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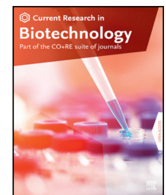
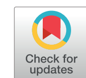


High titer fatty alcohol production in *Lipomyces starkeyi* by fed-batch fermentation



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ABSTRACT

Extracellular production of long chain fatty acid-based products, such as fatty alcohols, are being investigated as intermediates amenable to separations and catalytic upgrading to hydrocarbon fuels. We previously demonstrated that the oleaginous yeast, *Lipomyces starkeyi* expressing the fatty acyl-CoA reductase (*FAR*) gene from *Marinobacter aquaeolei* VT8, produced up to 770 mg/L of fatty alcohols. The *FAR* genes expressed in *L. starkeyi* transformants exhibited different fatty alcohol production levels. To better understand fatty alcohol production and potentially further increase fatty alcohol titer and yields, we investigated the expression levels, copy numbers, and enzymatic activities of the expressed *FAR* genes. Our results showed that fatty alcohol production is dependent on the above factors for higher titers of fatty alcohols. Fatty alcohol titer was further increased in the highest producing transformant by testing different basic process configurations (batch and fed-batch) with different initial nitrogen concentrations and feed rates. In fed-batch, a maximum of 4.2 g/L fatty alcohols were produced in 5-L bioreactor. The oleaginous yeast *L. starkeyi* can produce high titers of fatty alcohols particularly when periodically fed glucose and nitrogen.

1. Background

Recently, fatty alcohol, a fatty acid-derived product, in addition to lipids, have generated high interests among the researchers. This is not only because of the large demand for its applications in surfactants, detergents, and cosmetic products, but also because of its potential to be upgraded to liquid biofuels. Compared to intracellular triacylglycerides (TAG), fatty alcohols can be secreted from the cells reducing the cost and effort for recovery and upgrading to biofuels. Therefore, producing fatty alcohols is more economically viable than intracellular lipid accumulation as the costly extraction process can be eliminated in the fatty alcohol recovery process.

Currently, fatty alcohols are primarily derived from petroleum. Fatty alcohols can be biologically produced from fatty acyl-CoA by NAD(*P*)H-dependent fatty acyl-CoA reductase (*FAR*). Biological production of fatty alcohols has been reported in *Escherichia coli* and *Saccharomyces cerevisiae* by introduction of a single *FAR* gene (d'Espaux et al., 2017; Fillet et al., 2015; Liu et al., 2013; Liu et al., 2016; Runguphan and Keasling, 2014; Steen et al., 2010; Zheng et al., 2012). Due to well-established genetics and facile genetic manipulation, *S. cerevisiae* has been employed as a model yeast for fatty alcohol production. However, production titers of fatty alcohols have been relatively low despite various metabolic engineering strategies. Expressing the *TaFAR* gene and manipulating other related genes allowed *S. cerevisiae* to produce 655 mg/L hexadecanol through batch fermentation (Feng et al., 2015). The highest production titer thus far reported in *S. cerevisiae* is 1.2 g/L in shake flasks and 6 g/L via fed-batch fermentation (d'Espaux et al., 2017). This was a result of multiple metabolic engineering efforts including increasing *FAR* gene expression and deleting competing lipid synthesis pathway genes. So does the work with the low lipid-producing oleaginous yeast *Yarrowia lipolytica*. To achieve a decent fatty alcohol production, multiple genes like *ACC* (acetyl-CoA carboxylase), *FAS* (fatty acyl-CoA synthetase) and *ACL* (ATP-citrate lyase) had to be overexpressed in wild type *Y. lipolytica* to enhance the lipid synthesis in order to accumulate the fatty acyl-CoA, the precursor for fatty alcohol synthesis (Cordova et al., 2020; Wang et al., 2016a; Zhang et al., 2019). The relatively low production titer and yields of fatty alcohols in *Y. lipolytica* may be related to the limited native lipid synthesis capacity. In this context, high lipid-producing oleaginous yeasts might be better source for fatty alcohol production considering their strong de novo synthesis of fatty acids and lipids. Recent research on fatty alcohol production in oleaginous yeasts has

Abbreviations: FAR, fatty acyl-CoA reductase; TAG, triacylglycerides.

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demonstrated the potential of high lipid-producing oleaginous yeast to serve as hosts for fatty alcohol productions. In the high lipid-producing yeast *Lipomyces starkeyi*, simply by expressing one single fatty acyl-CoA reductase gene from *Marinobactor aquaeolei* VT8, 770 mg/L of fatty alcohols were produced after 144 h in shake flasks (Wang et al., 2016b). Fatty alcohols can easily reach a level of 1 g/L titer when glucose substrate concentration was increased from 3% to 5%. In another oleaginous yeast *Rhodosporidium toruloides,* by expressing the single *MaFAR* gene, 1.2 g/L of fatty alcohols were produced when grown on glucose in shake flasks and 8 g/L was achieved in a 7-L bioreactor with sucrose as a substrate (Fillet et al., 2015). In the most recent report, 1.7 g/L of fatty alcohols in batch fermentation was achieved in *L. starkeyi* with expression of a single *FAR* gene from *Mus musculus* (McNeil and Stuart, 2018). It is noteworthy that in the examples given above, fatty alcohol titers of ≥1 g/L were easily achieved in shake flasks from *L. starkeyi* or *R. toruloides* with the overexpression of a single *FAR* gene. This higher level of fatty alcohol production, which is much greater than with the overexpression of a single *FAR* gene in *S. cerevisiae* or *Y. lipolytica* suggests that high lipid-producing oleaginous yeasts have a stronger innate potential for producing fatty alcohols.

L. starkeyi can accumulate lipids exceeding 60% of their cellular dry weight, which are considered as alternatives to plant oils for biodiesel production (Ratledge and Wynn, 2002). In addition, *L. starkeyi* can grow on a wide variety of substrates, including glucose, xylose, mannose, and galactose (Sitepu et al., 2014; Wang et al., 2016b; Zhao et al., 2008). The case can be compared to the limited sugar-utilizing capacity of *Y. lipolytica* and *S. cerevisiae*, especially in the case of xylose, the second most common sugar component in lignocelluosic biomass feedstocks. These advantages make *L. starkeyi* a unique and attractive strain for further development as a liquid biofuels production host. However, *L. starkeyi* has not been well studied due to the lack of efficient genetic modification tools and insufficient knowledge of its genetics. Although the development of a lithium acetate transformation protocol (Calvey et al., 2014) and the recent success in *Agrobacterium*-mediated transformation (Dai et al., 2017; Lin et al., 2017) provided a tool for gene manipulation in *L. starkeyi*, it is still challenging to realize efficient multiple gene knockouts which is important for redirecting the carbon flux to fatty alcohol synthesis. To explore the possible ways to improve the fatty alcohol titer, in this study, we investigated the expression level, enzyme activity of *FAR* gene, and process options for increasing fatty alcohol production by *L. starkeyi*.

2. Materials and methods

2.1. Microorganisms and vectors

Lipomyces starkeyi NRRL Y-11557 was previously engineered to produce fatty alcohols by over-expression of the fatty acyl-CoA reductase (*FAR*) gene maqu_2220 (*MaFAR*_2220) from *Marinobacter aquaeolei* VT8 (Wang et al., 2016b). Briefly, the *Nat1* gene of *Streptomyces noursei* for resistance to nourseothricin was expressed using the native L. *starkeyi TDH3* promoter and *PGK1* terminator. The *MaFAR*_2220 gene was expressed by the native L. *starkeyi PYK1* promoter and *GAL1* terminator. The resulting plasmid was named pLS101–4.

2.2. Estimation of MaFAR_2220 gene copy number and transcription level in transformants

For real time qPCR to evaluate gene copy numbers, genomic DNA of the representative *L. starkeyi* transformants was extracted as described (Xu et al., 2017). The concentration of the extracted genomic DNA for qPCR was 20 ng/μL. In this study, the determination of gene copy number in selected transformants was done according to a method described by Weng et al.(Weng et al., 2004) using an ABI 7500 Real-Time PCR System (Thermo Fisher Scientific, Applied Biosystems, Grand Island, NY) and Power SYBR Green PCR Master Mix (Cat. no. 4367659, Applied Biosystems, Grand Island, NY). The endogenous eukaryotic initiation factor 5 (*eif5*) gene was used as a single-copy reference gene for comparison (Yamamoto et al., 2005). The qPCR reaction was performed as described (Xu et al., 2017) and the copy-number of the *MaFAR*_2220 gene versus the reference gene was calculated. For each genomic DNA sample, qPCR was run in triplicate and the copy numbers are presented as the mean with standard deviations < 0.2.

For evaluation of the transcription levels of *MaFAR*_2220, total RNA was extracted from ~60 to 80 mg (wet weight) cell pellets using Qiagen RNeasy Mini Kit (Valencia, CA). In the real time RT-PCR reaction, one microgram of purified total RNA was reverse-transcribed using High-Capacity cDNA Reverse Transcription Kit (Cat. no. 4368814, Applied Biosystems, Grand Island, NY) with random hexamers according to the manufacturer's instructions. Reactions were performed in triplicate. Relative transcription level was calculated from the Ct value of the reference or the *MaFAR*_2220 genes.

2.3. FAR enzyme activity assay

Activity of *MaFAR*_2220 expressed in *L. starkeyi* transformants was measured as described in literature (Willis et al., 2011). Briefly, the transformants were cultured for 48 h as described below. Cells were harvested by centrifugation followed by disruption by grinding in liquid nitrogen. Disrupted cell suspension (0.5 mL) was added to a reaction vessel along with 200 μM palmitoleyl-CoA and 800 μM NADPH in reaction buffer (pH 7.0) containing 20 mM Tris-HCl and 50 mM NaCl. Reactions were allowed to proceed for 1 h before extraction with 2 mL of dodecane. The dodecane-water mixture was separated by centrifugation and the dodecane phase was injected onto a GC for fatty alcohol quantitation as described below. All samples for analysis were run in triplicates and the activity data were presented as the mean.

2.4. Culturing MaFAR_2220 transformants for fatty alcohol production

The culture medium of *L. starkeyi FAR* transformants in 250 mL shake flask was as described in previous work (Wang et al., 2016b). The minimal medium contained (g/L): $(NH_4)_2SO_4$, 0.5; KH_2PO_4, 7.0; Na_2HPO_4, 2.0; $MgSO_4 \cdot 7H_2O$, 1.5; $CaCl_2 \cdot 2H_2O$, 0.1; $FeCl_3 \cdot 6H_2O$, 0.08; $ZnSO_4 \cdot 7H_2O$, 0.001; $CuSO_4 \cdot 5H_2O$, 0.0001; $CoCL_2 \cdot H_2O$, 0.0001; $MnSO_4 \cdot 5H_2O$, 0.0001. The carbon source was glucose (3% *w/v*). An overlay of 3 mL of dodecane was added to the cultures 48 h after the start of cultivation. For fatty alcohol quantitation, cultures were harvested after 6 d and centrifuged at 4000 rpm. The cell pellets, supernatant, and dodecane phase were separated after centrifugation.

Seed culture of the #6 transformant for inoculation of fermenters was prepared by picking a single colony into 25 mL of YPD broth in 125 mL baffled shake flasks at 30 °C and 220 rpm. After 36 h, 20 mL of culture was inoculated into 200 mL of the above mineral medium in a 1000 mL baffled shake flask and incubated in a shaker at 30 °C and 220 rpm.

Batch and fed-batch fermentation were performed in a 5-L bioreactor controlled by the BioFlo310 system (New Brunswick). 1.8 L of mineral medium having 5% glucose as the carbon source was inoculated with 200 mL of the seed culture grown for 36 h. The inoculated media in the bioreactor was then overlaid with 200 mL of dodecane. The fermentation culture was mixed at 300 rpm and purged with 1.0 vvm of sterile filtered air. The temperature and pH were maintained at 30 °C and 5.0 respectively. For fed batch fermentations, glucose or glucose and nitrogen were fed at various times as indicated on the figures. Periodic samples were taken for cell density, fatty alcohol, and glucose analysis.

2.5. GC–MS analysis for fatty alcohols

For the culture samples with dodecane overlay, the dodecane layer was recovered and was directly used for GC–MS analysis. For intracellular fatty alcohols in cell pellets, an extraction procedure similar to Steen et al' was used for extraction(Steen et al., 2010). The procedure was as described in previous work (Wang et al., 2016b).

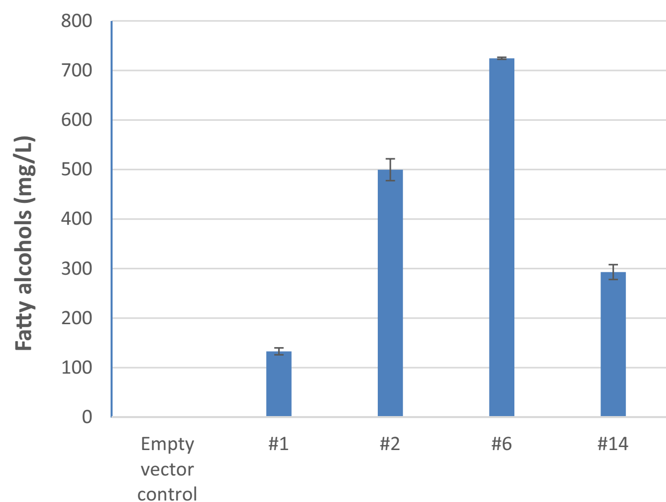

Fig. 1. Fatty alcohol production in selected FAR transformants.

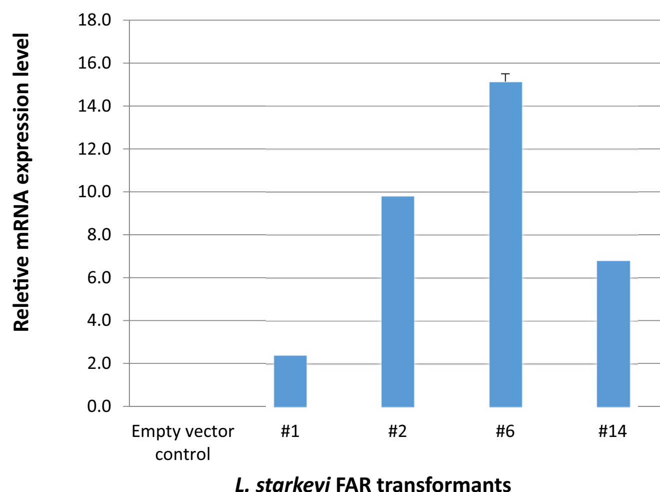

Fig. 2. mRNA expression levels of MaFAR_2220 in selected transformants.

Analysis for fatty alcohols was performed on a 7890 N Agilent GC system with an Agilent DB-Wax column, 30 m × 0.25 mm × 0.25 μm. The operation parameters were: 1 μL injection, split ratio 10:1, injector temp 250 °C, constant flow 1 mL/min He; oven ramp 100 °C for 1 min, 25 °C/min to 200 °C and hold for 1 min, 5 °C/min to 250 °C and hold for 7 min; FID detector at 280 °C, 40 mL/min H_2, 450 mL/min zero air, 30 mL/min makeup He, collection at 5 Hz. Saturated and unsaturated fatty alcohols were quantified using two fatty alcohol standards (NuChek GLC 32C and 34C). A similar program was run on the GC–MS to verify peak identities.

The fatty alcohol data from shake flask samples are referred to the fatty alcohols in dodecane phase unless otherwise noted. For bioreactor culture samples, we measured fatty alcohols in both dodecane and cell pellets. All samples in this study for fatty alcohol analysis were run in duplicates, and the standard deviations were < 0.3%.

3. Results and discussion

3.1. Characterization of L. starkeyi Transformants

3.1.1. Copy numbers and transcription levels of MaFAR_2220

We previously characterized 20 transformants as having different fatty alcohol production titers ranging from 0 to 770 mg/L (Wang et al., 2016b). Four transformants, #1, #2, #6, and #14, representing different production titers were selected for further study (Fig. 1). Real-time qPCR has been widely used to determine gene copy number in engineered organisms (Weng et al., 2004) (Liu et al., 2017; Schmittgen and Livak, 2008; Sekeli et al., 2013). These four transformants contained different copy numbers of the *MaFAR*_2220 gene compared to the single copy control eif5 gene (Yamamoto et al., 2005) (Table 1). These results suggest that gene copy number correlates with higher fatty alcohol titer. These results parallel typical strain engineering strategies in which inserting multiple copies of a gene of interest is a common and successful strategy for boosting production titers.

To determine the abundance of *MaFAR*_2220 mRNA in the transformants, RNA was extracted from the transformant strains and real-time RT-PCR analyses were conducted (Fig. 2). The results of this analysis parallel the results of the gene copy number analysis in which fatty alcohol titers correlate with gene copy numbers and mRNA levels.

Table 1
MaFAR_2220 gene copy numbers by qPCR in selected transformants.

	Transformant #1	Transformant #2	Transformant #6	Transformant #14
Gene copy number	1	4	6	3

qPCR was run in triplicate and the copy numbers are presented as the mean with standard deviations < 0.2.

3.1.2. FAR enzyme activity

In addition to gene copy numbers and mRNA expression levels, we also measured the activities of the *MaFAR*_2220 enzyme in selected transformants. Transformants #1, #6, and #14 representing relatively low, high, and medium fatty alcohol production levels respectively, were tested for enzyme activity. The highest enzyme activity of 33.5 mg/L/h was observed in the #6 transformant (Fig. 3), which is also the highest fatty alcohol producer. Similarly, enzyme activity in the #1 transformant, the lowest fatty alcohol producer among the three transformants, was barely detectable. This is most likely due to the lower expression of *MaFAR*_2220 in transformant #1.

3.2. Fatty alcohol production in bioreactors

To better control cultivation parameters, fatty alcohol production experiments were performed in bioreactors using the highest fatty alcohol producing #6 transformant. A single batch fermentation led to fatty alcohol production of nearly 1 g/L (dodecane phase) after 7 d (Fig. 4). Cell growth ceased and entered stationary phase after 5 d due to sugar depletion. The fermentation was allowed to continue given that we had previously observed fatty alcohols remaining inside cells after the completion of fermentation, and we want to understand if the intracellular fatty alcohols would

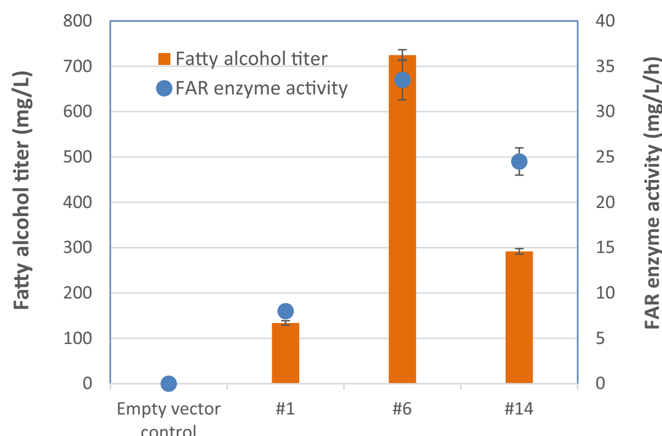

Fig. 3. MaFAR_2220 enzyme activities in selected transformants.

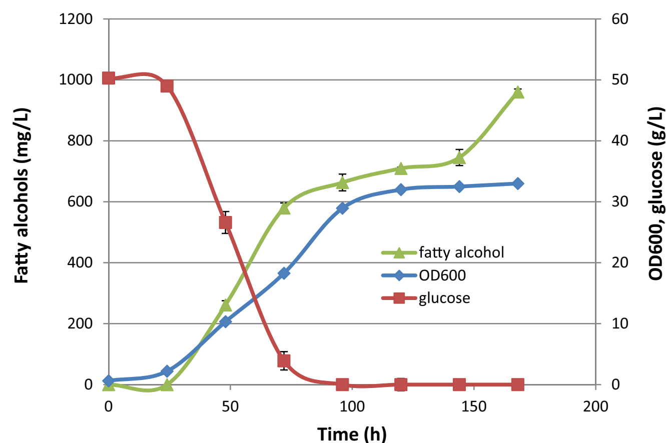

Fig. 4. Single batch production of fatty alcohols in 5-L bioreactor.

be secreted once carbon was exhausted. This continuation of the fermentation resulted in a sudden increase in fatty alcohol titer after approximately 48 h from the onset of stationary phase. Cells from the fermentation were then examined under the microscope (1000× magnification), where we noted that cell lysis was becoming prevalent. Thus, cell lysis in late stationary phase further increased fatty alcohol titer in prolonged stationary phase after depletion of glucose in the bioreactor.

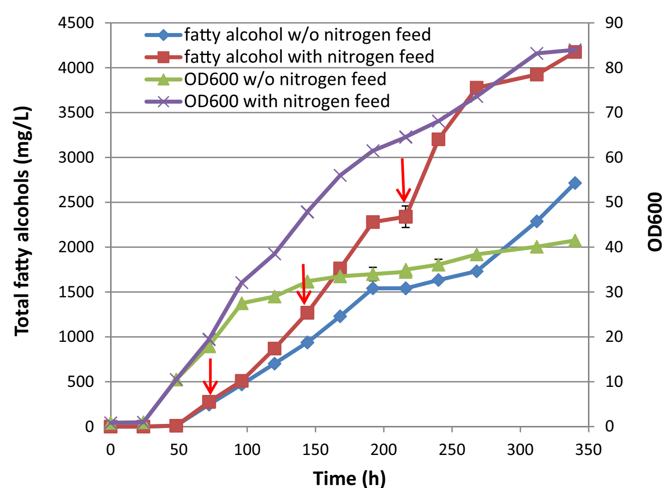

Fig. 5. Fatty alcohol production in fed-batch fermentations with and without nitrogen feeding. Arrows indicate feeding time points.

To achieve higher fatty alcohol production, fed-batch fermentations using two different feeding strategies were tested. Both fermentations started with 5% glucose as the carbon source and 0.5 g/L $(NH_4)_2SO_4$ as the nitrogen source. One fermentation strategy fed glucose at a concentration of 2.5% (i.e., adding 50 g of glucose to fermentation medium at each feeding), whereas the other strategy fed the same amount of glucose plus a limited amount of nitrogen (0.25 g/L $(NH_4)_2SO_4$, i.e., adding 0.5 g at each feeding). Compared to fermentation without nitrogen feeding, feeding glucose along with a limited amount of nitrogen proved to be an effective strategy for enhancing cell growth and fatty alcohol production (Fig. 5). This result suggests that it was essential to periodically replenish nitrogen to support cell growth and fatty alcohol production. We measured fatty alcohols both from the dodecane layer and cell pellets. The total fatty alcohol distributed among both the dodecane phase and intracellularly reached 4.2 g/L after 14 d (Fig. 6A, B) with approximately 80% being extracted during fermentation into the dodecane phase (Fig. 6B). This is thus far the highest observed fatty alcohol titer in L. *starkeyi* indicating that maintaining cell growth is important for continued fatty alcohol production. Maintaining cell growth by feeding a carbon source with a limited amount of nitrogen led to continued fatty alcohol production to achieve a high titer of fatty alcohols.

A 24-h lag in growth was initially observed. To test if an increased initial nitrogen content would increase initial growth rate and support high fatty alcohol production, a subsequent fermentation was initiated using a higher initial nitrogen concentration (10× higher nitrogen concentration of 5 g/L $(NH_4)_2SO_4$ compared to the fermentation depicted in Fig. 6) while glucose and $(NH_4)_2SO_4$ were fed in at the same concentration as previously. More cell mass was produced using this strategy and the lag phase was eliminated; however, less fatty alcohols (1650 mg/L) were produced (Fig. 7) compared to the previous fermentations using an initial limited nitrogen concentration. These results indicate that the nitrogen concentration is very important to achieving a high titer of fatty alcohols. Maintaining a low level of nitrogen concentration during fermentation (C/N ratio was 189 for fermentation with low initial nitrogen loading compared to C/N ratio of 41 with high initial nitrogen loading) would support cell growth while enhancing fatty alcohol synthesis with periodic doses of carbon and nitrogen.

Although a relatively high fatty alcohol titer was obtained (4.2 g/L) in the fed-batch fermentation, yield was low, 0.028 g fatty alcohol/g glucose which was far below the theoretical maximal yield (0.28 g fatty alcohol/g glucose). Carbons are mostly still directed to making cellular lipids. A down regulation of lipid synthesis is expected to further enhance fatty alcohol production and yield. This is what we will target in the following research along with the *lsku70* gene knockout to facilitate multiple gene deletions that has been demonstrated in *L. starkeyi* recently (Dai et al., 2019).

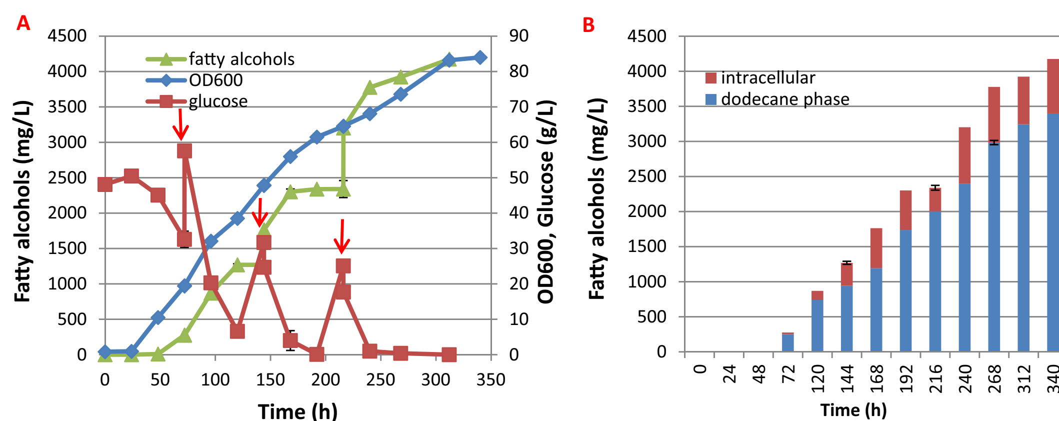

Fig. 6. Fatty alcohol production (A) and distribution (B) in fed-batch fermentation with nitrogen feeding. Arrows indicate feeding time points.

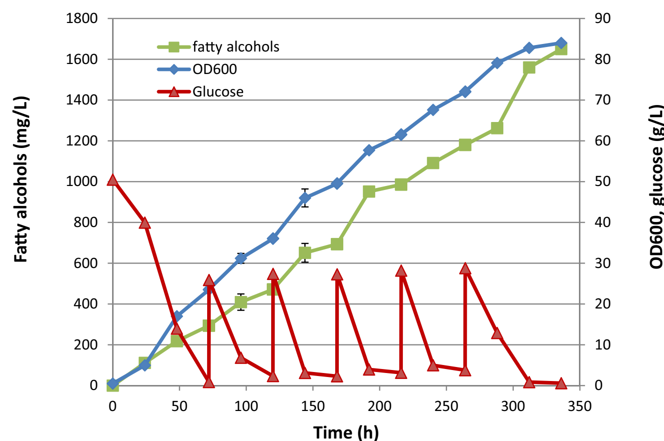

Fig. 7. Fatty alcohol production in fed-batch fermentation with a high initial nitrogen concentration.

4. Conclusions

Detailed studies of fermentation and subsequent fatty alcohol production designed to better understand the factors that accounted for the observed variability in production across our transformants were carried out. We observed that fatty alcohol production is dependent on gene expression level, copy number, and the activity of the *MaFAR*_2220 gene as expressed in *L. starkeyi*. Increasing gene copy number may further enhance fatty alcohol production. Process options were also explored to boost fatty alcohol production. Using a fed-batch strategy of periodic carbon and nitrogen feeding, we achieved the highest titers yet in *L. starkeyi*. In addition, we discovered that maintaining nitrogen level is important in fatty alcohol production. Thus, this study provides a baseline for further investigations into the genetic mechanism (e.g., transcriptomics, proteomics, metabolomics) of fatty alcohol production and the connection to TAG accumulation may lead to the identification of additional beneficial genetic targets in an effort to further improve production.

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Authors' contributions

WW, EK, HW, MZ, and MEH conceived and designed the experiments. WW, EK, HW, QX, and CYL designed the constructs, conducted genetic transformation, and transformants characterization. SVW conducted FAME and fatty alcohol analysis. TVW performed the bioreactor fermentation. WW and EK drafted the manuscript. HW, MZ, and MEH contributed to the manuscript preparation. All authors read and approved the final manuscript.

Declaration of competing interest

The authors declare no competing interests.

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