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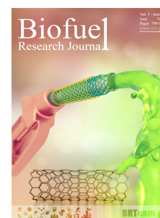

Original Research Paper

Lipid accumulation from glucose and xylose in an engineered, naturally oleaginous strain of *Saccharomyces cerevisiae*

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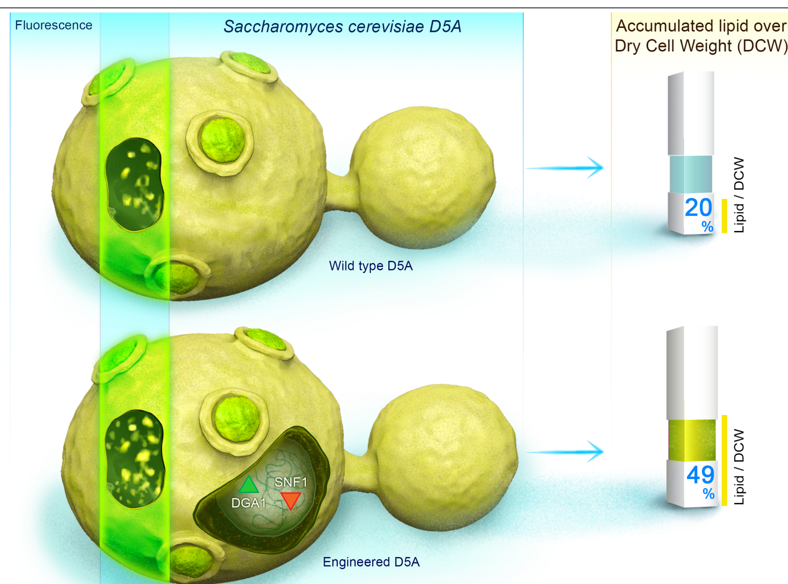
HIGHLIGHTS

- Discovered a strain of *Saccharomyces cerevisiae* that is naturally oleaginous.
- The engineered strain can utilize the biomass-derived sugars glucose and xylose to concurrently accumulate lipids and produce ethanol.
- Enables efficient use of biomass-derived sugars for biofuels production.

GRAPHICAL ABSTRACT



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ABSTRACT

Saccharomyces cerevisiae, a well-known industrial yeast for alcoholic fermentation, is not historically known to accumulate lipids. Four *S. cerevisiae* strains used in industrial applications were screened for their ability to accumulate neutral lipids. Only one, D5A, was found to accumulate up to 20% dry cell weight (dcw) lipids. This strain was further engineered by knocking out ADP-activated serine/threonine kinase (*SNF1*) which increased lipid accumulation to 35% dcw lipids. In addition, we engineered D5A to utilize xylose and found that D5A accumulates up to 37% dcw lipids from xylose as the sole carbon source. Further we over-expressed different diacylglycerol acyltransferase (*DGA1*) genes and boosted lipid accumulation to 50%. Fatty acid speciation showed that 94% of the extracted lipids consisted of 5 fatty acid species, C16:0 (palmitic), C16:1n7 (palmitoleic), C18:0 (stearic), C18:1n7 (vaccenic), and C18:1n9 (oleic), while the relative distributions changed depending on growth conditions. In addition, this strain accumulated lipids concurrently with ethanol production.

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1. Introduction

Saccharomyces cerevisiae is well known for robust ethanolic fermentation of various pre-treated lignocellulosic feedstocks for renewable fuel production. The two principle monomeric sugars released during pretreatment and enzymatic saccharification of these feedstocks are glucose and xylose. The *S. cerevisiae* strain D5A has previously been used to ferment pre-treated switchgrass, rice straw, distiller's grains, and lodgepole pine feedstocks (Bailey et al., 1982; Geddes et al., 2011) and was found to be tolerant to hydrolysate products present in pre-treated hardwoods and to 1% (v/v) butanol (Ranatunga et al., 1997; Knoshaug and Zhang, 2009). However, D5A, like all wild type *S. cerevisiae* strains, is unable to utilize xylose as a carbon source. Through the expression of the bacterial xylose isomerase (Karhumaa et al., 2006; Brat et al., 2009; de Figueiredo Vilela et al., 2013) or fungal xylose reductase and xylitol dehydrogenase pathways (Kotter et al., 1990; Bettiga et al., 2009; Bera et al., 2010), *S. cerevisiae* has been engineered to utilize xylose to produce ethanol.

The classical definition of an oleaginous yeast is one that accumulates greater than 20% dry cell weight (dcw) as lipids. *S. cerevisiae* is not known as being oleaginous and typically only accumulates 10-15% dcw lipids (Turcotte and Kosaric, 1989; Ratledge and Wynn, 2002; Kamisaka et al., 2007; Meng et al., 2009; Runguphan and Keasling, 2014; Greer et al., 2015) whereas oleaginous yeasts typically accumulate 25% to greater than 60% dcw lipids (Ageitos et al., 2011). Recently engineering efforts to increase lipid content have been made in oleaginous yeasts and one target has been the protein kinase *SNF1* gene. In *S. cerevisiae*, *SNF1* encodes an ADP-activated serine/threonine kinase (Mayer et al., 2011) that, in addition to its role in regulating carbon homeostasis and general stress responses, regulates genes involved in lipid synthesis and nitrogen metabolism (Usaite et al., 2009; Chumnanpuen et al., 2012) and has been shown to regulate the first committed step of fatty acid synthesis by directly phosphorylating and thus inactivating acetyl-CoA carboxylase (Mitchelhill et al., 1994; Woods et al., 1994). $Snf1^-$ mutants have also been shown to be defective in glycogen synthesis (Thompson-Jaeger et al., 1991; Hardie et al., 1998) and β-oxidation of lipids (Zaman et al., 2008; Usaite et al., 2009), and have shown up-regulation of genes involved in lipid biosynthesis particularly those leading to an increase in the acetyl-CoA pool and the downstream fatty acid synthases and glycerol-3-phosphate dehydrogenase that work in concert to turn that pool into neutral lipids (Seip et al., 2013). In addition, a *SNF1* knock-out in the oleaginous yeast *Yarrowia lipolytica* showed increased fatty acid accumulation over that of wild type in both nitrogen replete and deplete conditions (Seip et al., 2013).

Previous engineering efforts in *S. cerevisiae* showed a promising increase in lipid content by over-expressing a type 1 plant DGA (Greer et al., 2015). Total lipid production from glycerol was less than 12% dcw but represented nearly a doubling over empty vector control (Yu et al., 2013), and up to 17% dcw lipids, a four-fold increase by over-expression of *FAS1*, *FAS2*, and *ACC1* with small amounts (up to 4 g/L) of ethanol being produced (Runguphan and Keasling, 2014). Previous work has shown that a *SNF2* delete strain with over-expression of *DGA1* or a *DGA1* variant lacking the N-terminal 29 amino acids could accumulate lipids up to 45% and 50% dcw on 5% and 10% glucose, respectively (Kamisaka et al., 2007; Kamisaka et al., 2013). The gene *SNF2* codes for an ATPase subunit of the *SWI-SNF* chromatin remodeling complex (Peterson and Tamkun, 1995). Another engineering effort in *S. cerevisiae* showed a 63% increase in intracellular lipids by over-expression of native *GPD1* and *ACC1* from *Lipomyces starkeyi* (Wang et al., 2016).

Here we report the discovery of an oleaginous strain of *S. cerevisiae* which, through further genetic engineering, now utilizes the lignocellulosic biomass derived xylose monomer to produce ethanol concurrently with increased lipid accumulation through a *SNF1* knock-out and *DGA* over-expression. In this manuscript, we demonstrate high lipid accumulation in a *SNF*1 knock-out *S. cerevisiae* strain which is also capable of utilizing xylose presenting an opportunity to produce a lipid feedstock from cellulosic biomass.

2. Materials and Methods

2.1. Yeast strains, media, and seed culture growth conditions

The *S. cerevisiae* strains used in this study are listed in **Table 1**. Yeast strains were grown in YPD (Difco) at 30ºC with shaking at 225 rpm for seed culture generation and strain maintenance. For lipid accumulation, yeast strains were grown in yeast nitrogen base (YNB) (Sigma Y-0626) containing 5% glucose and 5 mM NH_4^+. For shake flask studies, cultures were grown at 30ºC with shaking at 225 rpm. Seed cultures were grown in 100 mL YPD in a 500 mL baffled shake flask overnight. For lipid accumulation, yeasts were grown in 300 mL of media in a 1 L baffled flask in duplicate inoculated with washed cells from an overnight YPD seed culture to an initial OD_{600} of 1. Cultures were incubated at 30ºC at 225 rpm. At each time point, 45 mL of culture was collected. From this sample, 5 mL were removed for OD_{600}, HPLC, and YSI analysis and the remaining 40 mL of culture was pelleted and frozen at -80ºC for *in-situ* FAME analysis.

Table 1.
S. cerevisiae strains used in this study.

Yeast strain	Relevant genotype	Reference
AGL	WT	Angel Yeast Co. Ltd.
D5A	WT	Bailey et al. (1982)
Fali	WT	Broin, Inc.
PE-2	WT	Basso et al. (2008)
BFY692	D5A; *GAL80*Δ::*Ps*XYL1-*Ps*XYL2-*Sc*XKS	This study
BFY709	BFY692; $Snf1^-$	This study
BFY742	BFY709; Sc*DGA1*	This study
BFY746	BFY709; ScDga1ΔNp	This study
BFY748	BFY709; Ls*DGA1*	This study

2.2. Genetic methods

The xylose reductase (*XYL1*), xylitol dehydrogenase (*XYL2*), and xylulose kinase (*XKS*) expression construct with 500 bp of GAL80 targeting flanking sequences was synthesized (DNA2.0). The *PGK1* promoter and *GAL10* terminator were used to drive *XYL1* expression. The *TDH3* promoter and *GAL2* terminator were used to drive *XYL2* expression and the *PGI1* promoter and *PDC1* terminator were used to drive *XKS1* expression. The construct was isolated from the vector with the restriction enzyme *NotI* and 1 µg of the gel-purified expression construct DNA was transformed into *S. cerevisiae* D5A for integration at *GAL80*.

The *SNF1* knockout was generated in two steps. Two *SNF1*-targeting constructs were built either having G418 or hygromycin B as the selection markers. For integrative targeting, 690 and 400 bp of sequence upstream and downstream of the *SNF1* locus, respectively, were added to either end of the constructs. In both constructs, the selection marker was driven by the *PGK1* promoter and the *GAL2* terminator.

The various DGA genes were PCR amplified (Sc*DGA1* (YOR245C)), or synthesized as a geneblock (Integrated DNA technologies Inc.) (Ls*DGA1* (synthesized with 3 introns removed, gm1.6201_g)). The Sc*DGA1*-truncated (Kamisaka et al., 2013) was constructed using PCR by amplifying the entire wild type Sc*DGA1* vector using primers starting on either side of the region to be eliminated and having 15-bp overlaps with each other to utilize Gibson assembly (New England Biolabs Inc., cat no. E2611S) to close the circle. All DGA genes were expressed by the *TDH3* promoter and *GAL2* terminator.

Transformation of linear or plasmid DNA into *S. cerevisiae* D5A was performed using a DMSO-enhanced lithium-acetate protocol (Hill et al., 1991) with the following modifications. Cells were initially washed in water. Six-hundred µL of PEG4000 solution was added and just prior to heat shocking, 70 µL DMSO was added. Cells were heat-shocked for 15 min at 42ºC and the last wash step was eliminated. Cells were re-suspended in 10 mM Tris-EDTA and plated on appropriate selective plates. Electrotransformation of *E. coli* Dh5α was performed as described (Invitrogen 11319-019) and plated on LB plates containing 100 µg/mL ampicillin. *E. coli* plasmid DNA was isolated using a plasmid spin mini-prep kit (Qiagen Inc., cat no. 27106).

2.3. Analytical methods

Concentrations of sugars and ethanol were measured using a high performance liquid chromatograph (HPLC) equipped with an HP refractive index detectors (Agilent Technologies, Palo Alto, CA). A Bio-Rad HPX-

87H organic acids column and H+ guard column (BioRad Laboratories, Hercules, CA) operated at 55 °C were used. The eluent was 0.01 N H_2SO_4 at a flow rate of 0.6 mL min^{-1}. Samples and standards were filtered through 0.45 mm nylon membrane syringe filters (Pall Corp., East Hills, NY) prior to injection onto the column.

Intracellular lipid accumulation was measured as fatty acid methyl esters (FAMEs) after *in situ* transesterification of the endogenous lipids to FAME followed by GC analysis as follows: 7 to 10 mg of lyophilized microbial biomass (dried overnight at 40°C under vacuum) was homogenized with 0.2 mL of chloroform:methanol (2:1 v/v), and the resulting solubilized lipids were transesterified *in situ* with 0.3 mL of HCl:methanol (5%, v/v) for 1 h at 85°C in the presence of tridecanoic acid (C13) methyl ester as an internal standard. FAMEs were extracted with 1 mL of hexane at room temperature for 1 h and analyzed by gas chromatography:flame ionization detection (GC:FID) on an Agilent (Santa Clara, CA, USA) 6890N with a DB-WAX column with dimensions 30 m × 0.25 mm i.d. and 0.25 µm film thickness. Individual fatty acids were identified by mass spectrometry for the location of the unsaturation of the fatty acid positional isomers. The FAMEs were quantified based on a 37-FAME calibration mixture (Supelco, certified reference material, CRM47885, Sigma-Aldrich, St. Louis, MO, USA) after normalizing for the internal standard. The sum of the individual fatty acids was calculated and expressed as weight % of dry biomass (Laurens et al., 2012).

2.4. Imaging

Images were acquired by staining a 100 uL cell suspension in water with 1 uL of Nile Red dissolved in acetone at 250 ug/mL. Cells and stain were allowed to incubate for 5 min prior to imaging. Images were collected on a Nikon epifluorescent microscope with 100× objective. Cells were illuminated by a mercury lamp with 480/40nm band pass excitation and 505 nm long pass emission filter sets.

3. Results and Discussion

3.1. Identification of naturally oleaginous S. cerevisiae

Four strains of *S. cerevisiae* previously used in industrial scale ethanol fermentations were characterized for lipid accumulation (**Fig. 1A**). Unique among these four strains was D5A which accumulated 20% FAME on a dcw basis in a nitrogen limited culture (5mM NH_4 initial concentration) compared to less than 15% dcw FAME for the other 3 strains. The rates of glucose utilization and ethanol production were similar among the four strains except for PE-2 which produced significantly more ethanol (**Fig. 1B**). PE-2 is known to give higher ethanol titers (Basso et al., 2008). Ethanol begins to disappear after 48 h due to the aerobic nature of the culture. The strains PE-2 and D5A also had lower final optical densities due to carbon being funneled to ethanol or lipids, respectively, rather than cell growth.

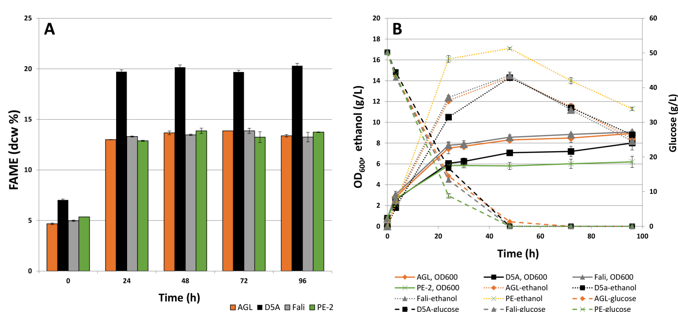

Fig.1. Comparison of industrial strains of *S. cerevisiae*. **A:** Lipid accumulation (% dcw FAME). **B:** Optical density, solid lines; glucose, dashed lines; and ethanol, dotted lines.

3.2. Engineering of S. cerevisiae for xylose utilization and increased lipid accumulation

With the identification of D5A as naturally oleaginous, we decided to further develop the strain for biofuels production by focusing on the strain's pentose sugar utilization. Typical *S. cerevisiae* is not natively competent to utilize xylose, thus we engineered D5A by integration of a three-gene xylose utilization expression cassette. An expression construct harboring *XYL1* and *XYL2* from *Pichia stipitis* and *XKS1* from *S. cerevisiae* was integrated at the chromosomal *GAL80* locus completely replacing the *GAL80* open reading frame to create strain BFY692. Complete replacement of *GAL80* was confirmed by PCR. Insertion at the *GAL80* locus served to remove Gal80p repression of the galactose catabolic genes when glucose is present providing active expression of the Gal2p transporter. The Gal2p transporter has been well characterized as a major transporter of the pentose sugars xylose and arabinose, as well as glucose and galactose (Hamacher et al., 2002; Sedlak and Ho, 2004; Salheimo et al., 2007; Knoshaug et al., 2015). Engineering of the *S. cerevisiae* D5A strain for xylose utilization has been previously described (Hector et al., 2011), however, our strategy differed in that we targeted integration of our three-gene expression cassette to knock-out a negative regulator of a key pentose transporter (Gal2p) to ensure effective expression of this transporter in the presence of glucose. We then further engineered BFY692 for increased lipid accumulation by deleting the global regulator *SNF1* as it has previously been shown to play an important role in lipid accumulation in both *S. cerevisiae* and *Y. lipolytica* (Chumnanpuen et al., 2012; Seip et al., 2013). This required a two-step process as D5A is a diploid yeast (Bailey et al., 1982). We first knocked out one copy using a *SNF1* targeted G418 resistance integration cassette, then the other copy using a similarly targeted Hygromycin B resistance integration cassette. Integration at one locus, then the other was confirmed by PCR. When grown for lipid accumulation in a nitrogen-limited culture, 34% dcw FAME accumulated in the double *SNF1* knock out strain BFY709 compared to 23% in the BFY692 parent (**Fig. 2A**). It was interesting to note that to realize an increase in lipid accumulation, both copies of *SNF1* had to be knocked out indicating that *SNF1* is a dominant gene, as there was no intermediate response with only one copy of *SNF1*. In these conditions, one copy of *SNF1* was adequate to perform its regulatory duties blocking increased lipid accumulation. In addition, though the double *SNF1* knock-out strain accumulates considerably more lipids than the parent, nearly the same amount of ethanol is produced by the end of the fermentation (**Fig. 2B**).

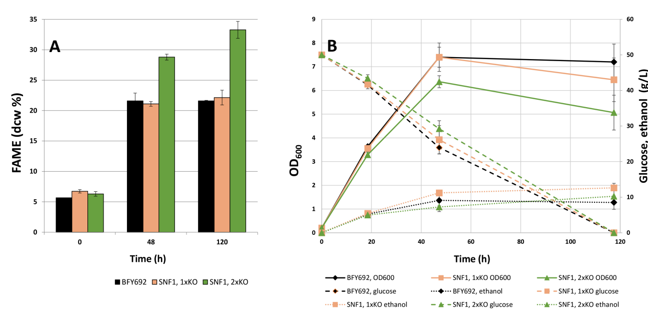

Fig.2. Lipid, optical density, and glucose utilization in aerobic shake flasks. *S. cerevisiae* strains: **A:** Lipid accumulation. **B:** Optical density, solid lines; glucose utilization, dashed lines; ethanol production, dotted lines.

Glucose utilization and growth rate between the parent strain and the single or double $Snf1^-$ strains were similar with the double $Snf1^-$ strain reaching a slightly lower OD_{600}. The lower cell density could possibly be explained by the funneling of more of the available carbon into lipid accumulation rather than cell growth in the double knock-out strain. It is

important to note that even though these were aerobic shake flasks, ethanol was produced and later metabolized to some degree, showing that ethanol and lipids can be produced simultaneously. With the decrease in glycogen synthesis and β-oxidation and the increase in the acetyl-CoA pool due to the loss of Snf1p regulation of these pathways, a plausible mechanism for the increase in lipid accumulation is that carbon flux now funnels into the *de-novo* lipid synthesis pathway while blocking lipids from oxidation.

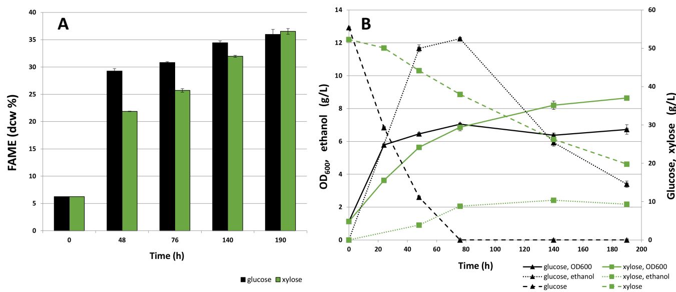

Fig.3. Lipid and ethanol production, growth, and sugar utilization by *S. cerevisiae* BFY709 in aerobic shake flasks. **A:** Lipid accumulation. **B:** Optical density, solid lines; sugar utilization, dashed lines; and ethanol production, dotted lines.

Strain BFY709 accumulated up to 36% FAME dcw from xylose as the sole carbon source (Fig. 3A). The accumulation of lipids from xylose took longer than when grown on glucose while ethanol production from xylose was much lower (Fig. 3B). Up to 0.5 g/L xylitol were also produced when grown on xylose. Cell density was slightly higher when grown on xylose while the consumption of xylose was considerably slower than the consumption of glucose with nearly half of the xylose still present after 190 h of incubation (Fig. 3B). This data suggests that carbon liberated from xylose is first shunted to lipid synthesis, with ethanol production being secondary. Using the introduced pathway, xylose is converted to D-xylulose-5-phosphate which then directly enters the pentose phosphate pathway (PPP). Glyceraldehyde-3-phosphate is readily isomerized to DHAP which can then enter the lipid pathway by conversion to glycerol-3-phosphate. Previous work has shown that *S. cerevisiae* has insufficient levels of TAL1 to efficiently ferment xylose (Walfridsson et al., 1995; Hasunuma et al., 2011), causing a reduced capacity of flux through the PPP and a build-up of sedoheptulose-7-phosphate (Kotter and Ciriacy, 1993). Reduced oxidative PPP flux improves ethanol yield (Jeppsson et al., 2002) suggesting that having a slower carbon flux through the PPP allows time for the lipid production pathway enzymes to siphon off carbon and shunt it to lipids rather than ethanol. However, previous research has also shown that sufficient flux through the PPP is needed for NADPH regeneration for increased lipid production in *Y. lipolytica* (Wasylenko et al., 2015). To further complicate this issue, it has also been shown that the non-oxidative PPP controls the rate of xylulose fermentation but not xylose fermentation (Johansson and Hahn-Hagerdal, 2002) suggesting that upstream barriers to xylose fermentation are in transport, xylose reductase, xylitol dehydrogenase, or xylulokinase. Clearly xylose (carbon) flux through the PPP is slow in our engineered strain and for unknown reasons leads to high lipid content (as high as glucose in the same time frame) at the expense of ethanol production even when the strain is unable to efficiently ferment all of the available xylose. The slower rate and inability to effectively utilize all of the xylose is likely due to exhaustion of the available nitrogen in the media as the fermentation was conducted in minimal media having an initial NH_4^+ concentration of only 5 mM. This low initial amount of NH_4^+ is typically reduced to undetectable levels within 6 h after inoculation (data not shown). In addition, ethanol production appears to cease while internal lipids continue to increase after 80 h. However, in aerobic flasks, it is possible that ethanol metabolism is occurring and thus reducing accumulation in the media though this is unlikely as xylose continues to decrease linearly. Given the complexities of possible carbon flux scenarios for glucose and xylose above, studies are underway to elucidate the basis for oleaginicity in this naturally oleaginous *S. cerevisiae* strain D5A. To visualize the internal lipids, Nile Red was used to stain lipid bodies within the cells after 76 h of growth on glucose or xylose (Fig. 4). Side-by-side images display two different focal planes within the same cells showing that multiple layers of lipid bodies exist throughout the cells but there were no large differences in lipid body size, number, or distribution observed between cells grown on glucose or xylose.

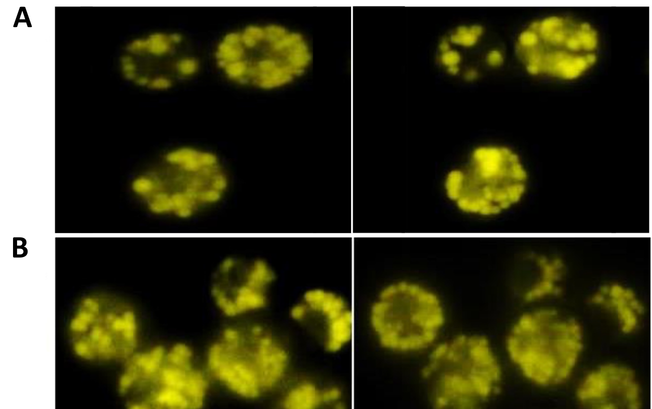

Fig.4. Nile Red stained accumulated lipids in cells of *S. cerevisiae* BFY709. **A:** glucose or **B:** xylose. The two panels show the same cells with different focal planes illustrating the different internal layering of stained lipid bodies.

To further increase lipids, we over-expressed DGA (BFY742, Sc*DGA1*), a truncated version (BFY746, ScDga1ΔNp, (Kamisaka et al., 2013)), and a DGA from *L. starkeyi* (BFY748, Ls*DGA1*) in strain BFY709 (Fig. 5). We observed up to 50% dcw FAME when over-expressing the native *DGA1* or the truncated version and 45% dcw FAME with *L. starkeyi DGA1*. High levels of lipids were also seen at an increased temperature of 37ºC though slightly lower than lipid levels at 30ºC. The optical densities ($OD_{600} < 8$), glucose utilization, and ethanol production (<14 g/L) for these strains were similar to those seen in previous experiments for D5A strains (data not shown).

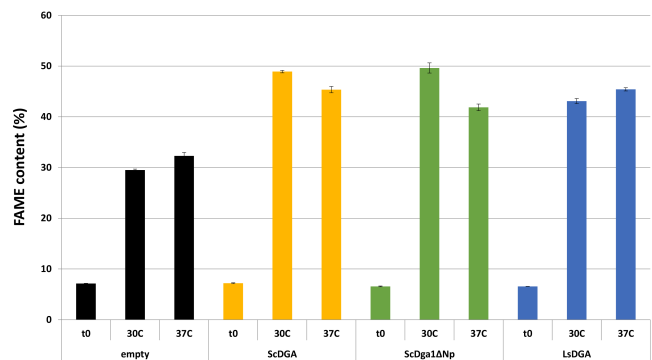

Fig.5. Maximum lipid content in D5A expressing different *DGA1* genes in seed cultures at t0 and 30 or 37ºC.

3.3. Fatty acid distribution in S. cerevisiae

The distribution of fatty acids changed over the course of the fermentations. In all, 21 different fatty acids species were found yet only 5

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fatty acid species, C16:0 (palmitic), C16:1n7 (palmitoleic), C18:0 (stearic), C18:1n7 (vaccenic), and C18:1n9 (oleic) made up >94% of the total fatty acids (**Fig. 6**). Over the course of the fermentations, when glucose was the sole carbon source in a defined media, C16:1n7 decreased, C18:0 and C18:1n9 increased, and C16:0 and C18:1n7 acids did not change appreciably. In contrast, when xylose was the sole carbon source in defined media, C16:1n7 and C18:1n9 decreased, C16:0 and C18:0 increased, while C18:1n7 remained unchanged signaling a small shift from unsaturated to saturated (**Fig. 6**). Our fatty acid speciation data agrees with previously reported values of C16:1 and C18:1 being the main species present (>72%), with C16:0 and C18:0 making up the bulk of the remainder (Kamisaka et al., 2013). The fatty acid speciation was different from that of oleaginous yeast of the *Lipomycetaceae* family in that C16:0 was typically between 15-50% whereas C16:1 was typically <10% for the majority of the strains with the exception of some species of *Myxozyma* (Van Rensburg et al., 1995). Recent fatty acid engineering efforts have shown the potential to change levels of different species of the fatty acids present in cells making possible designer ratios of lipids and fatty acid chain lengths (Tang et al., 2013) as well as efforts to produce free fatty acids, fatty alcohols, and fatty acid ethyl esters (Runguphan and Keasling, 2014). Clearly with the range of facile engineering tools and wealth of information available for *S. cerevisiae*, this yeast is being actively developed and has a huge potential for producing lipid-based fuels, designer lipids, fatty acids, and other hydrocarbon-based chemicals.

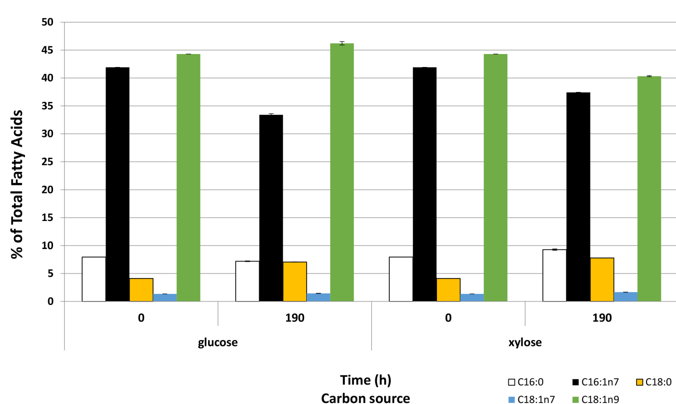

Fig.6. Changes in fatty acid speciation over time in glucose or xylose.

4. Conclusions

In conclusion, we have developed an oleaginous *S. cerevisiae* strain that concurrently produces high titers of ethanol with up to 50% lipids from either glucose or xylose. As interest in renewable biofuels continues to increase, genetic engineering efforts will continue in *S. cerevisiae* to develop next-generation biocatalysts with expanded product ranges from a variety of feedstock sugars.

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