L L-histidine (Merck), 2 mg/L DL-methionine (BDH), 2 mg/L DL-tryptophan (Merck), 1000 µL/L trace elements solution. The trace elements solution contained: 0.5 g/L boric acid (Chem-Lab), 62.5 mg/L $CuSO_4.5H_2O$ (Fluka), 333 mg/L $FeCl_3.6H_2O$ (Chem-Lab), 447 mg/L $MnSO_4.H_2O$ (Merck), 235 mg/L $Na_2MoO_4.2H_2O$ (Chem-Lab) and 712 mg/L $(Zn)_2SO_4.7H_2O$ (Chem-Lab). The microplate was incubated in a Synergy

HTX multi-mode microplate reader (BIOTEK) for 39 hours at 180 rpm and 30 °C, and the optical density was measured every 30 minutes. The PWS contributed to the turbidity. As a result, all measurements were corrected by subtracting a blank that consisted of 220 µL medium with the same v/v% PWS as the corresponding well.

2.2.3 Modelling

The growth curves showed non-ideal growth patterns, making estimation of the maximum specific growth rate (μ_m) with parametric models difficult. For this reason, the non-parametric approach of Kahm et al. was used that is based on a bootstrap method [20]. Briefly, the μ_m was estimated by fitting a cubic smoothing spline with 0.015 as the smoothing parameter to logarithmically transformed (ln(OD+1)) data with Matlab R2021a. The μ_m was found as the maximum of the first derivative of the smoothing spline. The initial cell concentrations were determined as the OD of the first points in the data sets. The duration of the lag phase was determined according to Baranyi and Roberts (1994) as the time at which the initial cell concentration intersected a line with a slope of μ_m. The algorithm was run 5000 times on random samples, with repetitions allowed, taken from the data. Finally, the average maximum specific growth rate (μ_m), the lag phase duration, and their standard deviation were calculated.

2.3 Growth inhibition by common lignin-derived inhibitors

2.3.1 Fermentation

Inoculum was prepared as before. The growth inhibition of four aromatics (vanillin, 4-hydroxybenzoic acid, 4-hydroxybenzaldehyde, and syringaldehyde) and PWS was evaluated. The concentrations were based on the work of Yaguchi et al. with *C. oleaginosum* [21]. To our best knowledge, no toxicity values of these compounds are

known for *Rhodotorula kratochvilovae.* Therefore, the same inhibitor concentrations were used for *R. kratochvilovae*.

Five stocks solutions of the inhibitors were prepared in YPD medium: 3 g/L vanillin (SAFC), 3 g/L syringaldehyde (Sigma-Aldrich), 5 g/L 4-hydroxybenzaldehyde (Aldrich), 5 g/L 4-hydroxybenzoic acid (Aldrich) and PWS. The PWS was supplemented to obtain 20 g/L peptone (Merck), 20 g/L glucose (VWR), 10 g/L yeast extract (Merck) in the sample. To eliminate the effect of pH, all stock solutions and the PWS were brought to pH 6.0. All solutions were filter sterilised over a 0.2 µm PES filter.

A linear concentration gradient over ten wells was obtained by diluting the stock solutions (1 to 50 times) with sterile YPD in a 96-well microplate (Greiner, 655095). The maximum specific growth rate on the YPD medium was also determined using 200 µL YPD medium without inhibitors. The influence of phenolics on the growth was evaluated in a rich medium (YPD) to exclude the influence of substrate specificity. Into each well, 20 µL of precultured cells were inoculated. The microplates were incubated for 120.5 hours in the microplate reader at 30 °C. The optical density was read every 0.5 hours using 220 µL of sterile YPD as a blank. The absorbance readings were corrected to a path length of 1 cm by multiplying by 1.5975.

2.3.2 Modelling

Again, the growth curves showed non-ideal growth patterns, and the method to estimate the μ_m described in the growth screening section above was used. The values of absolute and relative half-maximum inhibitory concentration (IC50) were estimated using the 4-parameter logistic model [22]. The four-parameter logistic model was fitted

to the estimated μ_m's using the *lsqnonlin* function in Matlab R2021a. As the 0% inhibition control, the average growth rate on yeast peptone dextrose was used. The 100% inhibition control was set at a μ_m of 0 h^{-1} [22].

2.4 Enzyme screening

The screening for ligninolytic and hydrolytic enzymes was done on yeast peptone dextrose (YPD) 2% agar (VWR) plates. Lignin peroxidase screening was performed according to Arboleda et al., using azure B as the indicator, but with 2% YPD agar instead of the malt extract agar [23]. The manganese peroxidase screening protocol was adapted from Bandounas et al. [24], briefly, the yeasts were grown on YPD 2% agar plates with 0.245 g/L methylene blue (Fluka). Similarly, the yeasts were screened for laccase activity on plates with 0.245 g/L ABTS (Sigma). The screening of xylanase was done according to Kalim and Ali (2016) with 0.1% xylan (Roth) agar plates and Congo red (Merck) as a dye. Cellulase screening was carried out in a similar way by replacing the xylan with carboxymethylcellulose (Fluka).

Most enzymes are only expressed when the necessary trace elements or inducing compounds are present. Therefore, YPD agar plates with the same dyes as above were made with 20 v/v% PWS to screen for induced ligninolytic activity. In addition, for laccase and manganese peroxidase screening, the addition of respectively 1 mM $CuSO_4$ (Fluka) and 1 mM $MnSO_4.H_2O$ (Merck) to the plates was investigated.

2.5 Fermentation in shake flasks

2.5.1 Medium and fermentation conditions

The inoculum was prepared as before, but the cells were washed with water and diluted to an OD of 0.4. PWS was supplemented with trace elements, and vitamins. The

185 composition was based on the work of Jiru et al. and Hassan et al. [25, 26]. One litre of
186 medium contained 2.0 g KH_2PO_4 (Merck), 1.5 g $MgSO_4.7H_2O$ (Fisher), 0.1 g
187 $CaCl_2.2H_2O$ (Chem-lab), 0.035 g $FeSO_4.7H_2O$ (Chemlab), 0.011 g $ZnSO_4.7H_2O$
188 (Chem-lab), 0.007 g $MnSO_4.H_2O$ (Merck), 0.002 g $CoCl.6H_2O$ (Chem-lab), 0.0013 g
189 $Na_2MoO_4.2H_2O$ (Chem-lab), 0.001 g $CuSO_4.5H_2O$ (Fluka), 12.5 µg myo-inositol
190 (Merck), 0.5 µg nicotinic acid (Sigma), 0.62 µg pantothenic acid (Sigma), 0.5 µg
191 thiamine (Sigma), 0.62 µg pyridoxine (Sigma), 0.013 µg biotin (Sigma), and 0.1 g/L of
192 NH_4Cl (Chem-lab). In contrast to the growth experiment, it was chosen to add vitamins
193 to the medium, as *C. oleaginosum* requires these for growth [27]. The pH of the
194 medium was adjusted with KOH (Chem-lab) to a pH of 6.0. Together with the nitrogen
195 present in the phenolic wastewater a C:N ratio of 216.7 g/g was reached. The
196 fermentation was performed in 500 mL Erlenmeyer flasks containing 100 mL PWS
197 medium. The Erlenmeyer flasks were inoculated with 5 mL washed cell suspension, and
198 incubated for 136.5±1.5 hours in a Sanyo Orbishake incubator at 30 °C and 150 rpm.

199 *2.5.2 Analytical techniques*

200 Biomass concentrations and substrate concentrations were determined daily. The
201 biomass concentrations was determined gravimetrically, as described previously [9].
202 Briefly, the biomass was harvested by centrifugation, washed twice with demineralised
203 water, and dried at 105 °C. The supernatant was used for the determination of the
204 monomeric substrates, i.e., aromatics (5-Hydroxymethylfurfural, furfural, 3,4-
205 dihydroxybenzaldehyde, 4-hydroxybenzoic acid, vanillic acid, syringic acid, vanillin,
206 and syringaldehyde), sugars (glucose and xylose), and organic acids (formic acid and
207 acetic acid) using HPLC, as described earlier [9]. The specific removal rate of these
208 compounds during the growth phase (q) was calculated for the *R. kratochvilovae* strains

and *C. oleaginosum*, using equation (1). With t_0 the time at which the growth phase started, t_1 the time at which the growth phase ended, X_0 the biomass concentration at time t_0, and X_1 the biomass concentration at time t1. The growth phase was defined as the phase where the cell concentration significantly increases. For *R. kratochvilovae* t_0 and t_1 were 0 and 15 hours, respectively. and for *C. oleaginosum* t_0 and t_1 were 42 and 91 hours, respectively.

$$q\left(\frac{mg}{g \cdot h}\right) = \frac{Concentration\ at\ t_0\left(\frac{mg}{L}\right) - Concentration\ at\ t_1\left(\frac{mg}{L}\right)}{(t_1(h) - t_0(h)) \cdot \left(X_0\left(\frac{g}{L}\right) + X_1\left(\frac{g}{L}\right)\right) \cdot 0.5} \qquad (1)$$

The lipid content, lipid titre, and dissolved organic carbon (DOC) concentrations were determined at the end of the fermentation. To this end, 10 mL fermentation broth was centrifuged for 10 min. at 3500 g (Sigma 3-16KL). The resulting supernatant was used for DOC determination. Briefly, the samples were filtered over a glass microfiber filter with a particle retention of 1.2 µm. Hereafter the DOC was determined by a Sievers InnovOx® (Suez, Trevose, PA, USA) [9]. The cell pellet was washed twice with demineralised water and lyophilised for 48 hours in a Christ freeze dryer. The cells were ground into a fine powder. Lipid extraction, and the determination of triacylglyceride, diacylglyceride, and monoacylglyceride content, concentration, and profile, were performed as described by Broos et al. [9].

2.6 Modelling

The growth curves were modelled using the logistic equation (Eq. 2), with N the biomass concentration, to obtain the μ_m and N_{max} using the *lsqnonlin*, and *ode45* function in Matlab R2021a.

$$\frac{dN}{dt} = \mu_m \cdot \left(1 - \frac{N}{N_{max}}\right) \cdot N \qquad (2)$$

2.7 Statistical analysis

Statistical analysis was performed using Matlab 2021a. The *normplot* and *adtest* function were used to assess the residuals' normality in the variance analysis (ANOVA). The assumption of equal variance of the groups in ANOVA was evaluated using the Barlett's test (*vartestn* function). When the residuals were normally distributed and groups had an equal variance, the *anova1* function was used for ANOVA. In this case, Tukey's honestly significant difference procedure was used for pairwise comparison between the groups. Welch's ANOVA was used when the groups did not have equal variances, but the residuals were normally distributed. Welch's ANOVA was performed using the *wanova* function developed by Penn [28].

3 Results and discussion

3.1 Growth screening

The performance of *C. oleaginosum* has been extensivsely researched and described in literature [17, 21]. However, the *R. kratochvilovae* strains are investigated for the first time and compared for their growth on PWS using a fast screening method. In the fast screening method, the growth of the five *R. kratochvilovae* strains was followed in the presence of seven different percentages of the phenolic waste stream (PWS 21.8 to 90.9 v/v%), supplemented with trace elements. After mathematical modelling, the maximum specific growth rate μ_m, and the duration of the lag phase were obtained and plotted against the v/v% PWS to investigate the growth differences (**Fig. 1**).

As can be observed in **Fig. 1a**, *R. kratochvilovae* EXF7516 or EXF3471 had the highest maximum growth rates in all conditions. The maximum growth rates (**Fig. 1a**) generally decreased with increasing PWS concentration due to the increasing toxicity. *R. kratochvilovae* strains EXF3697, EXF7516, and EXF3471, reached their lowest

maximum growth rate at 70 v/v% PWS. At low PWS concentrations, the growth inhibition by the PWS was limited, but little substrate was available. At high PWS concentrations, more substrate was available, but the growth inhibition of the PWS limited the growth. At an intermediate PWS concentration, i.e. 70 v/v%, growth was probably further limited by both a limited substrate concentration and a significant inhibition by the PWS. In **Fig. 1b,** the duration of the lag phase generally increased with increasing PWS concentration, as also the growth inhibition increased. *R. kratochvilovae* EXF7516 had the shortest lag phase for almost all PWS concentrations, indicating that the yeast is well suited for growth on PWS.

3.2 Simultaneous detoxification and lipid production

Batch fermentation in shake flasks was performed to assess the yeasts' potential to detoxify the PWS and produce lipids from it. In addition, their ligninolytic and hydrolytic enzyme activities and the growth inhibition of four lignin-derived compounds on *R. kratochvilovae* EXF7516 were investigated to further characterise the yeasts.

3.2.1 Fermentation in shake flasks on PWS

To obtain a high lipid concentration, a high biomass concentration is the first requirement. Therefore, the yeasts' growth in shake flask fermentations was evaluated.

The evolution of the biomass is shown in **Fig. 2**. All *R. kratochvilovae* strains had a similar growth pattern, with no, or a very short, lag phase. However, *C. oleaginosum* showed a long lag phase, indicating that only this yeasts needed to adapt to the PWS. The growth data were modelled using the logistic equation and the best fits are shown in **Fig. 2. Table 1** presents the obtained growth parameters, i.e., specific maximum growth

278 rates (μ_m), and maximum cell concentrations (N_{max}). The μ_m values of the investigated
279 *R. kratochvilovae* strains (**Table 1**) were up to three times larger than the μ_m of *C.*
280 *oleaginosum*, indicating their faster growth on PWS. Welch's ANOVA also revealed
281 significant differences between the average μ_m's with EXF7516 having a significantly
282 higher μ_m value than EXF11626, EXF3697, EXF3471, and *C. oleaginosum* ($p=0.0172$).
283 The maximum specific growth rates in the microplate growth assay (**Fig. 1a**) were
284 lower than in the shake flask experiment which may be due to the lack of vitamins in
285 the microplate growth assay's medium.

286 Other studies on *R. kratochvilovae*'s μ_m values are unavailable to our best knowledge.
287 However, Singh et al. studied the use of *Rhodosporidium toruloides* 9564^T (current
288 valid taxonomic designation *Rhodotorula toruloides*) for phenol degradation [29]. They
289 found a maximum specific growth rate of 0.0717 h^{-1}. Amirsadeghi et al. studied the use
290 of *Rhodotorula glutinis* ATCC15125 to produce lipids from pulp and paper effluents
291 supplemented with glucose, xylose, or glycerol [30]. They found μ_m values between
292 0.027 and 0.05 h^{-1}. The evaluated *R. kratochvilovae* strains have μ_m values two to four
293 times higher, with the highest value of 0.20 h^{-1} being obtained for *R. kratochvilovae*
294 EXF7516. It can be concluded that the investigated *R. kratochvilovae* strains are fast-
295 growing, which further indicates their suitability for the valorisation of lignocellulosic
296 wastewaters.

297 A maximum cell concentration on PWS (**Table 1**) between 2.9 g/L and 3.75 g/L was
298 reached for all *R. kratochvilovae* strains. ANOVA and Tukey's honestly significant
299 difference procedure showed that *C. oleaginosum* reached a significantly ($p<0.05$)
300 smaller maximum cell dry weight. Between the *R. kratochvilovae* strains, EXF9590,
301 EXF7516, EXF3697, and EXF3471, no significant difference ($p>0.05$) could be found.

Strain EXF11626 reached a significantly smaller maximum cell concentration than strains EXF9590 (p=0.0091) and EXF3697 (p=0.0486).

3.2.2 Growth inhibition by common lignin-derived phenolics

Due to differences in the lignocellulose source and pre-treatment technologies, the composition of phenolic waste streams will differ depending on their origin [4]. Therefore the growth inhibition of four phenolic compounds and the PWS were investigated for the fast-growing *R. kratochvilovae* strain, i.e., EXF7516 and *C. oleaginosum*.

The investigated phenolic compounds were chosen to represent the degradation products of the three monomers present in lignin, i.e. 4-hydroxybenzoic acid and 4-hydroxybenzaldehyde for p-coumaryl alcohol, vanillin for coniferyl alcohol, and syringaldehyde for sinapyl alcohol. In addition, it was chosen to use the phenolics in the aldehyde form, as the first step in their metabolic breakdown is typically the oxidation to the corresponding acid [21]. In addition, 4-hydroxybenzoic acid was included in the test to determine the difference in toxicity between the acidic and aldehyde form of the phenolics. The toxicity of the phenolic waste stream itself was also assessed. The applied concentration ranges were based on the work of Yaguchi et al. [21] with *C. oleaginosum*, and expanded where necessary. To the best of our knowledge, no toxicity values for these compounds are known for any *R. kratochvilovae* strain.

As shown in **Table 2,** the two most inhibitory phenolic compounds for *C. oleaginosum* were vanillin and 4-hydroxybenzaldehyde with an absolute IC50 of 0.26±0.06 g/L, and 0.4±0.6 g/L, respectively. This was followed by syringaldehyde (IC50 = 0.98±0.1 g/L) and 4-hydroxybenzoic acid, with the latter reducing the maximum specific growth rate

by almost 50% at the highest concentration evaluated (4.55 g/L). Higher concentrations of 4-hydroxybenzoic acid could not be investigated due to solubility limits. Interestingly, 4-hydroxybenzaldehyde was more inhibitory than 4-hydroxybenzoic acid. Only a small proportion of 4-hydroxybenzoic acid (pKa = 4.5) was in the protonated form as all the media were adjusted to pH 6.0. Therefore only a small part could diffuse through the cell membrane and cause a toxic effect. In contrast, all molecules of 4-benzaldehyde are always more apolar because they never contain a negative charge.

Yaguchi et al. determined the minimum inhibitory concentrations (MIC) of vanillin and 4-hydroxybenzoic acid to be 4.0 g/L and 0.2 g/L, respectively, for *C. oleaginosum* ATCC20509 [21]. These values are lower than expected from the IC50 values determined in our study. The reason for the reduced growth inhibition in this study is twofold: Firstly, the cells in this study were precultured for longer, i.e., 48 hours instead of 24 hours. After 48 hours of preculture, the cells reached the beginning of the stationary phase. Stationary phase cells have been shown to have increased resistance against various stresses, e.g., heat-shock [31]. Secondly, the pH of the media in the current study was adjusted to pH 6.0, while Yaguchi et al. did not report on the pH of the medium [21]. As mentioned above, the toxicity of 4-hydroxybenzoic acid is pH-dependent, making a direct comparison with the study by Yaguchi et al. difficult [21].

For *R. kratochvilovae,* IC50 values for lignin-derived inhibitors have not yet been published. However, Hu et al. studied the influence of 4-hydroxybenzoic acid, vanillin, and syringaldehyde on the growth and lipid production of *Rhodotorula toruloides* Y4 in a nitrogen-limited glucose medium [32]. Although they did not report IC50 values, Hu et al. observed almost complete growth inhibition at 1.2 g/L of 4-hydroxybenzoic acid and 1.8 g/L of vanillin. However, up to the maximum evaluated concentration of 1.8

g/L, complete growth inhibition by syringaldehyde was not observed. Their findings agree with the results obtained in this work for *R. kratochvilovae* EXF7516, as shown in the E-supplementary data.

When the dose-response curves of *R. kratochvilovae* EXF7516 are compared with those of *C. oleaginosum* (available in the E-supplementary data), it can generally be observed that *R. kratochvilovae* had lower growth rates than *C. oleaginosum*. Nevertheless, when comparing the IC50 values in **Table 2**, *C. oleaginosum* did not always have the highest IC50-value. *R. kratochvilovae* was more resistant to vanillin than *C. oleaginosum*, with an absolute IC50 value of 0.8±0.1 g/L, compared to 0.26±0.06 g/L for *C. oleaginosum*. Syringaldehyde was more growth-inhibiting to *R. kratochvilovae* than *C. oleaginosum*, with absolute IC50 values of 0.5±0.2 g/L and 1.0±0.1 g/L. 4-hydroxybenzaldehyde was equally growth-inhibiting with absolute IC50 values of 0.4±0.2 g/L and 0.4±0.6 g/L for *R. kratochvilovae* and *C. oleaginosum*, respectively. As with *C. oleaginosum*, 4-hydroxybenzoic acid had little inhibitory effect on the growth of *R. kratochvilovae*. The evaluated compounds' concentrations in the PWS were well below the IC50 values. Therefore no growth inhibition by 100% PWS was expected. However, the maximum growth rate decreased at 50 v/v% PWS. The higher-than-expected growth inhibition could be due to a synergistic effect between the inhibitors or other inhibiting compounds, i.e., furans, in the PWS [33]. It is unlikely that the organic acids would cause this toxicity as the PWS was adjusted to a pH of 6.0 [5].

The IC50 values were generally larger than the concentrations of syringaldehyde, vanillin, and 4-hydroxybenzaldehyde found in lignocellulosic hydrolysates [4]. The high IC50 values of the *R. kratochvilovae* strains make them suitable for detoxification,

372 although synergistic effects between the inhibitors may result in higher growth
373 inhibition.

374 *3.2.3 Ligninolytic, xylanase and cellulase activity screening*

375 The presence of ligninolytic and hydrolytic enzymes was investigated to assess the
376 yeasts' ability to degrade the non-monomeric substrates, i.e. lignin, xylan, and cellulose
377 fragments. Hereto, the yeasts were screened for lignocellulosic enzymes on agar plates
378 containing these non-monomeric substrates to evaluate their degradation.

379 Lignin peroxidase activity was observed by destaining of the agar plate for all yeasts
380 evaluated. However, for strains EXF3697, EXF3471, and EXF9590, adding PWS to the
381 agar plate led to increased decolourisation, indicating the PWS contains an inducer or
382 mediator of the enzyme. Also manganese peroxidase activity was detected for each
383 strain. Here, the addition of Mn^{2+}-ions increased the manganese peroxidase activity for
384 EXF3471 and EXF9590. Mn^{2+}-ions have been shown to stimulate manganese
385 peroxidase production by white rot fungi, and might have the same role here [3]. For *C.*
386 *oleaginosum,* adding PWS but no Mn^{2+}-ions, increased the manganese peroxidase
387 activity, indicating the presence of a manganese peroxidase inducer or mediator in the
388 PWS. The presence of manganese peroxidase activity correlates with the work of Yang
389 et al., who isolated *C. oleaginosum* and *Rhodotorula* strains from wastewater [34]. The
390 strains they isolated showed manganese peroxidase activity. The current study detected
391 no laccase activity for any of the investigated yeasts.

392 All yeasts, except *R. kratochvilovae* EXF11626, showed weak cellulase activity. In
393 contrast, weak xylanase activity was detected for all yeasts in this study. The weak
394 xylanase and cellulase activities expressed by the *R. kratochvilovae* strains were

consistent with the literature, where some *Rhodotorula* strains have been reported to possess xylanase [35] and cellulase [36, 37] activities.

3.2.4 Monomeric substrates

Fig. 3 shows the glucose, xylose, formic acid, and acetic acid concentrations over time. For all *R. kratochvilovae* strains, these substrates were mainly consumed during the first 15 hours of the fermentation, while for *C. oleaginosum,* the substrates were mainly consumed between 42 to 91 hours. For both yeasts, this corresponded to the growth phase (**Fig. 2**), as expected.

At the end of the fermentations of the *R. kratochvilovae* strains, the xylose concentration (**Fig. 3b**) started to increase. This is attributed to the hydrolysis of hemicellulose fragments present in the effluent [7], as confirmed by the xylanase activity revealed by the enzyme screening. Similarly, the increase in acetic acid concentration (**Fig. 3d**) for *R. kratochvilovae* strains EXF11626, EXF9590, and EXF7516 could be caused by hydrolysis of hemicellulose [38]. On the other hand, *C. oleaginosum* showed no increase in acetic acid concentration (**Fig. 3d**) and only a moderate increase in xylose concentration (**Fig. 3b**) at the end of the fermentation. The lower increases in xylose and acetic acid concentration, could be due to a faster conversion. The consumption of acetic acid by *C. oleaginosum* is in agreement with the work of Gong et al. They found that *C. oleaginosum* can co-consume glucose, xylose, and acetic acid [39]. The consumption of acetic acid by *R. kratochvilovae*, as observed in this work, has not yet been reported. However, several *Rhodotorula* species are known to metabolise acetate [40, 41].

417 The evolution of the aromatics during the fermentations is shown in **Fig. 4**. For *R.*
418 *kratochvilovae,* the aromatics were mostly degraded during the growth phase (**Fig. 2**),
419 similar to the sugars and organic acids (**Fig. 3**). Similarly, *C. oleaginosum* consumed
420 most of the aromatics during the growth phase. Only furfural and 4-hydroxybenzoic
421 acid were consumed before and after the growth phase, respectively. This indicates
422 sequential consumption of the aromatics by *C. oleaginosum*.

423 In contrast to the other aromatics, vanillic acid (**Fig. 4e**) and syringic acid (**Fig. 4f**) were
424 formed during the fermentation by both *C. oleaginosum* and *R. kratochvilovae*. The
425 formation of these compounds has also been observed for *R. kratochvilovae* EXF7516
426 by Broos et al. and may be linked to the degradation of (oligo)lignin [9, 42].

427 The *R. kratochvilovae* strains significantly reduced the total phenolic concentration
428 from 450 mg/L to as low as 17 mg/L, leaving mainly the newly formed vanillic acid and
429 syringic acid. In contrast, *C. oleaginosum* lowered the concentration of the phenolics to
430 only 257 mg/L. This high residual concentration is mainly attributed to the limited
431 4-hydroxybenzoic acid consumption by *C. oleaginosum,* and its sequential consumption
432 of the aromatics (**Fig. 4d**).

433 Assimilation of aromatics has been observed for many *Rhodotorula* species. *R. glutinis*
434 [43, 44], *R. toruloides* [32, 45, 46], *R. ruba* [47, 48], *R. kratochvilovae* [15, 16] and *R.*
435 *mucilaginosa* [49] have been found to degrade phenolic compounds such as
436 syringaldehyde, p-coumaric acid, ferulic acid, vanillin, vanillic acid,
437 4-hydroxybenzaldehyde, and 4-hydroxybenzoic acid. More specifically for *R.*
438 *kratochvilovae*, Patel et al. found that *R. kratochvilovae* could remove 94% of the
439 phenolics in pulp and paper industry effluent [15]. Furthermore, the yeast strain could

grow on 1 g/L phenol as the sole carbon source [16]. These results are similar to those found in our work, where *R. kratochvilovae* was shown to be a potent phenol degrader.

Yeasts from the *Cutaneotrichosporon genus* are also known to assimilate phenolics. They have been found to grow with phenol, resorcinol, p-coumarate, 4-hydroxybenzoic acid, syringate, or ferulate, as the sole carbon source [17, 45]. Furthermore, *C. oleaginosum* was found to accumulate 69.5% of its cell dry weight as lipids in a fed-batch using resorcinol as the sole carbon source [21]. Yaguchi et al. compared 36 oleaginous yeast strains for their aromatic assimilation. They found that, in general, *Cutaneotrichosporon* yeasts, e.g., *C. oleaginosum*, are best suited for assimilating a broad spectrum of aromatics. However, they did not investigate *R. kratochvilovae*, which was the best aromatic degrader under the conditions evaluated in the current work.

To further compare the yeasts, the specific substrate consumption rates (**Fig. 5**) during the growth phase, i.e., the first 15 hours for the *R. kratochvilovae* strains and between 42 to 91 hours for *C. oleaginosum*, were calculated. The specific consumption rates (**Fig. 5a**) of xylose, 4-hydroxybenzoic acid, formic acid, and acetic acid were higher than those of the other substrates (**Fig. 5b**) because their initial concentration in the PWS was at least ten times higher.

For vanillic acid and syringic acid, the consumption rates were negative because, as mentioned before, they were produced during the growth phase (**Fig. 4e and Fig. 4f**).

The differences in substrate consumption rates of both yeasts were further analysed using ANOVA and a Tukey-Kramer honestly significant difference (HSD) test. For glucose (**Fig. 5b**), 3,4-dihydroxybenzaldehyde (**Fig. 5b**), and 5-HMF (**Fig. 5b**), no

463 significant differences ($p>0.05$) were found between the yeasts evaluated. Xylose was
464 removed faster by *R. kratochvilovae* EXF11626 (q=19.7 mg/(g·h)) (p=0.0334) and
465 EXF3697 (q = 18.8 mg/(g·h)) (p=0.0334), compared to *C. oleaginosum*
466 (q = 7.2 mg/(g·h)). Between the *R. kratochvilovae* strains, no significant differences
467 ($p>0.05$) were found for the specific xylose consumption rate. Furfural was removed
468 significantly faster by the *R. kratochvilovae* strains EXF11626 (p = 0.0351), EXF3471
469 (p = 0.0044), and EXF9590 (p = 0.0316) (q = 0.45 to 0.64 mg/(g·h)), compared to *C.*
470 *oleaginosum* (q = 0.08 mg/(g·h)). No significant differences between the *R.*
471 *kratochvilovae* strains were found for furfural removal ($p>0.05$). Syringaldehyde was
472 also consumed faster by the *R. kratochvilovae* strains ($p<0.04$). *C. oleaginosum* started
473 to consume syringaldehyde in the middle of the growth phase (**Fig. 4h**), whereas the *R.*
474 *kratochvilovae* strains started to consume syringaldehyde immediately. This could
475 explain the slower consumption of syringaldehyde by *C. oleaginosum.*

476 The 4-hydroxybenzoic acid specific consumption rate (**Fig. 5a**) of *C. oleaginosum* was
477 negative, as its concentration increased during the growth phase (**Fig. 3c** and **Fig. 4d**).
478 In contrast, the *R. kratochvilovae* strains removed 4-hydroxybenzoic acid. For formic
479 acid, EXF11626 had a higher consumption rate ($p\leq0.03$) (q = 122 mg/(g·h)) than all
480 other yeast strains (q = 2.83 to 51 mg/(g·h)), except EXF7516 ($p>0.05$)
481 (q = 83 mg/(g·h)). The specific acetic acid consumption rate (**Fig. 5a**) was for *C.*
482 *oleaginosum* significantly ($p<0.03$) lower (q = 25 mg/(g·h)) than all other *R.*
483 *kratochvilovae* strains (q = 66-77 mg/(g·h)), except strain EXF3697 ($p>0.05$) (q = 55
484 mg/(g·h)). No significant differences ($p>0.05$) in the acetic acid consumption rate were
485 found between the *R. kratochvilovae* ($p>0.05$) strains.

3.2.5 Non-monomeric substrates

In contrast to the monomeric substrates, which were generally consumed by all strains, the non-monomeric DOC concentration (data available in E-supplementary data) did not change, indicating that the yeast strains did not consume them. This differs from the work of Broos et al., where both *C. oleaginosum* and *R. kratochvilovae* EXF7516 consumed non-monomeric substrates [9]. The difference can be attributed to a ten times higher cell concentration used in their work. Furthermore, the enzyme screening showed that all yeasts could express ligninolytic and hydrolytic enzymes, indicating that they have the potential to degrade the non-monomeric substrates. Further work should aim to increase the degradation of the non-monomeric substrates, as they account for 85 m/m% of the carbon present in the PWS.

3.2.6 Lipid production

Fig. 6a represents the lipid content reached after six days, at the end of the fermentation. The strain EXF9590 had a significantly lower lipid content than EXF7516 ($p=0.0285$), EXF3471 ($p=0.0059$), and *C. oleaginosum* ($p=0.0182$). No other significant differences ($p>0.05$) were found in the lipid content. For the lipid titre (**Fig. 6b**), it was found that *R. kratochvilovae* EXF3471 had a significantly higher lipid concentration than EXF9590 ($p=0.0075$) and *C. oleaginosum* ($p=0.0254$). Therefore, strain EXF3471 would be preferred for lipid production from the PWS. The lower lipid content than the generally accepted 20 m/m% CDW is due to the low amount (4.2 g/L) of readily degradable monomeric substrates.

The lipid yield obtained on PWS of up to 0.25 g lipid/g carbon consumed, which is equivalent to 0.12 g per g carbon source. The yield obtained in this work is significantly lower than the typical yield of 0.55 g lipid/g carbon (equivalent to 0.22 g lipid/g

glucose) obtained with glucose as a substrate [50]. This may be due to the presence of lignin-derived inhibitors, which increase the energy required for maintenance and thus substrate consumption.

The fatty acid profile of the produced microbial oil is important for its quality and application potential. For *R. kratochvilovae* EXF11626, EXF9590, EXF7516, EXF3697, EXF3471, the lipids consisted mainly of unsaturated fatty acids C18:1 (27 to 64%), and C18:2 (6 to 23%), and the saturated fatty acid C16:0 (13 to 25%), similar to olive oil [51]. While C18:1 was also the most abundant fatty acid for *C. oleaginosum*, in contrast to the *R. kratochvilovae* strains, it produced a significant amount of C17:1 and C18:0. The high levels of unsaturated fatty acids may be a response to the accumulation of reactive oxygen species (ROS) resulting from the presence of the phenolics [16] and other lignocellulose-derived compounds, such as 5-hydroxymethylfurfural [52].

4 Conclusion

R. kratochovilovae is a potent species for the detoxification of phenolic waste streams, as it grew up to four times faster than *C. oleaginosum* on the phenolic waste stream. It also removed the phenolic compounds to a residual concentration of 17 mg/L. Although the microbial oil production was limited, the fatty acid composition of *R. kratochvilovae* was generally similar to that of olive oil, allowing a wide range of applications for the microbial oil. Nevertheless, further process improvements are needed, in particular the use of non-monomeric substrates and phenolic compounds should be increased, as well as microbial oil production.

CRediT authorship contribution statement

Waut Broos: Conceptualization, Methodology, Formal Analysis, Investigation, Data Curation, Writing – Original Draft Preparation, Writing – Review & Editing, Visualisation, Project Administration, Funding Acquisition. **Iris Cornet:** Conceptualization**,** Methodology, Investigation, Writing – Review & Editing, Supervision, Project Administration, Funding Acquisition. **Jan Dries:** Conceptualisation, Supervision. **Siegfried E. Vlaeminck:** Conceptualization, Writing – Review & Editing, Supervision. **Nina Gunde-Cimerman:** Review & Editing, Resources. **Nikolett Wittner:** Methodology.

Appendix A . Supplementary data

E-supplementary data of this work can be found in the online version of the paper.

Data availability

Data will be made available on request.

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703

704 **Fig. legends**

705 **Fig. 1: Growth parameters obtained from growth modelling.** a) Maximum specific
706 growth rate (μ_m), and b) the duration of the lag phase, for the *R. kratochvilovae* strains
707 EXF7516, EXF3697, EXF9590, EXF3471, and EXF11626, at 21.8 to 90.9 v/v%
708 phenolic waste stream. Error bars represent the standard deviation derived from the
709 bootstrap method.

Fig. 2: Growth of *R. kratochvilovae* strains and *C. oleaginosum* on nitrogen-limited PWS. The points indicate the CDW concentration and its standard deviation (technical repeats, n=3). The curve shows the best fit of the logistic equation. The R^2 were all higher than 0.91. Two biological replications were performed with (-●-)replication 1, and (--▲--) replication 2.

Table 1: Growth parameters for growth on PWS: Maximum specific growth rates (μ_m), and b) maximum cell concentration (N_{max}), as estimated by the logistic model. $SD\mu_m$ represents the standard deviation on the μ_m (n = 2), while SD_{Nmax} represents the standard deviation on the N_{max} (n = 2).

Table 2: Absolute half-maximum inhibitory concentrations (IC50) for *C. oleaginosum* and *R. kratochvilovae* EXF7516. All IC50 values are expressed in g/L, unless otherwise noted.

Fig. 3: Sugars and organic acids concentrations with a) glucose, b) xylose, c) formic acid, and d) acetic acid over time. Two biological replications (rep1 and rep2) were performed.

Fig. 4: Concentrations of the aromatics during the fermentation with a) 5-HMF, b) furfural, c) 3,4-dihydroxybenzaldehyde, d) 4-hydroxybenzoic acid, e) vanillic acid, f) syringic acid, g) vanillin, h) syringaldehyde over time. The points represent the average of two biological replicates, with error bars representing the standard deviation.

Fig. 5: Specific consumption rates (q) during the growth phase with a) substrates with a high consumption rate, and b) substrates with a low consumption rate. Red stems represent the standard deviation (n=2).

Fig. 6: Microbial oil compositions and concentrations at the end of the fermentation, after 136.5±1.5 hours with a) the composition expressed as percentage CDW, and b) as lipid concentration (g/L), with (■) triacylglycerides, (■) diacylglycerides, (■) monoacylglycerides, and (■) free fatty acids. The error bars represent the standard deviation (n=2) on the total lipid content.

Tables and Figures

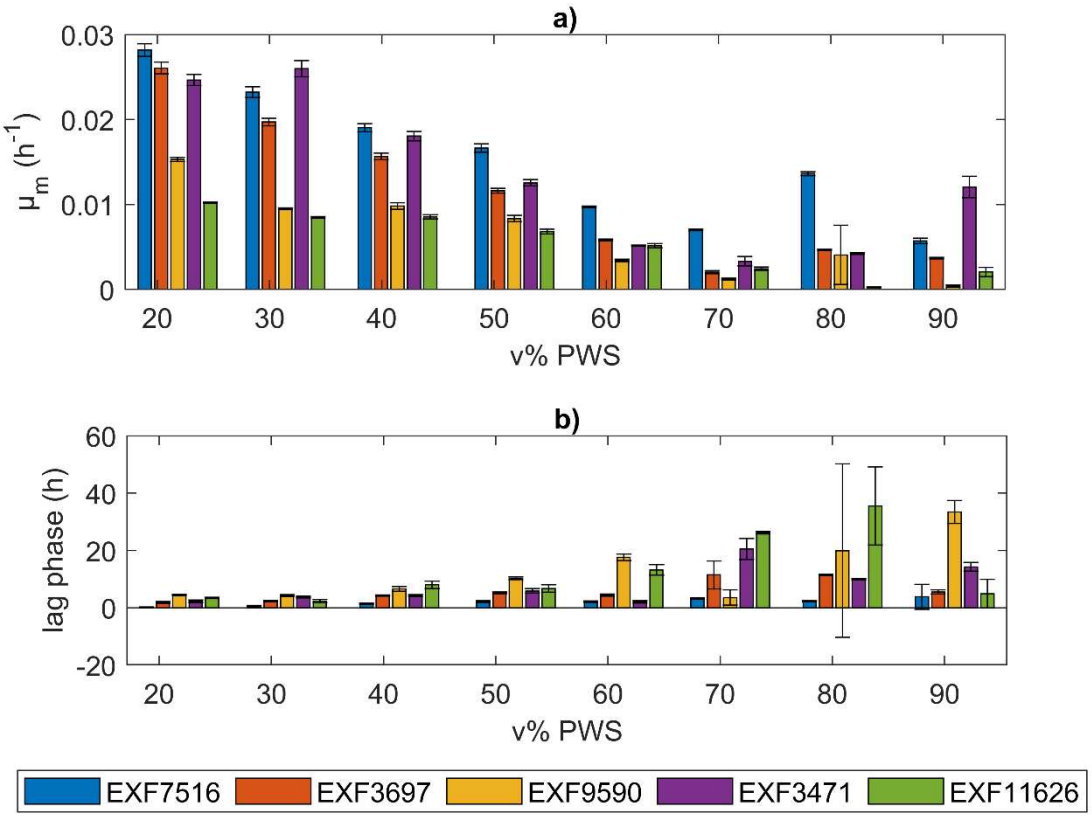
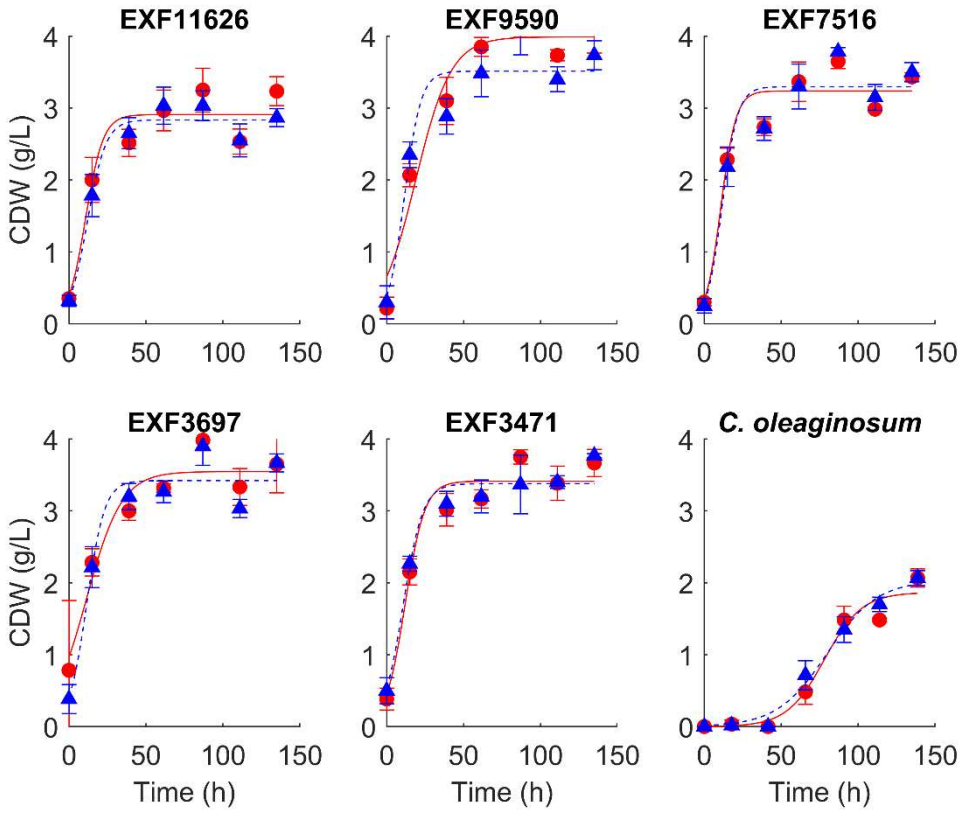


Fig. 1


EXF11626
EXF9590
EXF7516
EXF3697
EXF3471
C. oleaginosum
CDW (g/L)
Time (h)
0
1
2
3
4
0
50
100
150

741

742 **Fig. 2**

Strain	μ_m (h^{-1})	SD_{μ_m} (h^{-1})	N_{max} (g/L)	$SD_{N_{max}}$ (g/L)
R. kratochvilovae				
EXF11626	0.170	0.004	2.87	0.04
EXF9590	0.14	0.05	3.8	0.2
EXF7516	0.199	0.003	3.27	0.03
EXF3697	0.13	0.04	3.48	0.06
EXF3471	0.1559	0.0001	3.40	0.02
C. oleaginosum				
ATCC20509	0.07	0.01	1.95	0.08

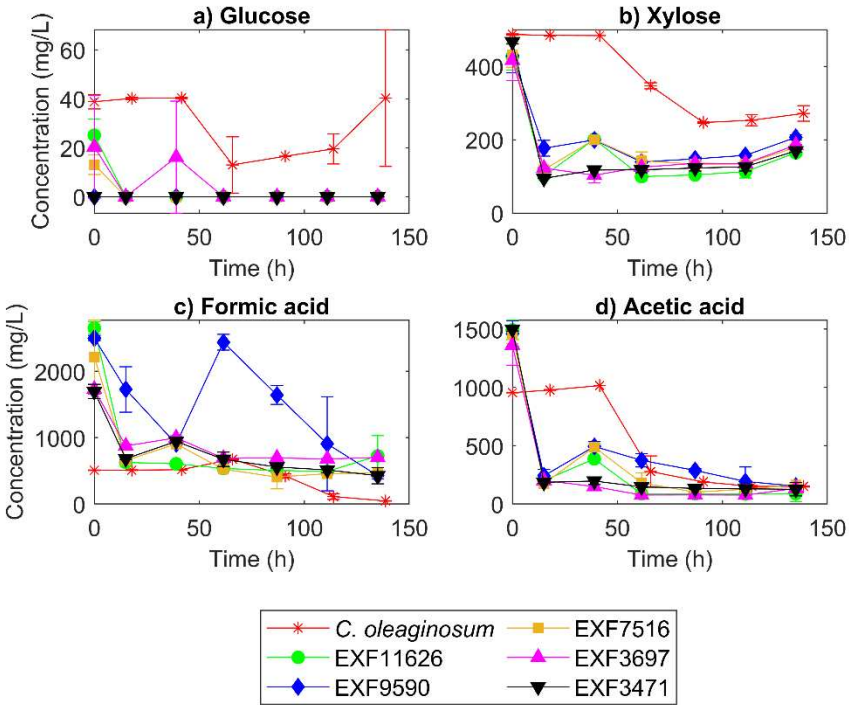
743

744 **Table 1**

Compound	IC50 *R. kratochvilovae* EXF7516	IC50 *C. oleaginosum* ATCC20509
4-Hydroxybenzaldehyde	0.4±0.2	0.4±0.6
4-Hydroxybenzoic acid	>4.55	>4.55
Syringaldehyde	0.5±0.2	1.0±0.1
Vanillin	0.8±0.1	0.26±0.06
PWS	>100%	>100%

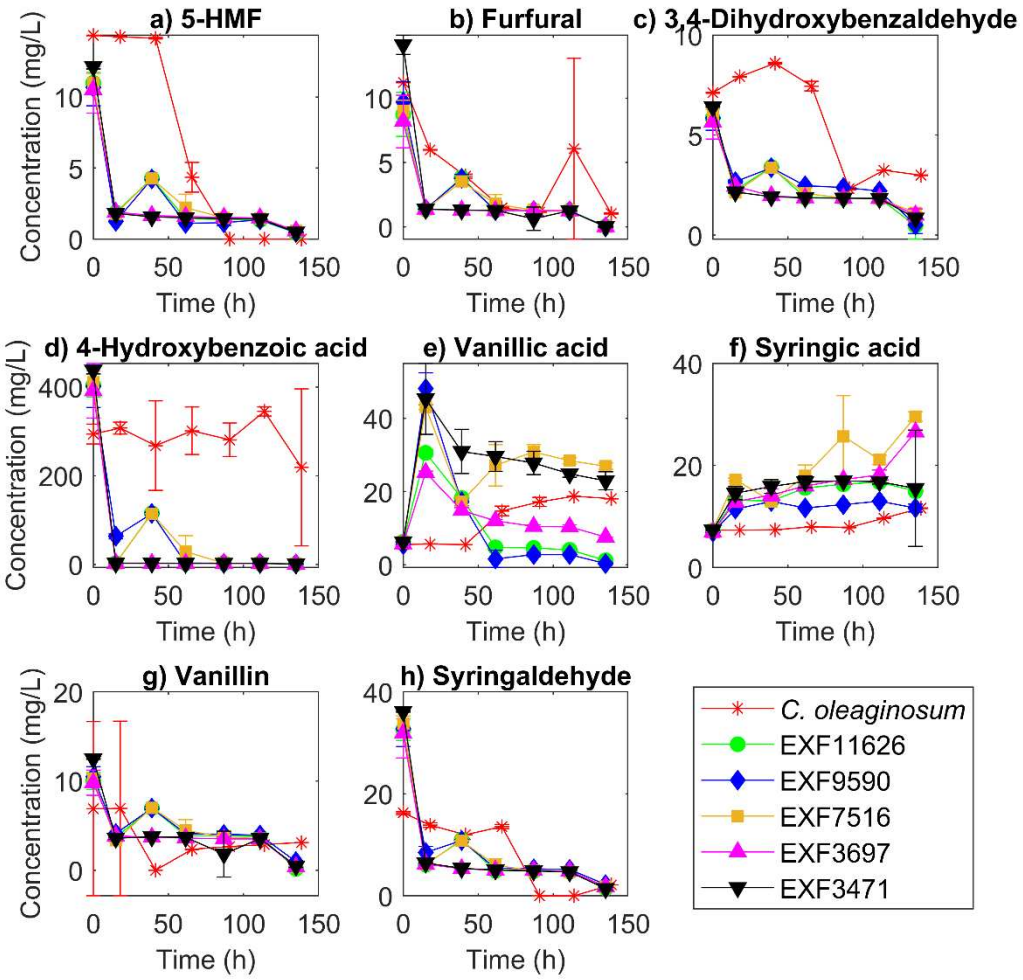
745

746 **Table 2**


a) Glucose
b) Xylose
c) Formic acid
d) Acetic acid
Concentration (mg/L)
Time (h)
C. oleaginosum
EXF11626
EXF9590
EXF7516
EXF3697
EXF3471

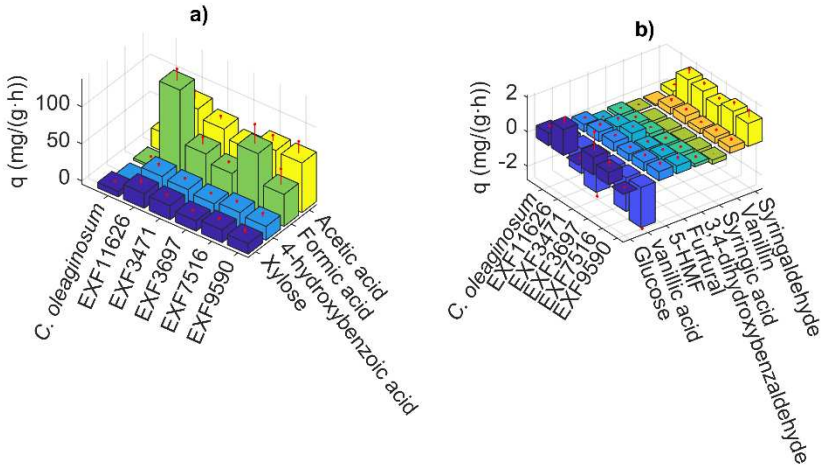
747

748 **Fig. 3**


a) 5-HMF
b) Furfural
c) 3,4-Dihydroxybenzaldehyde
d) 4-Hydroxybenzoic acid
e) Vanillic acid
f) Syringic acid
g) Vanillin
h) Syringaldehyde
Concentration (mg/L)
Time (h)
C. oleaginosum
EXF11626
EXF9590
EXF7516
EXF3697
EXF3471

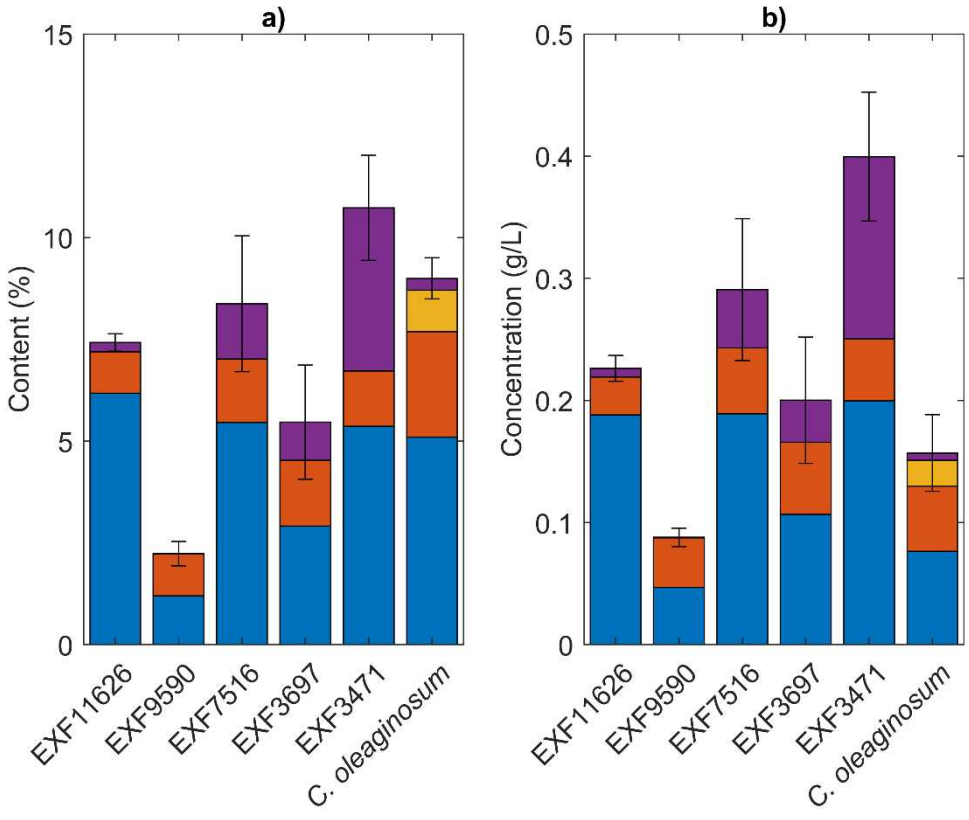
749

750 **Fig. 4**


a)
q (mg/(g·h))
100
50
0
C. oleaginosum
EXF11626
EXF3471
EXF3697
EXF7516
EXF9590
Acetic acid
Formic acid
4-hydroxybenzoic acid
Xylose
b)
q (mg/(g·h))
2
0
-2
C. oleaginosum
EXF11626
EXF3471
EXF3697
EXF7516
EXF9590
Glucose
vanillic acid
5-HMF
Furfural
3,4-dihydroxybenzaldehyde
Syrringic acid
Vanillin
Syringaldehyde

751

752 **Fig. 5**



753

754 **Fig. 6**

755