Sustainable production of lipids from cocoa fatty acid distillate fermentation driven by adaptive evolution in *Yarrowia lipolytica*

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Abstract

Single cell oil production using oleaginous yeasts is a promising alternative to animal and plant-derived lipids. But substrate costs for microbial fermentation are a major bottleneck. Using side streams as alternative to substrates like glucose, for growing yeast, is a potential cost-effective solution. By combining a previously reported process of growing yeasts on a solid cocoa fatty acid distillate side stream with adaptive evolution techniques, the growth of oleaginous yeast *Yarrowia lipolytica* was improved by 2-fold. The lipid titre was also boosted by more than 3-fold. Using transcriptomics, key genes were identified that are possibly involved in tailoring of lipid composition, side stream utilisation and enhancement of lipid titres. Candidate genes were also identified that might enable efficient growth and utilization of fatty acids and triacylglycerides found in cocoa fatty acid distillate. In summary, this research has improved the understanding of side stream utilisation for lipid production in oleaginous yeast.

Keywords: Single Cell Oil (SCO), Waste Lipids, RNA-seq, Oleaginous Yeasts, Valorization

Introduction

Lipids are ubiquitous components of dietary food, which not only serves as a source of essential nutrients, but they also generate food flavors and off-flavors (Shahidi and Hossain, 2022). Currently, most food oils and fats are derived from animal and plant sources. However, limited availability of agricultural land coupled with unsustainability of livestock farming has inspired global efforts to build alternative lipid production platforms. Microbial lipids or Single Cell Oil (SCO) are a promising sustainable alternative lipid production platform (Silva et al., 2023). The choice of the microorganism is critical for the success of a microbial lipid production platform. It should be a robust natural lipid producer that grows on a variety of substrates and is amenable to large scale fermentation. Oleaginous yeasts like *Yarrowia lipolytica, Rhodotorula toruloides, Lipomyces starkeyi and Rhodotorula glutinis* are good producers of lipids. Oleaginous yeasts can be up to 70% lipids in dry weight, of which 80-90% are triacylglycerols (TAGs) (Ageitos et al., 2011). The lipogenic pathway in the model organism *Y. lipolytica* has been extensively studied and subjected to a plethora of strain and process engineering strategies (Abdel-Mawgoud et al., 2018; Park and Nicaud, 2020).

Commercialization of microbial lipids can be limited by their high production costs. Lipid production by oleaginous microbes has traditionally been carried out extensively with glucose as a carbon source. However, glucose is an expensive substrate and is responsible for up to 80% of the medium costs and 60% of the production costs (Fei et al., 2011). Usage of waste side streams as substrates can be an effective strategy to lower production costs. *Y. lipolytica* has previously been shown to grow and produce lipids when fed with hydrophobic substrates like hydrocarbons, glycerol, waste cooking oil, industrial fats like stearin or volatile fatty acids (Fukuda, 2023; Gao et al., 2020;

Katre et al., 2017; Tsirigka et al., 2023). Majority of these studies thus far have focused on valorisation of hydrophobic substrates from the petrochemical industry, especially alkanes (Fickers et al., 2005). In contrast, residues from the agri-food sector have been relatively understudied and tailored more towards biodiesel production. Although there have been reports of cultivating *Y. lipolytica* in vegetable oils composed mainly of unsaturated fatty acids (Katre et al., 2017), studies on growth in solid lipid residues high in saturation are extremely sparse (Lad et al., 2022).

Fatty acid distillates (FAD) are solid lipid residues produced from deodorization of vegetable oils like palm oil or by cocoa degumming process. FADs can contain up to 85% fatty acids and hence can be an effective carbon source replacement (Radzuan et al., 2018; Tan et al., 2007). FADs have been used for various purposes such as production of animal feed, cosmetics and oleochemicals (Abdul Kapor et al., 2017). They have better theoretical conversion efficiency and shorter metabolic pathways to lipids, compared to sugar-based carbon sources (Dulermo et al., 2015; Gong et al., 2016; Lian et al., 2012; Onésime et al., 2022). Understanding the mechanism of transport of fatty acids into the cell and its subsequent activation has been an area of active research (Dulermo et al., 2015; Onésime et al., 2022). In a recent report, a solid side stream cocoa fatty acid distillate (CFAD), rich in saturated C16 and C18 fatty acids was used to produce microbial lipids. It was demonstrated using two different oleaginous yeasts, namely *R. toruloides* and *Y. lipolytica*. In *Y. lipolytica* strain, the biomass achieved in a bioreactor was 36.6 ± 1.9 g/L with a lipid titre of 3.4 ± 0.2 g/L and lipid yield of 0.17 ± 0.01 g/g. CFAD contains 95% lipids with 78% being Free Fatty Acid (FFA) and 17% TAG. It also has high amount of caffeine (5% by weight) (Peterson et al., 2023). As caffeine is a known growth inhibitor of yeast cells (Ruta and Farcasanu, 2020), higher amounts of CFAD waste could inhibit biomass production.

To enable the use of CFAD for large-scale single cell protein and lipid production, it is therefore necessary to relieve the growth inhibition caused by factors such as caffeine in the media.

Adaptive laboratory evolution (ALE) is a powerful technique to generate microbes with improved growth rates and stress tolerance in sub-optimal and unconventional media (Fernandes et al., 2023; Menegon et al., 2022). In ALE, the strains are exposed to prolonged stress or selection pressure (e.g., nutrient limitation, pH etc) through multiple generations. Over time the fitness of the population improves due to accrued beneficial mutations and rewiring of metabolism. The resulting populations are screened to isolate superior performing single colonies (Fernandes et al., 2023). Various studies have reported the use of ALE to grow microbes on non-conventional carbon sources (Liu et al., 2021; Menegon et al., 2022; Tsirigka et al., 2023). ALE of *Y. lipolytica* in the presence of 15% crude v/v glycerol as substrate resulted in a 3.6-fold increase of dry biomass and 1.6-fold increase of lipid concentration compared to the wild type strain. Differential gene expression analysis revealed key adaptation mechanisms and after 77 generations, 30% increase in lipid content was reported (Tsirigka et al., 2023).

In this work, lipid production in oleaginous yeast strain was improved using CFAD as carbon source without co-feeding ethanol. Using ALE technique, an evolved strain was developed that grows efficiently at higher concentrations of CFAD. Importantly, transcriptomic analysis of evolved strains was performed to generate insights into their mechanisms of adaptation and enhanced lipid production in high CFAD.

Methods and Materials

Strains, media and materials

Oleaginous yeast strains used in this study were *Rhodotorula toruloides* – strains ATCC 10788 and ATCC 20406 (American Type Culture Collection, Manassas, Virginia, United State of America) and *Yarrowia lipolytica* – strains CBS 2070 and CBS 2072 (*CBS*-KNAW *culture collection*, Westerdijk Fungal Biodiversity Institute, Utrecht, Netherlands). The Cocoa Fatty Acid Distillate (CFAD) medium used was made as follows: 10 g/L yeast extract, 20 g/L peptone, and different concentrations of CFAD side stream for ALE experiments. For Yeast Peptone Dextrose (YPD) medium which is the standard lab media for yeast growth, 10 g/L of yeast extract, 20 g/L peptone and 20 g/L glucose was used. Yeast extract and peptone were purchased from BD culture media, and D-Glucose and Agar from Sigma-Aldrich. Yeast DNA was extracted with YeaStar Genomic DNA Kit (Zymo Research) as per the manufacturer's protocol. Other reagents include dimethyl sulfoxide, and Phosphate Buffer Saline (PBS) diluted to 1X.

Cocoa fatty acid distillate media preparation

The CFAD side stream was obtained from Tiong Lam Supplies Pte. Ltd., Singapore. The feed was prepared as per previous report (Peterson et al., 2023) with some modifications. In short, the CFAD side stream was sterilised by autoclaving at 121^{o} C and 115 PSI for 20 minutes and stored at -20°C until further use. CFAD was weighed out at desired concentration in grams and then added to the autoclaved YP media. The media was prepared fresh every day before use. It was then placed in the 65°C oven for the solid to melt. The resulting CFAD containing media was vortexed to make a homogeneous mixture before use.

Adaptive Laboratory Evolution: Cell Passaging

Adaptive Laboratory Evolution was performed to improve the growth of oleaginous yeast strains. Initially, to provide mutagenic pressure, the strains were streaked on

YPD plates and subjected to ultraviolet light for a variable period (0, 10, or 30 seconds), and subsequently incubated overnight at 30°C. Single colonies were picked from the resulting plates and inoculated into fresh YPD media and incubated overnight at 30°C and 250 rpm shaking for recovery. 10% volume from this overnight culture was then transferred to media containing 10 g/L of CFAD. This was done to subject the strains to stress from the presence of CFAD components (i.e. caffeine). The cultures were passaged (10% by volume) into new CFAD containing media every 24 h to allow adaptation to the stress condition. Cell density was estimated by taking the optical density of overnight cultures at 600 nm. OD_{600nm} of overnight cultures was used to identify the cultures or populations with better growth compared to the wild type strains. After obtaining strains that performed better than the parental strain, they were passaged into a medium with higher concentration of CFAD. The CFAD concentration was increased by 10 g/L at every step up to a maximum of 40 g/L. Evolved strains were then characterized at 20 g/L of CFAD containing media to match the capabilities of the bioreactor, as previous efforts identified foaming limitations in bioreactors under higher CFAD substrate loading.

Comparison of parental strains with evolution strains

When required for comparison, parental strains were freshly streaked onto YPD plates and incubated at 30°C. A single colony was then inoculated into YPD media and incubated at 30°C, 250 rpm shaking overnight. The evolving populations and parental strain were first normalised to OD_{600nm} of 0.35 in both 1 mL YPD and 1 mL CFAD containing media at the passaging concentration, and then incubated overnight at 30°C, with 250 rpm shaking. The optical density was values at 600 nm were used to compare the growth of evolved strains and parental strains in YPD media and CFAD containing media.

Single Colony Isolation

Populations that had higher growth in terms of OD_{600nm} values than parental strains in CFAD containing media and had a higher growth rate in CFAD containing media than YPD media were selected for single colony isolation. This was done to isolate the best performing single colony candidates among the evolving population. The population was inoculated into 1 mL of CFAD containing media and incubated at 30°C and 250 rpm for 6 h. After 6 h, the cultures were diluted 2x, 10x or 50x and plated on YPD plates for overnight incubation at 30°C. Twenty distinct colonies were randomly selected from each population for further characterization. They were inoculated into 1 mL of CFAD containing media at the passaging concentration and compared based on overnight OD_{600nm} readings. Final evolved strains were selected based on higher OD_{600nm} readings compared to the wild type.

Stability Assay

Stability assays were performed on the isolated single colonies to confirm that the changes observed are stable even when subjected to removal of the stress. Selected strains were revived from glycerol stock onto YPD plates and incubated at 30°C overnight. Three colonies were randomly selected from each strain and inoculated into 1 mL of YPD media and incubated at 30°C, and 250 rpm. The OD_{600nm} reading was measured after 24 h. OD_{600nm} reading was normalised to 0.35 and freshly inoculated to YPD media again. Subculturing into fresh YPD media was repeated one more time, to subject multiple generations of these strains to stress free media. For the final passage, OD_{600nm} reading was normalised to 0.35 and reintroduced to CFAD 20 g/L containing media. The OD_{600nm} reading after 24 h growth was then used to determine the better performing strain. A total of 4 different strains that retained their ability to

grow well on CFAD was finalized for further fermentation characterizations.

Bioreactor fermentation

To study SCO production under optimal growth conditions, bioreactor studies were carried out. Prior to the fermentation in the bioreactor, 50 mL inoculum was grown in the CFAD media (0.28 g/L CFAD) and additional 10 g/L Tween 80 at 30 °C and 250 rpm for 24 h. Subsequently, the inoculum was transferred into 5 L bioreactor loaded with 2 L of CFAD media (20 g/L CFAD) and additional 10 g/L Tween 80 into a starting OD_{600nm} of 0.1. Fermentation was performed at pH 6.8, temperature of 30 °C, aeration at 1 L of compressed air/min and stirring at 900-1000 rpm. After 48 h of fermentation, the cells were harvested by centrifuging at 8000 rpm for 30 mins. The cell pellet and the remaining supernatant were subsequently freeze-dried separately to quantify crude lipid and fatty acid composition.

Fatty acid composition analysis

Lipid extraction, derivatization and quantification of fatty acids was performed as per previous report with only difference being the column used was a SUPELCO SP2560 fused silica capillary column (100 m x 0.25 mm x 0.20 um), with helium for a carrier gas (1.0 mL/min).

mRNA sequencing & differential gene expression analysis

To understand the changes in the transcriptome of the evolved strains, total mRNA sequencing and differential gene expression analysis was performed. The strains CBS 2072, EVO1 and EVO3 were streaked on YPD plates and 3 random colonies were selected from each for further experiments as biological replicates. A 10ml overnight culture was started in CFAD containing media (20 g/L CFAD). The following day, all

the biological replicates were sub-cultured into a fresh 50ml volume of the same media with a starting OD_{600nm} of 0.2. 2 ml of the culture was collected at 10 h and 24 h timepoints and cell pellet obtained through tabletop centrifugation at 4500 rpm for 5min at 4°C. The supernatant was discarded and the cell pellet flash frozen in liquid nitrogen. The samples were stored at -80°C until ready for RNA extraction. Total RNA from the cells were extracted using QIAGEN RNeasy Midi Kit according to the manufacturer's protocol. The extracted RNA was subsequently submitted to NovogeneAIT Genomics Singapore Pte Ltd for eukaryotic strand specific directional mRNA library preparation with polyA enrichment using NovaSeq PE150. After the samples passed quality control, the final sequencing data was analysed for differential gene expression (DGE), also by NovogeneAIT Genomics Singapore Pte Ltd. The original file from sequencing platform is transformed into sequenced reads using CASAVA base recognition (Base Calling). The reference genome used was *Yarrowia lipolytica* CLIB122. Alignment to this reference genome was performed using HISAT2. The DGE analysis between wild type strain and superior evolved strains was performed using a DESeq2 software. The p-values were corrected for FDR using Benjamini and Hochberg method. This generated the adjusted p-value (padj). The differentially expressed genes were determined with the parameters |log2(FoldChange)| >= 2 and padj <= 0.05. Novogene used the clusterProfiler software for GO-enrichment analysis. Relevant components of Figure 3 and 4 were generated using the NovoMagic, a free Novogene platform for data analysis.

Results and Discussion

1. Adaptive laboratory evolution for superior growth and lipid titre

ALE was applied as the principle tool to generate yeast strains adapted to perform better in CFAD medium. It was applied to four different oleaginous yeast strains (*Rhodotorula toruloides* ATCC 10788, ATCC 204091, *Yarrowia lipolytica* CBS 2070 and CBS 2072). These strains were grown on media containing with 20 g/L of CFAD. After strains were subjected to 79 passages, only one strain (*Y. lipolytica* CBS 2072) achieved superior growth compared to wild type strain. The remaining 3 strains did not show any marked improvement in growth even after multiple rounds of passaging (data not shown).

a. Comparison of growth on glucose vs side stream 20 g/L media

To provide a control as a starting reference for SCO production in *Yarrowia lipolytica* strain CBS 2072, it was grown on both standard lab media YPD and CFAD 20 g/L media. The biomass (cell dry weight) produced in CFAD 20 g/L was 4.1±0.3 g/L, which is about 45% lower (*p-value*<0.05) than the biomass generated in YPD (7.5±0.2 g/L) (Figure 1A, Table 1). Although the lipid content for growth in CFAD 20 g/L was twice than in YPD (at 12.3% of the biomass in CFAD compared to 6% in YPD), the final lipid titre were comparable in both conditions (Figure 1A). The associated lipid profile (Figure 1B) indicates that the higher lipid in CFAD is accompanied with higher amount of saturated fat especially C16:0 and C18:0 (*p-value*>0.05). This is most probably due to uptake of FFA from the media which is rich in saturated FFA. CFAD used in this study is the same as previous published report (Peterson et al., 2023). However, both cultures show a predominant proportion of unsaturated FFAs.

b. Superior growth in 20 g/L side stream media

Previous work has demonstrated that various oleaginous yeasts could be grown successfully at 10 g/L and 20 g/L CFAD media. However, to achieve enhance growth

lipid titres and yield, of strain *Y. lipolytica* CBS 2072, ALE was performed with 79 rounds of passages, with multiple populations of the strain passaged in parallel. After identifying populations with superior growth compared to wild type, the stability of the evolved phenotype was assessed by transferring them to YPD for 3 generations followed by CFAD 20 g/L. Two populations (697-5 and 697-20) showed enhanced growth in 20 g/L CFAD compared to wild type cells (*p-value* < 0.05, students t-test) (Figure 1C) and were selected for single colony isolation. Among the 20 colonies tested from each population, more than 90% of colonies demonstrated significantly higher OD_{600nm} (*p-value* < 0.05, students t-test), compared to the wild type in 20 g/L CFAD. Some colonies had 1.5-fold improvement in OD_{600nm} (see supplementary material). Based on these results, a total of four colonies were selected for further lipid and growth analysis. EVO1 and EVO2 were derived from population 697-5 while EVO3 and EVO4 were from population 697-20.

c. Fermentation in shake flasks

Shake flask fermentation of the evolved strains was carried out in a volume of 50 mL 20 g/L CFAD. The cell dry weight of all the four evolved strains was 2-fold more compared to that of the wild type strain (Figure 2A, Table 1). The improvement in biomass was accompanied by a 3-4 fold increase in lipid titre (Figure 2B). Lipid titre for EVO1 was 2.3±0.4 g/L as compared to 0.5±0.1 g/L for wild type strain. The percentage of total lipids w.r.t their biomass doubled in evolved strains compared to the wild type (Table 1). This indicates that these evolved strains take up more of the exogenous lipids from the media.

Lipid composition of superior evolved strains in shake flasks

The cell dry weight, lipid titre and lipid composition for the wild type and all 4 evolved strains are listed in Table 1 . Figure 2A also illustrates lipid composition for these tests,

290 and it can be seen that strain EVO2 had the highest percentage of unsaturation
291 (55.9±6.0%) and highest titre of unsaturated lipids (1.1±0.3 g/L). The strain EVO1 had
292 the highest lipid titre of 2.3±0.4 g/L, of which 38.2±8.5% is unsaturated lipid. The strain
293 EVO4 had a relatively lower lipid titre of 1.7±0.3 g/L, with the unsaturated lipid titre
294 being 0.9±0.1 g/L (*p-value*<0.05). In general, all the evolved strains had higher total
295 lipid titre and lower unsaturated lipid titre compared to wild type (*p-value* < 0.05) (Table
296 1, Figure 1B). It is interesting to note that, the overall percentages of C16:0 doubled in
297 EVO1, EVO3 and EVO4 compared to wild type, but these differences were not
298 statistically significant. It has been reported earlier that fatty acid binding proteins could
299 have chain length preferences (Dulermo et al., 2015). However, a high degree of
300 unsaturated FFAs indicates ex novo lipid synthesis occurs, with strain EVO2 showing
301 the highest percentage of C18:1 being produced (39.8±4.8%) among the evolved
302 strains. Thus, the ability to generate strains with higher growth capability and varied
303 fatty acid composition is demonstrated. These strains could be potentially used for
304 production of alternative lipids for food or feed with specific fatty acid profiles. For
305 example, EVO2 has close similarity with pork fat in terms of fatty acid composition (Wu
306 et al., 2022), while EVO1 contains a high proportion of saturated fatty acids (~60%)
307 and an enhanced C16:0/C18:0 ratio.

308 *d. Bioreactor fermentation of wild type and evolved strains*

309 The 5 L bioreactor run was performed for the wild type CBS 2072 and the evolved
310 strain EVO1 as it had the highest lipid titre and yield. The parameters of the
311 fermentation are listed in Table 1. The biomass achieved for the wild type and EVO1
312 in the bioreactor were not significantly different. However, the total lipid % and the lipid
313 yield was higher in EVO1, by 3-fold at 24 hour and 1.5-fold at 48 hours, compared to
314 the wild type. There was a 30% drop in the proportion of unsaturated fatty acids at

both timepoints in the evolved strain (Table 1). The overall titre of unsaturated lipids at 24 h for EVO1 was at 1.5±0.0 g/L. The behaviour of the evolved strain at 48 h is quite different in the 50 mL shake flask and the 5 L bioreactor. The lipid percentage of the biomass is lower in the bioreactor at 48 h (12.5±0.1%) compared to that of about 26.8±6.1% in shake flasks. However, the performance at 24 h was similar in both culture conditions with an observed improvement of 3-4 fold and high in saturated fat. Interestingly, even though CFAD is 40% C16:0 and 26% C18:0 in composition, higher amounts of C18:0 was observed in EVO1 compared to C16:0, indicating that there are chain length preferences in terms of fatty acid uptake. It is worthwhile to note that all differences observed in EVO1 from wild type values were significantly different (*p-value* < 0.05) (Table 1, Figure 2B). This is on par with the 3.1-fold improvement observed in extensively engineered strains grown on glucose (Xu et al., 2016). The cell dry weight yield ($Y_{X/S}$ g/g) for wild type (0.81±0.01) was comparable to that of EVO1 (0.78±0.02) at 48 h. Whereas, the apparent lipid yields ($Y_{L/S}$ g/g) evaluated at 48 h for wild type was 0.07±0.00 and EVO1 was 0.11±0.00 (Table 1). Hence both values were quite comparable. The lipid yield at 24 h was 0.25±0.00, which is double that of at 48 h. But it should be noted that the residual lipids were not measured at this timepoint (Table 1). Our yields are comparable to or better than recent reports of lipid production in *Y. lipolytica* cultured on volatile fatty acids (Gao et al., 2020) or on lignocellulosic biomass (Yook et al., 2020).

It is known that *Y. lipolytica* accumulates more lipids in the early stationary phase compared to late stationary phase (Carsanba et al., 2020; Papanikolaou et al., 2009). The differences in the shake flask and bioreactor lipid profiles could be due to differences in growth rate of *Yarrowia* cells in the two conditions. Hence a combination of various factors like strains and culture conditions could be used to achieve a variety

of lipid profiles. Unlike the previous report, ethanol co-feeding was not used for these bioreactor runs. Even though a different *Y. lipolytica* strain (CBS 2072) was used for this study, lipid titres were further improved at 20 g/L loadings in the evolved strain, compared to previous reported lipid titre of 3.4 g/L in *Y. lipolytica* CBS 2070. Other studies have shown that *Y. lipolytica* grown on FFA-rich food waste substrates such as palm fatty acid distillate or residual frying oil can generate a CDW of up to 12.45 g/L (Carsanba et al., 2020). These other side streams are high in palmitic acid and oleic acid and low in stearic acid. The evolved strains could also be grown on other such FFA-rich waste products, thus increasing the impact of these findings. Lipid titre improvement outperforms a recent report where *Y. lipolytica* strains were evolved to grow in waste streams like glycerol to produce lipids (Tsirigka et al., 2023). Though it should be noted that this was done in a minimal media containing glycerol unlike the adaptation experiments here which were done in rich media. The improvement in performance of evolved strains is also comparable to reports where lipid titre was improved under magnesium and nitrogen limitation (Daskalaki et al., 2019), and waste cooking oil (Katre et al., 2017).

2. Transcriptomics and Differential Gene Expression analysis of evolved strains

To understand the mechanism of adaptation of the evolved strains, differential transcriptomic analysis was performed. Both the wild type and evolved strains (EVO1 and EVO3) were culture in CFAD 20 g/L and and mRNA extraction performed after 10 h and 24 h of growth. The mRNA extracted and sequencing reads were of high quality (see supplementary material). EVO1 and EVO3 were analysed for Differential Gene Expression (DGE) compared to the wild type strain. At the 10 h growth timepoint, strain EVO1 had 502 differentially expressed genes (DEGs), while EVO3 had 42 DEGs. At the 24 h growth timepoint, EVO1 had 312 DEGs while EVO3 had 49 DEGs (Figure

3A). The Principal Component Analysis (PCA) plot for DEGs shows that the evolved strains vary distinctly from the wild type strain both at 10 h (Figure 3B) and 24 h (Figure 3C). In the PCA plot, the replicates of EVO1 and EVO3 are clustered separately compared to the wild type replicates. This indicates that the transcriptome of both the evolved strains are distinct or dissimilar from that of the wild type. The biological replicates for EVO1 were more consistent in terms of their expression profiles (Figure 3B, 3C). The Venn diagram (Figure 3D) shows the number of expressed genes in each of the three strains, that were either uniquely expressed or co-expressed. Specifically, 49 genes were expressed in both evolved strains and not in the wild type. A GO-term enrichment performed on these 49 genes at two timepoints show that they are involved in transmembrane transport, establishment of localization or are component of membrane (Figure 4A).

a. Differential Gene Expression analysis for strain EVO1 and EVO3

At 10 h, 247 genes were upregulated and 255 genes were downregulated. The GO-term enrichment performed on these DEGs shows that most of these belong to pathways like peptide metabolism, translation, lipid metabolism, glycerolipid and phospholipid metabolism, structural constituent of ribosome and part of kinetochore machinery (see supplementary material). At 24 h, 123 genes were upregulated and 189 genes were downregulated. The GO-term enrichment performed on these DEGs shows that most of these belong to pathways like oxidation reduction pathways, oxidoreductase activity, cofactor or coenzyme binding and transmembrane transport (see supplementary material). In contrast, for the strain EVO3, for both the timepoints, the GO term enrichment analysis indicated that the most enriched term is that of transmembrane transport. The number of DEG are lesser than for EVO1 (see supplementary material). This difference indicates that the two strains are employing

different mechanisms to adapt to CFAD. EVO1 could potentially be more focussed on higher utilization of FFA through differentially regulated cellular mechanisms while EVO3 could be doing so through differential transmembrane transport machinery.

b. Differentially expressed genes in fatty acid metabolic pathways

At 10 h, the strain EVO1 displayed higher expression of extracellular lipases lip2 (2.5-fold) and lip8 (7.5-fold) in comparison to the wild type (Table 2). This strongly suggests that EVO1 is actively breaking down the TAG in the media to glycerol and FFA to enable their uptake (Figure 4B). All the fatty acid degradation related genes like beta-oxidation genes (POX2, POX3, MFE, POT1, FAT3, FAT4 and Enoyl CoA hydratase) and intracellular lipases (lip15, lip17, TGL3, Lipid droplet lipase YALI0F16731) were repressed (Table 2). This shows that the strain is less actively breaking down intracellular fatty acids at 10 h. The exogenous FFA has to be activated to an acyl-CoA before it enters the beta-oxidation pathway. But the mechanism of this activation is still not clearly understood in *Y. lipolytica*. Recently 4 extracellular fatty-acid-binding proteins were discovered, but any upregulation of these genes were not seen in this study (Onésime et al., 2022). AAL9 is a putative acyl-CoA synthetase (Dulermo et al., 2016) with 2.5-fold higher expression in EVO1 suggesting that it might activate the exogenous FFA at the 10-hour timepoint. ARE1 and ARE2 genes, which causes esterification of sterol are significantly repressed (2-fold less) in evolved strains, indicating that steryl ester synthesis might be inhibited to channel more carbon flux into FAS. It has been shown that lipid productivity is increased by overexpressing genes like DGA1 (involved in TAG formation) and ACC1 (involved in fatty acid biosynthesis) (Wang et al., 2020). Deletion of genes like GUT1/2, ARE1/2 and β-oxidation pathway genes, has been shown to improve the lipid production in

oleaginous yeasts (Wang et al., 2020). Similar changes in expression of above listed genes were observed in this study (Table 2).

However at 24 h, there is higher expression of genes like FAA1 (1.5-fold), AAL1 (4.6-fold), AAL2 (4.9-fold) and AAL9 (5.4-fold), indicating higher fatty acid activation. Enoyl CoA hydratase has 3.2-fold higher expression, indicating that cells are breaking down some of the FFA into acetyl CoA for producing de-novo lipids. Interestingly, one of the intracellular lipases, lip4 was repressed by about 17.4-fold in evolved strain (Table 2). *Y. lipolytica* has about 16 reported intracellular lipases. Though the extracellular lipases are highly characterized, the intracellular lipases have been poorly studied. The data indicates that intracellular lipases lip4 and lip15 could play an important role in intracellular fatty acid accumulation and in countering FFA toxicity. Collectively, all these data indicate how the evolved strains might produce higher lipid amounts and offers several testable metabolic engineering strategies to improve lipid production.

c. Differentially expressed genes in important cellular activities

At 10 h, multiple subunits of the Ndc80-MIND-Spc7 complex and the Dam1 complex had significant elevation in expression in the evolved strain EVO1. The detailed list is provided in the supplemental material (see supplementary material). This complex is an essential component of the kinetochore assembly in yeast and plays a key role in cell cycle. Enhanced expression of the kinetochore complex genes in the evolved strain may suggest the presence of a microtubule-destabilising compound in the CFAD.

There is also an elevated expression of genes for 23S rRNA (23 fold), 16S rRNA (13 fold) and U3 sno RNA (3-4 fold) that are essential components of translation machinery. Other important genes like RPR1 which is a RNase that cleaves tRNA precursors to generate mature 5' ends and Scr1 which is involved in transporting

mature protein from ribosomes to endoplasmic reticulum have 5-fold higher expression in EVO1 at 10 h (see supplementary material). This might indicate that mRNA translation machinery is highly active in EVO1 which is consistent with its enhanced growth rate.

High levels of expression (5- to 20- fold) of mitochondria-encoded genes (see supplementary material) were observed in EVO1 at 10 h timepoint. As has been reported before, mtDNA damage can result in ROS overproduction which can lead to higher expression of mitochondrial genes (Al-Kafaji et al., 2016). High amounts of stress from the CFAD substrate can cause higher ROS production leading to this observation. Interestingly, RNY1 which encodes a RNase involved in breakdown of tRNA during oxidative stress (MacIntosh et al., 2001), was expressed about 30-fold higher in EVO1 at 10 h. These data are consistent with the hypothesis that higher uptake of CFAD media components into the cells could result in high oxidative stress.

There are various transporters and acid phosphatases that were significantly differentially expressed in EVO1 (see supplementary material). Ammonium transporter Mep2 has up to 54-fold decreased expression and phosphate transporter Pho89 has up to 58-fold elevated expression in EVO1. Mep2 is involved in nitrogen starvation response (Boeckstaens et al., 2007) whereas Pho89 is active in early phases of growth and under alkaline condition. Pi limitation can initiate RNA degradation, TAG biosynthesis and inhibit TCA cycle and ribosome biosynthesis, subsequently leading to lipid accumulation (Wang et al., 2018). Pho89 has been reported to be transcribed under condition of Pi limitation (Martinez and Persson, 1998). Thus Pho89 could be improving lipid accumulation.

At least 8 phosphatases including Pho2 were overexpressed in EVO1 with the increase ranging from 4-fold to 40-fold. Acid phosphatases in *Saccharomyces*

cerevisiae have been studied for decades and have been shown to be involved in regulation of various eukaryotic genes through phosphorylation and dephosphorylation (Sambuk et al., 2011). Phosphoregulation of the proteome might play a role in the adaptation of the evolved strains in the CFAD medium.

d. Effect of caffeine

Interestingly, two high affinity nicotinic acid plasma membrane permease like transporters were highly expressed (Tna1 like, 179-fold and cnsO like, 11-fold) in EVO1. In contrast another caffeine efflux pump, Caf5 like protein (YALI0F25597) was repressed by 15-fold in the evolved strain (see supplementary material). These results indicate how the strain might have adapted to higher concentrations of caffeine in the CFAD waste. Caffeine is known to arrest yeast cell cycle and disrupt DSB repair pathways. It is a known inhibitor of TORC1 and is known to increase chronological lifespan in yeast (Rallis et al., 2013). It is known to cause DNA damage and impairment of protein trafficking (Ruta and Farcasanu, 2020). DNA repair protein significantly upregulated in EVO1 are Rad53 (see supplementary material), Rad51 (2.5-fold) and Rad54 (2.2-fold). This suggests that the CFAD media might cause DNA damage necessitating cells to evolve mechanisms to overcome the stress.

e. Common differentially expressed genes in in EVO1 and EVO3

At 10 h, the glucose transport transcription regulator Rgt1 was upregulated in both the evolved strains significantly in comparison to the wild-type strain (Table 4). Rgt1 represses glucose transporters like HXT and is an important player in glucose metabolism. Rgt1 is repressed in presence of glucose (Kim et al., 2003). Increased expression of Rgt1 observed in the evolved strains is indicative of lack of glucose in the media. The phosphate transporter Pho89 is also highly upregulated in both the strains. Interestingly FAD1 (or OLE1) is upregulated in both evolved strains by about

3-fold (Table 4). This suggests that the evolved strains are taking up more exogenous fatty acids and OLE1 is converting the C16 and C18 acyl-CoAs to C16:1 and C18:1 acyl-CoAs. Subsequently these are incorporated into TAG and membrane lipids to deter caffeine toxicity. Also both the strains show reduces expression of lipase Lip15, indicating that the cells are not breaking down intracellular lipids at 10 h. Another interesting gene that is upregulated in both strains is a lysine acetyl transferase Lyc1 (YALI0E05533) (Table 4). Lysine acetyl transferases regulate various histone and non-histone proteins and regulate key cellular metabolic pathways (Patel et al., 2011). It could be regulating key cellular pathways that facilitate the adaptation to CFAD.

A recent review highlighted the importance of omics data that is lacking in sufficient amount for this microorganism along with fundamental knowledge of genetics, metabolism and cell biology (Abdel-Mawgoud et al., 2018). This transcriptomics data provides an insight into exogenous lipid metabolism, dynamics of lipase production in lipid rich environment and how *Yarrowia* responds to stress and improves its tolerance. Specifically at 10h, *Y. lipolytica* is accumulating lipids by upregulating extracellular lipases and downregulating intracellular lipases. It is also downregulating beta oxidation pathway (Figure 4C). Whereas at 24 h it is upregulating the beta oxidation genes and acyl-CoA activating genes to utilize the accumulated lipids (Figure 4C). Several key transporters are also differentially regulated as shown in Figure 4D. The ZRT1 like zinc transporter which is highly upregulated (>700-fold) could be a key factor in higher utilization of exogenous lipids, but this warrants further validation through reverse engineering. Differential expression of transporters like Mep2 and Pho89 could also be reversed engineered to further understand its effect on higher growth and lipid yield of the evolved strains. Hence potential mechanism was identified that might be enhancing the capability of the evolved strains to grow better in FFA-rich

514 CFAD media and improve lipid production. High lipid producing strains till date have
515 been obtained mostly through genetic engineering. The strain reported here is non-
516 GMO and the transcriptomic data provides candidate genes that could be tested
517 through genetic engineering to improve lipid productivity. Thus this work has generated
518 possible strategies for efficient utilization of side streams, paving the way for
519 sustainable lipid production in oleaginous yeast.
520
521 **Conclusions**
522 This work demonstrates that ALE techniques can be applied to improve the ability of
523 *Yarrowia lipolytica*, to grow on Cocoa Fatty Acid Distillate sidestream. ALE improved
524 biomass by >2-fold and lipid content by 3- to 4-fold in evolved strains. Lipid titre of 4.8
525 g/L with 32% unsaturation was achieved. Through transcriptomics, changes in
526 expression of genes like LIP4, LIP8, OLE1, AAL9 and MEP2 were identified which
527 appear to better equip evolved strains. In summary, strains produced were capable of
528 improved utilization of lipid-rich sidestreams, corroborated by enhanced growth and
529 lipid accumulation, thus paving way for sustainable production of microbial lipids.
530
531 **Data Availability**
532 Data generated during this study are included in this article, its supplemental and
533 Bioproject ID PRJNA1052669.
534
535 **Supplementary material**
536 E-supplementary data of this work can be found in online version of the paper.
537 Table A1, Table A2, Table A3, Figure A1, Figure A2
538

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Table and Figure legends

Table 1: Performance parameters for 50ml shake flask and 5L bioreactor fermentation of *Yarrowia lipolytica* strains wild type WT and evolved strains EVO1, EVO2, EVO3 and EVO4 (3 replicates). The reported values include cell dry weight (CDW g/L), lipids titre (Total lipids g/L), percent lipids in biomass (Total lipids %), fatty acid compositions of lipids (%FA; C16:0 palmitic acid; C16:1 palmitoleic acid; C18:0 stearic acid; C18:1 oleic acid; C18:2 linoleic acid), percentage (%) and titre (g/L) of unsaturated fats (Unsaturated FA) in the total lipids, cell dry weight yield ($Y_{X/S}$ g/g), lipids yield ($Y_{L/S}$ g/g).

Table 2: Differentially expressed genes in EVO1 related to fatty acid metabolism at 10 and 24 h growth timepoints. ID is the YALI identifier of the gene, Fold Change refers to folds of up or down(-) regulation w.r.t the wild type and p-value(adj) refers to adjusted p-value with Benjamini and Hochberg FDR calculation.

Figure 1: Growth and Adaptive laboratory evolution of *Yarrowia lipolytica* CBS 2072 strain. (* indicates *p-value*<0.05) **A**: Biomass (CDW) and lipid titre of wild type strain in YPD and CFAD 20 g/L media. **B**: Fatty acid composition of total lipids of wild type strain in YPD and CFAD 20 g/L media. **C**: OD_{600nm} for different evolving populations during stability assay.

Figure 2: **A**: Performance of *Yarrowia lipolytica* strains CBS 2072 (wild type) and evolved strains EVO1, EVO2, EVO3 and EVO4 during 50ml shake flask fermentation (3 replicates). (* indicates p-value<0.05 and ** indicates p-value<0.001) Values include cell dry weight in g/L, total lipid titre in g/L and Fatty acid composition of total lipids after 48 h growth. **B**: Performance of *Yarrowia lipolytica* strains CBS 2072 (wild type) and evolved strain EVO1 during 5L bioreactor fermentation (3 replicates). Values include cell dry weight in g/L, total lipid titre in g/L and Fatty acid composition of total lipids after 24h and 48 h growth.

Figure 3: DGE analysis for CBS 2072 (WT) vs evolved strains EVO1 and EVO3 after 10 h and 24 h of growth. **A**: Number of differentially expressed genes in both the strains compared to wild type (p_{adj} value <0.05 and $\log_2$ fold change >2). **B**: PCA plot for all biological replicates for all 3 strains at 10 h growth timepoint. **C**: PCA plot for all biological replicates for all 3 strains at 24 h growth timepoint. **D**: Venn diagram depicting number of genes expressed either uniquely in only one strain or in common in multiple strains after 10 h and 24 h of growth.

Figure 4: **A**: GO-term enrichment analysis for genes commonly expressed in both evolved strains EVO1 and EVO3 at 10 h and 24 h growth timepoints. Gene ratio refers to ratio of number of differentially expressed genes to total genes listed in the pathway. **B**: Fatty acid metabolism pathway and related pathways and how they are up and downregulated in the strain EVO1 at 10 h. Gene names indicated in red and green are downregulated and upregulated respectively in EVO1. **C**: Up- and down- regulation trends of genes at 10h and 24h in EVO1. **D**: Model for gene Down- and up- regulation in strain EVO1 at 10 h. Red and green indicates down- and up-regulated respectively.

Tables and Figures

Table 1

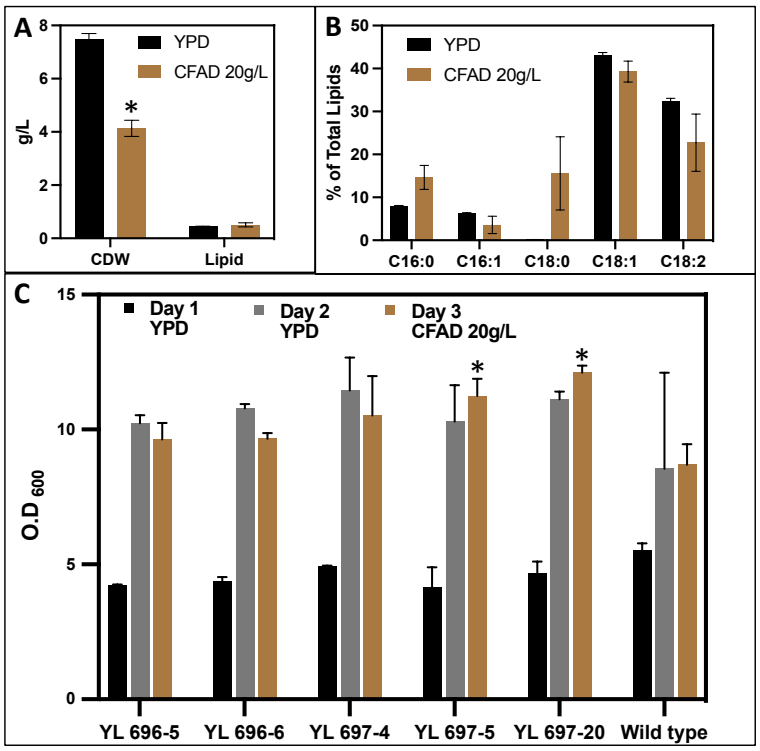
Strain	CDW (g/L)	Total Lipid Titre (g/L)	Total Lipid (%)	% FA of total lipids					Unsaturated FA		$Y_{X/S}$ (g/g)	$Y_{L/S}$ (g/g)
				C16:0	C16:1	C18:0	C18:1	C18:2	%	g/L		
Shake Flask experiments												
Grown in YPD media												
WT	7.5±0.2	0.4±0.0	6.0±0.1	8.0±0.1	6.3±0.1	0.2±0.0	43.0±0.8	32.4±0.7	83.2±0.2	0.4±0.0	0.37±0.01	0.30±0.00
Grown in CFAD 20 g/L media												
WT	4.1±0.3	0.5±0.1	12.3±1.9	14.6±2.8	3.6±2.0	15.6±8.5	39.3±2.4	22.7±6.7	65.8±11.1	0.3±0.0	0.22±0.02	0.03±0.00
EVO1	8.9±0.3	2.3±0.4	25.9±4.8	33.8±7.3	1.9±0.5	26.2±1.9	26.8±6.1	9.4±2.0	38.2±8.5	0.9±0.1	0.47±0.02	0.12±0.02
EVO2	9.1±1.3	1.9±0.5	20.7±2.9	24.5±2.6	2.7±0.6	17.7±4.1	39.8±4.8	13.3±1.7	55.9±6.0	1.1±0.3	0.48±0.07	0.10±0.03
EVO3	8.5±0.4	2.2±0.4	26.1±5.2	32.4±7.8	2.5±1.0	23.6±3.7	29.6±8.0	10.1±2.4	42.3±11.3	0.9±0.1	0.45±0.02	0.12±0.02
EVO4	8.4±0.5	1.7±0.3	20.3±2.8	28.8±6.8	3.3±1.1	18.1±4.9	35.3±8.3	12.9±2.4	51.3±11.7	0.9±0.1	0.43±0.03	0.09±0.02
5L Bioreactor experiments (Grown in CFAD 20 g/L media)												
WT: 24h	10.5±0.3	1.6±0.0	15.4±0.2	19.6±0.0	5.7±0.0	14.0±0.1	34.9±0.5	22.7±0.0	64.9±0.1	1.0±0.0	0.55±0.02	0.08±0.00
WT: 48h	15.4±0.2	1.3±0.0	8.3±0.1	10.6±0.1	6.3±0.0	2.3±0.0	45.7±0.0	31.0±0.0	86.5±0.1	1.1±0.0	0.81±0.01	0.07±0.00
EVO1: 24h	11.1±0.3	4.8±0.1	43.2±0.6	26.4±0.1	1.9±0.0	40.3±0.7	20.6±0.5	8.6±0.2	31.6±0.7	1.5±0.0	0.58±0.02	0.25±0.00
EVO1: 48h	14.9±0.3	1.9±0.0	12.5±0.1	12.8±0.0	3.1±0.0	29.7±0.5	32.2±0.3	18.8±0.1	55.7±0.5	1.0±0.0	0.78±0.02	0.11±0.00

754 Table 2
755

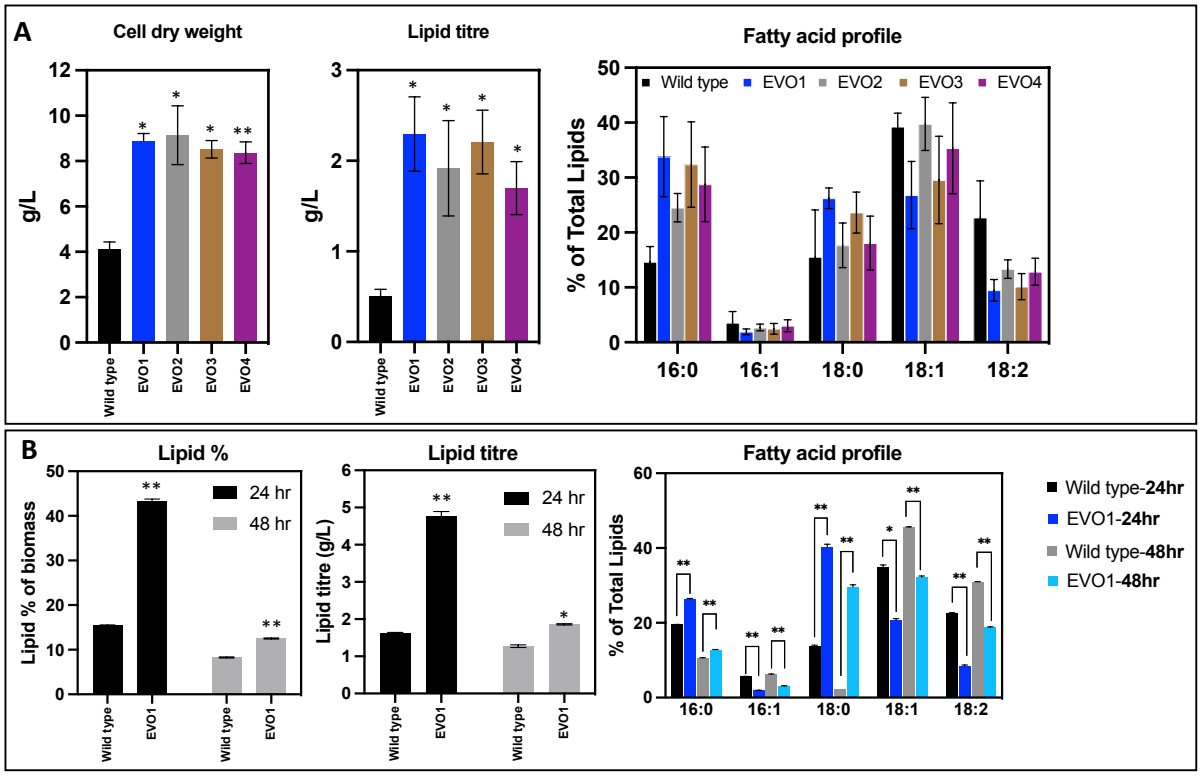
Gene	ID	Fold change	p-value(adj)
10 h			
lip2	YALI0A20350	2.3	7.67E-05
lip8	YALI0B09361	7.5	4.38E-14
lip15	YALI0E11561	-3.0	0.002634435
lip17	YALI0F32131	-2.4	0.039950119
TGL3	YALI0D17534	-1.4	0.011489975
Lipid droplet lipase	YALI0F16731	-3.2	0.000315773
PDC1	YALI0D10131	-3.5	0.00258608
ALD4	YALI0E00264	-2.1	0.038299461
ACC1	YALI0C11407	1.6	4.64564E-06
FAS1	YALI0B15059	1.4	0.007743357
FAS2	YALI0B19382	1.6	0.002054906
OLE1	YALI0C05951	2.6	7.98705E-05
ARE1	YALI0F06578	-2.1	0.029489565
ARE2	YALI0F06578	-2.1	0.029489565
MLS1	YALI0D19140	-3.2	0.037779739
ACO1 (Aconitase)	YALI0D09361	-1.9	0.000377481
Acyl coa dehyd	YALI0D15708	-1.9	1.66E-08
ADH2	YALI0E17787	-1.8	0.014993059
ADH3	YALI0A16379	3.4	6.87E-18
ADH4	YALI0A15147	1.8	0.001316218
GUT1	YALI0F00484	-2.4	1.85E-12
GUT2	YALI0B13970	-1.7	0.000779753
FAT3	YALI0B05456	-5.5	4.04E-12
FAT4	YALI0C09284	-3.9	1.89E-06
AAL9	YALI0A15103	2.5	1.20E-10
POX2	YALI0F10857	-2.0	0.000190723
POX3	YALI0D24750g	-1.5	0.015944723
MFE	YALI0E15378	-1.5	0.003891453
POT1	YALI0E18568	-1.5	0.019070121
Enoyl coa hyd	YALI0F22121	-3.2	1.85E-12
HFD3	YALI0A17875	1.9	0.004692095
24 h			
ALD4	YALI0E00264	-10.7	1.41E-06
ALD-like	YALI0D07942	6.0	0.000124452
ADH6	YALI0A15147	19.1	8.98E-08
ADH2	YALI0E07766	6.3	0.001010068
FAA1	YALI0D17864	1.5	0.028122957
Enoyl-CoA hydratase	YALI0D00671	3.2	0.000147368
YLJen1	YALI0C15488	4.6	0.011426711
AAL1	YALI0E11979	4.6	0.001089338
AAL2	YALI0A14234	4.9	0.020319007
AAL9	YALI0A15103	5.4	0.020443262
lip4	YALI0E08492	-17.4	0.011049796
Palmitoyl-protein thioesterase(PPT)	YALI0F14135	2.0	0.011574354

756

Figure 1

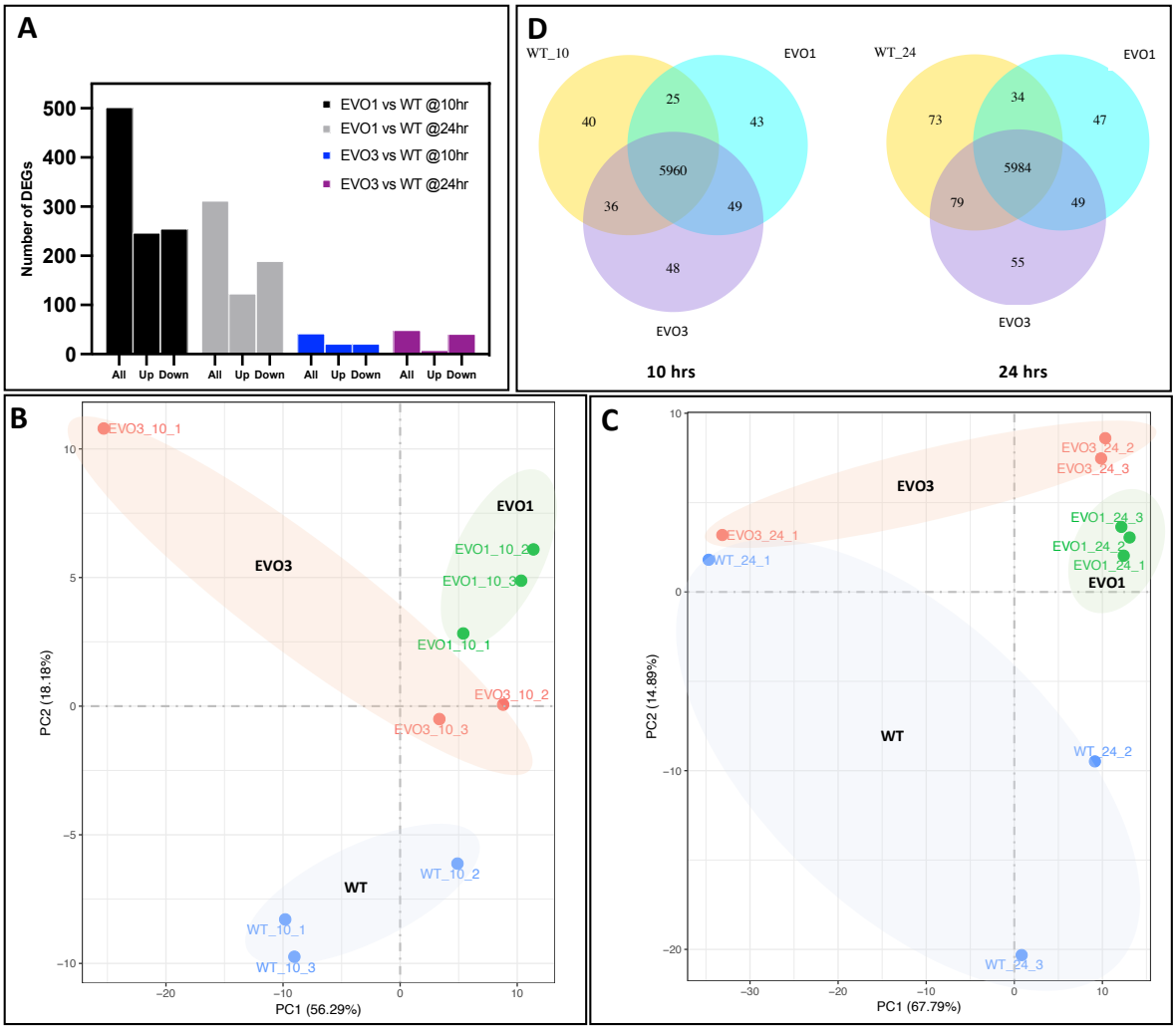

A
g/L
YPD
CFAD 20g/L
CDW
Lipid
B
% of Total Lipids
YPD
CFAD 20g/L
C16:0
C16:1
C18:0
C18:1
C18:2
C
O.D 600
Day 1 YPD
Day 2 YPD
Day 3 CFAD 20g/L
YL 696-5
YL 696-6
YL 697-4
YL 697-5
YL 697-20
Wild type

761 Figure 2
762



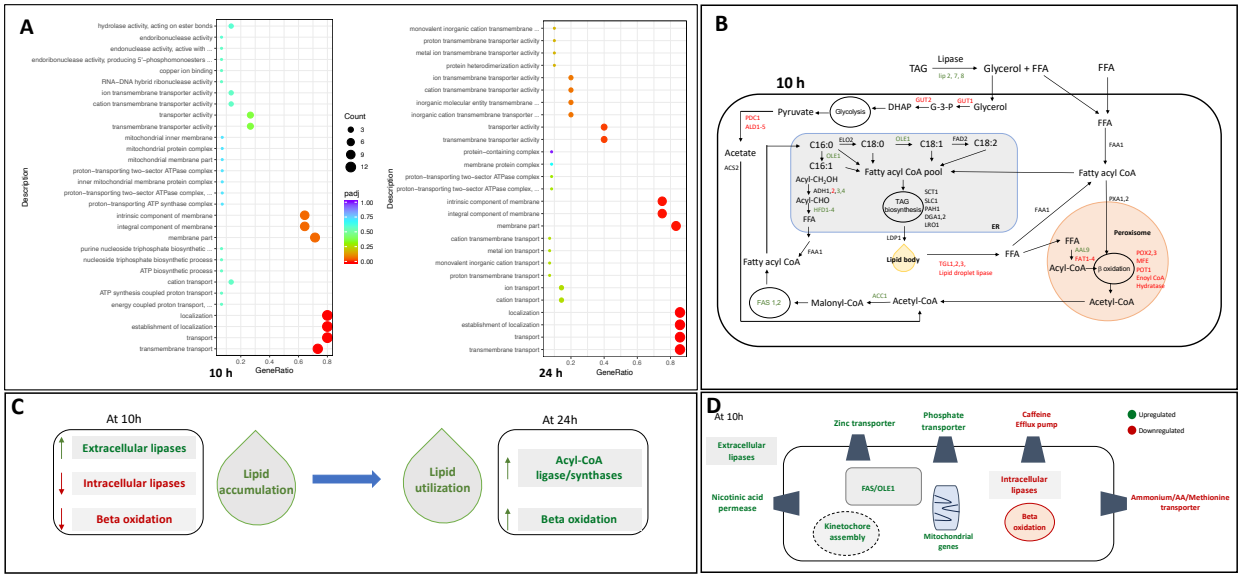
763
764

765 Figure 3:
766



767
768

Figure 4:


A
10 h
24 h
GeneRatio
Description
Count
padj
B
10 h
TAG
Lipase
Glycerol + FFA
FFA
Pyruvate
Glycolysis
DHAP
G-3-P
Glycerol
Acetate
C16:0
C18:0
C18:1
C18:2
C16:1
Fatty acyl CoA pool
Fatty acyl CoA
Acyl-CoA
Malonyl-CoA
Acetyl-CoA
Lipid body
ER
Peroxisome
C
At 10h
Extracellular lipases
Intracellular lipases
Beta oxidation
Lipid accumulation
Lipid utilization
At 24h
Acyl-CoA ligase/synthases
Beta oxidation
D
At 10h
Zinc transporter
Phosphate transporter
Caffeine Efflux pump
Upregulated
Downregulated
Extracellular lipases
Nicotinic acid permease
FAS/OLE1
Kinetochore assembly
Mitochondrial genes
Intracellular lipases
Beta oxidation
Ammonium/AA/Methionine transporter