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The evaluation of oleic acid alternatives for the biochemical production of 9-octadecenedioic acid.

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Highlights

- *Candida tropicalis* needs a long adaptation phase at high ethanol concentrations
- *Candida tropicalis* is severely intoxicated at low butanol concentrations
- Two feasible oleic acid alternatives were found for octadecenedioic acid production
- Butyl esters are rejected as substrates for microbial dicarboxylic acid production

Abstract

Despite considerable foaming problems, oleic acid is still the most commonly used substrate for the biochemical production of 9-octadecenedioic acid (DCA). In this paper, the suitability of alternative substrates such as oleyl alcohol, methyl-, ethyl- and butyl oleate, was investigated. First, the toxicity of the alternative substrates to the fermenting yeast *C. tropicalis* ATCC20962 was determined for different substrate concentrations. It was found that the addition of up to 100 gL^{-1} of the ester and alcohol substrates showed no growth influence. Since alcohols are expected side products when using ester substrates, the toxicity of the corresponding released alcohols was also evaluated. Methanol and ethanol showed no effect on the growth up to 36 gL^{-1} and 55 gL^{-1} respectively, whereas a half maximum inhibitory concentration (IC50) of 1.4 gL^{-1} for butanol was found. Using 20

gL^{-1} oleic acid, oleyl alcohol, methyl oleate and butyl oleate as substrate showed a yield of 14 gL^{-1}, 7 gL^{-1}, 5 gL^{-1} and 0.9 gL^{-1} 9-octadecenedioic acid respectively. A qualitative study showed that all four substrates were converted to 9-octadecenedioic acid. Oleic acid resulted in the highest yield whereas oleyl alcohol is the closest candidate for alternative DCA production.

Keywords

Candida tropicalis; Growth curve modelling; Long chain dicarboxylic acids; oleate esters; oleyl alcohol ; Toxicity tests

1. Introduction

Long-chain dicarboxylic acids (DCA) are high valuable building blocks for today's chemical industry. With a chemical formula of $HOOC(CH_2)_n COOH$, these chemicals are used in the production of numerous products and intermediates, for example, plastics, adhesives, powder coatings, perfumes and greases [1,2]. These industrially interesting compounds can be produced chemically by, for example, metathesis [3] or borane chemistry [4]. However, both ways show to be challenging due to the need for harsh reaction conditions and the formation of side products. A more sustainable approach is the biochemical conversion of an oil substrate to a long chain dicarboxylic acid. Many microorganisms have the enzyme system to oxidise the α,ω-positions of alkanes or fatty acids, i.e., Werner and Zibek published in their review a list of six yeasts that were researched for their long-chain DCA production [5]. Highest production was found when using a genetically modified *Candida tropicalis* ATCC-20962 yeast, which could reach DCA concentrations as high as 210 gL^{-1} when C12 substrates were fed. In this strain, the β-oxidation is blocked, thus preventing the intermediate metabolic degradation of the fatty acids and rendering a highly specific and selective long-chain DCA production [6]. Further improvement was attained by increasing the expression level of the genes coding for the first step in the ω-oxidation [7]. In more recent research several groups are working on

the implementation of the ω-oxidation pathway in microorganisms such as *E. coli* and *S. cerevisiae.* [8]

Additional increases in productivity and dicarboxylic acid concentrations could be achieved by adapting both the operating conditions and fermentation strategy. It is known that a slightly basic pH of the medium stimulates the secretion of DCA by the cell and, therefore, during the biotransformation, as strategy the pH should be kept higher than 8 [8,9,10]. When using the β-oxidation blocked *C. tropicalis* ATCC 20962 strain, it is necessary to supply a source of energy other than the lipids to the fermentation medium in order to keep the metabolism active. Therefore, glucose is usually added in fed-batch mode [10]. Also, the nature of oil substrate has a major influence on the process operation. In the past, several substrates have been evaluated for their bioconversion to long-chain DCA (C12-C22), ranging from alkanes [9], alcohols [11], vegetable oils [12], fatty acids [7], to methyl esters [11] and even epoxidized components [13]. All of these substrates showed satisfactory yields, depending on the starting concentrations of the applied substrate. Investigation of the impact of the substrates' chain length has shown that shorter chains, e.g. dodecanedioic acid, often show toxic effects leading to a decreased dicarboxylic acid production [14]. Longer chains have higher melting points making them solid at the fermentation temperature and therefore less accessible to the yeast. However, good production rates of dicarboxylic acids up to 22 carbon atoms have been reported [6]. During whole-cell biotransformation, the substrate's nature defines the final product which can range from C8 to C22 chain lengths. Unsaturated substrate chains will keep their double bonds resulting in unsaturated DCA molecules which is an extra benefit compared to chemical production where this possibility is lacking [15].

Despite the considerable amounts of research and the recently increased interest from industry for this biochemical process, the separate influences of the substrates and the operating conditions on the cell physiology are not yet fully characterised [16].

The present research has been conducted using the established, commercially available *Candida tropicalis* ATCC 20962 strain with blocked β-oxidation. The focus was on the effect of the difference in functionality at the α-end of the substrate carbon chain on DCA production, instead of the difference in the carbon chain length. Fatty acids are most commonly used in literature, however, as explained above, the bioconversion has to be carried out at a relatively high pH of 8.2. Due to the addition of sodium hydroxide for pH control, excessive foaming occurs when using, for example, oleic acid substrate. Lacking a reactive carboxylic group, oleyl alcohol, methyl oleate or butyl oleate application will circumvent this problem since the acid group is not available for ionic interactions and soap formation. In analogy, these substrates are expected to yield the same DCA product as oleic acid.

Another asset of using ester substrates is their lower melting point. The applied substrates are all in a liquid state at the fermentation temperature (30°C) and, therefore, show good emulsification properties in an aqueous medium. However, saturated or longer carbon chain fatty acids, e.g. stearic acid or erucic acid, have higher melting points (>30°C), which results in problems of clogging and diffusion. Ester substrates might be a solution to these problems as they have lower melting points compared to their fatty acids, e.g., butyl stearate with m.p. 27°C versus stearic acid with m.p. 69.3°C. Therefore, this shows opportunities to enhance the production of DCA from longer or saturated carbon chains. Experimental results on the butyl oleate (melting point of -26.4°C) applied in the present research can provide information on the feasibility of using butyl esters as a substrate.

The obtained yields are investigated in the perspective of the toxicity of applied substrates and their by-products. To our knowledge, this is the first time that the toxic effects of high concentrations of oleyl alcohol or alkyl oleate esters, as well as their corresponding alcohol side products, on the growth of *C. tropicalis* ATCC-20962 were investigated.

2.Materials and Methods

2.1 Culturing

Candida tropicalis ATCC 20962 was stored in 50 v% glycerol at -80°C. A loop was cultured on YPG agar plates containing 10 gL^{-1} of yeast extract, 20 gL^{-1} of peptone, 20 gL^{-1} of glucose and 20 gL^{-1} of agar. After inoculation, the plates were incubated for 2 days at 30°C and afterwards stored at 4°C for a maximum of 30 days. At the start of each experiment, a 100 mL preculture was prepared in YPD medium containing 10 gL^{-1} of yeast extract, 20 gL^{-1} peptone and 20 gL^{-1} glucose. This preculture was taken from the agar plate and was incubated at 30°C and shaken at 200 rpm inside a New Brunswick Innova 400 shaking incubator for 72 hours.

2.2 Toxicity tests

All toxicity tests were performed in 96-well plates using a BioTek Synergy microplate reader set at an orbital shaking speed of 365 cpm (orbit 2mm) and a temperature of 30°C. Every 10 minutes the OD_{600} was measured for each well.

2.2.1 Substrate toxicity tests

Toxicity at concentrations of 0, 10, 20, 40, 60, 80 and 100 gL^{-1} of oleyl alcohol, methyl oleate, ethyl oleate, butyl oleate and oleic acid were evaluated in threefold. To each well, 200 µL of YPD growth medium containing the corresponding concentration of fat substrate was added. Afterwards, 20 µL of the preculture suspension mentioned in section 2.1 was added to each well.

2.2.2 Alcohol toxicity tests

For methanol and butanol, concentrations of 0, 1.8, 3.6, 10.8, 18, 36 and 72 gL^{-1} were tested in threefold, whereas for ethanol additional experiments at 45, 55 and 65 gL^{-1} were performed. To each well the corresponding alcohol amount was added followed by the addition of growth medium (YPD) up to a total volume of 200 µL. Afterwards, 20 µL of cell suspension was added to each well.

2.3 Modelling the toxicity tests

The Baranyi & Roberts sigmoidal growth model [17] (Equations (1) and(2)) was fitted to the measuring points of each growth curve that were obtained from the substrate and alcohol toxicity tests using a nonlinear least-squares regression method of the software package Matlab (MathWorks). This model was chosen for its good description of lag, exponential and stationary growth phase.

$$\frac{dX}{dt} = \mu_{max} \frac{Q}{(1+Q)} \left(1 + \frac{X}{X_{max}}\right) X \qquad (1)$$

$$\frac{dQ}{dt} = \mu_{max} Q \qquad (2)$$

In these equations, X is the cell concentration (OD_{600}) and Q represents a dimensionless physiological state of the cell determining the lag phase. For each growth curve, the parameters maximal growth rate μ_{max} (min^{-1}), the maximal cell concentration X_{max} (OD) and the initial cell concentration X_0 were estimated. During modelling, the Jacobian was applied to calculate the parameters standard deviations for each separate growth curve. A benefit of using this model is the possibility to calculate the duration of the lag phase using Equation (3), which gives insight in whether or not the strain needs to adapt to the potentially toxic compounds.

$$t_{lag} = \frac{1}{\mu_{max}} \ln\left(1 + \frac{1}{Q_0}\right) \qquad (3)$$

In this equation, Q_0 is a parameter also estimated using Equations (1) and (2) representing the value for Q at the start of the fermentation.

With the average maximal growth rates estimated, the toxicity level can be determined using the half-maximal inhibitory concentration (IC50) method [18]. As with the substrates as well as methanol and ethanol, no clear negative effect on the maximal growth rate was observed, only on the experiments with butanol the IC50 was determined. Hereto, the maximal growth rates for the different alcohol concentrations were fitted to Equation (4) where Y (min^{-1}) is the response (specific

growth rate μ_{max}) and X is the alcohol concentration. a, b, c and d are estimated parameters determining the shape of the curve (see Equation (4)).

$$Y = \frac{a-d}{1+\left(\frac{X}{c}\right)^b} + d \quad (4)$$

'a' represents the upper asymptote of the curve (no inhibition) whereas d represents the lower asymptote of the curve (maximal inhibition). 'b' determines the slope of the linear part of the curve and finally c is the IC50.

2.4 Production of dicarboxylic acids

The DCA production fermentations were carried out in a sparger-aerated stirred Sartorius Biostat B-plus fermenter with a volume of 2 L and a fermentation strategy based on Lu et al. [13]. At the start of each experiment, 1 L of fermentation medium containing 20 gL^{-1} glucose, 3 gL^{-1} peptone, 6 gL^{-1} yeast extract, 7.2 gL^{-1} K_2HPO_4 and 9.3 gL^{-1} KH_2PO_4 was applied. 100 mL preculture was added to this medium. During the fermentation, the pH was controlled automatically using a 4M KOH solution. Since the pH only decreased naturally during the fermentation by consumption of glucose and production of DCA, no acid solution was necessary for pH regulation. During the first 24 h of fermentation, the pH was maintained at 6.5 (growth phase) and, after addition of 20 gL^{-1} fat substrate, adjusted to 8.2 during the conversion phase. The remaining settings of the fermenter were as follows: aeration 1.5 L/min (0.75 vvm), stirrer at 600 rpm, a temperature of 30°C. Daily, 20 mL of a 500 gL^{-1} glucose solution was added as a co-substrate to maintain the metabolism of the yeast. Samples were taken regularly and in triplicate. The experiments were carried out until no more substrate was present or when the increase of the DCA concentration was negligible.

2.5 Analysis of fermentation samples

All measurements were performed in threefold and the mean values and standard deviations were calculated.

Growth was monitored by measuring the dry cell weight. Hereto, 1 mL of sample was added to a dry and weighed microcentrifuge tube and centrifuged at 2500 g for 3 minutes. The supernatant was used for glucose measurement and the pellet was washed with demineralised water followed by a second centrifugation and washing step with ethyl acetate. After a third centrifugation step, the centrifuge tube was placed inside an oven at 120°C and dried for at least 5 hours. The dried samples were then weighed again and the weight of the empty centrifuge tube was subtracted resulting in the dry cell weight.

To measure the glucose consumption, the supernatant of the first centrifugation was filtered through a 0.2 µm Nylon filter before HPLC analysis. The HPLC system was an Agilent 1260 Infinity system using an Agilent HiPlex H ion exchange column and a refractive index detector. The elution method was isocratic with an 8 mmol/L H_2SO_4 solution in water and a flow rate of 0.6 mL/min.

GC-MS was used for both quantitative and qualitative analysis of the oil substrate and formed DCA products. 1 mL of the fermentation medium, including cells, was acidified to pH 2 using 35 % HCl and then extracted twice with 1 mL diethyl ether. Afterwards, the solvent was evaporated leaving a residue of oil substrate and DCA. To this residue, 0.5 mL of a 2 M H_2SO_4 in methanol solution was added as well as 0.5 mL of hexane and was vortexed for 10 minutes at 2500 rpm. Afterwards, the hexane layer was transferred to another tube and again 0.5 mL hexane was added to the residue mixture and vortexed for 10 minutes. The hexane layers were combined, now resulting in 1 mL of hexane containing methyl esters of the oil substrate and formed DCA. Next, the samples were diluted, spiked with methyl laurate as internal standard and analysed on GC-MS. The GC-MS system was a Shimadzu GC-2010 gas chromatograph equipped with a Phenomenex ZB-5MS column coupled to a GCMS-QP2010S mass spectrometer.

For infrared analysis, a Bruker LUMOS FT-IR ATR spectroscope was used. The purified samples were placed on the ATR diamond and measured at a frequency between 500 and 4000 cm^{-1}.

H-NMR was performed on the 9-octadecenedioic acid produced from the oleic acid substrate to confirm its structure. The equipment used was a Brucker NMR at 400 MHz.

2.6 ANOVA analysis

As the main goal of this paper is to compare the use of different fat substrates for the production of octadecenedioic acid (DCA), the obtained data and derived model parameters for the substrate need to be evaluated for significant differences. Therefore, an analysis of variance (ANOVA) was performed using Matlab (Mathworks) engineering software. All ANOVA results can be found in Tables S2-S10 in the supplementary information. The estimated specific growth rates (μ_{max}) and estimated X_{max} values pictured in Figure 1f and Figure 1g respectively were subjected to a two-way ANOVA to investigate whether or not the used substrate, the substrate concentration or the interaction between these two factors has a significant influence on the parameters μ_{max} or X_{max}. Growth curves of five different substrates at seven different oil substrate concentrations were evaluated in threefold, resulting in a total of 105 experiments and as many estimated μ_{max} and X_{max} values (as described in the modelling section 2.3). As these values are estimated model parameters, they also contain a standard error which has to be taken into account. Therefore to each value, a weight factor was given using the inverse variance weighting method. Here the weight factor is the inverse of the standard error, therefore values with a high standard error will carry less weight in the ANOVA whereas values with lower standard errors carry more weight.

To the data pictured in Figure 3b and Figure 3d, a one-way ANOVA was performed for both μ_{max} and lag time (t_{lag}) values. Here the same inverse variance weighting method was used. The data pictured in Figure 3f were not evaluated using ANOVA. They were fitted to the IC50 model structure (Equation 4) with estimated parameters and calculated standard deviations. The simulated model was plotted and a clear influence of the inhibitor concentration could be observed.

3 Results and discussion

3.1 Toxicity of the substrates

As the *C. tropicalis* ATCC-20962 yeast is genetically engineered and not able to perform β-oxidation, the fat substrates cannot be used as a carbon source for growth in cell count, therefore, only a minor effect on the growth rate is expected. However, at high concentrations, the substrates might become toxic, or cause a higher medium viscosity and lower oxygen solubility, thereby influencing the growth rates. To reveal these influences, growth curves were measured for different substrate concentrations and were fitted by the Baranyi and Roberts growth model (Equations 1 and 2). As plotting all measured data points would lead to unreadable figures, only a selection of measured data points and obtained growth curves after fitting is pictured in Figures 1a to 1e. It has to be noted that the used substrates are not soluble in water which can cause higher turbidity leading to a biased OD_{600} measurement. To evaluate if this is the case ANOVA was performed on the measured starting OD values of each experiment. Here the null hypothesis is that all values have the same mean and thus there is no influence by the substrate type or the substrate concentration on the OD. The obtained p-values for the substrate type, the substrate concentration and the interaction between the two are all higher than 0.05 (Table S2), therefore the null hypothesis is true and there is no significant effect on the OD measurement. For all experiments, typical growth curves were obtained, and, as can be observed in Figure 1a to 1e, the model describes the data very well. Considering biological variability, no major differences in the growth rates are observed for the different concentrations per substrate. The duration of the lag phases also did not increase for higher substrate concentrations, meaning that no adaptation period is needed at high substrate concentrations. One exception is the curve at 100 gL^{-1} of ethyl oleate in Figure 1d which is remarkably higher located than the lower substrate concentrations.

The small differences between the different growth curves are also reflected in the estimated values for the specific growth rate μ_{max} and maximal cell concentration X_{max}, which are pictured as a function of the substrate concentrations (Figure 1f and Figure 1g, respectively). In Figure 1f, it can be

observed that, at first sight, all experiments show similar specific growth rates, even at high substrate concentrations. To evaluate whether the used substrate and its concentration have a significant effect on the μ_{max} value, a two-way ANOVA was performed. Here the first null hypothesis was that there is no significant influence on the μ_{max} caused by the type of substrate. A second one was that there is no significant influence caused by the substrate concentration and a third one is that there is no significant influence caused by the interaction of the substrate type and the concentration. As for all variables (type of substrate, substrate concentration and the interaction) the p-values are lower than 0.05 (Table S3), the null hypotheses are false and it can be concluded that there is a significant effect on the μ_{max}. When comparing the X_{max} values (Figure 1g), again slight differences between the substrates at high concentrations can be observed. ANOVA again proved a significant influence of the applied substrate, the substrate concentration and the interaction between these two factors (Table S4). For oleic acid and butyl oleate the maximal OD (X_{max}) decreases with higher substrate concentrations whereas, for methyl oleate and ethyl oleate, a slight increase can be observed. From these toxicity tests, it can be concluded that the used substrate, as well as the substrate concentration, show a significant influence on both the maximum specific growth rate and the maximum cell concentration. One of the main reasons that cause this effect will be the change in oxygen solubility at higher substrate concentrations which is dependent on the viscosity of the substrates. As these experiments are carried out in microplates no oxygen control could be applied, therefore the dissolved oxygen will be different for each experiment. The main conclusion of these experiments, however, is that yeast growth is still possible at high substrate concentration opening up the possibility of using up to 100 gL^{-1}, in order to obtain high concentrations of produced DCA. Currently, the highest concentration of the dicarboxylic acid is achieved by using fed-batch methods, as performed by Funk et al. [10]. They produced 42 gL^{-1} DCA by feeding 1$g(Lh)^{-1}$ of oleic acid during the fermentation. The results in the present paper show that the substrate might also be added at high concentrations in batch mode without negative effects on the growth. Although these findings are very interesting, further investigation at higher concentration is not part of this paper. In the next

part, the toxicity of the formed alcohol side products when using methyl ester substrates will be under study.

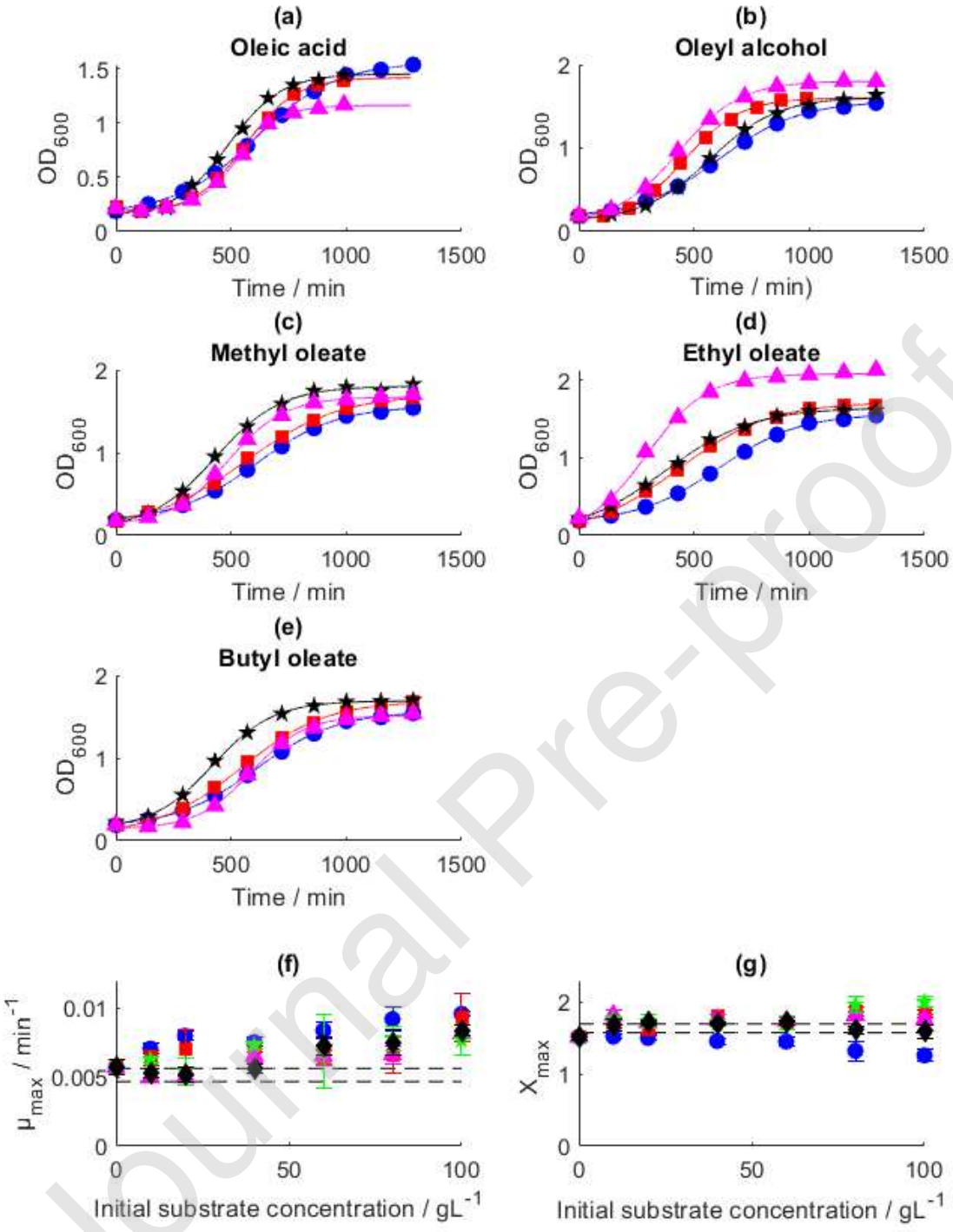

Figure 1: Selected growth curves of toxicity tests using oleic acid (a), oleyl alcohol (b), methyl oleate (c), ethyl oleate (d) and butyl oleate (e) for substrate concentrations of 0 gL^{-1} (●), 20 gL^{-1} (■), 60 gL^{-1} (★) and 100 gL^{-1} (▲). More concentrations and data points were measured than pictured here. The full lines on graphs (a),(b),(c),(d) and (e) are simulations of the fitted Baranyi & Roberts model. Estimated parameters μ_{max} (f) and X_{max} (g) for oleic acid (●), oleyl alcohol (■), methyl oleate (▲),

ethyl oleate (★) and butyl oleate (♦) for all performed experiments. The dashed lines in (f) and (g) represent the 95 % confidence interval of the estimated mean in the ANOVA study for the hypothesis that the parameters are not different.

3.2 Toxicity of the formed alcohols when using ester substrates

As will be mentioned later in Section 3.4, it was observed that *C. tropicalis* converts ester substrates into dicarboxylic acids, meaning that, at some point in the reaction pathway, the ester bond has to be hydrolysed into a fatty acid and an alcohol, the latter being a potential inhibitor. From literature it is known that *C. tropicalis* is able to produce lipases able to hydrolyse ester bonds [19]. This lipase catalysed reaction is also illustrated in Figure 2. Sugiharto et al. tested methyl decanoate as a substrate for the production of sebacic acid [20]. In their research, the hydrolysis is mentioned but the effect of the methanol is not investigated. Further on, James et al. proved the ability to hydrolyse butyl esters by *Candida* lipases [21]. Therefore, in the research presented here, the second set of experiments was performed investigating the inhibition and/or toxicity of the corresponding released alcohols of methyl oleate, ethyl oleate and butyl oleate, i.e. methanol, ethanol and butanol respectively, at different concentrations. A selection of the measured data points and fitted growth curves are pictured in Figures 3a, 3c and 3e. From Figure 3a, it can already be noticed that methanol seems to have no impact on the yeast growth which is also reflected in the estimated parameter μ_{max} and the calculated duration of the lag phase t_{lag} (see Figure 1b). The experiment where no methanol was added showed a specific growth rate of 0.014 ± 0.001 min^{-1} whereas the experiment with the highest concentration (72 gL^{-1}) still shows a μ_{max} of 0.012 ± 0.001 min^{-1}. To confirm if these values show significant differences, ANOVA was performed and it was found that increasing methanol concentrations showed a significant decreasing influence on the μ_{max} (Table S5). To evaluate up to which measured concentration there is no significant effect on the μ_{max} the values at the highest methanol concentrations were excluded from the ANOVA. When excluding the estimated μ_{max} values at 72 gL^{-1}, a p-value higher than 0.05 for the influence of the concentration was found (Table S6)

making this not significant. Therefore it can be concluded that there is no significant influence of methanol on the μ_{max} up to 36 gL^{-1}.

Investigation of the yeasts' adaptation time necessary before reaching the exponential growth phase by using Equation (3) provides a t_{lag} of 350 ± 24 min at a concentration of 72 gL^{-1} methanol, whereas this is only 230 ± 9 min when no methanol is added. ANOVA revealed that the influence of the methanol concentration on the duration of the lag phase is significant (Table S7). Considering the data pictured in Figure 3b, it seems that only the t_{lag} at 72 gL^{-1} is much higher compared to the other methanol concentrations. Therefore a second ANOVA was performed (Table S8), now excluding the three repeats at the highest methanol concentration. Interestingly there is no significant effect of the methanol concentration up to 36 gL^{-1}. A similar effect is observed in Figures 3c and 3d for the ethanol experiments where the μ_{max} and t_{lag} show small fluctuations up to an ethanol concentration of 55 gL^{-1}. It was determined that the estimated μ_{max} at 35.8 gL^{-1} was an outlier. For ethanol, the growth rate starts to decrease from 0.013 ± 0.001 min^{-1} to 0.007 ± 0.005 min^{-1} and the t_{lag} increases from 300 ± 15 min to 800 ± 127 min between a concentration of 55 gL^{-1} and 72 gL^{-1} respectively. After ANOVA it was found that the increasing ethanol concentration has a significant effect on both maximum specific growth rate (Table S9) and lag time (Table S11). From the data pictured in Figure 3d it is clear that the two last points (65 and 72 gL^{-1}) show a lower μ_{max} and a much higher t_{lag}. Therefore a second ANOVA was performed excluding these points. It was found now that up to 55 gL^{-1}, ethanol does not cause a significant effect on the μ_{max} (Table S10) or the t_{lag} (Table S12). The absolute maximal inhibition, i.e. no growth, was not obtained during these experiments. Therefore, the IC50 model could not be applied. As the lag time increases upon increasing ethanol concentration, this means that a longer adaptation period is necessary before the exponential growth phase is achieved. However, as the maximal specific growth rate is lower at these higher ethanol concentrations it can be concluded that there is still a toxic effect on the growth caused by ethanol which cannot be solved by a longer adaptation period. For butanol, presented in Figure 3e, a clear negative effect on the growth curve at low concentrations of 1.8 gL^{-1} can be observed. From a

concentration of 3.6 gL^{-1} on, little to no growth is observed which is also reflected in the estimated μ_{max} and t_{lag} (see Figure 3f). Both upper (no inhibition) and lower plateau (maximal inhibition/no growth) was measured here, therefore, the IC50 could be calculated and was determined at 1.42 ± 0.21 gL^{-1} of butanol. The other estimated parameters a, b and d of the IC50 model are presented in the supplementary information (Table S1).

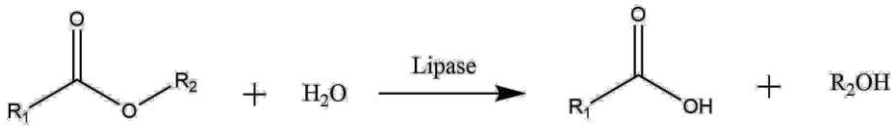

Figure 2: Lipase catalysed ester hydrolysis as performed by C. tropicalis

Butanol is clearly the most inhibiting compound for yeast growth as no growth was observed from an alcohol concentration higher than 3.6 gL^{-1} even after a very long adaptation period of more than 1000 min. In literature, this is also reported for other yeast strains, e.g. *Candida albicans* [22]. A possible explanation for the acute toxicity of butanol could be the fact that, due to its apolar nature, butanol can partition into cytoplasmic membranes and change the fluidity and normal functions of this membrane [23]. Methanol and ethanol, being more polar, take longer to act in the same way. Literature reveals several *Candida* strains that show the possibility to assimilate methanol [24], thereby producing alcohol oxidase and catalase, enzymes both also found in *Candida tropicalis,* possibly explaining the high tolerance for methanol and ethanol [25]. However, more research on this topic would be required.

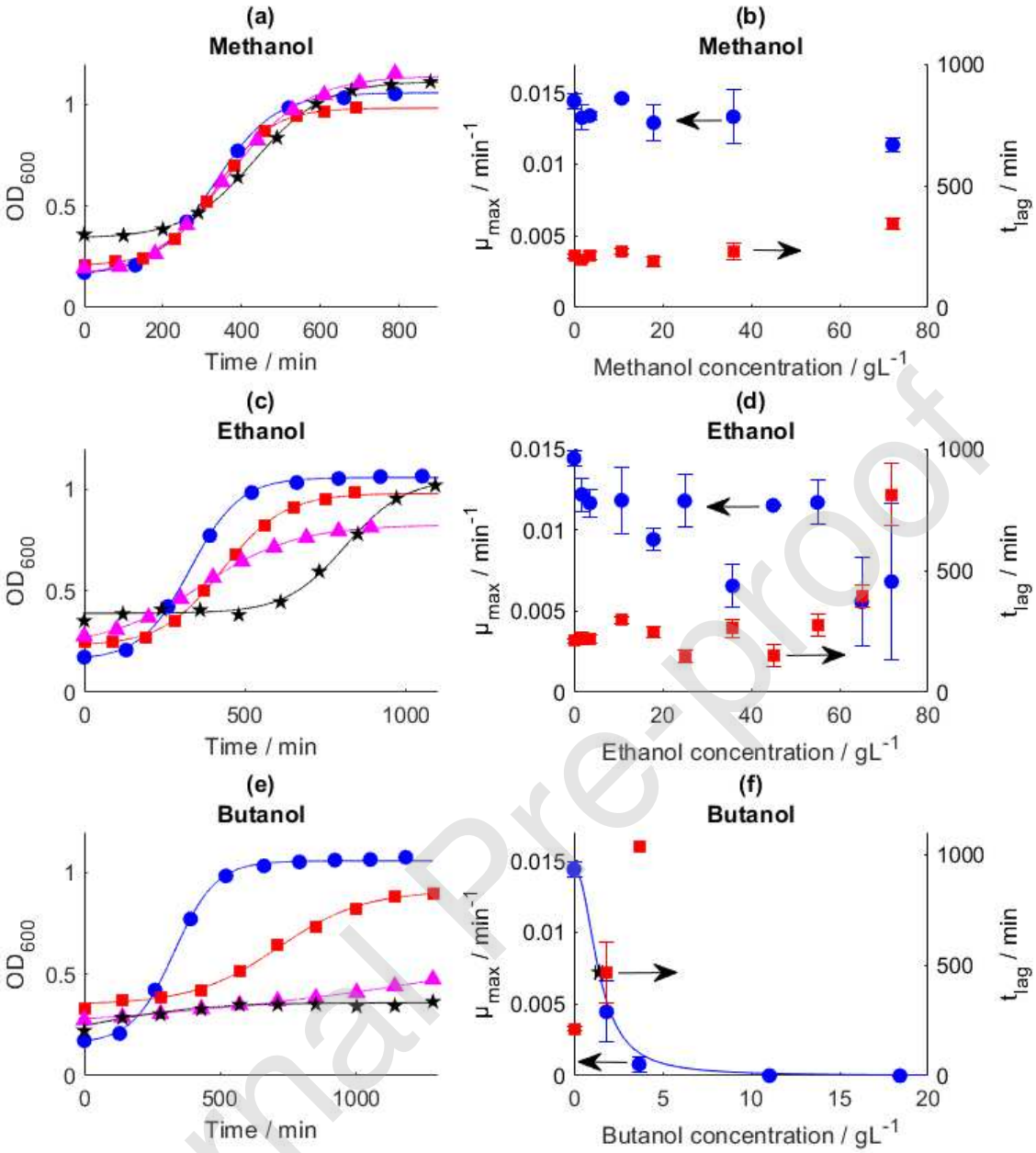

Figure 3: Measured growth curves during toxicity tests using methanol (a), ethanol (c) and butanol (e) as inhibitor. On graph (a) and (c) results for 0 gL^{-1} (●), 10.8 gL^{-1} (■), 36 gL^{-1}(▲) and 72 gL^{-1} (★) are pictured whereas on graph (e) results for 0 gL^{-1} (●), 1.8 gL^{-1} (■), 3.6 gL^{-1} (▲) and 10.8 gL^{-1} (★) are pictured, more concentrations and data points were obtained than shown. The full lines on graph (a),(c) and (e) are simulations of the fitted Baranyi & Roberts model. Estimated specific growth rate (μ_{max}) (●, left axis) and calculated lag phase (■, right axis) are pictured for methanol (b), ethanol (d) and butanol (f). On graph (f) the full line represents a simulation of the fitted IC50 model.

3.3 DCA production fermentations

DCA production fermentations were carried out using a starting concentration of 20 gL^{-1} oleic acid, oleyl alcohol, methyl oleate or butyl oleate. Ethyl oleate was not evaluated as not enough substrate was available. The evolutions of substrates, cells and DCA product are represented in Figure 4. As the experiments have different running times, the final DCA concentration was divided by the elapsed bioconversion time to calculate the productivity (Table 1). ANOVA was performed on the different productivity values and resulted to be significantly different (Table S13). Using this parameter, a good comparison between the experiments can be made.

After the initial growth phase of 24 h on glucose alone, all substrates lead to the production of DCA. Comparing the different substrates, the experiment with oleic acid substrate, presented in Figure 4b, resulted in the highest DCA concentration (13.6 gL^{-1}) after 48 h of bioconversion time, therefore also resulting in the highest productivity (0.28 $g(Lh)^{-1}$). Therefore, oleic acid is kinetically the most suitable for this production process. Lu et al. (2009) showed slightly higher results as they produced 17.3 gL^{-1} of DCA after 48h of bioconversion using the same yeast, substrate concentration and fermentation strategy [13]. As mentioned in Section 3.1, higher DCA concentrations were obtained using fed-batch strategies [10]. However, at a relatively low initial substrate concentration of 20 gL^{-1}, foaming was already observed. This problem will only worsen when increasing the substrate concentration to industrially relevant numbers, e.g. 100 gL^{-1}. Compared to a DCA titer of 13.6 gL^{-1} for oleic acid, using oleyl alcohol (Figure 4a) results in a significantly lower final DCA concentration of 7.1 gL^{-1} after 96 h of bioconversion, thus a productivity of 0.07 $g(Lh)^{-1}$. A positive point here is that no foaming was observed, as was the case when using oleic acid. The experiment using methyl oleate (Figure 4c) results in a DCA concentration of 5 gL^{-1} after a bioconversion time of 72h, a productivity of 0.07 $g(Lh)^{-1}$, which is a slightly lower titer but a similar productivity compared to oleyl alcohol. Therefore, also methyl oleate remains a feasible alternative for oleic acid. However, the experiment using butyl oleate as a substrate (Figure 4d) shows a totally different results, which was to be expected from the toxicity tests, as after 176 h of bioconversion only 1 gL^{-1} DCA was produced, which equals a

disappointing productivity of 5.30E-3 $g(Lh)^{-1}$. Therefore, butyloleate substrate is not suitable for industrial production. For both ester experiments no foaming was observed.

The slower reaction rate when using oleyl alcohol compared to oleic acid can be explained by two effects. The first one is a slower uptake of the oleyl alcohol by the cell compared to oleic acid. It is known that *C. tropicalis* is able to use either passive transport or protein-mediated transport systems for oleic acid depending on the substrate concentration [26]. Possibly oleyl alcohol can only profit from the passive transport systems as no oleyl alcohol transport proteins might be present, however, no proof for this was found in literature or this study. A second reason can be the fact that extra oxidation of the alcohol group is necessary when using oleyl alcohol, thus adding an extra step to the reaction pathway, slowing down the production.

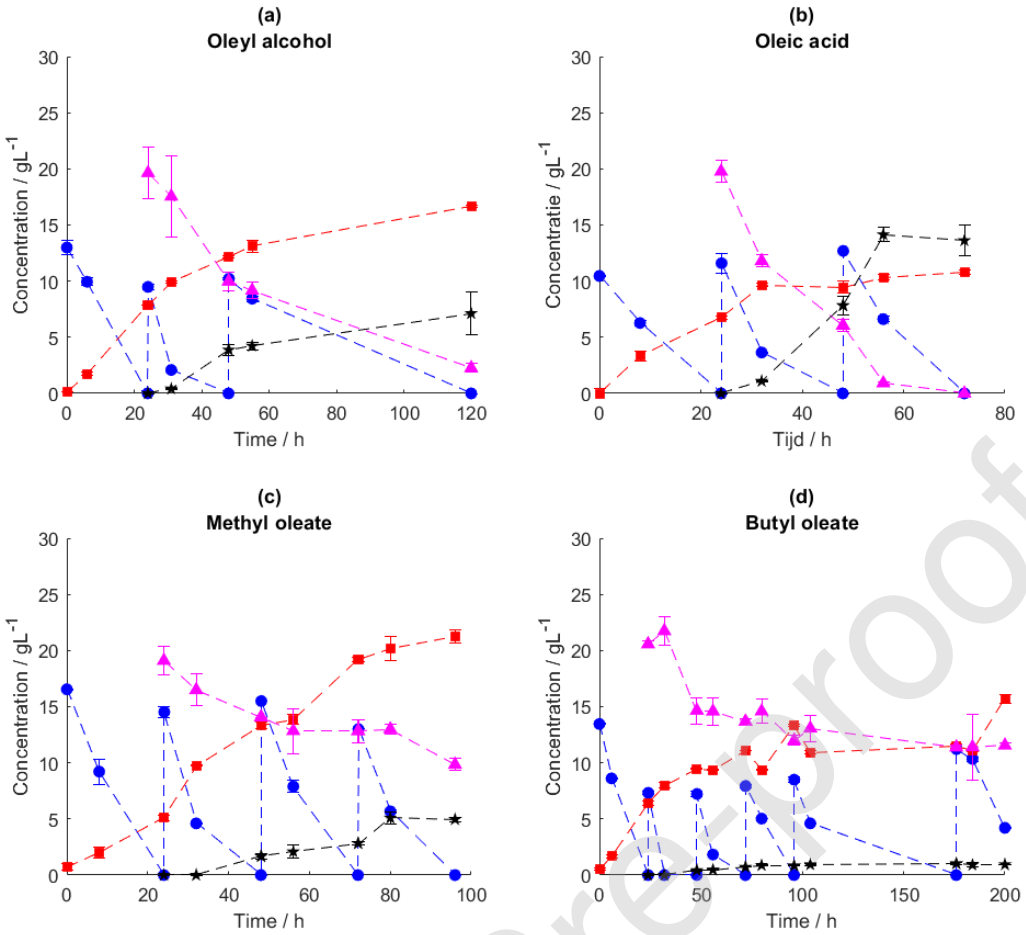

Figure 4: Concentrations of glucose (●), dry cell weight (■), oil substrate (▲) and DCA (★) product for the experiments with (a) oleyl alcohol, (b) oleic acid, (c), methyl oleate and (d) butyl oleate substrates.

According to the vast study of Rapp et al. *Candida tropicalis* is able to produce extracellular lipases and shows a good affinity towards triacylglycerides (TAG) [27]. Therefore the ester substrates will be subjected to extracellular hydrolysis prior to cell uptake. The result of this enzyme catalysed process will be an alcohol and a fatty acid, i.e. oleic acid (Figure 2). Since the released methanol, when using methyl oleate, shows no toxicity towards the yeast at the applied substrate concentrations, the released oleic acid profits from the same uptake and conversion rate as when actual oleic acid is fed as a substrate. From the butyl oleate consumption, the released butanol concentration was calculated to be 2 gL^{-1} which is higher than the IC50, thus resulting in severe intoxication of the yeast. This explains the low final DCA concentration of 1 gL^{-1}.

Extra remarks have to be made when looking into the mass balances of the different fermentations. Hereto, the initial and final experimental data of the four fermentations are applied for calculations for the yield factor using Equation (5) (see Table 1).

$$Yield\ factor = \frac{Produced\ DCA}{Consumed\ Substrate} \quad (5)$$

While it would be expected that the conversion of fatty acids equals the molar amount of DCA produced, the data show that 17.3 gL^{-1} of oleyl alcohol is converted into 7.1 gL^{-1} DCA. When taking into account the molar weights of oleyl alcohol and DCA, respectively 268.5 and 312.5 g/mol, a molar yield factor of 0.35 is found, meaning that only 35 % of the consumed oleyl alcohol will have been converted in DCA. As the yeast is not able to use hydrocarbons as an energy source, it could not have been degraded in the β-oxidation. According to Funk et al., the yeast is capable to store hydrocarbon substrates as TAG in lipid bodies during the DCA fermentation, which explains this observation [10]. In the same way, oleic acid (molar weight of 282.5 g/mol) will yield in 79 % conversion into DCA, which is in a similar range as the 70 % conversion that was described by Funk et al. for the same substrate [10]. Funk et al. concluded that increasing the feed rate of the oil substrate resulted in higher product yield. Our research proves that instead of a high rate addition rate, a batch addition results in an even 8% higher molar yield; When calculating the molar yield of DCA from the amount of consumed ester substrate, 0.55 (55 %) was found for methyl oleate and 0.10 for butyl oleate. ANOVA indicated that these yield factors for the different substrates are significantly different from each other (Table S14). The clear difference between the oleic acid and the other substrates leads to a prudent conclusion that more difficult substrates (like alcohols and esters) are less likely to be converted to DCA but will more likely accumulate inside the cell. Further on, it can be concluded that methyl oleate is the second most efficient substrate next to oleic acid.

Table 1: Overview of the initial and final data for the different substrates

	Unit	Oleyl alcohol	Oleic acid	Methyl oleate	Butyl oleate
Bioconversion time	h	96	48	72	176

DCA Productivity	$g(Lh)^{-1}$	0.07 ± 0.02	0.28 ± 0.03	0.07 ± 3.5E-3	5.30E-3 ± 0.20E-3
Yield factor		0.35 ± 0.14	0.79 ± 0.09	0.55 ± 0.16	0.10 ± 3.50E-3
Released alcohol calculated from substrate consumption	gL^{-1}	-	-	1.0 ± 0.2	2.0 ± 0.1

3.4 Qualitative analysis of the formed compounds

The formed 9-octadecenedioic acid produced from oleic acid was analysed using NMR to confirm its structure. Afterwards, GC-MS and FT-IR analyses were performed of this product as well as the fermentation products of the other oil substrates. In this way, it was confirmed that all used substrates resulted in the same desired product.

3.4.1 H-NMR analysis

The formed product from the oleic acid fermentation was analysed using H-NMR resulting in the following peaks: ^{1}H NMR (400 MHz, $CDCl_3$) δ: 5.35 (2H, t, J = 4.7 Hz), 2.35 (4H, t, J = 7.3 Hz), 2.00 (4H, dd, J = 5.6 Hz, J = 5,6 Hz), 1.64 (4H, m), 1.31 (16H, m). This is in accordance with the spectra found in other studies indicating that the produced compound is 9-octadecenedioic acid [28]. According to Warwel et al. the peak at δ=5.35 corresponds to the hydrogen atoms on carbon atom 9 and 10 (=C-) indicating that the double bond is still present [29].

3.4.2 GC-MS analysis

The purified products from each fermentation were derivatised to methyl esters and analysed by GC-MS. As all of the obtained mass spectra show high resemblance to the spectra found by Yi et al., this gives further certainty that the formed products are all the same 9-octadecenedioic acid molecules. The mass spectra are available in the supplementary information [30]. Typical high intensity peaks at m/z 41, 55, 67, 81, 248 , 276, 290 and 308 were observed in the spectra next to a low intensity peak at m/z 340, which equals the molar mass of 9-octadecenedioic acid dimethyl ester. The fact that all

mass spectra are the same confirms that the different substrates all produce the same dicarboxylic acid.

3.4.3 FT-IR

Since for FT-IR no derivatisation is needed, this might give a better look into the functional groups present in the formed products. All references in this section are from Silverstein and Webster [31]. When the infrared spectra of oleyl alcohol and the formed product from this substrate are compared (Figure 5a), it can be observed that a large peak around 1750 cm^{-1} is present in the spectrum of the formed product which is typical for the C=O stretch. Furthermore, a broad band, ranging from around 3300 to 2500 cm^{-1}, is observed which is caused by an O-H stretching absorption which is typical for an organic acid. Further on it can also be seen that a large band between 3500 and 3200 cm^{-1} is observed in the substrate spectrum and not in the product spectrum. This could be explained by O-H stretching vibrations of intermolecular hydrogen bonded molecules and is typical for end standing alcohols. Since this band is no longer present in the formed product and in combination with the peaks mentioned above, the conclusion can be made that a carboxylic group has been formed.

On Figure 5b, the IR spectra for methyl oleate and the formed DCA product from this substrate are compared. It can be seen that both show the peak for C=O stretch at around 1750 cm^{-1} but for methyl oleate, a shift of this peak is observed. It is known that esters have a carbonyl peak at lower frequencies compared to acids which is a first indication that the formed product contains only acid groups and no ester bonds. Further on, it can also be observed that the O-H stretch between 3200-2500 cm^{-1} is present in the product spectrum and not in that of the substrate which is a second indication that a dicarboxylic acid has been formed.

When the spectra of all formed products are compared in Figure 5c, it can be seen that these are very similar to each other. It is also clearly visible that all spectra perfectly align at the carbonyl peak around 1750 cm^{-1} from which it can be concluded that no esters are left in the formed products and

only acids remain. Therefore it is certain that both methyl oleate and butyl oleate are hydrolysed by the yeast cell resulting in the formation of methanol and butanol. From all previous information, it can be concluded that the formed products are all the desired 9-octadecenedioic acid.

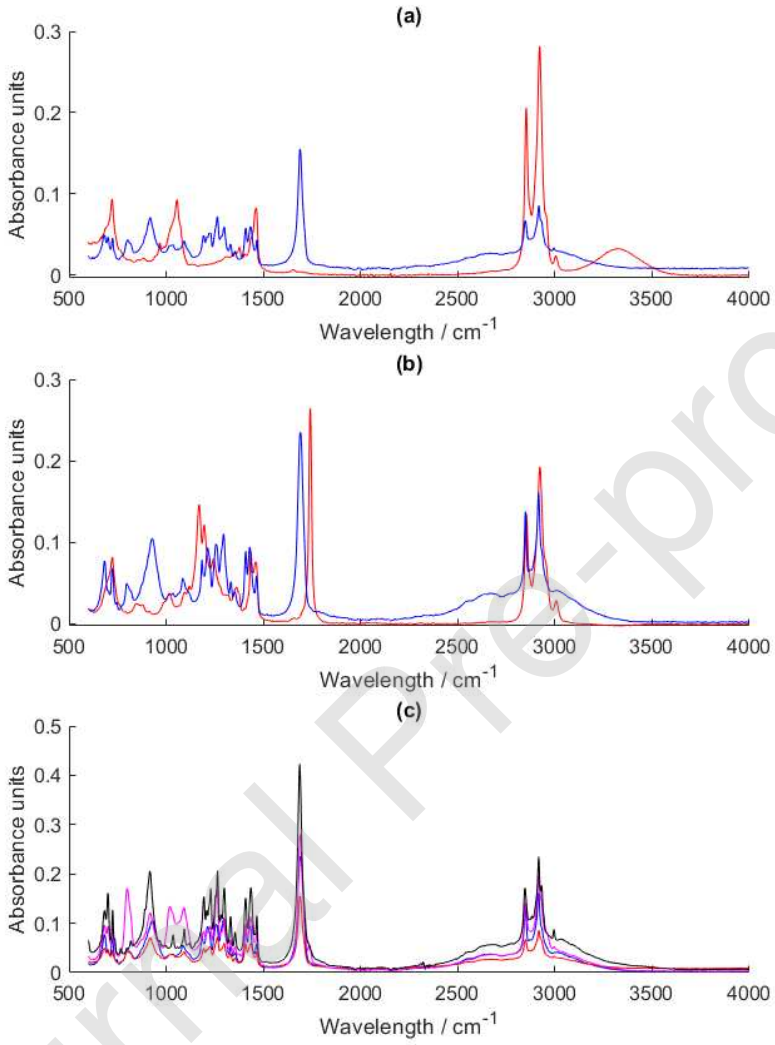

Figure 5: measured infrared spectra for (a) oleyl alcohol (red) and formed DCA product (blue), (b) methyl oleate (red) and formed DCA product (blue) as well as (c) a comparison of all formed DCA products.

4 Conclusion

C18:1 substrates with different α-functionalities were evaluated for their toxicity and suitability as a substrate for the biological production of 9-octadecenedioic acid, which is considered an important

chemical building block. All of the substrates were found to produce the target molecule, however, only oleyl alcohol and methyl oleate show to be feasible alternatives for oleic acid as they show little toxicity at high substrate concentrations up to 100 gL^{-1} and yield feasible DCA concentrations. Moreover, no toxic side products are produced as is the case when using ethyl- or butyl oleate. Future research should include the investigation of higher substrate concentrations and developing a mass balance to evaluate the glucose flow and potential storage of added substrate as intracellular lipids.

Credit Author statement

Jordy Bauwelinck: conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing-Original draft, Visualization,

Marc Wijnants: Writing – Review & Editing, Supervision

Serge Tavernier: Writing – Review & Editing, Supervision

Iris Cornet: Writing- Review & Editing, Supervision, Project administration, Data Curation,

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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