Direct Bioconversion of Sorghum Extract Sugars to Free Fatty Acids Using Metabolically Engineered *Escherichia coli* Strains: Value Addition to the Sorghum Bioenergy Crop

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Abstract

The unprecedented challenge of meeting the energy demand of growing economies has increased the need to produce hydrocarbon fuels using renewable sources. Among different platforms being researched these days, hydrocarbon biofuel and chemical production from free fatty acids (FFAs) have gained a lot of attention. The current study reports on value addition to sorghum energy crop through utilization of sorghum extract as a renewable carbon source for FFA production using genetically engineered *Escherichia coli*. In order to reduce the production cost of FFAs, direct sucrose utilizing *E. coli* strains were developed. The *E. coli* strain MG1655 with *fadD* mutant (named as ML103) either carrying the plasmid bearing the gene for acyl-ACP thioesterase (TE) from *Ricinus communis* (pXZ18) or a plasmid bearing a combination of the TE and the native (3R)-hydroxyacyl-ACP dehydrase gene (*fabZ*) (pXZ18Z) was modified to utilize sucrose by incorporating the pUR400 plasmid. The newly created strains utilized a mixture of pure sugars and sorghum extract efficiently. The 24h pH adjusted culture of ML103 pXZ18Z pUR400 produced a maximum of $6.18 \pm 0.52 \text{ g l}^{-1}$ from 30 g l^{-1} of pure sugar mixture and $2.95 \pm 0.04 \text{ g l}^{-1}$ with sorghum extract (15 g l^{-1} equivalent sugar concentration.). These data suggest that modified *Escherichia coli* strains were capable of directly utilizing sucrose and produce FFAs from it. Successful demonstration of direct bioconversion of sorghum extract to FFA was validated as a possible value addition of the sorghum bioenergy crop.

Key words: Free fatty acid; *Escherichia coli*; sorghum extract; hydrocarbon fuel; value addition

Abbreviations

Acyl-ACP-thioesterase	TE
acyl-acyl carrier protein	ACP
(3R)-hydroxyacyl-ACP dehydrase gene (<i>fabZ</i>)	pXZ18Z
Department of Energy	DOE
tetradecanoic acid	C14
Unsaturated hexadecanoic acid	C16:1
hexadecanoic acid	C16
phosphotransferase system	PTS
(3R)- hydroxyacyl-ACP dehydrase	<i>fabZ</i>
Luria-Bertani	LB
isopropyl- β -D-thiogalactopyranoside	IPTG
Free fatty acid	FFA

1.0. Background

Concerns about petroleum resources, energy cost and environmental effect have attracted significant attention to the production of sustainable renewable fuels and chemicals. Major efforts to this end have been focused on the microbial production of high-energy density fuels by cost-effective ‘consolidated bioprocesses’ of renewable materials [1]. Several energy crops are currently cultivated to produce biofuels where their yields vary between species, site of growth, type and environmental factors. Value addition to the lignocellulosic energy crops that are harvested as aerial biomass for energy production through developing high value biofuels or chemicals is always an attractive option. For instance, energy crops that are high in non-structural sugars provide additional value if these sugars are converted to high value chemicals or biofuels. Energy sorghum and energycane are both crops that provide producers with this option. This approach will be a win-win situation for energy crop valuation as well as providing a low cost carbon source for biofuel production.

One approach to using these sugars is through the production of FFAs. FFAs are composed of long alkyl chains and have been regarded as nature’s ‘petroleum’ for both chemical and energy storage functions of microbial cells [1]. Importance of producing FFAs as feedstock for advanced biofuel and high value chemical production has been explored previously [2-5]. High value chemicals such as synthetic lubricants ($\sim \$3.33 \text{ kg}^{-1}$) [6], C12-C14 alcohols ($\sim \$5.5$ to 17.3 kg^{-1}), and methyl ketones ($\sim 18.9 \text{ kg}^{-1}$) [7], etc. can be derived from FFAs, hence it is practical to produce FFAs from lignocellulosic biomass as compared to alcohol fuels. Under normal conditions, the biosynthesis of FFA is highly regulated [8, 9]. Unlike most oleaginous

microorganisms or plants, which accumulate a significant quantity of FFAs naturally, *E. coli* does not accumulate FFAs as intermediates in lipid biosynthesis [10]. Wild type *E. coli* strains produce FFAs mainly for the biosynthesis of lipids and cell membranes. In the present study, we selected *E. coli* as the working organism because of its ability to produce FFAs at higher yields after genetic modification (more than 85% of theoretical maximum, about 0.29 g g⁻¹ of FFAs per gm glucose utilized) [11, 12]. As compared *E. coli* oleaginous yeasts produce mainly triacylglycerol with maximum yield about 39-45% of the theoretical maximum [13]. Furthermore, unlike yeast, FFA biosynthesis of *E. coli* is of type II system which allows easier manipulation of the acyl-ACP thioesterases to produce FFA of various length. In yeast due to complicated cellular metabolism in addition to its complex regulatory mechanisms and cellular compartmentalization, a bacterial platform becomes attractive for FFA production [14]. Though yeast typically have ability to utilize sucrose, their ability to convert carbon flux into FFA is limited [14], hence modification of *E. coli* for sucrose utilization is considered an attractive strategy.

Figure 1 shows the proposed FFA biosynthesis pathway together with a simplified central aerobic metabolic pathway of *E. coli* using sucrose as a carbon source. Many *E. coli* strains are not able to utilize sucrose due to lack of an invertase, hence these strains require partial breakdown of sucrose [15] or sucrose containing carbon sources such as molasses, sorghum extract etc. Earlier, Schimitz et al. [16] and Wang et al. [17] reported that *E. coli* can be able to catabolize sucrose with incorporation of series of genes including *scrK*, encoding an ATP-dependent fructokinase, *scrY* encoding a sucrose-specific porin of the outer membrane, *scrA*

encoding the enzyme *Ilscr* of the phosphotransferase system for sucrose uptake, and *scrB* encoding an intracellular β -D-fructofuranoside fructohydrolase, which cleaves sucrose 6-phosphate into β -D-fructose and α -D-glucose 6-phosphate. Also, FFAs can be produced by introducing an acyl-acyl carrier protein (ACP) thioesterase (TE) gene (see Figure 1, dotted box) into *E. coli* [18]. The presence of the acyl-ACP thioesterase breaks the FFA elongation cycle and releases FFAs [19-21]. Furthermore, it has been reported that FFA overproduction induces the FFA degradative pathways [10].

Indeed, the ability to engineer *E. coli* to overproduce FFAs has been demonstrated [1, 18]. Literature reports show that different feedstocks such as glucose [11, 18], glycerol [22], woody hydrolysate [23], and switch grass derived sugars [24] are being used for production of FFAs using engineered *E. coli*. However, further refinements are urgently needed for making FFA-derived biofuels or chemicals economical at large-scale. There are two effective ways to reduce the cost of large-scale FFA production, the one is to enhance the yield of the target products through optimizing culture conditions, and the other one is to grow the cell in a low cost medium. Since the use of inexpensive carbohydrates is an effective method among other possible alternatives to reduce the cost of fermentation process, we have explored the opportunity of utilizing sorghum extract as a carbon source.

The United States Department of Energy (DOE) has identified four herbaceous bioenergy crops (switchgrass (*Panicum virgatum*), miscanthus (*Miscanthus x giganteus*), sugarcane (*Saccharum* spp.), and sorghum (*Sorghum bicolor*) to meet biomass production demand if lignocellulosic biomass is used for conversion. Of these crops, energy sorghum is a

highly productive, annual, drought tolerant C4 grass with an excellent genetic (diploid, inbreeding) and genomics platform [25, 26]. Like miscanthus and switchgrass, energy cane and energy sorghum produce large quantities of structural carbohydrates but they are also high moisture crops that produce significant quantities of non-structural carbohydrates [27].

In terms of biomass yields, energy sorghum is competitive with other herbaceous energy crops. Owens et al. [28] reported average yields for switchgrass across four diverse environments ranging from 4.85 to 10.6 MT ha⁻¹. In comparable studies in different environments, Gill et al. [27] reported energy sorghum yields range from 14.35 MT ha⁻¹ to 24.25 MT ha⁻¹ for the energy sorghum hybrid ES5200. In both cases, variation in yield was primarily associated with available soil moisture. A primary difference between switchgrass and energy sorghum is moisture content at harvest. At harvest, energy sorghum ranged from 65.5 to 76.3% moisture content [27]. While higher moisture content is considered as a negative in some conversion systems, the moisture in energy sorghum contains non-structural carbohydrates that can be easily extracted much like energy cane. At the same time the extraction process reduces the moisture content allowing use of bagasse as dry product. The actual non-structural carbohydrate content varies widely in energy sorghum, ranging from 4.1 to 19.3% brix content across 35 environments [27]. Sucrose composes most of this carbohydrate but significant quantities of fructose and glucose are also present and vary based on genotype [29]. These components represent an additional processing stream for industrial crop use through the conversion of sugars to FFAs that are not available in non-sugar producing crops such as switchgrass or miscanthus.

In this study, detailed investigations were performed for FFA production using *E. coli* strains containing the pUR400 plasmid [17] known to utilize sucrose. Our first hypothesis of current study was incorporation of a sucrose metabolizing pathway in a FFA producing *E. coli* strain would improve the capability of recombinant strains to utilize different carbon streams. This would allow elimination of the sucrose hydrolysis unit operation (e.g. otherwise very common process in corn based biofuel production process). The elimination of the sucrose hydrolysis unit operation will reduce cost of overall sucrose utilizing processes considerably and will allow direct conversion of sugar cane juice, beet root juice, sweet sorghum extract etc. to various biofuels and bioproducts by sucrose metabolizing organisms. The second hypothesis of the current study was, pH manipulation of different FFA producing cultures would significantly improve the FFA production ability and reduce acetate formation. To test these two hypotheses, we have constructed sucrose metabolizing strains and these strains were evaluated for their ability to produce FFAs using purified sorghum extract as a carbon source under various conditions. Preliminary studies were performed using model sugars and sugar mixtures to analyze and compare the most suitable conditions for FFA production. The pH manipulation study was performed to test the overall effect on FFA production and acetate formation. Further, a pH-stat control strategy was assessed on FFA producing recombinant *E. coli* cultures at fermenter scale using model and sorghum extract sugars as carbon sources.

2.0. Materials and Methods

2.1. Sweet sorghum extract preparation and purification

Sweet sorghum [*Sorghum bicolor* (L.) Moench], was produced by the Sorghum Improvement Program, Texas A&M AgriLife Research at the AgriLife Research Farm near College Station, Texas. Sweet sorghum cultivar was harvested when each genotype was in the early hard-dough stage, which is typically the growth stage for peak sugar content and production. After the panicles and leaves were removed, stems were crushed using a small three roller sugarcane mill to provide extract (Crown® CR100A, Brazil). The collected extract was immediately stored on ice and transported to Rice University, Houston Texas for further processing. At Rice University, extract was centrifuged twice at $10,000 \times g$ for 20 minutes and the supernatant was passed twice through Whatman No. 1 filter paper. Obtained clarified extract was filter sterilized using 0.22 μm cellulose acetate filter. The sterile extract was stored at $-20^{\circ}C$ until used.

2.1. Strains and plasmids

A derivative of *Escherichia coli* MG1655, ML103 (MG1655 *fadD*⁻ mutant) [11], was used throughout in this study (Table 1). The plasmids, pXZ18, carrying an acyl-ACP thioesterase (TE) gene from *Ricinus communis* [18] and pXZ18Z [12], carrying the native (3R)-hydroxyacyl-ACP dehydrase (*fabZ*) gene downstream of the TE gene from *R. communis* [30], were used in this study. A compatible plasmid, pUR400 (this plasmid was originally extracted from *Salmonella typhimurium*) containing the *scrK*, *Y*, *A*, *B*, and *R* genes allowing cells to convert sucrose to beta-D-fructose and alpha-D-glucose 6-phosphate was kindly provided by Dr. Kurt Schmid from the Universität Osnabrück, Germany [16]. All plasmid constructs were confirmed through DNA sequencing (Lone Star Labs, TX). A list of the strains and plasmids

used in this study is shown in Table 1.

2.2. Conjugation protocol to obtain sucrose utilizing strain

Luria-Bertani (LB) media supplemented with appropriate antibiotics was used in the conjugation experiments. Conjugation experiments were conducted at 37 °C. The strains ML103 pXZ18 and ML103 pXZ18Z (the ML103pXZ18 and ML103pXZ18Z strains were prepared by transforming pXZ18 and pXZ18Z plasmids into the ML103 strain as reported in one of our earlier studies [12]) each contain the ampicillin resistance marker and *E. coli* K12 pUR400 containing tetracycline marker were grown in 20ml LB and incubated aerobically at 37 °C (250 RPM) to an OD₆₀₀ of 0.4~0.6. To ensure that none of the resistance markers had been lost, 100 µl of each strain was plated on LB agar plates containing the appropriate antibiotic (*E. coli* K12 pUR400: 35 µg ml⁻¹ tetracycline; ML103 pXZ18: 100 µg ml⁻¹ ampicillin) and incubated overnight at 37 °C. From the remaining culture, cells were mixed at a ratio of 1 mL *E. coli* K12 pUR400 (donor strain): 9 mL ML103 pXZ18 or ML103 pXZ18Z (recipient strains) and incubated for 3 h at 37 °C. The conjugation mixture (1 mL of each) was diluted in sterilized water and serial dilutions (10⁻¹, 10⁻², and 10⁻³) were made, then the dilutions (100 µl) were plated on LB agar plates containing 35 µg ml⁻¹ tetracycline and 100 µg ml⁻¹ ampicillin. All plates were incubated at 37 °C overnight. Single colonies were restreaked on plates containing the mixture of ampicillin (100 µg ml⁻¹) and tetracycline (35 µg ml⁻¹). These conjugated product strains were used for further studies.

2.3. Inoculum preparation

The primary preculture was prepared by inoculating a single colony from a freshly grown plate into 5 mL of LB medium (per liter: tryptone 10 g, yeast extract 5 g, sodium chloride 10 g) supplemented with 100 mg l⁻¹ ampicillin (Fisher BioTech) and 35 mg l⁻¹ tetracycline in an orbital shaker (New Brunswick Scientific, NJ) operated at 250 RPM and 37 °C. After 8 hours of incubation, the cultures from 5ml culture tubes were transferred to fresh LB medium in 250 ml Erlenmeyer flask containing 50 ml media supplemented with 100 mg l⁻¹ ampicillin (Fisher BioTech) and 35 mg l⁻¹ tetracycline in an orbital shaker (New Brunswick Scientific, NJ) operated at 250 RPM and 37 °C to produce enough seed for inoculation at 1% v v⁻¹ level.

2.4. Shake flask cultures

LB broth medium supplemented with 15 g l⁻¹ of carbon source (either individual or mixture of pure sugars or sorghum extract as stated) and an appropriate quantity of ampicillin and tetracycline was used for aerobic cultivations. The expression of the acyl-ACP thioesterase was induced by the addition of isopropyl- β -D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. The culture was inoculated into a 250 ml flask containing 50 ml of the culture medium using an inoculation size of 1% (v v⁻¹). The cells were then cultivated in an orbital shaker (New Brunswick Scientific, NJ) operated at 250 RPM and 30 °C. Samples were taken at 24h intervals up to complete utilization of sugars for FFAs and metabolite analysis. For screening important cultivation parameters, Placket-Burman design was employed (for details please refer to supplementary file 1). Shake flask experiments were performed without

adjusting pH of cultures to 7.0 after 24h, unless stated otherwise.

2.5. The pH-stat control strategy for converting pure sugar mixture and sorghum extract to FFA

The pH-stat control strategy was evaluated for examining the effect of dynamic pH control on FFA production and acetate formation by recombinant *E. coli* strains. For this study, the 0.6L of fermentation medium (LB medium, pH 7.5 before autoclaving) was supplemented with the pure sugar mixture or sorghum extract mainly containing sucrose, glucose and fructose as carbon source. The fermentation was carried out at a constant pH of 7.0 (after it dropped from pH 7.5 to pH 7.0 during fermentation) and media was supplemented with 100 mg l⁻¹ ampicillin and 35 mg l⁻¹ tetracycline. The expression or the co-expression of the TE and the native (3R)-hydroxyacyl-ACP dehydrase (*fabZ*) (pXZ18Z) was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1mM. The cells were cultivated in a NBS BioFlo 110 (New Brunswick Scientific, NJ) 1.2 L fermenter vessel and dissolved oxygen was maintained at 40% by cascade mode using 1 vvm air flow and varying agitation between 150 RPM to 450 RPM. Samples were taken periodically for FFA and metabolite analysis.

2.6. Analytical techniques

Optical density was measured at 600 nm with a spectrophotometer (Beckman Coulter DU 800 UV/Vis Spectrophotometer, Beckman Coulter, Inc., Fullerton, CA); the culture was diluted to the linear range with 0.15 M NaCl. Previously established HPLC methodology was used to

analyze the extracellular metabolites [31, 32]. Briefly, 1 ml of culture was centrifuged and the supernatant was then filtered through a 0.2- μm syringe filter for HPLC analysis. The HPLC system (Shimadzu-20A Systems, Shimadzu, Colombia, MD) used was equipped with a cation-exchange column (HPX-87H, BioRad Labs, CA), a UV detector (Shimadzu SPD-20A) and a differential refractive index (RI) detector (Shimadzu RID-20A). A mobile phase of 2.5 mM H_2SO_4 solution at a 0.5 ml min^{-1} flow rate was used and the temperature was operated at 30 °C. Standards were prepared for sucrose, glucose, fructose, succinate, acetate, ethanol, lactate and pyruvate for both the RI detector and UV detector, and calibration curves were created. Metabolites such as glucose, acetate, ethanol and lactate were measured by the RI detector and pyruvate was measured by the UV detector at 210 nm.

2.7. FFA analysis

Cell cultures were harvested and 1 ml of fermentation broth was utilized for FFA analysis, as described earlier [33]. Authentic standards were used to quantify individual FFAs including tridecanoic acid (C13) and pentadecanoic acid (C15) and were added as internal standards in all samples. The FFA content of each sample was quantified by GC with FID detector. The GC-FID system was equipped with auto-injector and single DB-5MS column (30mx0.25mmx0.25 μm , Agilent Co., USA) directly connected to the flame ionization detector (FID). Helium was used as the carrier gas and the flow rate was set at 1ml min^{-1} . The oven temperature was initially held at 50°C for 2 min. Thereafter, the temperature was raised with a gradient of 4°C min^{-1} until the temperature reached 220°C. This temperature was then held for

10 minutes.

3.0. Results and Discussion

3.1. Production of FFAs using ML103 pUR400 pXZ18 on sucrose, glucose and fructose at different starting pH

From the viewpoint of industrial FFA production, it would be advantageous to grow *E. coli* on low cost substrates such as sucrose and specifically those obtained from renewable resources. However, most *E. coli* strains are able to utilize glucose and fructose readily, but lack the ability to metabolize sucrose, as a carbon source for biomass and FFA production due to the lack of an invertase [15]. Schmid et al. [16] reported expression of the 70 kb pUR400 plasmid which encodes the *scrK*, *Y*, *A*, *B*, and *R* genes allows *E. coli* to utilize sucrose successfully. The *scrK* gene encodes an ATP-dependent fructokinase, the *scrY* gene encodes a sucrose-specific porin of the outer membrane, the *scrA* gene codes for enzyme II^{scr} of the phosphotransferase system for sucrose uptake, and the *scrB* gene encodes an intracellular β -D-fructofuranoside fructohydrolase which cleaves sucrose 6-phosphate into β -D-fructose and α -D-glucose 6-phosphate. The regulation of the operon is controlled by the negative regulator ScrR.

The ML103 pXZ18 strain has been studied in detail earlier in our laboratory [18, 34], and its derivative ML103 pXZ18 pUR400 was utilized for further investigation. Preliminary investigations used sucrose, glucose and fructose as a carbon source at pH 7.0 and pH 7.5. After introduction of the pUR400 plasmid into a FFA producing host, the strain was observed to utilize sucrose efficiently. Table 2 shows that such a strain grown on sucrose as a carbon source

could produce 1.41 g l⁻¹ (with 16.51 g l⁻¹ sucrose utilization) and 1.22 g l⁻¹ (with 14.87 g l⁻¹ sucrose utilization) of FFAs at pH 7.5 and pH 7.0, respectively. At both initial pH values of 7.0 and 7.5, the optical density measured at 600 nm showed almost equal growth. Acetate accumulation was also very similar reaching 1.72 g l⁻¹ (pH 7.0) to 1.77 (pH 7.5); this concentration of acetate was enough to inhibit cell growth and FFA formation [22]. Generally, FFA synthesis in modified *E. coli* strains is balanced and coordinated distribution of acetyl-Co-A among TCA cycle, acetate formation and FFA synthesis. According to Zhang et al. [18] fine-tuning of the overexpression of TE is critical for FFA production to avoid accumulation of acetate in the culture which could inhibit overexpression of TE. In addition, *fabZ* overexpression will further improve FFA production. The other organic acids, succinate, formate and fumarate were accumulated at very low concentrations (data not shown), and the assumption is that their concentrations would be too low to exert any negative effects on ML103 pXZ18 pUR400. With sucrose as the carbon source, after 48h fructose, up to 2.54 g l⁻¹ with pH 7.5 and 2.44 g l⁻¹ with pH 7.0 was accumulated at significant level. This could be due to the metabolic pattern of sucrose with pUR400 plasmid, as sucrose-6-phosphate was identified as the primary product of the phosphoenol pyruvate (PEP)-dependent sucrose transport [16]. Plasmid pUR400 mediates the “peripheral” metabolism of sucrose in *E. coli* K-12 by furnishing the sucrose-specific, membrane-bound compound EII^{SCR} of the PTS and the hydrolyzing enzyme sucrose-6-phosphate hydrolase. The host contributes the general PTS proteins, including EI and HPr, and the additional factor, EIII^{glu,scr}, which is required for the activity of the sucrose transport system. The primary intracellular product of sucrose-6-

phosphate hydrolysis is fructose which is converted to fructose-6-phosphate by an ATP-dependent kinase [35]. Results of FFA production using sucrose as a carbon source revealed that formation of acetate could be a major obstacle to utilize engineered strain ML103 pXZ18 pUR400 or similar strains under the tested conditions.

In order to reduce acetate accumulation, glucose and fructose were tested as a replacement for sucrose in fermentation medium. It was observed that ML103 pXZ18 pUR400 could produce approximately 2.0 g l^{-1} of FFAs at both pH 7.5 and pH 7.0 after 48h (Table 2). These results corroborated an earlier finding, where ML103 pXZ18 produced around 2.0 g l^{-1} of FFAs when grown on glucose as a carbon source [18]. The FFA composition observed was tetradecanoic acid (C14) (47% of total FFAs), unsaturated hexadecanoic acid (C16:1) (33% of total FFAs) and hexadecanoic acid C16 (18% of total FFAs) in decreasing order, which was marginally different from the composition of FFAs formed by ML103 pXZ18 [18]. It was observed that incorporation of plasmid pUR400 increased the percentage of C14. At the same time, with 15 g l^{-1} of fructose in medium, 1.77 g l^{-1} and 1.68 g l^{-1} of FFAs were formed at pH 7.5 and pH 7.0 respectively. The FFA composition data with fructose as carbon source showed a similar profile to that of cultures grown on glucose. Further data analysis showed that acetate accumulation was negligible with glucose and fructose as carbon source as compared to sucrose only (Table 2). A mixture of glucose and fructose could resolve the problem of acetate accumulation with the strain ML103 pXZ18 pUR400, and notably FFA production was in the following order glucose > fructose > sucrose.

As observed during the above performed studies, individual carbon sources were shown

to have a different effect on FFA production during ML103 pXZ18 pUR400 fermentation.

Considering the influence of carbon source, we have performed studies with different combinations using Plackett-Burman design (details are provided in the supplementary file 1).

Result of all models showed fructose, glucose, sucrose and IPTG positively affected FFA production, while temperature, the interaction between glucose and fructose, or the interaction between glucose and temperature resulted in negative effects on FFA production

(supplementary file 1). With insight from Plackett-Burman design results, further studies were performed with pure sugar mixture of sucrose (50% w v⁻¹), glucose (25% w v⁻¹), fructose (25% w v⁻¹) and sorghum extract (natural mixture of sucrose, glucose, and fructose) at starting pH of 7.5 before autoclaving using ML103 pXZ18 pUR400 and ML103 pXZ18Z pUR400 strains.

3.2. Effect of pH manipulation (without and with pH adjustment to 7.5 after 24h of fermentation) on FFA production using pure sugar mixture and sorghum extract as a carbon source

Freshly transformed strains ML103 pXZ18 pUR400 and ML103 pXZ18Z pUR400 were tested for their ability to produce FFAs using a pure sugar mixture of sucrose, glucose and fructose at 15 and 30 g l⁻¹ and sorghum extract (15 g l⁻¹ total sugar equivalent) with and without pH manipulation. Figure 2a (data with dotted lines) shows that when ML103 pXZ18 pUR400 was grown on 15 and 30 g l⁻¹ pure sugar mixture it accumulated 2.33 and 2.86 g l⁻¹ of FFAs without pH manipulation. During this fermentation, acetate accumulation was negligible with 15 g l⁻¹, while about 2.50 g l⁻¹ was observed with 30 g l⁻¹ total sugar concentration (Figure 2b). We

observed that, at lower initial sugar concentration (15 g l^{-1}), the strain ML103 pXZ18 pUR400 efficiently utilized most of the sugar within 72 h (Figure 2c), but at higher initial sugar concentration (30 g l^{-1}), significant quantities of sucrose and fructose remained unutilized even at 96 h (Figure 2c). The optical density at 600 nm reached up to 5.44 and 11.68, with 15 g l^{-1} and 30 g l^{-1} sugar concentrations respectively (Figure 2c). The fermentation experiments performed using sorghum extract as a carbon source with ML103 pXZ18 pUR400 showed minimal FFA production (1.18 g l^{-1}), while considerable acetate and biomass accumulation was observed at level of 7.16 g l^{-1} (Figure 2b) and more than 10 OD, respectively. The low concentration of FFA observed could be due to high acetate accumulation during the fermentation period. Zhang et al., [18] earlier showed that strains accumulating a high concentration of acetate produced a very low concentration of FFAs. This could be due to diverting a portion of the strong pool of acetyl-Co-A to acetate instead of FFA production. Another report explaining manipulation of acetate pathways showed that if the acetyl-Co-A pool is being diverted to a particular product then it is possible to reduce acetate accumulation [36].

Instead of manipulating the acetate pathway, we thoroughly investigated FFA production fermentation process by monitoring pH change dynamically using an optical pH sensor (please refer to Figure S3 in supplementary file). We observed that, during exponential phase pH dropped to about pH 6.0 within 7 hours of fermentation. In the case of low initial sugar concentration (15 g l^{-1}) cultures accumulated a considerably lower concentration of acetate as discussed earlier (Figure 2b) and could be able to recover from the pH drop and subsequently

attain a pH value of more than pH 7.5 (data not shown). But in case of 30 g l⁻¹ initial sugar concentration and sorghum extraction fermentation, cultures were not able to recover completely and ended up producing more acetate and low FFA. This necessitated a change in the fermentation protocol. In the modified experiments, the culture pH was adjusted to 7.0 after 24h of incubation by manually adding sterile 1.5 N NaOH under aseptic conditions. Figures 2a (data with solid lines) illustrate the advantage of pH manipulation after 24h with pure sugar mixture and sorghum extract as carbon source. As seen in Figure 3a the total FFA production increased considerably from 2.33 g l⁻¹ to 2.50 g l⁻¹ with ML103 pXZ18 pUR400 at 15 g l⁻¹ pure sugar concentration. With the same strain, when fermentation was carried out with 30 g l⁻¹ and pH adjustment to 7.0 after 24h, FFA concentration increased 2 fold (Figure 2a, solid lines with filled triangular marker) as compared to a culture without pH adjustment (Figure 2a, dotted line with empty triangular marker). Similarly, when the experiments were performed using sorghum extract as carbon source (15 g l⁻¹ sugar concentration) and pH adjustment to 7.0 after 24h, results showed a two-fold increase in FFA production (2.92 g l⁻¹ FFA production as opposed to 1.18 g l⁻¹ FFA production when fermentation was performed without pH adjustment) with ML103 pXZ18 pUR400 culture. The 96h sugar utilization data (Figure 2d) with ML103 pXZ18 pUR400 showed significant improvement in utilization of most of the sugar components from the pure sugar mixture as well as with the sorghum extract after pH adjustment to 7.0 after 24h fermentation as compared to experiments performed without adjusting the pH (Figure 2c).

Like ML103 pXZ18 pUR400, we have also tested performance of the improved FFA producing strain ML103 pXZ18Z pUR400 by both strategies (with and without pH adjustment

after 24h of fermentation). The strain ML103 pXZ18Z pUR400 when grown on 15 and 30 g l⁻¹ sugar mixture, produced 3.10 and 3.23 g l⁻¹ FFAs (without pH manipulation), which is considerably higher than that of ML103 pXZ18 pUR400 strain (Figure 3a, dotted lines). Further, sorghum extract fermentation (without pH manipulation) of strain ML103 pXZ18Z pUR400 showed negligible (0.11 g l⁻¹) FFA production (Figure 3a, dotted line with empty square marker) and significant acetate accumulation (5.7 g l⁻¹) (Figure 3b, dotted line with empty square marker). In addition, similar to the ML103 pXZ18 pUR400 strain, ML103 pXZ18Z pUR400 also produced 2.20 g l⁻¹ of acetate with 30 g l⁻¹ of sugar concentration (Figure 3b, dotted line with empty triangular marker). After, pH manipulation, a similar trend was observed as that of the ML103 pXZ18 pUR400 strain, in that strain ML103 pXZ18Z pUR400 showed significant improvement in FFA accumulation (3.24 and 6.17 g l⁻¹ with 15 and 30 g l⁻¹ pure sugars, respectively, Figure 3a). This significant increase in FFA production could be due to improved efficiency of incorporated native (3R)-hydroxyacyl-ACP dehydrase (*fabZ*) in ML103 pXZ18Z pUR400 as compared to thioesterase (TE) present in ML103 pXZ18 pUR400 after pH adjustment. Also, as expected, the sorghum extract fermentation of strain ML103 pXZ18Z pUR400, produced about 2.95 g l⁻¹ of FFA as compared to 0.11 g l⁻¹ observed during the culture without pH adjustment (Figure 3a). As seen in Figure 3b, all the cultures with pH adjustment after 24h, produced negligible or no acetate. In contradiction to the ML103 pXZ18 pUR400 sugar utilization profile, we observed ML103 pXZ18Z pUR400 showed substantial concentration of residual sugars (mainly sucrose and fructose) in all cultures without pH manipulation (Figure 3c). Similar to the ML103 pXZ18 pUR400 culture, sugar utilization

improved significantly for the cultures of ML103 pXZ18Z pUR400 with pH manipulation (Figure 3d). The FFA production results obtained in the current study after pH manipulation are comparable to the earlier findings where studies were performed using glucose as sole carbon source [30]. With all the pH adjustment cultures, optical density measurement at 600 nm showed significant increases, reaching a maximum up to 17.09 in some cases (Figure 3d).

The FFA composition of both strains ML103 pXZ18Z pUR400 and ML103 pXZ18Z pUR400 with all tested conditions shows C14 and C16 (including saturated and unsaturated) as the major components of FFAs (Table 3). With pH manipulation, the C14 FFA concentration reached up to 0.88 g l⁻¹ and 1.07 g l⁻¹ with 15 g l⁻¹ and 30 g l⁻¹ pure sugar mixture, respectively for the ML103 pXZ18Z pUR400 culture. Under similar conditions, C14 FFAs produced by the strain ML103 pXZ18Z pUR400 was 1.21 g l⁻¹ and 1.60 g l⁻¹, and this showed a significant increase in the concentration of tetradecanoic acid as compared to ML103 pXZ18Z pUR400. The C16 FFA produced by both strains showed lower accumulation than that of C14 FFA in all the tested experimental conditions (Table 3). Earlier the same trend was observed by Wu et al. [23] when FFAs were produced using wood hydrolysate with strains ML190 pXZ18Z and ML103 pXZ18Z. Similarly, with sorghum extract as the carbon source, both strains ML103 pXZ18Z pUR400 and ML103 pXZ18Z pUR400 showed C14 FFA composition to be higher than that of C16 but lower than C16:1 (Table 3). The C18:1 FFA concentration was low as expected with both strains and under all conditions tested (Table 3). An observation of lower accumulation of C18:1 with all cultures is in agreement with earlier studies [18, 22, 34].

3.2. Effect of pH-stat control strategy on FFA production in bioreactor

The pH manipulation results obtained in section 3.2 motivated us to perform bioreactor studies with a pH-stat control strategy. The metabolically engineered *E. coli* strains were evaluated for FFA production using a pure sugar mixture and sorghum extract at bioreactor scale with strict pH control at 7.0. Figure 4 (a) shows the details of pure sugar mixture fermentation (at 15 g l⁻¹ starting sugar concentration) using strain ML103 pXZ18 pUR400. It was observed that maximum FFA accumulation reached up to 1.54 g l⁻¹ after 35h of fermentation, thereafter no significant increase in FFA production was observed. The FFA accumulation observed at bioreactor level with a culture of ML103 pXZ18 pUR400 was considerably lower than FFA production (2.5 g l⁻¹) observed with same culture and sugar concentration in shake flask experiments under pH manipulation strategy (Figure 2a). In addition, the concentration of FFA accumulated (around 2.0 g l⁻¹) by the parent strain ML103 pXZ18 using glucose as carbon source [18] is higher than present study (Figure 4a). Though sugars (sucrose and fructose) were available for the culture after 35h of fermentation, the strain was not able to convert residual sugars into FFAs. The optical density at 600 nm showed that, after 35h residual sugars were not converted into FFAs, however microbial growth kept increasing gradually. This could be due to, at a constant pH of 7.0 and high dissolved oxygen concentration, the microorganisms utilized sugars for supporting growth rather than FFAs formation. It was observed the pH-stat control strategy could have helped to reduce acetate, as a noticeably lower concentration of acetate was observed during growth phase (up to around 10h of incubation) and after that, microorganisms started utilizing it. A similar trend was observed when ML103 pXZ18 pUR400 was grown on

sorghum extract as carbon source in the bioreactor with the pH-stat control strategy. Maximum FFAs accumulated reached only up to 1.45 g l^{-1} after 24h (Figure 4b), however under the pH-stat control strategy, the strain ML103 pXZ18 pUR400 utilized sugars completely within 24h with no significant acetate formation (Figure 4b). The optical density of the culture reached 17.00 after 24h, this clearly showed that a considerable amount of carbon flux was diverted toward biomass formation under strict pH control (Figure 4b). As compared to ML103 pXZ18 pUR400, cultures of ML103 pXZ18Z pUR400 with pH-stat control strategy showed higher concentration of FFAs accumulation of about 2.67 g l^{-1} and 2.27 g l^{-1} with pure sugar mixture and sorghum extract, respectively (Figure 4c and d). This FFA production was higher than that of the earlier report of a ML103 pXZ18 culture [18] but was considerably lower than ML103 pXZ18Z [12] when fermentation was performed using glucose as a carbon source. Similar to ML103 pXZ18 pUR400, the pH-stat strategy of culture ML103 pXZ18Z pUR400 showed a similar trend for utilization of sugars from pure sugar mixture and sorghum extract, where cultures utilized all sugars within first 24h of incubation. The optical density (at 600 nm) reached up to 17.94 and 19.40 with pure sugar mixture and sorghum extract, respectively under pH-stat experiment of strain ML103 pXZ18Z pUR400. The FFA composition of fermentation performed using both strains showed a similar pattern under pH-stat control strategy (Table 3). An earlier study performed using woody biomass sugars as the carbon source showed total unsaturated FFA accumulation in the range of 15-45% [23], while current experiments showed slightly higher accumulation (26-52%) of unsaturated FFA.

4.0. Conclusions

The successful one-step conversion of sorghum extract sugars to FFA showed an added advantage of converting non-structural carbohydrates to biofuels and biochemicals. Plasmid pUR400 assisted ML103 pXZ18 and ML103 pXZ18Z cultures to take up and phosphorylate the disaccharide sucrose via the phosphoenolpyruvate-dependent carbohydrate phosphotransferase system (PTS). With incorporation of the pUR400 plasmid in strains, there is no need for sucrose prehydrolysis to monosaccharides to feed it as a carbon source for growing *E. coli* cultures. The combination of sucrose, glucose and fructose sugars could be efficiently utilized to produce FFAs at low cost. The sucrose metabolizing strains can produce FFA from sorghum extract as well as pure sugars with comparable efficiency. The pH manipulation of cultures after 24h fermentation improves FFA production significantly with a decrease in the accumulation of acetate. Sorghum extract could act as an excellent carbon source for microbial FFA production or other industrially important biomolecules and solvents.

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Tables

Table 1 List of strains and plasmids used.

Strain name	Relevant genotype or description	Source or Reference
<i>E.coli</i> MG1655 K12	<i>F⁻ lambda⁻ ilvG⁻ rfb⁻ rph⁻</i>	Blattner et al., [37]
ML 103	<i>F⁻ lambda⁻ ilvG⁻ rfb⁻ rph⁻ fadD⁻</i>	Li et al., [11]
Plasmid Name		
pXZ18	pTrc99a carries an acyl-ACP thioesterase gene from <i>Ricinus communis</i>	Zhang et al., [18]
pXZ18Z	pXZ18 carries (3R)-hydroxyacyl-ACP dehydrase gene (<i>fabZ</i>) from <i>E. coli</i>	San and Li [12]
pUR400	scrK, Y, A, B, and R genes, Tc ^r	Schmid et al., [16]

Table 2 FFA production using different individual carbon sources at 15 g l⁻¹ using ML103 pXZ18 pUR400 in LB medium at 30 °C and 250 rpm.

Carbon source	Starting pH	Incubation Time	Lipid (g l ⁻¹)	OD ₆₀₀	Sugar utilization (g l ⁻¹)			Acetate (g l ⁻¹)
					Sucrose	Glucose ^a	Fructose ^b	
Sucrose	7.0	24	0.90 ± 0.01	7.74 ± 0.19	11.03 ± 0.56	0.23 ± 0.05	0.00	ND
		48	1.22 ± 0.08	10.87 ± 0.18	14.87 ± 0.16	0.00	1.72 ± 0.07	1.72 ± 0.38
	7.5	24	0.84 ± 0.05	7.73 ± 0.23	12.24 ± 0.57	0.00	1.00 ± 0.05	ND
		48	1.41 ± 0.18	10.67 ± 0.04	16.51 ± 0.12	0.00	2.44 ± 0.07	1.77 ± 0.44
Glucose	7.0	24	1.28 ± 0.03	9.20 ± 0.04		10.98 ± 0.01		ND
		48	1.98 ± 0.05	11.04 ± 0.53		16.19 ± 0.02		0.09 ± 0.04
	7.5	24	1.13 ± 0.01	8.25 ± 0.16		11.01 ± 0.01		ND
		48	1.99 ± 0.04	11.34 ± 0.10		16.74 ± 0.01		0.16 ± 0.02
Fructose	7.0	24	1.08 ± 0.02	7.43 ± 0.23			11.07 ± 0.05	ND
		48	1.68 ± 0.03	7.44 ± 0.03			17.35 ± 0.02	ND
	7.5	24	1.01 ± 0.03	7.11 ± 0.42			10.27 ± 0.03	ND
		48	1.77 ± 0.08	7.56 ± 0.14			17.29 ± 0.01	ND

^a & ^b Sugar concentration reported is actually accumulation instead utilization in the case of sucrose as a carbon source; ND = not detected

Table 3 Composition of FFAs produced by strains ML103 pXZ18 pUR400 and ML103 pXZ18Z pUR400.

Strain	Culture condition	Initial sugar concentration (g l ⁻¹)	FFA composition (%)				Total unsaturated FFAs (%)
			C14	C16	C16:1	C18:1	
ML103 pXZ18 pUR400 [†]	SF, a	16.27	37.51	10.87	47.69	3.94	51.63
ML103 pXZ18 pUR400 [†]	SF, a	29.51	37.47	32.66	24.18	5.68	29.86
ML103 pXZ18Z pUR400 [†]	SF, a	16.13	38.16	31.07	23.84	6.93	30.77
ML103 pXZ18Z pUR400 [†]	SF, a	29.65	49.68	9.27	38.99	2.06	41.05
ML103 pXZ18 pUR400 [‡]	SF, a	17.29	28.76	18.86	43.75	8.63	52.38
ML103 pXZ18Z pUR400 [‡]	SF, a	16.76	11.94	4.20	77.01	6.85	83.86
ML103 pXZ18 pUR400 [†]	SF, b	16.13	34.38	30.49	27.74	7.38	35.12
ML103 pXZ18 pUR400 [†]	SF, b	29.22	38.66	34.50	21.03	5.80	26.83
ML103 pXZ18Z pUR400 [†]	SF, b	15.79	43.08	8.73	45.31	2.88	48.19
ML103 pXZ18Z pUR400 [†]	SF, b	28.57	45.65	10.17	42.03	2.15	44.18
ML103 pXZ18 pUR400 [‡]	SF, b	16.71	34.40	28.13	30.88	6.59	37.47
ML103 pXZ18Z pUR400 [‡]	SF, b	16.01	38.42	14.16	43.79	3.62	47.41
ML103 pXZ18 pUR400 [†]	Bio, c	17.88	33.75	23.64	37.91	4.70	42.61
ML103 pXZ18 pUR400 [‡]	Bio, c	16.79	33.75	23.64	37.91	4.70	42.61
ML103 pXZ18Z pUR400 [†]	Bio, c	17.33	38.92	20.59	33.39	7.10	40.49
ML103 pXZ18Z pUR400 [‡]	Bio, c	16.57	37.67	12.31	45.52	4.49	50.01

[†] Carbon source: pure sugar mixture; [‡] Carbon source: sorghum juice; SF, a: Shake flask culture without pH adjustment to 7.0 after 24h incubation; SF, b: Shake flask culture with pH adjustment to 7.0 after 24h incubation; Bio, c: Bioreactor culture with constant pH 7.00

Figure captions:

Fig. 1. Simplified central aerobic metabolic pathway of *E. coli* including the FFA production pathway

Fig. 2. pH manipulation study of ML103 pXZ18 pUR400 culture. With (data shown in solid lines) and without (data shown in dotted lines) pH manipulation profile of ML103 pXZ18 pUR400 fermentation for FFA production (a), acetate formation (b), sugar utilization with optical density (c), and (d). Note - SM: Sugar mixture, SE: Sugar extract, SM-S: Sugar mixture sucrose, SM-G: Sugar mixture glucose, SM-F: Sugar mixture fructose, SM-OD: Optical density of sugar mixture cultures, SE-OD: Optical density of sorghum extract cultures

Fig. 3. pH manipulation profile study of ML103 pXZ18Z pUR400 culture. The pH manipulation profile (with pH manipulation-shown in solid lines and without pH manipulation-shown in dotted lines) of ML103 pXZ18Z pUR400 for FFA production (a), acetate formation (b), sugar utilization with optical density (c), and (d). Note - SM: Sugar mixture, SE: Sugar extract, SM-S: Sugar mixture sucrose, SM-G: Sugar mixture glucose, SM-F: Sugar mixture fructose, SM-OD: Optical density of sugar mixture cultures, SE-OD: Optical density of sorghum extract cultures

Fig. 4. Evaluation of pH-stat control strategy for FFA production using sugar mixture and sorghum extract as a carbon source

Figure

Fig. 1.

Fig. 2.

Fig. 3.

Fig. 4.

Graphical abstract