Supporting Information

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Notes on Stoichiometry Analysis of Lipid Production on Acetate

Stoichiometry analysis provides valuable information in determining the theoretical maximum yields on triacylglycerol and nonlipid biomass synthesis as well as the trend of RQ (the ratio of carbon dioxide evolution rate to oxygen utilization rate) during fermentation. These indicators are important in evaluating the process efficiency toward lipid production. In the present study, the stoichiometry analysis for maximum lipid production was developed based on the following major assumptions:

i) The average elemental composition of triacylglycerols (TAGs) synthesized by *Y. lipolytica* is $C_{56}H_{102}O_6$ (based on the experimental data of fatty acid profiles).
ii) The average elemental composition of *Y. lipolytica* nonlipid biomass is $CH_{1.6}O_{0.54}N_{0.14}$ (27) (confirmed by CHNS measurements of *Y. lipolytica* biomass).
iii) Lipogenic NADPH comes primarily from the oxidative PPP (25).
iv) Carbon requirements for cell maintenance and minor byproducts are not considered as they account for only a minor percentage of the total requirement.
v) The maximum lipid content (gram lipids per gram biomass) achievable by *Y. lipolytica* cultured on acetate is 60%.

The stoichiometric analyses of lipid synthesis in *Y. lipolytica* are described as follows:

i) The average elemental composition of TAGs is $C_{56}H_{102}O_6$. Taking into account the numbers of Acetyl CoA (ACA), ATP, glycerol-3-phosphate (gly-3-P), and NADPH required for fatty acid elongation, fatty acid desaturation, and TAG synthesis (24), the overall equation can be obtained as follows:

$$53\ \mathrm{ACA} + 2\ \mathrm{Gly\text{-}3\text{-}P} + 47\ \mathrm{ATP} + 100\ \mathrm{NADPH} + 6\ \mathrm{O_2} \rightarrow 2\ \mathrm{C_{56}H_{102}O_6} + 12\ \mathrm{H_2O}. \quad \textbf{[S1]}$$

ii) ACA is synthesized from acetate by an ATP-driven reaction that is catalyzed by acetyl CoA synthetase (28):

$$\mathrm{Acetate} + \mathrm{CoA} + 2\mathrm{ATP} \rightarrow \mathrm{ACA}. \quad \textbf{[S2]}$$

iii) During lipogenic NADPH production from the oxidative PPP pathway, glucose-6-phosphate (G6P) is completely oxidized to CO_2:

$$\mathrm{G6P} \rightarrow 12\ \mathrm{NADPH} + 6\mathrm{CO_2}. \quad \textbf{[S3]}$$

iv) A number of metabolic activities including the glyoxylate cycle, transmembrane transport between cytoplasm and mitochondria, the TCA cycle, and gluconeogenesis are involved to generate glycerol-3-phosphate (Glyc3P) and G6P:
Cytosolic oxaloacetate (OAA) production via glyoxylate cycle:

$$\mathrm{citrate} + \mathrm{ACA} \rightarrow \mathrm{succinate} + \mathrm{OAA(c)} + \mathrm{NADH(c)}. \quad \textbf{[S4]}$$

Cytosolic citrate compensation from TCA cycle:

$$\mathrm{ACA} + \mathrm{succinate} \rightarrow \mathrm{citrate} + \mathrm{FADH_2(m)} + \mathrm{NADH(m)}. \quad \textbf{[S5]}$$

Therefore, the net effect of cytosolic OAA production is

$$2\ \mathrm{ACA} \rightarrow \mathrm{OAA} + \mathrm{FADH_2(m)} + \mathrm{NADH(m)} + \mathrm{NADH(c)}. \quad \textbf{[S6]}$$

From cytosolic OAA to Glyc3P:

$$\mathrm{OAA} + \mathrm{GTP} + \mathrm{ATP} + 2\ \mathrm{NADH(c)} \rightarrow \mathrm{Glyc3P} + \mathrm{CO_2}. \quad \textbf{[S7]}$$

Gluconeogenesis:

$$2\ \mathrm{OAA} + 2\ \mathrm{GTP} + 2\ \mathrm{ATP} + 2\ \mathrm{NADH(c)} \rightarrow \mathrm{G6P} + 2\ \mathrm{CO_2}. \quad \textbf{[S8]}$$

Therefore, the net effects of Glyc3P and G6P synthesis from acetate are

$$2\ \mathrm{ACA} + \mathrm{GTP} + \mathrm{ATP} + \mathrm{NADH(c)} \rightarrow \mathrm{Glyc3P} + \mathrm{CO_2} + \mathrm{FADH_2(m)} + \mathrm{NADH}, \quad \textbf{[S9]}$$

$$4\ \mathrm{ACA} + 2\ \mathrm{GTP} + 2\ \mathrm{ATP} \rightarrow \mathrm{G6P} + 2\ \mathrm{CO_2} + 2\ \mathrm{NADH(m)} + 2\ \mathrm{FADH_2(m)}. \quad \textbf{[S10]}$$

Taking into account the ATP production through the electron transport chain, and assuming that 1 GTP = 1 ATP, the above equations can be further simplified to

$$2\ \mathrm{ACA} + \mathrm{NADH(c)} + \mathrm{O_2} \rightarrow \mathrm{Glyc3P} + \mathrm{CO_2} + 2\ \mathrm{ATP}, \quad \textbf{[S11]}$$

$$4\ \mathrm{ACA} + 2\mathrm{O_2} \rightarrow \mathrm{G6P} + 2\ \mathrm{CO_2} + 4\ \mathrm{ATP}. \quad \textbf{[S12]}$$

v) ATP production via the TCA cycle is done as follows:
Cytosolic OAA generation via glyoxylate cycle and TCA cycle:

$$2\ \mathrm{ACA} \rightarrow \mathrm{OAA} + \mathrm{FADH_2(m)} + \mathrm{NADH(m)} + \mathrm{NADH(c)}. \quad \textbf{[S13]}$$

From cytosolic OAA to pyruvate:

$$\mathrm{OAA} \rightarrow \mathrm{Pyruvate} + \mathrm{CO_2}. \quad \textbf{[S14]}$$

Transmembrane transport of pyruvate:

$$\text{Pyruvate(c)} \rightarrow \text{Pyruvate(m)}. \quad \textbf{[S15]}$$

TCA cycle:

$$\text{Pyruvate(m)} \rightarrow 4\ \text{NADH(m)} + \text{FADH}_2\text{(m)} + 3\ \text{CO}_2 + \text{GTP(m)}. \quad \textbf{[S16]}$$

In the mitochondria, ATP is produced through the electron transport chain:

$$1\ \text{NADH} \rightarrow 2.5\ \text{ATP}$$
$$1\ \text{FADH}_2 \rightarrow 1.5\ \text{ATP}$$
$$1\ \text{GTP} \rightarrow 1\ \text{ATP}$$
$$1/2\text{O}_2 + 2\ \text{H}^+ + 2\text{e}^- \rightarrow \text{H}_2\text{O}.$$

Therefore, the net effect of ATP synthesis is

$$2\ \text{ACA} + 3.5\ \text{O}_2 \rightarrow 4\ \text{CO}_2 + 16.5\ \text{ATP} + \text{NADH(c)} + 7\ \text{H}_2\text{O}. \quad \textbf{[S17]}$$

Here we assume that part of the cytosolic NADH will be used as a reducing agent in the cytoplasm to reduce dihydroxyacetone phosphate to Glyc3p, and extra NADH will be used to generate ATP.

Multiplying **[S1]** by 1/2, **[S2]** by a, **[S3]** by 4.17, **[S11]** by 1, **[S12]** by 4.17, and **[S17]** by b and then summing all equations will result in

$$(26.5 + 2 + 16.68 + 2\text{b} - \text{a})\text{ACA} + (23.5 + 2\text{a} - 2 - 16.68 - 16.5\text{b} - (\text{b}-1)\times 2.5)\text{ATP} + (3 + 1 + 8.34 + 3.5\text{b} + (\text{b}-1)\times 0.5)\ \text{O}_2 + \text{a acetate} \rightarrow \text{C}_{56}\text{H}_{102}\text{O}_6 + (25 + 1 + 8.34 + 4\text{b})\text{CO}_2. \quad \textbf{[S18]}$$

Assigning the coefficient of ACA and ATP to be 0 (i.e., ACA and ATP are metabolically balanced), we obtain

$$\text{a} = 58.2,$$

$$\text{b} = 6.5.$$

Therefore, the stoichiometry equation for TAG synthesis is the following:

$$58.2\ \text{C}_2\text{H}_4\text{O}_2 + 37.8\ \text{O}_2 \rightarrow \text{C}_{56}\text{H}_{102}\text{O}_6 + 60.3\ \text{CO}_2 + 65.4\ \text{H}_2\text{O}. \quad \textbf{[S19]}$$

This results in the maximum lipid yield on acetate of 0.25 (g/g).

Nonlipid biomass production on acetate is

$$6.3\ \text{C}_2\text{H}_4\text{O}_2 \rightarrow 5\text{CH}_{1.6}\text{O}_{0.54}\text{N}_{0.14} + 7.6\ \text{CO}_2 \quad \textbf{[S20]}$$

(11). This results in the nonlipid biomass yield on acetate of 0.33 (g/g).

Thus, the overall process lipid yield $= \frac{60}{\frac{60}{0.25}+\frac{40}{0.33}} = 0.167$ (g/g).

Notes on the Effect of Carbon Starvation

In a constant pH process (pH set point is 7 in the present work), it is known that the amount of protons fed into the system equals the amount consumed:

$$F_1[H^+]_1 + k_2 \cdot dQN/dt = k_1 \cdot dQ_A/dt + F_p[H^+]_p. \quad \textbf{[S21]}$$

In this equation, k_1 represents the proportion of protons that are symported with acetate ions, k_2 represents the proportion of protons that are generated due to consumption of ammonium, F_1 is the feed rate of acetic acid (mL/h), F_p is the flowrate of the permeate (mL/h), $[H^+]_1$ is the proton concentration in the acetic acid feed (mol/L), $[H^+]_P$ is the proton concentration in permeate (mol/L), dQ_N/dt is the ammonium consumption rate (mmol/h), and dQ_A/dt is the acetate consumption rate (mmol/h).

At pH 7, proton outflow in permeate is negligible. Based on our experimental data, the term "$k_2 \cdot dQ_N/dt$" also can be neglected because its contribution is rather small in comparison with the term "$k_1 \cdot dQ_A/dt$." A simplified equation is obtained as follows:

$$F_1[H^+]_1 = k_1 \cdot dQ_A/dt. \quad \textbf{[S22]}$$

The coefficient k_1 can then be determined from regression of experimental data, which is 0.0046 ($R^2 = 0.9955$).

A variation of Eq. **S22** is

$$F_1C_1 \cdot \frac{[H^+]_1}{C_1} = F_1C_1 \cdot K = k_1 \cdot dQ_A/dt, \quad \textbf{[S23]}$$

in which C_1 represents the molar concentration of acetate, and the coefficient K is determined by the dissociation equilibrium of acetic acid. When k_1 is smaller than K, acetate feed cannot compensate its consumption.

In the present work, K is 0.006 for 30 g/L acetic acid, which explains why feeding 3% acetic acid in cascade with pH control cannot compensate for acetate consumption leading to carbon starvation as well as cell growth and lipid synthesis inhibition. In addition, when acetate concentration is above zero in the reactor, there is a carbon outflow from the permeate (F_pC_p). That is to say, the overall carbon fed into the system (F_1C_1) is partially lost in the permeate stream, which aggravates the carbon imbalance between feed and consumption.

Notes for Model 1

Calculation of Q_A.

$$\Delta Q_{A,t\rightarrow t+1} = \Delta X_{t\rightarrow t+1} \cdot \frac{1}{Y_{X/A,t+1}} = \Delta X_{t\rightarrow t+1} \cdot \frac{1}{Y_{X/A,t}} = \mu \cdot OD_t \cdot \frac{\Delta Q_{t-1\rightarrow t}}{\Delta OD_{t-1\rightarrow t}}, \quad \textbf{[S24]}$$

where X is biomass concentration, and $Y_{X/A}$ is biomass yield on substrate.

Proton Balance.

$$F_{A1}[H^+]_1 + F_{A2}[H^+]_2 - F_P[H^+]_P - k_1 \cdot \frac{dQ_A}{dt} + k_2 \cdot \frac{dQ_N}{dt} = 0, \quad \textbf{[S25]}$$

where k_1 is the proportion of protons that are symported with acetate ions, and k_2 is the proportion of protons that are generated due to consumption of ammonium.

Because the pH values of the acetic acid stream, sodium acetate stream, and fermentation broth are 2.6, 8.0, and 7.0, respectively, the proton concentration can be calculated as follows:

$$[H^+]_1 = 10^{-2.6},\ [H^+]_2 = 10^{-8},\ [H^+]_P = 10^{-7}.$$

Compared with the acetic acid feed stream, the proton contribution from the terms $F_{A2}[H^+]_2$ and $F_P[H^+]_P$ is negligible. The term $k_2 \cdot dQ_N/dt$ also can be neglected (*Notes on the Effect of Carbon Starvation*). Therefore, a simplified equation can be obtained,

$$F_1[H^+]_1 - k_1 \cdot dQ_A/dt = 0, \quad \textbf{[S26]}$$

where k_1 can now be obtained through the regression of experiment data.

Carbon Balance. When acetate concentration is above 0 and less than 2.5 g/L, an integral equation can be written as

$$-\frac{1}{F_{P,t\to t+1}} \cdot \ln \frac{F_{A1,t\to t+1}S_{A1} - \frac{dQ_A}{dt} - F_{P,t\to t+1}S_{A,t+1}}{F_{A1,t\to t+1}S_{A1} - \frac{dQ_A}{dt} - F_{P,t\to t+1}S_{A,t}} = -\frac{1}{V} \cdot \Delta t. \quad \textbf{[S27]}$$

According to this model, one would need to perform measurements of OD, acetate concentration in the fermentation broth, permeate volume, and the average acetate concentration in the permeate during the previous time interval. Using these results with a reasonable prediction of the acetate concentration for the next sampling time, F_{A1}, F_{A2}, and F_P can be solved.

When acetate concentration decreases to 0, equations for volume balance, proton mass balance, and carbon balance can be solved simultaneously for the variables F_{A1}, F_{A2}, and F_{A3}, as shown in Fig. S3.

Notes for Exhaust Gas Analysis

Theoretical RQ for Lipid Synthesis. Based on the stoichiometry analysis of *Y. lipolytica* lipid production on acetate (*Notes on Stoichiometry Analysis of Lipid Production on Acetate*), the overall equation for lipids' synthesis is

$$58.2\ C_2H_4O_2 + 37.8\ O_2 \to C_{56}H_{102}O_6 + 60.3\ CO_2 + 65.4\ H_2O. \quad \textbf{[S19]}$$

Therefore, the theoretical RQ for lipid synthesis is

$$RQ_L = 1.6.$$

RQ for Biomass Formation. Fig. S5 shows the time courses of RQ and lipid content. Cell culture methods are described in *Notes for Materials and Methods*. According to the experimental data, RQ stays less than 1.1 before lipid content reaches 20%.

CTR and Citrate Production. The stoichiometry analysis for citrate production is shown as follows:

$$\begin{aligned}
&\text{Acetate} + 2\text{ATP} \to \text{ACA(c)}\\
&2\ \text{ACA(c)} \to \text{succinate(c)} + \text{NADH}\\
&\text{succinate(c)} \to \text{succinate(m)}\\
&\text{succinate(c)} \to \text{OAA(m)} + \text{FADH}_2 + \text{NADH}\\
&\text{ACA(c)} \to \text{ACA(m)}\\
&\text{ACA(m)} + \text{OAA(m)} \to \text{citrate(m)}\\
&\text{citrate(m)} \to \text{citrate(c)}.
\end{aligned}$$

Taking into account the ATP production through the electron transport chain, the net equation for citrate production is

$$3\ \text{acetate} \to \text{citrate} + 0.5\ \text{ATP}. \quad \textbf{[S28]}$$

Notes for Materials and Methods

Bioreactor Conditions for Dilute Acetic Acid Fed-Batch Fermentation. The initial medium contained 30 g/L sodium acetate, 2.5 g/L yeast extract, 4.2 g/L YNB −AA −AS, and 2.4 g/L ammonium sulfate. The pH set point was 7.0. In the first 96 h, the feed consisted of 3% acetic acid and 1.5 g/L ammonium sulfate (C/N ratio = 32) to provide sufficient nitrogen for cell growth. Thereafter, only 3% acetic acid was fed. Acid feed was in cascade with the pH sensor to maintain the pH at 7.0. The fermentation process started with an initial volume of 1.0 L, and the tangential flow filtration started at 20 h when the volume increased to 1.5 L. The working volume remained at 1.5 L for the rest of the fermentation.

Bioreactor Conditions for the Joint Feeding of Dilute Acetic Acid and Sodium Acetate. The initial medium contained 30 g/L sodium acetate, 2.5 g/L yeast extract, 4.2 g/L YNB −AA −AS, and 2.4 g/L ammonium sulfate. In the first 72 h, the acid feed stream consisted of 3% acetic acid and 5 g/L ammonium sulfate (C/N ratio = 14) to provide sufficient nitrogen for cell growth. Thereafter, the feed consisted of 3% acetic acid and 0.65 g/L ammonium sulfate (C/N ratio = 102). Acid feed was in cascade with the pH sensor to maintain the pH at 7.0. Three percent acetate was fed starting from 42 h at a rate ranging from 5 mL/h to 20 mL/h to sustain the carbon requirements by the cells. The fermentation process began with an initial volume of 1.0 L, and the tangential flow filtration started at 24 h when the volume increased to 1.5 L. The working volume remained at 1.5 L for the rest of the fermentation.

Bioreactor Conditions for the First Model Based on Off-Line Analysis. The initial medium contained 41.7 g/L sodium acetate (equivalent to 30 g/L acetic acid), 5 g/L yeast extract, 8.4 g/L YNB −AA −AS, and 1.0 g/L ammonium sulfate. In the first 72 h, the acid feed stream consisted of 3% acetic acid and 5 g/L ammonium sulfate (C/N ratio = 14) to provide sufficient nitrogen for cell growth. Thereafter, the feed consisted of 3% acetic acid and 0.65 g/L ammonium sulfate (C/N ratio = 102). Three percent acetate was fed starting from 40 h when acetate concentration in the bioreactor decreased to 2.2 g/L. Feed rates were determined through the model 1 algorithm (Fig. S3). The fermentation process began with an initial volume of 0.8 L, and the tangential flow filtration started at 24 h when the volume increased to 1.5 L. The working volume remained at 1.5 L for the rest of the fermentation.

Bioreactor Conditions for the Second Model Based on On-Line Analysis. The initial medium contained 41.7 g/L sodium acetate, 5 g/L yeast extract, 8.4 g/L YNB −AA −AS, and 1.0 g/L ammonium sulfate. The acid feed stream consisted of 3% acetic acid. Three percent acetate was fed starting from 38 h when acetate concentration in the bioreactor decreased to 3 g/L. The nitrogen feed consisted of 47.5 g/L ammonium sulfate. Feed rates of acetic acid, sodium acetate stream, and ammonium sulfate were determined through the model 2 algorithm (Fig. 4). The fermentation process started with an initial volume of 0.8 L, and the tangential flow filtration started at 18 h when the volume increased to 1.5 L. The working volume remained at 1.5 L for the rest of the fermentation.

Bioreactor Conditions for the Optimized Model Combining both Carbon and Nitrogen Feed. The initial medium contained 41.7 g/L sodium acetate, 5 g/L yeast extract, 8.4 g/L YNB −AA −AS, and 1.0 g/L ammonium sulfate. The acid feed stream consisted of 3% acetic acid. Three percent acetate was fed starting from 30 h when acetate concentration decreased to 3 g/L. The nitrogen feed consisted of 47.5 g/L ammonium sulfate. Feed rates of acetic acid and sodium acetate were determined through the model 2 algorithm. Feed rate of ammonium sulfate was determined based on the online off-gas analysis and the feedback control algorithm (Fig. 7). The fermentation process began with an initial volume of 0.8 L, and the tangential flow filtration started at 18 h when the volume increased to 1.5 L. The working volume remained at 1.5 L for the rest of the fermentation.

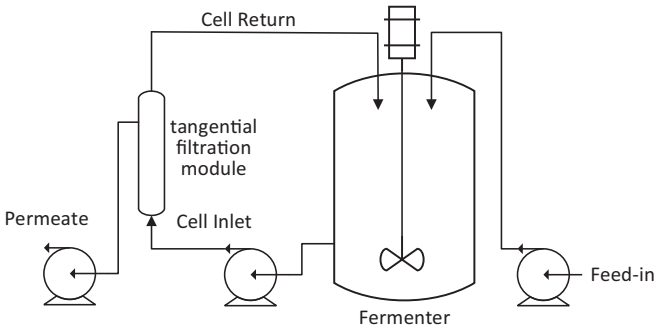

Fig. S1. Flowchart for lipid production with a cell recycling system. Carbon substrate is continuously fed into the reactor coupled to a tangential filtration module. Fermentation broth is pumped through a recycling loop and across a hollow-fiber membrane. Filtration is driven by the transmembrane pressure difference. Yeast cells are larger than the rated pore size of the filter (0.2 μm) and thus are retained in the retentate while soluble components of the fermentation broth pass through the membrane walls and flow out as the permeate.

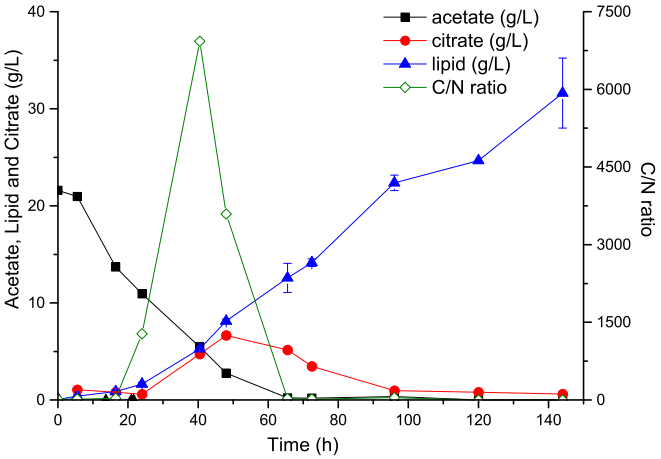

Fig. S2. A high C/N ratio causes citrate production.

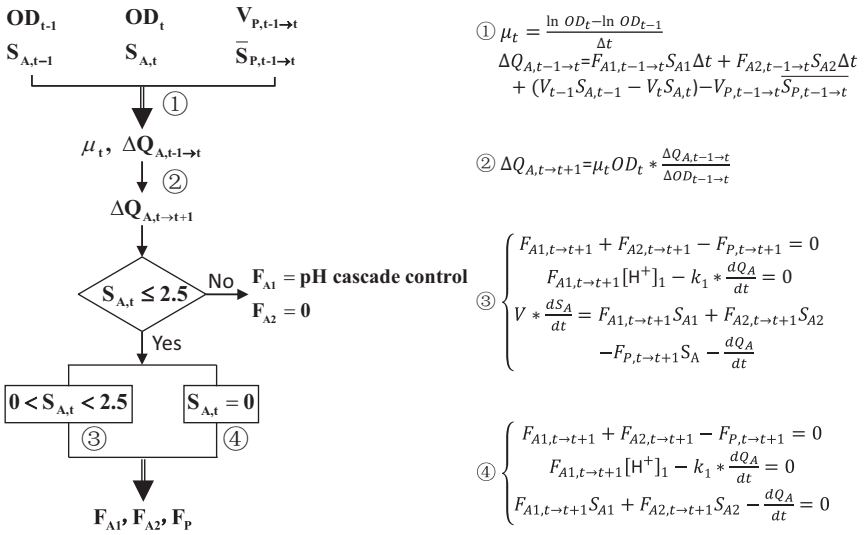

Fig. S3. Model 1 for the joint feeding of 3% acetic acid and sodium acetate based on off-line measurements. Variables used: F_{A1}, acetic acid feed rate (mL/h); F_{A2}, sodium acetate feed rate (mL/h); F_P, permeate flowrate (mL/h); $[H^+]_1$, proton concentration in the 3% acetic acid feed (measured by pH); k_1, coefficient obtained from regression; OD, optical density measured at 600 nm; Q_A, molar amount of acetate consumption (mmol); S_A, acetate concentration in the reactor (mol/L); S_{A1}, acetic acid feed concentration (0.5 mol/L); S_{A2}, sodium acetate feed concentration (0.5 mol/L); S_P, average acetate concentration in the permeate (mol/L); t, discrete time points of sampling; μ, specific growth rate (h^{-1}); V, working volume (mL); V_P, permeate volume (mL). For additional details, see *Notes for Model 1*.

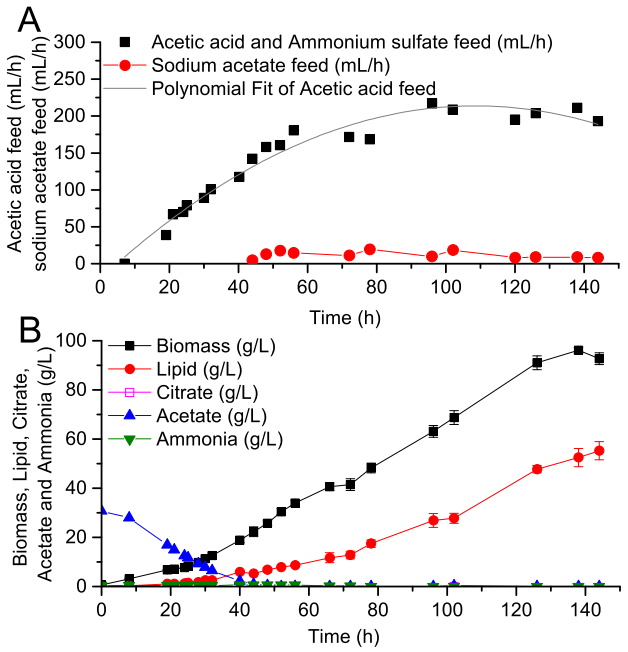

Fig. S4. Lipid production using a carbon-restrained mode by applying model 1. (*A*) Time courses for acetic acid, ammonium sulfate, and sodium acetate feed rates. Nitrogen was fed along with acetic acid. For the feed stream of acetic acid and ammonium sulfate, in the first 72 h, it consisted of 3% acetic acid and 5 g/L ammonium sulfate (C/N ratio = 14) to provide sufficient nitrogen for cell growth. Thereafter, the feed consisted of 3% acetic acid and 0.65 g/L ammonium sulfate (C/N ratio = 102). (*B*) Time courses for biomass and lipid production.

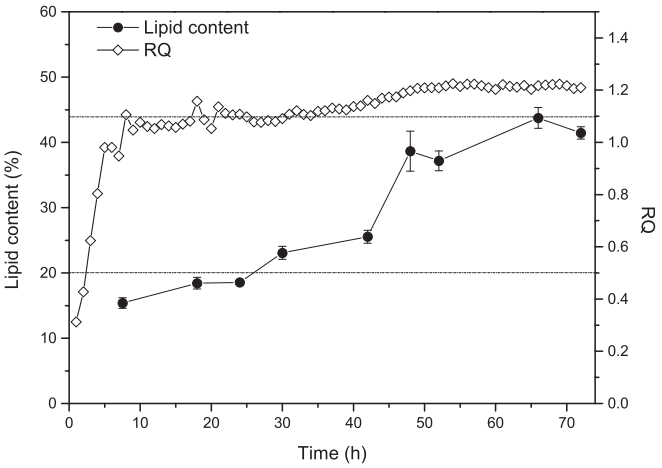

Fig. S5. Time courses for RQ and lipid content.