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Research Article

Glycerol conversion into a single cell oil by engineered *Yarrowia lipolytica*

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Oleaginous yeasts are considered as natural single cell oil producers. Engineering the lipid biosynthetic pathway has the potential to increase lipid accumulation by these yeasts. In *Yarrowia lipolytica*, three diacylglycerol acyltransferases encoded by *LRO1*, *DGA1*, and *DGA2* genes are involved in lipid formation. Strain JMY3580 was constructed by overexpressing *DGA2* gene in Q4 strain (*dga1Δ dga2Δ lro1Δ are1Δ*). Reconstruction of triacylglycerol synthesis pathway led to significant improvement in lipid accumulation. Strain JMY3580 accumulated over 40% of lipids in biomass, while lipid accumulation in wild-type strain was not able to exceed 20% when grown on a glycerol-based medium with carbon to nitrogen ratio of 90. Higher lipid accumulation (over 50%) was achieved in fed-batch grown cells when glycerol was added during cultivation. The best biomass yield was 18.5 g/L after 144 h with total fatty acid yield 9.9 g/L. Fatty acid composition was altered when *Dga2p* was the only diacylglycerol acyltransferase present in yeast cells, especially lower percentage of linoleic acid was present in lipids of JMY3580. Microbial oil prepared by conversion of glycerol by genetically engineered *Y. lipolytica* could be applied for various value-added products based on single cell oils.

Keywords: Glycerol / Lipids / Oleaginous yeast / Single cell oil / *Yarrowia lipolytica*

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1 Introduction

The origins of using microorganisms for single cell oil (SCO) production dates back to the beginning of 20th century. Since then, SCO production has become one of the important research areas in the biotechnology. In order to make SCO production economical, a low-cost feedstock should be employed. Nowadays, there is tendency to use various inedible feedstocks as substrates for SCO production [1–3]. Among others glycerol is considered as one of the suitable feedstocks, as well. Growing biodiesel production has resulted into large quantities of glycerol derived from vegetable oil transesterification, since it is produced in biodiesel manufacturing process as the main byproduct. Such an amount of produced glycerol is utterly independent of its demand, which has led to its use as a substrate for production of value added chemicals [4, 5]. For SCO production, the most important is natural ability of microorganisms to accumulate

significant amounts of storage lipids in cells. Storage lipid accumulation in these so-called oleaginous microorganisms can occur in two ways. De novo lipid accumulation is connected with synthesis of fatty acids (FA) from various hydrophilic carbon substrates. Ability to accumulate lipids is connected with specific carbon metabolism, which enables oleaginous microorganisms to direct carbon towards FA synthesis. Redirecting of carbon towards FA synthesis is usually triggered by exhaustion of nitrogen [6]. The other way is ex novo biosynthesis, and it is simply incorporation of extracellular FA into cells of oleaginous microorganism. It is not connected with nutrient depletion, the only limitation is ability to transport FA into the cell [7]. In the recent years, many metabolic engineering tools are used to study the lipid biosynthesis of microorganisms with the aim to improve lipid accumulation [8–11]. For this purpose, one of the most frequently used microorganisms is oleaginous yeast *Yarrowia lipolytica*. *Y. lipolytica* (formerly *Candida lipolytica*) was previously used as the source of proteins rather than SCO-producing yeast [12]. In many cases, this yeast was reported to secrete significant quantities of citric acid (CA) instead of massive lipid accumulation when nitrogen limited media was employed. This was observed particularly in nitrogen limited media with hydrophilic carbon sources [13–18]. On the other hand, when *Y. lipolytica* was grown in a media containing fats and oils considerable lipid accumulation was observed. This feature has been

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Abbreviations: CA, citric acid; DCW, dry cell weight; FA, fatty acid; SCO, single cell oil; SE, sterol esters; TAG, triacylglycerol; TFA, total fatty acids

Table 1. *E. coli* and *Y. lipolytica* strains and plasmids used in this study

Strain (host strain)	Plasmid, genotype	Reference
JME547 (DH5 α)	pUB4-Cre1 (2.1 kb Cre fragment in pUB4)	[23]
JME1132 (DH5 α)	JMP1132: JMP62- <i>pTEF-DGA2-URA3ex</i>	[26]
JME1822 (Mach1 TM)	JMP1822: JMP62- <i>pTEF-DGA2-LEU2ex</i>	[30]
W29	<i>MATA</i> , wild type	[31]
JMY1884	Q4, <i>pTEF-DGA2-URA3ex</i>	[26]
JMY3357	Q4, <i>pTEF-DGA2-URA3ex pTEF-DGA2-LEU2ex</i>	[30]
JMY3435	Q4, <i>pTEF-DGA2</i> $\times$ 2	[30]
JMY3513	Q4, <i>pTEF-DGA2</i> $\times$ 2 <i>pTEF-DGA2-URA3ex</i>	This work
JMY3580	Q4, <i>pTEF-DGA2</i> $\times$ 2 <i>pTEF-DGA2-URA3ex pTEF-DGA2-LEU2ex</i>	This work

considered as a very useful for conversion of low-value fatty substrates into “tailor-made” lipids such as cocoa-butter substitutes [19,20]. However, various wild-type strains identified as *Y. lipolytica* are not an absolutely uniform group of yeasts sharing exactly the same substrate conversion rate into storage lipids and extracellular metabolites such as organic acids and polyols [21]. Since many strains of *Y. lipolytica* accumulate significant amount of lipids only ex novo, when FA rich substrates are used, its true oleaginous character was questioned in the past [12]. Nowadays, *Y. lipolytica* is generally considered as an oleaginous yeast with ATP:citrate lyase activity encoded by genes *ACL1* and *ACL2* [22], although it possesses specific lipid metabolism divergent from other oleaginous yeasts. It represents a very convenient platform for remodeling biochemical pathways. *Y. lipolytica* is one of the yeasts with whole genome sequenced, which gave rise to many well-developed genetic engineering tools [23–25]. It has unique storage lipid metabolism due to presence of *DGA2* gene encoding DGAT1 acyl-CoA:diacylglycerol acyltransferase family protein [26]. Moreover, it is the yeast with potential to produce not only SCO, but also CA, polyols, enzymes (e.g. lipases and proteases), and decontaminate hydrocarbon polluted areas as well [15,27–29]. Here, we report conversion of glycerol into SCO by lipid overaccumulating *Y. lipolytica* strain constructed by multiple integration of the diacylglycerol acyltransferase *DGA2* gene into genome.

2 Materials and methods

2.1 Yeast strains, media composition, and culture conditions

Wild-type strain W29 (ATCC 20460) and strains derived from it were used (see Table 1). Rich yeast extract/peptone/dextrose (YPD) medium was used for inoculum preparation and contained 10 g/L yeast extract, 10 g/L peptone, and 20 g/L D-glucose. Composition of minimal medium (YNB) used for transformant selection was 1.7 g/L yeast nitrogen base without amino acids and ammonium sulfate, 5 g/L NH₄Cl, 50 mM phosphate buffer (pH 6.8), and 20 g/L glucose. For selection of prototrophic transformants, YNB agar plates with 20 g/L agar were used. For selection of auxotrophic transformants, leucine or uracil was added to YNB agar plates when required (0.1 g/L leucine or uracil). Agar plates for selection of marker excised cells were prepared as described by Fickers et al. [23]. Glycerol medium for lipid

Table 2. Primers used in this study

Primers	Sequence (5'→3')
DGA2 in	CTGCTTGTAAGTGTGAAGCCAG
Leu2 in	GAGCTCAACCACCAGCTGATC
Ura3 in	CCTTATCGGCAAGCTCAATG

accumulation contained 60 g/L glycerol, 1.5 g/L yeast extract, 0.5 g/L NH₄Cl, 7 g/L KH₂PO₄, 5 g/L Na₂HPO₄·12H₂O, 0.1 g/L CaCl₂, 1.5 g/L MgSO₄·7H₂O, 10 mg/L ZnSO₄·7H₂O, 0.6 mg/L FeCl₃·6H₂O, 0.07 mg/L MnSO₄·H₂O, 0.04 mg/L CuSO₄·5H₂O. Growth was carried out in 100 mL of glycerol medium in 500-mL baffled flasks. Flasks were inoculated with 24-h preculture to an optical density at 600 nm (OD₆₀₀) of 0.1. Cells were grown on orbital shaker at 120 rpm at 28°C. In fed-batch experiment, 72% (w/v) glycerol was added into flasks after 72 h of cell growth to introduce additional 60 g/L of glycerol. *Escherichia coli* cells were grown in Luria Bertani medium according to Sambrook and Russel [32].

2.2 Construction of multiple *DGA2* strain

Plasmids from *E. coli* JME1132 (*pTEF-DGA2-URA3ex*), and JME1822 (*pTEF-DGA2-LEU2ex*) were digested by *NotI* to release expression cassettes. Purified expression cassettes were used for the following transformations. Yeast cells were transformed by the lithium acetate method [33]. Strain JMY1884 (Q4-*DGA2* $\times$ 1, Leu[−]), constructed previously by Beopoulos et al. [26], was used for transformation with *pTEF-DGA2-LEU2ex* cassette to construct prototrophic strain JMY3357 (Q4-*DGA2* $\times$ 2). Subsequently auxotrophies were restored via *LEU2* and *URA3* marker excision in JMY3357 using the Cre-lox recombination system with the replicative plasmid pUB4-Cre1 from *E. coli* JME547 [23]. Resulting auxotrophic strain JMY3435 (Q4-*DGA2* $\times$ 2, Leu[−] Ura[−]) was transformed with *pTEF-DGA2-URA3ex* cassette yielding JMY3513 (Q4-*DGA2* $\times$ 3, Leu[−]), which was transformed with *pTEF-DGA2-LEU2ex* cassette to construct JMY3580 (Q4-*DGA2* $\times$ 4). Insertion of expression cassettes was confirmed by PCR (primers described in Table 2). All restriction enzymes were purchased from New England Biolabs (Ipswich, England). Genomic DNA was prepared according to Querol et al. [34]. Amplifications of DNA by PCR were performed in an Applied Biosystems 2720 thermal cycler with Taq DNA polymerase (Promega,

Figure 1. Kinetics of DCW ($\square$), glycerol (GloL, $\square$), lipid accumulation (TFA, $\bullet$) and citric acid (CA, $\blacktriangle$) secretion of *Y. lipolytica* strains W29 and JMY3580. Strains W29 (A, B) and JMY3580 (C, D) were cultivated on nitrogen limited glycerol medium with initial glycerol concentration 60 g/L. After 72 h additional 60 g/L of glycerol was added and cultivated until 144 h. Cells without feeding of glycerol during cultivation (A, C) were compared to glycerol fed cells (B, D). FA content is given as mean value of three independent experiments (SD < 10%).

Madison, WI). QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) was used for DNA fragments recovery from agarose gels.

2.3 Analytical methods

Samples were taken after three and six days. Cell suspensions were centrifuged ($2880 \times g$, 5 min), washed twice with saline, once with deionized water and freeze-dried. Freeze-dried cells were used for lipid analysis. Cells from cell suspensions (5 mL) were centrifuged, washed and dried until constant weight at 105°C to determine dry cell weight (DCW).

Glycerol and CA were determined by HPLC (Agilent) equipped with an Aminex HPX87H column (Bio-Rad) coupled to RI detector and UV detector as described by Lazar et al. [35].

FA from freeze-dried cells were transformed to FA methyl esters (FAMES) and analyzed as described previously [30]. Total cellular lipids were extracted by chloroform:methanol (2:1) solution [36] and analyzed by TLC on Merck TLC Silica gel 60 plates [30].

3 Results and discussion

3.1 Engineering yeasts for SCO production

Strain JMY3580 was derived from *Y. lipolytica* Q4 strain [26] by four consecutive *pTEF-DGA2* cassette insertions. Multiple copy insertion of *DGA2* has been already shown as an efficient strategy to improve lipid accumulation in *Y. lipolytica* [30]. In this study,

strain carrying four *DGA2* gene copies was constructed. The repeated insertion of *pTEF-DGA2-URA3ex* and *pTEF-DGA2-LEU2ex* cassettes resulted in prototrophic phenotype of yeast cells. Especially restoration of leucine biosynthesis was important, because functional leucine biosynthesis pathway is essential for normal cell growth and lipid accumulation in *Y. lipolytica* [37]. Thus, strain JMY3580 is prototrophic strain with no special requirements for growth factors compared to wild-type strain W29.

3.2 Growth and metabolite production on glycerol medium

Remodeling of triacylglycerol (TAG) biosynthesis pathway had major impact on yeast biomass production. After 72 h almost 57 g/L of glycerol were consumed by strain W29. Biomass yield of strain W29 was 12.4 g/L biomass, while strain JMY3580 yielded approximately 15.3 g/L biomass and consumed all glycerol in cultivation medium (Fig. 1A and C). The 1.2-fold increase in biomass yield of strain JMY3580 resulted predominantly from higher lipid accumulation expressed as total fatty acids (TFA) in biomass. Strain JMY3580 accumulated more than 40% of TFA in its biomass, while portion of TFA in wild-type strain did not exceed 20%. CA production by both strains was observed after 24 h of growth. CA productivity was not very significant and after 72 h both strains yielded about 1.5 g/L of CA. Not only CA secretion but also production of polyols was observed (see Table 3). Mannitol was the most abundant polyol secreted into cultivation medium. Wild-type strain has shown higher polyol

Table 3. Lipid, citric acid and polyol production on glycerol medium. Cells were grown on nitrogen limited 60 g/L glycerol medium for 72 h. After 72 h glycerol equal to concentration 60 g/L was added and cells were grown until 144 h. Cells grown in medium without addition of glycerol (*batch*) were compared to cells grown with glycerol addition (*fed-batch*). Consumed glycerol (Glo_{cons}), (DCW, TFA, citric acid (CA), erythritol (Ery), and mannitol (Man) are given as a mean value of three independent experiments (SD < 10%)

Strain	Time (h)	Glo _{cons} (g/L)	DCW (g/L)	TFA (g/L)	CA (g/L)	Ery (g/L)	Man (g/L)
W29	72	56.9	12.4	2.1	1.6	5.5	13.9
<i>batch</i>	144	60	10.9	1.6	2.3	0	13.8
<i>fed-batch</i>	144	100.6	11.4	1.9	4.7	20.6	24.5
JMY3580	72	60	15.3	7.4	1.5	1.4	10.4
<i>batch</i>	144	60	16.7	6.4	0.5	0	4.7
<i>fed-batch</i>	144	106.2	18.5	9.9	5.2	8.2	20.3

productivity than JMY3580. We assume that lower polyol productivity resulted from increased carbon flux toward neutral lipid biosynthesis. Glycerol consumption by the strain JMY3580 was faster which indicates that overexpression of diacylglycerol acyltransferase gene has impact on overall cellular metabolism. The comparable impact of genetic modification was observed by Celińska and Grajek, when glycerol catabolism was modified in *Y. lipolytica* A18 strain [38]. While JMY3580 consumed all glycerol after 72 h, strain W29 depleted glycerol 24 h later. After glycerol depletion decrease in TFA yield in biomass was observed in both strains (see Table 3). Interestingly, CA and polyol reutilization by yeast cells took place after glycerol was consumed. The similar behavior was observed by André et al. [21], although in their study CA and mannitol was reutilized when glycerol was still present in cultivation medium. Difference between W29 and JMY3580 in CA secretion and reutilization was observed. While JMY3580 started to reutilize CA immediately after glycerol depletion, W29 continued to produce CA until 120 h (Fig. 1A and C).

In order to avoid complete glycerol consumption and potentially increase lipid accumulation in yeast cells fed-batch cultivation was performed. Cells were grown in nitrogen limited glycerol medium with 60 g/L of glycerol. After 72 h amount of glycerol equal to concentration 60 g/L was added (Fig. 1B and D). Fed-batch was carried out to avoid carbon to nitrogen (C/N) ratio higher than 90 in the beginning of cultivation, because higher C/N ratios seems to promote CA formation at the expense of lipid accumulation [15,39]. Fed-batch cultivation was compared to cultivation, where carbon source was limited to only 60 g/L glycerol without addition of fresh glycerol. Addition of fresh glycerol had major influence on mutant strain JMY3580. In JMY3580 1.6-fold higher biomass yield was observed, compared to wild-type strain. Improvement in biomass yield of JMY3580 was connected mainly with elevated lipid accumulation. This strain produced almost 10 g/L of TFA what represents more than 50% of yeast biomass. Cells of strain W29 was not able to convert excess glycerol into cellular lipids as efficiently as cells of strain JMY3580. Addition of fresh glycerol did not influence the TFA yield in wild-type strain. Addition of fresh glycerol resulted in ongoing production of polyols and CA. While wild-type strain was better in polyol production compared to JMY3580, both strains were comparable in CA productivity. Glycerol consumption by both strains was comparable, although JMY3580 consumption capacity was slightly higher (see Table 3). Therefore, it can be concluded that remodeling of TAG biosyn-

thesis pathway by overexpression of *DGA2* gene allowed more efficient carbon flow from glycerol into storage lipids, at the expense of polyols productivity. According to the observation of Makri et al. [40], production of significant amount of CA (over 30 g/L) coupled with massive storage lipid degradation takes place in the late stationary phase in *Y. lipolytica* ACA-DC 50109. In our case, relatively low CA production about 5 g/L by both strains was observed. In strain JMY3580, CA was produced along with lipid accumulation and also polyol secretion when glycerol was available (Fig. 1C and D). Strain W29 showed the same CA productivity as JMY3580 and only moderate storage lipid degradation was observed after 144 h of cultivation in fed-batch as well as in batch mode (see Fig. 1A and C). In our experiments, pH was not regulated and dropped to value about 2.6 after 144 h of fed-batch cultivation. Such low pH has a negative effect on CA production [15] and favors rather production of polyols [41]. Controlling the pH around 5 was found to be effective for optimal glycerol conversion into CA by *Y. lipolytica* cells [18].

Various strains of *Y. lipolytica* show different performance on nitrogen limited medium with either sugars or glycerol as carbon sources. Significant lipid accumulation about 40% was observed when *Y. lipolytica* MUCL 28849 was grown on either glucose or glycerol combined with volatile FAs. Production of CA or polyols was not reported in this study [39]. Another type of hydrophilic substrate metabolism was observed for example in the study of Makri et al. when lipid turnover was observed during CA production phase of *Y. lipolytica* ACA-DC 50109 [40]. After nitrogen depletion lipids were initially stored and in late stationary phase, when significant CA production was observed, lipid degradation took place while simultaneously significant quantities of glycerol remained unconsumed in medium. Their study shows, that when higher glycerol concentration was employed, higher CA yield was observed at the expense of lipid yield. On the other hand, when *Y. lipolytica* ACA-DC 5033 was grown in nitrogen limited media with glycerol as a carbon substrate, simultaneous CA production and lipid accumulation was observed [21]. The same observation was made when strain *Y. lipolytica* A101 was grown in a glycerol medium with a concentration of glycerol 150 g/L [42]. Behavior of our modified strain JMY3580 and wild-type strain W29 was rather similar to strain ACA-DC 5033 in the study of André et al. [21] and A101 in the study of Dobrowolski et al. [42]. Both our strains were able to accumulate lipids with ongoing CA production. In our study as well as in study of Dobrowolski et al. [42], a CA reutilization was

Table 4. Lipid yields by different yeasts grown in glycerol-based media

Strain	Time (h)	Biomass (g/L)	Lipid content (%)	Lipid yield (g/L)	Growth mode	Reference
<i>C. curvatus</i> ATCC 20509	288	44.5	49	21.8	Fermentor	[44]
<i>C. curvatus</i> ATCC 20509	288	32.9	52	17.1	Fermentor	[45]
<i>C. curvatus</i> ATCC 20509	50	118	25	29.5	Fermentor	[46]
<i>R. toruloides</i> DSZM 4444	120	15	57	8	Fermentor	[47]
<i>R. glutinis</i> BCRC 21418	160	17.6	55	8.5	Fermentor	[48]
<i>Y. lipolytica</i> A101	120	7.9	26	2.1	Flask	[42]
<i>Y. lipolytica</i> NCYC3825	70	42	30	13	Fermentor	[38]
<i>Y. lipolytica</i> MUCL 28849	48	42.2	38.1	16.1	Fermentor	[39]
<i>R. babjevae</i>	120	9.4	35	3.3	Flask	[49]
<i>R. diobovatum</i>	120	11	64	7	Flask	[49]
<i>Y. lipolytica</i> JMY3580	144	18.5	53.7	9.9	Flask	This study

Table 5. FA profiles of *Y. lipolytica* strains cultivated for 144 h in glycerol medium (60 g/L glycerol) with (*fed-batch*) and without glycerol addition (*batch*). FA content is given as mean value of three independent experiments

Strain	Fatty acid profile (%)					
	C16:0	C16:1	C18:0	C18:1	C18:2	others
<i>batch</i>						
W29	12 ± 1.3	7 ± 0.3	9 ± 1.0	52 ± 1.3	13 ± 0.8	6 ± 0.2
JMY3580	17 ± 0.9	11 ± 0.6	9 ± 0.5	56 ± 1.4	5 ± 0.3	2 ± 0.1
<i>fed-batch</i>						
W29	15 ± 1.3	13 ± 3.5	6 ± 0.9	49 ± 1.6	11 ± 1.7	6 ± 0.4
JMY3580	17 ± 0.6	8 ± 0.8	12 ± 1.3	57 ± 1.0	3 ± 0.3	2 ± 0.1

observed after glycerol depletion. We did not observe massive lipid degradation in stationary phase of growth when carbon source was still present in medium, although in the case of W29 little decrease in TFA content was observed in last 48 h of growth (Fig. 1B). This observation is in contrast with studies in which storage lipids were degraded in stationary phase while substrate was still present [19, 40]. On the other hand, our study is in line with the other studies showing continuous lipid accumulation until substrate depletion [38, 42].

Genetic modifications resulting in overexpression of *DGA2* introduced important difference into JMY3580 compared to original strain W29. When W29 reached certain lipid level in cells it was not able to accumulate more lipids despite glycerol was still present in cultivation medium and a moderate lipid turnover was observed after 120 h (see Fig. 1B). However, JMY3580 was able to accumulate lipids continuously until 144 h and no lipid degradation was observed in stationary growth phase when glycerol was present in cultivation medium (see Fig. 1D). Moreover, strain JMY3580 produced less polyols, which are considered as metabolites competing with FA biosynthesis in *Y. lipolytica* [43]. Results achieved in this study were compared with other studies employing glycerol as a carbon source for SCO production. Table 4 summarizes biomass, lipid content, and lipid yield of different yeasts. Although many oleaginous yeasts with better lipid accumulation than *Y. lipolytica* are known (e.g. *Cryptococcus curvatus*, *Rhodospiridium toruloides*), *Y. lipolytica* has advantage of being easily engineered to produce “tailor-made” lipids with desired composition. Thus, lipids produced by *Y. lipolytica* presents an

economically viable alternative for valuable fats and oils from natural sources [50].

3.3 Lipid composition

Remodeling of TAG biosynthetic pathway in *Y. lipolytica* influenced FA composition of cellular lipids. Lipids of JMY3580 were slightly enriched with saturated palmitic acid (C16:0) compared to lipids of wild-type strain (see Table 5). Major FA of both strains was oleic acid (C18:1). This is a common feature of *Y. lipolytica* cells [51], unless extracellular FAs are used as a carbon source [52]. The major difference between FA profiles of W29 and JMY3580 was lower percentage of linoleic acid (C18:2) in lipids of JMY3580. Similar result was observed by Wang et al. [53], when lipid yield was improved by deletion of *MIG1* gene in *Yarrowia lipolytica* ACA-DC 5010. JMY3580 independently on culture conditions accumulated more C16:0 and less C18:2 than W29. This FA composition of JMY3580 could be a slight advantage if SCO should be turned into biodiesel afterwards. Although optimal FA composition for biodiesel based on FAME depends on many factors, higher fractions of mono-unsaturated FA and low levels of saturated FA are considered as optimal for minimizing cold flow problems and low levels of poly-unsaturated FA improve oxidation stability [54]. FA profiles of cells grown for 144 h either in fed-batch or without addition of glycerol are summarized in Table 5.

Differences in FA composition rise from altered TAG formation pathway in strain JMY3580. In wild-type strain, TAG synthesis is mediated by three proteins Dga1p, Dga2p, and Lro1p, with Dga1p having major contribution to TAG synthesis [26]. In JMY3580, only Dga2p is responsible for TAG synthesis. It is possible that Dga2p had preference for certain acyl-CoA and/or DAG, which could be an explanation of observed differences between JMY3580 and W29. However, no data about acyl-CoA or DAG specificities of TAG forming enzymes in *Y. lipolytica* are available and further research in this area is required. Another explanation of lower C18:2 percentages in lipids of strain JMY3580 is faster incorporation of synthesized FA into TAG. Rapid binding of FA into acylglycerols might have resulted in withdrawal of acyl chains from the influence of desaturases, which use acyl-CoA as a substrate [55]. However, it is well-known that diacylglycerol acyltransferases has an impact on TAG composition. Desired

composition of storage lipids could be achieved by overexpression of suitable diacylglycerol acyltransferase together with other enzymes (e.g. hydroxylases, desaturases) [11]. Such engineered *Y. lipolytica* strains are able to produce different valuable lipids containing unusual FA [50].

According to structural lipid analysis fraction of TAG in total lipids of strain JMY3580 was approximately 60–70% compared to 40–50% for the wild-type strain, depending on culture conditions. Increase in TFA accumulation of JMY3580 resulted in elevated TAG formation, because TAG are the major lipidic structure in which FA are stored in yeast cells [56]. TAG form a hydrophobic core of cellular compartment called lipid bodies (LB). Together with TAG also sterol esters (SE) are present in LB [57]. Level of SE was very low in total lipids of JMY3580. Only trace amounts of SE up to maximum 1% compared to 15% in wild type was observed in JMY3580 (see Fig. 2). Such SCO with low SE and total sterol content might be advantageous for biodiesel production. Sterols, SE, free FA, and phospholipids are considered as impurities in oils for biodiesel production [58]. Therefore, low levels of these compounds are desirable.

4 Concluding remarks

Y. lipolytica has shown as microorganism with great potential to become a cell factory for SCO production, which could have many potential applications (e.g. biodiesel, cocoa butter substitutes, source of unusual FA). *Y. lipolytica* has an advantage that it could be easily modified due to fully sequenced genome and already well-developed genetic engineering tools. Presented results suggest, that through combination of genetic engineering and optimization of cultivation process considerable production of SCO is achieved. A great advantage is possibility of using low-cost substrate as glycerol, and converting it into value-added products based on SCO.

Practical application

Engineered strain of *Yarrowia lipolytica* with improved lipid accumulation ability is a good tool for SCO production. This strain has the potential for conversion of low-cost substrates into value added products based on microbial oils. Glycerol is a substrate, which is readily utilized by *Y. lipolytica* cells. Moreover, the low price of glycerol makes it suitable for production of various value-added compounds. This study showed that by combining of metabolic engineering with suitable substrates and growth conditions high amount of SCO could be obtained.

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The authors have declared no conflict of interest.

Figure 2. Lipid separation by TLC for the wild type W29 and JMY3580 (Q4-DGA2 × 4). Lipids were extracted by modified procedure of Folch et al. [25]. Lipids were developed in hexane/ether/acetic acid 80/20/1 system. PL – polar lipids, DAG – diacylglycerols, ERG – ergosterol, FFA – free fatty acids, TAG – triacylglycerols, SE – sterol esters, SQ – squalene.

5 References

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