Contents lists available at ScienceDirect

Metabolic Engineering Communications

Direct production of fatty alcohols from glucose using engineered strains of *Yarrowia lipolytica*

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A B S T R A C T :

Fatty alcohols are important industrial oleochemicals with broad applications and a growing market. Here, we sought to engineer *Yarrowia lipolytica* to serve as a renewable source of fatty alcohols (specifically hexadecanol, heptadecanol, octadecanol, and oleyl alcohol) directly from glucose. Through screening four fatty acyl-CoA reductase (FAR) enzyme variants across two engineered background strains, we identified that MhFAR enabled the highest production. Further strain engineering, fed-batch flask cultivation, and extractive fermentation improved the fatty alcohol titer to 1.5 g/L. Scale-up of this strain in a 2L bioreactor led to 5.8 g/L total fatty alcohols at an average yield of 36 mg/g glucose with a maximum productivity of 39 mg/L hr. Finally, we utilized this fatty alcohol reductase to generate a customized fatty alcohol, linolenyl alcohol, from α-linolenic acid. Overall, this work demonstrates *Y. lipolytica* is a robust chassis for diverse fatty alcohol production and highlights the capacity to obtain high titers and yields from a purely minimal media formulation directly from glucose without the need for complex additives.

1. Introduction

Fatty alcohols (as defined as long hydrocarbons > C10 containing a terminal alcohol) are used in a wide range of applications in the cosmetic, lubricant/surfactant, personal care product, and pharmaceutical sectors (Fillet and Adrio, 2016; Adrio, 2017). More specifically, global demand for these molecules is currently estimated at over 2 million tons with an annual growth rate of 4.3% (Fillet and Adrio, 2016). This supply is traditionally sourced via catalytic hydrogenation of plant oils or petrochemicals (Fillet and Adrio, 2016). As a result, there is a need for more efficient, renewable processes as these chemical routes rely on fossil fuels or unsustainable farming practices (as seen in palm oil production) (Adrio, 2017).

As an alternative, fatty alcohols can be produced biologically through the activity of various enzymes (Willis et al., 2011). In particular, two main approaches have been explored for the *de novo* synthesis of fatty alcohols: (1) a stepwise conversion of fatty acyl-ACP to a fatty aldehyde which is subsequently reduced to an alcohol, and (2) the direct conversion from fatty acyl-CoA to fatty alcohol through the activity of a fatty acyl-CoA reductase (FAR) (Willis et al., 2011). In recent years, several groups have utilized both routes with varying success, as described below.

Efforts in *Escherichia coli* have mainly utilized the two-step pathway to produce both even and odd chain fatty alcohols utilizing a fatty acyl-ACP reductase. For example, heterologous expression of the *Orzya sativa* α-dioxygenase along with a modified thioesterase (TesA') and aldehyde reductase expression enabled production of 1.95 g/L of odd chain fatty alcohols (Cao et al., 2015). To produce even-chain fatty alcohols, heterologous expression of *Synechococcus elongatus* PCC7942 acyl-ACP reductase enabled 0.75 g/L fatty alcohols with further improvement to 1.9 g/L when paired with the aldehyde reductase YbbO (Liu et al., 2014; Fatma et al., 2016).

Outside of *E. coli*, especially in yeasts, research has focused on the single-step pathway enabled by the FAR enzyme and has resulted in higher overall titers. Expression of a *Mus musculus* FAR in *Saccharomyces cerevisiae* led to 6 g/L total fatty alcohol production in a fed-batch process (d'Espaux et al., 2017). Likewise, a similar workflow led to 1.7 g/L using *Lipomyces starkeyi* in a batch process (McNeil and Stuart, 2018). Using a different FAR enzyme (from the barn owl), 690 mg/L fatty alcohols were produced in *Yarrowia lipolytica* (Wang et al., 2016a,b). From literature, the most active FAR when expressed in yeast cells (based on highest achievable titers) comes from *Marinobacter hydrocarbonoclasticus* strain

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VT8 (also known as *Marinobacter aqueaolei* VT8) (Fillet et al., 2015; Wang et al., 2016a,b; Zhang et al., 2019). As evidence, this MhFAR (also known as Maqu_2220) enabled 770 mg/L fatty alcohols in *L. starkeyi*, 5.75 g/L in *Y. lipolytica* when grown on modified YPD media and 8 g/L in *Rhodosporidium toruloides* when grown in a sucrose fed-batch reactor (Fillet et al., 2015; Wang et al., 2016a,b; Zhang et al., 2019).

Given that fatty alcohols are produced from fatty acids and their precursors, it is not surprising that oleaginous yeasts have been explored as superior hosts for this particular class of molecules (Fillet et al., 2015; Wang et al., 2016a,b; McNeil and Stuart, 2018). To further improve fatty alcohol titers and yields, we selected *Y. lipolytica* as the host organism in this work. *Y. lipolytica* is a non-conventional yeast that is Generally Regarded as Safe, thus enabling use in industrial processes for products such as polyunsaturated fatty acids and citric acid (Xue et al., 2013; Groenewald et al., 2014). Moreover, a growing set of genetic tools along with sequence annotations (Blazeck et al., 2013; Liu and Alper, 2014; Curran et al., 2015; Wagner et al., 2018) have led to a sharp increase in the number of products demonstrated in this host (Blazeck et al., 2015; Abdel-Mawgoud et al., 2018; Markham, 2018b).

In this work, we sought to demonstrate high fatty alcohol production directly from chemically defined media. Specifically, many studies in the field have relied on the use of complex media additions (e.g. yeast extract, peptone, etc.) for higher titers, these additives prevent the true calculation of product yield due to unaccounted carbon. Here, we screen through four FAR enzymes across two of our pre-engineered, lipid producing *Y. lipolytica* strains and confirm MhFAR to be the most active. Next, we demonstrate an extractive fermentation process that can produce 1.5 g/L of fatty alcohols at the flask scale. Using a non-extractive bioreactor fermentation, we were able to achieve the highest published titer and maximum yield of fatty alcohols in *Y. lipolytica* at 5.8 g/L and 57 mg/g glucose, respectively. Finally, we demonstrate that this FAR can generate alternative fatty alcohols such as linolenyl alcohol.

2. Materials and methods

2.1. Plasmid and strain construction

The yeast strains used in this study, *Y. lipolytica* Po1f pex10 mfe1leucine$^+$ uracil$^+$ DGA1, named Po1fpmD for simplicity and *Y. lipolytica* L36DGA1 were isolated and characterized in previous work (Blazeck et al., 2014; Liu et al., 2015). Strain Po1fpmD was built by eliminating β-oxidation (mfe1) and peroxisome biogenesis (pex10) combined with diacylglycerol acyltransferase 1 (DGA1) overexpression (Blazeck et al., 2014). Strain L36DGA1 was generated from an isolated strain L36 which contained a mutant Mga2 regulator paired with overexpression of DGA1 (Liu et al., 2015). For production of alternative fatty alcohols, *Y. lipolytica* strain L36DGA1 3× RkD12-15 was used which overproduces α-linolenic acid (ALA) via three overexpressions of a dual functional Δ12/Δ15 desaturase (Cordova and Alper, 2018). A list of plasmids and primers used in this study is provided in Supplementary Tables 1 and 2. *E. coli* NEB10β competent cells were used for cloning and DNA propagation. Fatty acyl-CoA reductase (FAR) enzymes were selected from literature search and constructed through the biofoundry at Zymergen into *Y. lipolytica* strain CBS7504 ura3Δ ku70Δ. After construction, FAR enzymes were transferred to *Y. lipolytica* strains L36DGA1 and Po1fpmD for screening. To do so, FAR enzymes as well as their corresponding promoter and terminator were amplified using Polymerase Chain Reaction (PCR) with Q5 Hot Start High-Fidelity Polymerase (New England Biolabs) and inserted into a plasmid backbone using Gibson Assembly. Initial integration of FAR enzymes (using a nourseothricin-based plasmid) was confirmed via genomic DNA extraction and PCR confirmation. For the second integration of the MhFAR enzyme, a plasmid with resistance to mycophenolic acid was utilized (Wagner et al., 2018). The second integration of MhFAR was confirmed using PCR with primers in the mycophenolic acid resistance gene and MhFAR. As random integration was used in these experiments, several clones were selected and evaluated for production. Following analysis, clone(s) with highest production were isolated in biological triplicate for further analysis.

For expression in *Y. lipolytica*, the open reading frame of each FAR was codon optimized by Integrated DNA Technologies following the codon frequency table for *Y. lipolytica* generated by Kazusa DNA Research Institute and the synthesizability of DNA (as determined by IDT). The DNA sequences for all FAR enzymes is listed in Supplementary Table 3. FAR enzymes synthesized for this work were delivered as assembled transcriptional units containing pre-determined promoters and terminators. For HsFAR1 and AmFAR, the open reading frame was driven by the GPD1 promoter and the Lip2 terminator while MhFAR and AtFAR5 were driven by the promoter pYALI1_E09438g with the Lip1 terminator. The sequences of these promoters and terminators is listed in Supplementary Table 4. These expression cassettes were sub-cloned into existing plasmids for heterologous expression in *Y. lipolytica*. Constructs containing FAR enzymes were randomly integrated into the *Y. lipolytica* genome using high-efficiency electroporation as previously described (Markham, 2018a; Wagner et al., 2018). Colonies were isolated on agar plates containing 20 g/L agar (Teknova). Following previous work with *Y. lipolytica*, nourseothricin selection utilized 1000 mg/mL dissolved in sterile water in yeast peptone dextrose (YPD) media and mycophenolic acid selection required 100 mg/L mycophenolic acid (in DMSO) in minimal media (YNB, glucose) (Wagner et al., 2018). Isolated colonies were grown in YPD media and frozen in 20% glycerol.

2.2. Media conditions

E. coli was cultured at 30 °C in Lysogeny broth (Teknova) supplemented with 100 μg/mL ampicillin to maintain the desired plasmid and these lower temperatures were used to prevent mutations and increase plasmid yields. Tube and flask yeast cultures were grown in defined YSC media containing 40 g/L glucose and 5 g/L ammonium sulfate (0.79 g/L CSM-Complete, 6.7 g/L Yeast Nitrogen Base, 40 g/L glucose). YPD media was exclusively used for generating frozen stocks and preparing cells for transformation and contained 10 g/L yeast extract, 20 g/L peptone and 20 g/L glucose. *Y. lipolytica* tube fermentation was performed in 14 mL culture tubes containing 2 mL media incubated in a rotary drum at 28 °C. Flask fermentation was performed in unbaffled flasks containing 50 mL of media (in a 250 mL flask) with shaking at 225 rpm.

2.2.1. Extractive fermentation

To assess the impact of continuous extraction, fermentation utilized a dodecane overlay of 6 mL dodecane per 50 mL cell culture where noted. To minimize impacts on initial growth, dodecane was added after 24 h of flask fermentation. Dodecane (Sigma) was maintained as a separate phase forming an aqueous: organic emulsion.

2.3. Glucose analysis using HPLC

Glucose concentration was determined using High Performance Liquid Chromatography with an Aminex HPX-87P column. Supernatant from 1 mL of sample culture as filtered with a 0.2 μm Nylon filter before analysis using a Thermo Scientific Dionex Ultimate 3000 Rapid Separation LC system with RefractoMax 521 detector. Filtered and degassed water was used as the mobile phase with a flow rate of 0.6 mL/min. Column temperature was maintained at 85 °C. As necessary, supernatant samples were diluted with deionized water before filtration to stay within the linear range of the detector.

2.4. Fatty alcohol and fatty acid GC analysis

To detect and quantify both fatty alcohols and fatty acids, a 30 m, 0.32 mm, 0.25 μm DB-FATWAX-UI (Agilent) GC column was used. All equipment parameters were identical for both alcohols and acids except

for the ramp rate (and consequently the total run time). The injected volume was 1 μL (split ratio of 10) with Flame Ionization Detection on a Trace 1310 Gas Chromatograph (Thermo-Fisher). The initial oven temperature was 40 °C with a ramp to 250 °C as previously described (Liu et al., 2015). For fatty alcohols, the ramp rate was 3.0 °C/min while it was 5.0 °C/min for fatty acid methyl esters.

Fatty acid samples were analyzed as previously described following a Folch extraction (Folch et al., 1957; Cordova and Alper, 2018). Cell pellets were analyzed for lipid content and nonadecanoic acid was added as an internal standard at a final concentration of 0.1 g/L. After evaporation of solvent from lipid extractions, transesterification was performed at 85 °C with acidic methanol. The resulting fatty acid methyl esters were separated using 0.9% NaCl and hexane. The hexane layer was transferred to vials for GC analysis.

Fatty alcohols were extracted with ethyl acetate using a combined supernatant and cell pellet strategy previously described (Cao et al., 2015; Wang et al., 2016b). In brief, 300 μL of cell culture were collected and vortexed in ethyl acetate for 20 min. Silica beads were added to aid in cell lysis and nonadecane was added as an internal standard (10 mg/L final concentration). Samples were centrifuged at > 23,000 g for 10 min to separate ethyl acetate phase which was transferred for GC analysis. For experiments with a dodecane overlay, samples were briefly centrifuged after collection to isolate the dodecane layer which was directly analyzed using GC.

2.5. Bioreactor fermentation

For scale-up of fatty alcohol production, a New Brunswick Bioflo 115 Benchtop Bioreactor was used following previous work (Liu et al., 2015). Due to safety concerns, bioreactor fermentation did not include the dodecane extractive layer. Initial media contained 80 g/L glucose, 0.79 g/L CSM-Complete and 3.4 g/L Yeast Nitrogen Base. Following the previous study using the L36DGA1 parent strain, a glucose pulse of 80 g was added at approximately 72 h (Stock concentration of 600 g/L) (Liu et al., 2015). In this work, a second glucose pulse of 80 g was added at approximately 120 h to prevent glucose exhaustion. The initial cell density was 0.1 OD600 units with a total operating volume of 1.7 L.

Dissolved oxygen was maintained at or above 50% using an agitator cascade and pH was controlled to 5.0 using 1M NaOH as in previous literature (Fillet et al., 2015; Liu et al., 2015; d'Espaux et al., 2017). The pH control (base addition) balanced evaporative loss maintaining the volume at 1.7 L throughout the fermentation. Air was sparged into the bioreactor at a rate of 2.5 splm. Fermentation lasted for 10 days with samples collected daily for analysis. As necessary, sterile antifoam 204 (Sigma) was added. Biomass accumulation was determined for 1 mL of

cells by washing in PBS and completely evaporating any residual liquid at 90C for >1 h. All bioreactor samples (biomass, sugar, lipids, alcohols) were measured in duplicate.

2.6. Statistical analysis

All statistical analysis was carried out in R version 3.6.0, via R studio version 1.2.1335. Two-way ANOVA with Tukey's test was used to determine significance where noted.

3. Results

3.1. Evaluating FAR enzymes for fatty alcohol production in previously engineered Y. lipolytica

To enable fatty alcohol production in *Y. lipolytica*, we selected four FAR enzyme candidates to overexpress based on literature searches and sequence analysis. AmFAR from the honeybee (*Apis mellifera*) was selected as it was recently putatively identified as a FAR present in all segments of the bee body (Teerawanichpan et al., 2010). FAR activity using AmFAR was previously confirmed in *S. cerevisiae* with the highest efficiency seen on C18:0 (stearyl-CoA) substrate (Teerawanichpan et al., 2010). HsFAR was previously identified from *Homo sapiens* brain tissue and further studied using baculovirus delivery to insect cells with observed substrate specificity toward C16:0, C18:0, C18:1 and C18:2 acyl-CoAs (Cheng and Russell, 2004). AtFAR5 from *Arabidopsis thaliana* was selected based on its high production of fatty alcohols when screened in *S. cerevisiae* (Chacón et al., 2013). This FAR (FAR5 from Chacón et al.) is reported to favor C18:0 fatty acids rather than C16:0 (Chacón et al., 2013). The final FAR enzyme selected was MhFAR from *M. hydrocarbonoclasticus* strain VT8 (also known as *M. aqueaolei* VT8). This enzyme (also noted as Maqu_2220) has been widely studied in a variety of hosts including *Y. lipolytica, L. starkei, R. toruloides, S. cerevisiae,* and *E. coli* (Chacón et al., 2013; Wang et al., 2016b; Zhang et al., 2019; Fillet et al., 2015). In almost all studies, expression of this FAR enzyme led to the highest overall fatty alcohol production. In this work, FAR enzymes were codon-optimized prior to synthesis, assembly, and expression in two previously engineered strains, Po1fpmD and L36DGA1 (Blazeck et al., 2014; Liu et al., 2015). Both strains are suitable for high lipid accumulation (25 g/L in bioreactor) but differ in unsaturated fatty acid content producing between 93 and 67% unsaturated content for Po1fpmD and L36DGA1, respectively.

As shown in Fig. 1, total fatty alcohol production varied in both the Po1fpmD and L36DGA1 strains as a function of FAR enzyme expressed with the most significant production levels seen with the AtFAR5 and MhFAR. Reported fatty alcohol titers represent the total of intracellular
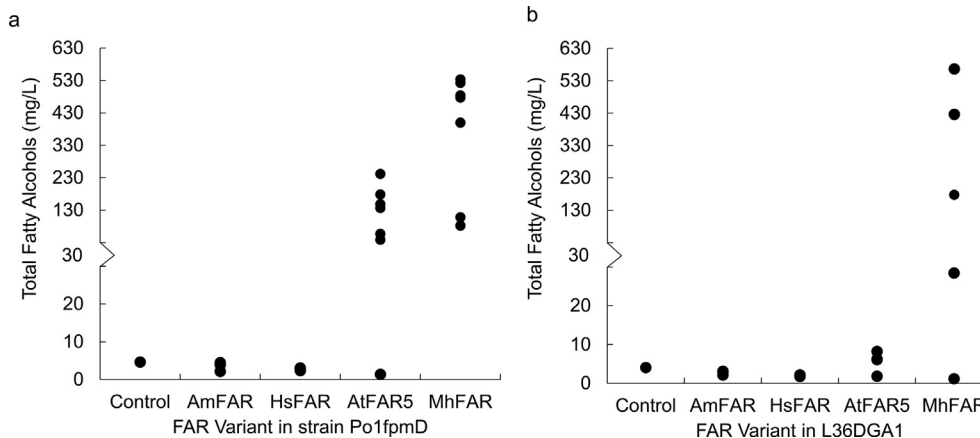

Fig. 1. *Clonal variation of FAR enzymes expressed in Y. lipolytica strains Po1fpmD and L36DGA1.* Each data point represents an individual clone obtained from the random integration transformation of the FAR into (a) Po1fpmD or (b) L36DGA1. This level of variation is expected due to random integrations of the FAR expression cassette used in this approach.

and extracellular fatty alcohols, however most production remained intracellular. Speciation of these fatty alcohols revealed the presence of hexadecanol (C16:0), heptadecanol (C17:0), octadecanol (C18:0), oleyl alcohol (C18:1) and linoleyl alcohol (C18:2) (Supplementary Fig. 1). This group of fatty alcohols directly matches the highly abundant fatty acid molecules present in *Y. lipolytica* except for palmitoleyl alcohol/palmitoleic acid (C16:1). Palmitoleyl alcohol was not observed in any FAR/strain combinations consistent with previous publications (Fillet et al., 2015; Wang et al., 2016b). A higher amount of oleyl alcohol was seen with the L36DGA1 strain which is congruent with its higher content of unsaturated fatty acids. Previous engineering efforts for fatty alcohol production observed similar trends with hexadecanol, octadecanol and oleyl alcohol as the predominantly observed species (Xu et al., 2016; d'Espaux et al., 2017).

It is important to note that the datapoints in Fig. 1 represents clonal variation occurring based on the use of random integrations for these FAR constructs. The parental strains Po1fpmD and L36DGA1 did generate detectable, albeit low levels of fatty alcohols at 3.8 and 4.8 mg/L, respectively. Two of the tested FAR enzymes, AmFAR and HsFAR did not enable production of fatty alcohols to levels higher than that of the basal level in the parental strains (Fig. 1). However, AtFAR5 and MhFAR did enable significant fatty alcohol production. Interestingly, the AtFAR5 enzyme enabled production of significantly more fatty alcohols in the Po1fpmD strain than in L36DGA1. Specifically, the average of the clones in Po1fpmD AtFAR5 clones produced an average of 114 mg/L while the L36DGA1 AtFAR5 clones produced only 5.6 mg/L total alcohols. As AtFAR5 generates predominately octadecanol (Supplementary Fig. 1), it is likely that the low availability of stearoyl-CoA in L36DGA1 prevent higher accumulation of fatty alcohols (Liu et al., 2015). As expected from prior literature, MhFAR enabled the highest production of fatty alcohols in both strains (d'Espaux et al., 2017; Zhang et al., 2019; Wang et al., 2016b) with clones producing over 400 mg/L of total fatty alcohols observed. As with before, the distribution of fatty alcohols trends with the total lipid distribution and thus the L36DGA1 strains accumulate a higher fraction of oleyl (C18:1) alcohol compared to the Po1fpmD strains (Supplementary Fig. 1). The top clones from both strain backgrounds was maintained in a biological triplicate and used for subsequent strain engineering.

3.2. Strain optimization and extractive fermentations for improved fatty alcohol production

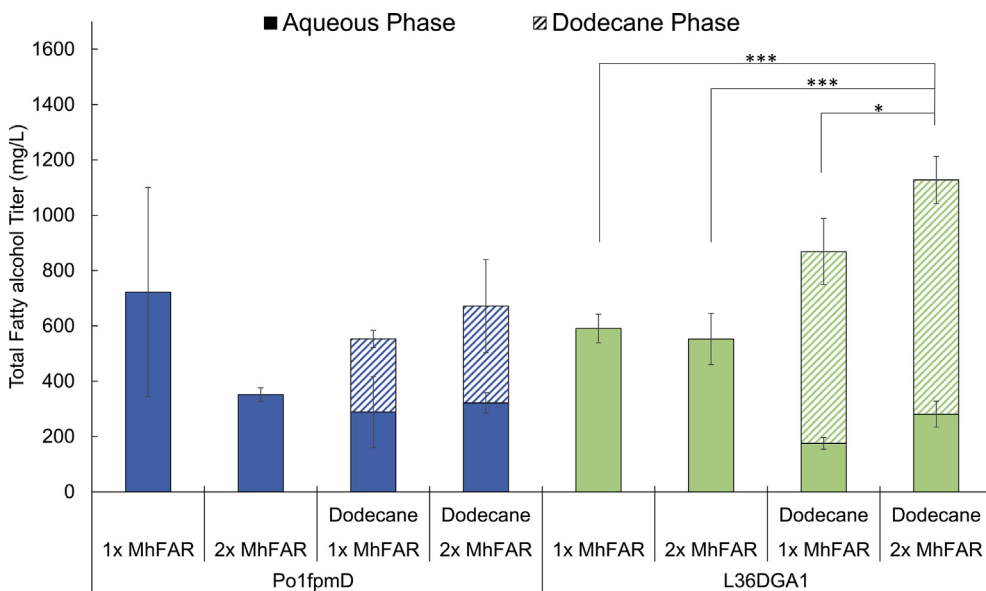To further improve fatty alcohol production, we sought to increase the copy number of MhFAR through a second transformation event using the mycophenolic acid resistance marker (Wagner et al., 2018). Initial tests with these strains (named 2× MhFAR compared with the prior strain noted as 1× MhFAR) are shown in Fig. 2. Somewhat surprisingly, this second integration event did not yield improvements during cultivation on YSC media and in fact, reduced fatty alcohol production in the case of Po1fpmD. To determine whether this result was due to culture conditions and/or strain limitations, we sought to evaluate the strains using an extractive dodecane overlay during fermentation, an approach that has been shown to improve the production of secreted oleochemicals while also easing downstream separations (Wang et al., 2016b; d'Espaux et al., 2017; McNeil and Stuart, 2018). To do so, the single and double integration strains were cultivated with a 12% dodecane overlay (6 mL per 50 mL culture added after 24 h). This experiment indeed confirmed the benefit of extractive fermentation (Fig. 2) and the impact of the second MhFAR integration resulted in significantly higher fatty alcohol production in the L36DGA strain under this condition, reaching 1.1 g/L. This improvement is due to the dodecane driving secretion providing a driving force for higher production capacity and net flux which can only be observed in the context of the second MhFAR transformation event. While the Po1fpmD-based strains did not respond positively to the second integration event, a similar, slight improvement in production was seen under the extractive fermentation condition with approximately 50% fatty alcohol secretion. Outside of the improvement in titer, the dodecane overlay provides bioprocess benefits by increasing product secretion and resulted in up to 75% extracellular fatty alcohol content (Fig. 2), thus enabling a more simplified downstream processing that bypasses cell lysis.

Next, we sought to evaluate whether there were any biases associated with the use of a dodecane overlay in the fatty alcohol distribution. To this end, we did not observe any difference in the composition of fatty alcohols to fatty acids correspondence in the L36DGA1 2× MhFAR as shown by the matrices in Fig. 3 or in the Po1fpmD 2× MhFAR strain (Supplementary Fig. 2). Since we conducted this experiment using a defined (YSC) media, product yield can be accurately calculated directly from glucose as shown in Fig. 4. Several of the strain-overlay combinations were able to achieve yields around 29 mg fatty alcohols per g glucose consumed. Given that the L36DGA1 2× MhFAR strain demonstrated the highest fatty alcohol titer, efficient glucose utilization, and a high yield, we selected this strain for additional optimization.

Next, a spiked-batch fermentation was conducted to further improve fatty alcohol production given that all the initial glucose (40 g/L) was nearly consumed in L36DGA1 2× MhFAR culture with a dodecane overlay experiment conducted above. To do so, a pulse of concentrated

Fig. 2. *Total fatty alcohol titer evaluating the impact of second integration of MhFAR enzyme and dodecane overlay.* Po1fpmD strains showed no significant differences in total fatty alcohol production across conditions. L36DGA1 based strains shows an improved fatty alcohol profile based on genetics and conditions. Aqueous phase represents the combined intracellular and extracellular fatty alcohols in the water phase. Error bars represent the standard deviation of biological triplicate experiments. Significance is denoted as: *p < 0.05, ***p < 0.005 (tested via two-way ANOVA).
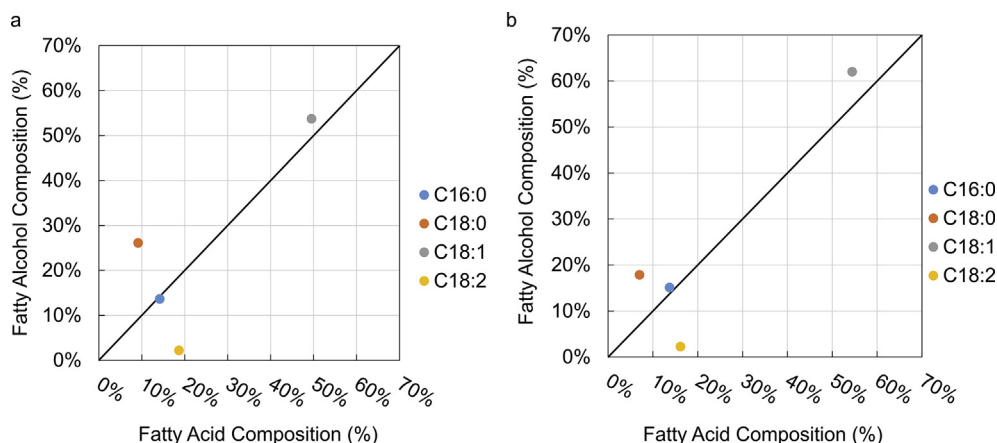
Fig. 3. *Fatty acid: Fatty alcohol ratio matrix for strain L36DGA1 2x MhFAR cultured with and without extractive fermentation.* The fatty acid to fatty alcohol ratio is maintained between (a) normal fermentation conditions and (b) extractive fermentation with a dodecane overlay. Experimental data represents the average values of a biological triplicate experiment.
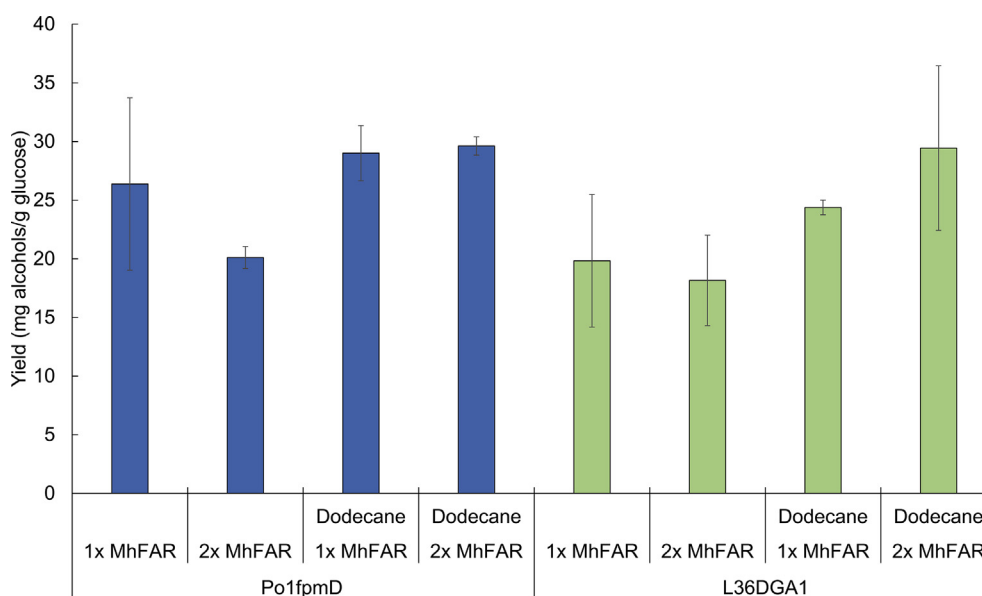

Fig. 4. *Fatty alcohol yield with multiple integrations and extractive fermentation using YSC media.* Yields were calculated directly from glucose due to the use of minimal media. Error bars represent the standard deviation of biological triplicate experiments. Yields do not significantly vary across groups as tested by a two-way ANOVA test.

glucose (equivalent to 40 g/L) was added after 3 days of flask fermentation. As shown in Fig. 5, this additional fed glucose significantly improved final fatty alcohol production from 1.2 g/L to 1.5 g/L when using the dodecane overlay. A similar improvement was not observed for the culture grown without the overlay. As shown in Table 1, the fatty alcohol yield varies from 20.6 to nearly 40 mg/g glucose across this experiment with the highest yield observed in the dodecane overlay experiment with a glucose pulse. Residual glucose concentration at each time point is shown in Supplementary Fig. 3. Interestingly, the maximum fatty alcohol productivity occurred at 3 days (72 h) of flask fermentation reaching 14 mg/L hr whereas maximum lipid accumulation was reached after 5 days in flask fermentation (data not shown). Ultimately, using this strain engineering and fermentation condition approach, overall fatty alcohol titer reached 1.5 g/L in flask fermentation.

3.3. Bioreactor production of fatty alcohols

As a final demonstration of fatty alcohol production capacity, we evaluated the performance of the L36DGA1 2× MhFAR strain in a controlled, 2L bioreactor. Due to safety concerns, extractive fermentation could not be carried out at this scale due to dodecane flammability and existing equipment incompatibility. Nevertheless, we observed that fatty alcohol production was dramatically improved using this a fed-batch fermentation (two glucose pulses, 80 g each) with pH and dissolved oxygen control (control curves shown in Supplementary Fig. 4) As shown in Fig. 6, fatty alcohols were continuously produced throughout the fermentation reaching a maximum titer of 5.8 g/L. Table 2 provides the titer and distribution of individual fatty alcohol species observed at the end of bioreactor fermentation. The strategy of two glucose pulses prevented glucose exhaustion and led to a consistent biomass accumulation throughout the fermentation. The total fatty alcohol yield (Supplementary Fig. 5) reached a maximum of 57 mg/g glucose consumed during the fermentation and maintained at a value above 35 mg/g for the remainder of the fermentation. Most of the fatty alcohols were produced within the first half of the fermentation run where the bioreactor reached a maximum specific productivity of 39 mg fatty alcohols/L hr with an overall averaged productivity of 24 mg/L hr throughout the run.

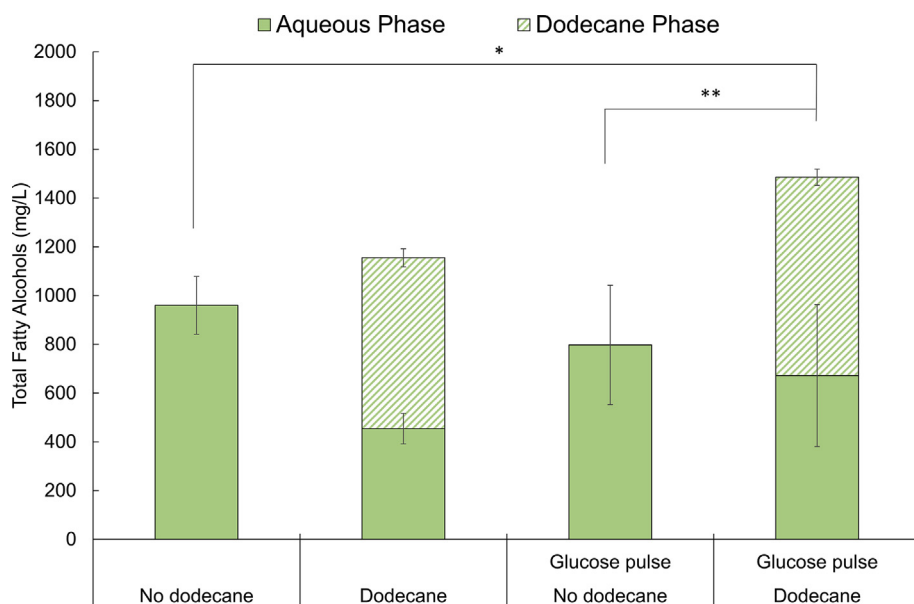Despite the parental strain being previously engineered for lipid

Fig. 5. *Fatty alcohol production and localization using dodecane extraction and/or a glucose pulse.* A glucose pulse paired with extractive fermentation leads to highest fatty alcohol production in flasks. Data shown represents final time point tested for this flask fermentation (117 h). Aqueous phase represents the combined intracellular and extracellular fatty alcohols in the water phase. Error bars represent the standard deviation of biological triplicate experiments. Significance is denoted as: *$p < 0.05$, **$p < 0.01$ (tested via two-way ANOVA).

Table 1

Fatty alcohol yield and productivity from strain L36DGA1 2x MhFAR in flask fermentation. Fatty alcohol yield is calculated from consumed glucose. Errors values presented represent the standard deviation of biological triplicates.

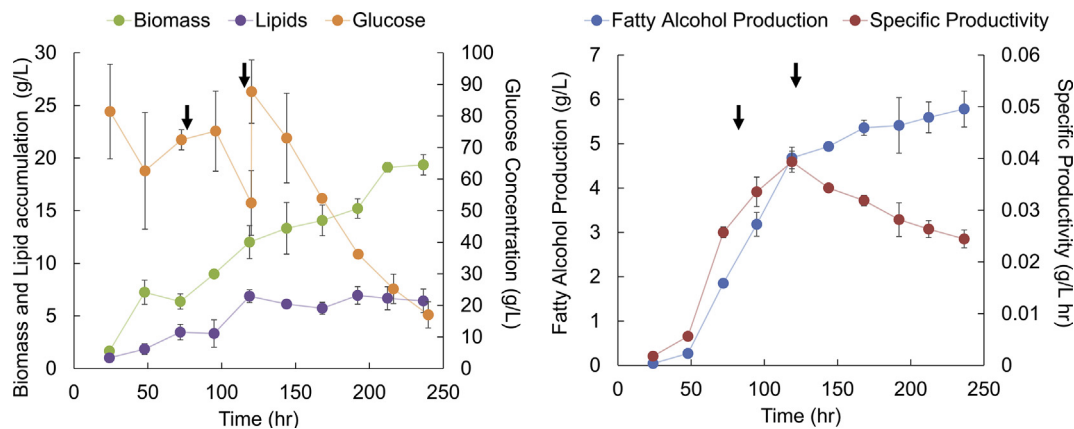|		Fatty Alcohol Yield (mg/g consumed glucose)			Fatty Alcohol Productivity (mg/L hr)		
		24 h	72 h	117 h	24 h	72 h	117 h
No dodecane		32.9 ± 4.7	35.0 ± 4.9	37.8 ± 12.6	5.1 ± 0.7	11.9 ± 1.7	8.2 ± 1.0
Dodecane		29.2 ± 4.1	31.5 ± 0.7	28.9 ± 2.0	4.6 ± 0.3	12.5 ± 0.1	9.8 ± 0.7
No dodecane	Glucose pulse	25.8 ± 4.4	32.5 ± 4.3	30.1 ± 11.6	4.6 ± 0.2	11.2 ± 1.6	6.8 ± 2.1
Dodecane	Glucose pulse	20.6 ± 13.7	34.9 ± 1.2	39.4 ± 12.2	4.7 ± 0.5	14.0 ± 2.5	12.7 ± 2.3

Fig. 6. *Fed-batch bioreactor of strain L36DGA1 2x MhFAR with two glucose pulses, pH 5.0.* Controlled bioreactor fermentation data enables 5.8 g/L of fatty alcohol production directly from minimal glucose media. (a) Growth parameters show biomass accumulation paired with a corresponding decrease in measured glucose concentration. Arrows represent the addition of 80 g glucose at 72 and 120 h. (b) Fatty alcohol titer and specific productivity demonstrate high level production over the course of the fermentation with a peak productivity at 5 days. Error bars represent the standard deviation of biological duplicate experiment.

overproduction, the total lipids accumulated reached a plateau of 6.5 g/L. This total lipid titer is significantly lower than previously reported for the parent strain (25 g/L), likely due to the different conditions used here to optimize for fatty alcohols (Liu et al., 2015). Previous optimization identified pH 3.5 with one glucose pulse as ideal for lipid overproduction, here we used pH 5.0 with two glucose pulses to increase fatty alcohol titer (Liu et al., 2015). As with the small-scale cultures, the fatty acid: fatty alcohol ratio was maintained between species for this bioreactor run (Supplementary Fig. 6). Citric acid was also quantified as *Y. lipolytica* is known to produce this compound and other organic acids during fermentation (Supplementary Fig. 7). Citric acid steadily accumulates

during growth on glucose and can be consumed after glucose depletion in longer fermentations (Markham et al., 2018a). Collectively, these results demonstrate production of nearly 6 g/L total fatty alcohols at an average yield of 36 mg/g glucose and maximum productivity of 39 mg/L hr, representing the highest production in this host.

3.4. Production of customized fatty alcohol products

To further diversify the speciation of fatty alcohols in *Y. lipolytica*, we sought to evaluate the capacity of MhFAR to produce alternative fatty alcohols. To do so, we utilized a previously engineered strain in our lab

Table 2

Titer and distribution of each fatty alcohol compound during bioreactor fermentation at the end of fermentation (240 h). Errors values presented represent the standard deviation of biological duplicate.

	Fatty Alcohol Titer (g/L)	Fatty Alcohol Distribution (%)
Hexadecanol (C16:0)	1.10 ± 0.10	19.1 ± 0.3
Heptadecanol (C17:0)	0.08 ± 0.01	1.3 ± 0.0
Octadecanol (C18:0)	1.76 ± 0.12	30.5 ± 0.2
Oleyl alcohol (C18:1)	2.63 ± 0.19	45.2 ± 0.3
Linoleyl alcohol (C18:2)	0.17 ± 0.00	2.9 ± 0.2
Total	**5.8 ± 0.40**	**100%**

(L36DGA1 3xRkD12-15), developed to produce α-linolenic acid at nearly a third of the total fatty acid pool (Cordova and Alper, 2018). Coupled with low temperature fermentation, α-linolenic acid was produced at 1.5 g/L in bioreactor scale up (Cordova and Alper, 2018). Heterologous expression of MhFAR in this strain background likewise enabled fatty alcohol production. As shown in Supplementary Fig. 8, a single transformation of MhFAR resulted in 105 mg/L total fatty alcohol production at the test tube scale. Unique to this strain, we observed linolenyl alcohol (generated from α-linolenic acid) at a level of 2 mg/L (representing 1.8% of the total fatty alcohol pool). Supplementary Fig. 9 shows that while MhFAR prefers C16, C18, and C18:1 as substrates, it can be used for production of customized fatty alcohols (such as C18:3 in this case), albeit at lower efficiency.

4. Discussion

In this study, we enabled the highest reported titer and yield of fatty alcohols in *Y. lipolytica* in a condition that was directly from glucose without the need for complex media additives. By initially evaluating four potential FAR enzymes in two unique strain backgrounds, we were able to re-confirm that MhFAR was the most active. Moreover, we demonstrated that the distribution of generated fatty alcohols is indeed related to the starting lipid composition for most common fatty acids found in the cell (Fig. 2, Supplementary Figs. 1 and 2). To the best of our knowledge, this is the first work to consider the relationship between fatty acids and their corresponding alcohols in two differing strains of the same species, thus providing insight into the substrate specificity of MhFAR. In *Y. lipolytica*, MhFAR prefers saturated fatty acyl-CoA substrates over unsaturated (Fig. 2, Supplementary Fig. 2), although it is still capable of generating high levels of oleyl alcohol when oleic acid represents the major lipid species (such as the case in strain L36DGA1). The production of alcohols from polyunsaturated fatty acids is not highly efficient by this enzyme as seen in the case of linolenyl alcohol. While the titer of linolenyl alcohol was low (2 mg/L), this molecule can be useful as an antibiotic and a component of phenol esterification reactions (Crout et al., 1982; Sabally et al., 2005). This overall comparison stresses the importance of selecting the correct combination of strain and enzyme for a given application.

The utility of multiple integrations was only seen in more optimal media conditions, as is evident with the extractive fermentation data. In particular, the presence of the dodecane overlay enabled an improved fatty alcohol production in L36DGA1 2× MhFAR and resulted in more than 75% of the fatty alcohols to be secreted. While bioreactor fermentation did not include an extractive layer due to safety concerns, we anticipate further increases in titer are possible under these conditions. Not only does the extractive layer improve production, it also has significant process advantages and can enable continuous processing and separation of products.

Finally, the high titers and yields reported in this work of nearly 6 g/L and 36 mg/g are the highest reported in *Y. lipolytica*. It should be noted that although high titers of total fatty alcohols have been reported in *Y. lipolytica* (5.75 g/L), these experiments utilized modified YPD media

(Zhang et al., 2019). Likewise, d'Espaux et al. generated 6.0 g/L total fatty alcohols in *S. cerevisiae* extensively utilizing YPD media despite subsequently using a yield calculation only on glucose as the presumed carbon source (d'Espaux et al., 2017). Not only is complex media undesirable, it is also hard to calculate a true product yield from carbon without accounting for the contents of peptone and yeast extract. Fillet et al. reports a fatty alcohol yield of 40 mg/g from *R. toruloides* generating the highest overall titer reported of 8 g/L. However, this process utilizes sucrose and a more complex additive, corn steep liquor. Conversely, this work utilized only chemical defined media and reports product yields exceeding 35 mg/g glucose while also producing a high titer.

As another metric in evaluating production capacity, specific titer (fatty alcohol titer per cell density) is shown in Supplementary Fig. 10. Research efforts with *E. coli* and *S. cerevisiae* produced 16 and 70 mg/L/OD$_{600}$, respectively (Cao et al., 2015; d'Espaux et al., 2017). Likewise, previous work using MhFAR in *L. starkeyi* and *Y. lipolytica* enabled 51 and 24 mg/L/OD$_{600}$ (Wang et al., 2016b; Xu et al., 2016). In contrast, this work enabled 87 mg/L/OD$_{600}$ indicating the higher production capacity per cell. While additional optimization (dodecane overlay, C/N ratio, etc) can further improve production, this work shows that the same high titers are possible using chemically defined YSC media in a fed-batch fermentation. Thus, this work and strains developed provide a starting point toward sustainable production for a growing fatty alcohol market and demand.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mec.2019.e00105.

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