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Engineering *Rhodosporidium toruloides* with a membrane transporter facilitates production and separation of carotenoids and lipids in a bi-phasic culture

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Abstract The oleaginous yeast *Rhodosporidium toruloides* has great biotechnological potential. It accumulates a high amount of lipids which can be used for biofuels and also produces carotenoids which are valuable in the food and pharmaceutical industry. However, the location of these two hydrophobic products in the cell membrane prohibits its efficient harvesting and separation. Here, the transporter Pdr10 was engineered into *R. toruloides* and cultured in two-phase media containing oil. This enabled the production and in situ export of carotenoids into the oil and concurrent separation from intracellular lipids in the cells. When Pdr10 strain was cultured in the two-phase media, carotenoids and fatty acids yield increased from 1.9 to 2.9 μg/mg and 0.07 to 0.09 mg/mg, respectively. A total of 1.8 μg/mg carotenoids was exported by Pdr10 strain, as compared to 0.3 μg/mg in the wild type. In the Pdr10 strain, the composition of carotenoids and fatty acid it produced also changed. Torulene became the major carotene produced instead of torularhodin. Also, the unsaturated fatty acid C18:2 became the dominant fatty acid produced instead of the saturated C16:0, which was similar to the grape seed oil used in the two-phase media. This indicated that oil was being consumed by the cells, which was supported by the increased intracellular glycerol levels detected by gas chromatography-mass spectrometry (GC-MS). Our approach represents an easy and greener extraction method which could serve to increase the yield and facilitate separation of carotenoids and fatty acids.

Keywords *Rhodosporidium toruloides* · ABC transporter · Carotenoids · Lipids · Extraction · Separation

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Introduction

Microorganisms are commonly used for the production of a wide variety of valuable compounds, such as carotenoids and lipids for biofuels (Mata-Gomez et al. 2014; Peralta-Yahya et al. 2012). To liberate the desired product, there is usually a need to first disrupt the cell physically or by a chemical method, then extract and purify the product. It is this downstream step of extraction and purification, which usually raises the overall cost of production greatly (Mata-Gomez et al. 2014; Mosier et al. 2005; Sheehan et al. 1998).

Carotenoids and lipids are hydrophobic compounds which are located in the lipid bi-layer of the cell membrane (Gruszecki and Strzałka 2005). This is an obstacle for the easy recovery of pure carotenoid and lipid extracts. At present, limited information exists on extracting and separating these compounds. From palm oil samples, which are carotenoids and lipid rich, the method of trans-esterification to harvest lipids prior to recovery of carotenoids was explored. However, the study showed that the subsequent step which required temperature of 60 °C led to carotene degradation (Chuang

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and Brunner 2006). Another study investigated freezing a lipid-rich sample first to solidify the lipids, followed by filtration to separate the lipids from carotenoids successfully removed 90 % of lipids from the sample. However, the method also required an additional saponification step which led to losses in carotenoid recovery (Rodriguez-Amaya and Kimura 2004). Furthermore, the current extraction methods for lipids and carotenoids also require the use of organic solvents such as chloroform, methanol, or hexane, which are environmentally unfriendly (Li et al. 2014; Park et al. 2007).

Few micoorganisms have the ability to produce both lipids and carotenoids. *Rhodosporidium toruloides* (*R. toruloides*) is a robust oleaginous yeast which can naturally produce both. It is able to accumulate up to 60 % of its dry weight in lipids, which can be used as biofuels (Xu et al. 2012). The carotenoids *R. toruloides* produces are β-carotene, torulene, and torularhodin (Buzzini et al. 2007). All of these carotenoids are valuable in the food and pharmaceutical industry, as they are precursors of vitamin A, and their reddish orange pigments are used as food dye or colorants (Frengova and Beshkova 2009). Hence, although there is great biotechnological potential of *R. toruloides*, an efficient method to harvest and separate its carotenoids and lipids needs to be developed.

Agrobacterium tumefaciens-mediated transformation (ATMT) was recently developed as a genetic tool for this oleaginous yeast (Liu et al. 2013). Using this method, we transformed the gene encoding for the membrane transporter, pleiotropic drug resistance (Pdr) 10 from *Saccharomyces cerevisiae* into *R. toruloides*, as it has been shown to export β-carotene in *S. cerevisiae* (Verwaal et al. 2010). We demonstrate that when this modified strain of *R. toruloides*, Pdr10 strain, was cultured in a two-phase system, it facilitated the continuous export of carotenoids in situ without the need for any extraction steps. This further allowed the concurrent separation of carotenoids from the intracellular lipids. The use of this transporter in *R. toruloides* in the two-phase media also resulted in the enhanced production of both carotenoids and lipids.

Materials and methods

Microorganism and culture conditions

We used *R. toruloides* CBS 5490 in this work (Lee et al. 2014). It was cultivated in YPG media containing 60 g/L glycerol, 10 g/L yeast extract, and 20 g/L peptone and maintained on YPG agar plates containing 20 g/L agar. For in situ export of carotenoids, 20 % (*vol*/*vol*) grape seed oil was added to the culture media. Fermentation was carried out for 12 days in shake flasks in a total volume of 50 ml, shaking at 250 rpm, at 30 °C. *A. tumefaciens* were cultivated in LB media at 28 °C. All strains, plasmids and primers, and their source are listed in Table 1.

Transformation of *R. toruloides* by ATMT

Pdr10 gene was synthesized in a pUC57 plasmid by Genscript (Singapore), with codon optimization for *R. toruloides*. The gene was digested by restriction enzymes purchased from New England Bio labs (NEB, MA, USA). Next, the gene was ligated into the binary plasmid pRH2031 which contains a hygromycin expression cassette and electroporated into *A. tumefaciens*. The gene was transformed into *R. toruloides* via ATMT, performed accordingly to (Liu et al. 2013). To confirm successful transformation of *R. toruloides*, hygromycin-resistant colonies, which contained the binary plasmid carrying the Pdr10 gene, were grown on YPG agar plate supplemented with hygromycin and cefotaxime to inhibit growth of *A. tumefaciens*. Next, fungal colony PCR was carried out using oligonucleotide primers synthesized by 1st BASE Pte Ltd. (Singapore) (primer sequence listed in Table 1). The PCR products were separated on a 1 % (*w*/*v*) agarose gel. Colonies exhibiting a 2324-bp fragment were considered positive transformants for Pdr10. An empty vector without the Pdr10 gene was also transformed into *R. toruloides* using ATMT (control).

Analysis of intracellular and exported carotenoids

At the end of fermentation on day 12, culture media was centrifuged at 10,000×*g* for 10 min. To analyze for exported carotenoids, the oil phase was analyzed by high performance liquid chromatography (HPLC). As the oil fraction was dense, it was diluted with acetone in the ratio 750 μl:500 μl. For intracellular carotenoids analysis, 1 ml of washed cell pellet was broken with glass beads by a bead grinder (MP Biomedicals) and extracted in 250 μl of acetone. This was repeated 4 times, until the pellet was colorless, in a total of 1 ml acetone. Both the acetone extract and oil phase were analyzed using HPLC (Agilent 1100 Series instrument). Samples were separated on a LiChrospher 100 RP-18 column (5 μm; 250 mm × 4 mm, Merck). The mobile phase used was a gradient of 70 to 100 % acetone in MilliQ water, and the flow rate was 0.5 ml/min. The total run time was 110 min. Detection was performed at 450 nm according to (Weber et al. 2007). Carotene standards from CaroteNature (Switzerland) were used to identify and quantitate the carotenoids based on the retention time and area. Carotenoids were normalized to cell dry weight (μg/mg). Cell dry weight was quantified by washing and drying the cells of 1 ml culture broth in an oven set at 70 °C, until constant dry cell weight (DCW). A standard curve was made correlating optical density (OD) at 600 nm to dry cell weight.

Table 1 All strains, plasmids, and primers used in this study

Strain	Description	Source/ref
Wild type	*R. toruloides* strain CBS 5490	CBS-KNAW
Pdr10	Pdr10 gene transformed into wild type with plasmid pRH2031	This study
A. tumefaciens	AGL-1 strain used for ATMT transformation	(Baumgartner et al. 2010)
ABC transporter		Organism
Original Pdr10 sequence	NCBI accession no. NP_014973.1/NM_001183748.1.	*S. cerevisiae*
Codon optimized Pdr10 sequence	DDBJ accession no. LC031821	*R. toruloides*
Plasmid		
pRH2031	Contains a hygromycin gene expression cassette	(Liu et al. 2013)
Primers (5′ → 3′)		
Pdr10	Forward: CTCGCCTACATCATCTTCT Reverse: AGGAGTGGGTTTGGGATT	

Extraction and trans-esterification of lipids into FAME

Washed cells of equal OD 10 at the end of fermentation on day 12 were resuspended in 1000 μl of 0.9 % NaCl solution and acidified with 200 μl of acetic acid. Ten microliters of 10 mg/ml heptadecanoic acid (Sigma) was added as an internal standard to account for sample losses. The cells were broken using glass beads by a bead grinder (MP Biomedicals). Lipids were extracted using an adjusted chloroform-methanol method (Bligh and Dyer 1959). Three milliliters of chloroform-methanol in the ratio 2:1 was added, vortexed vigorously for 1 min, and centrifuged at 10,000×*g* for 10 min at 4 °C. The lower chloroform layer containing lipids were collected. To further extract remaining lipids, an additional 3 ml of chloroform-methanol in the ratio 2:1 was added. Then, the chloroform extracts were combined, and rotary evaporated to dryness using a TurboVapH LV Concentration Workstation. For trans-esterification, 500 μl 10 % BF3-methanol (Fluka, 15,716) was added, and the sample was incubated at 95 °C for 20 min. Next, 300 μl saturated NaCl (26 % saturated in water at 20 °C) and 300 μl hexane was added, vortexed vigorously, and centrifuged at top speed for 5 min. The upper hexane layer was taken for fatty acid methyl ester (FAME) analysis. FAME was normalized to cell dry weight (mg/mg).

Fatty acid methyl esters analysis by GC-MS

FAME analysis was performed according to previous work (Horák et al. 2009). An Agilent Technologies 7890A-5975C GC-MS system fitted with a HP-5MS column (30 m × 0.250 mm i.d.; film thickness, 0.25 μm; Agilent) was used. The carrier gas was helium, supplied at 1.1 ml/min. The inlet source temperature were set at 250 °C, and MS source temperatures at 230 °C. Oven temperature was maintained at 80 °C for 1 min, and then ramped to 250 °C at a rate of 7 °C/min, and held at 250 °C for 10 min. Full scan mode was used from 35 to 600 m/z. Quantification was carried out using FAME standards (Supelco, 18,918).

Intracellular metabolites analysis by GC-MS

One milliliter of fermentation culture on day 1, 4, and 7 were harvested. Cells of equal OD 10 were washed carefully with MilliQ water three times, measured spectrophotometrically, quenched in 800 μl ice cold methanol, and lyzed using glass beads and a bead grinder. Ten microliters of 2 mg/ml ribitol dissolved in MilliQ water was added to each sample as an internal standard to correct for any loss of metabolite during the extraction process. The samples were prepared accordingly to (Wang et al. 2010). All samples for GC-MS were run within 24 h. One microliter of sample was injected using Shimadzu QP 2010 Plus GC-MS system. The oven temperature was kept at 75 °C for 4 min and was raised 4 °C every min, to a final temperature of 280 °C and held for 2 min. Mass spectra were recorded from 35 to 600 m/z with a scan time of 0.2 s.

Results

Transformation of the Pdr10 gene via ATMT into *R. toruloides*

ATMT was used as the method to transform *R. toruloides* with the Pdr10 gene. As shown in Fig. 1a, growth of red *R. toruloides* colonies containing the successfully transformed vector with the Pdr10 gene could grow on the agar plates supplemented with hygromycin. Growth of colonies was also observed for control, transformed with an empty vector with no Pdr10 gene, due to the expression of the hygromycin gene cassette on the vector. For the wild type, *R. toruloides* without

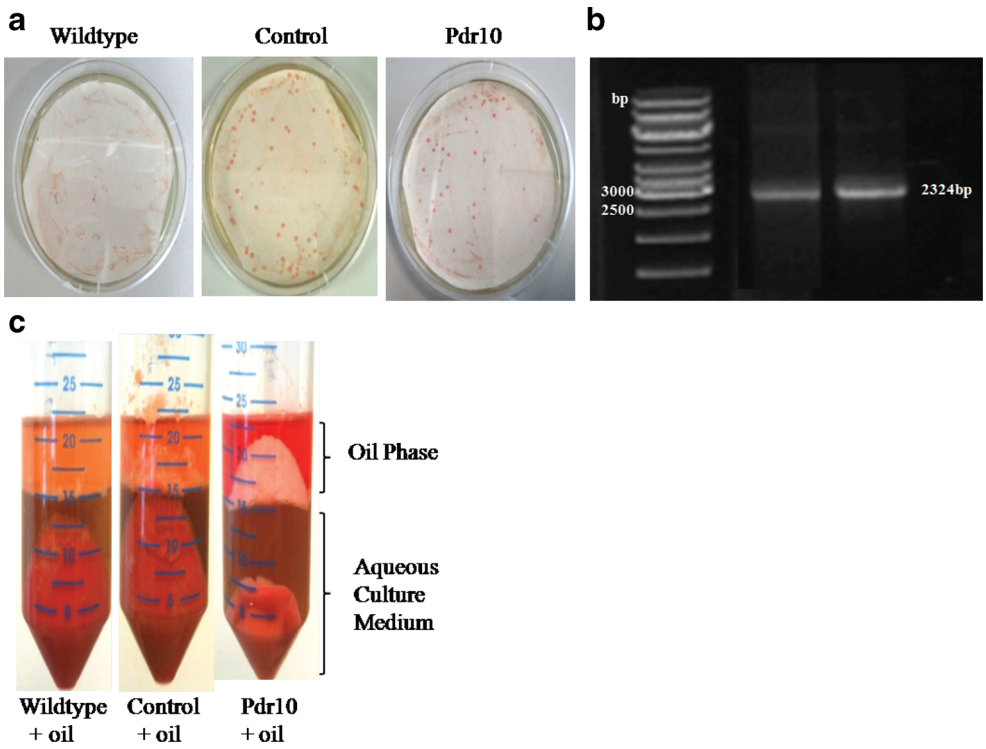

Fig. 1 **a** Growth of *R. toruloides* colonies on agar plates without (wild type) and with ATMT transformation of empty vector (control), and Pdr10 gene into *R. toruloides* (Pdr10). **b** *Left*: the Pdr10 gene as a positive control. *Right*: colony PCR detection of a colony shows a 2324-bp band which is the same size as the control, indicating the colony is positive for the presence of Pdr10 gene. **c** After centrifugation at the end of fermentation, the oil phase is seen at the *top*, and aqueous culture media at the *bottom* containing the cell pellet

any transformation did not yield colonies (Fig. 1a). To confirm successful transformation without picking up mutations in the Pdr10 strain, five colonies were picked and sent for sequencing. All five did not contain mutations. Colony PCR using Pdr10-specific primers were also used to detect the presence of the gene for the five colonies. The PCR results showed all five colonies contained the gene. Figure 1b shows an example of a positive colony containing the 2324-bp band, indicating the presence of the gene in *R. toruloides*. All transformants behaved the same. Expression of the Pdr10 gene was also checked on a protein level using immunofluorescence (Figure S1).

Pdr10 strain cultured in the two-phase media increased carotenoids production

The export of carotenoids into the oil phase by the Pdr10 transporter was quantitated using HPLC. The production of intracellular carotenoids was also quantitated by HPLC. As shown in Table 2, in the absence of the two-phase media, carotenoid production was comparable between the wild type (0.9 μg/mg) and Pdr10 (0.8 μg/mg) strains. No carotenoids were exported into the aqueous culture media (not detected as shown in Table 2). Using the two-phase media, the intracellular carotenoid production was higher in wild type (1.6 μg/mg) as compared to Pdr10 (1.1 μg/mg). However, only 0.3 μg/mg of the total carotenoids were exported by the wild type strain as compared to 1.8 μg/mg by the Pdr10 strain. The control behaved like the wild type, as a similar total of 0.3 μg/mg carotenoids were exported. The higher amount of carotenoids which are present in the oil phase can be visually observed in Fig. 1c after centrifugation of the two-phase culture media, as reflected in the more pigmented oil phase belonging to the Pdr10 strain. Overall, the use of a two-phase media increased total carotenoids production from 1.9 μg/mg in the wild type to 2.9 μg/mg in the Pdr10 strain.

Pdr10 transporter exports torulene with the greatest efficiency

As can be seen in the peaks detected using HPLC in Fig. 2a, b, the three carotenoids: torularhodin, torulene, and β-carotene, were exported by the wild type and Pdr10 strains into the oil phase. Using standards for quantitation, we found that in the wild type, the amount of torulene exported was negligable, and only minute amounts of β-carotene (0.2 μg/mg) and torularhodin (0.1 μg/mg) were exported (Fig. 2b). In comparison, torulene was exported in large amounts (1.1 μg/mg), followed by β-carotene (0.6 μg/mg) and torularhodin (0.1 μg/mg) by the Pdr10 strain (Fig. 2b).

Pdr10 strain cultured in the two-phase media increased total fatty acids production

Besides carotenoids, *R. toruloides* is an oleaginous yeast capable of producing large amounts of lipids. At the end of fermentation, total intracellular fatty acids were extracted from the cells and quantitated using GC-MS. In Fig. 3, the four

Table 2 Quantitation of total carotenoids production at the end of fermentation

(μg/mg)		Wild type	Control	PDR10	Wild type + oil	Control + oil	PDR10 + oil
Intracellular	Torularhodin	0.6 ± 0.09	0.5 ± 0.03	0.5 ± 0.02	0.8 ± 0.09	0.8 ± 0.04	0.3 ± 0.02
	Torulene	0.1 ± 0.01	0.1 ± 0.01	0.1 ± 0.02	0.3 ± 0.02	0.3 ± 0.03	0.4 ± 0.05
	β-carotene	0.2 ± 0.03	0.2 ± 0.02	0.2 ± 0.03	0.5 ± 0.01	0.5 ± 0.01	0.4 ± 0.01
Exported	Torularhodin	ND	ND	ND	0.1 ± 0.02	0.1 ± 0.04	0.1 ± 0.02
	Torulene	ND	ND	ND	0.0 ± 0.0	0.0 ± 0.0	1.1 ± 0.1
	β -carotene	ND	ND	ND	0.2 ± 0.04	0.2 ± 0.01	0.6 ± 0.03
Total		0.9 ± 0.04	0.8 ± 0.03	0.8 ± 0.02	1.9 ± 0.05	1.9 ± 0.03	2.9 ± 0.03

ND not detected

types of fatty acids produced were the saturated palmitic acid (C16:0) and stearic acid (C18:0), and unsaturated oleic acid (C18:1) and linoleic acid (C18:2) in the wild type, control, and Pdr10 strains. In the absence of the two-phase media, total fatty acid production was higher in the wild type (0.05 mg/mg), as compared to Pdr10 (0.02 mg/mg). However, in the presence of the two-phase media, the total fatty acid production increased in the wild type to 0.07 mg/mg and 0.09 mg/mg in the Pdr10 strain. The control produced similar amount of fatty acids as wild type, 0.05 mg/mg without the two-phase media, and 0.07 mg/mg fatty acids with oil phase (Fig. 3).

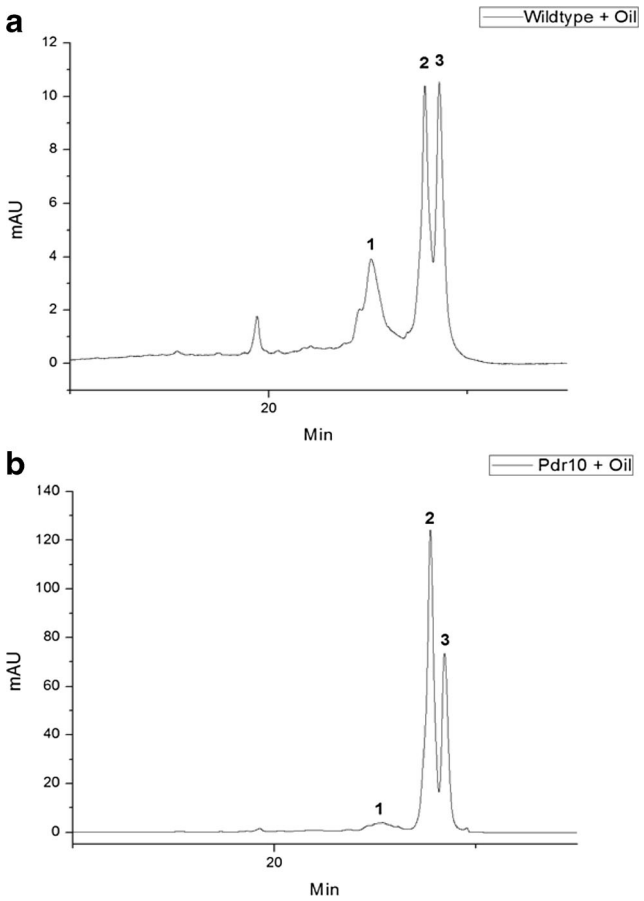

Fig. 2 Quantitation and detection of exported carotenoids into the oil phase by HPLC in **a** wildtype and **b** Pdr10 expressing cells. *1*, torularhodin; *2*, torulene; *3*, β-carotene

Effect of the two-phase media on the composition of fatty acid in *R. toruloides*

The most distinct observation was the change in the composition of fatty acids produced (Fig. 3). The dominant type of fatty acids produced by wild type, control, and Pdr10 was the saturated fatty acids, C16:0 and C18:0. However, in the presence of the two-phase media, the dominant type of fatty acid produced was no longer the saturated fatty acids C16:0 and C18:0, due to the increase in the production of the unsaturated fatty acid C18:2, in the Pdr10 strain. Overall, the amount of total unsaturated fatty acids, C18:1 and C18:2, become the majority of the total fatty acids which was produced in the Pdr10 strain (Fig. 3).

Two-phase culture media serves as an additional carbon source for *R. toruloides*

To explain the increased carotenoid and fatty acid production in the wild type and Pdr10 strains in the two-phase media, we hypothesize that the oil was being taken up into the cells and consumed. To explore this, we used metabolite profiling by GC-MS and characterized the intracellular metabolites produced on day 1, 4, and 7 of fermentation. Days 1, 4, and 7 were chosen to represent the early, mid, and late exponential growth phase, before the cells enter stationary phase on day 8 (data not shown), when metabolite production would start to slow down. As seen in Fig. 4, we found four interesting differential metabolites. In Fig. 4a, levels of glycerol which is a carbohydrate decreased rapidly in the wild type and Pdr10 cells without the two-phase media and was depleted by day 7. A reverse trend was seen, in the presence of the two-phase media, where glycerol levels did not deplete but continued to increase, until they reached a stationary level from day 4 onwards, for both wild type and Pdr10 strains. The highest

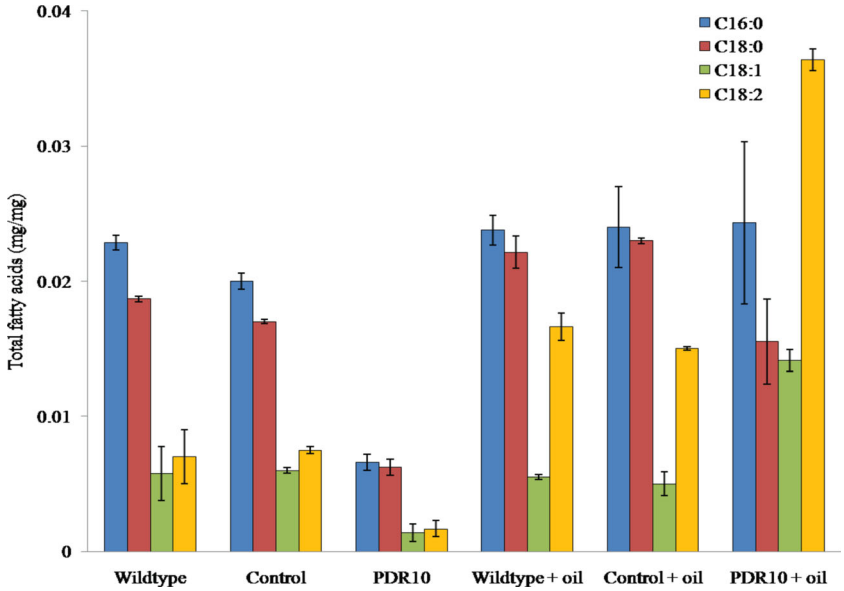

Fig. 3 Total fatty acid production at the end of fermentation for wild type, control and Pdr10 with and without the two-phase media culture

amounts of glycerol were found in the Pdr10 strain cultured with the two-phase media. The levels of the amino acid glycine (Gly) were also elevated, in both wild type and Pdr10 cells in the presence of the two-phase media (Fig. 4b). Malic acid is a metabolite which is involved in the tricarboxylic acid (TCA) cycle, and it level was found to be comparable in all groups (Fig. 4c). Another metabolite belonging to the TCA cycle and glyoxylate cycle, succinic acid, was found to be increased in the Pdr10 strain cultured with the two-phase media, as compared to the other groups.

Discussion

This work describes a systematic investigation into an easy extraction and separation method for carotenoids and lipids in *R. toruloides*. Despite the high biotechnological potential of *R. toruloides*, few publications have developed an efficient extraction method for lipids and carotenoids for this microorganism. Hence, we transformed the Pdr10 transporter gene from *S. cerevisiae*, which was shown to export β-carotene in *S. cerevisiae*, into *R. toruloides* to create the Pdr10 strain. In our study, no carotenoids were exported into strictly aqueous media (Table 2). This is consistent with a previous study which showed that hydrophobic carotenoids required a hydrophobic phase such as oil in order to be exported (Verwaal et al. 2010). The control with an empty vector behaved similarly to the wild type strain (Table 2 and Figs. 1 and 3).

In the wild type, carotenoids were also exported into the oil phase, indicating that *R. toruloides* has its own similar innate type of transporters (Table 2). The family of ATP-binding cassette (ABC) transporters is a highly conserved set of proteins, found in all kingdoms of life, including humans and yeasts (Higgins 1992). In addition, a proteomic study found proteins belonging to the ABC transporter family in *R. toruloides* (Zhu et al. 2012). Previously, it was reported that the Pdr10 transporter was specific to export the carotenoid β-carotene and not phytoene (Verwaal et al. 2010). In our study, Pdr10 seemed to export all three carotenoids: torularhodin, torulene, and β-carotene, with the greatest efficiency for torulene. However, the amount of exported torularhodin was comparable to the wild type (Table 2). Therefore, it was difficult to say if the export of torularhodin was due to the Pdr10 transporter or to the innate transporters belonging to *R. toruloides*, and further investigations are needed.

Interestingly, carotenoid and lipid production was increased when the wild type and Pdr10 strains were cultured in the two-phase media. It was also noted that the composition of fatty acids in Pdr10 strain changed. The unsaturated linoleic acid (C18:2) was strikingly increased and became the major fatty acid instead of the saturated stearic acid C16:0 (Fig. 3). This reflected the composition of grape seed oil, which was used in the two-phase media, whose dominant fatty acid is also the unsaturated linoleic acid C18:2 (Pande and Akoh 2010). Our data suggested that uptake of the oil was taking place and consumed by *R. toruloides*.

To investigate if oil was being consumed by *R. toruloides*, metabolite profiling by GC-MS was carried out. For all groups with or without the two-phase media, glycerol was used as the carbon source. Glycerol is able to enter the cells through diffusion or active transport (Chatzifragkou et al. 2010) and undergoes glycolysis to produce acetyl-CoA, a compound which is a precursor for many pathways. It was found that the depleted intracellular glycerol levels decreased steadily in wild type and Pdr10 strain without the two-phase culture media. As the cells utilize the glycerol for growth and other metabolic

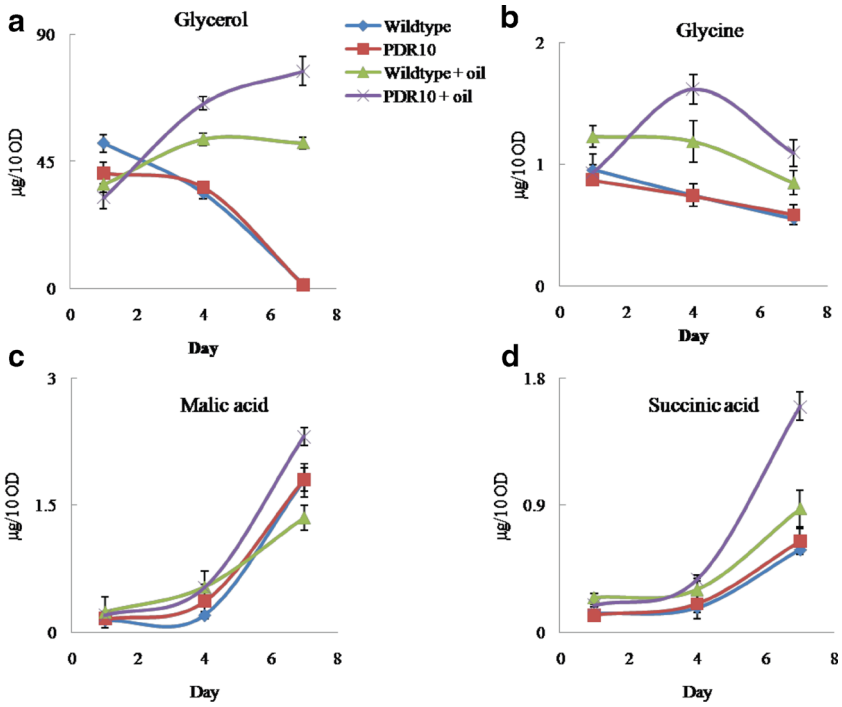

Fig. 4 Metabolite profiling for all groups on day 1, 4, and 7 of fermentation quantitated and identified using GC-MS.

processes, this explains the depleting intracellular glycerol levels. On the other hand, glycerol levels did not decrease for wild type and Pdr10 strain cultured in the two-phase media containing grape seed oil (Fig. 4a).

For *R. toruloides* to use oil as a substrate, the oil must first by hydrolyzed. Oil mainly consists of triglycerides (TAGs), which cannot enter the cell directly. TAGs can be hydrolyzed to glycerol and free fatty acids by enzymes such as lipases (Fig. 5) (De Marchi et al. 2012; Vakhlu and Kour 2006). A secreted lipase was detected in a proteomic study of *R. toruloides* (Zhu et al. 2012). This suggests that *R. toruloides* produced a lipase to hydrolyze the grape seed oil into glycerol and free fatty acids, where both could subsequently be uptaken into the cell. This explains the stable intracellular glycerol levels in wild type and Pdr10 in the two-phase media in Fig. 4a. We postulate that the extra glycerol provided from hydrolysis of the grape seed oil provided more acetyl-CoA from glycolysis, which could enter the carotenoids and fatty acid synthesis pathways (Fig. 5). This may account for the increased carotenoids (Table 2) and fatty acid production (Fig. 3) seen in wild type and Pdr10 strain in the two-phase media. The production of extracellular lipase to utilize hydrophobic substrates is seen in another oleaginous yeast *Yarrowia lipolytica*, which was demonstrated to hydrolyze and uptake triglycerides as a carbon source (Fickers et al. 2005).

Glycine is an amino acid which can be synthesized from the compound glyceraldehyde-3-phosphate in the glycolysis pathway (Fig. 5). Its level was increased in the wild type and Pdr10 strain, when the two-phase media was used (Fig. 4b). This data suggests glycerol derived from the oil entered glycolysis. Malic acid is a compound involved in the TCA cycle. There was no difference in malic acid levels in the wild type and Pdr10 strain, with or without the two-phase media (Fig. 4c). Succinic acid levels were found to be elevated in the

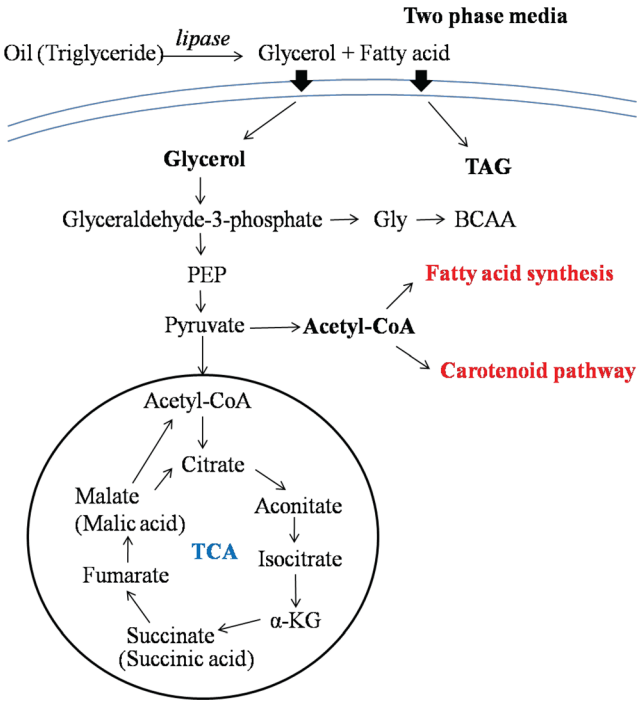

Fig. 5 The uptake of glycerol and fatty acids, and metabolic pathways of *R. toruloides* cultured in the two-phase media. *Gly* glycine, *PEP* phosphoenolpyruvate, *BCAA* branched chain amino acid, *α-KG* α-ketoglutarate

presence of the two-phase media, for both wild type and Pdr10 strains (Fig. 4d), although further studies are needed.

Therefore, our results suggest the increase in carotenoid and fatty acid production seen in the two-phase culture in both wild type + oil and Pdr10 strains, as compared to without oil is due to the hydrolysis of oil by *R. toruloides* via a secreted lipase. We hypothesize that the glycerol derived from oil provides more acetyl-CoA to enter the carotenoids and fatty acids pathway. In addition, the fatty acids derived from oil enter the cell through active transport or diffusion and are stored as TAG in lipid bodies (Fig. 5) (Fickers et al. 2005; Kohlwein and Paltauf 1984).

To the best of our knowledge, this is the first study to provide a method for harvesting and separating two lipid-like compounds, carotenoids and lipids from *R. toruloides*, by expressing a transporter. Our approach reduces the multiple steps required in traditional methods and may be applied to other organisms producing carotenoids and lipids. The two-phase culture using oil for in situ extraction and export of carotenoids is also a more environmentally friendly method, than the traditional organic solvents which are used to extract carotenoids. To decrease cost, other types of hydrophobic substrates or fats such as waste cooking oil (Katre et al. 2012), or scrap fish oil (Guo and Ota 2000), may also be explored. In this study, we used the traditional glass beads and chloroform-methanol method for the subsequent lipid extraction. In future, other more green methods for lipid extraction may be explored to create an overall total green harvesting and separation method for carotenoids and lipids.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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