Optimizing induction conditions for production of biodiesel in A2A *E.coli* strain

Masha Alekseeva, Sosie Yorki, Olayemi Ajao

Biodiesel is a sustainable transportation fuel composed of fatty acid alkyl esters that are industrially produced from plant or animal oils in an economically inefficient process[1]. A promising alternative for scaling up biodiesel production is microbial fermentation, however, the yields of currently existing technologies are not sufficient for use in the industry[2]. Screening culturing conditions is a potential approach to address these yield shortcomings. Here, we developed a reproducible protocol for fatty acid ethyl ester (FAEE) extraction from bacterial cultures to ensure consistency of the separation step in our production process. We then tested the effects of culturing conditions such as cell density at induction and inducer molecule concentration in order to maximize FAEE yield from fermentation of recombinant *E.coli.* Optimal culturing process conditions are an OD_{600} value of 0.4 at induction, an IPTG concentration of 0.1mM, at a culturing temperature of 25°C after induction and a 26-hour culturing time. Although we did not improve upon the yield reported for A2A strain in the existing literature, we saw a 4-fold improvement in the specific yield upon induction at lower OD_{600} and an almost a two-fold increase in the total yield for cultures induced with lower concentration of IPTG. Our results provide a promising foundation for standardizing induction conditions in the studies aiming at further optimization and scale-up of FAEE production in recombinant *E.coli*.

Production of sustainable and economic fuels is a priority for the scientific community in order to reduce global fossil fuel dependence and slow down the climate change[1]. One of the most appealing renewable transport fuel alternatives is biodiesel, a biofuel composed of fatty acid alkyl esters. The main large-scale production method is a transesterification reaction of vegetable oils or animal fats with short-chain alcohols such as methanol, ethanol or propanol. However, the biomass processing to obtain the feedstock oil is energy intensive and costly, limiting the scale at which biodiesel can be produced[1]. To overcome current challenges in biodiesel manufacturing, genetic engineering of microbial strains and fermentation can be used for development of an easily scalable and economically viable production method[2,3]. Previously, Steen et al. demonstrated a method of engineering *E. coli* to produce FAEEs, or biodiesel, directly from low-cost glucose and xylan feedstocks[3]. A2A bacterial strain capable of FAEE production is engineered to express genes from fatty acid and ethanol biosynthetic pathways simultaneously, taking advantage of the microbial catalysis simplicity. However, a reported value of 9.4% of theoretical product yield is still too low for the industrial application of this bioprocess[4].

To improve the FAEE yield, it is necessary to understand which conditions in the fermentation and experimental setup significantly affect yield. We tested different culturing conditions to characterize their effects on the FAEE production in A2A *E.coli* strain. Our results suggest significant impact of the OD_{600nm} value at induction as well as IPTG concentration on the FAEE yield, with both variables being inversely correlated with the yield.

The biodiesel production process requires the use of reliable and consistent product separation and quantification methods to avoid error and product loss. Thus, we also attempted to develop a reproducible FAEE extraction and quantification protocol. Existence of such standardized protocol would aid in any future studies on FAEE producing bacterial cultures. After examining methods used in previous studies[2,3,5], we designed and tested the first protocol for the extraction using ethyl acetate as the hydrophobic solvent and gas chromatography-mass spectrometry (GCMS) for FAEE identification and quantification. The data gathered using the initial protocol showed large relative standard deviation (RSD) above 30% for both induced and uninduced cultures suggesting an error during the extraction or quantification step of the process (Supplementary Fig. 1). Additionally, due to chosen dilution regimes, some of the samples resulted in very noisy total ion chromatograms with no distinct peaks that could be matched to FAEE standards, which could not be used for reliable quantification (Supplementary Fig. 2).

The changes introduced for the second iteration of the extraction protocol worked to confirm the success of induction as well as the separations step of our process (Supplementary Fig.1). We chose different dilution concentrations in order for predicted FAEE yield to fall within the instrumental detection limit. We also reduced the duration and intensity for vortexing and centrifugation steps of the protocol, hypothesizing that longer times would contribute to higher mixing between FAEEs and other lipid compounds present in the sample which would result in a substantial number of split peaks. Implementation of the changes resulted in significant statistical differences being observed between induced and uninduced culture samples, with respective RSD of 9.3 and 10.8% for technical replicates. Multiple experimental data obtained using the final protocol showed similar or lower RSD for technical replicates (Supplementary Table 2), which suggests that our protocol outlines an efficient and reproducible method for FAEE extraction from bacterial cells.

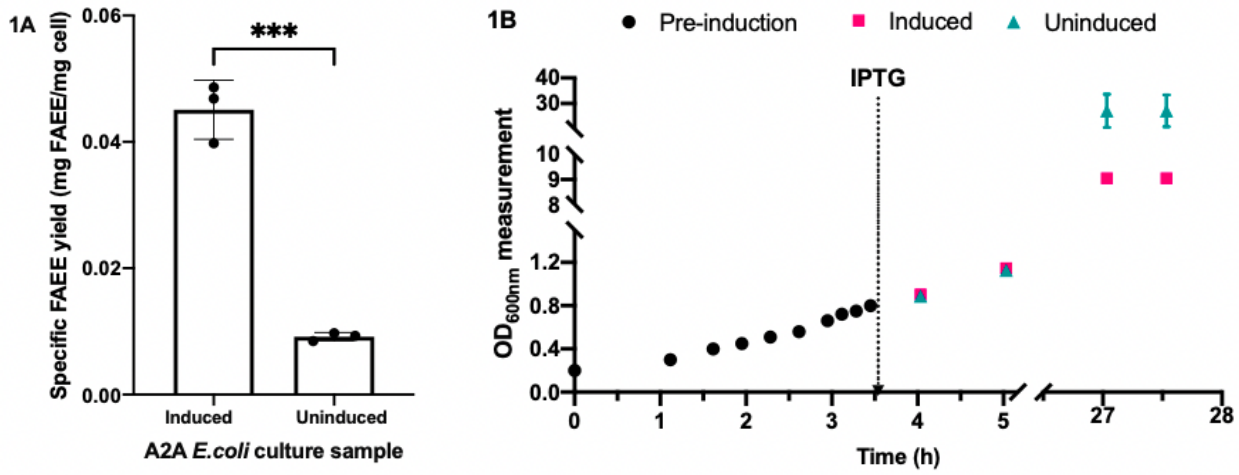


Figure 1. Induction with IPTG increases FAEE yield between induced and uninduced cultures and does not affect growth shortly after induction. A) Bacteria were grown together at 37°C pre-induction and split at OD_{600nm}=0.8 into biological triplicates for each type of culture. Subsequently, all samples were incubated at 25 °C with shaking. Values and error bars represent data points and standard deviation. Asterisks denote the significant differences between indicated samples using a two-tailed, equal variance t test *(*P<0.05, **P<0.01, ***P<0.001*). B) Three biological replicates were split into induced and uninduced cultures at the time indicated by dashed line. X-axis right segment shows data from 24 hours after induction. Data points represent average OD value, error bars represent standard deviation.

Expression of FAEE-synthetic pathways in the recombinant A2A *E. coli* was engineered to be under the transcriptional control of the *lacUV5* promoter, inducible by addition of isopropyl-β-D-1-thiogalactopyranoside (IPTG)[3]. The *lac* promoter is one of the most common and well-characterized methods to overproduce foreign proteins in bacteria[6]. Total product yield (g product/L) in such system would depend on the specific cell yield (g product/g cell) as well as the cell density (g cell/L). High levels of gene expression and related protein synthesis divert metabolic and energy resources from cellular growth, reducing the cell yield. In order to prevent cell growth inhibition by the high levels of product biosynthesis, it is important to add the inducer after obtaining a high concentration of cells in the culture[7].

We used spectrophotometry to measure cell density before and after the induction to monitor bacterial culture growth and investigate how it would affect the final amount of biodiesel product. The effect of IPTG addition on FAEE production and growth is shown in Figure 1. There is an observable four-fold increase in FAEE specific yield upon induction, confirming that the bacterial culture expresses necessary engineered pathways. However, the observed average post-induction FAEE titer of 97.9 mg/L culture was around 4 times smaller than the value reported in the literature for this strain under similar culturing conditions[3].

Logistic curves with transformed growth data were used to obtain growth rate and doubling times for pre- and post-induction culture samples in the exponential growth phase. An average growth rate constant for induced cultures was 0.096 hr^{-1} with an average doubling time equal to 7.2 hours, whereas the values for uninduced cultures were 0.14 hr^{-1} and 4.8 hours respectively (Supplementary Table 1). The calculated growth constants as well as the growth curve (Fig.1B) show that the growth slows down at long times after induction, which is consistent with our expectations for this process as we would want the majority of cellular resources to be diverted to FAEE biosynthesis rather than bacterial colony growth.

To further increase FAEE production and investigate the effects of induction variables on the product yield, we tested three different IPTG concentrations (low - 0.01 mM, medium - 0.1 mM, and high - 1mM). Results of the experiment with variable IPTG concentration display a clear trend between FAEE production and inducer quantity (Fig.2).

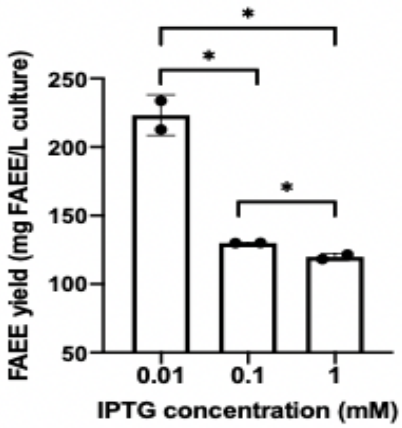


Figure 2. Increased FAEE production at lower IPTG concentrations. Bacterial cultures were grown together at 37°C, split at OD of 0.8 into biological duplicates and induced with variable IPTG concentration. Asterisks denote the significant differences between indicated samples using a two-tailed, equal variance *t* test *(*P<0.05, **P<0.01*). Bars show mean value, error bars show standard deviation.

Our data strongly suggests an optimal IPTG concentration of 10μM compared to higher concentrations for achieving a maximized FAEE yield with almost a two-fold yield increase. Moreover, these results are consistent with observations previously reported in the literature for recombinant bacteria induced with variable IPTG amount[5-7]. Medium or high IPTG concentrations direct more cellular resources towards transcription and protein expression than to FAEE production resulting in potential overwhelming of biosynthetic machinery, protein misfolding, cell deaths, and lower biodiesel yield as a consequence.

While variable concentration of IPTG would allow some control over the metabolic load imposed on the cell by foreign gene expression, the product yield also depends on the induction time during the bacterial growth cycle[6,7]. In general, if the cellular growth and viability decreases post-induction, it would be advantageous to induce in late-exponential or stationary phase[6]. However, optimal induction time can vary based on the limiting factor for FAEE production pathways. We tested several induction OD_{600nm} values and compared their effects on the specific FAEE yield as well as bacterial growth parameters (Fig 3. and Table 1).

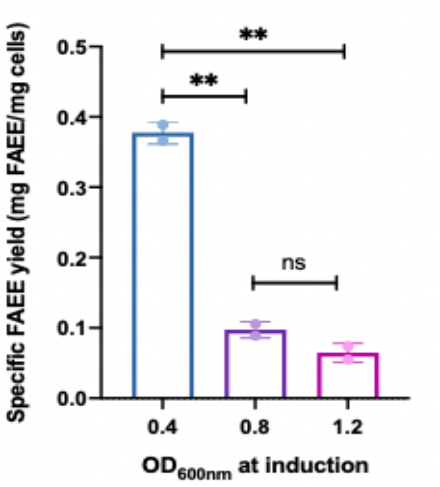


Figure 3. Specific FAEE yield is higher at lower induction cell densities. Data shown includes averaged results from an experiment with biological duplicates induced at each of the three OD values. All cultures were grown at 37ºC pre-induction, split and induced with IPTG upon reaching the target OD, and incubated for 24 hours after induction at 25ºC with shaking. Asterisks denote the significant differences between indicated samples using a two-tailed, equal variance *t* test *(*P<0.05, ns P>0.05*). Bars represent the mean value, error bars show standard deviation.

Table 1. Growth rate parameters for cultures induced at different cell densities.

	Average Growth Rate (hr^{-1})	Average Doubling Time (hr)
Pre-induction cultures	0.403	1.728
Post-induction @ OD 0.4	0.159	4.365
Post-induction @ OD 0.8	0.185	3.756
Post-induction @ OD 1.2	0.14	4.974

(No statistical differences between growth rates for these cultures were found using unpaired, two-tailed *t* test)

The trends we observed were contradictory to our initial hypothesis that a higher cellular density at induction would result in a higher biodiesel yield due to a larger number of cells simultaneously producing FAEEs. Our data shows the cultures induced at lower ODs having higher total and specific FAEE yields. Samples induced at OD of 0.4 had the highest average titer equal to 223 mg FAEE/L culture, which is 3.2% of theoretical yield compared to only 1.7-1.8% yield for cultures induced at other ODs. Our data suggests that FAEE production might be limited by the availability of nutrients in the medium as well as cellular growth rates in the stationary phase. Since more cells would enter stationary phase when induction happens at higher ODs, their growth and biosynthetic activity might be lower as a result of nutrient depletion and natural decrease of bacterial metabolic rates as the culture nears the death phase of growth cycle[6]. Thus, upon induction, these cell cultures would exhibit lower specific and total yields compared to cultures induced at lower ODs (in the mid-exponential phase), since those cultures would have an optimal balance between growth and metabolic activity as they enter stationary phase.

This work contributes to the development of an economically viable method for FAEE production in A2A *E.coli* strain. We designed a reproducible FAEE extraction and quantification method that results in consistent product recovery and low coefficient of variation for the obtained data. This standardized protocol was used in experiments with variable induction conditions to characterize IPTG and induction OD_{600} (cell density) effects on FAEE yield and bacterial growth. As expected, IPTG concentration increased and correlated inversely with the FAEE yield. A similar trend was observed in an experiment with variable induction OD. Our findings indicate that specific FAEE yield increases with decreasing induction cell density as measured by OD_{600nm}. These results will be valuable for experimental setup design in future microbial biofuel production research as well as development of industrial scale-ups. Future extensions of this work could include more replicates for experiments with variable induction cell density or IPTG concentration. Another valuable research path would be to manipulate both variables in tandem and observe the changes in FAEE yield.

Supplementary Information

References

1. Hill, J., Nelson, E., Tilman, D., Polasky, S. & Tiffany, D. Environmental, economic, and energetic costs and benefits of biodiesel and ethanol biofuels. *Proc. Natl Acad. Sci*. *USA* **103**,11206–11210 (2006).
2. Duan Y, Zhu Z, Cai K, Tan X, Lu X. *De novo* Biosynthesis of Biodiesel by *Escherichia coli* in Optimized Fed-Batch Cultivation. PLoS ONE 6(5): e20265. (2011).
3. Steen, E., Kang, Y., Bokinsky, G. *et al.* Microbial production of fatty-acid-derived fuels and chemicals from plant biomass. *Nature* **463,** 559–562. (2010).
4. Huffer S., Roche C.M., Blanch H.W., Clark D.S. Escherichia coli for biofuel production: bridging the gap from promise to practice. *Trends in Biotechnology,* 30(10), 538-545. (2012).
5. Steen, E. Constructing and engineering fatty acid metabolic pathways for the production of fuels and chemicals. PhD Dissertation, University of California, Berkeley. (2010).
6. Donovan, R. S., Robinson, C. W., & Glick, B. R. Review: Optimizing inducer and culture conditions for expression of foreign proteins under the control of the lac promoter. *Journal of Industrial Microbiology and Biotechnology*, *16*(3), 145–154. (1996).
7. Sivashanmugam, A., Murray, V., Cui, C., Zhang, Y., Wang, J., & Li, Q. Practical protocols for production of very high yields of recombinant proteins using Escherichia coli. *Protein science : a publication of the Protein Society*, *18*(5), 936–948. (2009).
8. Huang, W., Zhang, Y. Analysis of biofuels production from sugar based on three criteria: Thermodynamics, bioenergetics, and product separation. *Energy & Environmental Science* 4, 784-792 (2010).
9. ECMDB statistics. (n.d.). Retrieved February 28, 2021, from https://ecmdb.ca/e_coli_stats.
10. Volkmer B, Heinemann M. Condition-dependent cell volume and concentration of Escherichia coli to facilitate data conversion for systems biology modeling. *PLoS One* ;6(7):e23126. doi:10.1371/journal.pone.0023126. (2011).

Methods

FAEE extraction

Homogenization of *the E. coli* induced and uninduced cultures was performed by thawing the cell cultures (for X minutes) and mixing induced and uninduced culture triplicates into two 15mL culture samples respectively. Then, the combined induced and uninduced cultures were split into six 5mL aliquots, three induced and three uninduced, to control for variations between cultures. A 1:1 v/v ratio of EtOAc:culture was added to the aliquots. Each aliquot was vortexed at the 8 setting on the Fisher Scientific Vortex Mixer for 15 seconds in an upright position, alternating between vortexing induced and uninduced cultures. The aliquots were then centrifuged (Thermo Scientific Sorvall Legend XTR) at 4000 x g for 5 minutes. The aqueous bottom layer was discarded and the top ethyl acetate layer was filtered through a glass pipette containing cotton overlaid with sodium sulfate to remove any remaining water. 1 mL of the dried ethyl acetate layer was placed in a GCMS vial for FAEE quantification and analysis using an Agilent 7890A machine.

Quantitative analysis with gas chromatography-mass spectrometry

The GCMS ramp was as follows: an initial temperature of 70°C was held for 1 minute, then the temperature increased to 300°C at a rate of 25°C/min and was held at 300°C for 1 minute. The carrier gas used was He with an injection volume of 1 µL, flow rate of 0.5 mL/min, velocity of 16.4 cm/s, and pressure of 8 psi. The column was a 30 m x 250 µm x 0.25 µm HP-5ms Ultra Inert. For the MS, a split ratio of 1:10 and split flow of 10.048 mL/min was used. The split injection port was held at 250°C. The peak area quantification was performed with Agilent Mass Hunter Quantitative Analysis software. Standard curves for finding concentration were created using Sigma Aldrich C4-C24 Saturated FAEEs standard and allowed for reliable quantification in the range from 0-45 mg FAEE/L culture. Peaks that corresponded to saturated FAEEs were chosen based on retention time and mass spectrum correspondence between the standard and the sample. The common peaks for quantification were for ethyl dodecanoate, ethyl myristate, and ethyl palmitate. Unsaturated FAEEs were not quantified due to the absence of necessary standard curves.

Bacterial Strain Used

E. coli A2A strain was engineered via overexpression of thioesterase (‘tesA), pyruvate decarboxylase (pdc), alcohol dehydrogenase (adhB), FadD enzyme (fadD (M335I)), two copies of wax ester synthase (atfA) and knockout of FadE enzyme (ΔfadE)[4].

Culturing and induction protocol

The recombinant bacteria *E. coli* A2A were plated on LB-agar plates with ampicillin (Amp), tetracycline (Tet), and chloramphenicol (Cam) and incubated at 37ºC for 24 hours. Single colonies were inoculated in LB media with Amp, Tet, Cam and incubated at 37ºC for 8-9 hours with agitation. Starter LB culture was diluted 50x into M9, supplemented with 2% glucose, 1% casamino acids, Amp, Tet, and Cam, and incubated at 37ºC with agitation. Starter M9 culture was further diluted 50X into M9 supplemented as mentioned above and incubated at 37ºC with agitation with samples taken every hour to measure optical density (OD_{600nm}) until it reached a target value of 0.8 after around 4 hours. Then, 25μL of 1M IPTG was added to one set of cultures to induce FAEE production. Both induced and uninduced cultures were incubated at 25ºC for 24 hours after induction. Final cell density measurements were taken 24-26 hours after induction.

Testing variable IPTG concentrations

Bacterial cultures were started as described in the above protocol and shaken at 225rpm, 37ºC with samples taken every 20 minutes to measure optical density until it reached 0.8. Solutions of target IPTG concentrations were prepared as follows: to make 100mM IPTG stock, 30μL of 1M IPTG solution was mixed with 270μL of water; to make 10 mM IPTG stock, 30μL of 100mM IPTG stock was mixed with 270μL of water. For induction, the cultures were split into three sets of biological duplicates and 25μL of 1M, 100mM, and 10mM IPTG solutions were added to the cultures. Induced cultures were transferred to 225 rpm shaker at 25ºC. Optical density measurements were taken every 30 minutes for the first 3-4 hours after induction. Final optical density measurements were taken 24 hours after induction before FAEE extraction using the protocol above.

Testing variable induction OD_{600nm}

Cultures were started as described earlier and placed in a shaker at 37ºC with optical density measurements taken every 20 minutes. Once the OD_{600nm} value reaches 0.4, the culture was split into three flasks with equal volume. One of the flasks was then split into two biological replicates, induced with 25μL of 1M IPTG solution, and placed in a shaker at 225 rpm, 25ºC. OD_{600nm} measurements were taken every 20 minutes for the two remaining uninduced flasks. Analogously, the cultures were induced and moved to 25ºC shaker upon reaching target values (OD_{600nm} of 0.8 and 1.2). 24 hours after induction for each type of culture, final optical density measurements were taken.

Calculations:

Calculation 1. Theoretical yield of FAEEs produced by E. coli cells.
Assume palmitate ethyl ester (PEE), a saturated C16 FAEE, can serve as an average for all saturated FAEEs in solution. Assume all glucose is used by *E. coli* cells and that all glucose goes to FAEE production. Approximation of molecular equation[8]:
4.5glucose + 16NAD+ + 14NADPH + 2ADP + 2Pi PEE + 16NADH + 14NADP+ + 9CO2 + 9H2O + 2 ATP
(1 mol PEE/4.5 mol glucose)*(1 mol glucose/180 g glucose)*(284.5 g PEE/mol PEE) = 0.35 g PEE/g glucose
Using 2% glucose in M9 media, where 2% is 2 g glucose/100 mL media
(0.35 g FAEE/g glucose)*(2 g glucose/100 mL)*(1000 mL/L) = 7 g FAEE/L cell culture
To find % theoretical yield, total FAEE yield (g FAEE/L culture) was divided by theoretical yield of 7 g FAEE/L Culture.

Calculation 2. Specific yield of FAEE, measured as mg FAEE/mg *E. coli* cells.
Calculations made assuming 8x108 cells/(mL1 OD600)[10] and an *E. coli* cell dry weight of 3x10-13 g cell/mol[9].
Sample calculation where x = mg FAEE/L:
(x mg FAEE/L)*(1/final OD600)*(1 L/1000 mL)*(OD600 1.0/8x108cellsmL)*(1/3x10-13gmol)*(1 g/1000 mg) = y mg FAEE/mg cell. We chose to use specific yield as out metric for productivity since it would allow us to directly compare the biosynthetic capabilities between cultures as it controls for variation in the cell culture size. Since all cultures were grown roughly the same time, it is not necessary to control for time in the calculation to compare different samples. Variation in specific yield would reflect the differences in the FAEE producing abilities of different samples.

Calculation 3. Specific growth rate and doubling time.
The specific growth rates (μ) were calculated using the cell mass balance equation for batch processes:

$$dX/dt = X\mu$$

Integrating from X_0 (initial cell concentration) to X and from $t = 0$ to t, it gives:

$$X = X_0 exp(\mu t)$$

$$ln(X/X_0) = \mu t.$$

Using this equation, and taking *X* as the absorbance measurements (O.D.) at 600 nm, linear adjustments were made to the plots during the exponential growth phase prior to induction and after IPTG addition. For post-induction calculations, data points at times soon (less than 30 minutes) after induction were excluded assuming induction and subsequent pathway expression would not happen for some time after inducer addition.
The specific growth rates were obtained from the linear fits with the correlation coefficient R^2>0.9.

Doubling time was found using $X = 2X_0$:

$$ln(2X_0/X_0) = \mu t_d$$
$$t_d = ln(2)/\mu = 0.693/\mu$$

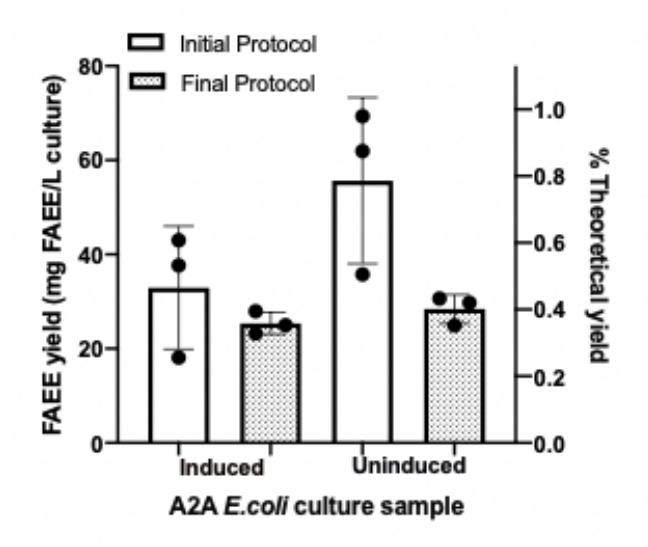
Calculation 4. Calculation of relative standard deviation (RSD).
Relative standard deviation, RSD, defined as:

$$RSD = S/x$$

RSDs were calculated using FAEE yield data. S represents the standard deviation of a sample. x represents a sample's mean concentration of FAEEs (mg FAEE/L culture).

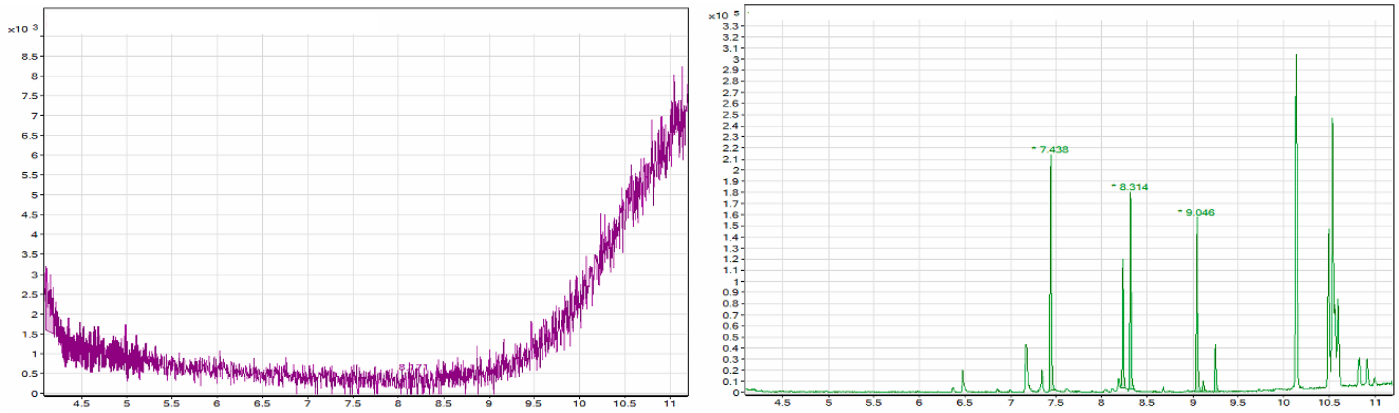
Supplementary Figures

Supplementary Figure 1. Comparison of initial and final extraction protocols shows lower relative standard deviation (RSD) for the final protocol.



Data shown are results from two experiments with technical triplicates for each type of culture. Bacterial cultures were grown at 37°C, split into induced and uninduced cultures upon IPTG addition, and placed into a 25°C shaker post-induction. The bars represent mean values, error bars show standard deviation. Two-sample, two-tailed *t* test p>0.05 for both protocols.

Supplementary Figure 2. Total Ion Chromatograms of FAEE extract samples with no dilution (green, on the left) and 50x dilution (purple, on the right). Data shown are results of GCMS sampling for two technical replicates of cultures induced with . All diluted samples in the experiment showed a lot of noise in the respective TiCs which hindered data analysis for these samples.



Supplementary Table 1. Growth rate parameters for induced and uninduced cultures show slowing down of bacterial growth upon induction with IPTG.

	Average Growth Rate (hr^{-1})	Average doubling time, t_d (hr)
Pre-induction	0.406	1.71
Induced cultures	0.0961	4.81
Uninduced cultures	0.144	7.21

Supplementary Table 2. Relative standard deviation (RSD) calculated using total yield measurements for technical replicates after FAEE extraction.

Experiment	RSD
Induction with 25μL of 1M IPTG at OD = 0.8	
• Induced cultures	0.1
• Uninduced cultures	0.18
Induction at variable OD_{600nm}	
• Induced at 0.4	0.066
• Induced at 0.8	0.019
• Induced at 1.2	0.02
Induced with variable IPTG	
• Induced with 0.01mM	0.09
• Induced with 0.1mM	0.021
• Induced with 1mM	0.02