Production of microbial lipids utilizing volatile fatty acids derived from wastepaper: A biorefinery approach for biodiesel production

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Abstract

Volatile fatty acids (VFAs) derived from organic wastes are being considered as low-cost feedstock for microbial lipid production as a valuable alternative to plant derived oils/biodiesel. In this study, VFAs were produced from anaerobic open culture fermentation of wastepaper and subsequently, used as feedstock for lipid production by *Cryptococcus curvatus*. Total VFAs, yield and productivity achieved from waste office paper (WOP) and newspaper (WNP) were 17.3 and 10.2 g/L, 0.17 and 0.10 g/g TS, and 0.86 and 0.51 g/L/day, respectively. Biomass, lipid content and productivity achieved utilizing VFAs of WOP and WNP were 4.3 and 2.9 g/L, 41.2 and 27.7% DCW, and 0.037 and 0.033 g/L/h, respectively. The dominance of fatty acids such as oleic, palmitic, stearic and linoleic acid in the lipids suggests a high level of similarity with plant/vegetable oils used for biodiesel production. Therefore, VFAs derived from wastepaper could be potentially used as feedstock to produce microbial lipids towards cost-effective production of biodiesel.

Keywords: Biodiesel; Volatile fatty acids; Wastepaper; Anaerobic open culture fermentation; Oleaginous yeast; Microbial lipids.

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1. Introduction

Currently, the world is facing global challenges such as fossil fuel depletion and climate change caused by global warming due to the increase of greenhouse gasses (GHGs) emissions from fossil fuels [1, 2]. Biodiesel, fatty acids alkyl ester, can be obtained by transesterification of triacylglycerol from edible vegetable oils (i.e. soybean, sunflower and palm oil), fats and cooling oil wastes, has several advantages than conventional petroleum-based diesel fuel such as non-sulfur oxide emission, sustainability and potential for carbon reduction [3]. However, the use of these edible oils as a feedstock for biodiesel limiting its commercialization as it competes with food production and increase the cost of raw material such as vegetables and agricultural commodities [4]. Microbial lipids produced by oleaginous microorganisms, which accumulates 20-80% of their dry weight in the form of lipids under nutrient-limitation conditions, are the most promising alternative non-edible lipid source for sustainable production of biodiesel [5-8]. However, the production cost of microbial lipids remains as a major limiting factor due to the carbon sources used for production, which is estimated to be about 80% of the total medium cost and it contributes to over 60% of the total production costs while using glucose as a carbon source [9]. A potential solution to reduce the production cost is to utilize low-cost or waste biomass that can be used as a substrate for microbial lipid production [8, 10, 11].

Volatile fatty acids (VFAs) are linear short-chain fatty acids (C2 – C5), which includes acetic, propionic, butyric, isobutyric, valeric, isovaleric and 2-methylbutyric acid are intermediate products of anaerobic digestion (AD), have been extensively investigated for production of various bio-based materials, using a so-called VFAs platform [12-14]. The major advantages of VFAs platform for bio-based materials production are the absence of requirement for sterilization or addition of enzymes to hydrolyze, and the availability of feedstock in

substantial quantities [15].VFAs are considered as a potential carbon source for lipid production using oleaginous yeast as it requires only shorter transformation pathway (VFAs into acetyl-CoA, which is used for biosynthesis of lipids) with high theoretical lipid conversion efficiency [16, 17].

Microbial lipids production utilizing lignocellulosic biomass has received increasing interest in recent days, as an alternative solution for large-scale production of biodiesel [7, 18, 19]. Wastepaper, a major component of municipal and industrial solid wastes, accounts more than 35% of total lignocellulosic wastes is being considered as a promising feedstock for biofuels due to its sustainability and abundance [20, 21]. Recently, wastepaper has been used as feedstock for production of various valuable bio-products such as bioethanol and PHAs [7, 20, 22]. Utilization of wastepaper for production of microbial lipids is a promising alternative biorefinery approach for large-scale production of biodiesel, which is not only reduces the cost of production, but also provides alternative route for waste management [23]. Hence, in this study we aim to investigate the conversion of wastepaper into VFAs through anaerobic open culture fermentation (OCF) and subsequently, use the VFAs as possible feedstock for production of microbial lipids as an alternative to industrial production of biodiesel.

2. **Materials and methods**

All chemicals and reagents used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA) or as indicated.

2.1. Inocula

2.1.1. Inoculum for VFAs production

Waste activated sludge (WAS) obtained from local wastewater treatment plant was passed through a sieve (18 mesh) and heat treated at 90^{o} C for 20 min to inactivate methanogens.

77 The raw sludge contained 1.59 ± 0.01 g/L total suspended solid (TSS) and 1.36 ±0.01 g/L
78 volatile suspended solid (VSS). Further, WAS purged with N_2 gas for 10 min, pH maintained at
79 5.4 - 5.6 using 2N HCl and NaOH, incubated in a shaker incubator at 35^{o}C and 200 rpm was
80 used as a inoculum for VFA production.

81 *2.1.2. Inoculum for microbial lipid production*

82 The oleaginous yeast, *Cryptococcus curvatus* DSM 70022 obtained from *DSMZ*
83 *(Germany)* was propagated on YPD agar slants in every two weeks (yeast extract 10; peptone 10;
84 glucose 20; agar 15 (g/L), pH 6.0, 30^{o} C). For seed culture, *C. curvatus* was inoculated into 50
85 mL of YPD medium in 250 mL flask and incubated at 30^{o} C, 200 rpm for 36 h. Afterwards, the
86 cultures were grown in a medium containing 10 g/L acetate, 2 g/L propionate, 1 g/L butyrate, 1
87 g/L peptone, and 1 g/L yeast extract (pH - 5.5) for 24 h and used as a inoculum for lipid
88 production.

89 *2.2. Feasibility of C. curvatus for lipid production using VFAs*

90 The feasibility of *C. curavtus* for lipid production using VFAs was investigated using
91 synthetic VFAs [mixture of acetic acid (AA), propionic acid (PA) and butyric acid (BA)]. The
92 influence of VFAs on lipid accumulation was studied by comparing the initial concentration and
93 ratio of each VFA in the mixture. The effect of initial VFAs concentration was evaluated using 2,
94 5 and 10 g/L at a ratio of 5:1:4. Effects of various VFAs ratio (AA: BA: PA) (5:1:4, 5:2:3, 6:2:2
95 and 6:1:3) on lipid accumulation was investigated at an initial concentration of 5 g/L VFAs. The
96 effect of various nitrogen sources and their combination (1:1) on lipid production was
97 investigated using various inorganic [$(NH_4)_2SO_4$, NH_4Cl, NH_4NO_3, $NaNO_3$ and KNO_3] and
98 organic nitrogen sources [yeast extract (10% N, w/w)] and peptone (14% N, w/w)]. The initial

pH of the medium was adjusted to 5.5 using 2N HCl and NaOH, and the C/N ratio was maintained at 40 under all tested concentrations.

2.3. Feedstock pretreatment for VFAs production

The feedstock for VFAs production such as waste office paper (WOP) and waste newspaper (WNP) were shredded into a small pieces (2 x 6 mm) and subjected to pretreatment by mixing with 0.5% H_2O_2 (5% w/v) and then autoclave at 121^oC for 30 min. The solid residue was collected by centrifugation (Eppendorf-5810R, Germany) at 5000 *xg* for 10 min, washed 3 - 4 times repeatedly with deionized water until obtain neutral pH, dried at 60^oC for 24 h and used as substrate for anaerobic digestion.

2.4. VFAs production from wastepaper by OCF

Anaerobic open culture fermentation (OCF) was carried out in 250 mL reactors (serum bottles with seals) with 100 mL of anaerobic fermenter medium [modified RAMM medium containing 1; yeast extract, 0.27; KH_2PO_4, 0.35; K_2HPO_4, 0.53; NH_4Cl, 0.1; $MgCl{\cdot}6H_2O$, 0.075; $CaCl2{\cdot}2H_2O$ and 10; $NaHCO_3$] with the solid loading of 10% (w/v) pretreated WOP and WNP. $NaHCO_3$ was added to the medium separately as an alkaline buffer. The trace element solution (DSMZ 320, 0.1% v/v) and vitamin solution (DSMZ 503, 0.1% (v/v) was added to the fermentation medium and the pH was adjusted to 8.0 (2 N HCl and NaOH). 2-mercaptoethanesulfonate (BES) (12 mM) was used as a methanogens inhibitor. The reactors were seeded with 10% (v/v) inoculum, purged with nitrogen gas for 10 min, sealed with rubber stopper with crimp aluminum seals and incubated at 30^o C with 100 rpm for 4 weeks. After incubation, the broth from OCF was centrifuged (Eppendorf-5810R, Germany) at 10,000 *xg* for 10 min and the supernatant was subjected to struvite precipitation at 1: 1: 1.1 (Mg^{2+}: NH^{4+}-N:

PO_4^{3-} -P) molar concentration to achieve C/N ratio of 40 and subsequently used for lipid production.

2.5. Lipid production using VFAs produced from wastepaper

Lipid production was carried out in 250 mL conical flasks containing 50 mL of VFAs broth obtained from OCF. No additional nutrients were added. Seed cultures were grown for 24 h in synthetic VFAs media until reaching an Optical Density of 1 at a wavelength of 600 nm (OD_{600}). Flasks were inoculated with 1% (v/v) seed cultures subsequently grown for 72 h at 30 ^oC and 200 rpm. Aliquots (5 mL) were withdrawn at regular intervals (12 h) and used to determine cell biomass, lipid production and residual VFAs in the medium. Biomass was estimated gravimetrically by centrifuging the culture broth (5 mL) at 5000 *xg* for 10 min at 4^oC, washed with deionized water and dried at 60^oC for 24 h and expressed as cell dry cell weight (g DCW/L).

2.6. Analytical methods

Total Solids (TS) were determined by drying at 105 ^oC overnight and volatile solids (VS) were determined by ashing at 550 ^oC for 3 h [24]. Total nitrogen (TN) and ammonium nitrogen (NH_3-N) were measured using a standard method [24]. The concentrations of VFAs were analyzed using an HPLC (Agilent Technology 1100 series) equipped with Aminex HPX-87H column (BIORAD INC., USA), using refractive index (RI) and diode array detectors (DAD). The mobile phase was 0.004 M H_2SO_4 at a flow rate was 0.6 mL/min and the column temperature was maintained constant at 50^oC. Samples were filter through 0.2µm (PVDF) syringe filter (Millipore, USA) and subsequently used for analysis.

Lipids extraction from dried biomass was done by the method of Folch et al. [25]. Briefly, 10 mg of dried biomass was digested using 3.2 mL of 4M HCl at 55^{o}C for 2 h and extracted with 8 mL of chloroform/methanol (2:1, v/v), vortexed for 2 - 3min and centrifuged at 2,000 *xg* for 5 min. Further, the extracted solution was purged with nitrogen gas to evaporate the solvents and the lipids were measured and expressed as g/L.

Fatty acid methyl esters (FAME) were prepared using 2.8 M H_2SO_4 in methanol containing (10 mL/L) nonadecanoic acid (C19:0) as an internal standard and heated at 100°C for 4 h. After cooling to room temperature, 1 mL of distilled water was added, vortexed for 3 min and centrifuged at 2,000 *xg* for 1 min for organic phase separation. FAMEs were analyzed by GC (Agilent 7890A, USA) equipped with flame ionization detector (FID) and FAMEWAX column (30 m x 320 µm x 0.25 µm) using helium as a carrier gas. The injector was kept at 280^{o}C with an injection volume of 1 µL with a split ratio at 30. The initial oven temperature was set at 120^{o}C. The oven temperature was increased at a heating rate of 3^{o} C/min up to 240^{o}C and held for 20 min. The temperature of the detector was set at 250^{o}C. Fatty acids were calculated relative to their weight compared to total lipids in biomass and expressed as percentage (% total lipids).

3. Results and discussion

3.1. Effect of VFAs concentration and ratio on biomass and lipid production

The effects of initial concentration of VFAs on biomass and lipid production was investigated and presented in Table 1. Biomass and lipid yield achieved was 1.62 ± 0.06 and 0.587 ± 0.004, 2.78 ± 0.08 and 0.781± 0.008, and 4.19 ± 0.11 and 0.712 ± 0.005 g/L, and lipid content achieved was 36.3 ± 0.21, 28.1 ± 0.18 and 17.0 ± 0.20% with 2, 5 and 10 g/L VFAs concentration, respectively. These results evidenced that *C. curvatus* was able to grow even at

10g/L VFAs concentration; however, there was a considerable decrease in lipid production reported with increasing the concentration VFAs, which clearly suggesting that the higher concentration of VFAs promotes the cell growth than lipid accumulation. Thus, the initial concentration of 5 g/L VFAs was considered as most suitable resulting in high yield of biomass and lipid production compared to the other tested VFAs concentration. Park et al. [12] reported that the yeast, *C. curvatus* was not able to utilize 8 g/L of VFAs; however, lipid production was elevated with increasing VFAs to 6 g/L. Several other studies also reported that there was a significant inhibition on lipid production when VFAs concentration increased above 5 g/L [26, 27].

The effect of different ratio of VFAs on biomass and lipid production was investigated with four different ratios such as 5:1:4, 5:2:3, 6:2:2 and 6:1:3 (AA: BA: PA). The cell biomass and lipid yield achieved was ranged between 3.62 ± 0.10 and 4.38 ± 0.08 g/L, and 0.85 ± 0.004 and 1.69 ± 0.010g/L, respectively (Table.1). The results suggested that maximum biomass (4.38 ± 0.08 g/L), lipid production (1.69 ± 0.010g/L) and lipid yield coefficient (0.338 g/g) was achieved with the VFAs ratio of 6:1:3. Our results showed that high content of acetic acid (AA) in VFAs mixture greatly promotes the cell biomass and lipid productivity; hence, acetic acid is more favorable for high productivity than butyric and propionic acids. Liu et al. [28] reported that high content of AA in VFAs mixture (6:3:1) increased the biomass and productivity by *C. curvatus* utilizing WAS- derived VFAs through sequencing batch fermentation strategy.

3.2. Effect of various nitrogen sources on lipid accumulation

Several studies suggested that lipid accumulation using VFAs were greatly influenced by nitrogen source used for production. In this study, effect of various nitrogen sources was

investigated using synthetic VFAs at 5 g/L with C/N ratio 40 as presented in Table 2. The cell biomass achieved from the nitrogen sources such as ammonium sulphate, ammonium chloride, ammonium nitrate, sodium nitrate, potassium nitrate, yeast extract and peptone were 3.60 ± 0.13, 3.69 ± 0.14, 3.71 ± 0.16, 1.49 ± 0.08, 1.36 ± 0.08, 3.24 ± 0.15 and 4.17 ± 0.12 (g/L), respectively. The lipid yield (g/L) and lipid content (%) achieved using ammonium sulphate, ammonium chloride, ammonium nitrate, sodium nitrate, potassium nitrate, yeast extract and peptone were 0.236 ± 0.006 and 6.5 ± 0.18, 0.456 ± 0.008 and 12.4 ± 0.08, 0.416 ± 0.006 and 11.2 ± 0.12, 0.256 ± 0.005 and 17.2 ± 0.14, 0.139 ± 0.004 and 10.2 ± 0.08, 0.790 ± 0.006 and 24.4 ± 0.16, and 0.556 ± 0.008 and 13.3 ± 0.10, respectively. The results of combined addition of organic and inorganic nitrogen (1:1) suggested that maximum cell biomass (5.53 ± 0.09 g/L), lipid yield (1.724 ± 0.008 g/L) and lipid content (31.2 ± 0.13) was achieved with the combination of ammonium nitrate and yeast extract. These results clearly suggested that the nitrogen sources playing an important role in cell biomass and lipid production, and the combined addition of ammonium nitrate and yeast extract (1:1) was most suitable for high yield of lipids while using VFAs as a carbon source. Several other studies also reported that the combination of both organic and inorganic nitrogen sources were significantly higher than the yield achieved while using organic/inorganic nitrogen alone with carbon sources [7, 29].

3.3. VFAs production from wastepaper through OCF

The VFAs production profile from WOP and WNP by OCF suggested that VFAs production started at day 2 and reached maximum after 20 days (Fig. 1). VFAs produced from WOP and WNP were 17.28 ± 0.67 and 10.23 ± 0.52 g/L with the total nitrogen of 106.16 ± 2.34 and 82.62 ±1.52 mg/L, respectively. The VFAs yield and productivity achieved from WOP and WNP were 0.173 g/g TS and 0.864 g/L/day, and 0.102 g/g TS and 0.512 g/L/day, respectively.

Sawatdeenarunat et al. [30] (2017) achieved a VFAs yield of 107.25 ± 2.19 mg/gVS from anaerobic digestion of Napier grass using micro oxygenation. Park et al. [12] reported that 8.12 g/L of VFAs obtained from rice straw after 2 weeks of anaerobic fermentation with NH_3-N and total-N content of 75.16 mg/L and 129.33 mg/L, respectively.

Figure 2 shows the composition of VFAs produced from WOP and WNP. Results suggests that acetic, butyric and propionic acids were produced equally at the earlier stages (4 days); however, acetic acid was remained as most dominated thereafter followed by propionic and butyric acid. The composition of VFAs was 53.4 and 48.6 % AA, 35.6 and 35.8 % PA, and 11.0 and 17.3 % BA with WOP and WNP, respectively. Our results are consistent compared to previous studies where a similar trend was observed with the dominance of acetic, propionic, and butyric acids during anaerobic digestion of various waste biomasses [12, 30].

3.4. Lipid production utilizing VFAs produced from wastepaper

Lipid production using the VFAs derived from anaerobic OCF of WOP and WNP was carried out by growing the oleaginous yeast, *C. curvatus* for 72 h at 30^{o}C without any additional nutrients at a C/N ratio of 40. During the batch cultivation, cell biomass production was increased constantly from the beginning and reached maximum at 48 and 24 h with WOP and WNP, respectively (Fig.3 a & b). Lipid accumulation was also increased with time and reached maximum at 48 h without any further increase. Biomass, lipid yield and lipid content achieved from the VFAs of WOP and WNP were 4.32 ± 0.24 and 2.91 ± 0.23 g/L, 1.78 ± 0.12 and 0.80 ± 0.06 g/L, and 41.2 ± 0.62 and 27.7 ± 0.36 %, respectively (Table 3). The lipid coefficient achieved was 0.11 ± 0.02 and 0.08 ± 0.02 g/g VFA with the productivity of 0.037 ± 0.004 and 0.033 ± 0.006 g/L/h from VFAs of WOP and WNP, respectively. The results suggested that the

cell biomass and lipid yield achieved from VFAs of WOP was comparatively higher than the VFAs of WNP, due to the high yield of VFAs and compositional variation between WOP and WNP. Xu et al. [4] achieved 2.5 g/L biomass with lipid productivity of 0.272 g/L/d from *C. curvatus* utilizing VFAs from anaerobic digestion of macroalgae.

The results of VFAs consumption during batch cultivation suggested that all of the three VFAs were started to be utilized from the beginning of fermentation, and were completely exhausted within 72 and 60 h with the VFAs of WOP and WNP, respectively (Fig. 4). The results suggested that acetic acid was mainly utilized up to 36 h followed by butyric and propionic acids. Though biomass and lipid production were increased with decreasing VFAs concentration in the medium, rate of production was comparatively high during assimilation of acetic acid than other VFAs. Previous studies also suggested that high proportion of acetic acid was more advantageous for the synthesis of microbial lipids and cell mass production than butyric and propionic acids [31, 32]. These results also indicated that *C. curvatus* is able to utilize all three kinds of acid simultaneously, but preferably acetic acid than propionic and butyric acids due to the variation in metabolic fate of each single VFAs [17, 33]. Acetic acid can be directly transformed to acetyl-coenzyme A (CoA), which can be used to synthesize microbial oils. Contrarily, propionate, an odd-chain carboxylic acid, is converted to propionyl CoA and then enters the tricarboxylic acid (TCA) cycle via methylmalonyl-CoA interconversion to succinyl-CoA. On the other hand, butyrate undergoes β-oxidation to produce acetoacetyl-CoA which is further transformed into acetyl-CoA [17, 33].

3.5. Fatty acid profile of microbial lipids produced by C. curvatus utilizing VFAs

Fatty acid profile analysis of the lipids produced by *C. curvatus* suggested that C18 fatty acids (stearic, oleic, linoleic acid) were dominated (80%), followed by C16 fatty acid (palmitic acid) (15%). The results suggested that the oleic acid (52.64 ± 1.32 and 50.65 ± 1.82%) was the most abundant fatty acid followed by palmitic acid (16.42 ± 1.16 and 15.18 ± 0.82%), stearic acid (15.26 ± 0.78 and 14.41 ± 0.69%) and linoleic acid (12.25 ± 0.82 and 12.16 ± 0.71%) in lipids produced from VFAs of WOP and WNP, respectively. Several other studies were also reported that palmitic acid, stearic acid, and oleic acid were the major fatty acids of lipids produced by *C. curvatus* using VFAs derived from various sources [4, 12]. Thus, the long chain saturated and unsaturated fatty acids (C16 and C18) are the main components of the lipid, which is similar to the typical plant/vegetable oils, suggesting its potential to use as a feedstock for large scale production of biodiesel.

4. Conclusions

Utilization of wastepaper for production of microbial lipids to use as feedstock for biodiesel aims to open new avenues for cost-effective production of biofuels through biorefinery concept. Moreover, this biorefinery approach offers a potential valuable and alternative route for management of wastepaper. Importantly, VFAs derived from wastepaper were used for lipid production without the need to supply any additional nutrients. Biomass (4.3 g DCW/L) and lipid accumulation (41 %) achieved in this study was comparatively higher than other studies utilizing VFAs from various sources. Fatty acid profiles of lipids produced were comparable to plant/vegetable oils used for biodiesel production, and hence, VFAs derived from wastepaper could be a potential feedstock for microbial lipids production to use as non-edible lipid source for biodiesel. However, further investigations will be needed to ensure process scale up feasibility and sustainable production.

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Production of microbial lipids utilizing volatile fatty acids derived from wastepaper: A biorefinery approach for biodiesel production

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Abstract

Volatile fatty acids (VFAs) derived from organic wastes are being considered as low-cost feedstock for microbial lipid production as a valuable alternative to plant derived oils/biodiesel. In this study, VFAs were produced from anaerobic open culture fermentation of wastepaper and subsequently, used as feedstock for lipid production by *Cryptococcus curvatus*. Total VFAs, yield and productivity achieved from waste office paper (WOP) and newspaper (WNP) were 17.3 and 10.2 g/L, 0.17 and 0.10 g/g TS, and 0.86 and 0.51 g/L/day, respectively. Biomass, lipid content and productivity achieved utilizing VFAs of WOP and WNP were 4.3 and 2.9 g/L, 41.2 and 27.7% DCW, and 0.037 and 0.033 g/L/h, respectively. The dominance of fatty acids such as oleic, palmitic, stearic and linoleic acid in the lipids suggests a high level of similarity with plant/vegetable oils used for biodiesel production. Therefore, VFAs derived from wastepaper could be potentially used as feedstock to produce microbial lipids towards cost-effective production of biodiesel.

Keywords: Biodiesel; Volatile fatty acids; Wastepaper; Anaerobic open culture fermentation; Oleaginous yeast; Microbial lipids.

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1. Introduction

Currently, the world is facing global challenges such as fossil fuel depletion and climate change caused by global warming due to the increase of greenhouse gasses (GHGs) emissions from fossil fuels [1, 2]. Biodiesel, fatty acids alkyl ester, can be obtained by transesterification of triacylglycerol from edible vegetable oils (i.e. soybean, sunflower and palm oil), fats and cooling oil wastes, has several advantages than conventional petroleum-based diesel fuel such as non-sulfur oxide emission, sustainability and potential for carbon reduction [3]. However, the use of these edible oils as a feedstock for biodiesel limiting its commercialization as it competes with food production and increase the cost of raw material such as vegetables and agricultural commodities [4]. Microbial lipids produced by oleaginous microorganisms, which accumulates 20-80% of their dry weight in the form of lipids under nutrient-limitation conditions, are the most promising alternative non-edible lipid source for sustainable production of biodiesel [5-8]. However, the production cost of microbial lipids remains as a major limiting factor due to the carbon sources used for production, which is estimated to be about 80% of the total medium cost and it contributes to over 60% of the total production costs while using glucose as a carbon source [9]. A potential solution to reduce the production cost is to utilize low-cost or waste biomass that can be used as a substrate for microbial lipid production [8, 10, 11].

Volatile fatty acids (VFAs) are linear short-chain fatty acids (C2 – C5), which includes acetic, propionic, butyric, isobutyric, valeric, isovaleric and 2-methylbutyric acid are intermediate products of anaerobic digestion (AD), have been extensively investigated for production of various bio-based materials, using a so-called VFAs platform [12-14]. The major advantages of VFAs platform for bio-based materials production are the absence of requirement for sterilization or addition of enzymes to hydrolyze, and the availability of feedstock in

substantial quantities [15].VFAs are considered as a potential carbon source for lipid production using oleaginous yeast as it requires only shorter transformation pathway (VFAs into acetyl-CoA, which is used for biosynthesis of lipids) with high theoretical lipid conversion efficiency [16, 17].

Microbial lipids production utilizing lignocellulosic biomass has received increasing interest in recent days, as an alternative solution for large-scale production of biodiesel [7, 18, 19]. Wastepaper, a major component of municipal and industrial solid wastes, accounts more than 35% of total lignocellulosic wastes is being considered as a promising feedstock for biofuels due to its sustainability and abundance [20, 21]. Recently, wastepaper has been used as feedstock for production of various valuable bio-products such as bioethanol and PHAs [7, 20, 22]. Utilization of wastepaper for production of microbial lipids is a promising alternative biorefinery approach for large-scale production of biodiesel, which is not only reduces the cost of production, but also provides alternative route for waste management [23]. Hence, in this study we aim to investigate the conversion of wastepaper into VFAs through anaerobic open culture fermentation (OCF) and subsequently, use the VFAs as possible feedstock for production of microbial lipids as an alternative to industrial production of biodiesel.

2. **Materials and methods**

All chemicals and reagents used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA) or as indicated.

2.1. Inocula

2.1.1. Inoculum for VFAs production

Waste activated sludge (WAS) obtained from local wastewater treatment plant was passed through a sieve (18 mesh) and heat treated at 90^{o} C for 20 min to inactivate methanogens.

77 The raw sludge contained 1.59 ± 0.01 g/L total suspended solid (TSS) and 1.36 ±0.01 g/L
78 volatile suspended solid (VSS). Further, WAS purged with N_2 gas for 10 min, pH maintained at
79 5.4 - 5.6 using 2N HCl and NaOH, incubated in a shaker incubator at 35^{o}C and 200 rpm was
80 used as a inoculum for VFA production.

81 *2.1.2. Inoculum for microbial lipid production*

82 The oleaginous yeast, *Cryptococcus curvatus* DSM 70022 obtained from *DSMZ*
83 *(Germany)* was propagated on YPD agar slants in every two weeks (yeast extract 10; peptone 10;
84 glucose 20; agar 15 (g/L), pH 6.0, 30^{o} C). For seed culture, *C. curvatus* was inoculated into 50
85 mL of YPD medium in 250 mL flask and incubated at 30^{o} C, 200 rpm for 36 h. Afterwards, the
86 cultures were grown in a medium containing 10 g/L acetate, 2 g/L propionate, 1 g/L butyrate, 1
87 g/L peptone, and 1 g/L yeast extract (pH - 5.5) for 24 h and used as a inoculum for lipid
88 production.

89 *2.2. Feasibility of C. curvatus for lipid production using VFAs*

90 The feasibility of *C. curavtus* for lipid production using VFAs was investigated using
91 synthetic VFAs [mixture of acetic acid (AA), propionic acid (PA) and butyric acid (BA)]. The
92 influence of VFAs on lipid accumulation was studied by comparing the initial concentration and
93 ratio of each VFA in the mixture. The effect of initial VFAs concentration was evaluated using 2,
94 5 and 10 g/L at a ratio of 5:1:4. Effects of various VFAs ratio (AA: BA: PA) (5:1:4, 5:2:3, 6:2:2
95 and 6:1:3) on lipid accumulation was investigated at an initial concentration of 5 g/L VFAs. The
96 effect of various nitrogen sources and their combination (1:1) on lipid production was
97 investigated using various inorganic [$(NH_4)_2SO_4$, NH_4Cl, NH_4NO_3, $NaNO_3$ and KNO_3] and
98 organic nitrogen sources [yeast extract (10% N, w/w)] and peptone (14% N, w/w)]. The initial

pH of the medium was adjusted to 5.5 using 2N HCl and NaOH, and the C/N ratio was maintained at 40 under all tested concentrations.

2.3. Feedstock pretreatment for VFAs production

The feedstock for VFAs production such as waste office paper (WOP) and waste newspaper (WNP) were shredded into a small pieces (2 x 6 mm) and subjected to pretreatment by mixing with 0.5% H_2O_2 (5% w/v) and then autoclave at 121^oC for 30 min. The solid residue was collected by centrifugation (Eppendorf-5810R, Germany) at 5000 *xg* for 10 min, washed 3 - 4 times repeatedly with deionized water until obtain neutral pH, dried at 60^oC for 24 h and used as substrate for anaerobic digestion.

2.4. VFAs production from wastepaper by OCF

Anaerobic open culture fermentation (OCF) was carried out in 250 mL reactors (serum bottles with seals) with 100 mL of anaerobic fermenter medium [modified RAMM medium containing 1; yeast extract, 0.27; KH_2PO_4, 0.35; K_2HPO_4, 0.53; NH_4Cl, 0.1; $MgCl{\cdot}6H_2O$, 0.075; $CaCl2{\cdot}2H_2O$ and 10; $NaHCO_3$] with the solid loading of 10% (w/v) pretreated WOP and WNP. $NaHCO_3$ was added to the medium separately as an alkaline buffer. The trace element solution (DSMZ 320, 0.1% v/v) and vitamin solution (DSMZ 503, 0.1% (v/v) was added to the fermentation medium and the pH was adjusted to 8.0 (2 N HCl and NaOH). 2-mercaptoethanesulfonate (BES) (12 mM) was used as a methanogens inhibitor. The reactors were seeded with 10% (v/v) inoculum, purged with nitrogen gas for 10 min, sealed with rubber stopper with crimp aluminum seals and incubated at 30^o C with 100 rpm for 4 weeks. After incubation, the broth from OCF was centrifuged (Eppendorf-5810R, Germany) at 10,000 *xg* for 10 min and the supernatant was subjected to struvite precipitation at 1: 1: 1.1 (Mg^{2+}: NH^{4+}-N:

PO_4^{3-} -P) molar concentration to achieve C/N ratio of 40 and subsequently used for lipid production.

2.5. Lipid production using VFAs produced from wastepaper

Lipid production was carried out in 250 mL conical flasks containing 50 mL of VFAs broth obtained from OCF. No additional nutrients were added. Seed cultures were grown for 24 h in synthetic VFAs media until reaching an Optical Density of 1 at a wavelength of 600 nm (OD_{600}). Flasks were inoculated with 1% (v/v) seed cultures subsequently grown for 72 h at 30 ^oC and 200 rpm. Aliquots (5 mL) were withdrawn at regular intervals (12 h) and used to determine cell biomass, lipid production and residual VFAs in the medium. Biomass was estimated gravimetrically by centrifuging the culture broth (5 mL) at 5000 *xg* for 10 min at 4^oC, washed with deionized water and dried at 60^oC for 24 h and expressed as cell dry cell weight (g DCW/L).

2.6. Analytical methods

Total Solids (TS) were determined by drying at 105 ^oC overnight and volatile solids (VS) were determined by ashing at 550 ^oC for 3 h [24]. Total nitrogen (TN) and ammonium nitrogen (NH_3-N) were measured using a standard method [24]. The concentrations of VFAs were analyzed using an HPLC (Agilent Technology 1100 series) equipped with Aminex HPX-87H column (BIORAD INC., USA), using refractive index (RI) and diode array detectors (DAD). The mobile phase was 0.004 M H_2SO_4 at a flow rate was 0.6 mL/min and the column temperature was maintained constant at 50^oC. Samples were filter through 0.2µm (PVDF) syringe filter (Millipore, USA) and subsequently used for analysis.

Lipids extraction from dried biomass was done by the method of Folch et al. [25]. Briefly, 10 mg of dried biomass was digested using 3.2 mL of 4M HCl at 55^{o}C for 2 h and extracted with 8 mL of chloroform/methanol (2:1, v/v), vortexed for 2 - 3min and centrifuged at 2,000 *xg* for 5 min. Further, the extracted solution was purged with nitrogen gas to evaporate the solvents and the lipids were measured and expressed as g/L.

Fatty acid methyl esters (FAME) were prepared using 2.8 M H_2SO_4 in methanol containing (10 mL/L) nonadecanoic acid (C19:0) as an internal standard and heated at 100°C for 4 h. After cooling to room temperature, 1 mL of distilled water was added, vortexed for 3 min and centrifuged at 2,000 *xg* for 1 min for organic phase separation. FAMEs were analyzed by GC (Agilent 7890A, USA) equipped with flame ionization detector (FID) and FAMEWAX column (30 m x 320 µm x 0.25 µm) using helium as a carrier gas. The injector was kept at 280^{o}C with an injection volume of 1 µL with a split ratio at 30. The initial oven temperature was set at 120^{o}C. The oven temperature was increased at a heating rate of 3^{o} C/min up to 240^{o}C and held for 20 min. The temperature of the detector was set at 250^{o}C. Fatty acids were calculated relative to their weight compared to total lipids in biomass and expressed as percentage (% total lipids).

3. Results and discussion

3.1. Effect of VFAs concentration and ratio on biomass and lipid production

The effects of initial concentration of VFAs on biomass and lipid production was investigated and presented in Table 1. Biomass and lipid yield achieved was 1.62 ± 0.06 and 0.587 ± 0.004, 2.78 ± 0.08 and 0.781± 0.008, and 4.19 ± 0.11 and 0.712 ± 0.005 g/L, and lipid content achieved was 36.3 ± 0.21, 28.1 ± 0.18 and 17.0 ± 0.20% with 2, 5 and 10 g/L VFAs concentration, respectively. These results evidenced that *C. curvatus* was able to grow even at

10g/L VFAs concentration; however, there was a considerable decrease in lipid production reported with increasing the concentration VFAs, which clearly suggesting that the higher concentration of VFAs promotes the cell growth than lipid accumulation. Thus, the initial concentration of 5 g/L VFAs was considered as most suitable resulting in high yield of biomass and lipid production compared to the other tested VFAs concentration. Park et al. [12] reported that the yeast, *C. curvatus* was not able to utilize 8 g/L of VFAs; however, lipid production was elevated with increasing VFAs to 6 g/L. Several other studies also reported that there was a significant inhibition on lipid production when VFAs concentration increased above 5 g/L [26, 27].

The effect of different ratio of VFAs on biomass and lipid production was investigated with four different ratios such as 5:1:4, 5:2:3, 6:2:2 and 6:1:3 (AA: BA: PA). The cell biomass and lipid yield achieved was ranged between 3.62 ± 0.10 and 4.38 ± 0.08 g/L, and 0.85 ± 0.004 and 1.69 ± 0.010g/L, respectively (Table.1). The results suggested that maximum biomass (4.38 ± 0.08 g/L), lipid production (1.69 ± 0.010g/L) and lipid yield coefficient (0.338 g/g) was achieved with the VFAs ratio of 6:1:3. Our results showed that high content of acetic acid (AA) in VFAs mixture greatly promotes the cell biomass and lipid productivity; hence, acetic acid is more favorable for high productivity than butyric and propionic acids. Liu et al. [28] reported that high content of AA in VFAs mixture (6:3:1) increased the biomass and productivity by *C. curvatus* utilizing WAS- derived VFAs through sequencing batch fermentation strategy.

3.2. Effect of various nitrogen sources on lipid accumulation

Several studies suggested that lipid accumulation using VFAs were greatly influenced by nitrogen source used for production. In this study, effect of various nitrogen sources was

investigated using synthetic VFAs at 5 g/L with C/N ratio of 40 as presented in Table 2. The cell biomass achieved from the nitrogen sources such as ammonium sulphate, ammonium chloride, ammonium nitrate, sodium nitrate, potassium nitrate, yeast extract and peptone were 3.60 ± 0.13, 3.69 ± 0.14, 3.71 ± 0.16, 1.49 ± 0.08, 1.36 ± 0.08, 3.24 ± 0.15 and 4.17 ± 0.12 (g/L), respectively. The lipid yield (g/L) and lipid content (%) achieved using ammonium sulphate, ammonium chloride, ammonium nitrate, sodium nitrate, potassium nitrate, yeast extract and peptone were 0.236 ± 0.006 and 6.5 ± 0.18, 0.456 ± 0.008 and 12.4 ± 0.08, 0.416 ± 0.006 and 11.2 ± 0.12, 0.256 ± 0.005 and 17.2 ± 0.14, 0.139 ± 0.004 and 10.2 ± 0.08, 0.790 ± 0.006 and 24.4 ± 0.16, and 0.556 ± 0.008 and 13.3 ± 0.10, respectively. The results of combined addition of organic and inorganic nitrogen (1:1) suggested that maximum cell biomass (5.53 ± 0.09 g/L), lipid yield (1.724 ± 0.008 g/L) and lipid content (31.2 ± 0.13) was achieved with the combination of ammonium nitrate and yeast extract. These results clearly suggested that the nitrogen sources playing an important role in cell biomass and lipid production, and the combined addition of ammonium nitrate and yeast extract (1:1) was most suitable for high yield of lipids while using VFAs as a carbon source. Several other studies also reported that the combination of both organic and inorganic nitrogen sources were significantly higher than the yield achieved while using organic/inorganic nitrogen alone with carbon sources [7, 29].

3.3. VFAs production from wastepaper through OCF

The VFAs production profile from WOP and WNP by OCF suggested that VFAs production started at day 2 and reached maximum after 20 days (Fig. 1). VFAs produced from WOP and WNP were 17.28 ± 0.67 and 10.23 ± 0.52 g/L with the total nitrogen of 106.16 ± 2.34 and 82.62 ±1.52 mg/L, respectively. The VFAs yield and productivity achieved from WOP and WNP were 0.173 g/g TS and 0.864 g/L/day, and 0.102 g/g TS and 0.512 g/L/day, respectively.

209 Sawatdeenarunat et al. [30] (2017) achieved a VFAs yield of 107.25 ± 2.19 mg/gVS from
210 anaerobic digestion of Napier grass using micro oxygenation. Park et al. [12] reported that 8.12
211 g/L of VFAs obtained from rice straw after 2 weeks of anaerobic fermentation with NH_3-N and
212 total-N content of 75.16 mg/L and 129.33 mg/L, respectively.

213 Figure 2 shows the composition of VFAs produced from WOP and WNP. Results
214 suggests that acetic, butyric and propionic acids were produced equally at the earlier stages (4
215 days); however, acetic acid was remained as most dominated thereafter followed by propionic
216 and butyric acid. The composition of VFAs was 53.4 and 48.6 % AA, 35.6 and 35.8 % PA, and
217 11.0 and 17.3 % BA with WOP and WNP, respectively. Our results are consistent compared to
218 previous studies where a similar trend was observed with the dominance of acetic, propionic, and
219 butyric acids during anaerobic digestion of various waste biomasses [12, 30].

220 *3.4. Lipid production utilizing VFAs produced from wastepaper*

221 Lipid production using the VFAs derived from anaerobic OCF of WOP and WNP was
222 carried out by growing the oleaginous yeast, *C. curvatus* for 72 h at 30^{o}C without any additional
223 nutrients at a C/N ratio of 40. During the batch cultivation, cell biomass production was
224 increased constantly from the beginning and reached maximum at 48 and 24 h with WOP and
225 WNP, respectively (Fig.3 a & b). Lipid accumulation was also increased with time and reached
226 maximum at 48 h without any further increase. Biomass, lipid yield and lipid content achieved
227 from the VFAs of WOP and WNP were 4.32 ± 0.24 and 2.91 ± 0.23 g/L, 1.78 ± 0.12 and 0.80 ±
228 0.06 g/L, and 41.2 ± 0.62 and 27.7 ± 0.36 %, respectively (Table 3). The lipid coefficient
229 achieved was 0.11 ± 0.02 and 0.08 ± 0.02 g/g VFA with the productivity of 0.037 ± 0.004 and
230 0.033 ± 0.006 g/L/h from VFAs of WOP and WNP, respectively. The results suggested that the

231 cell biomass and lipid yield achieved from VFAs of WOP was comparatively higher than the
232 VFAs of WNP, due to the high yield of VFAs and compositional variation between WOP and
233 WNP. Xu et al. [4] achieved 2.5 g/L biomass with lipid productivity of 0.272 g/L/d from *C.*
234 *curvatus* utilizing VFAs from anaerobic digestion of macroalgae.

235 The results of VFAs consumption during batch cultivation suggested that all of the three
236 VFAs were started to be utilized from the beginning of fermentation, and were completely
237 exhausted within 72 and 60 h with the VFAs of WOP and WNP, respectively (Fig. 4). The
238 results suggested that acetic acid was mainly utilized up to 36 h followed by butyric and
239 propionic acids. Though biomass and lipid production were increased with decreasing VFAs
240 concentration in the medium, rate of production was comparatively high during assimilation of
241 acetic acid than other VFAs. Previous studies also suggested that high proportion of acetic acid
242 was more advantageous for the synthesis of microbial lipids and cell mass production than
243 butyric and propionic acids [31, 32]. These results also indicated that *C. curvatus* is able to
244 utilize all three kinds of acid simultaneously, but preferably acetic acid than propionic and
245 butyric acids due to the variation in metabolic fate of each single VFAs [17, 33]. Acetic acid can
246 be directly transformed to acetyl-coenzyme A (CoA), which can be used to synthesize microbial
247 oils. Contrarily, propionate, an odd-chain carboxylic acid, is converted to propionyl CoA and
248 then enters the tricarboxylic acid (TCA) cycle via methylmalonyl-CoA interconversion to
249 succinyl-CoA. On the other hand, butyrate undergoes β-oxidation to produce acetoacetyl-CoA
250 which is further transformed into acetyl-CoA [17, 33].

251 *3.5. Fatty acid profile of microbial lipids produced by C. curvatus utilizing VFAs*

Fatty acid profile analysis of the lipids produced by *C. curvatus* suggested that C18 fatty acids (stearic, oleic, linoleic acid) were dominated (80%), followed by C16 fatty acid (palmitic acid) (15%). The results suggested that the oleic acid (52.64 ± 1.32 and 50.65 ± 1.82%) was the most abundant fatty acid followed by palmitic acid (16.42 ± 1.16 and 15.18 ± 0.82%), stearic acid (15.26 ± 0.78 and 14.41 ± 0.69%) and linoleic acid (12.25 ± 0.82 and 12.16 ± 0.71%) in lipids produced from VFAs of WOP and WNP, respectively. Several other studies were also reported that palmitic acid, stearic acid, and oleic acid were the major fatty acids of lipids produced by *C. curvatus* using VFAs derived from various sources [4, 12]. Thus, the long chain saturated and unsaturated fatty acids (C16 and C18) are the main components of the lipid, which is similar to the typical plant/vegetable oils, suggesting its potential to use as a feedstock for large scale production of biodiesel.

4. Conclusions

Utilization of wastepaper for production of microbial lipids to use as feedstock for biodiesel aims to open new avenues for cost-effective production of biofuels through biorefinery concept. Moreover, this biorefinery approach offers a potential valuable and alternative route for management of wastepaper. Importantly, VFAs derived from wastepaper were used for lipid production without the need to supply any additional nutrients. Biomass (4.3 g DCW/L) and lipid accumulation (41 %) achieved in this study was comparatively higher than other studies utilizing VFAs from various sources. Fatty acid profiles of lipids produced were comparable to plant/vegetable oils used for biodiesel production, and hence, VFAs derived from wastepaper could be a potential feedstock for microbial lipids production to use as non-edible lipid source for biodiesel. However, further investigations will be needed to ensure process scale up feasibility and sustainable production.

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Table

Table 1 Effect of initial concentrations and ratio of VFAs on biomass and lipid production (C/N ratio: 40). Results are presented using mean ± SD, n=3

VFAs	Biomass (g/L)	Lipid yield (g/L)	Lipid content (%)	$Y_{x/s}$ (g/g)
Concentrations (g/L)				
2	1.62 ± 0.06	0.587 ± 0.004	36.3 ± 0.21	0.294
5	2.78 ± 0.08	0.781 ± 0.008	28.1 ± 0.18	0.156
10	4.19 ± 0.11	0.712 ± 0.005	17.0 ± 0.20	0.071
Ratio				
5:1:4	3.62 ± 0.10	0.85 ± 0.004	23.6 ± 0.23	0.170
5:2:3	3.78 ± 0.08	0.99 ± 0.008	26.2 ± 0.18	0.262
6:2:2	4.16 ± 0.12	1.25 ± 0.008	30.1 ± 0.22	0.250
6:1:3	4.38 ± 0.08	1.69 ± 0.010	38.6 ± 0.19	0.338

$Y_{x/s}$ - Lipid yield coefficient, g lipid/g VFAs

Table 2 Effect of various nitrogen sources on biomass and lipid production using synthetic VFAs as carbon source at 5 g/L (C/N ratio: 40). Results are presented using mean ± SD, n=3.

Nitrogen sources	**Biomass (g/L)**	**Lipid Yield (g/L)**	**Lipid content (%)**	$Y_{x/s}$ **(g/g)**
$(NH_4)_2SO_4$	3.60 ± 0.13	0.236 ± 0.006	6.5 ± 0.18	0.047 ± 0.002
NH_4Cl	3.69 ± 0.14	0.456 ± 0.008	12.4 ± 0.08	0.091 ± 0.004
NH_4NO_3	3.71 ± 0.16	0.416 ± 0.006	11.2 ± 0.12	0.083 ± 0.006
$NaNO_3$	1.49 ± 0.08	0.256 ± 0.005	17.2 ± 0.14	0.051 ± 0.004
KNO_3	1.36 ± 0.08	0.139 ± 0.004	10.2 ± 0.08	0.028 ± 0.003
Yeast extract (YE)	3.24 ± 0.15	0.790 ± 0.006	24.4 ± 0.16	0.158 ± 0.005
Peptone	4.17 ± 0.12	0.556 ± 0.008	13.3 ± 0.10	0.111 ± 0.003
$(NH_4)_2SO_4$ + YE	3.42 ± 0.21	0.360 ± 0.004	10.5 ± 0.11	0.072 ± 0.004
NH_4Cl + YE	4.68 ± 0.16	0.828 ± 0.006	17.7 ± 0.08	0.166 ± 0.006
NH_4NO_3 + YE	5.53 ± 0.09	1.724 ± 0.008	31.2 ± 0.13	0.345 ± 0.008
$NaNO_3$ + YE	0.76 ± 0.05	0.112 ± 0.004	14.7 ± 0.10	0.022 ± 0.006
KNO_3+ YE	1.20 ± 0.08	0.199 ± 0.006	16.6 ± 0.12	0.040 ± 0.002

$Y_{x/s}$ - Lipid yield coefficient, g lipid / g VFAs

Table 3 Biomass, lipid yield, lipid content, lipid coefficient and productivity of *C. curvatus* from VFAs derived from anaerobic open culture fermentation of waste office paper (WOP) and waste newspaper (WNP). Results are presented using mean ± SD, n=3.

Substrate	Biomass (g/L)	Lipid yield (g/L)	Lipid content (%)	Lipid coefficient (g/g VFA)	Lipid productivity (g/L/h)
WOP	4.32 ± 0.24	1.78 ± 0.12	41.2 ± 0.62	0.11 ± 0.02	0.037 ± 0.004
WNP	2.91 ± 0.23	0.80 ± 0.06	27.7 ± 0.36	0.08 ± 0.02	0.033 ± 0.006

Table 4 Fatty acid profile of lipids from volatile fatty acids (VFAs) derived from anaerobic open culture fermentation of waste office paper (WOP) and waste newspaper (WNP). Results are presented using mean ± SD, n=3.

Fatty acids	**VFAs**	
	WOP	**WNP**
Palmitic acid (C16:0)	16.42 ± 1.16	15.18 ± 0.82
Stearic acid (C18:0)	15.26 ± 0.78	14.41 ± 0.69
Oleic Acid (C18:1)	52.64 ± 1.32	50.65 ± 1.82
Linoleic acid (C18:2)	12.25 ± 0.82	12.16 ± 0.71

Figure Captions

Fig. 1 Total volatile fatty acids (TVFAs) production during anaerobic open culture fermentation (OCF) of waste office paper (WOP) and waste newspaper (WNP). Results are presented using mean ± SD, n=3.

Fig. 2 Composition of volatile fatty acids (VFAs) produced during OCF of (a) waste office paper (WOP) and (b) waste newspaper (WNP). Results are presented using mean ± SD, n=3. (PA: Propionic Acid, BA: Butyric acid, AA: Acetic acid)

Fig. 3 Biomass production, lipid yield and content during batch cultivation of *C. curvatus* from volatile fatty acids (VFAs) of waste office paper (WOP) and waste newspaper (WNP) from OCF. Results are presented using mean ± SD, n=3.

Fig. 4 Consumption of each volatile fatty acids (VFAs) vs cell biomass production by *C. curvatus* utilizing VFAs of (a) waste office paper (WOP) and (b) waste newspaper (WNP). Results are presented using mean ± SD, n=3.

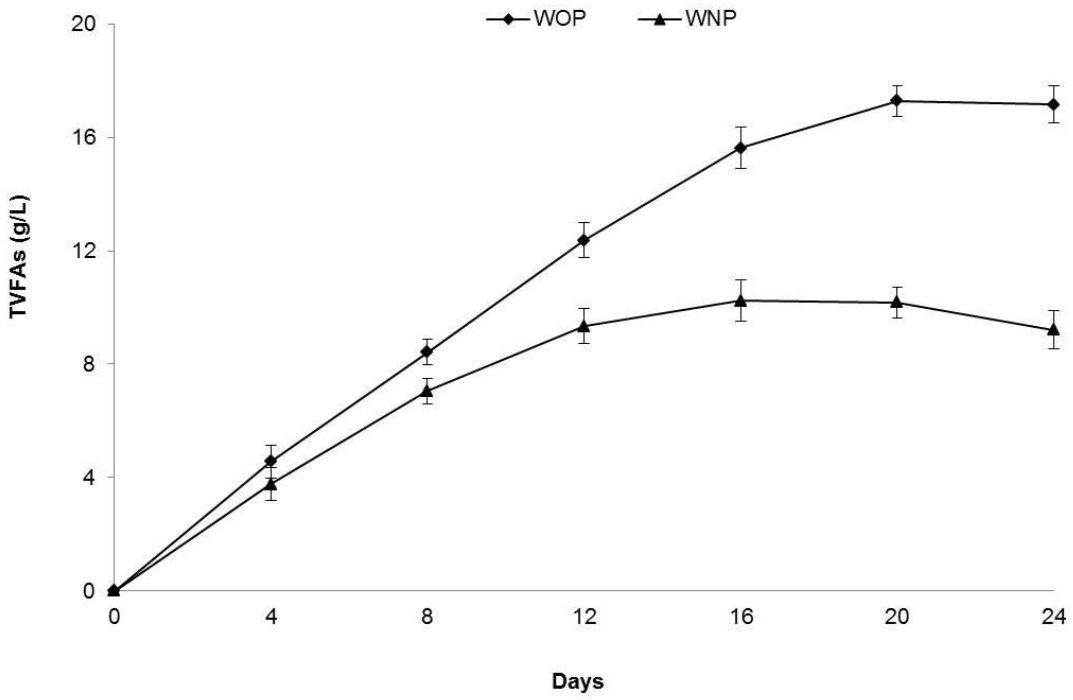

Fig. 1

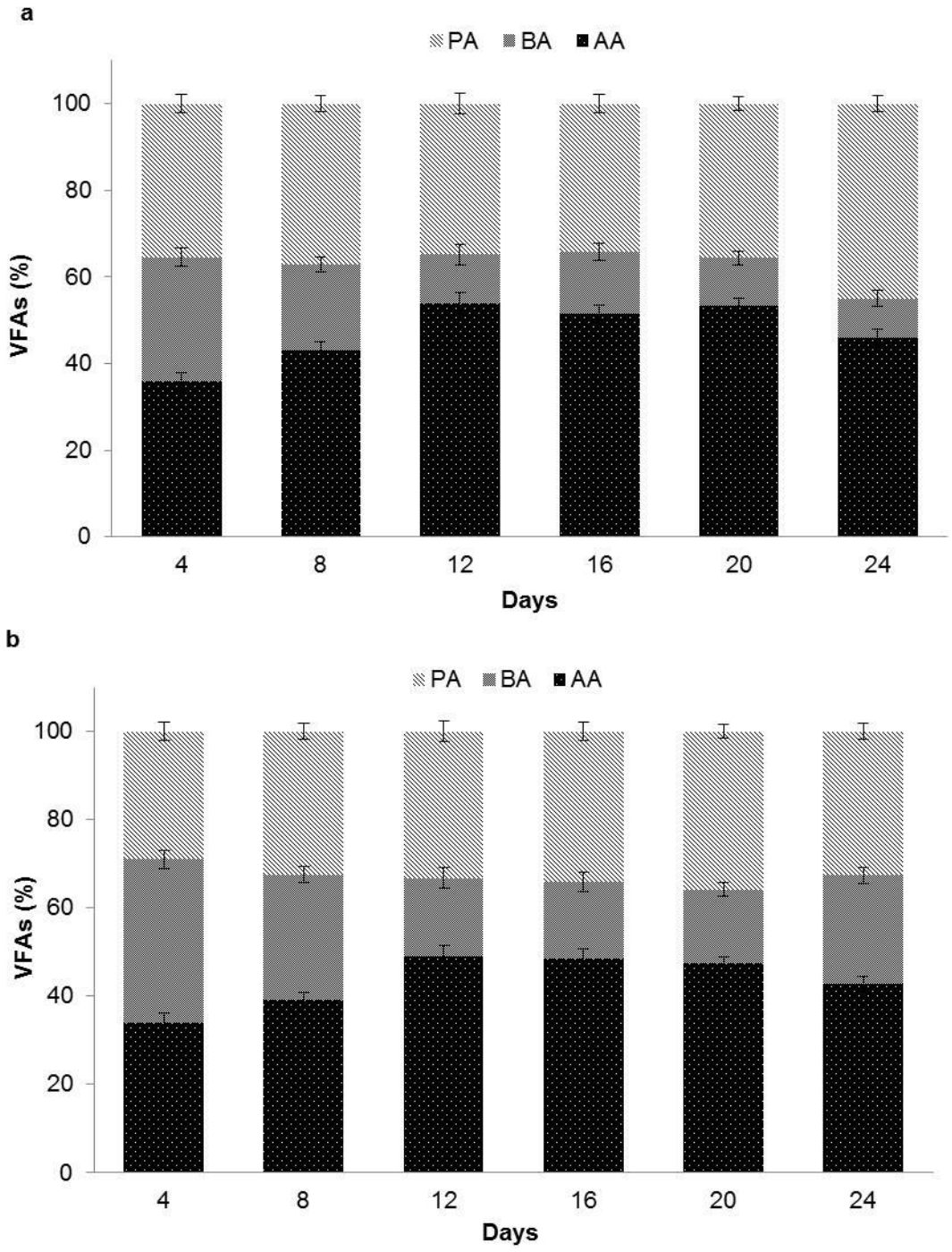
a
PA
BA
AA
100
80
60
40
20
0
VFAs (%)
4
8
12
16
20
24
Days
b
PA
BA
AA
100
80
60
40
20
0
VFAs (%)
4
8
12
16
20
24
Days

Fig. 2

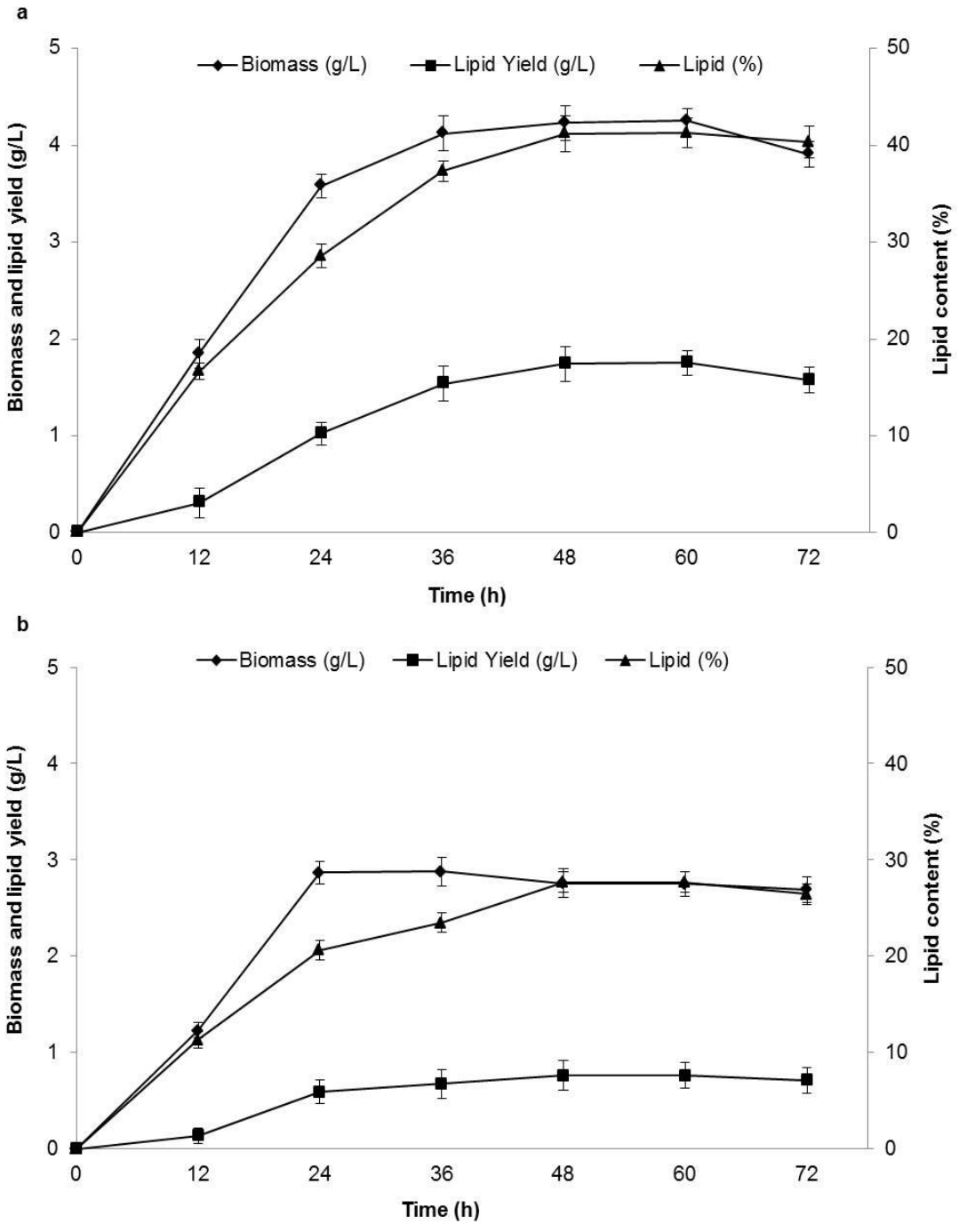
a
Biomass (g/L)
Lipid Yield (g/L)
Lipid (%)
Biomass and lipid yield (g/L)
Lipid content (%)
Time (h)
0
12
24
36
48
60
72
b
Biomass (g/L)
Lipid Yield (g/L)
Lipid (%)
Biomass and lipid yield (g/L)
Lipid content (%)
Time (h)

Fig. 3

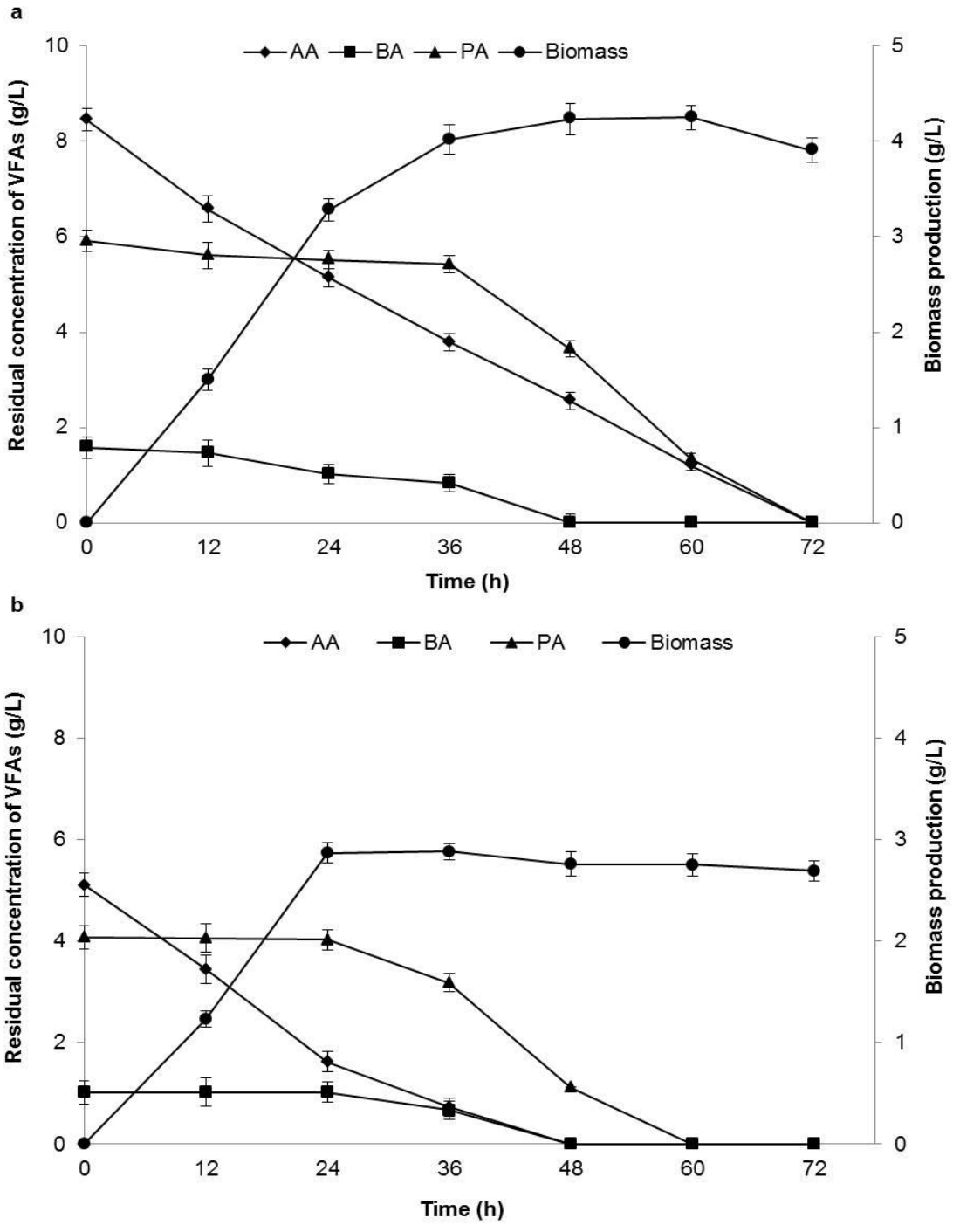
a
AA
BA
PA
Biomass
Residual concentration of VFAs (g/L)
Biomass production (g/L)
Time (h)
b
AA
BA
PA
Biomass
Residual concentration of VFAs (g/L)
Biomass production (g/L)
Time (h)

Fig. 4

Declaration of Interests

- We, all the authors, declare that we have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT Author Statement

Neelamegam Annamalai: Design, Conceptualization, Methodology, Manuscript preparation, Revision, **Nallusamy Sivakumar:** Design and Supervision: **Alfred Fernandez-Castane:** Design, Review and Editing, **Piotr Oleskowicz-Popiel**: Design, Review