Enhanced Microbial Oil Production by Activated Sludge Microorganisms via Co-Fermentation of Glucose and Xylose

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DOI 10.1002/aic.14169
Published online July 24, 2013 in Wiley Online Library (wileyonlinelibrary.com)

The co-fermentation of glucose and xylose by activated sludge microorganisms for the production of microbial oils for use as biodiesel feedstock was investigated. Various carbon sources at initial concentration of 60 g/L and C:N ratio 70:1 were investigated: xylose, glucose, and 2:1 and 1:2 (by mass) glucose/xylose mixtures. Oil accumulation ranged between 12 to 22% CDW, the highest of which was obtained when xylose was the sole substrate used. Kinetic modeling of the fermentation data showed that specific growth and oil accumulation rates were similar in all substrate types and the lipid coefficient ranged from 0.02 to 0.06 g/g of sugar consumed. The fatty acid methyl ester yield and composition of the lipids showed their suitability for conversion to biodiesel. Based on the results, lignocellulose sugars could be used as fermentation substrates by activated sludge microorganisms for enhancing the oil content of sewage sludge for its use as a sustainable biofuel feedstock source. © 2013 American Institute of Chemical Engineers *AIChE J*, 59: 4036–4044, 2013

Keywords: Fermentation, lipids, biodiesel, sewage sludge, lignocellulose, mathematical modeling

Introduction

The use of microbial oils produced by oleaginous microorganisms as an alternative lipid feedstock for biodiesel production has been studied only recently.[1–4] Oleaginous microorganisms are capable of accumulating 20–80% of their biomass as lipids.[5] This accumulation occurs as a response to an environmental stress, such as limitation of a key nutrient (usually nitrogen) relative to an excess supply of the carbon source leading to the conversion of the excess carbon into storage lipid for later mobilization by the microbial cells. Lipids similar to vegetable oils can be synthesized by certain oleaginous yeasts,[6] fungi,[7] and algae[8] which can be subsequently extracted and converted to biodiesel. Lipid accumulation has also been reported in a few bacterial strains belonging to the actinomycetes group such as *Mycobacterium, Streptomyces, Rhodococcus,* and *Nocardia*.[9,10] These microbial oils appear to be more advantageous over vegetable oils and animal fats for biodiesel production because of their relatively shorter generation times, less labor and land requirements, minimal influence of location, season, and climate on yields and quality, and the relative ease of fermentation process scale-up.[11] However, some investigators contended the economic feasibility of this process, and emphasized several drawbacks such as the theoretical yield limitations, costs associated with the carbon source needed for the cultivation of these oleaginous microorganisms, large-scale fermentation equipment capital and operating costs, the potential for contamination of large-scale oleaginous microbial cultures, and the treatment and disposal of spent culture broth and cell biomass residues after oil extraction.[12] Therefore, considerable research has to be conducted in order to resolve these issues and improve the economic competitiveness of microbial oils for their use as a renewable biodiesel feedstock.

In order to address the issues related to the costs of fermentation substrates, the utilization of low-cost and abundant waste materials such as lignocellulosic biomass from agricultural and forestry residues and municipal solid wastes as sources of fermentable sugars have been considered.[4,13] 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.[14] Lignocellulose is composed of the biopolymers cellulose, hemicellulose, and lignin; hence for it to be more effectively utilized by fermenting microorganisms, it needs to undergo hydrolysis to release the hexose and pentose monosaccharide sugars that make up cellulose and hemicellulose.[14] Most lignocellulose hydrolyzates obtained under mild hydrolysis conditions contain higher levels of xylose than glucose since hemicelluloses are easier to degrade than cellulose. Therefore, the oleaginous microorganisms must be able to utilize pentose sugars such as xylose in addition to

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the hexose sugars. Despite the abundance of xylose in lignocellulose hydrolyzates, it was only recently that many investigators have looked into lipid accumulation by oleaginous microorganisms using pure xylose, synthetic hydrolyzate sugar mixtures with varying xylose proportions, or xylose-rich biomass hydrolyzates as fermentation substrates. Earlier studies on the yeast *Candida curvata* conducted by Evans et al. have shown maximum lipid accumulation of up to 49% of its dry cell weight using different sugars as carbon sources, including xylose.[15] The study conducted by Zhao et al. showed that *Lipomyces starkeyi* can accumulate up to 61.5 wt % of its biomass as lipids using a 2:1 glucose/xylose substrate mixture.[16] More recently, Gong et al. obtained roughly 50 wt % lipid accumulation by the same yeast strain on substrate mixtures of glucose, xylose, and cellobiose.[17] *Trichosporon cutanaeum* was also shown to accumulate lipids of up to 59 wt % with a lipid coefficient of 0.17 g/g sugar using a 2:1 glucose/xylose mixture and 39.2 wt % lipids and 0.15 g/g lipid coefficient after fermentation of corn stover hydrolyzate with a higher concentration of xylose.[18] The fungi *Mortierella isabellina* and *Cunninghamella echinulata* were also shown to accumulate up to 65.5 and 57.7 wt % of their dry biomass as lipids, respectively when grown on xylose-containing media.[19] These studies produced excellent results in terms of xylose utilization and microbial oil yield by pure cultures of selected oleaginous yeast and fungi strains. However, the requirement for sterile conditions during the fermentation process will require additional capital and operating costs when translated into a commercial scale. Furthermore, in order to achieve true economic feasibility while using waste materials such as agroforestry residues as carbon sources, a well-established network or supply chain has to provide these materials into the fermentation facility with minimal collection and transportation costs.[13]

Recently, an innovative technology seeking to address these challenges that is designed to exploit mixed microbial cultures in natural environments such as sewage sludge in fermentation processes for the production of microbial oils was presented. A series of initial studies investigated lipid extraction and direct transesterification of raw sewage sludges containing adsorbed oils and lipid-containing microbial cells resulting into oil yields of 7–10 wt % and biodiesel yields of up to 2–5 wt %.[20–23] In the most recent study aimed at increasing the oil and biodiesel yields from sewage sludge, activated sludge microorganisms were cultivated in aerobic bioreactors operating at initial C:N ratio 70:1 and 60 g/L glucose loading to elicit oleaginous behavior among the constituents of this natural microbial consortium. Sludge biomass with increased lipid content and biodiesel yield of around 17.5 ± 3.9 wt % and 10.2 ± 2.0 wt %, respectively were generated—significantly higher than those obtained from raw sewage activated sludge.[24] Additionally, molecular analysis established that the increased oil content of the sludge could be attributed to a population shift in the activated sludge microbial community toward microorganisms that could potentially exhibit oleaginous behavior but are yet to be identified.[24]

This process could pose the following cost advantages: Using mixed cultures such as activated sludge could eliminate the need for media sterilization and assist in the implementation of continuous cultures with minimal risk of contamination. Furthermore, there a potential for scaling-up the process using available municipal wastewater treatment plant infrastructures and utilizing the existing supply chain transporting nutrient and carbon-rich municipal and industrial wastewaters which can be used as growth media and agroforestry lignocellulosic residues and industrial and municipal solid wastes as carbon sources into the wastewater treatment/fermentation plant. The problem regarding the disposal of spent fermentation broth and cellular debris after lipid extraction could also be solved by utilizing the existing anaerobic digestion operations available in many sewage treatment plants for treatment of these wastes or via recycling of the cell debris as fertilizers or soil amendments. This process could have a two-fold advantage in terms of enhancing the income potential and environmental sustainability of wastewater treatment facilities: (1) adding value to sewage sludge by increasing its oil content which leads to the (2) reduction of sewage sludge biosolids disposed in landfills or by incineration. These advantages could potentially overcome the relatively lower lipid yields of the activated sludge microorganisms when compared to pure oleaginous strains.

The objective of the current study was to evaluate xylose as a sole carbon source or combined with glucose for the growth and oil accumulation by activated sludge microorganisms in aerobic fermentation cultures and serve as a proof-of-concept regarding the potential of utilizing lignocellulose biomass residues as a source of fermentable sugars for the enhanced generation of microbial oils by activated sludge mixed cultures. 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 microbial lipid extracts were then characterized to determine their suitability as biofuel feedstock.

Materials and Methods

Inocula

Activated sludge obtained from the Hilliard Fletcher Wastewater Treatment Plant located in Tuscaloosa, AL, USA was used as the source of microorganisms and inocula for the fermentation experiments. The activated sludges were generated under low carbon and nutrient loading conditions as evident in the sewage influent quality data:

$$\mathrm{pH} = 7.0 \pm 0.1$$

$$\mathrm{BOD}_5 = 133 \pm 48\,\mathrm{mg/L}$$

$$\mathrm{COD} = 396 \pm 94\,\mathrm{mg/L}$$

$$\mathrm{NH_4^+ - N} = 22 \pm 3\,\mathrm{mg/L}$$

$$\mathrm{NO_2^- - N} = 4.2 \pm 0.9\,\mathrm{mg/L}$$

$$\mathrm{PO_4^{3-} - P} = 8.9 \pm 1.1\,\mathrm{mg/L}$$

$$\mathrm{C:N\ ratio} = 23\ (\mathrm{Carbon\ as\ total\ COD})$$

Grab samples of the sludge were collected, combined into a single composite mixture, and maintained in a 3-L glass jar with mixing and aeration at room temperature for at most 24 h prior to the fermentation experiments.

Fermentation experiments

The batch fermentation experiments were conducted on BIOFLO 310 bioreactors (New Brunswick Scientific - Eppendorf, Edison, NJ) with working volume of 3 L. A modified synthetic wastewater recipe 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.[25] The synthetic wastewater medium was supplemented with ammonium sulfate (1.62 g/L) as the nitrogen source and four different combinations of glucose and xylose mixtures corresponding to a constant initial carbon-to-nitrogen ratio (C:N) of 70:1 and total carbon source concentration of 60 g/L. These were: glucose only, xylose only, 2:1 glucose/xylose mass ratio, and 1:2 glucose/xylose mass ratio. The fermenter vessel containing the medium was sterilized by autoclaving at 121°C and 20 psig for 20 min to prevent cross-contaminating other microbial culture experiments being conducted on the same bioreactor equipment. The sugars were autoclaved separately to prevent caramelization and mixed aseptically with the rest of the media after cooling to room temperature. The initial pH of the media was then set to 6.5 using sterile 5M NaOH. Aerobic batch fermentation was commenced by inoculating the sterile medium with 20% (v/v) of the activated sludge maintained by aeration. The pH of the culture was uncontrolled and monitored throughout the duration of the fermentation using a built-in pH probe. The temperature of the culture was controlled at 25 ± 1°C using a platinum 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 to 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 in the next section. Four replicates of the fermentation experiments for each treatment were conducted within 1–2 weeks apart to minimize the variability of the initial activated sludge characteristics.

Analytical methods

Cell biomass concentration was determined by centrifuging 30-mL samples of the culture broth at 3,400 × *g* for 20 min. The cell pellets were 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 then weighed. The results were reported as cell dry mass concentration in g/L.

Residual glucose and xylose in the broth supernatant were measured using a YSI 2700 Biochemistry Analyzer (YSI Life Sciences, Yellow Springs, OH, USA) equipped with glucose and xylose oxidase membrane probes. 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), a CG16 guard column (50 × 4 mm), and a conductivity detector.

Lipids in the activated sludge biomass were extracted and weighed using the method of Bligh and Dyer[26] and the values were reported as the gravimetric lipid yield in percent of the cell dry weight (% CDW). The fatty acid methyl ester (FAME) yield and composition of the lipid extracts were determined by transesterification of the samples using a 2% H_2SO_4 in methanol solution followed by gas chromatography (GC) analysis of the resulting FAMEs using an Agilent 6890N Gas Chromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped with a Restek 11023 Stabilawax DA 30 m × 0.25 mm ID capillary column with a 0.25-μm film thickness and a flame ionization detector.

Kinetic modeling

The fermentation kinetics of microbial oil production by the activated sludge microorganisms was analyzed using the model developed previously by Economou et al. for oil production by the oleaginous fungus *Mortierella isabellina*.[27,28] The balance equations for the non-lipid (fat-free) biomass, lipids, sugars, and nitrogen (as ammonium) are, respectively:

$$\frac{dX}{dt} = \mu_{SN} X \tag{1}$$

$$\frac{dL}{dt} = q_L X \tag{2}$$

$$-\frac{dS}{dt} = \left(\mu_{SN}\frac{1}{Y_{X/S}} - q_L\frac{1}{Y_{L/S}}\right)X \tag{3}$$

$$-\frac{dN}{dt} = \mu_{SN}\frac{1}{Y_{X/N}}X \tag{4}$$

In the aforementioned equations, X is the non-lipid biomass concentration (g/L), L is the lipid concentration (g/L), S is the assimilable (S at time t minus equilibrium S) sugar concentration (g/L), N is the assimilable ammonium-nitrogen concentration (g/L), $Y_{X/S}$ (g of non-lipid biomass produced/g of sugars consumed), $Y_{L/S}$ (g of lipids produced/g of sugar consumed), $Y_{X/L}$ (g of non-lipid biomass produced/g of lipids produced), and $Y_{X/N}$ (g of nonlipid biomass produced/g of ammonium-nitrogen consumed) are the yield coefficients, μ_{SN} is the specific growth rate on sugars and nitrogen (1/h), and q_L is the specific lipid accumulation rate of the biomass (g lipids/g non-lipid biomass-h).

The specific growth rate on sugars and nitrogen (μ_{SN}) and specific rate of lipid production (q_L) were assumed to follow a double-substrate limitation model with sugar inhibition by the Andrews' equation

$$\mu_{SN}(S,N) = \mu_{SNmax}\frac{S}{K_S + S + \frac{S^2}{K_{i1}}}\frac{N}{K_N + N} \tag{5}$$

$$q_L(S,N) = q_{Lmax}\frac{S}{K_{LS} + S + \frac{S^2}{K_{i2}}}\frac{k_2}{k_2 + N} \tag{6}$$

where μ_{SNmax} is the maximum specific growth rate (1/h), q_{Lmax} is the maximum specific lipid accumulation rate (g lipids/g non-lipid biomass-h), K_S, K_{LS}, K_N are the saturation constants (g/L), K_{i1} and K_{i2} are the inhibition constants for non-lipid biomass and lipids, respectively (g/L), and k_2 is the constant that describes inhibition of lipid accumulation by high levels of nitrogen (g/L).

Values of the fermentation kinetic parameters were calculated from the experimental data using Mathcad Prime 2.0 (PTC Inc., Needham, MA, USA). Initial estimates of the parameters were obtained via regression and fitting of the integrated forms of Eqs. 1–4, and the values were optimized by minimization of the residual sum of squares. Model predictions were then obtained using the Runge-Kutta-Fehlberg

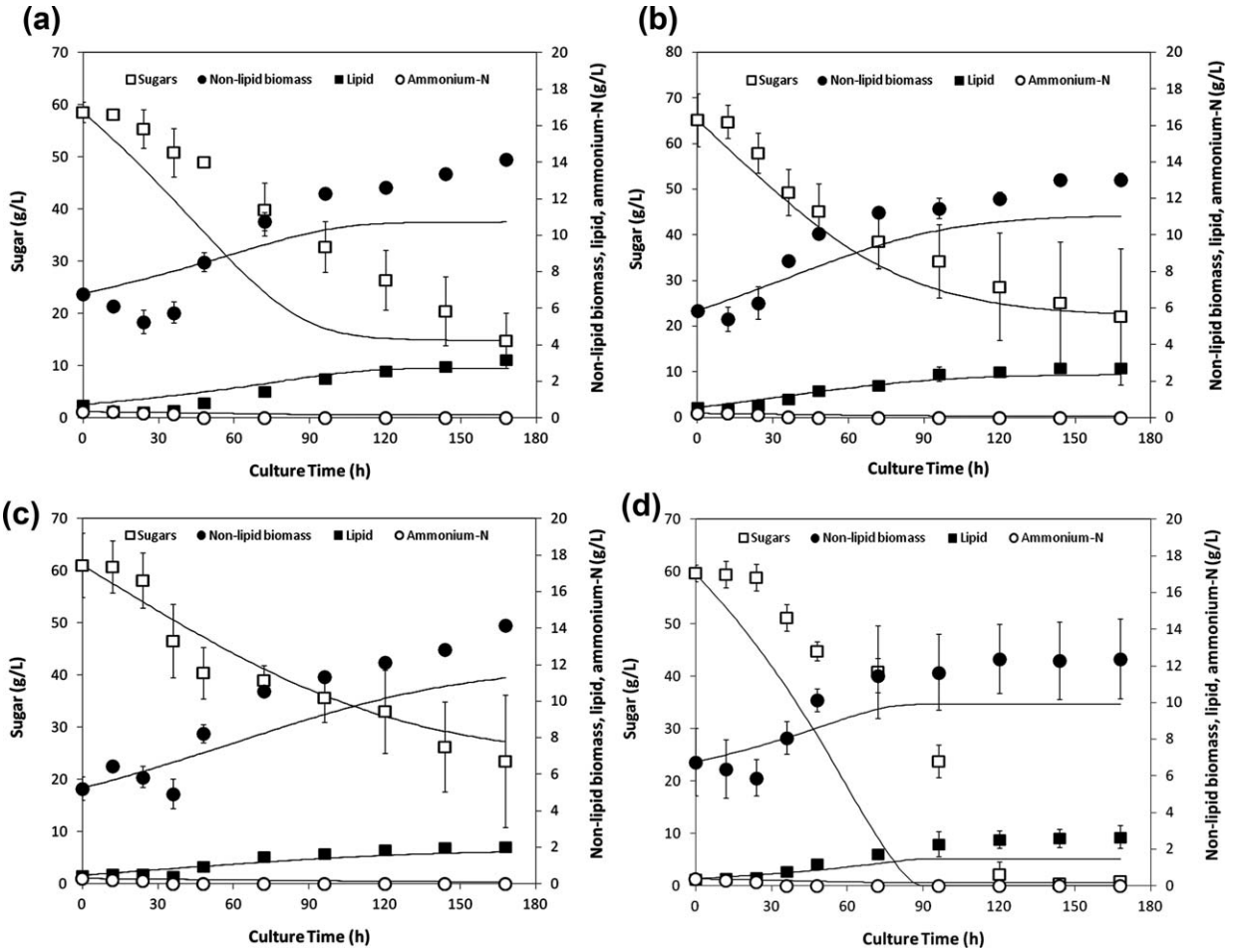

Figure 1. Fermentation experiments on growth and lipid production by activated sludge microbial cultures using various carbon sources: (a) pure xylose, (b) 2:1 ratio (by mass) glucose-to-xylose, (c) 1:2 ratio (by mass) glucose-to-xylose, and (d) pure glucose.

Points represent experimental data and lines represent model predictions.

(RK45) algorithm of the ordinary differential equations program of Polymath 6.1 (CACHE Corp., Austin, TX, USA) and plotted along with the experimental data for comparison.

Results

Fermentation experiments

Under normal operating conditions in wastewater treatment plants, activated sludges are generated in low carbon and nutrient conditions; hence, these "raw" sludges have relatively low initial lipid contents and biodiesel yields as shown in a previous study.[20] It was hypothesized that in order to increase the amount of microbial oils that can be extracted from activated sludge that can be converted to biodiesel, the oleaginous activity of certain fractions of the activated sludge microbial consortium could be triggered via cultivation in a high C:N ratio medium using lignocellulose biomass sugars as fermentation substrate. Fermentation experiments were conducted to test this hypothesis by cultivating activated sludge microorganisms in a high C:N ratio synthetic wastewater media with glucose, xylose, and glucose/xylose mixtures as substrates and the resulting fermentation profiles are shown in Figure 1a–1d. Mathematical modeling of the fermentation data was conducted and the calculated values of the kinetic parameters are summarized in Table 1. All error bars in the figures represent the standard deviations of the responses from the mean.

Figure 1a shows the experimental data of total sugars, total nitrogen, nonlipid biomass, and lipids plotted against fermentation time and the corresponding model predictions for the activated sludge microbial culture fed with pure xylose. It can be seen that growth and lipid accumulation commenced after a lag phase wherein slight reductions in both non-lipid biomass and lipids were observed within the first 48 h of fermentation. These reductions that occurred while xylose consumption appeared to stall in the first 24 h could be attributed to the acclimation of the activated sludge microorganisms to xylose by synthesis of enzymes for xylose utilization or by the shifting of the microbial population, in which a fraction of the microorganisms more acclimated for xylose utilization proliferated and dominated while others declined similar to the results of our previous investigation.[24] Growth and lipid accumulation then commenced to reach average maximum levels of 14 and 3.2 g/L of non-lipid biomass and lipid, respectively. Over the course of these experiments, an average maximum lipid content of $21.5 \pm 3.6\%$ CDW of the enhanced sludge was achieved, which is more than twice as much the initial lipid content of raw activated sludge and higher than those achieved when using glucose as sole carbon source. Similar growth trends were observed in the treatments with 2:1 glucose/xylose (Figure 1b), 1:2

Table 1. Batch Fermentation Kinetic Parameter Estimates for Growth, Lipid Accumulation, and Sugar and Nitrogen Consumption by Activated Sludge Microorganisms Compared with Literature Values on Pure Cultures of Oleaginous Microorganisms

		Literature values		
Parameters	Present work	*Mortierella isabellina* on sweet sorghum extract[27]	*Mortierella isabellina* on rice hull hydrolyzate[28]	*Thamnidium elegans* on glycerol[30]
μ_{SNmax} (h^{-1})	0.023	0.566	0.468	0.089
q_{Lmax} (g/L h)	0.007	0.785	0.298	0.028
$Y_{X/S}$ (g/g)	[a]0.089 (0.169)[e] [b]0.122 (0.179) [c]0.179 (0.238) [d]0.053 (0.098)	0.345	0.354	0.08
$Y_{L/S}$ (g/g)	0.02 – 0.06	0.242	0.215	0.43
$Y_{X/N}$ (g/g)	[a]22.7 [b]32.7 [c]31.6 [d]14.9 [a]22.7	18.209	23.129	3.93
K_S (g/L)	[a]81.104 [b]80.015 [c]78.461 [d]49.97	1.256	3.582	20
K_{LS} (g/L)	[a]10.079 [b]94.919 [c]319.9 [d]0.399	69.269	93.785	–
K_N (g/L)	–	0.184	0.085	0.15
k_2 (g/L)	0.022	835.2	880.839	–
K_{i1} (g/L)	[a]19.437 [b]89.48 [c]79.163 [d]18.656	20.981	6.509	–
K_{i2} (g/L)	[a]50.669 [b]80.574 [c]80 [d]80	0.399	2.023	–

[a]Using pure xylose as substrate.
[b]Using 2:1 ratio (by mass) glucose and xylose as substrate.
[c]Using 1:2 ratio (by mass) glucose and xylose as substrate.
[d]Using pure glucose as substrate.
[e]Values in parenthesis were calculated from experimental data.

glucose/xylose (Figure 1c), and pure glucose (Figure 1d), although the lag periods of growth, lipid accumulation, and sugar consumption were 24 h shorter when glucose is at a higher proportion in the substrate. In these treatments, growth and oil accumulation achieved similar levels among the treatments with no statistical differences, reaching maximum non-lipid biomass and lipid concentrations of 12–14 g/L and 2–3 g/L, respectively. These values corresponded to cellular oil contents of around 13– 17% CDW.

The ammonium-nitrogen utilization patterns in Figure 1 showed complete utilization after 48 h of fermentation, regardless of the substrate type used. This depletion appeared to coincide with the onset of oil production in the cultures. Furthermore, it was observed that in the treatments with xylose, there were considerable amounts of residual sugars in the culture even after 7 days of fermentation. Hence, the assimilable sugar or the difference between the instantaneous sugar concentration and the experimental residual level was used in the kinetic modeling. Approximately 20–25% of the initial sugar concentration remained in the culture broth in the fermentation runs utilizing pure xylose as substrate whereas around 18 and 13% of the initial sugar concentration remained at the 2:1 and 1:2 glucose/xylose substrate treatments, respectively. When used as sole carbon source, glucose was fully consumed within 120 h of fermentation. Figure 2a and 2b shows the glucose and xylose concentration profiles as a function of fermentation time in these treatment runs utilizing the glucose/xylose substrate mixtures. Unlike the reported results of Hu et al.[18] on the simultaneous utilization of glucose and xylose by the yeast *Trichosporon cutaneum,* the activated sludge microorganisms exhibited a sequential utilization of glucose followed by xylose, regardless of their relative proportions similar to the findings of Zhao et al.[16] on the yeast *Lipomyces starkeyi*. This could be an indication of the preferential utilization of glucose by the activated sludge microorganisms initially for assembly of enzymes for xylose metabolism. The sequential substrate utilization pattern could also be related to the apparent diauxic growth patterns shown in Figures 1b and 1c for the fermentation runs utilizing glucose/xylose substrate mixtures in which the non-lipid biomass concentration profiles slightly exhibited dual lag phases: one at the start of the fermentation run and the other occurring at the fermentation time when the residual glucose concentration has been reduced to levels lower than the residual xylose concentration (120 h).

Estimation of kinetic parameters and model validation

Table 1 summarizes the calculated parameter values resulting from the kinetic modeling of the fermentation process for microbial oil accumulation by activated sludge

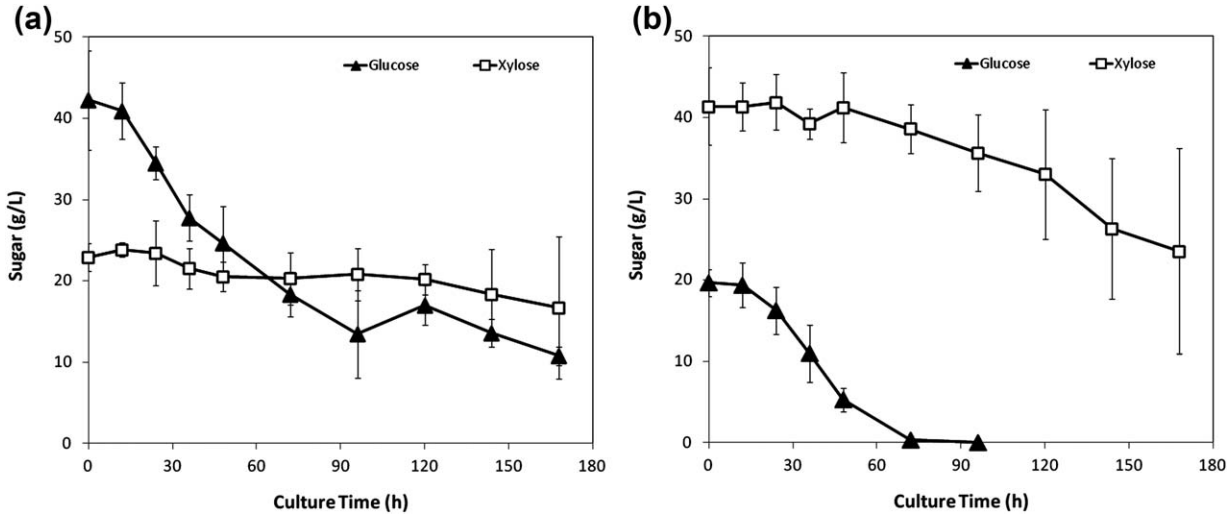

Figure 2. Comparison of glucose and xylose utilization patterns in batch fermentation cultures of activated sludge microorganisms with glucose-to-xylose ratios of (a) 2:1 and (b) 1:2.

microorganisms using glucose, xylose, and mixtures thereof as carbon source. For all the cases investigated, the calculated maximum specific growth and lipid accumulation rates were very similar, having average values of 0.023 and 0.007 h^{-1}, respectively. In terms of the yield coefficients, the experimental and predicted $Y_{L/S}$ values were not statistically different among all the treatments investigated, ranging from 2–6% of total sugar utilized, although the fermentation run utilizing xylose and glucose/xylose mixtures produced the higher lipid yield values. Comparison of the $Y_{X/S}$ and $Y_{X/N}$ values showed the highest non-lipid biomass yield on sugars a 1:2 glucose/xylose fermentation substrate, while slightly higher non-lipid biomass yields were observed when 2:1 glucose/xylose substrate was used. Predicted $Y_{X/N}$ values matched the experimental data well, while the model underestimated the actual experimental $Y_{X/S}$ levels. Table 1 shows that the calculated specific rates and yield values for activated sludge microorganisms were all noticeably lower that the values obtained in the studies utilizing the same modeling approach for pure oleaginous fungi cultures, although in some cases, non-lipid biomass yields were similar. This was expected, as the mixed microbial culture of activated sludge would require a fraction of the initial substrate supply for acclimation and population shifting before eventually establishing a continuous and stable growth and lipid accumulation process. Conducting fed-batch or continuous fermentation using the sugar-acclimated activated sludge microbial cultures could potentially lead to higher oil yields.

Comparison of the saturation constants for sugars (K_S, K_{LS}) in Table 1 shows relatively higher values of these parameters in fermentation runs with xylose and glucose/xylose mixtures as substrates than with glucose alone, implying an initially lower affinity of the initial microbial community in activated sludge to xylose. This could have been eventually alleviated as the microbial consortium shifted to a community more acclimated toward xylose as the fermentation progressed. These values were mostly higher that the saturation constants of the pure oleaginous fungi presented in Table 1, although activated sludge microorganisms fed with pure glucose and xylose appeared to have a relatively higher affinity for these sugars for lipid accumulation. On the other hand, the calculated K_N values for activated sludge microorganisms were close to zero and several orders of magnitude lower than the estimates for *Mortierella isabellina*. This was expected, as the initial activated sludge consortium is more adapted for ammonium-nitrogen consumption in biooxidation processes in municipal wastewater treatment. Furthermore, the calculated inhibition constants (K_{i1} and K_{i2}) for activated sludge were relatively similar for all the cases investigated in this study, with the exception of the K_{i1} values for pure glucose and xylose. The reason for this is unclear but the lower K_{i1} values in these treatments could imply that growth of the activated sludge microbial consortium was inhibited when using a single type of carbon source. Also, the relatively higher inhibition constants of the activated sludge microorganisms compared to *Mortierella isabellina* might imply a higher tolerance or inhibition threshold of the former towards high sugar concentrations.

As shown in Figures 1a–1d, the model used in this study was only able to provide an overall average fit to the experimental data. Model predictions of oil accumulation correlated well with the experimental data ($R^2 = 0.8$–0.95) in all cases except in the treatment in which glucose was the sole carbon source (Figure 1d). Residual sugar predictions fit the experimental values well ($R^2 = 0.93$) on the treatments utilizing glucose/xylose mixtures as fermentation substrates (Figures 1b and 1c). However, the treatments utilizing pure glucose and pure xylose showed higher predicted residual sugar utilization rates (Figures 1a and 1d), hence, the predicted residual sugar levels were lower than the experimental values. In terms of microbial growth, the model appeared to simulate the growth curve of non-lipid biomass production data satisfactorily and predict equilibrium concentrations at the stationary phase in all the treatments investigated but failed to simulate the lag growth phase within the first 36–48 h of fermentation. The ammonium-nitrogen levels predicted by the model also did not match the experimental data, especially at the point of nitrogen depletion at 48 h of fermentation; however, the relative magnitudes of the values are very small to be visualized in the fermentation profiles presented in Figure 1. Regardless, the kinetic model used in this study was chosen for its theoretical bases and assumptions that accurately describe the microbial oil accumulation process, at least in pure cultures of oleaginous microorganisms. Other models or modifications of the current one that would take into account certain conditions observed in this study such

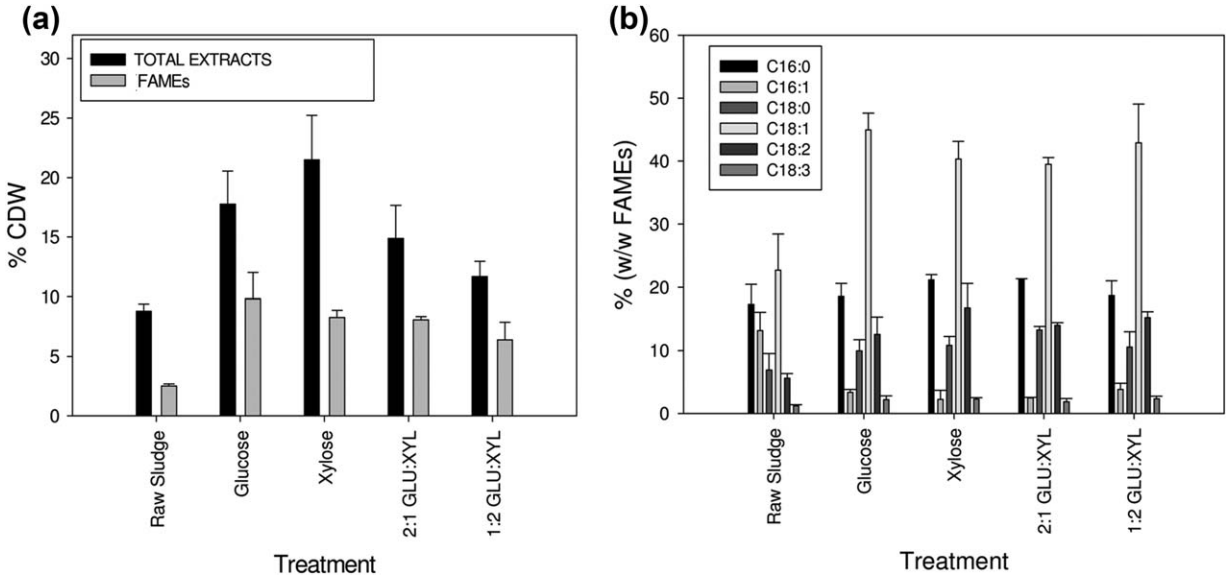

Figure 3. Analysis of activated sludge microbial lipids produced via glucose and xylose fermentation: (a) percentage of total lipid extract and transesterification yield based on cell dry weight (CDW), and (b) composition of fatty acid methyl esters in the transesterified activated sludge microbial lipids.

as diauxic growth and other unknown mechanisms in activated sludge mixed cultures affecting microbial oil production will be explored in forthcoming studies.

Analysis of FAME yield and composition

The results of the characterization of the lipid extracts obtained from activated sludge biomass harvested after 7 d of fermentation are shown in Figure 3. Figure 3a shows that the transesterification (biodiesel) yield consistently accounted for approximately 40–60% (w/w) of the total lipid extract or roughly 8– 20% of the cell dry weight regardless of the type of substrate used, which is higher than that seen in raw activated sludge (25 wt %. of extracts and 2–3% cell dry weight). Based on the error bars in the chart, there appears to be no significant difference among the biodiesel yields per dry mass of enhanced sludge biomass in each of the treatments (type of substrate) investigated.

In terms of the composition of the FAMEs produced by transesterification of the lipids, Figure 3b shows that palmitic (C16:0) and oleic acid (C18:1) methyl esters were the dominant FAME residues in the biodiesel derived from raw activated sludge lipids, consisting approximately 20 and 25% of the total FAMEs, respectively. However, after cultivation of the activated sludge in media containing glucose, xylose, or glucose-xylose mixtures, the oleic acid methyl ester levels significantly increased to 35–50% of the total FAMEs. On the other hand, the saturated fatty acid methyl ester levels, majority of which is attributed to palmitic acid ME (C16:0), were unaffected by the fermentation process and remained around 18–20%. Similar results were seen in previous studies involving pure oleaginous yeasts such as *Candida curvata*[15] and *Lipomyces starkeyi,*[16] and fungi such as *Mortierella isabellina, Cunninghamella echinulata,*[29] and *Thamnidium elegans*[30] which shows the suitability of microbial oils from oleaginous microorganisms and those from the enhanced activated sludge microorganisms used in this study as a renewable biodiesel feedstock.[31] The fatty acid compositions of the microbial oils were comparable with the target soybean oil composition to produce biodiesel with improved cold flow, ignition quality (cetane number), oxidative stability, and presumably reduced nitrogen oxide emissions due to relatively low levels of linolenic acid (C18:3).[32]

Discussion

The results show that activated sludge microorganisms cultivated in high C:N ratio media with xylose and glucose-xylose mixtures as fermentation substrates were able to utilize these sugars for the production of microbial oils which can be used as biodiesel feedstock. This is advantageous for a potential industrial-scale utilization of sugars from lignocellulosic biomass for lipids-to-biodiesel production as the activated sludge microorganisms may not require genetic modification to enhance xylose utilization, unlike most ethanologenic yeasts such as *Saccharomyces cerevisiae*. Furthermore, mild hydrolysis conditions may be applied to lignocellulose biomass as xylose could be more easily released from hemicellulose than glucose from cellulose, reducing the risk of forming byproduct compounds such as furans and organic acids in the hydrolyzate that could severely inhibit the growth and oil accumulation of oleaginous microorganisms. Although the oil content and yields of the activated sludge microorganisms were relatively lower than those reported for pure cultures of oleaginous yeasts and fungi, they represent a significant improvement compared to raw activated sludges in terms of oil content, transesterification yield, and consistency and suitability of its fatty acid composition for biofuels applications independent of the source of the sludge, wastewater, and substrates and the prevailing climate and weather conditions.

The findings that activated sludge microorganisms were able to utilize xylose as fermentation substrate for microbial lipid accumulation and produce slightly higher amounts of microbial oils than when using glucose could be explained by the metabolic pathways for xylose utilization in microorganisms. The utilization of xylose requires additional biochemical steps relative to glycolysis as well as an added set of enzymes.[33] In bacteria, which constitutes majority of the activated sludge microbiota, a xylose isomerase (XI) converts xylose to xylulose. In yeasts however, xylose is converted into

xylitol and, subsequently, to xylulose in reactions catalyzed by xylose reductase (XR) and xylitol dehydrogenase (XH), respectively with NADPH and NAD^+ acting as cofactors. The xylulose produced is then phosphorylated to form xylulose-5-phosphate (D-Xylu-5-P) and is either metabolized through the pentose phosphate pathway or the phosphoketolase reaction pathway.[34] The pentose phosphate pathway generates reducing equivalents in the form of NADPH needed for fatty acid biosynthesis and also the intermediates glyceraldehyde-3-phosphate (G-3-P) and fructose-6-phosphate (F-6-P), which are utilized in glycolysis for the production of pyruvate. Pyruvate is then converted to acetyl-CoA building blocks needed in the fatty acid and TAG biosynthetic pathways in oil-accumulating microorganisms[35] at a yield of approximately 1.0 mol of acetyl-CoA per 100 g of xylose produced.[34] On the other hand, the phosphoketolase reaction pathway utilizes phosphoketolase enzymes for the conversion of the D-Xylu-5-P intermediate directly into G-3-P and acetyl phosphate (acetyl-P).[36] Similarly, the G-3-P produced is shuttled to glycolysis where it is converted to pyruvate and to acetyl-CoA. The fewer reaction steps via the phosphoketolase pathway results in a higher acetyl-CoA yield of around 1.2 mol per 100 g of xylose.[34] Because of the relatively higher oil yield when using xylose than glucose as substrate, it could be assumed that the dominant microorganisms in the activated sludge consortium metabolized xylose primarily through the phosphoketolase reaction pathway.

Microorganisms that do not ferment xylose such as native *Saccharomyces cerevisiae* lack the genes encoded for XR and XH, and, hence, would require genetic or metabolic engineering to allow for the expression of these enzymes needed for xylose utilization.[37] In this study, the native activated sludge microorganisms were able to utilize xylose after a lag period. The synthesis of the XR and XH enzymes needed for xylose utilization could have occurred in this lag period and this would have caused the relatively slower xylose uptake rate than that of glucose, which is readily metabolized through glycolysis. The positive effect of this slower xylose uptake, however, is a more efficient channeling of carbon from xylose to lipid biosynthesis. Because of the relatively easier metabolism of glucose, it could have been readily used for other functions such as biosynthesis of nonlipid cellular materials, respiration, and cell maintenance instead of lipid generation.

The natural ability of activated sludge microorganisms to utilize pentose sugars such as xylose will be advantageous for the commercial scale application of activated sludge cultures for lipid accumulation using mixed sugar substrates such as lignocellulose hydrolyzates as they require no costly genetic modification procedures.[35] The results also suggest an opportunity to integrate lignocellulosic ethanol production with lipid accumulation by activated sludge. The former could convert the glucose from lignocellulose into ethanol, while the latter could use the remaining xylose for lipid accumulation. Adaptation of the activated sludge microbial population to mixed sugar substrates at the onset of xylose utilization and optimization of fermentation conditions will be necessary to further improve lipid yield using glucose-xylose mixtures as fermentation substrates.

Conclusions

The use of pentose sugars, primarily xylose, as a sole or co-substrate with glucose for growth and microbial oil production by activated sludge microorganisms was investigated. The sludge microbial oils were analyzed for transesterification yield and fatty acid methyl ester composition to determine their suitability for use as biodiesel feedstock. Based on the results, it can be concluded that the activated sludge microorganisms are able to utilize xylose and glucose/xylose mixtures for growth and oil accumulation at comparable or even higher yields than using glucose alone. The "enhanced" activated sludge acquired improved lipid and biodiesel yields, and a consistent fatty acid composition more suitable for a biodiesel lipid feedstock than lipids extracted from raw activated sludge. The fermentation kinetics model used in this study only provided an average fit with the experimental data, hence other models will be considered in forthcoming studies. The calculated values of the kinetic and yield parameters for growth and lipid accumulation by activated sludge microorganisms generally imply lower performance compared with the pure oleaginous yeast and fungi strains. However, the findings of this study highlight the great potential of establishing a commercial scale process using available municipal wastewater treatment plant infrastructure and sugars from renewable lignocellulose biomass in agroforestry and municipal solid waste residues as substrates for the production of renewable biofuel feedstock. This could have the advantages of adding value to sewage sludge as an added income source for municipalities, and the reduction of biosolids disposed in landfills or by incineration to enhance the environmental sustainability of traditional municipal sewage treatment processes.

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Manuscript received Mar. 19, 2012, and revision received May. 16, 2013.