1 **TITLE PAGE**

2 *Title :*

3 Microbial conversion of synthetic and food waste-derived volatile fatty acids to lipids

4 *Authors :* Shashwat Vajpeyi¹, Kartik Chandran^{1,*}

5 *Affiliation :*

6 ¹:Department of Earth and Environmental Engineering, Columbia University, New
7 York, NY 10027

8 *Correspondent footnote:*

9 Kartik Chandran, Department of Earth and Environmental Engineering, Columbia
10 University, 500 West 120th Street, New York, NY 10027. Phone : (212) 854 9027,
11 fax : (212) 854 7081, email : kc2288@columbia.edu, URL :
12 www.columbia.edu/~kc2288

13 *Running title:*

14 Microbial conversion of VFA to lipids

15

Abstract

Lipid accumulation in the oleaginous yeast *Cryptococcus albidus* was evaluated using mixtures of volatile fatty acids (VFA) as substrates. In general, batch growth under nitrogen limitation led to higher lipid accumulation using synthetic VFA. During batch growth, an initial COD:N ratio of 25:1 mg COD:mg-N led to maximum intracellular lipid accumulation ($28.33 \pm 0.74\%$ g/g dry cell weight), which is the maximum reported for *C. albidus* using VFA as the carbon source, without compromising growth kinetics. At this feed COD:N ratio, chemostat cultures fed with synthetic VFA yielded statistically similar intracellular lipid content as batch cultures ($29.88 \pm 1.92\%$, g/g). However, batch cultures fed with VFA produced from the fermentation of food waste, yielded a lower lipid content ($14.99 \pm 0.06\%$, g/g). The lipid composition obtained with synthetic and food-waste-derived VFA was similar to commercial biodiesel feedstock. We therefore demonstrate the feasibility of linking biochemical waste treatment and biofuel production using VFA as key intermediates.

Keywords: Volatile fatty acids, microbial lipids, biodiesel, biofuel, fermentation

1. Introduction

During the past decade, global biofuel production has tripled. As a result, biofuels have come under particular scrutiny owing to the significant resource stress they exert in terms of substrates, including agricultural products and water. Biofuels such as bio-ethanol and biodiesel have contributed to an increase in the food prices either directly through use of food crops towards bio-ethanol production or indirectly through use of agricultural land mass for cultivation of oil crops for biodiesel production (Bouriazos et al., 2014). Accordingly, alternate substrates for improving the longer term sustainability of biofuel production need to be explored. In this study, we evaluated the use of volatile fatty acids (VFA), for lipid production (and subsequent conversion to biodiesel) by oleaginous (oil-producing or accumulating) microorganisms. Oleaginous microorganisms can biologically convert a wide array of carbon sources into lipids and can accumulate lipids in excess of 65% of their dry cell mass. Oleaginous microorganisms have been employed for lipid production with a wide variety of commercial carbon sources including glucose (Hansson & Dostálek, 1986a), starch, sucrose, xylose (Li et al., 2010) ethanol and lactose (Papanikolaou et al., 2010). However, none of these strategies have so far proven viable for commercial biodiesel production either due to the high cost of the substrate, glucose, which accounts for about 80% of the total production cost (Fei et al., 2011). Among alternate less expensive carbon sources for lipid production are volatile fatty acids (VFA). VFA are especially attractive, since they can be produced by anaerobic fermentation of different nonedible feed stocks such as organic waste streams including waste activated sludge (Zhang et al., 2009), food waste (Lim et al., 2008), dairy waste (Demirel & Yenigun, 2004), municipal solid waste (Sans et al., 1995) among others. Traditionally, anaerobic treatment of organic waste has been focused on biogas production through methanogenesis.

Alternately, VFA produced at wastewater treatment plants have mainly been considered for use in biological nutrient removal operations. Herein, we explored the possibility of re-directing the VFA to lipids by the oleaginous yeast *Cryptococcus albidus*, for downstream use in biodiesel production.

The principal objectives of this study were to determine lipid and cell yields and specific growth rates of *C. albidus* batch cultures subjected to various degrees of nitrogen limitation and different carbon sources and to characterize the speciation of the accumulated lipids and evaluate the potential for use as a commercial biodiesel feedstock.

2. Materials and Methods

2.1. Batch cultivation of C. albidus cultures

Cryptococcus albidus (ATCC 10672) was obtained from American Type Culture Collection (ATCC, Manassas, VA). *C. albidus* cultures were propagated through a monthly subculture on liquid yeast mold (YM) broth (BD Difco, Sparks, MD). The nutrient medium for all the experiments contained, per liter: 3g KH₂PO₄, 1g MgSO₄·7H₂O, 15 mg FeCl₃·6H₂O, and 7.5 mg ZnSO₄·7H₂O, 0.5 mg CuSO₄·5H₂O (ACS grade chemicals, Fisher Scientific, Pittsburgh, PA). Ammonium chloride (NH₄Cl) was used as the nitrogen source and VFA were used as the sole carbon and energy source. All the equipment and solutions for microbial cultivation were steam sterilized at 121°C and 15 psi for 15 min. VFA were sterilized using a 0.2 µm syringe filter (VWR scientific, Bridgeport, NJ) and added to the autoclaved medium upon cooling to prevent losses by volatilization. 24-hour-old *C. albidus* cultures in the late log phase grown on YM broth were used as the inoculum (10% v/v). Batch cultivation was performed in aerobic 6.0 L glass reactors (Bellco glass, Vineland, NJ) at a temperature of 23°C to stationary phase (96 hours, data not shown). Reactor pH was measured using an autoclavable pH electrode (Cole Parmer, Vernon

hills, IL) and automatically controlled (Jenco 3676, San Diego, CA) at 6.0 ± 0.1 through automated addition of 2.0 mol/L HCl or NaOH solutions. The above temperature and the pH were chosen based upon a previous study by (Fei et al., 2011). Sterile lab air (filtered through 0.2 μ m filter, Millipore MTGR 05010 Bedford, MA) was supplied at 360 L/h to keep the dissolved oxygen concentration near saturation.

2.2. Effect of nitrogen concentration during batch growth

The initial VFA concentration was kept constant at 6500 mg COD/L and five different initial nitrogen concentrations of 1300, 260, 130, 52 and 26 mg $\text{NH}_3\text{-N/L}$ were tested which correspond to an initial COD:N ratio of 5:1, 25:1, 50:1, 125:1 and 250:1 respectively.

2.3. Effect of different carbon sources and VFA concentrations on batch growth

VFA produced from anaerobic fermentation of food waste and a synthetic 5:1:4 VFA mixture of acetic: propionic: butyric acid (expressed as COD, which was reflective of fermenter output) were used as carbon and energy sources in distinct experiments. The impact of nitrogen limitation on batch cultures was tested using three different initial synthetic VFA concentrations of 2600, 6500 and 10,000 mg COD/L, while keeping the initial ammonia-nitrogen concentration at 260 mg-N/L.

2.4. Chemostat cultivation

Duplicate chemostats ($V = 6\text{L}$) were operated at a hydraulic retention time (HRT) of 3 days. The influent VFA and nitrogen concentrations were 6500 mg COD/L and 260 mg-N/L corresponding to the COD:N ratio of 25:1 obtained from batch cultivation described above. The remaining growth medium composition and all other operation conditions were same as for the batch cultures.

2.5 Cell growth and reactor performance measurement

20 mL samples were periodically withdrawn from the batch and chemostat reactors using a sterile sampling port assembly. Cell concentrations were measured as absorbance of the culture broth at 600 nm (OD₆₀₀, Genesys-20 spectrophotometer, Thermoscientific, Waltham, MA). Cell samples were centrifuged (10,000 rpm, 9133 x g, 10 min) and filtered using a 0.2 µm syringe filter (VWR scientific, Bridgeport, NJ) for VFA and nitrogen measurements. VFA concentrations were determined using colorimetry (Hach volatile acids reagent set (2244700). Ammonia concentrations were measured using an ion-selective electrode (Thermofisher scientific, Waltham, MA).

For the batch cultures, maximum specific cell growth rate (µ_m), the specific VFA and nitrogen uptake rates (sVUR and sNUR) and the relative nutrient uptake ratio (ΔCOD:ΔN) were calculated using following equations:

$$\ln\left(\frac{OD_{exp}}{OD_o}\right) = \mu_m * t_{exp} \quad \text{Equation (1)}$$

$$sVUR = \frac{1}{X_f} * \frac{COD_o - COD_f}{t_f} \quad \text{Equation(2)}$$

$$sNUR = \frac{1}{X_f} * \frac{N_o - N_f}{t_f} \quad \text{Equation (3)}$$

$$\frac{\Delta COD}{\Delta N} = \frac{COD_o - COD_f}{N_o - N_f} \quad \text{Equation(4)}$$

where OD, COD, N, X and t represent the optical density at 600nm, VFA concentration in mg COD/L, ammonia concentration (mg-N/L), cell concentration (mg/L) and time of incubation, respectively. The subscripts 'o', 'exp' and 'f' represent the concentrations at the initiation, end of exponential phase and end of batch incubation, respectively. The end of exponential growth phase was inferred visually from the time series profiles of OD during batch growth. The end of the incubation was defined as the time point past which VFA, NH₃-N and cell densities (as OD) were nearly invariant.

VFA composition was determined using gas chromatography with flame ionization detection (GC-FID) with MXT-1 column, 60m, 0.53mm ID (Restek , Bellfonte, PA) . The flame ionization detector was operated at 240°C with an initial temperature of 150°C for 5 min subsequently ramping up 10°C/min to 240°C.

2.6 Anaerobic fermentation of food waste

Anaerobic fermentation was performed in a 6.0 L-jacketed vessel (Bellco glass, Vineland, NJ) maintained at 37°C by recirculating water from a hot water bath. The pH in the fermenter was adjusted at 6.5 by automated addition of 1:1 1M NaHCO₃ and 1M NaOH solution. The anaerobic fermenter was operated at a HRT of 2 days to limit methanogenesis and enhance production of VFA. To prepare the feed, food waste was obtained from the Columbia University cafeteria. The total solids (TS) and total volatile solids (TVS) content in the raw undiluted food waste were determined gravimetrically by drying 200 mg food waste in a furnace (Thermoscientific, Asheville, NC) at 105°C and 550°C respectively until no further decrease in the weight was observed. The total and soluble COD (tCOD and sCOD, respectively), total nitrogen (TN) and VFA concentrations were determined using colorimetry (Hach kits 2125915, 2672245 & 2244700, Loveland, CO). The COD loading rate of the fermenter was 13000 mg COD/L/day. The food waste was blended in a mixer and suitably diluted using de-ionized (DI) water to achieve a final concentration of 26000 mg COD/L. The fermentate was centrifuged at 10,000 rpm for 20 min (Beckman Coulter, Avanti J 26, Pasadena, CA) and the supernatant was collected and sterilized using a 0.2 µm syringe filter (VWR scientific, Bridgeport, NJ) to be used as the carbon source for *C. albidus* cultivation.

2.7. Lipid extraction and determination of fatty acid composition

Lipids were extracted according to the Folch wash method(Folch et al., 1957) with some

modifications. Briefly, cell pellet was lysed by ultrasonication at 70 Hz for 10 min. followed by mechanical disruption using 0.4-0.5 mm glass beads for 30 min on a vortex mixer. Lipids were extracted using a 2:1 chloroform:methanol mixture, the purified chloroform layer was evaporated to dryness under a N₂ stream. The lipids were immediately weighed and transesterified using 12% boron trifluoride (BF₃) and the fatty acid methyl esters (FAME) components were analyzed using SRI instruments 8610C GC-FID (Torrance, CA) with MXT-WAX column, 30m, 0.53mmID (Restek , Bellfonte, PA). The injector temperature was 40°C, ramped at 6°C to 200°C and then to 250°C at 15°C/min.

3. Results and discussion

3.1.Effect of nitrogen concentration on the lipid and biomass yield

The relative initial carbon to nitrogen ratio (COD:N) has been reported to affect the extent of intracellular lipid accumulation(Immelman et al., 1997). In our experiments, nitrogen was considered growth limiting if the supplied nitrogen was exhausted before the VFA. Herein, nitrogen limiting conditions (NH₃-N ≤ 260 mg/L, COD:N ≥ 25:1) resulted in statistically higher intracellular lipid content ($p=0.03$). The maximum intracellular lipid content ($28.81\pm2.06\%$) was achieved at an excessively high COD:N ratio of 250:1, but this occurred at the expense of a lower kinetics ($\mu_m = 0.02\pm0.00\text{ h}^{-1}$) (Table I). In contrast, when excess nitrogen (COD:N = 5:1) was fed, the growth kinetics ($0.04\pm0.00\text{ h}^{-1}$) were higher ($p=0.02$) but the intracellular lipid content was significantly lower ($19.59\pm0.16\%$, $p=0.03$). Of the different COD:N ratios tested, a COD:N ratio of 25:1 was the most favorable for maximizing both intracellular lipid accumulation ($28.33\pm0.74\%$ w/w) and growth kinetics ($0.04\pm0.00\text{ h}^{-1}$). To date, this is the highest reported degree of lipid accumulation in *C. albidus* using VFA as the carbon source. Previous studies have reported similar optimal COD:N ratios in different oleaginous species

under diverse growth conditions. COD:N ratios of 24:1, 26:1 and 27:1 were reported for optimal lipid production by the oleaginous yeast *Rhodospiridium toruloides* on distillery wastewater (Ling et al., 2014), oleaginous fungus *Cunninghamella echinulata* on tomato hydrolysate waste (Fakas et al., 2008) and oleaginous fungi *Mucor circinelloides* on acetate (Immelman et al., 1997) respectively. These results suggest that the metabolic pathways for lipid accumulation across different oleaginous species might be triggered under similar degree of nutrient limitation, despite differences in their stoichiometric demands.

The different COD:N ratios also impacted the relative COD and nitrogen uptake ratio, $\Delta\text{COD}/\Delta\text{N}$ (w/w, Equation 4). At non-limiting feed NH_3 concentrations (COD:N =5:1), the relative uptake ratio ($\Delta\text{COD}/\Delta\text{N}$) was 33.10 ± 7.24 g COD:gN. Based on the empirical formula of $\text{CH}_{1.65}\text{O}_{0.54}\text{N}_{0.14}$ (von Stockar & Liu, 1999), the stoichiometric COD:N ratio of yeast cells is 16.94 g COD:g N). Therefore, under non-limiting nitrogen supply, the fraction of carbon supplied, which is assimilated is roughly 51% (COD:COD), well in keeping with the calculated theoretical yield of microbial growth on acetate (50% COD/COD for a electron capture efficiency of 50%) (Rittmann & McCarty, 2001).

However, at progressively nitrogen limiting conditions (increasing initial COD:N ratios), the cells exhibited an increase in the relative $\Delta\text{COD}/\Delta\text{N}$ uptake ratio. At an initial COD:N ratio of 25:1, the $\Delta\text{COD}/\Delta\text{N}$ increased by 141% to 79.84 ± 8.76 w/w ($p=0.03$) and even further to 186.69 ± 28.31 w/w for an initial COD:N ratio of 250:1. These results show that *C. albidus* cultures responded to nitrogen limitation by taking up disproportionately higher levels of organic carbon, potentially for intracellular storage. This finding was supported further by the COD and nitrogen based biomass yield coefficients ($Y_{\text{X}/\text{COD}}$ and $Y_{\text{X}/\text{N}}$) obtained from the batch experiments. Essentially, under different degrees of nitrogen limitation, the extent of COD

consumption (5639.29 ± 483.09 mg/L) and observed yield of biomass per gram of COD consumed ($Y_{X/COD} = 0.19 \pm 0.01$ g/g, which includes biomass carbon and storage carbon) was found to be statistically invariant (Figure 1). In contrast, the yield coefficient $Y_{X/N}$ was 5.5 fold higher for a feed COD:N ratio of 250:1, compared to 5:1 (Figure 1). Similar observations were reported previously in nitrogen starved and nitrogen sufficient cultures of *Chlorella vulgaris* using a range of nitrate concentrations as the nitrogen source. It was observed that progressive nitrogen limitation did not cause a proportional decrease in the biomass concentration but the yield coefficient, $Y_{X/N}$, increased from 1.6 to 10 when initial assimilative N-source (nitrate, in that case) was decreased from 2000 mg-N/L to 40 mg-N/L (Griffiths et al., 2014). These trends in $Y_{X/N}$ suggest that under nitrogen limitation and exhaustion, the same unit carbon was consumed. However, the consumed carbon was channeled towards intracellular carbonaceous storage compounds, rather than to more cellular biomass. Therefore, *C. albidus* responds to nitrogen limitation by altering the relative substrate uptake efficiency ($\Delta COD/\Delta N$) and increasing intracellular carbon storage including lipid storage.

The response of *C. albidus* to nitrogen limitation can be explained by certain key metabolic reactions involved in carbon and nitrogen assimilation. Following nitrogen exhaustion, the enzyme adenosine monophosphate (AMP) deaminase (EC 3.5.4.6), which catalyzes the conversion of AMP to inosine monophosphate (IMP) and ammonium, is activated to scavenge intracellular nitrogen (Ratledge, 2002). This depletion of AMP causes allosteric inhibition of the activity of the enzyme isocitrate dehydrogenase (IDH, EC 1.1.1.41), which catalyzes the oxidative decarboxylation of isocitrate to α -ketoglutarate during the tricarboxylic acid (TCA) cycle (Ratledge, 2004). α -ketoglutarate is a nitrogen transporter and forms the backbone of the glutamate family of amino acids and is essential for nitrogen assimilation in the cells. At limiting

nitrogen concentrations, the flux through the TCA cycle slows down, resulting in buildup of citrate inside the mitochondrion (Liu et al., 2011). Consequently, citrate is transported outside the mitochondria and is cleaved by the enzyme ATP: Citrate lyase (ACL, EC 2.3.3.8) to form acetyl co-A and the excess acetyl co-A is directed towards fatty acid synthesis for storage (Ratledge, 2004). Indeed, in nitrogen limited cultures of oleaginous yeast *Lipomyces starkeyi* intracellular citrate concentrations increased by ~80% and the specific activity of IDH enzyme dropped from 0.023 to 0.005 U/g once the nitrogen source was exhausted (Tang et al., 2009). In another study, the N-content of the *Chlorella vulgaris* decreased from 9% to 0.5% of the dry cell weight upon nitrogen exhaustion (Griffiths et al., 2014). A down-regulation of IDH enzyme and a concomitant increase in the levels of ACL enzyme and intracellular lipids under nitrogen starved conditions were also observed in *Chlamydomonas reinhardtii* cultures (Wase et al., 2014). Therefore, these are several consistent lines of evidence that nitrogen limitation is an effective lipogenesis inducer in the oleaginous microorganisms, as experimentally observed during our study.

3.2. The effect of initial VFA concentrations on the lipid and biomass yield

As described above, at an initial VFA concentration of 2600 mg COD/L (COD:N= 10:1), the cell growth was limited by the carbon source (and not nitrogen). The VFA mixture was completely exhausted in 48h, following which the cells entered stationary phase (data not shown). The relative nutrient uptake ratio, $\Delta\text{COD}/\Delta\text{N}$ (62.23 ± 1.00 w/w) was statistically similar ($p=0.11$) to that of the batch with excess nitrogen (COD:N = 5:1). The specific VFA and nitrogen utilization rates ($\text{sVUR} = 65.25 \pm 0.15 \text{ h}^{-1}$ and $\text{sNUR} = 1.06 \pm 0.00 \text{ h}^{-1}$) were also similar ($p=0.07$ and 0.46) to the batch cultures at the feed COD:N ratios of 5:1 and 10:1. In contrast, when the initial VFA concentration was increased to 10,000 mg COD/L, no cell growth or lipid accumulation was

1 observed presumably due to substrate inhibition on account of toxicity of acetic acid at higher
2 concentrations. High concentrations of acetate interfere with the membrane transport of the
3 phosphates (Georgina Rodrigues, 2000). Acetate was shown to inhibit the growth of the
4 oleaginous yeast *Brettanomyces bruxellensis* at concentrations higher than 2100 mg COD/L and
5 growth was completely inhibited at acetate concentrations higher than 4200 mg COD/L (Yahara
6 et al., 2007). However, we found that the *C. albidus* was not inhibited by even higher initial
7 acetate concentrations as high as 3250 mg COD/L (calculated as 50% of total initial COD of
8 6500 mg/L). This further suggests that *C. albidus* might also be a good candidate for high
9 throughput lipid production as it can efficiently convert high strength organic waste streams to
10 lipids, without the need for substrate dilution.

11 3.3. Differential VFA utilization rates

12 Acetate was consumed more rapidly with a maximum specific substrate utilization rate of
13 47.91 ± 4.24 mg-acetate/g biomass/h compared to 3.97 ± 1.54 mg-propionate/g biomass/h for
14 propionate and 7.42 ± 3.01 mg-butyrate/g biomass/h for butyrate. Acetate was completely
15 exhausted by the end of the exponential growth phase and propionic and butyric acid were
16 utilized only after the depletion of acetate (Figure 2). The trends herein are in good agreement
17 with those from previous studies, in which higher specific uptake rates have been reported for
18 acetate (1.50 ± 0.14 d⁻¹) compared to propionate (0.07 ± 0.00 d⁻¹) and butyrate (0.09 ± 0.06 d⁻¹) in the
19 mixed bacterial cultures engaged in polyhydroxyalkanoate production (Fradinho et al., 2014).
20 Slower consumption rates of longer chain acids compared to acetate may be attributed to their
21 different metabolic fate after intake. Propionate, an odd chain carboxylic acid, cannot be cleaved
22 directly to acetyl-CoA and it must be converted to propionyl-CoA and enter the TCA cycle via
23 methylmalonyl-CoA inter-conversion to succinyl-CoA. The rate of propionate metabolism is

1 therefore likely controlled by the rate of its decarboxylation of acetyl-CoA. In case of butyrate,
2 the higher number of biochemical transformations that it must undergo including β -oxidation to
3 acetoacetyl-CoA and then further cleavage to acetyl-CoA may be the cause of its slower
4 uptake(Fradinho et al., 2014). Therefore, in mixed substrate systems, acetate will likely be
5 preferably used for growth over other VFA (Albuquerque et al., 2013). The factors influencing
6 the VFA output from fermentation are fairly well understood and a predominantly acetate
7 product is possible through process manipulations which might lead to improved lipid yield
8 downstream.

9 3.4. Fatty acid composition of the lipids accumulated by *C. albidus* in batch cultures

10 In all cases with synthetic VFA, the fatty acids accumulated were predominantly palmitic
11 (C16:0), oleic (C18:1), and linoleic acid (C18:2), corresponding to those of soybean and jatropha
12 oil, which are used as feedstock for biodiesel production in the States and the European
13 Union(Seo et al., 2014) . The fatty acid composition in the nitrogen limited batches (COD:N $\geq$
14 25:1) was statistically similar ($p=0.43$) to palmitic (C16:0), oleic (C18:1), linoleic (C18:2) acid
15 at $27\pm1.58\%$, $50\pm1.32\%$ and $15\pm1.29\%$ respectively (Table II). In general, similar fatty acid
16 compositions were reported for yeast *Cryptococcus curvatus* (Chi et al., 2011), yeast
17 *Rhodotorula mucilaginosa* (Li et al., 2010) and for algae *Laminaria japonica* (Xu et al.) under
18 different growth medium and conditions. This suggests that the metabolic pathways of lipid
19 accumulation could be conserved across the oleaginous species across different feedstocks and
20 growth conditions, which makes *Cryptococcus albidus* a good candidate for biodiesel production
21 from highly diverse organic waste streams.

22 The composition of fatty acids also plays a crucial role in the quality of biodiesel that can be
23 produced. To achieve the best compromise between the cold flow properties *i.e.* the tendency of

1 fuel to solidify at lower temperatures and the oxidative stability *i.e.* the susceptibility of
2 unsaturated fats to self-oxidation, the optimum feedstock should have relatively low levels of
3 saturated and poly unsaturated fats and high levels of monounsaturated fats (Hoekman et al.,
4 2012). We found that the microbial lipids obtained from the VFA contained about 70%
5 unsaturated fats with **a** linoleic acid (C18:2) content at about 15%, which is very similar to the
6 commercial feedstock used for biodiesel production (~75% unsaturated fats in jatropha oil).
7 Therefore, in addition to the high degree of lipid accumulation of *C. albidus* when grown on
8 VFA with nitrogen limitation, the appropriate composition of the accumulated lipids further
9 renders this process highly suitable for biodiesel production.

3.5. Effect of different carbon sources on the lipid and biomass yield

Raw food waste and fermentate composition. The raw food waste used to prepare the
fermenter feed had a total solids and total volatile solids content of (TS = 20.10±4.63% w/w) and
(TVS = 16.22±0.15% w/w) respectively. The tCOD, sCOD, TN and VFA concentrations in the
raw food waste were 312±30.88 g/Kg, 79.6±18 g/Kg, 15.6±0.7 g/Kg and 6.2±3.9 g/Kg
respectively. The food waste fermentation process was concluded after 24 d of continuous
operation at a 2d HRT. At this point, the average fermentate COD concentration was
27065±7048 mg/L, which consisted on an average of 52.6±10.9% soluble COD (sCOD) while
the sCOD consisted of 71.3±22.7% VFA on average. The conversion of the influent COD load to
VFA was 38.7±12.9% and the VFA concentration in the fermentate was 10068±3367 mg
COD/L. The most common VFA present in the fermentate were acetate (51.5±0.6%), propionate
(13.8±1.4%) and butyrate (34.6±0.1%), all expressed as mass.

Batch growth on both synthetic VFA and the sterilized VFA stream from the anaerobic food-waste fermenter resulted in similar biomass concentration ($p=0.11$, Table III). However, the intracellular lipid concentration ($14.9\pm0.1\%$) and the maximum specific growth rate ($0.021\pm0.00\text{ h}^{-1}$) were statistically lower for fermentate VFA ($p=0.02$, Table III). Similarly, in a previous study, the oleaginous yeast *Cryptococcus curvatus* also showed poor cell growth and a decrease in the intracellular lipid content (13.5%) when using food waste fermentation effluent as the growth medium (Chi et al., 2011), pointing to the presence of as yet unknown inhibitors in the fermentate. Another possible cause of lower lipid yield could be the high protein content of the food waste, which can effectively lower the COD:N ratio below the favorable range ($\text{COD:N}\geq 25$). Based on our previous results, food waste fermentate TKN concentrations were in the range $1410.8\pm516.2\text{ mg-N/L}$ (Ljupka Arsova, 2010) resulting in an effective feed COD:N ratio in our *C. albidus* batch cultures of 2.87:1. Although not attempted in this study, the observed differences in the kinetics (μ_m) and extent of lipid accumulation between synthetic VFA and VFA present in fermentate point to the necessity for independent optimization of lipid production from different waste streams, which is not entirely unexpected.

In general, the relative content of unsaturated lipids (proportion of lipids with 1,2 or 3 carbon-carbon multiple bonds), using synthetic VFA at all initial COD:N ratios of 5:1 (76.7 ± 0.5), 25:1 (68.5 ± 0.4), 50:1 (69.9 ± 0.7), 125:1 (69.7 ± 1.0) and 250:1 (66.7 ± 0.7) was higher than on fermentate (43.1 ± 2.1), as shown in Table II. Previously, (Hansson & Dostálek, 1986b) reported that the degree of unsaturation of fatty acids in chemostat *C. albidus* cultures is directly proportional to the specific growth rate. While this argument certainly holds for this study, given the lower maximum specific growth rate (Table III) and corresponding lower degree of lipid

unsaturation (Table II) with fermentate, more metabolic level studies are needed to elucidate specific mechanisms of differential lipid speciation.

3.6. Biomass and lipid production during chemostat cultivation

At an operational HRT of 3 days ($D = 0.33 \text{ d}^{-1}$), the steady state biomass concentration was $1.02 \pm 0.07 \text{ g/L}$ and the intracellular lipid content was $29.88 \pm 1.92\%$. The biomass yield coefficients normalized to influent COD and nitrogen concentrations ($Y_{X/\text{COD}} = 0.18 \pm 0.01$ and $Y_{X/N} = 9.05 \pm 0.29$) were statistically similar to those in the batch culture ($p=0.18$ and 0.52 , respectively). The composition of the lipids produced was also largely similar to those from the batch cultures (Table II). Chemostat cultures could be advantageous over the batch cultures since any transient changes in the available COD:N ratio can be eliminated under the steady state operation and the cells can be maintained under continuous and sustained nitrogen limitation. Furthermore, chemostat operation can also allow the cell growth rate (μ) to be manipulated through the dilution rate, which can be used to optimize kinetics and yields of lipid production. Although not optimized herein, an increase in intracellular lipid content with decreasing dilution rates (increasing HRT values) has been shown previously using glucose and complex organics, but not with VFA (Hansson & Dostálek, 1986b).

3.7 Techno-economic assessment of lipid production:

The production of lipids by first converting non-lipid carbon to VFA as key intermediates by anaerobic fermentation is more advantageous from than just focusing on the inherent lipid content of organic waste streams such as food waste, sewage sludge or fecal sludge. In our experiments, we were able to achieve an overall conversion of $20.29 \text{ mg lipid/ g-COD}$ from food waste influent with a corresponding lipid yield from VFA of $52.4 \text{ mg lipid/gVFA-COD}$.

1 The choice of the feedstock for lipid production can account for up to 80% of the total biodiesel
 2 production cost (Fei et al., 2011). Further, the cost of lipid production itself can vary
 3 significantly between microorganisms and feedstocks. For lipid production using yeast based
 4 processes, (Seo et al., 2014) reported a final lipid cost of \$2.19/kg lipids produced using
 5 *Cryptococcus curvatus* fed with algal residue, while (Fei et al., 2011) reported \$0.49/kg and
 6 \$3.15/kg from *C. albidus* growing on VFA and glucose respectively. In comparison, the cost of
 7 current commercial feed stock like Jatropha and soybean oil is \$0.76/kg and \$0.77/kg (Seo et al.,
 8 2014). Using the lipid yield coefficient ($Y_{LS} = 40.96$ kg lipid/ ton VFA) for VFA produced from
 9 food waste as the carbon source and a VFA production cost of \$30-\$100/ ton (Fei et al., 2011),
 10 the overall lipid production cost from food waste was US\$ 0.81-2.53/kg.
 11 Correspondingly, for our process, the overall cost of biodiesel production for a 10 million gallon
 12 per year system as modeled after (Haas et al., 2006) equaled \$0.71-\$2.23 /L (as summarized
 13 along with added assumptions in Table IV). In contrast, for algal biodiesel, (Ratlidge & Cohen,
 14 2008) reported a very high cost of \$21/L for algal biodiesel, while (Davis et al., 2011) reported
 15 the unit cost to be \$2.25/L. It must be noted that this analysis does not even take into account the
 16 positive impact on the environment considering the reduced discharge of food waste (or other
 17 organic wastes) without treatment. In sum, the production of lipids from waste derived VFA
 18 using oleaginous yeast such as *C. albidus* offers an economically viable prospect for sustainably
 19 linking waste management and biofuel production, while minimizing reliance on food crop for
 20 energy use.

4. Conclusions

Batch cultivation of *C. albidus* was optimized to maximize intracellular lipid accumulation, by imposing nitrogen limitation at initial VFA concentrations as high as 6500 mg COD/L. In general, the produced microbial lipids were of similar composition to soybean and jatropha oil, both of which are used for commercial biodiesel production. In sum, we demonstrate the potential for a VFA based flexible microbial platform for the bioconversion of organic ‘waste’ streams to lipids. This proposed link to ‘waste’ derived lipids could also lead to more sustainable pathways for biofuel production than through agricultural resources alone.

5. Acknowledgements

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6. Tables and figures

Table I. The effect of different initial COD:N ratio on the biomass growth, specific growth rate and lipid production by *C. albidus*. The initial VFA concentration was 6500 mg COD/L in all experiments and the initial ammonia-N concentrations were varied.

Initial COD:N ratio	5:1	25:1	50:1	125:1	250:1
Biomass concentration (g/L)	1.3±0.0	1.1±0.1	1.0±0.0	1.1±0.0	0.97±0.0
Lipid concentration (g/L)	0.26±0.0	0.32±0.0	0.21±0.0	0.27±0.0	0.27±0.0
% Intracellular lipid accumulation (g/g)	19.6±0.2	28.3±0.7	21.3±0.1	24.1±0.2	28.8±2.1
$Y_{L/\Delta COD}$ (mg/g)	44.1±0.7	52.4±0.9	32.1±1.1	40.8±0.0	41.2±2.0
Maximum specific growth rate μ_m (h⁻¹)	0.041±0.0	0.040±0.0	0.040±0.0	0.038±0.0	0.023±0.0
sVUR (mg-COD/g-cell/h)	64.2±1.1	88.7±3.8	91.4±15.9	91.4±17.2	86.5±20.3
sNUR (mg-N/g-cell/h)	1.8±0.3	1.3±0.3	1.2±0.1	0.96±0.0	0.4±0.1
$\Delta COD/\Delta N$ (g/g)	33.1±7.2	79.8±8.8	80.1±7.1	100.7±19.3	186.7±28.3

Table II. Percentage composition of lipids obtained under different cultivation conditions

Lipids	Batch with different initial COD:N					VFA from food waste	Chemostat
	5:1	25:1	50:1	125:1	250:1	fermentation	
C 14:0	0.28±0.0	0.21±0.0	1.5±1.7	1.5±1.7	0.19±0.1	10±0.4	0.52±0.2
C 16:0	20.8±1.9	28.7±1.4	26.4±1.3	27.3±0.1	29.9±0.7	31.4±1.7	36.8±0.9
C 16:1	0.24±0.1	0.57±0.2	0.69±0.2	0.61±0.1	0.89±0.0	2.0±0.0	0.96±0.4
C 18:0	2.2±0.1	2.4±0.1	2.2±0.3	1.6±0.6	3.1±0.1	4.0±0.0	2.7±2.0
C 18:1	66.3±2.4	50.4±1.2	50.5±0.7	52.8±2.6	49.8±0.1	36.2±2.3	37.4±3.2
C 18:2	9.1±0.4	15.3±0.7	16.7±0.2	14.0±3.5	13.9±0.7	3.5±0.4	19.1±0.3
C 18:3	0.9±0.1	1.9±0.2	1.7±0.4	1.9±0.1	1.7±0.1	0.9±0.0	2.3±0.3
C 20:0	ND	ND	ND	ND	ND	11.5±0.8	ND
C 20:1	0.15±0.0	0.35±0.1	0.33±0.0	0.33±0.0	0.42±0.0	0.52±0.0	0.29±0.1

Table III. Effect of different COD sources on cell growth and lipid accumulation by *C. albidus*.

Carbon source	Synthetic VFA mixture (initial COD:N=25:1)	VFA from anaerobic fermenter
Biomass Concentration (g/L)	1.1±0.1	0.96±0.0
Maximum specific growth rate μ_m (h ⁻¹)	0.040±0.0	0.021±0.0
% Intracellular lipid accumulation (g/g)	28.3±0.7	14.9±0.1
$Y_{L/\Delta COD}$ (mg/g)	52.4±0.9	30.8±0.6

Table IV. Summary of unit costs for 10 million gallons/year biodiesel production using *C. albidus*. All costs are in US\$ unless otherwise mentioned

Costs for biodiesel production	Cost of VFA production	
Carbon source cost	\$30/ton	\$100/ton
Lipid yield from <i>C. albidus</i> (kg lipid/ton VFA)	40.96	40.96
Lipid cost (\$/lb)	0.33	1.11
Raw material cost ^a (\$/L biodiesel)	0.68	2.20
Utilities cost ^b (\$/L biodiesel)	0.012	0.012
Labor cost ^c (\$/L biodiesel)	0.013	0.013
Supplies cost ^d (\$/L biodiesel)	0.004	0.004
General works cost ^e (\$/L biodiesel)	0.003	0.003
Depreciation@10% capital cost/year (\$/L biodiesel)	0.03	0.03
Glycerol value ^f (\$/L biodiesel)	-0.034	-0.034
Gross cost ^g (\$/L biodiesel)	0.71	2.23
Gross cost ^h (\$/Kg biodiesel)	0.81	2.53

^a Raw material includes cost of lipid, methanol, sodium methoxide, hydrochloric acid, sodium hydroxide and water.

^b Utilities including gas and electricity (0.06\$/kWh), taken from US energy Information Administration(USEIA).

^c Labor includes operating, maintenance, supervisory and fringe benefit costs.

^d Supplies include operating and maintenance supplies.

^e General works include administrative costs, property insurance and taxes.

^f Glycerol co-product when sold as 80% crude solution at a market value of 0.15\$/lb

^g Sum of all the costs minus the co-product value.

^h Calculated from density of biodiesel 0.88 kg/L reported by (Alptekin & Canakci, 2008)

Figure 1. Effect of initial nitrogen concentration on the COD based biomass yield coefficient and nitrogen based biomass yield coefficient. The initial VFA concentration was 6500 mg COD/L in all experiments. Yield coefficients expressed in weight of biomass produced per gram COD consumed. Error bars represent standard deviation of three replicate measurements.

Figure 2. Rate of consumption of different VFA in batch cultures of *C. albidus*. The initial VFA and nitrogen concentrations were 6500 mg-COD/L and 260 mg-N/L, respectively. Error bars represent standard deviation of three replicate measurements.

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