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Jordy Bauwelinck[1], Michel Caluwe[1], Marc Wijnants[1], Nikolett Wittner[1], Waut Broos[1], Jan Dries[1], Veerle Akkermans[1], Serge Tavernier[2] and Iris Cornet[1]

[1]Biochemical Wastewater Valorization and Engineering (BioWAVE), Faculty of applied engineering, University of Antwerp, Groenenborgerlaan 171 Wilrijk, Belgium

[2]Intelligence in processes, Advanced catalysts & Solvents (iPRACS), Faculty of applied engineering, University of Antwerp, Groenenborgerlaan 171 Wilrijk, Belgium

Corresponding author: Iris Cornet: iris.cornet@uantwerpen.be

Abstract

Two high-fat containing side streams from the chocolate industry were evaluated as an alternative renewable substrate for biochemical long-chain dicarboxylic acid (DCA) production by *Candida tropicalis.* Cleaning water from liquid chocolate transport and dissolved air flotation (DAF) sludge from the wastewater treatment plant contained 29 and 18 m% fat respectively. The addition of up to 100 $g.L^{-1}$ of the 'chocolate water' and 80 % (v/v) of DAF sludge had no negative effects on both growth and DCA production. In a fed-batch reactor, a DCA concentration of 5.8 $g.L^{-1}$ was obtained after 120 h and 47.5% of the consumed fats were converted to DCA upon glucose addition when

using chocolate water. Using DAF sludge, a DCA concentration of 4.9 $g.L^{-1}$ was obtained after 72 h, equalling 37.5 % of the consumed fats. This research shows the potential of the researched side streams as a substrate for DCA production.

Keywords

Bioconversion; Bioprocess modelling; *Candida tropicalis* ; long-chain dicarboxylic acids; Wastewater valorisation;

1. Introduction

The search for renewable resources is one of the most important tasks of today's chemical industry. As fossil raw materials are getting more scarce and expensive, there is a high need for alternatives. First-generation renewable resources for oil-derived chemicals are found in the form of vegetable oils extracted from rapeseed or soybeans [1]. These oils are highly suitable for the production of biodiesel and also for the production of valuable platform molecules, such as long-chain dicarboxylic acids [2] or biosurfactants [3]. However, it becomes clear that there are several issues when using this feedstock, making them not as sustainable as expected [4]. Therefore in recent years, second and next generation feedstock have been researched, resulting in the biorefinery concept where mostly waste biomass has been used as a renewable feedstock for the chemical industry [5]. Next to this, also resources from wastewater treatment plants are studied to produce valuable raw materials for the production of platform molecules, at the same time purifying the wastewater from the production process [6]. As an example, biodiesel can be immediately produced through transesterification of oils found in domestic wastewater [7], or these oils can be used as a feedstock for biotechnological processes e.g. the production of algal biomass and lipids [8].

In this paper, we investigate wastewater from the food industry for its application as a feedstock for the bio-based production of long-chain α,ω-dicarboxylic acids (DCA) by an oleaginous yeast. With their structural formula of $HOOC(CH_2)_nCOOH$, $n \geq 12$, long-chain DCA are used as a monomer for the

production of high-grade polymers, as waxing agents in lubricants, as an emulsifier in cosmetics and numerous other applications [2,9–11]. The global market size of C18 dicarboxylic acid was valued at USD 6.6 billion in 2016 and is expected to reach USD 9.4 billion by 2024[12]. Currently, there are two main production strategies for these high-value chemicals. The first one is chemical production, but the harsh reaction conditions, expensive catalysts and the formation of side products make it economically and ecologically less interesting. The different chemical production strategies with their disadvantages were reviewed by Stempfle et al [13]. More sustainable technology is the biochemical production strategy where a hydrophobic substrate, e.g. fatty acids, is converted to DCA by a specific yeast strain. *Candida tropicalis* is the most widely used yeast strain for this purpose because it is capable to perform an ω-oxidation, i.e. the terminal methyl group of fatty acids and alkanes is oxidised to a carboxylic group [2]. Extensive research on this pathway related to DCA production has been performed which was reviewed by Huf et al. and Werner et al. [2,11]. Gene expression and repression studies of the enzymes in the ω-oxidation pathway were performed by Funk et al. [14]. In this study, *C. tropicalis* ATCC20962, a commercially available genetically engineered yeast, was used. The POX genes coding for acyl-CoA-oxidase, i.e. the first enzyme of the β-oxidation, are disrupted in this strain. In this way, the yeast becomes incapable of shortening fatty acid chains therefore unable to produce energy from fatty acids [15]. This results in the fatty acids being converted into high dicarboxylic acid instead. The DCA productivity and yield will be increased and the resulting DCA will have the same carbon chain length as the fatty acid substrate. Also, the degree of unsaturation of the substrate's acyl chain is not altered during the DCA production process. Since the yeast is not able to produce energy from fatty acids, an energy supplying co-substrate has to be added which is done in the form of glucose[14]. In other studies process parameters such as temperature, pH and aeration were investigated[16–18]. The optimal values (30°C, pH 8.2) from these publications are implemented in the presented manuscript.

In literature, several substrates have been investigated for the production of long-chain DCA ranging from alkanes, fatty acids, fatty acid esters, fatty alcohols and vegetable oils [2,19–21] Most of these

substrates are already valuable because they are pure chemicals derived from vegetable oil and can be applied in other production processes, e.g. food additives, pharmaceuticals, etc. Therefore alternative sources for fatty acids or vegetable oils that are not in competition with the food industry and don't require extensive purification should be investigated. Food industry wastewater can contain high amounts of fat which are currently treated as waste. The fat content is highly dependent on the type of process but typical amounts range between 250-5000 $mg.L^{-1}$ for the seafood industry and 100-2000 $mg.L^{-1}$ for the meat industry [22]. These fats could still be valuable substrates for biotechnological processes such as the production of DCA. In this research, two side streams from a chocolate tank truck cleaning company in Belgium were evaluated as a feedstock for DCA production without any form of isolation or purification of the fats. These side streams are less expensive compared to the purified substrates, e.g. fatty acids, and are not in competition with the food industry. Chocolate waste has been chosen because it is unlikely to contain any toxic components for the yeast while it also contains nutrients (sugar, protein) for cell growth. To our knowledge this has not been investigated before and the valorisation potential of this side stream is therefore of great novelty

In this proof of concept study, the DCA yields of the two types of side streams from the chocolate tank cleaning as hydrophobic substrates for DCA production are compared with each other as well with the current benchmark substrate, i.e. oleic acid.

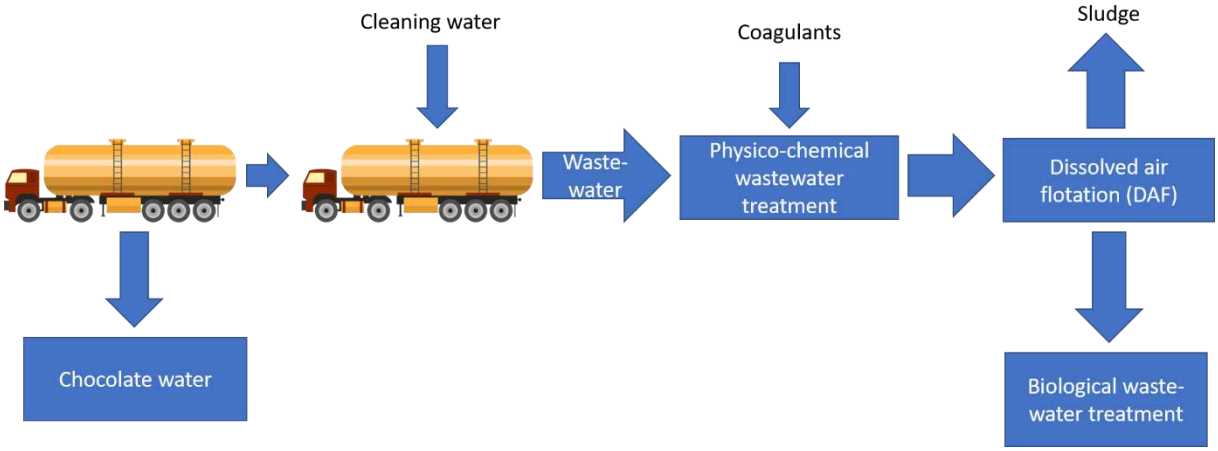

Figure 1: Overview of the wastewater treatment of the tank truck cleaning company from which the samples were obtained.

2. Materials & Methods

2.1 Culturing

Candida tropicalis ATCC20962 yeast was cultured as described in Bauwelinck et al [23]. In brief, the strain was stored in 50 % (v/v) glycerol at -80°C. A loop was cultured on YPG agar plates containing 10 $g.L^{-1}$ of yeast extract, 20 $g.L^{-1}$ of peptone, 20 $g.L^{-1}$ of glucose and 20 $g.L^{-1}$ agar. These plates were incubated for 2 days at 30 °C and afterwards stored at 4°C. Before each experiment, a 100 mL preculture was prepared in a growth medium containing 10 $g.L^{-1}$ yeast extract, 20 $g.L^{-1}$ peptone and 20 $g.L^{-1}$ glucose by inoculating from the agar plates. This preculture was incubated at 30°C and shaken at 200 rpm for 24 h.

2.2 Chocolate water experiments

Figure 1 shows an overview of the company's wastewater treatment. Tank trucks deliver liquid chocolate to the chocolate factory, but a residual amount of chocolate remains which is removed by using only water and stored in a buffer tank before the final cleaning is carried out. This results in a highly viscous paste known at the company as "chocolate water", of which a large part can be sold as cattle feed because it does not contain any cleaning agents. Since the chocolate water was a highly

viscous paste it was mostly treated as a solid. Therefore, all dosages were weighed and are indicated in $g.L^{-1}$.

2.2.1 Shake flask experiments

Four chocolate water concentrations were evaluated for fermentation to DCA in duplicate. Therefore, 2 g, 4 g, 6 g and 10 g of the chocolate water paste was dissolved in 90 mL fermentation medium containing 10 $g.L^{-1}$ glucose, 3 $g.L^{-1}$ peptone, 6 $g.L^{-1}$ yeast extract, 7.2 $g.L^{-1}$ K_2HPO_4 and 9.3 $g.L^{-1}$ KH_2PO_4 and brought into 500 mL baffled shake flasks. The shake flasks were sterilised at 121°C for 20 minutes and, after cooling, inoculated with 10 mL of the same preculture, prepared as mentioned in Section 2.1. This resulted in chocolate water concentrations of 20, 40, 60 and 100 $g.L^{-1}$. The pH of the media was adjusted to 8.2 using 4M KOH and the shake flasks were incubated at 30°C and shaken at 200 rpm. Daily, the pH was adjusted to the optimal value of 8.2 [17] and an extra dose of 2 mL 500 $g.L^{-1}$ glucose solution was added to each flask in order to maintain the metabolism of the yeast. The dry weight content of the medium was measured at the beginning of the fermentation and the end after 120 h. The DCA concentration of the final fermentation medium was measured in each flask.

2.2.2 Batch reactor experiment

To evaluate the yeast's capability to produce DCA from the chocolate water substrate, a batch experiment was performed. The reactor experiments were carried out in a 2.5 L sparger-aerated stirred BioFlo 110 computer-controlled fermenter New Brunswick Scientific, Edison, NJ, USA). 900 mL of the fermentation medium, mentioned in Section 2.2.1, was added to the fermenter. This medium also contained 100 g of chocolate water which after inoculation will result in a chocolate water concentration of 100 $g.L^{-1}$. The fermenter with the medium was sterilised at 121°C for 20 min and afterwards inoculated with 100 mL of preculture. As was learned from previous experiments, excessive foaming occurs during the fermentation, therefore silicone antifoam (Chem-Lab) was added continuously at a rate of 1 $mL.h^{-1}$ [23]. During the first 48 h of the fermentation, the pH was controlled at 8.2. However, in an attempt to hydrolyse the sucrose present in the chocolate water,

the pH was reduced to 2 from 48 to 72 h of fermentation and increased again to 8.2 after 72 h until the end of the fermentation. This is also pictured in Figure 3C.

2.2.3 Fed-batch reactor experiment with glucose dosage

900 mL of the same fermentation medium as mentioned in Section 2.2.1 which contained 20 $g.L^{-1}$ of chocolate water was added to the fermenter and sterilised at 121°C for 20 min. To start the fermentation, 100 mL of preculture, as described in Section 2.1, was added. During fermentation, the pH was continuously controlled at 8.2 using 4 M KOH and 2 M H_2SO_4. The fermenter was aerated at 1.5 $L.min^{-1}$ (1.5 vvm) and stirred at 600 rpm. To ensure that the yeast was able to maintain its metabolism, glucose was dosed at a rate of 0.4 $g.L^{-1}.h^{-1}$ as was found to be optimal by Funk et al [14]. As the fat substrate was almost entirely consumed after the first 24 h, each day 100 mL of a 200 $g.L^{-1}$ sterilised watery chocolate paste was added to the fermenter. Each day a sample was taken from the fermenter and analysed according to the methods described in Section 2.4.

2.3 DAF sludge experiments

After discharging the residual chocolate, cleaning water is added to the tank truck (Figure 1) and will undergo a wastewater treatment process. One of the steps here is dissolved air flotation (DAF) which separates the fats and suspended solids from the water through the principle of flotation. The sludge floating on top of the tank has a high-fat content, making it possibly an interesting substrate for DCA production. In contrast to the chocolate water, this DAF sludge can contain traces of coagulant polymers and cleaning agents. Therefore, this DAF sludge has no value and is usually burnt after drying or anaerobically digested to biogas [24]. Research has been performed on this side stream for the production of biodiesel; however, the lipids were first purified from the sludge which increases the cost of the process [25]. In the present research, the feeding of the sludge without any pretreatment was investigated. The DAF sludge was also highly viscous, however still liquid. Therefore, all dosages were measured volumetrically and are indicated in vol%.

2.3.1 Shake flask experiments

As in the experiment described in Section 2.2.1, also DAF sludge was added to the medium at the four different concentrations and fermentations were performed together with a blank experiment without added DAF sludge. Also here the pH was daily adjusted to 8.2 and 2 mL of 500 $g.L^{-1}$ glucose solution was added to maintain the yeast's metabolism. The colony forming units were determined after 24 h and after 120 h. At the end of the experiment, the DCA concentration was measured in each flask.

2.3.2 Reactor experiments

Two similar reactor experiments using DAF sludge were performed, one with and one without a continuous antifoam dosage of 1 $mL.L^{-1}.h^{-1}$. 800 mL of the DAF sludge was added to the fermenter in combination with 100 mL of fermentation medium with the same composition as in 2.2.3. Glucose was dosed at 0.4 $g.L^{-1}.h^{-1}$. pH and aeration were controlled as in section 2.2.3.

2.4. Analysis

The dry weight measurement and GC-FID analysis were the same as in Bauwelinck et al. [23]. GC-FID was used to measure the fatty acid profile of free fatty acids and the concentration of long-chain dicarboxylic acids. Gel permeation chromatography (GPC) was used to measure the number of triacylglycerides (TAG) in the medium. 1 mL of fermentation sample was extracted twice with diethyl ether. Afterwards, the ether was evaporated leaving the extracted hydrophobic components as residue. Hereafter, 1 mL of tetrahydrofuran (THF) was added and the sample was filtered through a 0.2 µm PTFE syringe filter into an HPLC vial. The samples were analysed on an Agilent HPLC system equipped with a Phenomenex 500 Å in series with a Phenomenex 100 Å gel permeation chromatography column with Tetrahydrofuran (THF) as mobile phase at a flow rate of 1 $mLmin^{-1}$. This method is optimised for the separation of free fatty acids, mono-, di- and triglycerides.

To measure the sugar concentration, a fermentation sample 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 heated at 70°C. The elution method was isocratic with a mobile phase of 8 mmol.L^{-1} H_2SO_4 in water and a flow rate of 0.6 $mLmin^{-1}$.

All measurements were performed in triplicate enabling the calculation of average values and standard errors. For cell counting, colony forming units (CFU) were determined by making several dilutions of a fermentation sample and adding 100 µL of this dilution to a YPG agar plate and spreading with a triangular spatula. After 24 h of incubation at 30°C, the colonies were counted manually.

2.5 Modelling and yield calculation

The CFU data from the fed-batch chocolate water reactor experiment were modelled using the Baranyi & Roberts sigmoidal model [26]. The *lsqnonlin* function of the Optimisation module of Matlab (MathWorks) was used to fit Equations (1) and (2) to the obtained CFU data pictured in Figure 3a. The acquired mathematical model with corresponding estimated parameters was simulated as presented in FIG….

$$\frac{dlogN}{dt} = \mu_{max}\frac{Q}{(1+Q)}\left(1-10^{logN-logN_{max}}\right)\frac{1}{ln10} \quad (1)$$

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

Here N is the cell count ($CFUmL^{-1}$), N_{max} is the maximal cell count ($CFUmL^{-1}$), μ_{max} is the specific growth rate (h^{-1}) and Q is a dimensionless variable determining the lag phase.

The dicarboxylic acid production was modelled using a linear variant of this model. Equations (3) and (4) were used to fit the dicarboxylic acid data points from the fed-batch chocolate water experiment.

$$\frac{dP}{dt} = r_{max}\frac{Q_P}{(1+Q_P)}\left(1-\frac{P}{P_{max}}\right) \quad (3)$$

$$\frac{dQ_P}{dt} = r_{max}Q_P \quad (4)$$

Here P is the dicarboxylic acid product concentration ($g.L^{-1}$), P_{max} is the maximal dicarboxylic acid concentration at the end of the experiment ($g.L^{-1}$), r_{max} is the volumetric dicarboxylic acid production rate ($g.L^{-1}.h^{-1}$) and Q_P is a dimensionless variable determining the lag phase without production.

Equation (5) was used for the calculation of the conversion yield from consumed TAG to produced DCA. The TAG that are consumed but not converted to DCA are stored as intracellular lipids.

$$Conversion\ Yield = \frac{Produced\ DCA}{Consumed\ TAG} \cdot 100\ \% \qquad (5)$$

3. Results & Discussion

As chocolate water and DAF sludge are industrial side streams, there is no exact recipe, and the composition changes over time. However, when a side stream is used from the same company with a stable continuous production process, differences in composition are expected to be minimal. It is still important to characterise the received samples for fat, sugar and dry weight content prior to the fermentation experiments. The results of these analyses are presented in Table 1.

3.1 Chocolate water as a high fat containing feedstock

The chocolate water contained 29 m% of fat, which makes it a potentially feasible substrate for the production of long-chain dicarboxylic acids (Table 1). However, the residual, non-fat containing, dry mass of 10 m% in this waste stream will lead to a higher fermentation medium viscosity and potentially lower oxygen solubility, possibly limiting the dicarboxylic acid production. From the fatty acid profile, it could be found that the side stream mainly contained oleic acid (C18:1) next to minor amounts of palmitic acid (C16:0), stearic acid (C18:0) and linoleic acid (C18:2).

Table 1: Composition of the Chocolate water and DAF sludge used in this study. Due to the nature of the substrate, all amounts of chocolate water were determined gravimetrically whereas the DAF sludge was measured volumetrically. This causes the difference in units.

	Chocolate water	**DAF sludge**

Residual dry mass (105 °C)	10 ± 3 %(m/m)	57 ± 5 $g.L^{-1}$
Total fats	29 ± 4 m%	18 ± 1 $g.L^{-1}$
Sugars (Sucrose)	43 ± 1 m%	NA
Fatty acid profile		
C16:0	12.9 ± 0.1 % (m/m)	39 ± 1 % (m/m)
C18:2	14.7 ± 0.1 % (m/m)	7 ± 1 % (m/m)
C18:1	62.1 ± 0.3 % (m/m)	26 ± 1 % (m/m)
C18:0	10.1 ± 0.1 % (m/m)	29 ± 1 % (m/m)

3.1.1 No toxicity of chocolate water in shake flask experiments

Shake flask experiments with different dosages of chocolate water were performed in order to evaluate the effect of the substrate level on the yeast growth and DCA production. Because this side stream is normally sold as cattle feed, it can be assumed that no toxic components that inhibit the yeast growth are present. However, higher concentrations of the chocolate water will certainly lead to higher medium viscosities, resulting in a lower oxygen solubility. In Figure 2a the measured dry weights for the different shake flasks are pictured. The initial dry weight (blue) increases proportionally to the higher chocolate water dosage, which is to be expected because the substrate has a dry mass of 9 m% of itself. This initial dry weight is measured after inoculation. Since all shake flasks are inoculated from the same preculture, differences in dry weight caused by inoculated cell mass are minimal. The dry weight increases during fermentation because the cells grow on the sugars added (10 $g.L^{-1}$) or present in the medium. It can be noticed that the measured dry weight at the end of the fermentation (after 120 h) has increased for all chocolate water dosages, also for the higher ones. To evaluate whether there is a significant inhibiting or growth-promoting effect on the growth due to a higher chocolate water dosage, a one-way ANOVA has been performed on the total dry weight increase (upper red part in Figure 2a) as this represents the actual cell growth. Any potential consumption of dry weight particles by the yeast was not taken into account. This resulted

241 in a 95 % certainty that the higher chocolate water dosage had no significant effect on the dry weight
242 increase (see supplementary data). Therefore, it could be concluded that there is no major effect on
243 cell growth. It was also evaluated if the higher sucrose concentration due to higher chocolate
244 dosages improved the yeast growth (see Table 1). As this is not the case, this gives the first indication
245 that sucrose might not be consumed by the yeast. This will also be investigated in the following
246 experiments.

247 Additionally to the growth, also the dicarboxylic acid production was investigated during these
248 experiments. Here it is expected that a higher dosage of the chocolate water results in a higher
249 dicarboxylic acid (DCA) concentration as more fat substrate is available. However, there is no
250 knowledge of the availability for the yeast of the triacylglycerides (TAG) in this particular substrate
251 mixture and whether inhibitory compounds are present. For comparison purposes, the highest
252 chocolate water dosage was chosen at 100 $g.L^{-1}$ as this represents a fat concentration of 29 $g.L^{-1}$
253 which is comparable to the 20 $g.L^{-1}$ substrate concentration used in previous work [23]. However,
254 when the substrate has no negative effect on the yeast, possibly higher amounts could have been
255 used, as in literature also higher concentrations (50 and 30 $mL.L^{-1}$) of pure triacylglycerides were fed
256 to *C. tropicalis* mutants with a positive effect on dicarboxylic acid production [21]. Therefore, no
257 substrate inhibition caused by the present fats themselves is expected here. In Figure 2b, the
258 measured DCA concentrations after 120 h of fermentation time are pictured as a function of the
259 chocolate water dosage. The experiments with 20, 40 and 60 $g.L^{-1}$ show relatively similar total DCA
260 yields between 0.05 and 0.12 $g.L^{-1}$ whereas the experiments with 100 $g.L^{-1}$ show a higher DCA yield of
261 0.27 $g.L^{-1}$. These yields might seem very low, however, the impact of lacking continuous pH control
262 and efficient aeration in shake flasks cannot be underestimated. In a reactor the yields are expected
263 to be much higher which will be investigated further on in this article. Using ANOVA, it was proven
264 that the effect of the chocolate dosage was only significant when 100 $g.L^{-1}$ concentration was
265 applied. In Figure 2b, the dicarboxylic acid profile at the end of the fermentation shows that the
266 composition of the DCA product is similar to the fatty acid composition of the chocolate water

substrate, i.e. C18:1 is the most abundant whereas C16:0 and C18:0 are present in somewhat equal amounts and C18:2 is the least abundant.

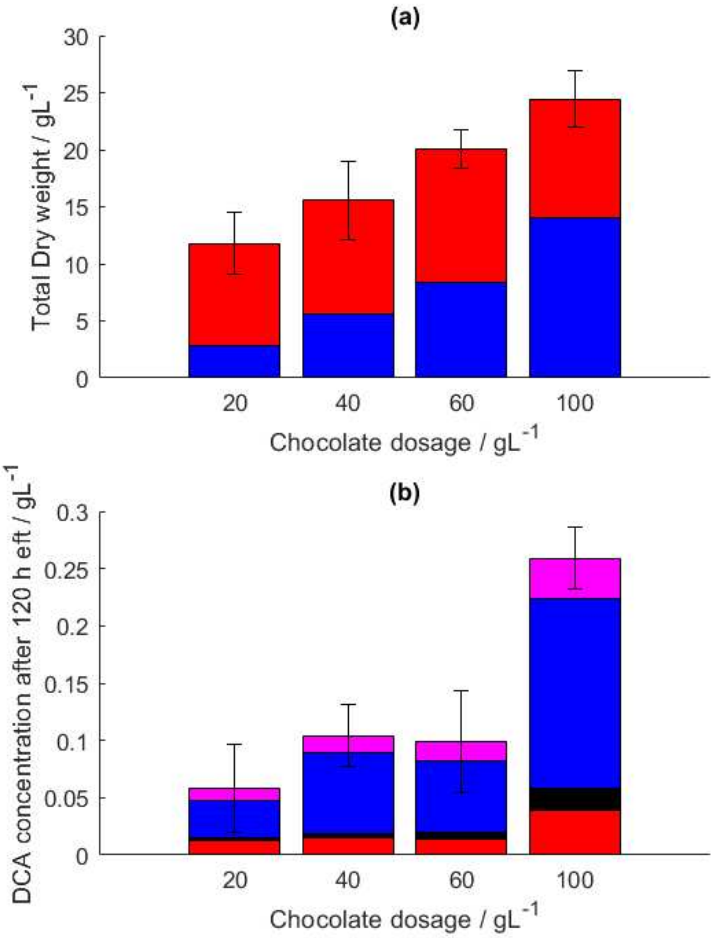

Figure 2: Follow-up of shake flask experiments for different chocolate water concentrations, (a) (blue) initial Dry weight in the shake flask and (red) increase in Dry weight in the shake flask after 120 h, and (b) Dicarboxylic acid (DCA) concentration at the end of the fermentation. Each block represents a different DCA: C16:0 ■, C18:2 ■, C18:1 ■ and C18:0 ■ . 'eft' stands for elapsed fermentation time.

3.1.2 Chocolate water sugars cannot be consumed by the yeast

After successful bioconversions in the shake flasks, a reactor experiment was set up with controlled pH and aeration. In Section 3.1.1 it was proven that there is no effect on the yeast growth and DCA production in the presence of a high chocolate water dosage up to 100 $g.L^{-1}$, therefore this high concentration was used in the fermenter experiment. As is known from the composition analysis of the chocolate water (Table 1), the substrate contains a high amount of sugars itself (43 m%) in the

280 form of sucrose. To test whether this sugar could be used for yeast growth and maintenance of the
281 cell metabolism, a batch test was performed where 100 $g.L^{-1}$ of the chocolate water was fed without
282 extra glucose dosage, eliminating this extra cost in a potential production process. The results of
283 yeast viability and growth are illustrated in Figure 3a. During the first 24 h of the experiment, only a
284 small increase in both dry weight (DW) and CFU was observed. As the shake flask experiment with
285 100 $g.L^{-1}$ of chocolate water dosage where glucose was added showed a large increase in DW, the
286 low growth in this reactor experiment will be caused by available carbon substrate shortage. Figure
287 3c shows the measured sugar concentrations as a function of time. First, it has to be noted that the
288 HPLC measurement method utilises 0.8 mM H_2SO_4 as the mobile phase and the column is heated at
289 75°C. The combination of the acidic liquid and the high temperature causes sucrose to be hydrolysed
290 into glucose and fructose. Therefore, when sucrose is analysed, this results in two peaks on the
291 chromatogram, one for glucose and one for fructose. To evaluate whether sucrose was fully
292 hydrolysed during the analysis, a sample containing 3 $g.L^{-1}$ pure sucrose was analysed and resulted in
293 a measured concentration of 1.5 $g.L^{-1}$ glucose and 1.5 $g.L^{-1}$ fructose. Looking at the data pictured in
294 Figure 3c it can be observed that at the beginning of the fermentation the measured glucose
295 concentration is higher compared to the measured fructose concentration. This is caused by extra
296 unconsumed glucose added with the preculture. After 24 h the values for glucose and fructose are
297 equal again meaning that sucrose is the only sugar left in the medium and which will remain like this
298 during the rest of the fermentation. The sugar concentration stays rather stable between 24 h and
299 120 h of fermentation, this in combination with the low growth leads to the conclusion that the
300 sugars present cannot be used as a carbon substrate for the yeast. In literature, several long-chain
301 dicarboxylic acid production experiments using *C. tropicalis* mutants growing on sucrose are
302 described[17,27,28]. However, no information on the sucrose metabolism was found for the
303 ATCC20962 strain as glucose is always added as a co-substrate. It is however known that there are
304 several *C. tropicalis* strains lacking α-glucosidase and are therefore not capable of metabolising
305 sucrose [29]. Although it is not proven that the ATCC20962 strain lacks these enzymes, it is more

306 than likely based on these results. In an attempt to hydrolyse the present sucrose in the medium into
307 glucose and fructose, between 48 h and 72 h of fermentation time, the pH of the medium was
308 decreased from 8.2 (the optimal pH for DCA production [17]) to 2, which is the same pH as 8 mM
309 H_2SO_4. From previous unpublished experiments, it was known that *C. tropicalis* is able to survive
310 acidic conditions when sufficient nutrients are present. However, as can be observed in Figure 3a,
311 this is not the case for the carbon starved culture in our experiment as no colonies were formed on
312 the plates when inoculated after 120 h of fermentation. Also, no extra sugar was consumed after 120
313 h leading to the conclusion that there was no hydrolysis of sucrose.

314 From Figure 3b, it can be observed that the TAG present in the medium were consumed very quickly
315 (28.5 $g.L^{-1}$ in 24 h), however, only a limited amount of DCA (1.2 $g.L^{-1}$) was produced which remained
316 stable during the next 96 h of fermentation. Using the GPC data, also the total amount of free fatty
317 acids present in the medium could be calculated and is pictured in Figure 3b. After 24 h, 10 $g.L^{-1}$ of
318 free fatty acids were found in the medium, this is the result of fast hydrolysis of the TAG present in
319 the fermentation medium by the yeast. However as there is no consumable carbon substrate
320 present, the yeast is not able to convert these fatty acids into DCA.

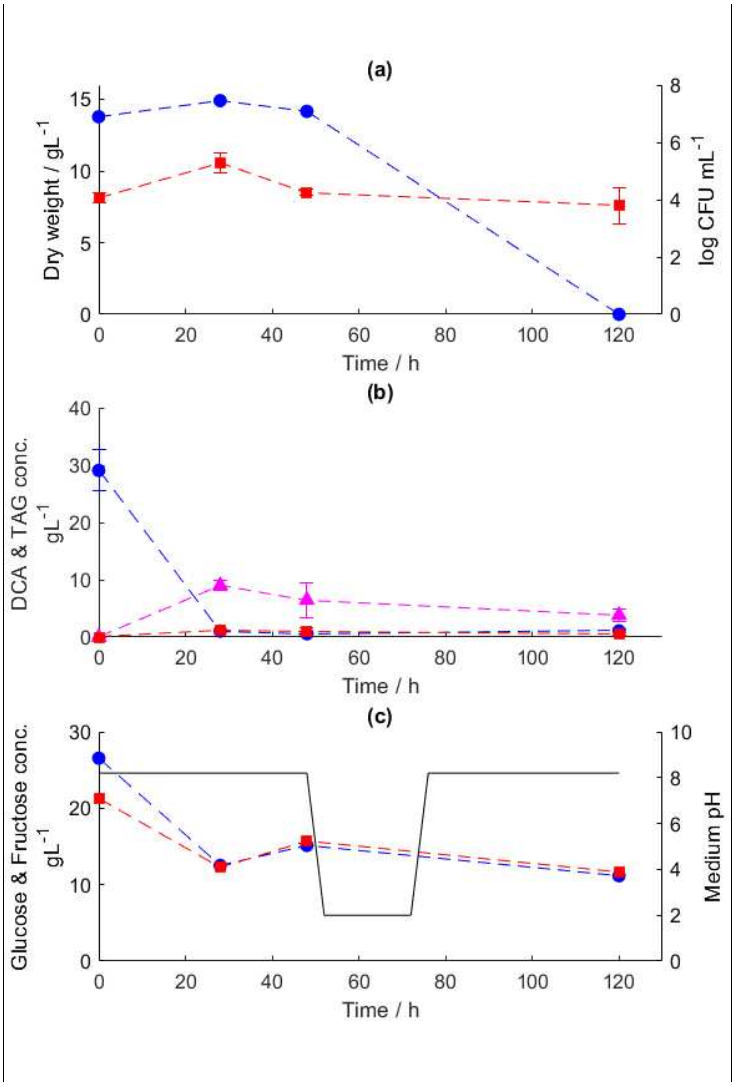

Figure 3: Batch reactor experiment on chocolate water (a) Dry weight of the medium(■) and colony forming units (●) (b) Extracellular triacylglyceride (TAG) (●), extracellular free fatty acid concentration (▲) and long chain dicarboxylic acid concentration (■). (c) Measured extracellular fructose (■) and glucose (●)concentrations with error bars. The full black line represents the medium pH.

3.1.3 DCA production from Chocolate water in a fed batch reactor

Since it is clear that sucrose cannot be consumed by the *C. tropicalis* ATCC 20962 yeast, a second reactor experiment was performed where glucose was added in fed batch mode. Since the 100 $g.L^{-1}$ chocolate water dosage already caused a viscous medium, in the present experiment, a safe lower concentration of 20 $g.L^{-1}$ chocolate water was chosen. As the initial added 1.9 $g.L^{-1}$ TAG were all consumed after 24 h of fermentation, a fed-batch strategy was applied where 5 times 20 $g.L^{-1}$ chocolate water was added to the reactor, resulting in a total dosage of 100 $g.L^{-1}$. Figure 4a presents

333 the yeast growth during the fermentation. It can be observed that the yeast shows exponential
334 growth during the first 30 h of fermentation (at high glucose concentration around 10 $g.L^{-1}$) and
335 afterwards remains stable and viable around 10^{13} $CFUmL^{-1}$. This is in clear contrast to the batch
336 experiment pictured in Figure 3 where no growth and cell death after 120 h was observed. Therefore
337 it is clear that addition of glucose improves the fermentation. The full blue line pictured in Figure 3a
338 represents a simulation of Equations (1) and (2) fitted on the measured data. The N_{max} was
339 determined to be 13.2 ± 0.2 $log(CFU)mL^{-1}$. As only one data point is available in the exponential
340 phase, no reliable specific growth rate could be determined, therefore the simulation was made
341 using the μ_{max} value of 0.68 ± 0.04 h^{-1} obtained for *Candida tropicalis* ATCC20962 on glucose in
342 previous unpublished research. The dry weight (DW) presented in Figure 4a increases during the
343 fermentation which is caused by both cell growth and the addition of fresh chocolate water
344 substrate. The dashed line between the data points in Figure 4b represents the DW increase for the
345 actual fermentation. What is important to notice here is that even though the number of colony
346 forming units does not increase any more after 40 h of fermentation there is still an increase in DW
347 which cannot be caused by growth in cell count. However, as is known from the literature, *C.*
348 *tropicalis* can accumulate intracellular lipids, therefore this is most likely the cause of the increasing
349 DW [14]. This accumulation can go up to 40 m% of the cell dry weight [30].

350 The measured triacylglyceride (TAG) and dicarboxylic acid (DCA) concentrations in the medium are
351 pictured in Figure 4b. At the start of the fermentation, the TAGs were rapidly consumed, therefore
352 an extra chocolate water dosage was added every 24 h. Due to the heterogenic nature of the
353 chocolate water, there are some differences in the TAG amount added by each substrate dosage.
354 After the first 24 h, 1.9 $g.L^{-1}$ of TAG was consumed and between 24 h and 48 h, 5 $g.L^{-1}$ was consumed.
355 During this period also the first DCAs were produced. However, the TAG consumption seemed to
356 slow down after 48 h and came to an end after 96 h as the TAG concentration remained the same.
357 This is also noticed in the DCA production with between 24 h and 96 h an almost linear increase in
358 total DCA production whereas after 96 h the concentration remains constant. It is unclear why the

359 production stops although there can be several reasons. Feedback inhibition by the formed DCA
360 product cannot occur as much higher concentrations than 5.8 $g.L^{-1}$ were realised in the literature
361 [19]. The high viscosity of the medium (visually observed) due to the high DW, in combination with a
362 high cell count, will result in lower oxygen accessibility for the yeast. As was proven by Huang et al.
363 oxygen supply is of high importance for the ω-oxidation, therefore the lower oxygen accessibility
364 could explain the decrease in DCA production[18]. Additionally, increased foaming was observed
365 during the fermentation and antifoam was added, however, after 120 h the foaming became so
366 excessive that the reactor overflowed and the experiment had to be stopped. This is also an issue
367 that is observed when utilising benchmark substrates e.g. oleic acids that are commercially used in
368 biochemical DCA production and should therefore not prevent industrial scale up[23]. When
369 calculating the conversion yield it can be observed that for the total added amount of 100 $g.L^{-1}$
370 chocolate water in the reactor, a DCA concentration of 6.5 $g.L^{-1}$ was obtained. Based on the
371 measured TAG data (Figure 4b) the total fat dosage can be calculated as 24.5 $g.L^{-1}$, of which 12.2 $g.L^{-1}$
372 was consumed. Using Equation (5) the yield was calculated to be 53.2 % which is lower compared to
373 the 72 to 79 % obtained when using pure oleic acid [14,23]. As the cost of this chocolate water
374 substrate is much lower compared to pure oleic acid, this chocolate water may still be a feasible new
375 feedstock for the production of high valuable long-chain dicarboxylic acids.

376 Because the TAG substrate contains different fatty acids, also the produced DCA is a mixture of
377 different carbon chains. Therefore the DCA concentrations pictured in Figure 3b are the sum of the
378 C16:0, C18:2, C18:1 and C18:0 dicarboxylic acid concentrations. The separate concentrations are
379 shown in Figure 3c and the sigmoidal DCA production model (Equations 3 and 4) was fitted to the
380 data points, resulting in the volumetric production rate r_{max} [$g.L^{-1}.h^{-1}$] and the maximal concentration
381 P_{max} [$g.L^{-1}$] which are shown in Table 2.

382 *Table 2: Overview of the estimated parameters using Equations (3) and (4) on the measured data in Figure 3c as well as the*

383 *calculated conversion yield using Equation (5).*

DCA	r_{max} [$g.L^{-1}.h^{-1}$]	P_{max} [$g.L^{-1}$]	Conversion Yield
C16:0	0.07 ± 0.01	1.18 ± 0.06	41.1 %
C18:2	0.06 ± 0.01	0.25 ± 0.01	13.5 %
C18:1	0.10 ± 0.02	3.74 ± 0.39	56.0 %
C18:0	0.07 ± 0.01	0.96 ± 0.04	41.3 %
Total DCA	0.17 ± 0.04	6.46 ± 0.75	53.2 %

384

385 The obtained values for r_{max} for the different DCA chains are relatively similar, only the value for
386 C18:1 DCA is slightly, though not significantly, higher with an average of 0.10 $g.L^{-1}.h^{-1}$. These are
387 lower compared to the pure oleic acid substrate as this usually results in an r_{max} of 0.5 $g.L^{-1}.h^{-1}$[11,31].
388 Even the total DCA production rate (0.17 $g.L^{-1}.h^{-1}$) is lower compared to this value. From the
389 chocolate water fatty acid profile (Table 1), it is known that oleic acid (C18:1) is the most abundant
390 fatty acid in the substrate. According to general substrate limiting kinetics, this could explain the
391 highest r_{max} for C18:1 DCA, which would also explain the lower reaction rates for C16:0, C18:0 and
392 C18:2 DCA. The same trend is reflected in the values for P_{max} where C18:1 DCA shows the highest
393 final concentration, C16:0 and C18:0 DCA are similar and C18:2 DCA yields the lowest concentration.
394 Using an adapted form of Equation (5) where the consumed TAG is replaced by the specified fatty
395 acid consumed (e.g. oleic acid for C18:1 DCA), the conversion yield for each fatty acid can be
396 calculated as presented in Table 2. Oleic acid (C18:1) is the most efficiently conversed as 56.0 % of
397 the added oleic acid chains, present in the TAG, were converted to dicarboxylic acids. Palmitic acid
398 (C16:0) and stearic acid (C18:1) showed relatively similar yields and linoleic acid (C18:2) shows the
399 worst conversion yield. This is in line with the results for r_{max} and P_{max}.

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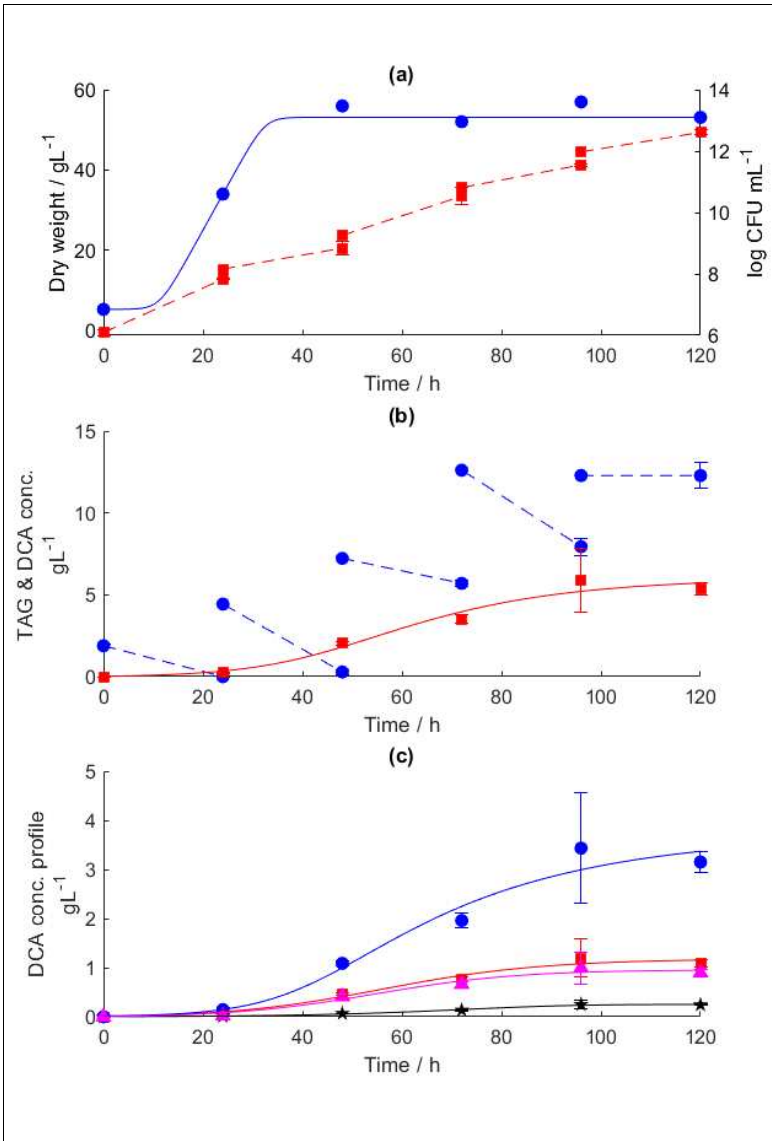

Figure 4: Fed-batch bioreactor experiment of chocolate water with (a) dry weight in the medium (■) and colony forming units (●), (b) Extracellular triacylglyceride (TAG) (●) and long chain dicarboxylic acid concentration (■) and (c) Separate dicarboxylic acid concentrations, C16:0 (■), C18:2 (★), C18:1 (●) and C18:0 (▲). The full lines represent fitted models to the experimental data.

3.1.4 Chocolate water is a valuable feedstock for DCA production

Inedible chocolate residue from a chocolate transportation company was tested for its potential application as a substrate for long-chain dicarboxylic acid production. This so-called chocolate water can be added in high concentrations up to at least 100 $g.L^{-1}$ without severe toxic effects or negative impact on yeast growth. However, when using *C. tropicalis* ATCC20962, the sugars present in the chocolate water cannot be used as a carbon source and extra glucose has to be fed to the

412 fermentation, increasing the production cost. Using a fed-batch strategy, a conversion of 23.7 % TAG
413 to DCA was obtained at a DCA concentration of 5.8 $g.L^{-1}$ after 120 h. This is lower compared to a pure
414 vegetable oil derived substrate like oleic acid (up to 40 $g.L^{-1}$), however, the low-value chocolate side
415 stream can be valorised in this way[11]. When yeast strains that are capable of utilising sucrose as a
416 carbon source would be applied as starting microorganism for knock-out of the β-oxidation pathway,
417 this could eliminate the need for extra glucose dosage, therefore reducing the cost of the production
418 process and making this chocolate water a feasible feedstock. Alternatively, *Candida tropicalis*
419 ATCC20962 could be genetically modified for additional alpha-glucosidase activity, thus enabling the
420 conversion of sucrose. Otherwise, a pre-treatment of the chocolate water where the sucrose is
421 hydrolysed prior to fermentation could be performed. This will increase production cost and time
422 when acid hydrolysis is performed due to an extra step. However, when enzymatic hydrolysis is
423 applied, the cost will be increased by the additional need of the enzyme but will lower the glucose
424 substrate cost. When simultaneous hydrolysis and fermentation is applied, this will not increase the
425 investment costs nor process time. Additionally, the slower release of available sugar will benefit the
426 process because high glucose concentrations prohibit DCA production and stimulate TAG
427 accumulation[14]. The chocolate water also contains essential nutrients, e.g. proteins, which are
428 currently added to the medium in the form of yeast extract and peptone. As the focus of this proof of
429 concept study was on the suitability of the substrate to produce DCA, no conclusions on the
430 consumption of the chocolate proteins can be made. However, this is an interesting topic for future
431 research as this would eliminate the extra cost of yeast extract or peptone addition. A final question
432 is whether this production concept is an economically more viable method of treating the chocolate
433 water compared to use as cattle feed. A detailed techno-economic study would provide more insight.

434 3.2 Dissolved air flotation (DAF) sludge as a fat containing substrate

435 A second side stream evaluated for its applicability as a feedstock for long-chain dicarboxylic acid
436 production was the sludge of a dissolved air flotation (DAF) wastewater treatment unit. As this
437 sludge was a very viscous liquid, but not as paste-like as the chocolate water, all dosages were

438 measured volumetrically. To gain a better idea of the valorisation potential of this DAF sludge, a
439 compositional analysis was executed from which the results are illustrated in Table 1. The residual
440 dry mass is 9 m% lower compared to the chocolate water which explains why this DAF sludge side
441 stream is less viscous. A disadvantage is the much lower fat content compared to the chocolate
442 water, therefore a higher amount of DAF sludge will have to be added in order to have sufficient TAG
443 substrate and obtain significant DCA concentrations. When looking at the fatty acid profile, palmitic
444 acid (C16:0) was found to be the most abundant followed by stearic acid (C18:0) and oleic acid
445 (C18:1). Linoleic acid (C18:2) was the least abundant. During the applied DAF wastewater treatment,
446 coagulation polymers (polyelectrolytes) were added in order to decrease the suspended solids in the
447 water. These polymers are, in small amounts, present in the flotation sludge and it has to be
448 investigated if they can inhibit the yeast growth. Also, small amounts of cleaning agents can be
449 present in the sludge which can also cause a toxic effect. Therefore, several batch experiments at
450 different DAF sludge dosages were conducted in shake flasks to investigate their effect on yeast
451 growth.

452 ### 3.2.1 No toxicity of DAF sludge in shake flask experiments

453 During the shake flask experiments different dosages of the DAF sludge ranging from 0 % (v/v) to 80
454 % (v/v) were applied. Due to the high dosage of DAF sludge, the dry weight (DW) was already very
455 high at the beginning of the experiment (11 to 44 $g.L^{-1}$ depending on the dosage applied) Therefore
456 the relative increase in DW due to yeast growth will be small, resulting in a large measurement error.
457 Counting the colony forming units after 24 h of fermentation was chosen as the preferred method
458 for investigating the toxicity of the DAF sludge to the yeast. From Figure 5a it can be observed that all
459 experiments show similar viability, i.e. around 10^9 colony forming units per mL after 24 h of
460 fermentation. To test whether there is a significant effect on the yeast viability by the increasing DAF
461 sludge concentration, a one-way ANOVA was set up. From this analysis, it is concluded with 95 %
462 certainty that the increase in $CFUmL^{-1}$ is the same for all experiments and that the cell concentration
463 is therefore comparable. From this, it can be concluded that there is no toxic effect caused by any of

464 the sludge concentrations applied. This is a positive result because it means that the DAF sludge can
465 be added at the highest possible amount of fat substrate in the fermentation medium.

466 The shake flask experiments were run for 120 h in total and at the end, the DCA content was
467 measured. The results are drawn in Figure 5b. With the blank experiment (0 % (v/v) DAF), no DCA
468 was found, which was expected as there was no fat substrate present. As expected, it can be
469 distinguished that for increasing DAF sludge concentration when more fat substrate is present, the
470 DCA concentration increases. From this experiment, it is important to conclude that the yeast is able
471 to convert the fats present in the DAF sludge into long-chain dicarboxylic acids. In shake flasks only
472 low concentrations up to 0.36 $g.L^{-1}$ DCA were obtained, this is expected to be higher in reactor
473 experiments due to better mixing, pH control and aeration. Another important observation is that
474 the DCA fatty acid profile differs from that in the DAF sludge substrate. In the substrate, C16:0 is the
475 most abundant, however, in the shake flasks, C18:1 DCA is mostly present, followed by C16:0 and
476 C18:0. As was observed in Table 2 for the chocolate water substrate, C18:1 DCA showed the highest
477 production rate (10 $g.L^{-1}.h^{-1}$), however, it was concluded that this was probably caused due to the
478 higher C18:1 concentration in the chocolate water substrate. In the DAF sludge experiments, this is
479 not the case as lower concentrations are present, but C18:1 still shows the highest production. This
480 indicates that the yeast prefers this chain over the other chains present in the medium. Further
481 investigation will be accomplished in a batch reactor.

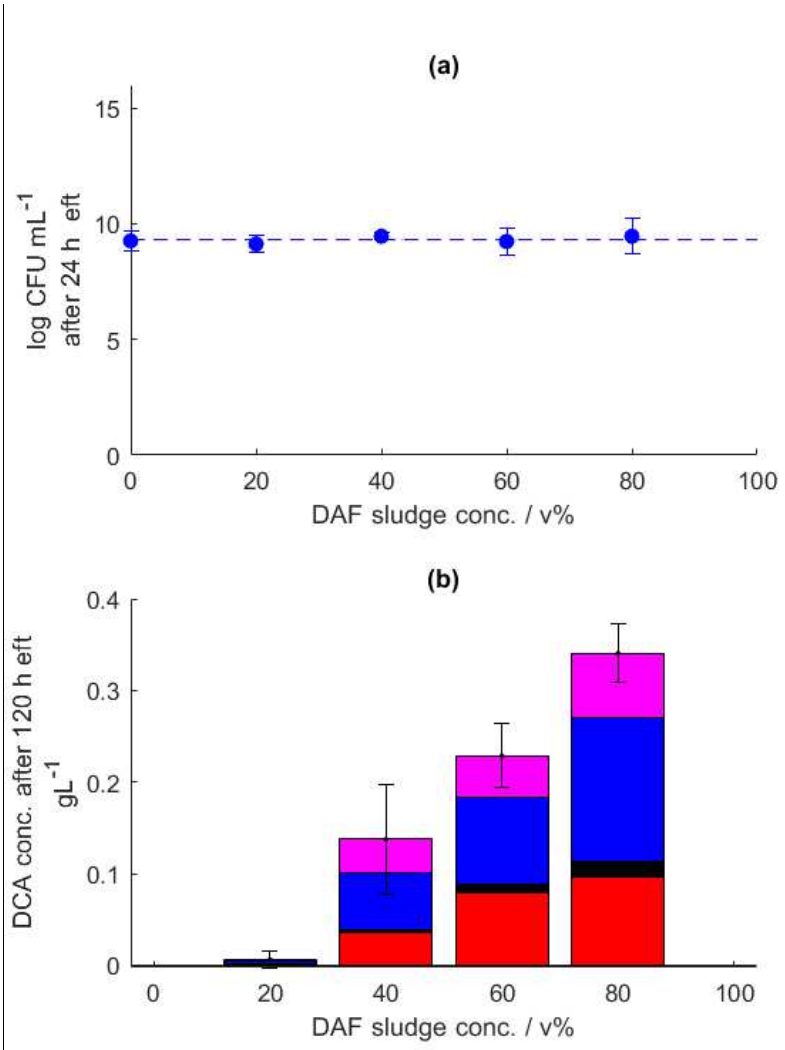

Figure 4: Shake flask experiments with different concentrations of DAF sludge, (a) Colony forming units after 24 h of fermentation. The dashed line represents the mean of all data samples. (b) DCA concentration at the end of the fermentation. Each block represents a different DCA: C16:0 ■, C18:2 ■, C18:1 ■ and C18:0 ■.

3.2.2 Antifoam addition necessary in a high density reactor experiment

Two reactor experiments were carried out containing 80 % (v/v) of DAF sludge, one with antifoam dosage and one without. Next to this also glucose was fed continuously at 0.4 $g.L^{-1}.h^{-1}$. In Figure 6, the graphs on the left represent the experiment with the antifoam dosage and the graphs on the right the experiment without. It was noticed that the antifoam caused the medium to be more homogenous and less viscous which led to a better sampling compared to the experiment where no antifoam was added. In Figure 6a the $CFU.mL^{-1}$ increase during the first 24 h and stay rather similar

493 during the fermentation indicating that the yeast does not suffer from the high DAF sludge dosage.
494 The DW is already very high at the beginning of the fermentation (47 $g.L^{-1}$) and rises steadily during
495 the fermentation, indicating yeast growth or lipid accumulation in the yeast. On the other hand, the
496 experiment without antifoam dosage showed already excessive foaming after 24 h and the medium
497 turned to be highly viscous. Moreover, some sedimentation at the bottom of the fermenter was
498 found, therefore the sampling accuracy was limited. This is reflected in the data for DW and CFU of
499 this experiment (Figure 6b) which resulted in some measured data with high standard deviations and
500 fluctuations. However, it can be concluded again that the yeast is still viable under these
501 circumstances as high, although fluctuating, cell counts were measured, up to 10^{12} $CFU.mL^{-1}$. This is
502 higher compared to the experiment with antifoam dosage (10^{9} $CFU.mL^{-1}$), although the latter showed
503 much more reliable data due to better sampling without foam formation. As there is less difference
504 in the DCA yield between the experiment with and without antifoam (Figures 6e and 6f), the practical
505 improvements from using antifoam (better sampling, lower viscosity, no foam, etc.) outweigh the
506 potential lower cell count. Taking the standard deviation into account the initial fat substrate dosage
507 for both experiments was comparable with an average of 18 $g.L^{-1}$ (Figures 6c and 6d). If antifoam was
508 dosed, DCA production started earlier because after 24 h a higher concentration was measured
509 compared to the experiment without antifoam. Another effect that is similar with and without
510 antifoam is that after 48 h the DCA concentration reaches an optimum and afterwards decreases
511 again. As the *C. tropicalis* ATCC20962 yeast is not able to consume DCA for energy there should be no
512 decrease in DCA concentration. This decrease can be caused by the interference of some
513 components in the medium with the DCA measurement. Nonetheless, at the end of the
514 fermentation, a total DCA concentration of 4.9 $g.L^{-1}$ and 2.9 $g.L^{-1}$ was measured for the experiment
515 with and without antifoam respectively. Using Equation (5) the conversion yield can be calculated for
516 both experiments and resulted in 37.5 % and 20.4 % for the experiment with and without antifoam
517 respectively. This is lower compared to the chocolate water and is mainly due to the higher DW,
518 leading to the high viscosity of the medium and inferior oxygen transfer. Because of the different

519 evolution with a decrease at the end for the different produced DCA chains as a function of time (see
520 Figure 6e and 6f) and because less experimental data in time were obtained compared to the
521 chocolate water fed-batch experiment, no modelling was performed here. From Figures 6e and 6f, it
522 can be observed that C18:1 and C16:0 show similar DCA production rates and concentrations
523 whereas the C18:0 DCA production rate is slightly slower and shows lower DCA concentrations,
524 followed by C18:2 which shows the lowest DCA production rate and concentration. The latter is
525 expected as this is also the least present in the substrate. As C16:0 is the most abundant fatty acid in
526 the DAF sludge it would be expected to have the highest reaction rate and yield. However, C18:1 still
527 shows the highest conversion yield (Table 3). Therefore, also other factors are expected to determine
528 the reaction rate and efficiency of a specific DCA carbon chain. Eschenfeldt et al. (2003) discovered
529 that the CYP52A17 enzyme, responsible for the first oxidation step in the ω-oxidation pathway,
530 shows lower reaction rates with increasing chain length, however, longer unsaturated fatty acids
531 (e.g. C18:1) showed higher conversion rates compared to shorter saturated fatty acids. This is in line
532 with the results presented here. Therefore it can be concluded that the effect of a double bond in the
533 chain on the conversion rate is much higher compared to increasing chain length, and makes it a
534 much more suitable substrate. One contradiction here is that, when following this theory, C18:2 DCA
535 should be the best substrate as it has the highest degree of unsaturation. However, due to the low
536 concentration of this chain in the substrates, it is most likely that the kinetic effect described in
537 Section 3.1.2 has a higher impact compared to the substrate preferences of the CYP52A17 enzyme.

538 *Table 3: Conversion yields for the separate carbon chains.*

Carbon chain	With antifoam	Without antifoam
C16:0	30.7 %	23.2 %
C18:2	27.2 %	16.7 %
C18:1	41.9 %	29.0 %
C18:0	31.6 %	22.7 %

This experiment shows that DAF sludge can be used as a valuable fat feedstock for the production of long-chain dicarboxylic acids. Compared to the chocolate water side stream the yields and DCA titers are lower. Also compared to pure substrates (e.g. oleic acid) the yields are much lower therefore DAF sludge is less suitable as a substrate. Still, these experiments are an example of a potential production process for the valorisation of DAF sludge side streams. Potential improvements to increase the yield could be to first extract the fats from the DAF sludge, then feeding them to the yeast which will lead to a lower medium DW content thus a lower viscosity. However, adding this extra purification step will increase the cost of the process.

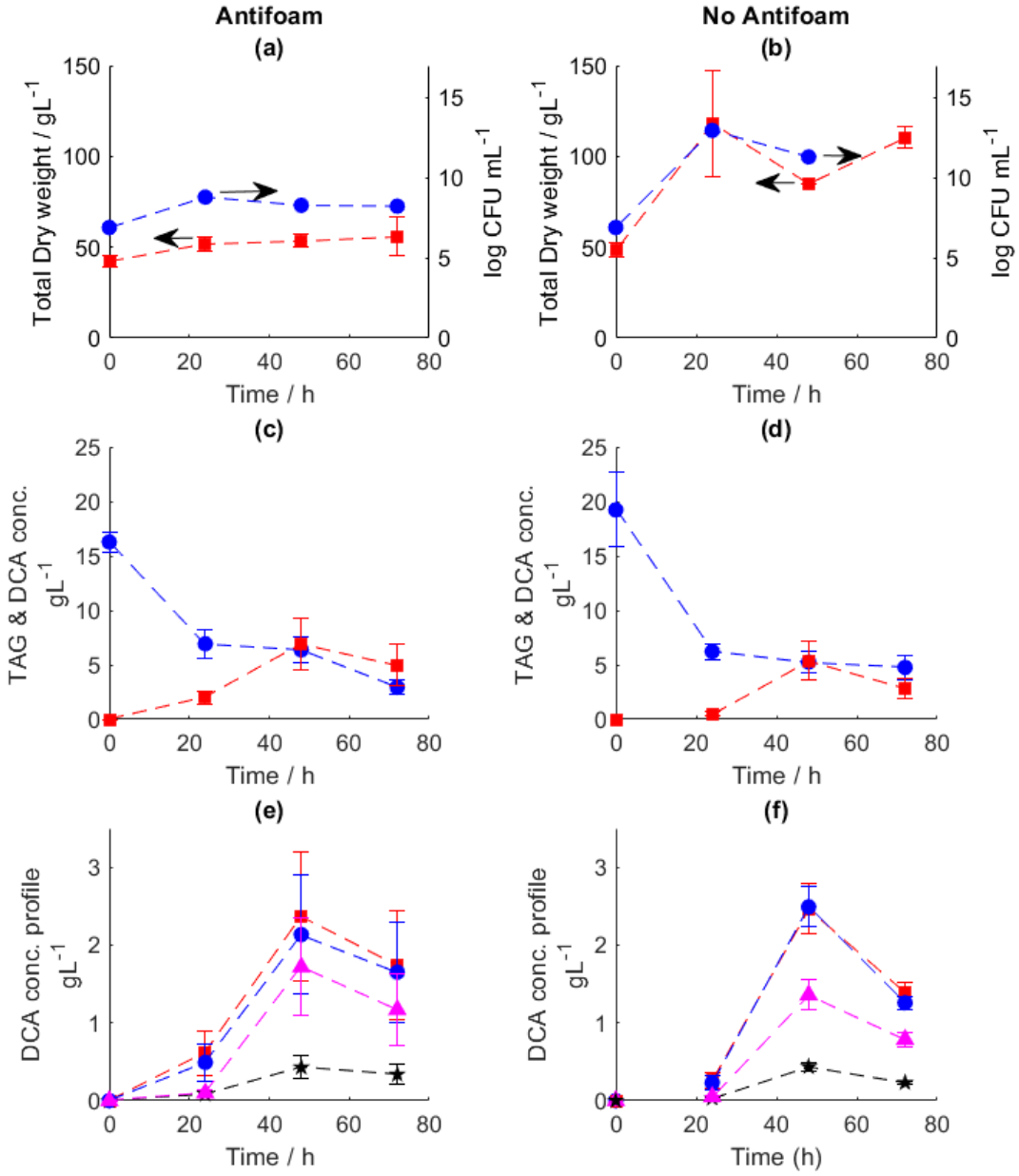

Figure 5: DAF sludge experiments, (left) with antifoam dosage, (right) without antifoam dosage. (a-b) dry weight (■) and colony forming units (●) (c-d) Extracellular triacylglyceride (TAG) (●) and long chain dicarboxylic acid concentration (■)(e-f) Separate C16:0 (■), C18:2 (★), C18:1 (●) and C18:0 (▲) dicarboxylic acid concentrations

4. Conclusion

The suitability of two chocolate factory side streams as a substrate for microbial long-chain dicarboxylic acid production was evaluated. Chocolate water shows to be a valuable feedstock for this process as no toxic effects up to 100 $g.L^{-1}$ occurred and reasonable concentrations of long-chain

dicarboxylic acids were obtained. However, it is unfortunate that the high amount of sugar still present in the side stream cannot be metabolised by *C. tropicalis* ATCC20962 and therefore extra glucose had to be added during the fermentation, increasing the cost. A second source, namely Dissolved air flotation (DAF) sludge, also showed no growth inhibiting effects up to 80 % (v/v) dosage and long-chain dicarboxylic acids were obtained. Due to the lower fat concentration in the DAF sludge, a very high amount (80 % (v/v)) was added to the fermenter to obtain feasible yields which resulted in very high dry weight concentrations and a highly viscous medium. Moreover, antifoam needed to be added to prevent excessive foaming as well as glucose to maintain the cell's metabolism, all increasing the production cost. These two side streams show a high potential for the valorisation of a low-value side stream to highly valuable chemical building blocks using industrial biotechnology. Technically it is possible to produce dicarboxylic acids from these side streams, however, more research and optimisation are necessary to obtain higher product concentrations and an economically viable production process. In future research similar side streams could be looked at as well.

Supplementary material

Supplementary information can be found in the online version of the paper.

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