Research Article

Received: 12 August 2011 Revised: 3 October 2011 Accepted: 4 October 2011 Published online in Wiley Online Library:

(wileyonlinelibrary.com) DOI 10.1002/jctb.2760

Effect of acetic acid on lipid accumulation by glucose-fed activated sludge cultures†

Andro Mondala, Rafael Hernandez,* Todd French,* Linda McFarland, Darrell Sparks, William Holmes and Monica Haque

Abstract

BACKGROUND: The effect of acetic acid, a lignocellulose hydrolysis by-product, on lipid accumulation by activated sludge cultures grown on glucose was investigated. This was done to assess the possible application of lignocellulose as low-cost and renewable fermentation substrates for biofuel feedstock production.

RESULTS: Biomass yield was reduced by around 54% at a 2 g L^{-1} acetic acid dosage but was increased by around 18% at 10 g L^{-1} acetic acid dosage relative to the control run. The final gravimetric lipid contents at 2 and 10 g L^{-1} acetic acid levels were 12.5 ± 0.7% and 8.8 ± 3.2% w/w, respectively, which were lower than the control (17.8 ± 2.8% w/w). However, biodiesel yields from activated sludge grown with acetic acid (5.6 ± 0.6% w/w for 2 g L^{-1} acetic acid and 4.2 ± 3.0% w/w for 10 g L^{-1} acetic acid) were higher than in raw activated sludge (1–2% w/w). The fatty acid profiles of the accumulated lipids were similar with conventional plant oil biodiesel feedstocks.

CONCLUSIONS: Acetic acid enhanced biomass production by activated sludge at high levels but reduced lipid production. Further studies are needed to enhance acetic acid utilization by activated sludge microorganisms for lipid biosynthesis.

Keywords: lignocellulose; inhibitors; fermentation; lipids; biodiesel; mixed cultures

INTRODUCTION

The use of oleaginous microorganisms as alternative lipid feedstock sources for biodiesel production has only recently been studied.[1–4] Oleaginous microorganisms are bacteria, yeast, fungi, and heterotrophic algae species capable of accumulating 20–80% of their biomass as lipids.[5] This accumulation is usually triggered as a response to stress such as the limitation of a key nutrient (usually nitrogen) relative to an excess supply of the carbon source.[5] The most commonly utilized carbon sources are pure sugars such as glucose, sucrose, lactose, and xylose.[6] However, in order to reduce the cost of producing biodiesel from microbial lipids at the commercial scale, other sugar sources that are low cost and abundant must be used. Lignocellulosic biomass obtained from agricultural wastes, forestry residues, energy crops, or municipal solid wastes have been considered as excellent candidates for use as a renewable source of sugars for fermentation processes to produce biofuels such as bioethanol[7–9] and more recently biodiesel feedstock lipids.[2,4,10,11]

Lignocellulose is considered to be the most abundant and renewable substance in the biosphere, accounting for nearly 50% of all the biomass in the world or approximately 10–50 billion tons.[12] It is composed of cellulose, hemicellulose, and lignin and is usually pretreated by acid hydrolysis to release the hexose and pentose monosaccharide sugars that make up the cellulose and hemicellulose components for more effective utilization by fermenting microorganisms.[13] However, the release of these sugars is accompanied by the formation of various chemical by-products which are known to exert inhibitory effects on microbial growth and metabolism.[14] Among these compounds are organic acids such as acetic acid, which is typically the most abundant organic acid found in lignocellulose hydrolyzate. Acetic acid is released via the cleavage of acetyl groups from hemicellulose such as acetylxylan under mild pretreatment conditions.[15] In addition, it is also a by-product of fermentation; hence its concentrations in microbial cultures do not depend solely on the severity of the hydrolysis reaction used to pretreat the biomass substrate.[16] The observed effects of acetic acid in microbial fermentations for biofuels production are more complex than other inhibitor compounds such as furfural. For instance, acetic acid has been shown to decrease biomass and ethanol yields in ethanologenic yeasts.[15,17] However in some cases, relatively higher acetic acid levels at 9 g L^{-1} (150 mmol L^{-1}) stimulated ethanol yield and production rate.[18] Similarly, it was reported that acetic acid levels of up to 70 mmol L^{-1} had negligible effects on lipid production by the oleaginous yeast *Rhodosporidium toruloides* and at 120 mmol L^{-1} of acetic acid in the culture, even reached a high lipid content of 68% by wt[19] Therefore, it is important to fully study the effects

* *Correspondence to: Rafael Hernandez and Todd French, Mailing Address: 323 Presidents Circle, Mississippi State, MS 39762, USA. E-mail: rhernandez@che.msstate.edu, french@che.msstate.edu*

† *Presented at the 101st Annual Meeting of the American Oil Chemists Society (AOCS), May 19, 2010, Phoenix, AZ, U.S.A. and at the 2009 American Institute of Chemical Engineers (AIChE) Annual Meeting, Nov. 12, 2009, Nashville, TN, U.S.A.*

Renewable Fuels and Chemicals Laboratory, Dave C. Swalm School of Chemical Engineering, Mississippi State University, Mississippi State, MS 39762 USA

of acetic acid on microorganisms related to biofuel production so that hydrolyzate pretreatment steps and fermentation strategies could be developed and evaluated to ensure a more efficient utilization of lignocellulose sugars.

In a previous study, our group has demonstrated the novel application of activated sludge microbial cultures obtained from municipal sewage treatment plants to produce biofuel feedstock lipids.[20] The cost advantages of using mixed cultures such as activated sludge include eliminating the need for media sterilization and the implementation of continuous cultures with minimal risk of contamination.[21] Therefore, the objective of this study was to determine the effect of acetic acid on growth and lipid accumulation by activated sludge microbial communities using glucose as the primary carbon source. Fermentation kinetics and yield parameters were obtained using established models to aid in the interpretation of the data and for their potential use in large scale bioreactor design. The lipid extracts produced were then characterized to determine their suitability as biofuel feedstock.

MATERIALS AND METHODS

Inocula

Activated sludge obtained from a treatment plant located in Tuscaloosa, AL, USA treating municipal wastewater was used as inocula for the microbial cultures in this study. While the characteristics of the wastewater influent fluctuated periodically, the typical influent quality data were:

$$\text{pH} = 7.0 \pm 0.1$$
$$\text{BOD}_5 = 133 \pm 48 \text{ mg L}^{-1}$$
$$\text{COD} = 396 \pm 94 \text{ mg L}^{-1}$$
$$\text{NH}_4^+\text{-N} = 22 \pm 3 \text{ mg L}^{-1}$$
$$\text{NO}_2\text{-N} = 4.2 \pm 0.9 \text{ mg L}^{-1}$$
$$\text{PO}_4^{\ 3}\text{-P} = 8.9 \pm 1.1 \text{ mg L}^{-1}$$
$$\text{C:N ratio} = 23 \text{ (Carbon as total COD)}$$

The activated sludge grab samples were transferred and combined into a single composite mixture and maintained in a 3 L gas jar with mixing and aeration at room temperature for 24 h.

Batch fermentation experiments

The batch experiments were conducted using BIOFLO 310 bioreactors (New Brunswick Scientific – Eppendorf, Edison, NJ) with working volumes of 3 L. A modified synthetic wastewater recipe[22] containing (per L of DI water) 0.15 g gelatin, 0.21 g starch, 0.07 g yeast extract, 0.01 g casamino acids, 1.5 g NaH_2PO_4, 1.0 g K_2HPO_4, and 5 mL trace mineral solution was used as the cultivation medium for the activated sludge. Glucose (60 g L^{-1}) and ammonium sulfate (1.62 g L^{-1}) were added as carbon and nitrogen sources, respectively corresponding to a constant initial carbon-to-nitrogen ratio (C:N) of 70:1 based on mass. The medium was sterilized by autoclaving at 121 °C, 20 psig for 20 min. Glucose was autoclaved separately to prevent caramelization and mixed aseptically with the rest of the media after cooling to room temperature. Two concentration levels of acetic acid 2 g L^{-1} (33 mmol L^{-1}) and 10 g L^{-1} (167 mmol L^{-1}) were selected and tested based on average low and high levels in lignocellulose hydrolyzates across a wide variety of biomass types and hydrolysis reaction conditions found in literature.[7,8,10,23–25] Acetic acid was added aseptically into the cooled and sterile media immediately before the start of the fermentation run. The initial pH of the media was then set to 6.5 using sterile 5 mol L^{-1} NaOH. Aerobic batch fermentation was initiated by inoculating the sterile media with 20% (v/v) of the maintained activated sludge. The pH of the culture was uncontrolled and monitored throughout the duration of the experiment using a built-in pH probe. The temperature of the culture was controlled at 25 ± 1 °C using a platinum resistance temperature detector (RTD) and a water jacket in the fermenter vessel. Foaming was prevented by the automatic addition of a 1:10 dilution of non-oil, polypropylene-based Antifoam 204 concentrate (Sigma Aldrich, St Louis, MO, USA). Air filtered through a 0.45 µm HEPA vent filter (Whatman, Kent, UK) was bubbled into the culture at 1 vvm (volume of air per volume of media per minute). Agitation rate was set at 300 RPM from 0–24 h of fermentation and then increased to 400 RPM at 24 h and 500 RPM at 48 h in order to maintain a minimum dissolved oxygen level of 20% saturation throughout the experiment. The total incubation time for the batch culture was 7 days and culture broth samples were collected at regular time intervals via an air-lock sampling port in the fermenter vessel and then processed and analyzed according to the methods outlined below.

Analytical methods

Cell biomass concentration was determined by centrifuging 30 mL fermentation broth samples at 3400 *g* for 20 min. The cell pellets produced were then washed with 20 mL of 0.85% (v/v) saline solution and stored in a −80 °C freezer for at least 2 h. The frozen cell pellets were then freeze-dried using a Freezone 6 Bulk Tray Freeze Dryer (Labconco Corp., Kansas City, MO, USA) and weighed. The results were reported as cell dry mass concentration in g L^{-1}.

Residual glucose in the supernatant was measured by using a YSI 2700 Biochemistry Analyzer (YSI Life Sciences, Yellow Springs, OH, USA) equipped with a glucose oxidase membrane (YSI Inc. (http://www.ysilifesciences.com)). Residual ammonium-nitrogen (NH_4^+-N) was determined using an ICS 3000 ion chromatograph (Dionex Corp., Sunnyvale, CA) equipped with an IonPac CS16 cation exchange analytical column (250 × 4 mm) and CG16 guard column (50 × 4 mm) and a conductivity detector using a method described elsewhere (DIONEX Corp. (http://www.dionex.com)).

Lipids in the raw and cultivated activated sludge biomass were extracted using the method of Bligh and Dyer.[26] The resulting lipid extracts were weighed and the values were reported as the gravimetric lipid yield (% CDW). Biodiesel yield and fatty acid profile of the lipid extracts were estimated by a transesterification and gas chromatography-flame ionization detection (GC-FID) analysis method described in an earlier paper.[20]

Residual acetic acid concentrations in the culture were measured using solvent extraction and subsequent analysis of the extracts by GC-FID based on a method described elsewhere.[27] Fermentation broth supernatant samples (0.5 mL) were transferred into microcentrifuge tubes and acidified by the addition of 0.5 mL of 50% (v/v) H_2SO_4 in order to shift the acetic acid dissociation equilibrium towards the formation of the protonated acid which is more hydrophobic. Afterwards, 1 mL of Optima® grade chloroform (Fisher Scientific, Pittsburgh, PA, USA) containing 1 g L^{-1} of hexanoic acid (Sigma Aldrich, St Louis, MO, USA) was pipetted into the acidified sample to extract the protonated acetic acid, and 0.5 g of NaCl was added in order to enhance the separation

of the organic and aqueous phases. The contents were then mixed by gently inverting the tubes 18 times in 10 s, after which the tubes were centrifuged at 13 400 *g* for 30 s using a Model 5415D Microcentrifuge (Eppendorf NA, Hauppauge, NY, USA). After centrifugation, the bottom (chloroform) layer was withdrawn using a 10-inch Pasteur pipette and transferred into an autosampler vial with a glass insert. The samples were then analyzed using an Agilent 6890N Gas Chromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped with a flame ionization detector, and a Restek 11 023 Stabilawax DA 30 m × 0.25 mm ID capillary column with a 0.25 µm film thickness and operated at an oven temperature of 10 °C min^{-1} to a maximum of 260 °C. The instrument was calibrated using a standard solution containing known amounts of acetic acid based on the response factor of the internal standard hexanoic acid. The average retention time of acetic acid was found to be 10.5 min.

Kinetic modeling

The fermentation kinetics was modeled in order to aid in the interpretation of the data trends using the following equations as applied elsewhere.[28–30] Non-lipid biomass production was characterized using the Logistic model:

$$\frac{dX}{dt} = \mu_{max} X(t)\left(1 - \frac{X(t)}{X_{max}}\right) \quad (1)$$

where X (g L^{-1}) is the non lipid biomass concentration in the fermentation broth at any time t (h), μ_{max} (h^{-1}) is the maximum specific growth rate, X_{max} (g L^{-1}) is the maximum non lipid biomass concentration attainable corresponding to the maximum carrying capacity of the culture.

Lipid accumulation was described by a Luedeking–Piret type equation (Equation (2)) expressing the rate of lipid production as a function of the instantaneous non-lipid biomass concentration X and the non lipid biomass production rate:

$$\frac{dP}{dt} = nX + m\frac{dX}{dt} \quad (2)$$

where P (g L^{-1}) is the lipid concentration in the fermentation broth at any time t (h), and m and n are empirical constants used for the prediction of the instantaneous lipid concentration in the culture.

Glucose utilization was also modeled by another Luedeking–Piret type equation (Equation (3)) where the rate of glucose utilization is expressed as a function of the instantaneous non-lipid biomass growth, lipid formation rate, and a cell maintenance coefficient term:

$$-\frac{dS}{dt} = \frac{1}{Y_{X/S}}\frac{dX}{dt} + \frac{1}{Y_{P/S}}\frac{dP}{dt} + k_e X \quad (3)$$

where S (g L^{-1}) is the residual glucose concentration in the fermentation broth at any time t (h), $Y_{X/S}$ and $Y_{P/S}$ (g g^{-1} glucose consumed) refer to the non lipid biomass and lipid yield coefficients, respectively, and k_e is the maintenance coefficient. Estimation and optimization of the kinetic parameter values were conducted using the methods described in a previous paper.[20] The calculated kinetic parameters are summarized in Table 1 and based on the R^2 values, the model fit the fermentation data from the control and treatment runs satisfactorily.

Table 1. Effect of acetic acid on fermentation kinetics and yield parameter estimates for lipid accumulation by activated sludge cultures

	Acetic acid concentration (g L^{-1})		
Parameters	0 (Control)	2	10
Non-lipid biomass			
μ_{max}, (h^{-1})	0.027 ± 0.018	0.029 ± 0.017[†]	0.030 ± 0.010
X_0 (g L^{-1})	5.54 ± 1.51	5.05 ± 0.64	4.21 ± 1.20
X_{max} (g L^{-1})	12.9 ± 1.8	8.09 ± 0.55	16.6 ± 1.80
R^2	0.91	0.93	0.96
Lipid			
m	0.150	0.112	0.646
n	8.20 × 10^{-4}	1.58 × 10^{-4}	5.28 × 10^{-4}
P_{max} (g L^{-1})	2.67 ± 0.05	1.15 ± 0.003	1.65 ± 0.04
Max. lipid content (% CDW)	17.8 ± 2.8	12.5 ± 0.7	8.8 ± 3.2
Lipid productivity (g L^{-1} h^{-1})	0.014 ± 0.003	0.005 ± 0.002	0.004 ± 0.003
R^2	0.96	0.90	0.81
Total carbon sources			
$Y_{X/S}$ Overall (g g^{-1})*	0.095 ± 0.032	0.044 ± 0.012	0.112 ± 0.072
$Y_{X/S}$ Growth phase (g g^{-1})	0.228 ± 0.093	0.096 ± 0.014	0.061 ± 0.020
$Y_{X/S}$ Lipid phase (g g^{-1})	0.050 ± 0.028	0.023 ± 0.008	0.186 ± 0.071
$Y_{P/S}$ Overall (g g^{-1})	0.039 ± 0.008	0.011 ± 0.001	0.010 ± 0.008
$Y_{P/S}$ Growth phase (g g^{-1})	0.057 ± 0.016	0.006 ± 0.002	0.018 ± 0.011
$Y_{P/S}$ Lipid phase (g g^{-1})	0.033 ± 0.008	0.014 ± 0.002	0.007 ± 0.001
k_e (g/g·h)	0.019	0.079	0.007
R^2	0.94	0.97	0.97

[†] Errors represent the 95% confidence limits.
* Yield coefficients are based on amount of total carbon substrates consumed.

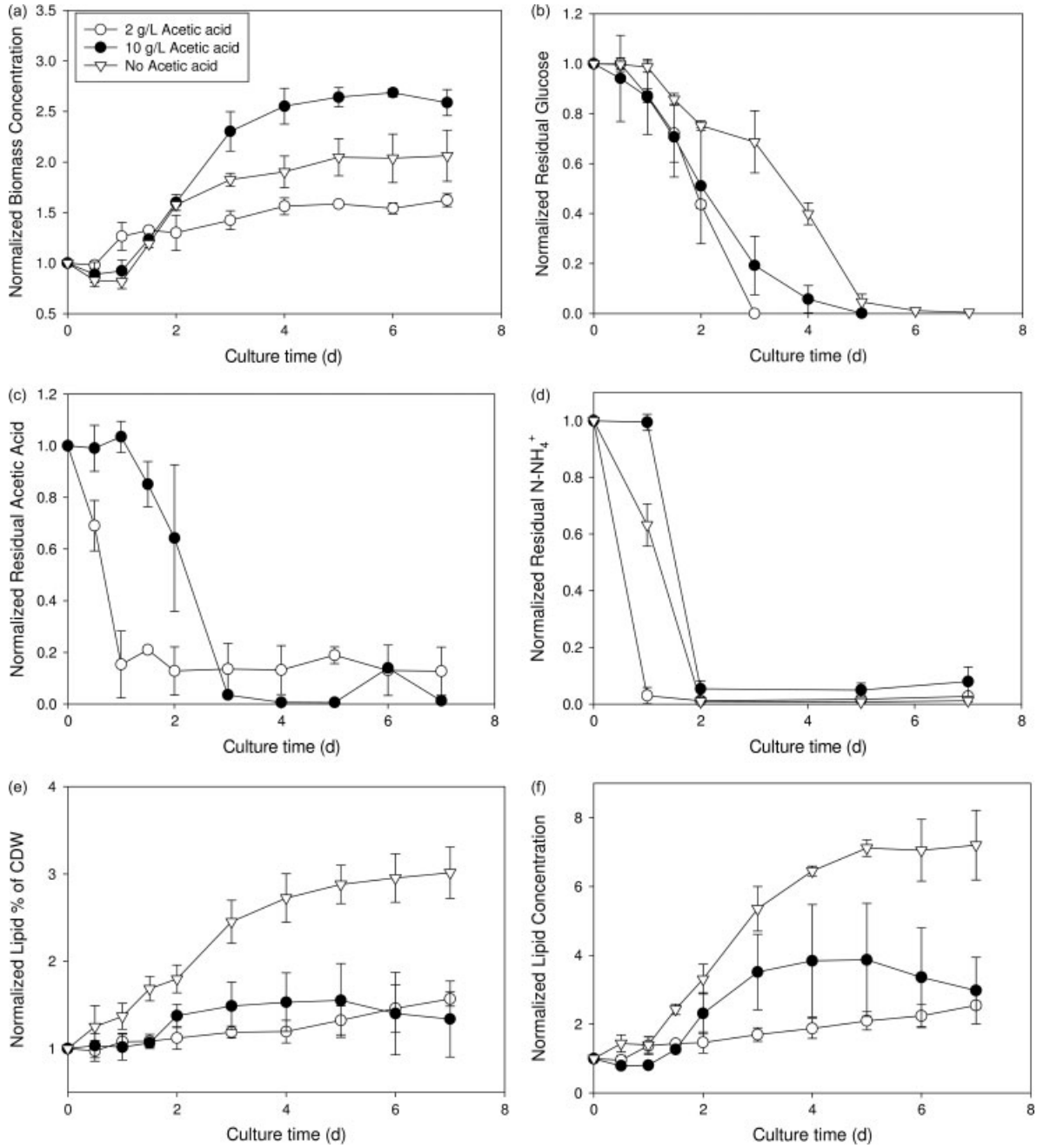

Figure 1. Effect of acetic acid on fermentation profiles of activated sludge microbial cultures for enhanced lipid accumulation.

RESULTS AND DISCUSSION

Fermentation profiles

To evaluate biomass and lipid production by activated sludge microorganisms in the presence of acetic acid, batch fermentation experiments were conducted using synthetic media containing glucose as the primary carbon source and dosed with known amounts of acetic acid. Control runs containing only glucose were conducted alongside the treatments. Four replicate experiments were conducted 1–2 weeks apart to take into account some variability in the initial activated sludge characteristics. The fermentation profiles obtained from the experiments were plotted as normalized values relative to the initial levels. All error bars in the figures represent the 95% confidence limits of the mean responses.

Figure 1 shows a summary of the fermentation profiles of activated sludge glucose-fed batch cultures for enhanced lipid production dosed with acetic acid. As shown in Fig. 1(a), the overall biomass production by activated sludge microorganisms appeared to be reduced by a 2 g L^{-1} (33 mmol L^{-1}) acetic acid loading but was significantly increased by a 10 g L^{-1} (167 mmol L^{-1}) acetic acid dose relative to the control run. A 12 h lag phase was observed at the 2 g L^{-1} acetic acid treatment run, followed by a short exponential phase of growth and a leveling-off of the biomass concentration after 24 h. On the other hand, both the control and 10 g L^{-1} acetic acid treatment run showed a 24 h lag period, after which the exponential growth phase was observed up to 2 days in the control run and 3 days in the 10 g L^{-1} acetic acid treatment run. Although μ_{max} estimates

shown in Table 1 were not significantly different between the control and acetic acid treatment runs, $Y_{X/S}$ (based on the total amount of carbon sources, glucose and acetic acid) and X_{max} were reduced by 54 and 37% (2 g L^{-1} acetic acid treatment run) respectively, at the 2 g L^{-1} acetic acid run; and increased by 18 and 29%, respectively, at the 10 g L^{-1} acetic acid treatment run (Table 1). Another interesting observation shown in Table 1 is that the majority of the substrate conversion to biomass occurred during the early exponential phase ($Y_{X/S}$ at growth phase $>$ $Y_{X/S}$ at stationary phase) for 2 g L^{-1} acetic acid treatment run compared with the control. The opposite effect was observed at the 10 g L^{-1} acetic acid treatment run, which could be due to the initial lag in biomass growth in favor of acetic acid detoxification.

The glucose consumption rates (Fig. 1(b)) observed in the acetic acid treatment runs were also found to be higher than in the control run. A short lag period of up to 24 h in glucose uptake was seen in the control run, whereas in both high and low acetic acid levels tested, glucose consumption commenced almost immediately after inoculation. Glucose was fully consumed after only 3 days on the 2 g L^{-1} acetic acid treatment run and after 5 days on the 10 g L^{-1} acetic acid treatment run. In terms of the acetic acid uptake profiles (Fig. 1(c)), a fast acetic acid uptake rate was observed in the 2 g L^{-1} acetic acid treatment run, while at the higher acetic acid dose of 10 g L^{-1}, a longer lag period of approximately 24 h was observed after which the acetic acid concentration dropped drastically after 3 d. In both acetic acid treatment levels tested, the observed equilibrium residual acetic acid concentration was around 0.5–1 g L^{-1}. The ammonium-nitrogen (NH_4^+-N) consumption trends (Fig. 1(d)) also somewhat reflected the biomass production profiles, as the lag period in NH_4^+-N consumption at the 10 g L^{-1} acetic acid treatment run coincided with the lag period in biomass growth. After this lag period, an increased rate of biomass growth at the exponential phase was observed and corresponded with the rapid consumption of the residual NH_4^+-N in the culture. Since the initial NH_4^+-N supply in the culture is constant, the faster rate of NH_4^+-N depletion on the 2 g L^{-1} acetic acid treatment run relative to the 10 g L^{-1} acetic acid treatment run and control could be related to its possible use in the acetic acid detoxification process or cell maintenance by the activated sludge microorganisms.

As opposed to the observed enhancement in biomass production due to high acetic acid levels ($\geq$10 g L^{-1}), lipid accumulation by activated sludge was apparently inhibited by the presence of acetic acid at both the high and low levels investigated. As shown in Fig. 1(e), the gravimetric lipid content (% CDW) of activated sludge biomass grown in the presence of acetic acid increased by no more than 50% of the initial value in raw activated sludge whereas in the control run with no acetic acid supplied, the lipid content of activated sludge biomass generated after 7 d was thrice the initial value. Different trends in lipid accumulation were observed between the two levels of acetic acid investigated. With the addition of 2 g L^{-1} acetic acid in the activated sludge culture, biomass lipid contents increased gradually to reach the maximum of 12.5 $\pm$ 0.7% CDW after 7 days. On the 10 g L^{-1} acetic acid treatment run, biomass lipid content increased from 7 to 9% CDW between 36 and 48 h of fermentation and gradually increased to a maximum level of 10.2 $\pm$ 4.3% CDW after 5 d, after which it decreased to the final lipid content of 8.09 $\pm$ 0.55% CDW. In the absence of glucose or a carbon source the microorganisms become idle in terms of lipids production, or consume the accumulated lipids for cell maintenance. The data suggest that semi-batch or continuous glucose addition could further increase lipid yields.

The resulting overall growth phase and stationary (lipid accumulation) phase $Y_{P/S}$ values shown in Table 1 were reduced by around 70–90% on the acetic acid treatment runs relative to the control run. The lipid concentration profiles (Fig. 1(f)), which describe the net effect of total biomass yield and gravimetric lipid content, showed similar trends to the total biomass production (Fig. 1(a)) and gravimetric lipid yield profiles (Fig. 1(e)). The maximum lipid concentrations (P_{max}) attained in the culture with 2 and 10 g L^{-1} acetic acid loadings were reduced by 57% (1.2 g L^{-1}) and 38% (1.7 g L^{-1}), respectively, compared with the control run (2.7 g L^{-1}) as shown in Table 1. Furthermore, the values of the empirical parameters m and n for the lipid accumulation model showed that the majority of the lipid yield in the acetic acid-fed cultures were biomass growth-associated since $m > n$ (Table 1).

Based on these findings, it can be generalized that low acetic acid level inhibited biomass growth while a relatively high acetic acid dosage appeared to enhance biomass growth in activated sludge microorganisms growing in glucose. This finding was similar to the results obtained from experiments on the oleaginous yeast *Trichosporon cutaneum* 2.1374, wherein a 3.68 g L^{-1} maximum cell mass concentration was obtained at the fermentation run with 5 g L^{-1} acetic acid compared with 2.75 g L^{-1} in media without acetic acid.[4] However, the activated sludge cultures did not exhibit an enhancement in lipid yields unlike some pure oleaginous yeast cultures mentioned in literature. For instance, lipid accumulation by the oleaginous yeast *Trichosporon cutaneum* 2.1374 was slightly improved with the addition of 5 g L^{-1} acetic acid (1.12 g L^{-1}) compared with no acetic acid (1.09 g L^{-1}).[4] Moreover, lipid contents were as high as 68% in *Rhodosporidium toruloides Y4* grown the presence of acetic acid levels between 4.2 and 7.2 g L^{-1}.[15]

The seemingly complex effect of acetic acid on the fermentation trends exhibited by activated sludge microorganisms growing under lipid accumulation conditions could be explained in terms of the nature of acetic acid in aqueous media as well as the supposed acetic acid detoxification response exhibited by various microorganisms as summarized in Fig. 2. In aqueous media, acetic acid undergoes dissociation into acetate (k_1) according to the equilibrium reaction:

$$\underset{\text{acetic acid}}{CH_3COOH} + H_2O \leftrightarrow \underset{\text{acetate}}{CH_3COO^-} + H_3O^+ \quad (4)$$

which has an equilibrium constant (K_a) of 1.8×10^{-5} or a pK_a of 4.75 at 25 °C. It is generally understood based on previous studies on ethanologenic yeasts that the inhibitory effect of acetic acid is related to the hydrophobicity of the undissociated/protonated acetic acid (CH_3COOH), which can diffuse through the microbial cell membranes (k_2) and dissociate in the cytosol (k_3), causing a decrease in intracellular pH, and potentially cause inhibition in cell growth and metabolism due to the acidic pH in the cytosol.[12,14,31,32] This shows the importance of the pH of the culture as a critical variable in fermentations involving organic acids, as adjusting the initial pH of acetic acid-containing media to above its pK_a (4.75) could reduce the concentration of the toxic CH_3COOH by shifting the equilibrium of the dissociation reaction (Equation (4)) to the right towards the formation of acetate (CH_3COO^-), which is less inhibitory that acetic acid and in some cases could be utilized as a supplementary carbon source.[15] The initial pH of all the experiments conducted in this study was set at 6.5. As mentioned above the pH was not controlled after initiation of experiments.

With regard to the acetic acid that crossed the plasma membrane into the inside of the microbial cell, two mechanisms have been

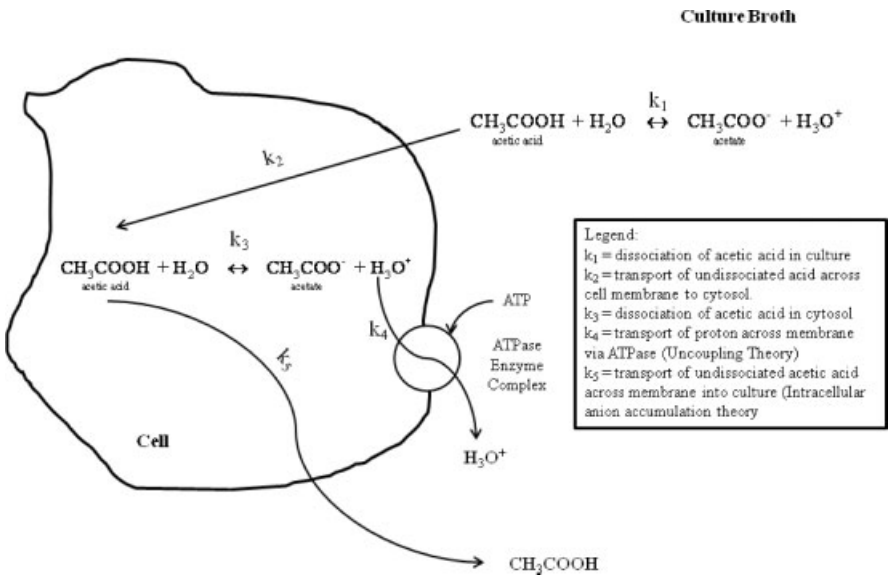

Figure 2. Possible dissociation and transport routes of acetic acid in microbial cultures.

mentioned in the literature in an attempt to explain its inhibitory effects on microbial growth and metabolism: the uncoupling theory and the intracellular anion accumulation theory.[33] The uncoupling theory states that in order to maintain a neutral intracellular pH to combat the inflow of CH_3COOH into the cytoplasm, protons (represented by the hydronium ion, H_3O^+) are pumped across the cell membrane by the action of the ATPase enzyme located at the plasma membrane at the expense of ATP hydrolysis, causing a reduction in the flux of ATP that could have been used for biosynthesis of cellular materials.[34] This phenomenon is represented in Fig. 2 as the process labeled k_4. The ejection of protons from the cell could cause a build-up of H_3O^+ in the fermentation broth and shift the equilibrium of k_1 (Fig. 2) to the left and decrease the pH of the culture. On the other hand, the intracellular anion accumulation suggests that acetic acid toxicity is caused by the build-up of CH_3COO^- inside the microbial cells due to the dissociation of CH_3COOH diffused through the cell membrane into the inside of the cell (k_3 in Fig. 2). Acetate could not diffuse through the cell membrane because of its polarity so its accumulation inside the cell causes the equilibrium of the reaction k_3 (Fig. 2) to shift to the left and cause accumulation of the undissociated acetic acid as well. In order to alleviate this stress, the most probable response of the cell is to eject the undissociated acetic acid across the cell membrane into the fermentation broth (k_5 in Fig. 2). The build-up of CH_3COOH in the culture could then shift the equilibrium of k_1 (Fig. 2) to the right (dissociation into CH_3COO^-) and increase the pH of the culture.

Based on the pH responses of the acetic acid-dosed activated sludge cultures shown in Fig. 3, it can be inferred that the detoxification mechanism based on the uncoupling theory was dominant in the low acetic acid-dosed culture (2 g L^{-1}) due to the reduction of pH while the mechanism based on the intracellular anion accumulation theory was dominant in the high acetic acid-dosed culture (10 g L^{-1}) due to the observed increase in the culture pH. The most probable reason behind this phenomenon is the fact that due to the relatively high amounts of acetic acid and acetate in the 10 g L^{-1} acetic acid treatment run, process k_5 (Fig. 2) was more dominant and occurred at a faster rate such that it overshadowed the effect of the ATPase proton pump (k_4).

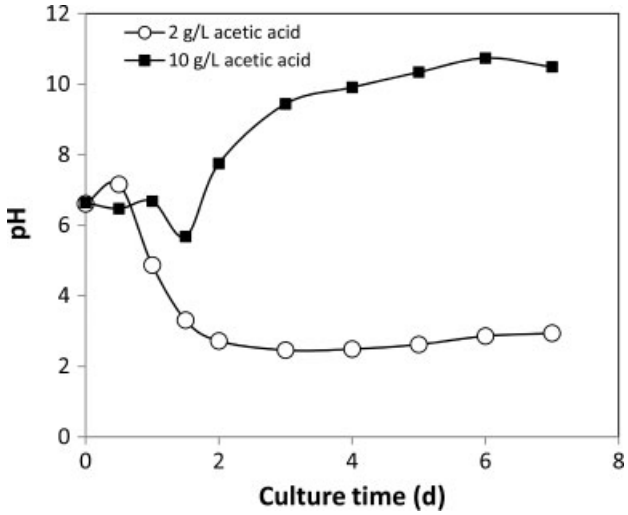

Figure 3. Variation in the pH of acetic acid-dosed activated sludge cultures as a function of culture time.

This could have caused the equilibrium of k_1 to shift towards the formation of acetate (a strong conjugate base to the weak acid CH_3COOH). Under this treatment, acetic acid was probably continuously assimilated and ejected by the cells along with a build-up of acetate in the cytosol, which could explain the observed 24 h lag period in acetic acid uptake shown in Fig. 1(c). This lag period could also represent the time span wherein the activated sludge microorganisms were building enzymes needed for acetate utilization and metabolism at the expense of glucose utilization (Fig. 1(b)) without any observed biomass (Fig. 1(a)) and lipid (Fig. 1(e)) production. Eventually, the microorganisms were able to utilize acetate possibly in the production of non-lipid biomass materials but not storage lipids such as triacylglyerides, and equilibrium levels of the processes were achieved towards the stationary phase. It should be noted, however, that the analysis method employed in this study takes into account roughly the total amount of CH_3COOH/CH_3COO^- species in the culture, as the samples were acidified strongly to convert CH_3COO^- into

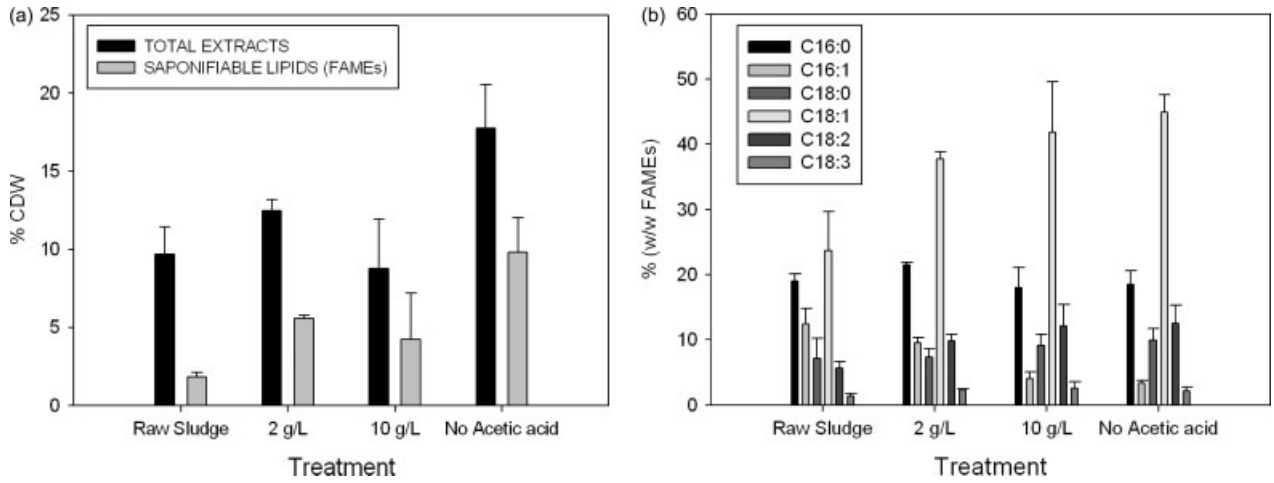

Figure 4. Effect of acetic acid on saponifiable lipid yield (a) and fatty acid profile (b) of lipids derived from glucose-grown activated sludge biomass.

CH_3COOH, which was extracted from the aqueous broth and dissolved in chloroform for GC analysis.

On the other hand, it appears that due to the relatively smaller amounts of the acetic acid species on the 2 g L^{-1} acetic acid treatment run, both the proton pumping mechanism of the ATPase enzyme (k_4) and the ejection of excess CH_3COOH exhibited roughly the same magnitude of effect in the fermentation culture. A small increase in the culture pH on the 2 g L^{-1} acetic acid treatment run (Fig. 3) could indicate an initial build-up of acetate inside the cell due to process k_5 (Fig. 2) but was then followed by a sharp drop in the pH as the equilibrium of the intracellular dissociation of acetic acid (k_3 in Fig. 2) shifted to the right due to the possible utilization of acetate by the microbial cells for biosynthesis and metabolism. As a result, more hydronium ions/protons were probably released into the cytosol and are subsequently ejected by the cell via the action of ATPase (k_4) into the culture. This then causes a shift in the equilibrium of k_1 to the left, forming more undissociated acid and lowering the culture pH until the equilibrium pH and CH_3COOH/CH_3COO^- concentration (Fig. 1(c)) were reached. Although an additional carbon source was available and was probably utilized by the activated sludge microorganisms, the reduced amount of ATP available for biosynthesis and used instead for the proton pump detoxification mechanism probably caused the observed reduction of overall biomass and lipid production compared with the control run and with an excess of acetic acid (10 g L^{-1} treatment) in the culture (Fig. 1(a)). This, however, was unlike the response of some pure oleaginous microorganism strains such as the yeast *Rhodosporidium toruloides,* which showed improved lipid accumulation due to the increase in the C : N ratio in the medium which makes culture conditions more favorable for lipid accumulation.[19] Although some small increases in the lipid content with fermentation time was observed in the acetic acid treatment runs, these were lower than those seen in the control experiments with no acetic acid in the medium. As a mixed culture of various types of microorganisms, many complex environmental and micro-ecological factors could have contributed to the suppression of the activity of lipid biosynthesis enzymes as an effect of the presence of acetic acid in the culture. More detailed metabolic and genetic studies on the active microbial components of the activated sludge microbial population will be needed to confirm this theory.

Lipid and FAME Analysis

The results of the characterization of the lipid extracts obtained from activated sludge biomass harvested after 7 days of cultivation are shown in Fig. 4. As shown in Fig. 4(a) and previously in Fig 1(e), the highest total lipid yield of activated sludge cultures was obtained on the control run at 17.8 ± 2.8 while those of the 2 g L^{-1} and 10 g L^{-1} acetic acid treatment runs were 12.5 ± 0.7 and 8.8 ± 3.2% w/w, respectively. Although this is the case, the saponifiable fraction of the lipid extract, or those that can be converted to methyl esters (biodiesel), consistently accounted for approximately 50–60% (w/w) of the total lipids regardless of the level of acetic acid in the culture. This is a noticeable improvement compared with raw activated sludge (25%). Overall, the final biodiesel yields from activated sludge grown in the presence of acetic acid after 7 days (5.6 ± 0.6% w/w total biomass at 2 g L^{-1} and 4.2 ± 3.0% w/w total biomass at 10 g L^{-1} acetic acid) were higher than the biodiesel yield from raw activated sludge, which averages between 1–2% w/w of the total sludge biomass. In terms of the fatty acid distribution of the lipid extracts, Fig. 4(b) shows a very similar fatty acid profile between raw and cultivated sludge biomass that has been grown with or without acetic acid, with the exception of a significantly higher concentration of oleic acid (C18 : 1) in the latter. Similar results were observed in the oleaginous yeast *R. toruloides,* wherein oleic acid was likewise the major fatty acid (40–53% of total fatty acids) regardless of the concentration of acetic acid in the media.[19]

The fatty acid profiles obtained in this study were similar to plant oils used for biodiesel production such as soybean oil; hence lipids produced by activated sludge microorganisms from lignocellulose hydrolyzate has great potential as biodiesel feedstock. The resulting biodiesel will have improved cold flow, ignition quality (cetane number), oxidative stability, and presumably reduced nitrogen oxide emissions due to very low levels of linolenic acid (C18 : 3).[35] Additionally, the final fatty acid profile of the lipids obtained from the cultivated (enhanced) sludge was also found to be consistent regardless of the number of replicate experiments performed. This was unlike raw activated sludge in which over long residence times in the wastewater treatment facility, the fatty acid composition may have experienced variations caused by a number of variables in the environment such as prevailing weather conditions, organic loading of wastewater, dissolved oxygen, and others. This finding is important because it could allow for the large

scale production of lipid and biodiesel with consistent quality from wastewater biorefineries employing the proposed fermentation process.

CONCLUSIONS

The effect of acetic acid, a major lignocellulose biomass hydrolysis by-product, on the growth and lipid accumulation of activated sludge cultures using glucose as the primary carbon source was investigated. $Y_{X/S}$ (based on the total amount of carbon sources, glucose and acetic acid) and X_{max} were reduced by 54 and 37% on the 2 g L^{-1} acetic acid treatment run, respectively; and increased by 18 and 29%, respectively, on the 10 g L^{-1} acetic acid treatment run. This enhancement in biomass production has been attributed to the possible utilization of acetate as an additional carbon source for non lipid biomass production. However, the maximum gravimetric lipid content of the cultivated sludges was lower at 12.5 $\pm$ 0.7% and 8.8 $\pm$ 3.2% w/w for 2 and 10 g L^{-1} acetic acid levels, respectively, compared with that obtained in the control experiments (17.8 $\pm$ 2.8% w/w). Unlike pure oleaginous yeasts, the activated sludge microorganisms were not able to fully exploit acetate for lipid biosynthesis, possibly due to the complexity of the combined metabolic activities of the various members of the activated sludge microflora as well as competing processes for energy (ATP) and carbon substrate such as the complex acetic acid detoxification mechanisms possibly exhibited by the activated sludge microorganisms. Despite the negative effect of acetic acid dosing on total gravimetric lipid yields, the saponifiable fraction of the lipids (biodiesel yield) produced in the presence of acetic acid was higher than that from raw activated sludge. The fatty acid composition of theses lipids was also consistent regardless of acetic acid concentration and inoculum batches in fermentation media and indicates their suitability to produce biodiesel.

Based on the results obtained in this study, lignocellulose biomass hydrolyzates could be used as fermentation substrates for activated sludge microbial cultures created specifically for the production of lipids for biodiesel production. However, experimental conditions and fermentation modes need to be optimized to further enhance lipid yields and overcome the inhibitory effects of acetic acid on lipid production when using biomass hydrolyzates as substrates.

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