Doctoral Dissertation

Production of preferable high-oleic acid yeast lipid as an alternative source of biodiesel in the oleaginous yeast *Rhodosporidium toruloides*

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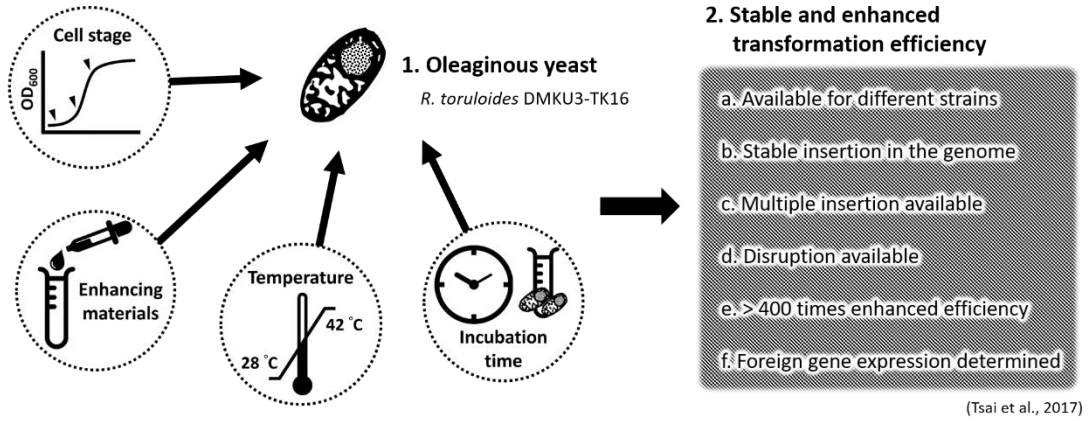
CONTENT

ABSTRACT

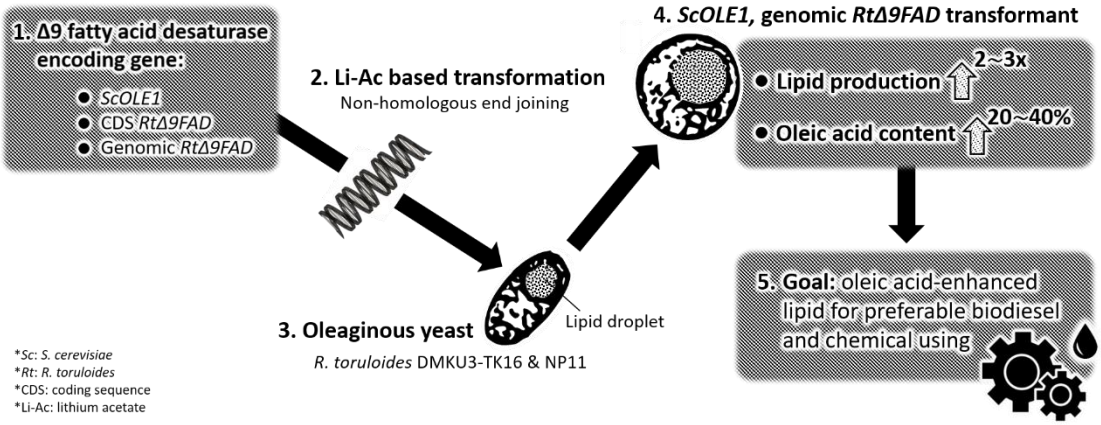
The oil plants provide a sufficient source of renewable lipid production for fuel and chemical supplies as an alternative to the depleting fossil source, but the environmental effect from these plants' cropping is a concern. *Rhodosporidium toruloides*, a promising lipid producer is thus considered as an alternative for renewable lipid production. However, lack of a sufficient and efficient transformation method makes further genetic manipulation of this organism difficult. I here developed a new transformation system for *R. toruloides* by using a lithium acetate based method. We succeed in applying linear DNA fragment containing the target gene expression cassette into the genome, and the transformation efficiency was enhanced 417-fold compared to the initial trial. This newly developed method is thus simple, time-saving, and useful for introduction of an exogenous gene into *R. toruloides* (*Rt*) strains. Further, we attempted to obtain an oleic acid (OA; C18:1)-enriched lipid with desired oil property for fuel and chemical uses. Increasing lipid production was observed in *Saccharomyces cerevisiae OLE1* (*ScOLE1*) and *Rt* genomic Δ*9 fatty acid desaturase* (*RtΔ9FAD*) gene-overexpressing *R. toruloides* strains. The *ScOLE1* transformant output OA content >70% of total lipid, fivefold more in total amount. A different enhancing effects from the protein coding sequence and genomic sequence of *RtΔ9FAD* gene were also observed. Overall, I established a sufficient transformation system for *R. toruloides* and thus resulted in *Δ9FAD* gene overexpression to obtain OA-enriched lipid as a candidate source of designed biodiesel and lipid-related chemicals.

GRAPHICAL OVERVIEW



(Tsai et al., 2017)

I. Establishment of a Li-Ac based transformation system



*Sc: S. cerevisiae
*Rt: R. toruloides
*CDS: coding sequence
*Li-Ac: lithium acetate

II. Enhanced OA content and lipid amount for the preferable yeast lipid

LIST OF ABBREVIATIONS

LA: linoleic acid

LNA: linolenic acid

MA: myristic acid

OA: oleic acid

PA: palmitic acid

POA: palmitoleic acid

STA: stearic acid

AA: amino acid

ATMT: *Agrobacterium*-mediated transformation

CDS: coding sequence

DCW: dry cell weight

DMSO: dimethyl sulfoxide

FAD: fatty acid desaturase

FAEE: fatty acid ethyl ester

FAME: fatty acid methyl ester

GC: gas chromatography

GPD: glyceraldehyde-3-phosphate dehydrogenase

HR: homologous recombination

LD: lipid droplet

Li-Ac: lithium acetate

NHEJ: non-homologous end joining

SCD: stearoyl-CoA desaturase

ssDNA: salmon sperm DNA

CHAPTER 1

General introduction

1.1 Background: needs of renewable lipid sources

Lipid is a crucial factor for the life of organisms. It can serve for energy storage (triacylglycerol), contribute to membrane architecture (phospholipid, cholesterol, waxes, sphingolipid) and act as chemical messenger (diacylglycerol, steroid derivatives, sphingolipid, cholesterol). As energy resources for living creatures, lipid (>90% is triacylglycerol) can be accumulated in lipid droplets (LDs), also termed as fat globules, oil bodies, or lipid particles. Triacylglycerol is the ester of glycerol and three fatty acids, which is constituted by long aliphatic chain with carboxylic acid group. Nowadays, fatty acids are usually produced by hydrolyzing triacylglycerol industrially (Houde *et al*., 2004; Kamal *et al*., 2015; Kavitha *et al*., 2010; Ngaosuwan *et al*., 2009), and the purified fatty acids can be utilized for industrial applications (**Figs. 1.1-a, b, c**).

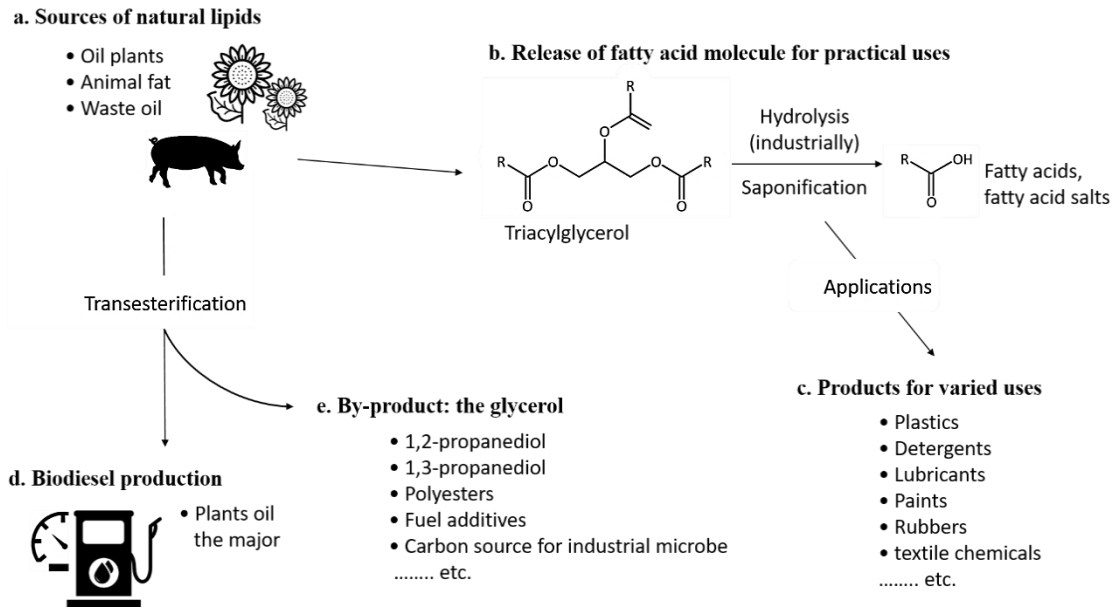


Fig. 1.1-a: The production process and applications of natural lipids.

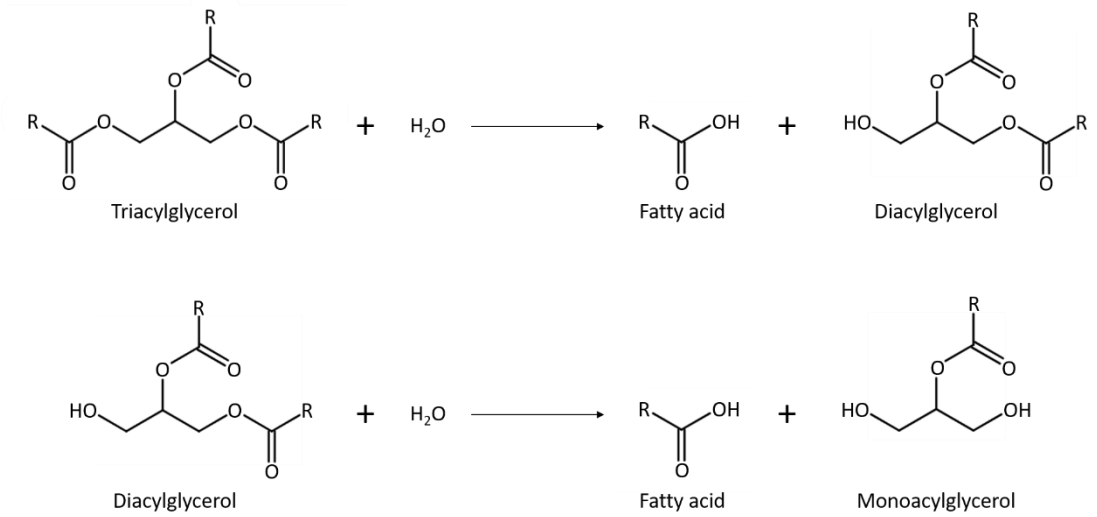


Fig. 1.1-b: Hydrolysis of triacylglycerol.

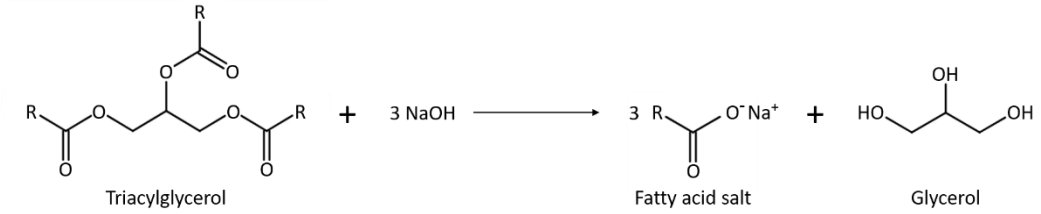


Fig. 1.1-c: Saponification of triacylglycerol.

Many commercial uses as important additives of fatty acids and their derivatives have been known since last century such as organic detergents, rubber (oleic acid), paints, plastics (azelaic acid derived from oleic acid, ricinoleic acid), lubricants, inks, metalworking fluids, textile chemicals, cosmetics (highly refined stearic, palmitic, myristic, lauric acid…etc), food additives….etc. (Ruston, 1952). Because of the shortage of fossil fuels and the well-established environmental pollution, one of these alternative sources is oil plant-derived lipid, which generates less environment-disturbing effects than fossil sources. Such lipids are already being used as environmental friendly biofuels (Knothe *et al*., 2006; Knothe, 2008; Vasudeban and Briggs, 2008). In addition, lipids from microorganisms are considered to be an alternative source of renewable fuels for replacing petroleum-derived fuels (Beller *et*

al., 2015; dos Santos *et al*., 2015; Kannengiesser *et al*., 2015; Sarsekeyeva *et al*., 2015; Sheng and Feng, 2015; Lennen and Pfleger, 2013; Steen *et al*., 2010;) even it is still on the developing stage.

1.2 Current status: lipid production from oil plants

Commercially produced fatty acids are mostly derived from animals, plants and marine fats that called natural fatty acids. The global market of fatty acids was predicted probably to reach $13 billion in 2017 (Yaakob *et al*., 2014) which revealed the potential value. As mentioned above, except being industrial feedstock, fatty acid could be also considered as a kind of resources for renewable fuel supplying. However, harvesting fats from animals and marine sources is too expensive to support for supplying renewable fuel. Oil seed crops (e.g. lipid from corn, sunflower, soybean, canola...etc.) are the major lipid resource of renewable fuel and chemical feedstock because of their ability to store solar energy, lower CO_2 emissions and practicality of being cultured in fields (Bansal and Durrett, 2015; Vega-Sánchez and Ronald, 2010). Nowadays, the supplying of plant derived fuel has resulted in competition with food, higher prices and environmental concerns associated with their production (Hill *et al*., 2006; Steen *et al*., 2010; Sudhakar *et al*., 2011). The production of plant-derived lipids involves several issues regarding its impacts on the environment such as water consumption, and pesticide administration (resulting in contamination on land and in the water, destruction of the primeval forest), plus questions regarding the limitation of field sizes, sun or artificial light requirements, and even the climate factors (Hill *et al*., 2006; Steen *et al*., 2010; Sudhakar *et al*., 2011; Williams *et al*., 2009). Hence, to fit the increasing demand of lipids, the development of stable, controllable and scalable fatty acid production system is needed, while microorganisms based lipid-producing system seems to be a sustainable route (**Fig. 1.2**).

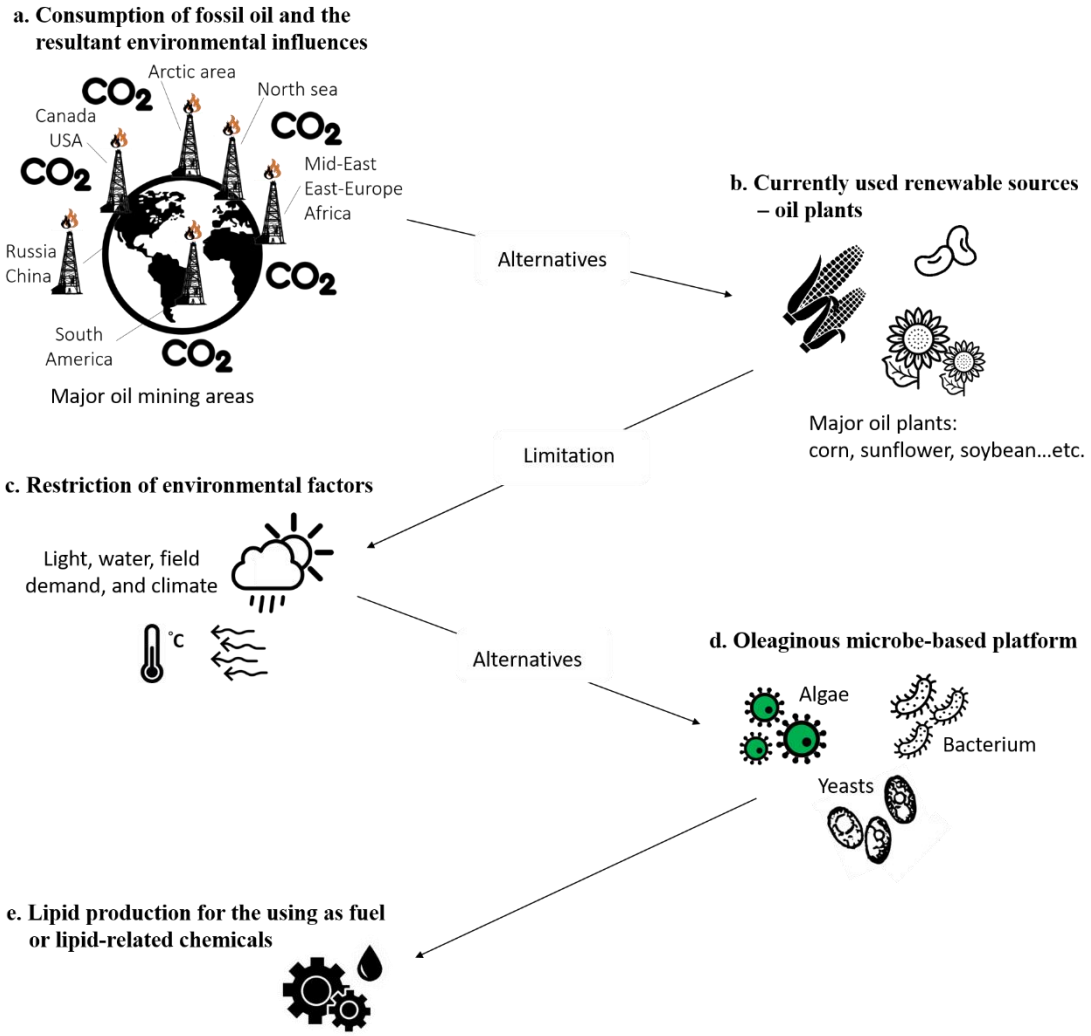


Fig. 1.2: Current status of plant-derived lipids and other renewable alternatives.

1.3 Alternatives: oleaginous yeasts-based platform

In the nature, some microorganisms have lipid-producing ability to store high amount lipids such like bacteria, microalgae and yeasts, those of which can be considered for new lipid sources as the raw materials of fatty acid production (Ageitos *et al*., 2011; Anahas *et al*., 2014; Taleb *et al*., 2014; Erdrich, 2014). During the last few years, it has been demonstrated that *Escherichia coli* and microalgae could be converted to lipid producing system. Microalgae can utilize CO_2 and solar energy to grow and produce lipids, however, like plants, the culture of microalgae is also limited by environmental temperature, light, water, and high cost of lipid recovery (Clarens *et al*.,

2017; Marrone *et al*., 2017; Richardson *et al*., 2010; Slade *et al*., 2013; Subramanian *et al*., 2013; Ullah *et al*., 2014). Biomass fermentation carried out by *E. coli* for fatty acid production was reported (Steen *et al*., 2010). Nevertheless, the *E. coli* has relatively low cellular productivity of lipid (approximately 9.7% of biomass). Lipid-producing yeast which can produce lipid over 20% of dry cell weight (DCW) was called oleaginous yeast, and the oleaginous yeast genera includes *Candida*, *Cryptococcus*, *Lipomyces*, *Yarrowia*, *Rhodosporidium*, *Rhodotorula* and *Trichonsporon* (Ageitos *et al*., 2011). As previous study presented, microalgae can accumulate lipid by 20−50% of DCW, while some oleaginous yeasts have been reported to accumulate lipids up to 80% of DCW (Wahlen *et al*., 2013). Also, compared to microalgae, yeast is less affected from environmental factors. With the high content of single cell oil and several advantages in culturing condition, oleaginous yeast is thought to be a great platform for fatty acid production (**Fig. 1.3**). While, the greenhouse gases produced from yeast culture process might be a point to be concerned in a comparison with plant-based system.

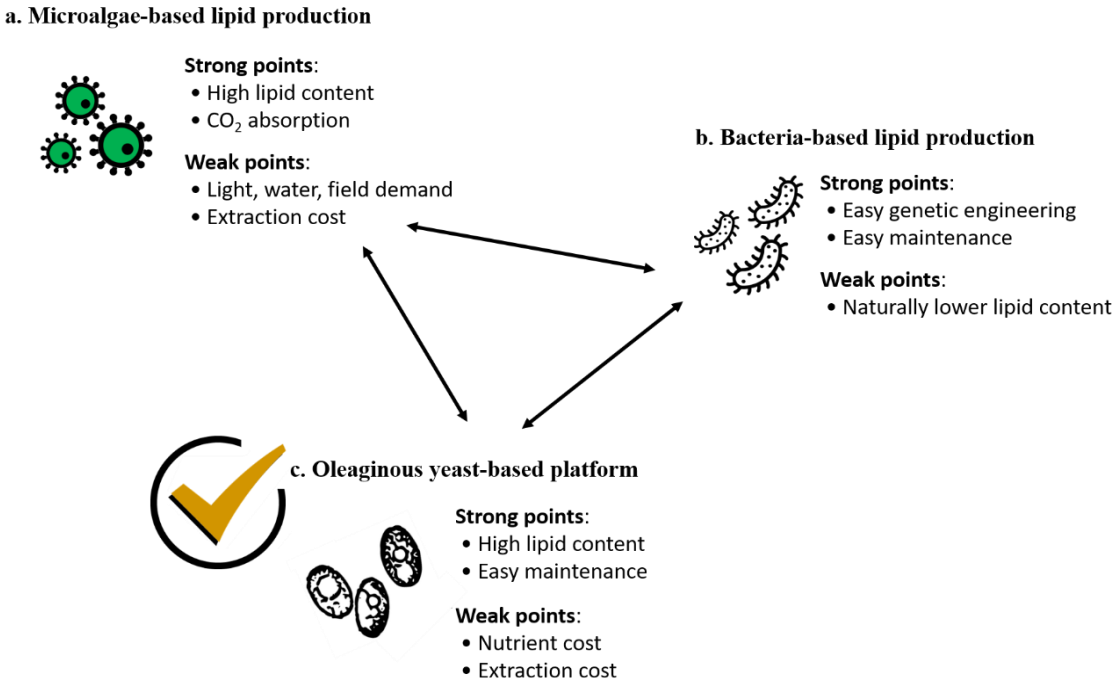


Fig. 1.3: Microbe-based platforms for lipid production.

1.4 Prospect: *Rhodosporidium toruloides* as workhorses

In the last few decades, a robust and pink-colored yeast, *Rhodosporidium toruloides* was found as an oleaginous yeast (accumulate lipids more than 70% of its DCW). Due to this great lipid-accumulating ability, *R. toruloides* has attracted the sight of scientists in field of lipid production (**Fig. 1.4**). *R. toruloides* belongs to basidiomycetous yeast and it is formerly known as *Rhodotorula glutinis* or *Rhodotorula gracilis* (Zhu *et al*., 2013). During these years, the genome sequence of two *R. toruloides* strains MTCC 457 (Taxonomy ID: 1165933) and NP11 (Taxonomy ID: 1130832) have been reported and widely used for the study of genetic engineering (Kumar *et al*., 2012; Zhu *et al*., 2012). To further utilize the lipid-accumulating ability, *R. toruloides* has been being researched (e.g. culturing conditions, feeding substances) for lipid production in recent years. In 2007, *R. toruloides* Y4 (diploid type of NP11) has shown that it is capable of being cultured with high cell density (Li *et al*., 2007) during cultivation period. Besides, the tolerance of *R. toruloides* Y4 to lignocellulose derived cell-growth inhibitors (such like *p*-hydroxybenzaldehyde, vanillin, furfural and its derivatives) in biomass based fermentation has been described (Hu *et al*., 2009) and Y4 strain was also known to utilize extracts and hydrolysates of *Jerusalem artichoke* (a perennial herbaceous plant) as low cost feeding material (Zhao *et al*., 2010). Moreover, another strain *R. toruloides* Y2 was found to be able to produce lipid in the treatment of wastewater generated industrially (Ling *et al*., 2013; Zhou *et al*., 2013). In earlier 2015, Tchakouteu *et al.* demonstrated the capability of *R. toruloides* NRRL Y-27012 to use biodiesel-derived glycerol as feeding substrate to achieve lipid production. Additionally, with the presence of ethanol in cultivating broth, *R. toruloides* was reported to be able to intracellularly convert triacylglycerol to fatty acid ethyl esters (FAEEs) the useful and practical biofuel molecules (Jin *et al*., 2013). Heretofore, *R. toruloides* has shown the potential to be cultivated intensively and fed by low cost substances towards lipid

production. Except for those known *R. toruloides* strains, a *R. toruloides* strain named *R. toruloides* DMKU3-TK16 (TK16) was isolated from soil in Thailand by enrichment technique in nitrogen-limited medium. As previous study has presented, TK16 can accumulate lipid about 70% of its DCW (Kraisintu *et al*., 2010). According to the approved high lipid-accumulating ability, TK16 was surveyed as a potential candidate for alternative resource of microbial-lipid production.

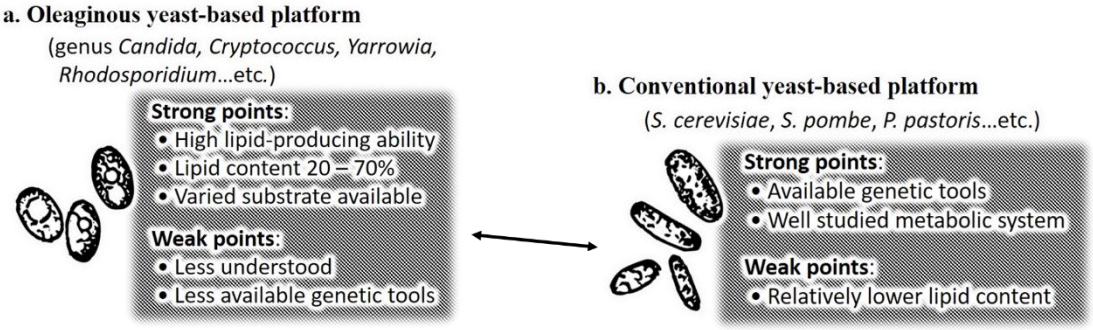


Fig. 1.4: The use of *Rhodosporidium toruloides* as workhorses.

1.5 Objective

With the promising lipid-accumulating ability, basidiomycetous yeast, the oleaginous *R. toruloides* is a prospective model for the production of industrially-valuable fatty acid. Yet, only two transformation systems have been reported for genetic manipulation in *R. toruloides* strains (Tully and Gilbert, 1985; Lin *et al*., 2014). Therefore, this study aims to establish an efficient system for *R. toruloides* transformation with TK16 as the working model. Moreover, the *R. toruloides* will be investigated for the production of preferable yeast lipid towards the uses as diesel fuel or lipid related chemicals.

To achieve the genetic engineering in *R. toruloides*, an accessible and stable tool is needed. However, to the date of present study started, only a transformation system done by *Agrobacterium*-mediated transformation (ATMT) has been reported could

stably act in *R. toruloides* (Lin *et al*., 2014). In chapter 2, I tried to establish a genetic engineering system based on the lithium acetate (Li-Ac) method which a generally known way that used for transformation in conventional yeasts. Without a need of using cell wall digesting enzyme for protoplast preparation, the Li-Ac based method is much easier to access genetic engineering with only the treatment of intact cells. Each parameter such as applying DNA amount, incubation effect of the mixture, and temperature treatment will be discussed later.

OA-enriched lipid was currently known as suitable material for the diesel using or for the production of lipid-related chemicals. *R. toruloides* was then chosen for the producing platform to generate an OA-enriched lipid. In chapter 3, the lipid-producing capability of *R. toruloides* was utilized for the production of preferable yeast lipid. The gene which is supposed to be responsible for OA production in *R. toruloides* will be analyzed for function determination and the production of desired lipid. The possibly different effect that may be derived from protein coding sequence (CDS) and genomic sequence was also examined.

CHAPTER 2

Development of a genetic engineering system for *R. toruloides*

2.1 Background: transformation of *Rhodosporidium toruloides* since 1980s

To investigate and engineer yeasts for further research or application, transformation system is very important and necessary for genetic modification. So far, most transformation systems have been developed for the commonly-used model yeast strains *S. cerevisiae* and *S. pombe* (e.g. homologous recombination, episomal and integrative vectors, electroporation…etc.). However, the development of transformation system for those *R. toruloides* strains is still immature and only a few methods have been reported (**Table 1**).

R. toruloides strain	Research achievement	Applied transformation method	Reference
MRE333, MS7013, MS7054, MS7563	First transformation trial of *R. toruloides*	Protoplast mediated method	Tully and Gilbert, 1985
NP11	Genome reported, transformation by antibiotic selection	ATMT mediated method	Zhu *et al.*, 2012; Lin *et al.*, 2014
MTCC457	Genome reported	None	Kumar *et al.*, 2012
Y4 (NP11 diploid type)	Tolerance of high cell density and biomass derived culture	None	Li *et al.*, 2007; Hu *et al.*, 2009; Zhao *et al.*, 2010
Y2	Lipid produce in industrially generated wastewater	None	Ling *et al.*, 2013; Zhou *et al.*, 2013
ATCC10657	*GPD1* promoter identified	None	Liu *et al.*, 2013
NRRL Y-27012	Utilization of biodiesel-derived glycerol	None	Tchakouteu *et al., 2015*

Table 1: Transformation achievement of referred *R. toruloides* strains until 2017.

By protoplast mediated transformation with the presence of polyethylene glycol (PEG), *R. toruloides* had been transformed with transformation frequencies as approximately 1 x 10^3 transformants/μg of DNA (plasmid). However, unstable plasmid replication and locus integration were observed from obtained transformants (Tully and Gilbert, 1985) indicating the system has not been established well yet. *Agrobacterium*-mediated transformation (ATMT) was then tried to introduce exogenous gene in *R. toruloides* NP11 genome and antibiotic resistant transformants were successfully

obtained (Lin *et al*., 2014) .

2.2 Objective: A stable and efficient transformation system is needed

Thus far, only a few methods had been presented to engage transformation in *R. toruloides* successfully, but the instability restricted the gene introducing and application range. Also, both of the applied systems need additional preparation of protoplast by cell digestion or *Agrobacterium* strains for yeast transformation (**Fig. 2.2.1**). Therefore, a more efficient system is still highly required (**Fig. 2.2.2**).

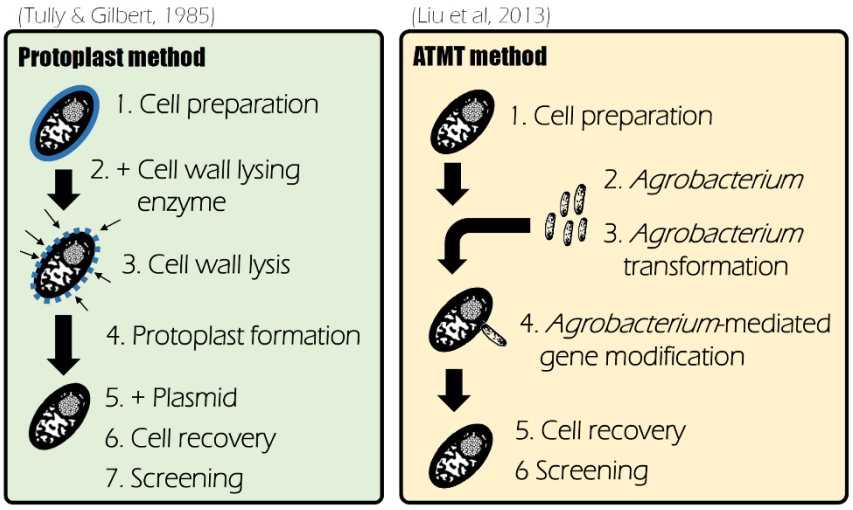


Fig. 2.2.1: *R. toruloides* transformation flow of protoplast and ATMT method.

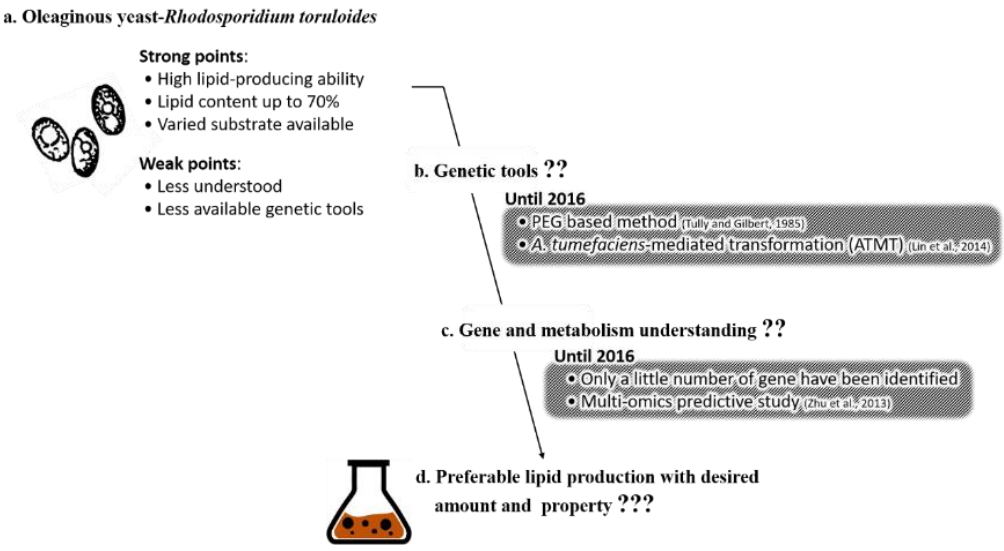


Fig. 2.2.2: Current problems of *R. toruloides* research remain to be solved.

2.3 Results: the development lithium-acetate based transformation system

2.3.1 Zeocin sensitivity of *R. toruloides* DMKU3-TK16

Bleomycin was isolated from *S. verticillatus* in 1966 (Umezawa *et al*.) and known as a glycopeptide antibiotic (Omoto *et al*., 1972; Fujii *et al*., 1973), it has anticancer ability and can inhibit replication of viruses, bacteria, fungi, and mammalian cells by its DNA damaging effect (Moore, 1982). This bleomycin induced suppression of cell growth was caused by inhibiting DNA synthesis with free radicals producing DNA single-strand breaks at 3'-4' bonds in deoxyribose (Dorr, 1992; Hecht, 2000). To more understand the effect of bleomycin repressed growth in fungi, *S. cerevisiae* has thus been used for investigating cell damage and mutagenic mechanism (Moore, 1978; Moore *et al*., 1980; Keszenman *et al*., 1992). Also, bleomycinm was reported to occur cell wall damage trigger cell toxicity in *S. cerevisiae* (Lim *et al*., 1995) but only DNA breaking. As a bleomycin category antibiotics, Zeocin has been used as a selective marker for yeast screening in such like *S. cerevisiae, P. pastoris, S. pombe* and *C. glabrata* (Johansson and Hahn-Hägerdal, 2002; Alderton *et al*., 2006; Sunga *et al*., 2008; Sugano *et al*., 2010; Benko and Zhao, 2011). Bleomycin was successfully examined for *R. toruloides* NP11 transformants screening (Lin *et al*., 2014). For this reason, I tried to examine the effect of Zeocin, a bleomycin category antibiotic for selection of *R. toruloides* DMKU3-TK16 transformants. In this experiment, TK16 showed significant sensitivity as 50 μg/mL or higher concentration of Zeocin (**Fig. 2.3.1**), indicated that Zeocin could be competent for the selection of TK16 transformants. However, large numbers of Zeocin-resistant mutants occurred when performing transformation by treating with 50 μg/mL of Zeocin for selection. Thence, the Zeocin concentration was substituted by 150 μg/mL for higher selectivity, and 150 μg/mL of Zeocin was employed for all of the following experiments.

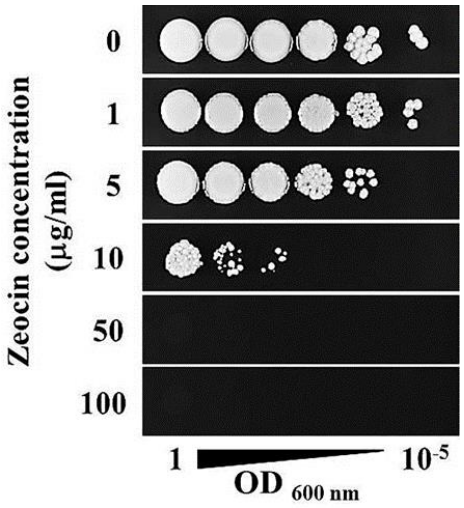


Fig. 2.3.1: Sensitivity to Zeocin.

Cells were grown in YM liquid broth medium at 28°C overnight, and serial dilutions of each culture were spotted onto YM plates containing serial concentrations of Zeocin ranging from 0 to 100 μg/mL.

2.3.2 Schematic design of the transformation efficiency trial

In this work, the construct pP_{GPD}-Shble which carrying the selective marker gene *Sh ble* (*GPD1* promoter-*Sh ble*) expression cassette was used as template for DNA fragment preparation by PCR (**Fig. 2.3.2-a**). Briefly, TK16 was inoculated in flask for pre-culture then the cultured cells were collected for incubation with transformation mixture which containing DNA fragment of expression cassette**.** Finally, those incubated TK16 cells were proceeded to recovery cultivation and plating on Zeocin (150 μg/mL) containing medium as selection (**Figs. 2.3.2-b, c**). The Li-Ac transformation mixture applied here mainly referred and modified from transformation systems for *S. cerevisiae* and *S. pombe* with the cell permeabilization resulted by PEG and Li-Ac (Ito *et al*., 1983; Gietz *et al*., 1995; Gietz and Woods, 2001; Gietz and Woods, 2006; Morita and Takegawa, 2004) (**Fig. 2.3.2-d**). Under the regulation of *GPD1* promoter from *R. toruloides* ATCC10657 (Liu *et al*., 2013), *Sh ble* cassette was used as selection marker to carry out transformation and the following efficiency tests. By the basically designed method (Kanazawa, 2014), transformation was carried out with 20 μg of DNA fragment which embodied *Sh ble* cassette and only 2 colonies on average appeared in the first trial. The given transformation efficiency was too low to utilize for routine experiment in TK16.

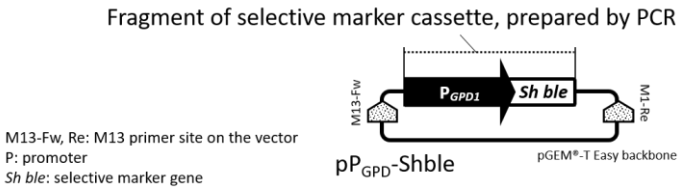


Fig. 2.3.2-a: Illustration of the selective marker gene carrying cassette in a vector. The selective marker gene was under the control of *GPD1* promoter. The DNA fragment of cassette was prepared by PCR for the trial of transformation efficiency.

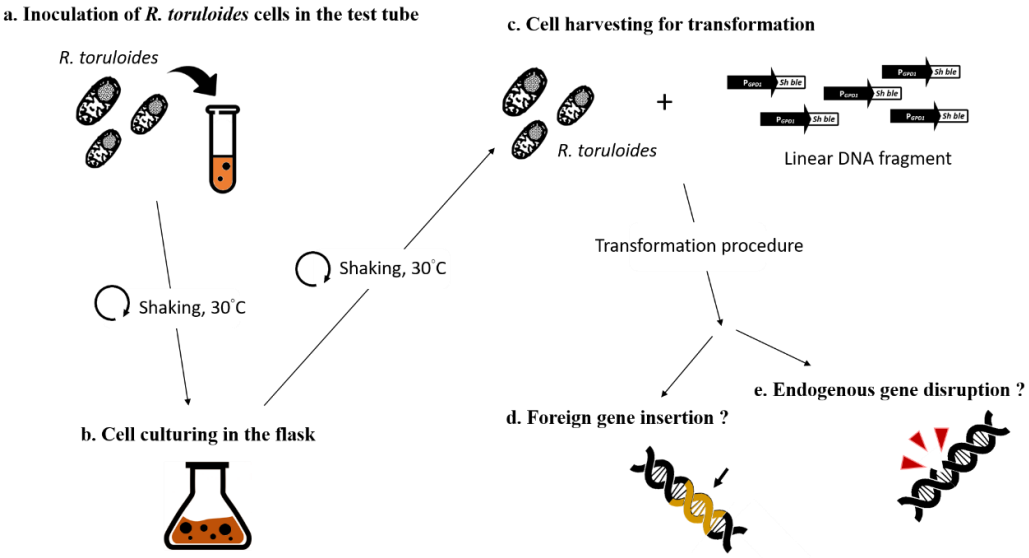


Fig. 2.3.2-b: Basic experimental flow of transformation process in TK16.

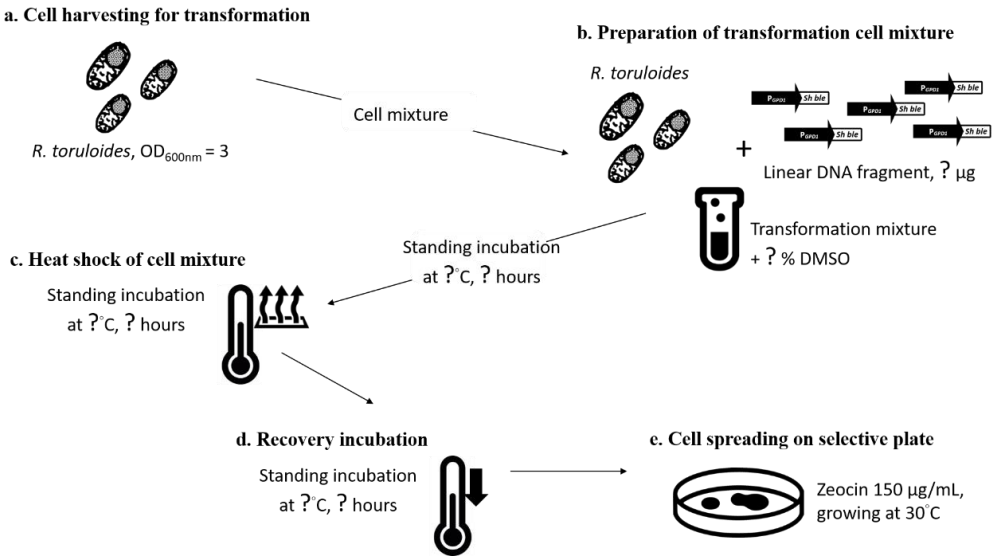


Fig. 2.3.2-c: Parameters for the optimization of transformation efficiency in TK16. Parameters in the transformation process will be examined, i.e., the amount of DNA fragment, incubation period at each step, and the heat shock temperature.

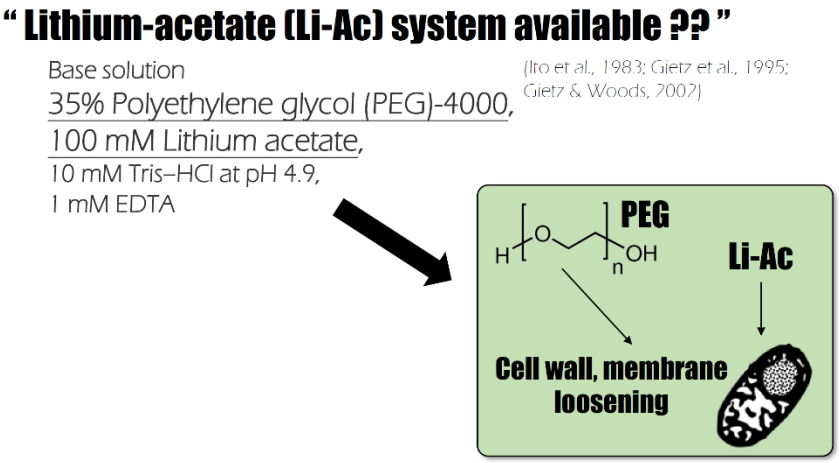


Fig. 2.3.2-d: Hypothesis of Li-Ac system for *R. toruloides* transformation.

2.3.3 Transformation efficiency of TK16 affected by heat shock temperature and DNA amount

To investigate the effect of applied DNA concentration in transformation, DNA was tested with an increment of concentration by 0, 1, 10, 20, 30, 40 and 50 μg in each reaction mixture (100 μL). As the result revealed, 1 μg DNA was unable to afford any transformant. Moreover, higher number of colonies was obtained when higher DNA concentration was applied, which 50 μg provided the highest transformation efficiency (**Fig. 2.3.3**) in this test against other DNA concentrations. Heat shock treatment at various temperatures (28°C, 37°C and 42°C) was examined also. Different heat shock temperatures affected transformation efficiency, especially the group heat shocked by 37°C showed higher efficiency than others. The highest transformation efficiency was obtained when 50 μg of DNA was treated, particularly the 37°C-treated group appeared 43 colonies on average (**Fig. 2.3.3**).

According to these consequents, 1 μg of DNA was too low and at least 20 μg was needed to obtain colonies for all temperatures treating. In addition, 37°C is an optimized temperature for TK16 transformation. This optimized cell treating condition was used for any later efficiency test.

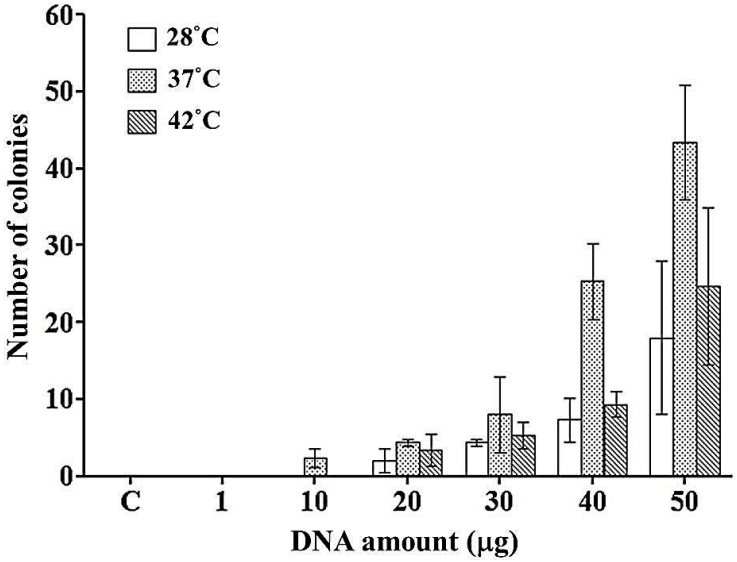


Fig. 2.3.3: Effects of the linear DNA fragment amounts and the temperatures for the heat shock treatment on TK16 transformation efficiency.

The P_{GPD}-*Sh ble* fragments were amplified and used for the transformation at various amounts (0, 1, 10, 20, 30, 40 and 50 μg for each reaction) with heat shock treatment at the various temperatures (28°C, 37°C and 42°C). The data are the mean ± SD of the number of colonies counted from at least three individual plates.

2.3.4 Effect of incubation time in transformation mixture before heat shock

In the transformation process, cells should be incubated in the transformation mixture for PEG-4000 mediated cell permeabilization before heat shock. Therefore, the incubation period in the mixture should affect the permeability of treated cells towards influencing transformation efficiency (**Fig. 2.3.4**).

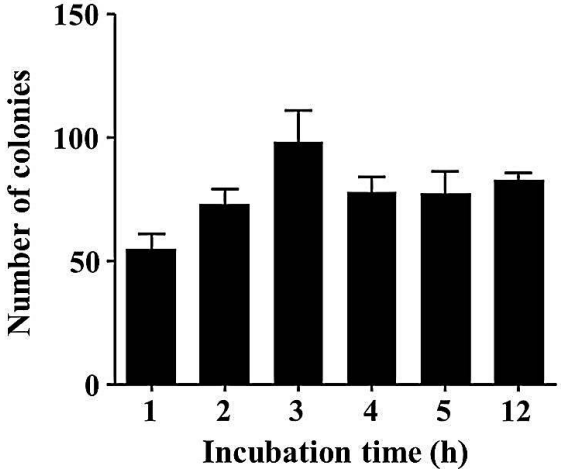


Fig. 2.3.4: Effects of incubation time with transformation mixture

Transformation mixtures were subjected to a 28°C-incubation period for 1, 2, 3, 4, 5 and 12 h before heat shock treatment. The data are the mean ± SD of the number of colonies counted from at least three individual plates.

TK16 cells were incubated with transformation mixture and 50 μg DNA for different durations (1, 2, 3, 4, 5 and 12 h) before heat shock then processed to the following steps. The colony number showed an increasing trend in the range between 1 and 3 h incubation. These 3 groups represented colony number by 55, 73 and 93 on average respectively. However, colony number did not continue to raise larger with longer incubation time. These groups showed stationary number of colonies compared with 3 h incubated cells against the shortest one. This result indicated a suitable range for incubation time before heat shock.

2.3.5 Transformation efficiency elevated by addition of DMSO and ssDNA

Currently transformation system and tool have been mostly developed for commonly used model yeasts *S. cerevisiae* and *S. pombe*. DMSO and ssDNA were known to improve transformation efficiency by addition into the transformation mixture for cell incubation (Schiestl and Gietz, 1989; Hill *et al*., 1991). To obtain enhanced transformation efficiency in TK16, DMSO and ssDNA were investigated in this experiment. In the pre-incubation step, transformation was treated with 10% DMSO (v/v) or 100 μg of ssDNA. Both of 10% DMSO and ssDNA improved transformation efficiency with 37°C heat shock, and especially the 10% DMSO could provide increment of colony number more than 400 times compared to the first trial (**Figs. 2.3.5-a, b**). Size of ssDNA is one of the factors for its enhancing ability, therefore, ssDNA fragments larger than 10 kbp and 7 kbp were tested in the experiment. The ssDNA with larger size enhanced efficiency more than the short one (**Fig. 2.3.5-c**). Both DMSO and ssDNA can improve transformation efficiency in TK16, however, the simultaneous treating of DMSO and ssDNA did not afford higher efficiency (**Fig. 2.3.5-d**). Consequently, addition of 10% DMSO in transformation mixture should be a better assistance to TK16 transformation than ssDNA.

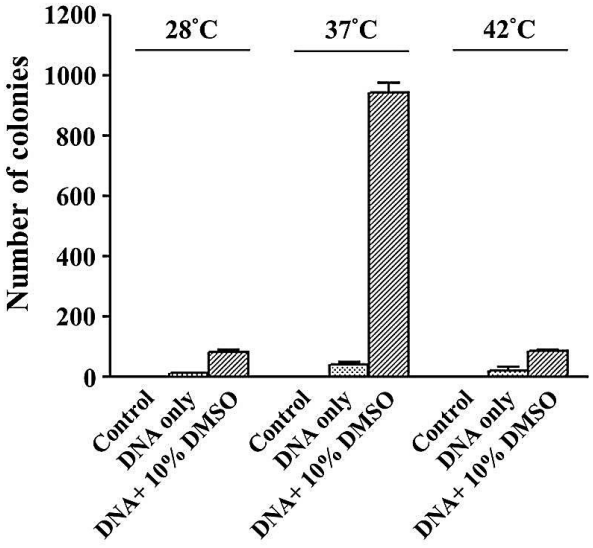


Fig. 2.3.5-a: DMSO treatment before heat shock improved transformation efficiency.

DMSO was added into transformation mixture to incubate TK16 cells with DNA fragment by 10% of total volume. Three hours incubated cells were then treated by different heat shock temperatures 28°C, 37°C and 42°C.

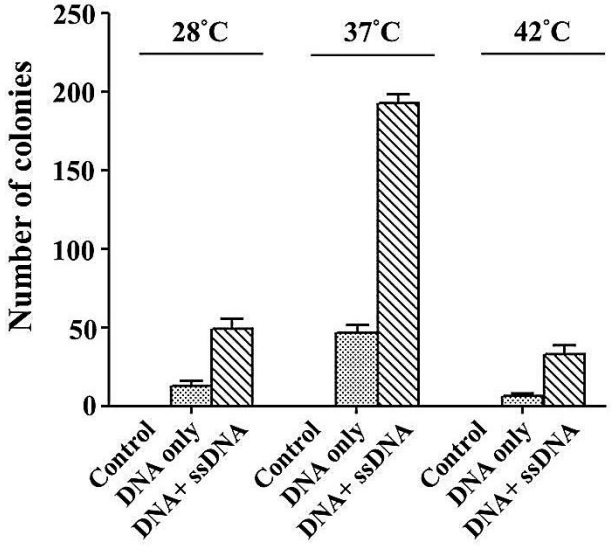


Fig. 2.3.5-b: Salmon sperm carrier DNA (ssDNA) improved transformation efficiency.

Similarly, ssDNA was also tested for its ability to improve TK16 transformation efficiency. Same as the DMSO trail, ssDNA was added into transformation mixture before heat shock with concentration of 100 μg. The addition of ssDNA enhanced transformation efficiency for all three groups, and 37°C heat shocked TK16 still showed highest colony number against the other two groups (28°C and 42°C).

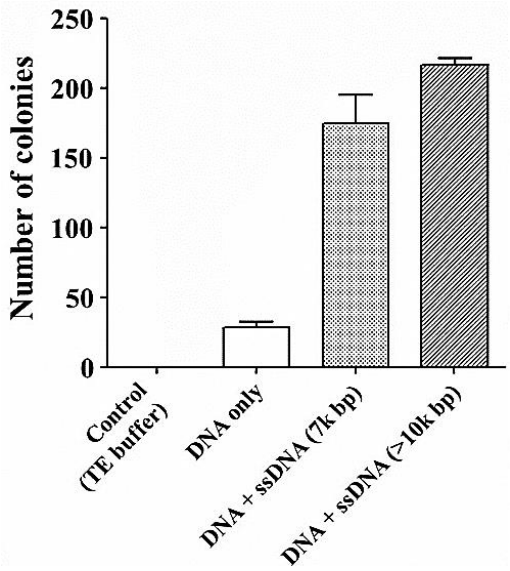


Fig. 2.3.5-c: Transformation enhancing ability of ssDNA was affected by fragment size.

Shorter ssDNA (7 kbp) was examined for transformation improving. Both two sizes ssDNA improved TK16 transformation efficiency, but the strength of shorter one was reduced in comparison with longer one (>10 kbp).

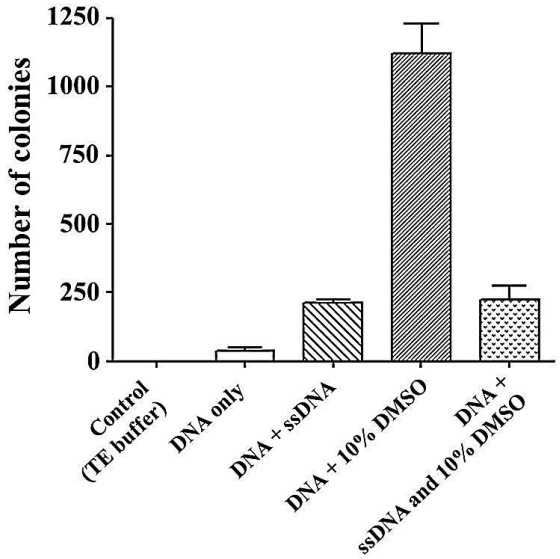


Fig. 2.3.5-d: Synergistic effect between DMSO and ssDNA in TK16 transformation.

In this experiment, ssDNA and DMSO were examined for synergistic effect. From the result revealed, both DMSO and ssDNA can improve transformation efficiency respectively, especially DMSO treated TK16 showed extremely higher efficiency than others. Nevertheless, treating DMSO and ssDNA simultaneously did not increase efficiency and even only showed similar level as ssDNA have had.

2.3.6 Effect of shaking incubation after heat shock step

After heat shock treatment and cooling down, YM medium was added into transformation mixture then all the mixed mixture was transferred to test tube for recovery cultivation. The recovery cultivation was carried out with shaking for 6 h before plating on selective plate. This recovery step for treated cells was considered to be important to influence yeast transformation efficiency (Tripp *et al*., 2013). Hence, TK16 cells were tested by different periods of recovery cultivation (0, 1, 2, 3, 4, 5, 6). The number of colonies significantly increased along with longer incubation time of 6-h cultured cells compared to the starter (**Fig. 2.3.6**). These results clearly indicated the recovery cultivation after heat shock step can affect transformation efficiency strongly.

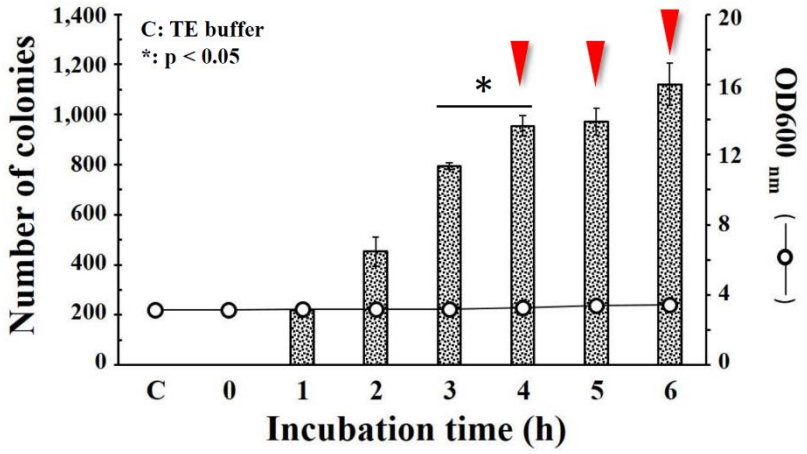


Fig. 2.3.6: Effect of cultivation time after cells being heat shocked.

After heat shock step, TK16 cells were further cultivated with medium addition in the test tube with shaking. For the trial of the effect of incubation time, treated cells were cultivated for 0, 1, 2, 3, 4, 5 and 6 h. An increasing trend was observed obviously along with the longer cultivation time, significantly.

2.3.7 NHEJ mediated gene insertion and its stability in TK16 genome

Without sufficient, replicative or stable episomal plasmid for transformation in *R. toruloides* strains, genome integrating system mediated by NHEJ (Abdel-Banat *et al*., 2010) might be a solution for genetic manipulation in TK16. With the selection by high concentration of Zeocin (150 µg/mL), once the colony resisted Zeocin toxicity and grew

on the selective plate, it was recognized as a possible *Sh ble* transformant. To determine the insertion of *Sh ble* cassette and confirm its stability, I analyzed obtained colonies with colony PCR and Southern blot. Appeared colonies were cultivated on non-selective plate until full growth for 10 generations then refreshed on Zeocin selective plate again. These colonies still showed Zeocin resistance and the *Sh ble* expression cassette could be detected by colony PCR (**Fig. 2.3.7-a**) from these transformants.

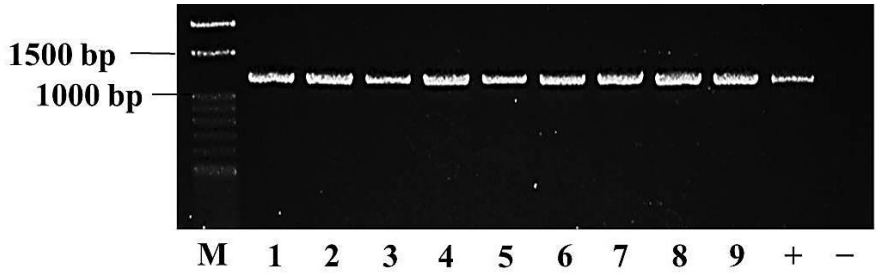


Fig. 2.3.7-a: Colony PCR result of *Sh ble* transformants.

M: marker, 1 to 9: each number represents a distinct transformant, +: positive control signal from p*GPD1-Sh ble* carrying vector, −: wild-type TK16 colony. All Sh ble transformants showed clear PCR signal of p*GPD1-Sh ble* fragment indicated that the stable gene existence in transformants.

To further confirm the genome insertion of *Sh ble* gene in TK16 genome, Southern blot was applied with the *Sh ble* fragment (375 bp) as a probe. X-ray film detection showed various fragment size and signal density from restrict enzyme digested TK16 genome. Accordingly, the *Sh ble* expression cassette was suggested to insert randomly and disturbed by different copy numbers in the genomes of distinct transformants (**Fig. 2.3.7-b**).

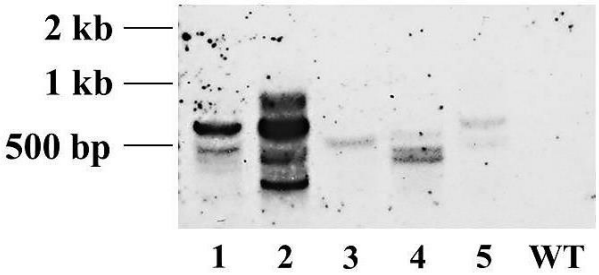


Fig. 2.3.7-b: Southern blot analysis for *Sh ble* gene insertion.

WT: wild-type TK16, 1 to 5: each number represents a distinct transformant. Different sized signal from Southern blot analysis demonstrated the random insertion of *Sh ble* cassette and gene integration into TK16 genome was mediated by NHEJ (non-homologous end joining).

2.3.8 Successful EGFP expression in TK16 transformants

To apply the developed transformation method in TK16 for heterologous protein expression, EGFP was utilized as a model protein for expression trial. The DNA fragment for transformation consisted of EGFP expression cassette which was ligated after a selective marker, the *Sh ble* cassette (**Fig. 2.3.8-a**). As the efficiency had been presented by *Sh ble* cassette conducted transformation, result of EGFP fragment employed transformation showed similarly high efficiency in application (**Fig. 2.3.8-b**) even with larger size.

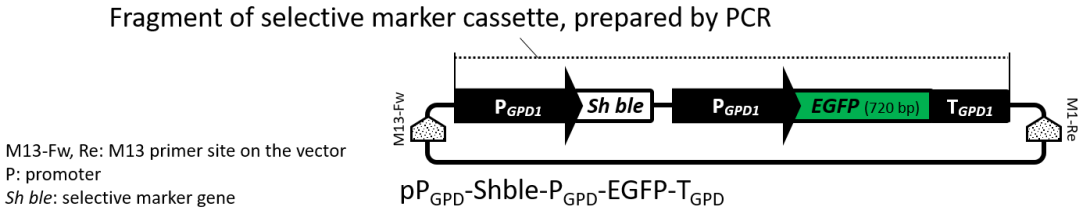


Fig. 2.3.8-a: Illustration of the *EGFP* gene carrying cassette in a vector.

The EGFP gene was under the control of *GPD1* promoter, and the DNA fragment of cassette was prepared by PCR then be used for the trial of transformation efficiency.

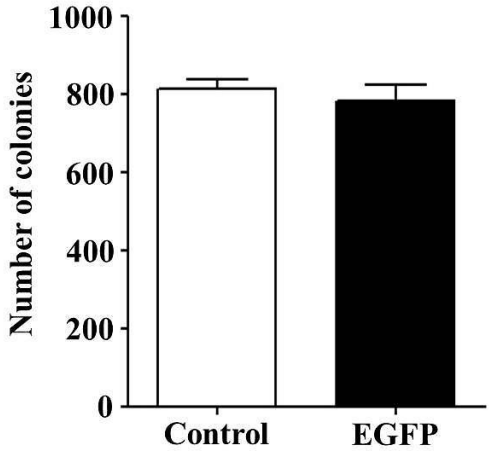


Fig. 2.3.8-b: Transfo rmation efficiency of EGFP expression cassette.

EGFP expression cassette with a larger size was utilized to do transformation in TK16, and the result still showed a similar transformation efficiency compared with the control (*Sh ble* cassette) presented.

The insertion of EGFP expression cassette was confirmed by colony PCR as

mentioned above and the EGFP protein production was examined by microscopic observation and Western blot. The green fluorescence signal of heterologous EGFP was found to be distributed in cytoplasm other than nuclei under microscope in EGFP transformants (**Fig. 2.3.8-c**). Moreover, the heterologous EGFP protein in EGFP transformants was detected by Western blot analysis clearly with a molecular mass of 27 kDa, but none in *Sh ble* cassette transformants (**Fig. 2.3.8-d**). These results demonstrated the developed system successfully introduced heterologous EGFP gene into TK16 and led to protein production, strongly revealing the possibility for introduction and expression of other exogenous proteins in TK16.

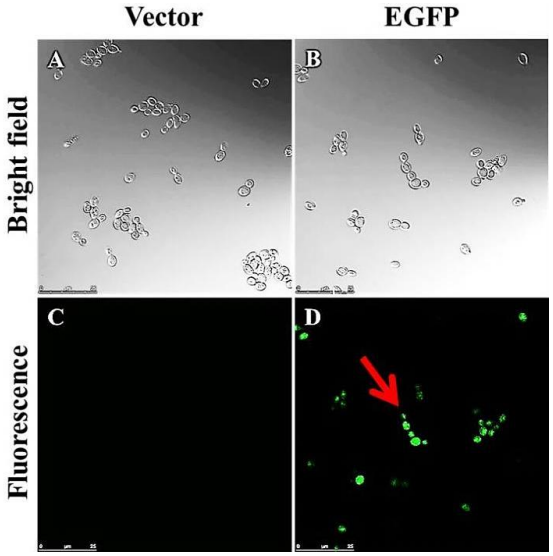


Fig. 2.3.8-c: Observation for green fluorescence expressed in EGFP transformants.

Green fluorescent signal was clearly obtained from EGFP transformant under microscope but not Sh ble cassette carrying only one (vector).

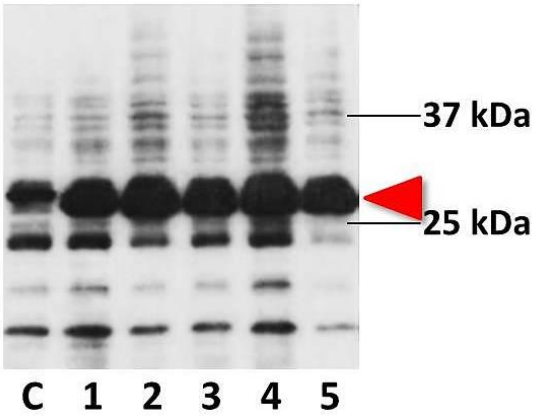


Fig. 2.3.8-d: Western blot analysis for EGFP protein production in TK16 transformants.

C: wild-type TK16 displayed as control, 1-5: each number represents a distinct transformant. Clear signal indicated the 27 kDa EGFP protein was successfully produced in all chosen transformants.

2.3.9 Application of developed transformation system in other *R. toruloides* strains

Protoplast mediated *R. toruloides* transformation method has been carried out in strain MRE333 and MS7013 (Tully and Gilbert, 1985). While, the failed trial of protoplast mediated method in *R. toruloides* NP11 was reported (Lin *et al*., 2014). These results declared the formerly developed transformation systems of *R. toruloides* may have uncertainty for application across different strains. To determine whether the newly developed lithium acetate method can be applied in different *R. toruloides* strains, transformation was conducted in NP11 and ATCC10657 by the developed method in this study. Similarly, NP11 and ATCC10657 were examined for Zeocin sensitivity on selective plate and both of them showed sensitivity to Zeocin by treating with 50 μg/mL or higher concentration (**Fig. 2.3.9-a**).

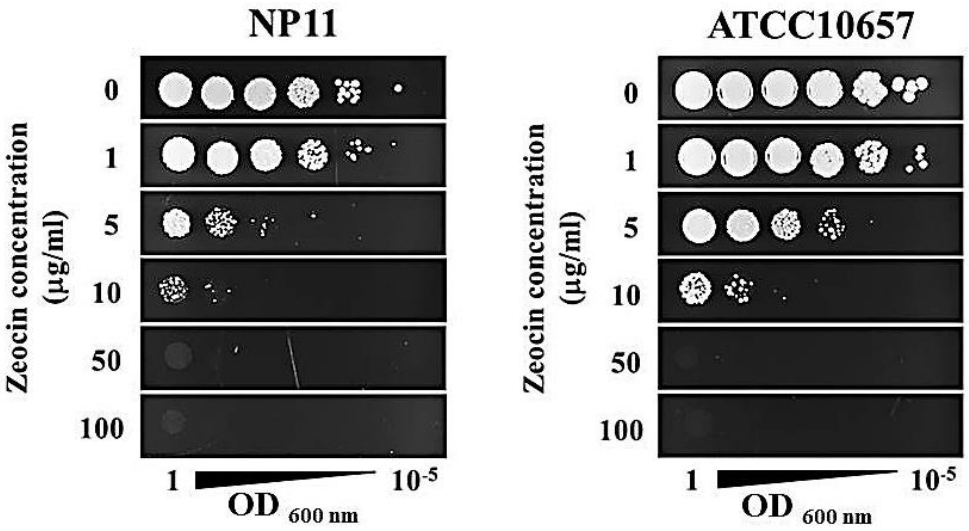


Fig. 2.3.9-a: Zeocin sensitivity of *R. toruloides* NP11 and ATCC10657

R. toruloides NP11 and ATCC10657 were examined for Zeocin sensitivity, both of them showed sensitivity since 50 μg/mL of Zeocin was used. However, trial for transformants screening appeared a lot of Zeocin-resistant mutant by 50 μg/mL of Zeocin administration, thus, higher concentration was applied for the further experiments.

A lot of mutants appeared by treating 50 μg/mL of Zeocin, transformation was carried out with 150 μg/mL Zeocin selection. *Sh ble* gene was successfully introduced

in both *R. toruloides* strains with the optimized conditions described before. However, once shortened the cultivation time to 3 h after heat shock, transformation efficiency reduced significantly, especially NP11 which obtained no colony under this treatment. This result showed a lower efficiency in NP11 and ATCC10657 compared to TK16 (**Fig. 2.3.9-b**), however, it still strongly exhibit the feasibility to apply the newly developed Li-Ac based transformation system in different *R. toruloides* strains.

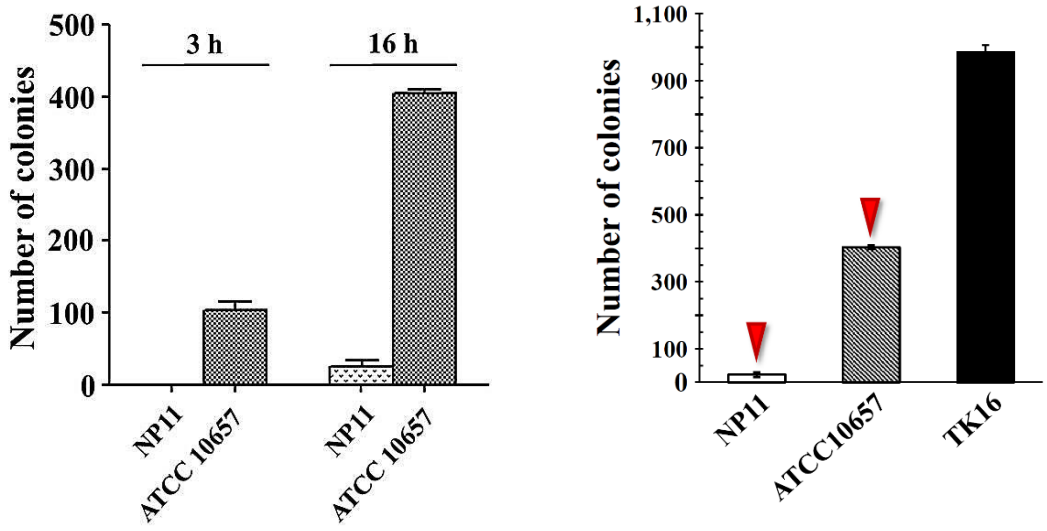


Fig. 2.3.9-b: *R. toruloides* strain NP11 and ATCC10657 transformation with the present method.

R. toruloides NP11 and ATCC10657 were successfully transformed by developed transformation system. Both strains were tested by different cultivation time after heat shock step, but in the 3 h cultivation test group, only ATCC10657 can grow but none from NP11.

2.3.10 Effect of growth phase on transformation efficiency

For yeast transformation (e.g., *S. cerevisiae* or *S. pombe*), mostly the log-phase or early stationary-phase cells are chosen for the process to gain high efficiency (Kawai *et al*., 2010; Tripp *et al*., 2013). To this end, all efficient transformation results shown above were achieved by TK16 cells at log-phase (OD_{600} = 3) (**Fig. 2.3.10-a**).

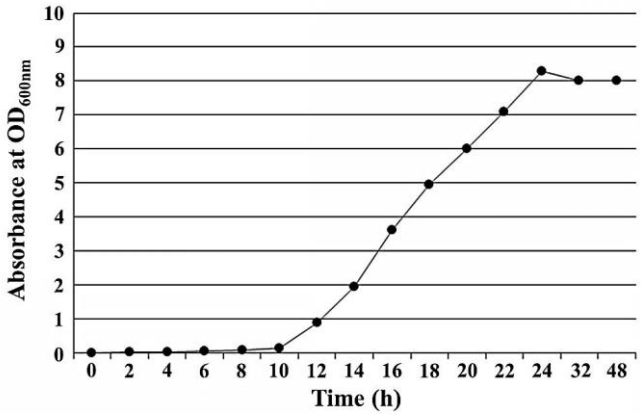


Fig. 2.3.10-a: The growth curve of TK16.

However, cells from different growth phases may have distinct properties that could influence transformation efficiency. Therefore, cells from pre-culture step with 16, 32 and 48 h cultivation were collected to perform transformation by developed method. Cells from each group were all adjusted to OD_{600} of 3 then were applied to transformation. Surprisingly, 48 h pre-cultured TK16 had the best efficiency against others (**Fig. 2.3.10-b**).

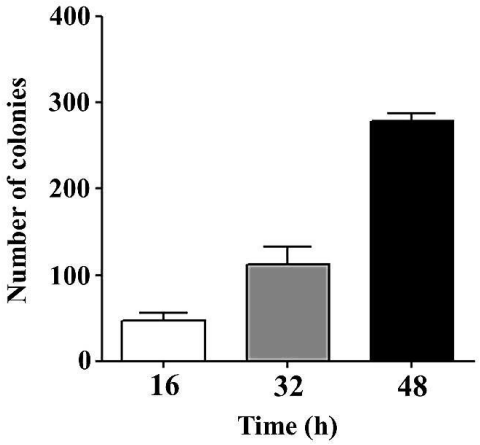


Fig. 2.3.10-b: Effect of cell growth phase on transformation efficiency.

TK16 cells from different growth phase (16, 32 and 42 h pre-culture) were collected for transformation test. With the developed transformation system, the cells from 48 h pre-culture presented higher efficiency compared to others.

2.3.11 Terminator insertion after *Sh ble* gene caused low transformation efficiency and loss of downstream gene

With the developed transformation system, I may introduce foreign genes into TK16 to produce industrially valuable fatty acids. For this goal, we introduced *Claviceps purpurea* oleate Δ12-hydroxylase gene (*CpFAH12*) and *Arabdopsis thaliana* 3-ketoacyl-CoA synthase 18 (also named fatty acid elongase 1, *FAE1*) in TK16 to produce ricinoleic acid (Yazawa *et al*., 2013 & 2014) and erucic acid (Ghanevati and Jaworski, 2002) respectively. However, no ricinoleic acid and erucic acid productions were observed from GC analysis. Insertion of a terminator after selective marker (*Sh ble* gene) may be one of the options for the appropriate expression of target that locates at the downstream site on the expression cassette. Terminator can stabilize mRNA structure towards correcting and improving gene expression (Mischo *et al*., 2013; Geisberg *et al*., 2014). As shown previously in this study, there was no terminator inserted between *Sh ble* cassette and EGFP cassette (**Figs. 2.3.2-a, 2.3.8-a**) or even between *Sh ble* cassette and *FAH12, FAE1* cassette. EGFP was chosen as model for expression level test with *GPD1* terminator (isolated from *R. toruloides* ATCC10657) insertion after *Sh ble* gene. *GPD1* terminator ligated *Sh ble* expression cassette (*GPD1* promoter-*Sh ble-GPD1* terminator) was successfully introduced in TK16, but transformation efficiency was found reducing obviously (**Fig. 2.3.11**).

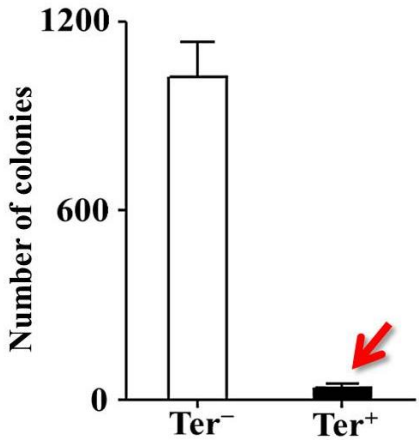


Fig. 2.3.11: Low transformation efficiency was observed by terminator insertion.

Red arrow indicates the colony number from cells which were treated by terminator ligated *Sh ble* expression cassette (pGPD1-Sh ble-terGPD1). The insertion of terminator led to lower transformation efficiency compared to the control (white bar: pGPD1-Sh ble transformant).

In addition, EGFP transformants also showed low transformation efficiency and the

loss of EGFP cassette at the downstream of *GPD1* terminator ligated *Sh ble* cassette. The same situation happened by replacing *GPD1* terminator with *CYC1* terminator (from pYES2) or *NOS* terminator (from pBI121), and yet, the reason is still unclear. Another solution to manipulate expression of multiple genes in the same time might be related to the orientation of genes. In the case of *S. cerevisiae*, direction of different genes can regulate expression level during simultaneous expression (Ishii *et al*., 2014). To examine the effect of gene orientation, the experiment is still in the progress.

2.3.12 Disruption of endogenous *URA3* gene

For processing the genetic engineering in industrially useful yeast, applicable system of endogenous gene may be also necessary for a comprehensive metabolic manipulation. With the developed method, the endogenous *URA3* gene, a commonly used auxotrophic marker in yeast research, was disrupted. The *URA3* gene disruption cassette (2,700 bp) consisting 1,502-bp *URA3* locus fragment interrupted with *Sh ble* cassette (1,198 bp) was constructed (**Fig. 2.3.12-a**), and was introduced into TK16. The transformants were screened on three kinds of agar plates including YM agar with Zeocin, SD agar plate with 5-FOA and the SD-ura for counter-selection. The dual Zeocin- and 5-FOA-resistants were further examined by PCR with specific primers (TK16guraF and TK16guraR) that bound to the up- and down-stream regions of the introduced *URA3* gene disruption cassette in the TK16 genome (**Fig. 2.3.12-a**).

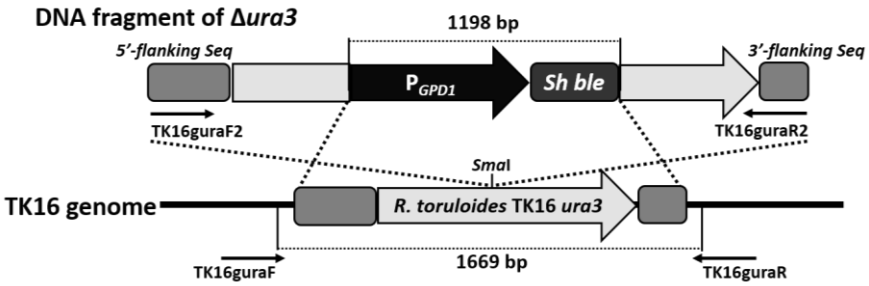


Fig. 2.3.12-a: Schematic illustration of *URA3* gene disruption strategy.

P_{GPD1}: *R. toruloides* ATCC 10657 GPD1 promoter, *Sh ble*: *S. hindustanus* bleomycin-resistance gene, TK16guraF2 and TK16guraR2: primer for preparation of fragment, TK16guraF and TK16guraR: primer for PCR confirmation of *Sh ble* cassette interrupted *URA3* locus from genomic DNA.

PCR results with predicted size of 2,867 bp using genomic DNA from all ten dual resistants examined demonstrated that the *URA3* locus was successfully replaced with the heterologous *URA3* gene disruption cassette by probable homologous recombination (**Fig. 2.3.12-b**). A simultaneous confirmation for phenotype of wild-type TK16 and different transformants on each selective media certainly supported the result of endogenous *URA3* gene disruption (**Fig. 2.3.12-c**).

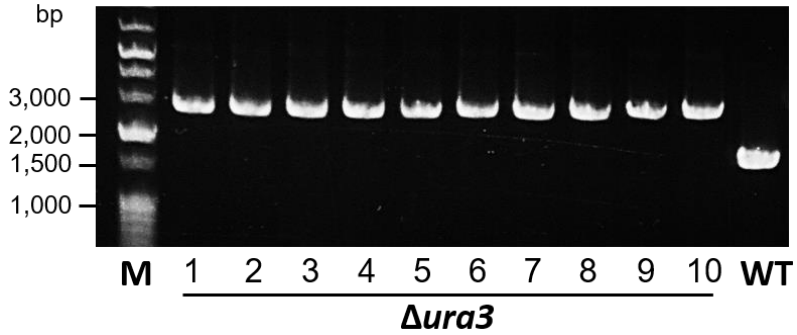


Fig. 2.3.12-b: PCR results of putative Δ*ura3* transformants.

Genomic DNA was extracted for PCR analysis and the band shift at nearly 3,000 bp indicates the successful interruption of *URA3* locus, WT: wild type.

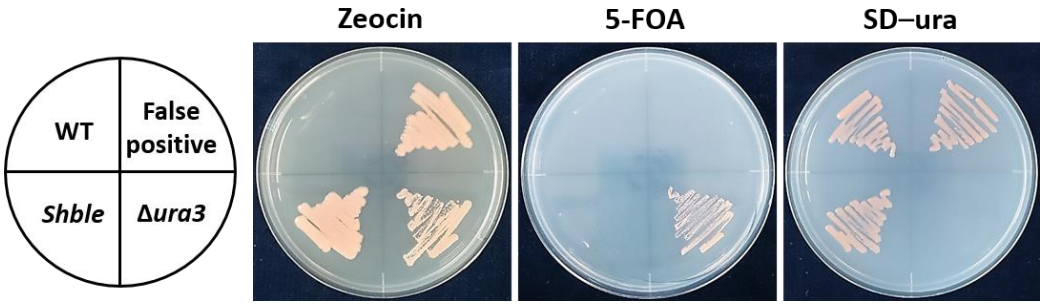


Fig. 2.3.12-c: TK16 phenotype confirmation.

Wild type (WT), *Sh ble* transformant (*Shble*), the *URA3* gene disruption cassette randomly integrated Zeocin-resistant transformant (False positive), the transformant whose *URA3* locus was successfully replaced with the *UAR3* gene disruption cassette (Δ*ura3*).

2.4 Summary: a sufficient system for *R. toruloides* transformation

By the developed transformation system, transformation efficiency of *R. toruloides* DMKU3-TK16 was improved (**Fig. 2.4-a**), and a maximum enhancement was obtained by 2.5 x 10^3-fold increment of colony number (**Fig. 2.4-b**). Incorporating with the successes in heterologous EGFP expression and application of different *R. toruloides* strains, this lithium acetate based transformation system should promote further research and engineering of *R. toruloides* strains in the future (**Fig. 2.4-c**).

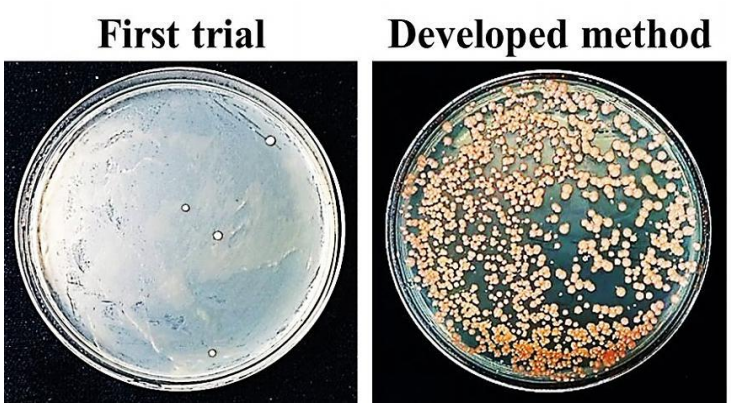


Fig. 2.4-a: Colony appearance of transformed TK16 by different trail.
TK16 transformed by developed transformation system showed much higher number of colonies.

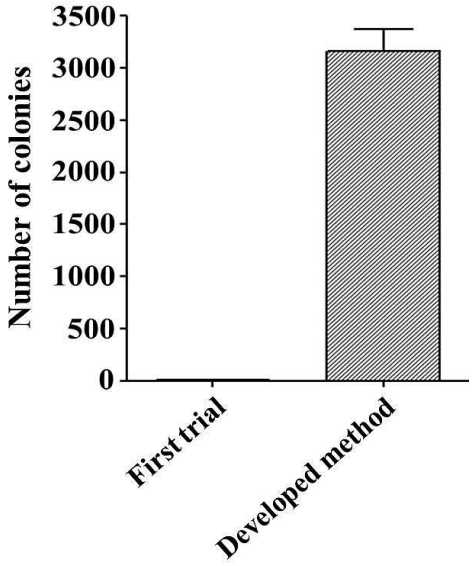


Fig. 2.4-b: Transformation efficiency of TK16 carried out by developed system in comparison with first trial.

A maximum enhancement was obtained by 2.5 x 10^3-fold increment of colony number on average against first trial.

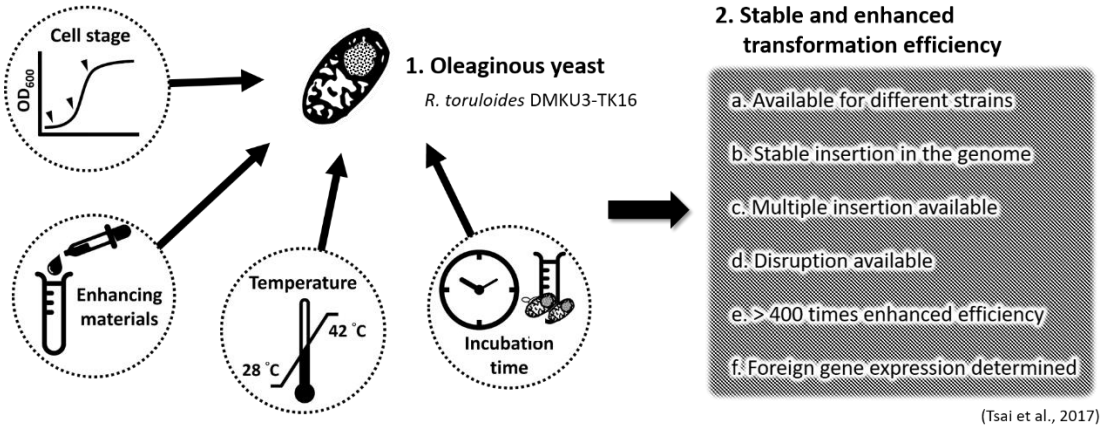


Fig. 2.4-c: Establishment of a Li-Ac based transformation system.

2.5 Discussions

2.5.1 Transformation in TK16

So far, a few kinds of transformation methods have been developed for *R. toruloides*. In 1985, Tully and Gilbert reported to transform *R. toruloides* by protoplast mediated system as the first successful transformation trial of *R. toruloides*. It was suggested that the transformant may have arisen by a cross-over event between plasmid and chromosome. Besides, the 2μ origin of replication of *S. cerevisiae* was shown not to function in *R. toruloides* (Tully and Gilbert, 1985) which may cause the unstable replication of introduced plasmid. This situation reflected the problem of unstable transformants obtained after selection. With the same purpose to utilize *R. toruloides*, Lin *et al.* tried to transform *R. toruloides* NP11 by protoplast mediated method, unfortunately, they failed to repeat the transformation system for arising transformants. Hence, ATMT was examined for *R. toruloides* NP11 transformation and provided stable transformants with antibiotic selection (Lin *et al*., 2014). These experiments have shown developed systems for transformation in *R. toruloides*, but more efficient system

is still needed.

Lithium acetate-based method is one of the popular systems for *S. cerevisiae* transformation. In this method, the presence of PEG is essential for successful transformation in intact yeast cells. Lithium acetate is not the sole contributor but it can enhance transformation efficiency accompanied with heat shock treatment. Notably, the cells harvested from mid-log phase were tested to show the highest transformation efficiency (Ito *et al*., 1983; Kawai *et al*., 2010). With lithium acetate-based method, there is no request to prepare protoplast, *Agrobacterium* or other special equipment through complicated procedures toward carrying out transformation. Due to its simpler and faster process, lithium acetate-based method has been popularly used for transformation in *S. cerevisiae* or some other yeasts. This study is therefore an attempt to have adequate transformation efficiency in *Rhodosporidium* sp. by the lithium acetate-based method.

To reach sufficient transformation efficiency with lithium acetate-based method in *R. toruloides* DMKU3-TK16, several steps in the process were examined: (1) applied DNA concentration; (2) heat shock temperature; (3) incubation period with lithium acetate and PEG based transformation mixture; (4) recovery after heat shock and (5) effect of cell growth phase. Because there is no episomal vector now available for *R. toruloides* transformation, target gene containing DNA fragments were employed to perform transformation. Notably, this transformation was considered to be conducted by the event of NHEJ. Various DNA concentrations (0, 1, 10 20, 30, 40, 50 μg) were examined and the result showed a highest efficiency by treating of 50 μg DNA with 37°C heat shock (**Fig. 2.3.3**). Incubation of intact yeast cells with transformation mixture is also suggested to be an important pretreating step for transformation, hence the incubation step was modified by longer incubation period and addition of assistant reagents (DMSO, ssDNA) (Hill *et al*., 1991; Kawai *et al*., 2010; Schiestl and Gietz,

1989). With the trial of different incubation periods, 3 h incubated test gave the best improvement (**Fig. 2.3.4**) which probably owning to the suitable permeabilization level was achieved. DMSO and ssDNA could enhance transformation efficiency by different levels, especially 10% DMSO was approved to enhance efficiency dramatically (**Figs. 2.3.5-a, b**). However they showed no synergistic effect and only increased efficiency with the nearly level given by ssDNA but DMSO (**Fig. 2.3.5-c**). DMSO was reported to have less effect in lithium acetate/single strand carrier DNA/PEG method (Gietz *et al*., 1995), and this could be the reason for no synergistic effect obtained from simultaneous treating of DMSO and denatured ssDNA, yet the detail of mechanism is still unknown.

Since heat shock was employed to enhance transformation efficiency, recovery of cells after heat shock is cared for arising transformants. Heat shocked TK16 cells were cooled down at room temperature (neither on ice nor higher than 28°C) for 5 min or no transformant will be obtained. With addition of medium, incubated mixture was transferred to test tube for culturing overnight with shaking before plating onto selective plate. Time course result indicated a few h cultivation still successfully appeared transformants with a slightly increasing trend to the longer cultivation time, however, hundreds folds increment was found after overnight cultivation for 16 h (**Fig. 2.3.6**). It was suggested that the transformed cells kept generating progeny during long time cultivation to show such a increasing of colony number. To elucidate this question, an experiment for knowing the changing of cell number should be executed. So far, an efficient lithium acetate based transformation system has been established for TK16, even the administration of high concentration DNA (50 μg for high efficiency shown above) is still a barrier for arriving simpler and materials conserving system. Nevertheless, sufficient transformation efficiency has been achieved with 20 μg or lower DNA concentration for obtaining transformants, which is a great improvement

compared to the first trial.

Mostly, efficient yeast transformation was achieved by cells at mid-log phase (Kawai *et al*., 2010). In the earlier part of this work, those successful transformation results gained with high efficiency were also performed by mid-log TK16 cells (**Fig. 2.3.10-a**). Surprisingly, with the trial of cells collected from different phases, cells obtained from 48 h pre-culture (stationary phase) was found to supply higher efficiency in this study (**Fig. 2.3.10-b**). With colony-forming unit (CFU) experiment, the 48 h cultured cells has higher cell number under the same OD_{600} value (OD_{600} = 3). By considering the reaction model of lithium acetate based transformation, the influence of cell wall or endocytotic membrane invagination (Kawai *et al*., 2010) between different growth phases may have some effects as well.

With this newly developed lithium acetate based transformation system, I have successfully introduced *Sh ble* and *EGFP* expression cassette in TK16. To access the stability of gene insertion, *Sh ble* transformants were analyzed by colony PCR and Southern blot. After the passage of 10 generations on non-selective plate, no revertant was observed and *Sh ble* gene still could be detected from all transformants by colony PCR (**Fig. 2.3.7-a**). A further confirmation by Southern blot also demonstrated the gene integration in TK16 genome (**Fig. 2.3.7-b**). By these results, high mitotic stability of gene insertion was approved, and it was suggested to be carried out by NHEJ due to no homologous sequence was applied in using fragments for transformation. Yet, the proposed NHEJ event in TK16 should be further investigated by the gene which is responsible for double strand DNA breaks repairing, e.g. *Ku70* (Abdel-Banat *et al*., 2010).

2.5.2 Application of newly developed transformation system in *R. toruloides* sp.

By the newly developed transformation system, TK16 could be transformed with

greatly improved efficiency (maximum obtained by 2.5 x 10^3-fold), but it has not been tested in different *R. toruloides* strains. In recent years, *R. toruloides* NP11 and ATCC1067 were widely utilized for *R. toruloides* research (Koh *et al*., 2014; Lin *et al*., 2014; Liu *et al*., 2013; Zhu *et al*., 2012) which should be suitable models for the application test of lithium acetate-based system. As those optimized conditions described before, both NP11 and ATCC10657 were successfully transformed with *Sh ble* cassette and showed Zeocin resistance by growing on selective plate (150 μg/mg Zeocin containing). Strain ATCC10657 was transformed with nearly half of the efficiency which TK16 have had, in contrast, only a small amount of colonies were found as NP11 transformant. ATCC10657 has been reported to be genetically closer to *Rhodotorula glutinis* ATCC204091 than NP11 which only 77% identity was acquired (Lin *et al*., 2014). I may hypothesize TK16 has the higher genetic identity to *Rhodotorula glutinis* strains, and the TK16 property based transformation system therefore resulted in different efficiency in NP11 and ATCC10657. However, more researches and evidences are required to prove it.

2.5.3 Heterologous gene expression in TK16

In order to know the possibility of foreign gene expression, TK16 was transformed with the selective marker linked EGFP expression cassette (*GPD1* promoter-*Sh ble*-*GPD1*-promoter-*EGFP*-*GPD1* terminator) (**Fig. 2.3.8-a**) for expression test. EGFP fragment was successfully applied for high efficiency even with larger size compared to *Sh ble* cassette (*GPD1* promoter-*Sh ble*-*GPD1*) (**Fig. 2.3.8-b**). Green fluorescent signal was also clearly observed in EGFP transfomrants under microscope (**Fig. 2.3.8-c**) and the protein production was detected by western blot (**Fig. 2.3.8-d**). The EGFP expression indicated possibility to express foreign genes in TK16. According to the high lipid-producing ability of TK16, I tried to introduce *CpFAH12* and *AtFAE1* genes

in TK16 respectively for ricinoleic acid and erucic acid production. Ricinoleic acid can be utilized for the production of lubricants, nylon, dyes, inks, soaps, adhesives, plasticizers (Holic *et al*., 2012), macrolactones and polyesters (Slivniak and Domb, 2005). Besdies, erucic acid can be considered as industrial feedstocks for the production of manufacturing plastics, nylon13-13 and high temperature lubricants (Guan *et al*., 2014; Li *et al*., 2012). However, by western blot and GC analysis, unfortunately there was no significant protein and fatty acid production being detected from *CpFAH12* and *AtFAE1* transformants. It was suggested that gene expression level was too low to produce recognizable amount of target product. To resolve this problem, I tried to modify the constructed fragment by terminator insertion and altering the orientation of linked cassettes. Appropriate terminator was known to help upstream gene expression (Mischo *et al*., 2013; Geisberg *et al*., 2014), but there was no terminator installed after *Sh ble* gene in both *Sh ble* cassette and *Sh ble* cassette linked EGFP transforming fragment (**Fig. 2.3.8-a**). Therefore, *GPD1* terminator from *R. toruloides* ATCC10657 was ligated just after *Sh ble* gene to construct new fragments for transformation. However, once the terminator inserted fragment was applied, transformation efficiency reduced obviously (**Fig. 2.3.11**). In addition, terminator-inserted EGFP trnasformants were found to lose downstream EGFP expression cassette, even replacing *GPD1* terminator by *CYC1* or *NOS* terminator. The reason of terminator related low transformation efficiency and unstable gene insertion is still unclear and under investigation. Meanwhile, altering orientation of expression cassettes was also considered as a possible solution (Ishii *et al*., 2014) and the research is progressing.

To this day, except for selective markers (Km^R, *Ble* transformant reported by Lin *et al*. in 2014, *Sh ble* transformant represented in this work) and model protein (EGFP), there is still no very successful heterologous gene expression for industrial product production in *R. toruloides*. Hence the investigation of how to express protein with

desired activity and improved expression level is very important for TK16 towards fatty acid production, or even for the further application in industry. In order to have successful protein expression and enhanced production level, several topics should be concerned, (1) promoters; (2) terminators; (3) selective markers; (4) vector maintenance and copy number; (5) codon usage.

(1) Promoters: once the target gene was chosen, promoter is usually an important point to be considered for promoting gene expression in the next step. Many promoters have been used to direct foreign gene expression successfully in *S. cerevisiae.* For example: efficient *GAP* (glyceraldehyde-3-phosphate dehydrogenase, also called *GPD1*) and *PGK* (phosphoglycerate kinase) promoter from the glycolytic pathway have been used. Also, in other commonly used yeasts, such like *P. pastoris*, *TEF* promoter (translation elongation factor 1) was approved with its high efficiency (Ahn *et al.*, 2007) except for *GAP* (Waterham *et al*., 1997) and *PGK* (de Almeida *et al*., 2005) promoter. In addition to these constitutive promoters, inducible promoter is also able to direct protein expression or even to enhance higher production level, e.g. *GAL1* promoter (Johnston, 1987), *ADH* (dehydrogenase, alcohol dehydrogenase) (Shuster, 1989) in *S. cerevisiae* and *AOX1* (alcohol oxidase) promoter in *P. pastoris* (Tschopp *et al*., 1987). Different from those commonly used model yeasts, there were only *GPD1* (Liu *et al*., 2013) and *PGK* (Lin *et al*., 2014) promoter have been tested to drive protein expression in *R. toruloides* strains. With the less number of defined promoters, it is very difficult to utilize them for the purpose to express all desired proteins, especially there was no promoter has been isolated from TK16. To find efficient promoters for TK16, I may need to characterize native promoters from TK16 which referred sequences of those well-known yeast promoters or directly from protein expression profile. Meanwhile, those well studied promoters from *S. cerevisiae* or *P. pastoris* should be also examined for their function in TK16.

(2) Terminators: terminator is an essential component in expression cassette and can influence protein output by controlling synthesis termination and stability of mRNA. Appropriate terminator can help completion and correcting transcription then further enhance protein production. Suitable combination of promoter and terminator in the expression cassette is one of key points for successful foreign protein production. However, as mentioned before, I am facing the problem of low transformation efficiency and loss of downstream cassette caused by terminator insertion. The way to resolve this problem is by modifying the direction of separate cassettes and it is under investigation.

(3) Selective markers: selective markers are yeast genes that complement auxotrophic mutations in host strain. For example, the *LEU2* and *URA3* genes are respectively used in strains that are *leu2* or *ura3*. In addition to maintain transformants with auxotrophic complementation, positive selection is also available due to the resistance to bioactive compounds such like G418 (Webster and Dickson, 1983) or Zeocin resistant transformants have been shown in this study. These frequently used selective markers allow strains maintenance in laboratory, however, sometimes they have unexpected effect on metabolism of host and influence protein expression. Especially those strains aimed for multiple genes expression which were usually transformed by several different selective markers may have undesired effect.

(4) Vector maintenance and copy number: to obtain desired protein production, vector which carrying target genes plays an important role in transformation. Unlike the genome integration, some episomal plasmid can afford 10 to 200 copies (e.g. plasmid YRp1 and pJDB219) (Berry *et al*., 1987; Wu *et al*., 1989) of target expression cassette per cell which then led to enhanced production level. The episomal vector may depend on its replication efficiency and relatively easy to be recovered, contrarily, the host genome integrated system could supply more stable maintenance of strains and

some integrative vectors have capacity with 100 copies. However, without sufficient episomal or integrative vector for *R. toruloides*, the transformation in TK16 should rely on NHEJ mediated gene insertion with the DNA fragment. With the host genome integration, the selection of high copy number strain is a way to gain higher production level. For example, I can increase the concentration of Zeocin (400 μg/mL or higher) to directly screen high resistance strains from single copy strains then examine the copy number by Southern blot analysis.

(5) Codon usage: based on the effort of bioinformatics, nowadays I can assume the codon preference in various yeast strains. To express heterologous gene in host yeast, codon optimization is a point should be considered. Some examples have shown the improvement of protein production through codon optimization towards express foreign genes in prokaryotic or eukaryotic expression systems (Kotula and Curtis, 1991; Krynetski *et al*., 1995; Liu *et al*., 2014; Sinclair and Choy, 2002; Yadava and Ockenhouse, 2003). In order to express target fatty acids in TK16 by introducing plant genes, codon optimization may provide enhanced effect.

CHAPTER 3

High-oleic acid lipid production in *R. toruloides*

3.1 Background: oleic acid (OA, C18:1) in current plant-derived lipid products

In efforts to determine the precise relationship between the fatty acid composition of plant-derived lipids and their performance as biodiesel fuel, a focus of the research has been to identify the effects of different fatty acids (Ferrari, 2005; Knothe, 2005; Knothe *et al*., 2006; Knothe, 2008; Tsakraklides *et al*., 2018). Monounsaturated fatty acids (MUFAs), especially the oleic acid (OA, C18:1) enriched plant-derived lipid, offer better oxidative stability, cold flow ability, and using performance as lubricants or biodiesel compared to non-MUFA enriched lipids (Ferrari, 2005; Knothe, 2005; Knothe *et al*., 2006; Knothe, 2008). Moreover, a high OA content (i.e., 60–90% of the total lipid) in a plant-derived biodiesel fuel was reported to produce less polluting products such as CO or NO_x after being used (Knothe *et al*., 2006; Knothe, 2008; Tsakraklides *et al*., 2018). Plant-derived OA-enriched lipids have therefore become a preferable alternative as a renewable lipid source. However, the production of plant-derived lipids involves several issues regarding its impacts on the environment such as water consumption, and pesticide administration (resulting in contamination on land and in the water), plus questions regarding the limitation of field sizes, sun or artificial light requirements, and even the climate factors (Hill *et al*., 2006; Williams *et al*., 2009; Steen *et al*., 2010).

3.2 Current statements: OA production form yeasts

Oleaginous yeasts discovered from different living environments have the ability to use varied substrates including industrial waste water or biomass towards a lipid synthesis with less environmental-restricting factors compared to oil plants, and such

yeasts may therefore serve as lipid-producing workhorses (Li *et al*., 2008; Beopoulos, 2009; Ageitos *et al*., 2011; Kosa & Ragauskas, 2011; Sitepu *et al*., 2014; Tchakouteu *et al*., 2015; Shi & Zhao, 2017; Cho & Park, 2018). In addition, flexible, controllable and trackable genetic tools will enable the oleaginous yeasts to become a feasible platform for lipid production for practical uses in the future (Liang and Jiang, 2013; Fillet and Adrio, 2016; Adrio, 2017; Shi and Zhao, 2017). However, the OA content that produced from conventional or oleaginous yeasts thus far has been limited (approx. 40–50% maximum), which is lower than the OA level (≥ 60%) in the oil plant-derived products (Knothe, 2008; Fillet *et al*., 2017; Uprety and Rakshit, 2017; Polburee *et al*., 2018; Tsakraklides *et al*., 2018; Yuzbasheva *et al*., 2018).

Yazawa *et al*. achieved efficient OA accumulation with increased ethanol tolerance in *Saccharomyces cerevisiae* with an overexpression of *rat elongase* (*rELO*) gene, and the resultant proportion of OA is around 40% of the total lipid; however their system does not seem to provide an aim or a sufficient method of producing OA-enriched lipid (2011). Several attempts to enhance the lipid production in the well-studied oleaginous yeast *Yarrowia lipolytica* resulted in limited levels of OA-enriched lipids (Qiao *et al*., 2015; Ledesma-Amaro and Nicaud, 2016; Gao *et al*., 2018). A very recent work that obtained an OA level over 90% in *Y. lipolytica* again emphasized the importance of OA-enriched lipid for the industrial purposes (Tsakraklides *et al*., 2018). Compared to *Y. lipolytica*, the oleaginous yeast *Rhodosporidium toruloides* is not yet understood well enough for the realization of its potential, due in part to the limitations of available investigative tools.

3.3 Objective: alternative source of sufficient high-OA lipid production

The OA ratio produced from present oleaginous yeast system is still relatively lower than the ratios of the currently used plant-derived products. Moreover, the exact

function of *R. toruloides* Δ9FAD remains to be established. Therefore, I conducted a functional examination and attempted to enhance the OA production in *R. toruloides* by overexpressing *ScOLE1* and the homologue *RtΔ9FAD* genes. With the use of a previously established transformation system, *ScOLE1* and *RtΔ9FAD* genes were introduced individually in both the NP11 and TK16 strains for the production of OA-enriched lipid.

3.4 Results: high-OA lipid production in *R. toruloides*

3.4.1 Background: Δ9 fatty acid desaturase produces OA

R. toruloides belongs to subphylum Pucciniomycotina in the phylum Basidiomycota (basidiomycotaetes). It is known as a producer of carotenoids or enzymes, and it is now being intensively studied and used for lipid production based on its promising lipid productivity ((Zhu *et al*., 2012; Tai and Stephanopoulos, 2013; Sambles *et al*., 2017; Park *et al*., 2017; Coradetti *et al*., 2018). *R. toruloides* strains were shown to be able to accumulate high amount of lipids under the nitrogen-limited conditions and to naturally produce OA at a relatively higher ratio in total lipid compared to other yeasts.

In addition to providing better lipid properties for biodiesel and chemical uses, OA is also an important precursor for the further synthesis of valuable polyunsaturated fatty acids (PUFAs) in oleaginous yeasts (Yazawa *et al*., 2009; Sakuradani, 2010; Uemura, 2012; Buček *et al*., 2014; Wang *et al*., 2016). *OLE1* gene, with synonyms also known as *stearoyl-CoA desaturase* (*SCD*) or Δ*9-fatty acid desaturase* (Δ*9FAD*) gene, encodes Δ9-fatty acid desaturase (Δ9FAD), which catalyzes the double bond formation between carbons 9 and 10 of palmitic acid (PA; C16) and stearic acid (STA; C18) to synthesize palmitoleic acid (POA; C16: l) and OA (Stukey *et al*., 1989; Cook and McMaster, 2002; Xue *et al*., 2016). The majority of Δ9FAD-synthesized product (POA or OA) is highly

host-dependent. Only a few *OLE1* homologues from oleaginous yeasts have been isolated and characterized for their lipid- or OA-related production capacity (Meesters and Eggink, 1996; Qiao *et al*., 2015; Ledesma-Amaro and Nicaud, 2016; Zhang *et al*., 2016; Díaz *et al*., 2018; Tsakraklides *et al*., 2018). Zhang *et al*. (2016) demonstrated that the overexpression of the own *SCD* gene in *R. toruloides* resulted in enhanced lipid production, but the OA ratio is still relatively lower than the ratios of the currently used plant-derived products. Moreover, the function of *R. toruloides* Δ9FAD remains to be established.

3.4.2 Sequence analysis of *R. toruloides* NP11 Δ9-fatty acid desaturase

The amino acid (AA) sequence of putative *R. toruloides* NP11 Δ9FAD (*Rt*Δ9FAD) (XP_016270987) was obtained from the NCBI protein database (Zhu *et al*., 2012). The putative *Rt*Δ9FAD gene encodes a polypeptide of 545 AAs with a predicted molecular mass of 60.8 kDa (Gasteiger *et al*., 2003; Artimo *et al*., 2012). A characterized Δ9FAD from *Cutaneotrichosporon curvatus* (CAA71449.1) was shown to share 64% similarity with *Rt*Δ9FAD (Meesters and Eggink, 1996). Herein, we analyzed the AA sequence of *Rt*Δ9FAD with several Δ9FAD homologous proteins, i.e., the characterized *Sc*Δ9FAD protein (AAA34826.1) (**Figs. 3.4.2-a**).

The relevancy shown by the phylogenetic tree in Figure 3.3.2-a illustrates the clear distinctions among yeast, fungal, and plant Δ9 desaturases and the further distinction from the outgroup yeast Δ12 desaturases. In the phylogenetic tree, *Rt*Δ9FAD was relatively close to the characterized Δ9FAD from *C. curvatus* (CAA71449.1) (Meesters and Eggink, 1996). The *Sc*Δ9FAD was grouped with another characterized Δ9FAD from *Ogataea angusta* (BAA11837.1), a yeast from the subphylum Pezizomycotina (Anamnart *et al*., 1997). The alignment analysis compared FADs from *S. cerevisiae* (Stukey *et al*., 1990), *Mortierella alpine* (Sakuradani *et al*., 1999), *O. angusta*

(Anamnart *et al*., 1997), *Trichophyton equinum*, and *Ustilaginoidea virens* (**Fig. 3.4.2-b**). The putative *Rt*Δ9FAD was divided into an OLE1 region (fatty acid desaturase region) and a Cyt-b5 region (cytochrome b5-like Heme/Steroid binding domain and nitrate reductase) according to previous studies (Cook and McMaster, 2002; Lou and Shanklin, 2010; Bai *et al*., 2015). These Δ9FADs shared two highly conserved histidine sequences, HRXHHR and HNFHH (dashed underlining) in the hypothetical OLE1 region that would be expected to be responsible for the function of Δ9 fatty acid desaturase.

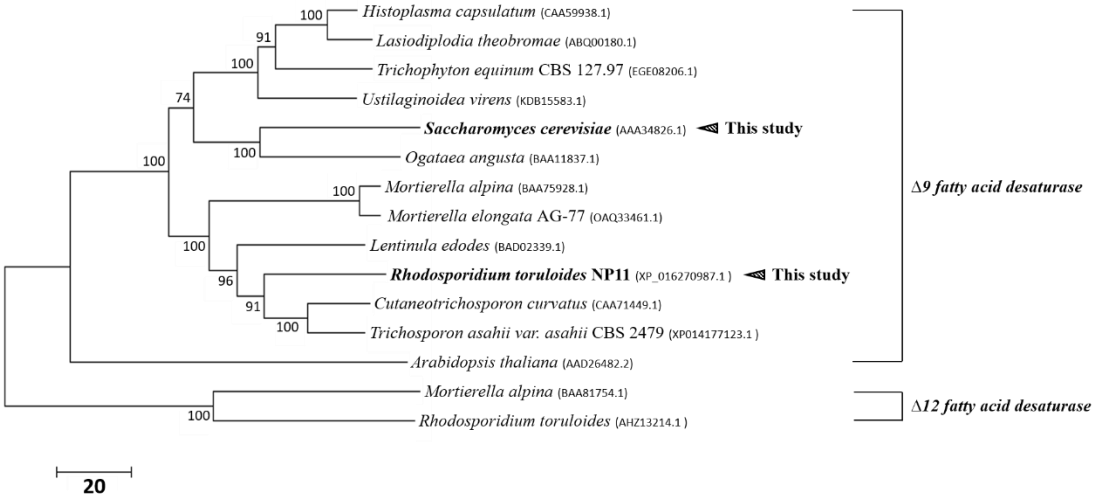


Fig. 3.4.2-a. Phylogenetic tree of the evolutionary relationships of Δ9 fatty acid desaturase protein orthologues.

Molecular phylogeny shared by the characterized and deduced Δ9FAD protein homologues, with Δ12FADs (fatty acid desaturase) being an outgroup. The multiple alignment was performed and the phylogenetic tree was constructed using the neighbor-joining algorithm of MEGA7. Branch lengths are proportional to the phylogenetic distances, with the numbers representing the frequency which was replicated after 1000 bootstrap iterations.

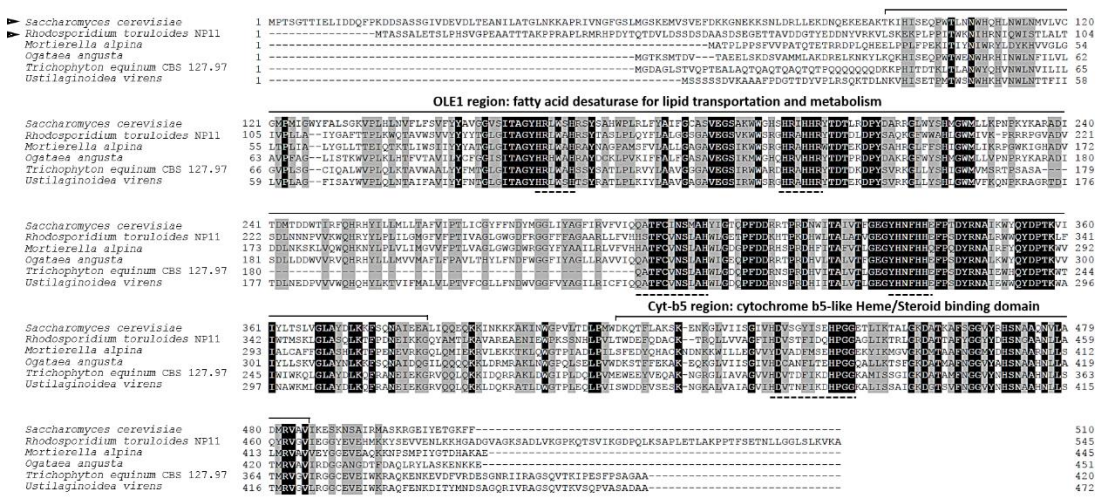


Fig. 3.4.2-b. Protein sequence alignment and analysis of *R. toruloides* NP11 Δ9FAD.

Identical residues are boxed in black, and the conservative areas are shaded in gray. Two hypothetical regions (OLE1 and Cyt-b5 region) are briefly classified separately, with dashed underlining indicating the conservative histidine-involved domains.

In addition, other three domains with a conserved histidine residue (dashed underlining) were observed in both the OLE1 and Cyt-b5 regions; these domains might also serve as the active area of each region (Shanklin *et al*., 1994; MacKenzie *et al*., 2002). The *Rt*Δ9FAD protein was also predicted to have four possible transmembrane domains (**Fig. 3.4.2-c**). *Rt*Δ9FAD was thus suggested to be a functional homologue of *Sc*Δ9FAD.

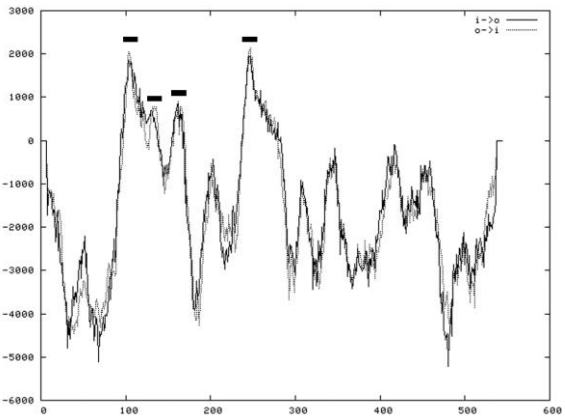


Fig. 3.4.2-c. Transmembrane domain prediction of *R. toruloides* NP11 Δ9 desaturase performed by TMpred Server.

Black bars: the predicted sites of transmembrane domain.

3.4.3 The functional complementation of *S. cerevisiae ole1* disruptant by *Rt*Δ*9FAD* gene

The Δ9FAD is generally known to have the function of catalyzing the fatty acids PA (C16) and STA (C18) to POA (C16:1) and OA (C18:1) respectively in mammals, yeasts, and plants. To examine the function of *Rt*Δ9FAD, we introduced the Δ*9FAD* gene cloned from NP11 into the *S. cerevisiae ole1* disruptant BY4389 (*Scole1*Δ) for a complementary test (**Fig. 3.4.3-a**). The *Scole1*Δ strain was incapable of surviving on the plate without OA supplement (**Fig. 3.4.3-a**, upper left), but the cell survival was restored by the overexpression of *ScOLE1* gene as a reference strain under the galactose induction (upper right).

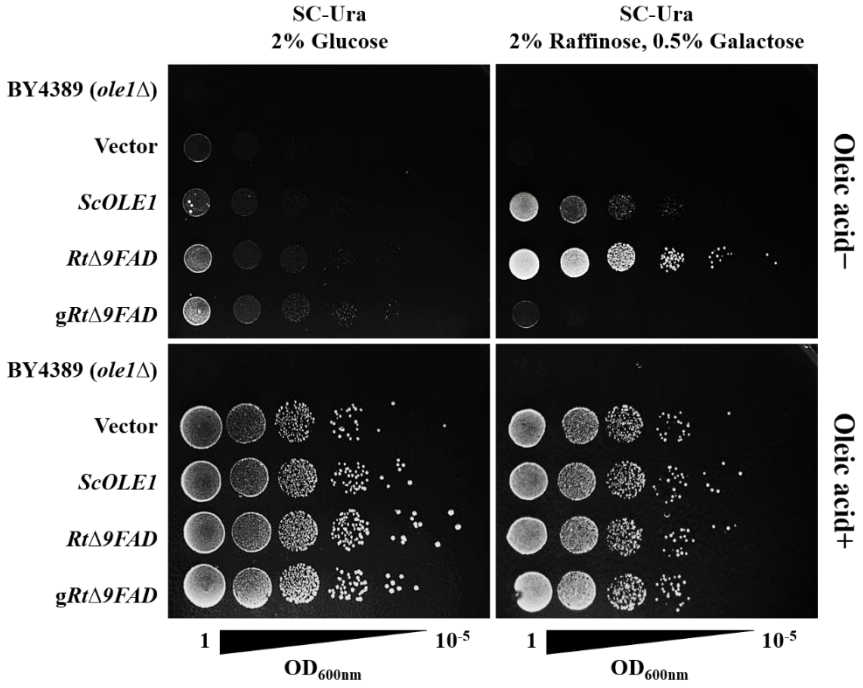


Fig. 3.4.3-a: Spot assay of *Rt*Δ9FAD complementing cell survival.

Live/dead assay among the background of *Scole1*Δ strain BY4389, comparing the function of overexpressing pYES2 empty vector, *ScOLE1*, *Rt*Δ*9FAD* (CDS) and g*Rt*Δ*9FAD* (genomic). The experiment was conducted on the SC-Ura medium with or without OA supplement.

Different from *ScOLE1* gene, the *Rt*Δ*9FAD* gene has a genomic sequence that inserted with several introns. While the possibly resulted effect have never been reported, *Rt*Δ*9FAD* gene was thus overexpressed for complementation in *Scole1*Δ following the introduction of the coding sequence (CDS) or genomic sequence, presented by *Rt*Δ*9FAD* and g*Rt*Δ*9FAD* respectively. The genomic *Rt*Δ*9FAD* sequence was unable to save the OA deficiency-induced cell death. Contrarily, the CDS *Rt*Δ*9FAD* expression restored the cell survival and showed an even better growth effect compared with the reference group (*ScOLE1*).

We also investigated the fatty acid composition of extracted lipid from each strain (**Fig. 3.4.3-b**).

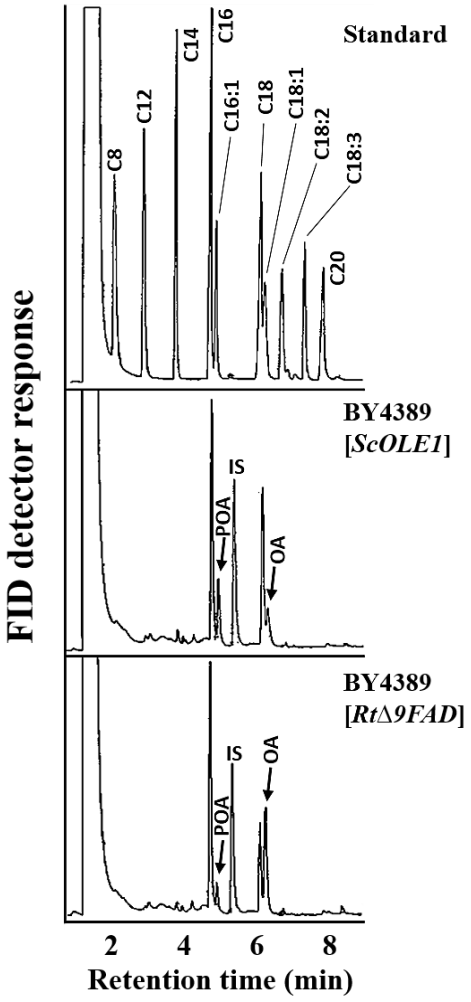


Fig. 3.4.3-b: Gas chromatography analysis of fatty acid composition in *ScOLE1* or *Rt*Δ*9FAD* overexpressing *Scole1*Δ.

The peaks of POA and OA were identified by a comparison with the commercially available fatty acid methyl ester standard and are indicated by *black arrows*.

IS: internal standard, heptadecanoic acid methyl ester (C17).

Without OA, the empty vector pYES2-transformed *Scole1*Δ could not grow in YM broth. As the reference, *Scole1*Δ carrying pYES2-ScOLE1 grew and showed the regained production of POA and OA. Likewise, the *Rt*Δ*9FAD* expression also restored POA production and significantly enhanced the OA accumulation. Quantitatively, the production of PA was not significantly affected in the *Rt*Δ*9FAD* transformant, but the POA level (4.4%) was 2.3-fold less than the reference (10.2%) (**Fig. 3.4.3-c**). The proportion of STA was 20.9% along with an OA ratio at 25.2%, which was greater than the OA level from the reference. These results demonstrated the PA and STA converting function of *Rt*Δ9FAD and also revealed the different substrate preference from each applied Δ9FAD in the *S. cerevisiae* host background.

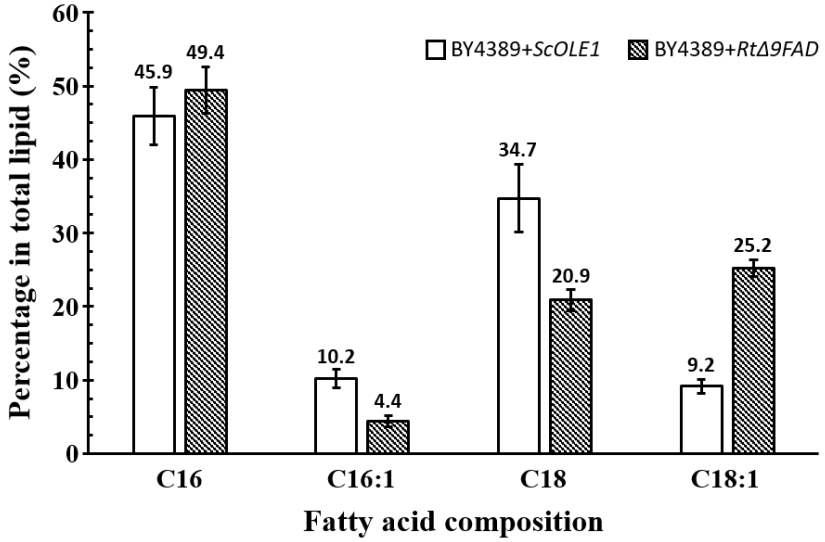


Fig. 3.4.3-c: Quantitative measurement of fatty acid profile in percent fraction. Numbers are the occupied ratio in total lipid of each indicated fatty acid. C16, palmitic acid, PA; C16:1, palmitoleic acid, POA; C18, stearic acid, STA; C18:1, oleic acid, OA. The values are the average of triplicate cultures. Error bar: SD.

3.4.4 Introduction of *ScOLE1* and *Rt*Δ*9FAD* gene increased lipid production in *R. toruloides* strains

In order to produce an OA-enriched lipid with higher lipid productivity, I then introduced *ScOLE1* gene, and both the CDS and the genomic sequence of *Rt*Δ*9FAD* genes into *R. toruloides* strains NP11 and TK16. The effect on cell growth of gene insertion was examined during the lipid accumulating condition, and most of the cell growth was not obviously affected (**Fig. 3.4.4-a**).

To identify the optimal period of desired lipid production, I inspected the lipid production in a time-dependent experiment (**Fig. 3.4.4-b**). With both the TK16 and NP11 background, the *ScOLE1* and g*Rt*Δ*9FAD* transformants began to show increased lipid production since cultivation started in the nitrogen-limited environment for lipid accumulation after 24 h. I observed that the stationary period of lipid accumulation started about at 96 h and reached the maximal amount at 120 h after the initiation of lipid accumulation in both *R. toruloides* backgrounds, and thereafter a slight decline of the lipid amount was also observed at 144 h that may reflect to the nutrient deficiency in the medium and the consumption of lipid storage to maintain the cell survival.

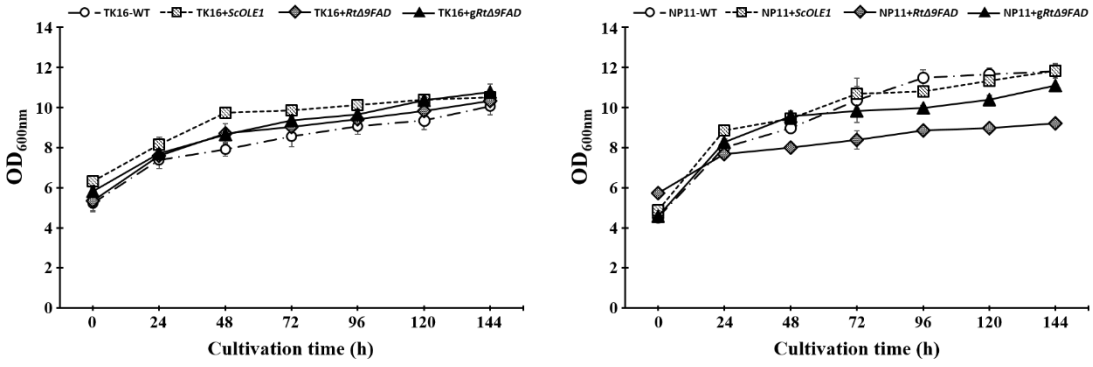


Fig. 3.4.4-a: Growth effects of *ScOLE1* and *Rt*Δ*9FAD* gene insertion on *R. toruloides* strains during lipid accumulating condition.

The growth of the transformants compared with each wild-type strain was mostly equivalent. TK16 (left) and NP11 (right) transformants cell density was determined by absorbance OD_{600nm}. WT: wild-type strain; +*ScOLE1*: *ScOLE1* transformant; + *Rt*Δ*9FAD*: *Rt*Δ*9FAD* transformant; + g*Rt*Δ*9FAD*: g*Rt*Δ*9FAD* transformant. The presented values are the average of triplicate cultures. Error bars: SD.

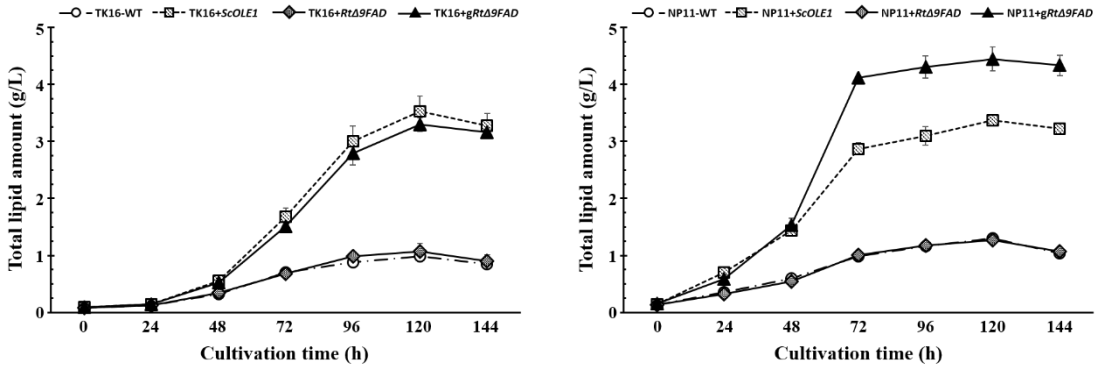


Fig. 3.4.4-b: Time-dependent examination of lipid production and comparison among the wild type and transformants.

Total lipid amount determined quantitatively in TK16 (left) and NP11 (right) transformants. WT: wild-type strain; +*ScOLE1*: *ScOLE1* transformant; +*Rt*Δ*9FAD*: *Rt*Δ*9FAD* transformant; +g*Rt*Δ*9FAD*: g*Rt*Δ*9FAD* transformant. The presented values are the average of triplicate cultures. Error bars: SD.

Compared to the wild type, the *ScOLE1* and g*Rt*Δ*9FAD* transformants showed approx. threefold lipid increases in both the NP11 and TK16 background. The lipid productivity (μg/mg) at each time point was determined, and the probable period of lipid production was obtained with the highest efficiency at 96–120 h in transformants (**Fig. 3.4.4-c**).

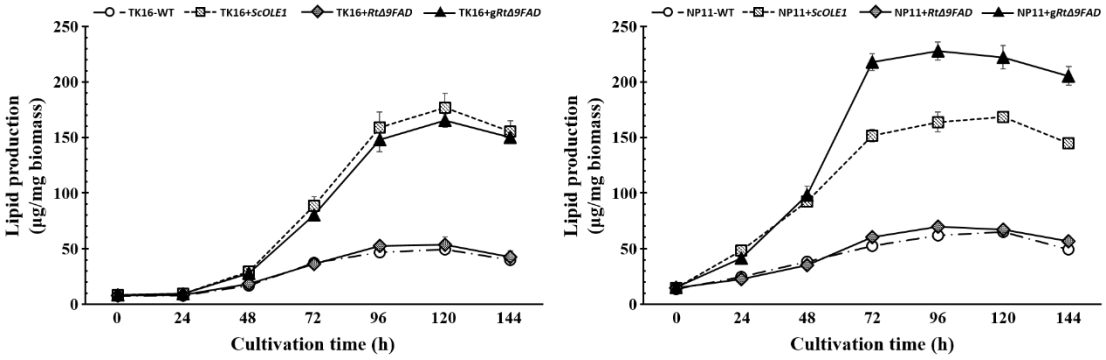


Fig. 3.4.4-c: Corresponding lipid productivity at each observed time point.

Lipid productivity of all strains measured quantitatively in TK16 (left) and NP11 (right) transformants measured quantitatively in TK16 (left) and NP11 (right) transformants. WT: wild-type strain; +*ScOLE1*: *ScOLE1* transformant; +*Rt*Δ*9FAD*: *Rt*Δ*9FAD* transformant; +g*Rt*Δ*9FAD*: g*Rt*Δ*9FAD* transformant. The presented values are the average of triplicate cultures. Error bars: SD.

All transformants were also directly observed by microscopy with lipid staining (**Figs. 3.4.4-d, e**).

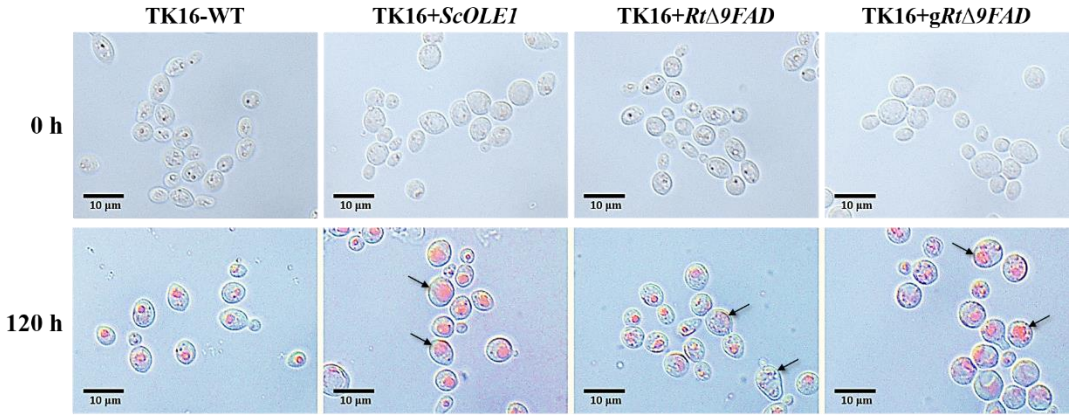


Fig. 3.4.4-d: Lipid droplet observation in wild-type TK16 and transformants by microscopy.

The lipid droplets were stained by Sudan IV, and the cells were observed at 0 and 122 h from a bright field. Scale bar: 10 μm. WT: wild-type strain; +*ScOLE1*: *ScOLE1* transformant; +*RtΔ9FAD*: *RtΔ9FAD* transformant; +g*RtΔ9FAD*: g*RtΔ9FAD* transformant.

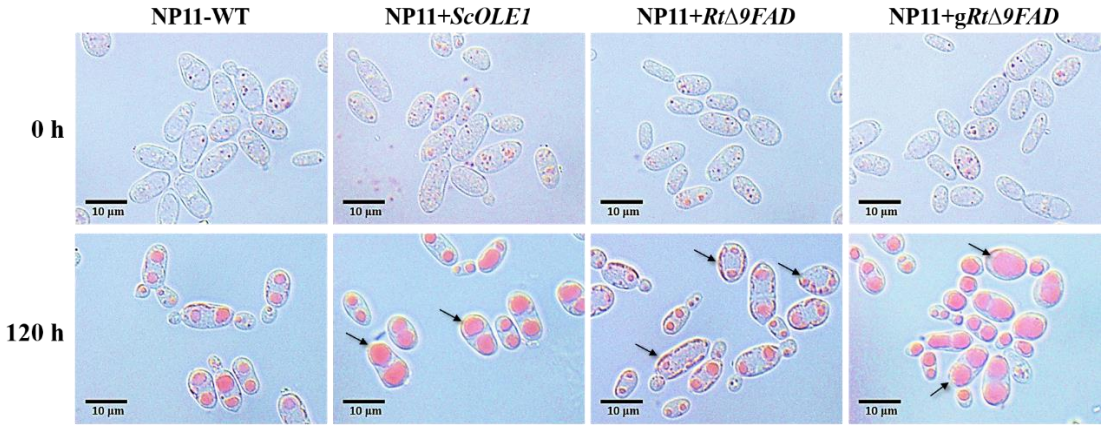


Fig. 3.3.4-e: Lipid droplet observation in wild-type NP11 and transformants by microscopy.

The lipid droplets were stained by Sudan IV, and the cells were observed at 0 and 122 h from a bright field. Scale bar: 10 μm. WT: wild-type strain; +*ScOLE1*: *ScOLE1* transformant; +*RtΔ9FAD*: *RtΔ9FAD* transformant; +g*RtΔ9FAD*: g*RtΔ9FAD* transformant.

Yeast cells were collected at 0 and 120 h, and apparent lipid droplets could be seen after 120 h of lipid accumulation. The *ScOLE1* and g*Rt*Δ*9FAD* transformants displayed larger lipid droplets than the wild type and *Rt*Δ*9FAD* transformed groups, and we observed different appearances of the lipid droplets (separated small droplets distributed around the cell) from *Rt*Δ*9FAD* transformants compared to the others (condensed large droplets). Taking these results together, we chose 120-h cultivation for lipid accumulation in the subsequent experiments.

3.4.5 *ScOLE1* gene expression significantly increased the OA content in lipids

The ultimate goal of this study was the production of lipid with enhanced OA content, and we therefore examined the fatty acid composition of the transformants towards our purpose (**Fig. 3.4.5-a**).

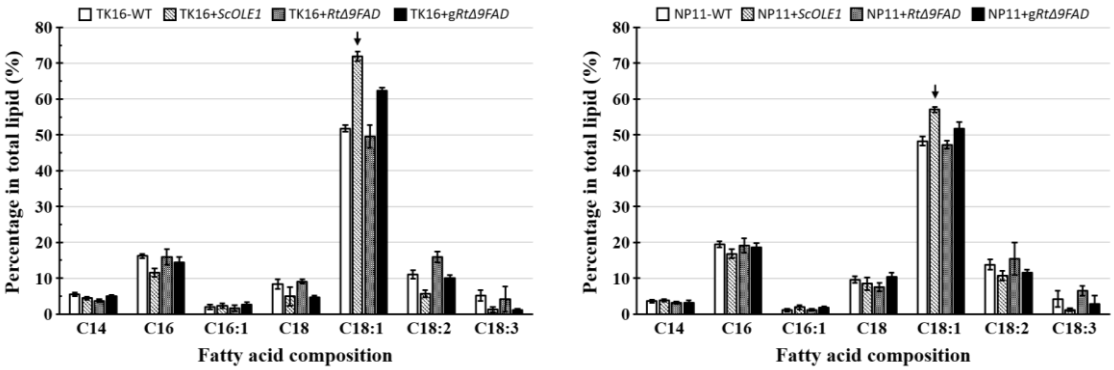


Fig. 3.4.5-a: *ScOLE1* and *Rt*Δ*9FAD* gene overexpression in the *R. toruloides* TK16 and NP11 resultant lipid and oleic acid production.

Fatty acid composition of all strains measured quantitatively in TK16 (left) and NP11 (right) transformants from 122-h culture. The fatty acid profile is presented as the percent fraction for each indicated fatty acid. C14, myristic acid, MA; C16, palmitic acid, PA; C16:1, palmitoleic acid, POA; C18, stearic acid, STA; C18:1, oleic acid, OA; C18:2, linoleic acid, LA; C18:3, linolenic acid, LNA. WT: wild-type strain; +*ScOLE1*: *ScOLE1* transformant; +*Rt*Δ*9FAD*: *Rt*Δ*9FAD* transformant; +g*Rt*Δ*9FAD*: g*Rt*Δ*9FAD* transformant.

The results of our fatty acid analysis showed that the lipid produced in the *ScOLE1* TK16 transformant contained OA at 72% of the total lipid, which was higher than the ratios produced in other yeast-originated lipids. The g*Rt*Δ*9FAD* TK16 transformant was able to produce lipid with the OA ratio of 62% of the total lipid, but a similar effect was not observed in the *Rt*Δ*9FAD* TK16 transformant.

Compared to the wild-type TK16, the OA content was enhanced by 40% in the *ScOLE1* transformant and 20% in the g*Rt*Δ*9FAD* transformant. With the NP11 background, the *ScOLE1* transformant also produced lipid at 57% OA compared to wild type, reaching approx. 18% enhancement, but the effect was not significant in the g*Rt*Δ*9FAD* transformant. We also found that the *Rt*Δ*9FAD* transformants did not clearly enhance the produced lipid amount or OA content in the TK16 or NP11 background. Combining the enhanced lipid-producing ability and OA level, the *ScOLE1* and g*Rt*Δ*9FAD* transformants furnished higher lipid and OA production. Particularly, the *ScOLE1* TK16 transformant was able to produce lipid with a threefold total amount and fivefold increase in the OA content for the efficient production of OA-enriched lipid at the desired level (**Fig. 3.4.5-b**).

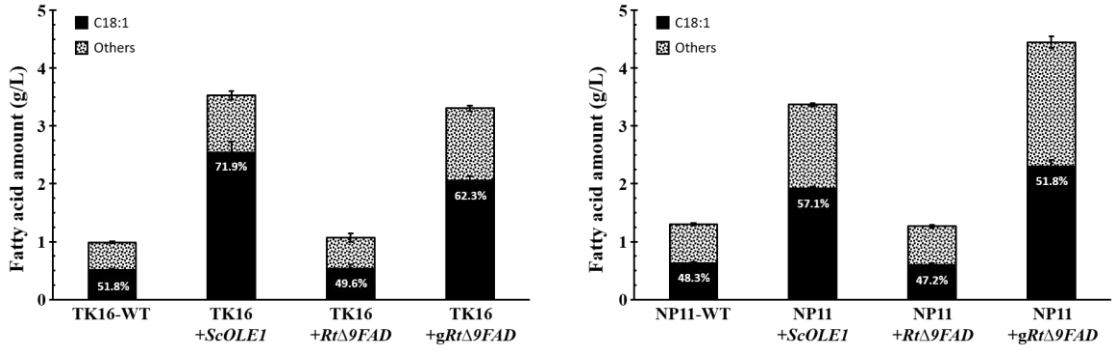


Fig. 3.4.5-b: *ScOLE1* and *Rt*Δ*9FAD* gene overexpression in the *R. toruloides* resultant lipid and oleic acid production.

Lipid content with OA in the percent fraction from 122-h culture. *Black bar with numbers*: The OA occupied ratio in the final output lipid. WT: wild-type strain; +*ScOLE1*: *ScOLE1* transformant; +*Rt*Δ*9FAD*: *Rt*Δ*9FAD* transformant;

+g*Rt*Δ*9FAD*: g*Rt*Δ*9FAD* transformant. The presented values are the average of triplicate cultures. Error bars: SD.

3.4.6 The expression level of *Rt*Δ*9FAD* gene in the *R. toruloides* transformants

Considering that using the CDS and the genomic sequence of *Rt*Δ*9FAD* gene resulted in notably different effects on lipid production, I conducted a qPCR to investigate whether the level of transcription contributed to these effects (**Fig. 3.4.6**). The genomic sequence conferred a higher transcriptional level among the wild-type and CDS transformants. A nearly threefold higher level was detected in the g*Rt*Δ*9FAD* TK16 transformants against the CDS-introduced group, and approx. threefold and twofold higher levels were detected in the NP11 transformants compared to the wild-type and CDS transformants. A similar result was obtained with a different primer pair at the 5' end of the target sequence (data not shown). These results indicated that the genomic sequence did provide a relatively higher transcriptional level, and that this might thence lead to the distinct enhancing effect on lipid production.

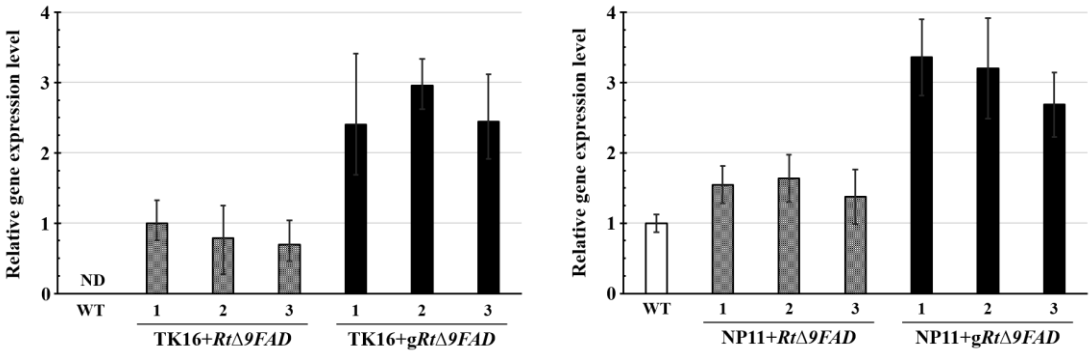


Fig. 3.4.6: Investigation of the *Rt*Δ*9FAD* gene expression level in transformants. The mRNA expression level was normalized with *URA3* expression, which was used as the internal standard. In the TK16 strain, one of the *Rt*Δ*9FAD* CDS transformants (transformant 1) was selected for reference. With the NP11 background, the wild type (WT) was selected for reference. Each number indicates an independent clone. ND: non-detected. The presented values are the average of triplicate cultures. Error bars: SD.

3.5 Summary: high-OA lipid production from Δ9 FAD transformants

In summary, we sought to obtain a high OA content lipid content to produce preferable biodiesel and lipid-related chemicals. In this work, I performed an AA sequence analysis and examined the function of *RtΔ9FAD* gene as the candidate gene toward OA-enriched lipid production. By applying *ScOLE1* and *RtΔ9FAD* genes in the oleaginous yeast *R. toruloides*, we achieved increased lipid production with the desired OA content. We also observed an interesting expression effect by introducing a genomic sequence that could be an useful example for the future manipulation of endogenous genes in *R. toruloides* (**Fig. 3.5**).

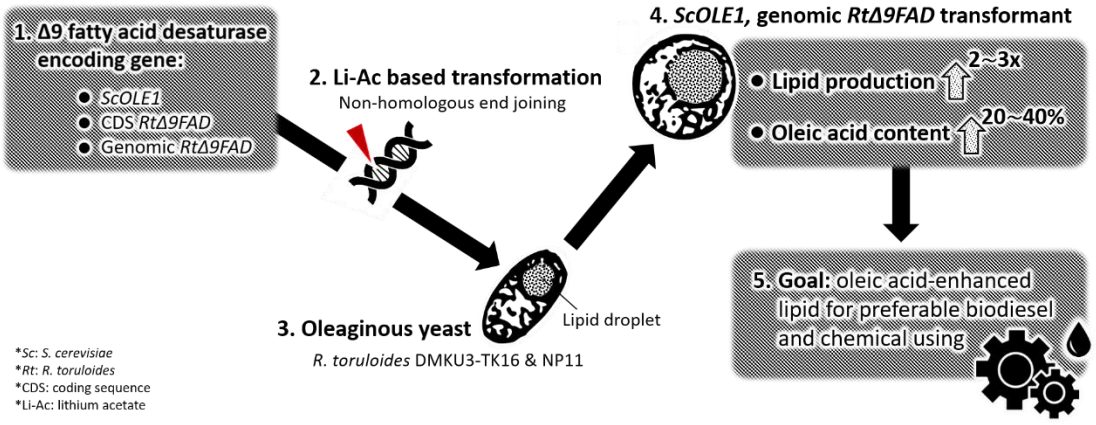


Fig. 3.5: Enhanced OA content and lipid amount for the preferable yeast lipid.

3.6 Discussions

3.6.1 The production of high OA lipid

High OA plant-derived lipids have better applicability with preferable stability and less air pollutants, and the low melting point of OA methyl ester maintains its capability in the cold environments (Knothe, 2005; Knothe *et al*., 2006; Knothe, 2008). An increased OA content was observed to enhance engine performance in soybean oil-

derived biodiesel (Ferrari, 2005). A recent work in *Y. lipolytica* also has emphasized the importance of high OA containing lipid towards using (Tsakraklides et al, 2018). The intensive cropping of oil plants has environmental influences, and lipid-rich algae are considered another conductive option that involves less pressure from the requirements of renewable oil sources, but the use of lipid-rich algae is also limited by light and water demands and the high cost of oil recovery (Richardson *et al.*, 2010; Clarens *et al*., 2011; Slade and Bauen, 2013; Ullah *et al*., 2014; Marrone *et al*., 2017). Oleaginous yeasts provide an effective platform without requiring similar restrictions on the process of production, and a feasible system of genetic engineering could provide designable products. We aimed to produce OA-enriched lipid from the oleaginous yeast *R. toruloides* by an overexpression of *ScOLE1* and *Rt*Δ*9FAD* genes.

Some studies of *Y. lipolytica* and *R. toruloides* have applied endogenous Δ*9FAD* homologues in the course of lipid production and enhanced the lipid amount. However, most of those effort did not significantly improve the OA level or further discuss the function of oleaginous Δ*9FAD* gene (Qiao *et al*., 2015; Ledesma-Amaro and Nicaud, 2016; Zhang *et al*., 2016; Díaz *et al*., 2018). Zhang *et al*. overexpressed native *SCD* gene in *R. toruloides* IFO0880 to increase the lipid production by 28% of total lipid (g/L), which was described as a limited enhancement (2016). Here, we analyzed the gene itself and used the CDS and the genomic sequence individually for the comparison. Consequently, threefold increased lipid production was obtained with enhanced OA content.

3.6.2 Function of applied Δ9FADs

Several histidine domains were identified in the *Rt*Δ9FAD protein sequence and are speculated to be the active sites (**Figs. 3.4.2-b, c**). The *Rt*Δ*9FAD* gene complemented the production of POA and OA and restored the survival of *Scole1*Δ without OA

supplementation (**Fig. 3.4.3-a**). Interestingly, the efficiency of OA production was higher than that of POA in *Rt*Δ*9FAD* transformed *Scole1*Δ, which was found to be different from the *ScOLE1* transformant (**Figs. 3.4.3-b, c**). This result indicated that the substrate preference of Δ9FAD might be strongly related to the gene origin. Such phenomena of distinct substrate preferences among Δ9FADs were observed from Δ9FAD isomers in different models, i.e., the mouse SCD1-4 and *M. alpina* ole1p, ole2p and Δ9-3 (Wongwathanarat *et al*., 1999; Zheng *et al*., 2001; MacKenzie *et al*., 2002; Miyazaki *et al*., 2006).

After our functional examination, we then introduced *ScOLE1* and *Rt*Δ*9FAD* genes into the *R. toruloides* TK16 and NP11 strains to obtain the production of OA-enriched lipid. Under the nitrogen limited condition, both strains gained the highest level of lipid accumulation about 120 h cultivation after culturing started, afterwards a slight decline of lipid content was observed (**Fig. 3.4.4-a**). Unexpectedly, the *ScOLE1* gene (which is rarely discussed when enhancing lipid production is considered) drove the *R. toruloides* transformants to give higher lipid amounts, which the lipid production were increased by 3- and 2.5-fold compared to the wild-type TK16 and NP11 (**Fig. 3.4.4-b**). The genomic sequence of *Rt*Δ*9FAD* gene also provided an enhancing effect on lipid production, but this effect was not clearly observed when a CDS fragment was used as the expression target (**Fig. 3.4.4-b**). Although some Δ9FADs are known to be related to the obesity in mammal models or to lipid production in yeasts, the exact mechanism underlying their enhancement of yeast lipid production is still not fully understood (Jones *et al*., 1996; Dobrzyn and Ntambi, 2005; Qiao *et al*., 2015; Ledesma-Amaro and Nicaud, 2016; Zhang *et al*., 2016; Díaz *et al*., 2018; Gao *et al*., 2018).

In the present study's microscopic observations, the *ScOLE1* and g*Rt*Δ*9FAD* gene transformants displayed larger lipid droplets (**Figs. 3.4.4-d, e**). The phenomena of Δ9FAD overexpression in oleaginous yeasts thus increasing lipid accumulation have

been observed and possible mechanisms have recently been proposed (Zhu *et al*., 2012; Zhang *et al*., 2016; Sambles *et al*., 2017; Coradetti *et al*., 2018). As previous studies revealed, Δ9FAD has shown a strong relationship with stability of the cellular membrane by contributing the membrane fluidity (Brock *et al*., 2007; Boutet *et al*., 2009; Li *et al*., 2009; Shi *et al*., 2013; Rohwedder *et al*., 2014; Covino *et al*., 2016; Fang *et al*., 2016). In addition, a research on human SCD revealed its role in the conversion of MUFAs toward the safe storage of fatty acids, thus protecting the cell from damage from saturated fatty acids (Li *et al*., 2009). We propose that a probable mechanism is as follows: the enhanced membrane integrity and incorporation capability of a lipid droplet itself by Δ9FAD overexpression might also allow higher lipid accumulation beyond the natural limitation.

3.6.3 Change of fatty acid composition in *R. toruloides* transformants

We analyzed the lipid from the *ScOLE1* and g*Rt*Δ*9FAD* gene transformants of the *R. toruloides* strains to increase the understanding of the fatty acid composition (**Figs. 3.4.5-a, b**). The results of our analyses demonstrated that the *ScOLE1* transformants produced OA-enriched lipid with higher OA ratios than the other groups especially. Since we did not disrupt the *fatty acid desaturase 2* (*FAD2*) gene which is responsible for double bond formation on Δ12 carbon, the linoleic acid (LA; C18:2) amount increased along with the OA reduction during longer cultivation periods, and the high-OA lipid could be obtained at the time point before LA was highly synthesized with the current culture conditions. We are continuing to investigate the function of *RtFAD2* gene and the effects on the host itself.

Interestingly, we also observed that the *ScOLE1* in the *R. toruloides* background seemed to have less effect on POA synthesis, which may indicate an effect of substrate abundance (**Figs. 3.4.3-b and 3.4.5-a**). As previous researches have indicated, the

OLE1 gene expression could be feedback regulated by existence of oleic acid and also meditated by membrane-bound transcriptional regulators (i.e. Spt23, Mga2) (Zhang *et al*., 1999; Martin *et al*., 2007; Covino *et al*., 2016). However, the expression cassette carrying gene of our interest in this work was under the control of exogenous promoter and this fact may exclude the possibility of regulating factors among promoter elements. Studies of *Sc*Ole1p identified the multiple factors that could regulate *OLE1* gene expression, revealing that the regulation of *OLE1* gene or Ole1p may not be easily assumed with a single factor (Martin *et al*., 2007). Accordingly, it may be suggested that the OA production in the g*Rt*Δ*9FAD* transformants is regulated by an endogenous homeostasis system of fatty acid composition control, which has not been characterized well in *R. toruloides*. But this effect may less affected the heterologous *ScOLE1* gene. However, there is no clear evidence of this to date.

3.6.4 The intronic effect observed from *Rt*Δ*9FAD* gene

Our application of the CDS and the genomic sequence of *Rt*Δ*9FAD* gene in *R. toruloides* led to a significant discrepancy in lipid production. Although we have determined the resulting influence on the transcriptional level, the reasons underlying such a difference between each sequence remain unclear (**Fig. 3.4.6**). In eukaryotic systems, intronic factors can dramatically affect the gene expression in various ways. An intron can mediate the gene expression by providing DNA accessibility, enhancing the initiation and activity of RNA polymerase II, or even regulating pre-mRNA processing, splicing, mRNA localization, and downstream RNA metabolism and alter the protein association in the post-splicing stage (Le Hir *et al*., 2003; Rose, 2008). The Δ*9FAD* gene of the industrial strain *M. alpina* was found to have one intron, but its intronic effect has not been described (Sakuradani *et al*., 1999), and such factor has never been studied in *R. toruloides* Δ*9FAD*.

The gene sequence driven effects have been studied on *ScOLE1* gene ScOle1p mRNA transcribe stability from protein coding sequence which provide information how genetic elements may regulate the expression of a *FAD* gene in varied ways. Deletion analysis of elements on transmembrane sequences and cytochrome b5 domain within the protein coding region of Ole1p revealed these elements are essential for stabilizing the transcript (Vemula *et al*., 2003). Moreover, the researches on unsaturated fatty acids mediated mRNA decay showed degrading exosomal activity of exosome degradation to regulate the transcribe mRNA stability (van Hoof *et al*., 2000; Wang *et al*., 2005). Furthermore, these mechanisms were suggested to be related with transcriptional factors Spt23 or Mga2 that thus suggested may not only play a role as transcriptional regulator (Kandasamy *et al*., 2004). These studies have given us the present model of studying on Δ9FAD protein that encoded by *ScOLE1* gene. However, the lack of intron in *ScOLE1* gene has limited providing the applicable information about intronic effect.

With the present *R. toruloides* background, we examined the region-specific mRNA stability by qRT-PCR with the 5'- and 3'-region specific primer pairs, but the results obtained were not clearly different (data not shown). Therefore, the resulting change in the expression level seems not be directly derived from distinct mRNA stability. Due to the lack of similar studies, it is apparent that a more extensive understanding of how genomic and intronic elements are involved in gene expression is necessary for the further genetic manipulation of the *R. toruloides* platform in the future.

CHAPTER 4

General conclusions and discussions

4.1 Current results: sufficient high-OA lipid supply

Owing to the depletion and related environmental influences of fossil oil mining and petroleum industry establishment, renewable lipid sources become an alternative for reducing the consumption of our planet. Oil plants, microalgae, bacteria and oleaginous yeasts provides varied platform to supply green energy and lipid related materials. By determining the fatty acid composition with product property, OA-enriched lipid is therefore currently known to be a preferable material as biodiesel or chemical feedstock.

In chapter 2, to exploit the potential of the oleaginous yeast *R. toruloides*, we have conducted the development of a transformation method. The lithium acetate (Li-Ac) based transformation method could provide a time-saving process with stable gene integration. By treating the intact cell, genome insertion and disruption is now achievable in *R, toruloides*. Our study presented decisive parameters of the whole process such as administering DNA amount, additive using and identifying temperature effects that could be adjusted for method optimization in varied *R. toruloides* strains. This sufficient transformation method gives the way forth to the genetic engineering in *R. toruloides*.

In chapter 3, with the developed method, we have created *R. toruloides* strains with enhanced OA content (>70% of total lipid) by an introduction of Δ*9FAD* encoding genes in *R. toruloides* strains. Moreover, the lipid productivity was improved to obtain a 3-fold increased amount of OA-enrich lipid. On the course of producing OA-enriched lipid with higher amount was thus achieved in *R. toruloides*. I also have observed interesting results of varied product that caused by the using of CDS and genomic

sequence, which has never been reported in the oleaginous yeast Δ*9FAD* genes implied us the unknown mechanism yet to be declared.

In chapter 4, concluding the present study, I established a sufficient method for the genetic engineering in *R. toruloides* and therefore I was able to introduce desired gene for the production of OA-enriched lipid.

4.2 Future prospects

R. toruloides is a hot host in the field of oleaginous yeast study during the current status because of its promising lipid productivity. To exactly regulate the metabolic engineering process in *R. toruloides* platform, the present system of genetic manipulation is yet sufficient enough although several transformation system have been reported. A system could provide stable insertion or disruption simultaneously of multiple genes is still highly required. Moreover, some works tend to decrypt the metabolic network with multi-omics platform and did provide useful information for the future study. While, for the practical uses a metabolic engineering system still needs to be established and understood. However, the lack of examination on those deduced genes remains their exact functions unclear and thus limits the progress. It will be necessary to conduct more identifications of genetic mechanism or each independent gene if we would continue to explore the applicability of *R. toruloides*.

In case of the present study, the downstream regulation of OA is still unclear, the understanding of related genes such as FAD2 is also crucial point to further enhance the OA producing efficiency in the final product. Additionally, the intronic effect might be also a point to be utilized for improved gene overexpression in *R. toruloides*. This efforts would be expected to provide benefits on the development of green energy and renewable materials.

5. MATERIALS AND METHODS

5.1 Microorganisms and medium

Rhodosporidium toruloides DMKU3-TK16 was obtained from the Department of Microbiology, Faculty of Science, Kasetsart University, Thailand (Kraisintu *et al*., 2010). *Rhodosporidium toruloides* ATCC 10657 and NP11 were obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA). The *R. toruloides* strains were grown in YM broth (0.3% yeast extract, 0.3% malt extract, 0.5% peptone and 1% glucose) for cell preparation and on YM agar plate for routine maintenance. *R. toruloides* strains were cultivated in nitrogen-limited medium (0.075% yeast extract, 0.055% $(NH_4)_2SO_4$, 0.04% KH_2PO_4, 0.2% $MgSO_4 \cdot 7H_2O$ and 7% glucose) for lipid production.

Saccharomyces cerevisiae BY4389 (*MATa*, *ole1*Δ*::LEU2*, *ura3-52*, *his4*) strain was obtained from the National Bioresource Project-Yeast (NBRP-Yeast) at Osaka University, Japan. *S. cerevisiae* BY4389 strain was grown in YM broth or on the YM agar plates with a supply of 1% oleic acid (*v/v*) and 0.5% Tween 20 (*v/v*) for routine maintenance. *S. cerevisiae* transformants carrying pYES plasmid were grown in synthetic complete medium lacking uracil (SC-Ura) supplemented with 1% oleic acid and 2% glucose, or medium without oleic acid, 2% raffinose and 0.5% galactose was used for the induction of a target gene in the complementary experiment.

Two kinds of SD agar plate (2% glucose, 6.7 g/L yeast nitrogen base w/o amino acids, 3% agar, 20 mg/L histidine, 120 mg/L leucine, 60 mg/L lysine, 20 mg/L arginine, 20 mg/L tryptophan, 20 mg/L tyrosine, 40 mg/L threonine, 20 mg/L methionine 50 mg/L phenylalanine, 20 mg/L uracil, and 20 mg/L adenine) with 0.2% (*w/v*) 5-fluoroorotic acid (5-FOA) and without uracil (SD–ura) were used for screening of TK16 Δ*ura3* transformants. *Escherichia coli* DH5α was used for all cloning procedures.

5.2 Plasmid construction

The construction of an expression cassette for target gene overexpression was done as described (**Figs. 5.2-a, b, c, d**).

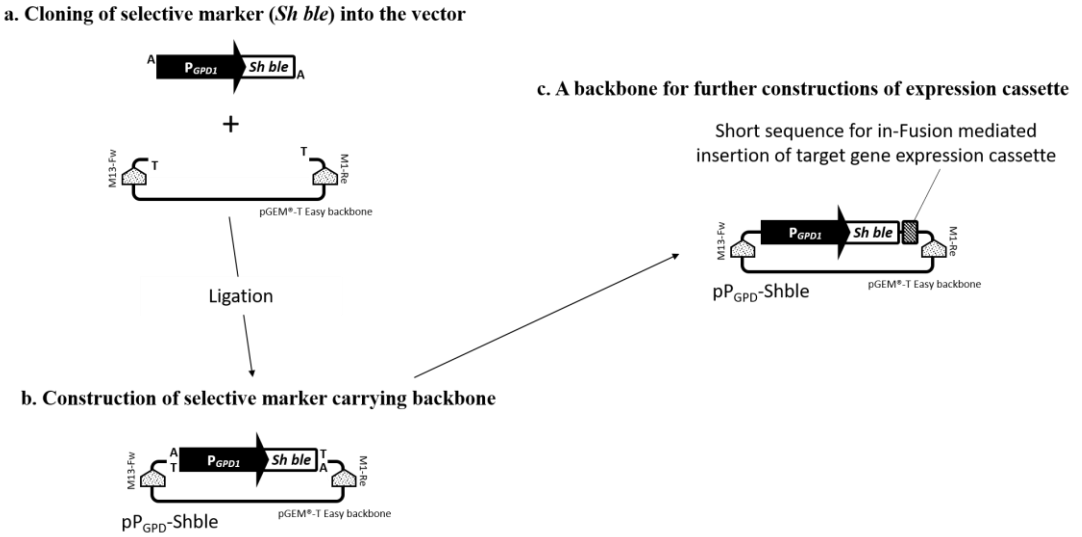


Fig. 5.2-a: The construction of *Streptoalloteichus hindustanus* bleomycin-resistance (*Sh ble*) gene expression cassette in a vector.

The *Sh ble* gene was cloned under the control of *GPD1* promoter, and the cassette was preserved in a pGEM®-T easy vector by TA-cloning. P: promoter.

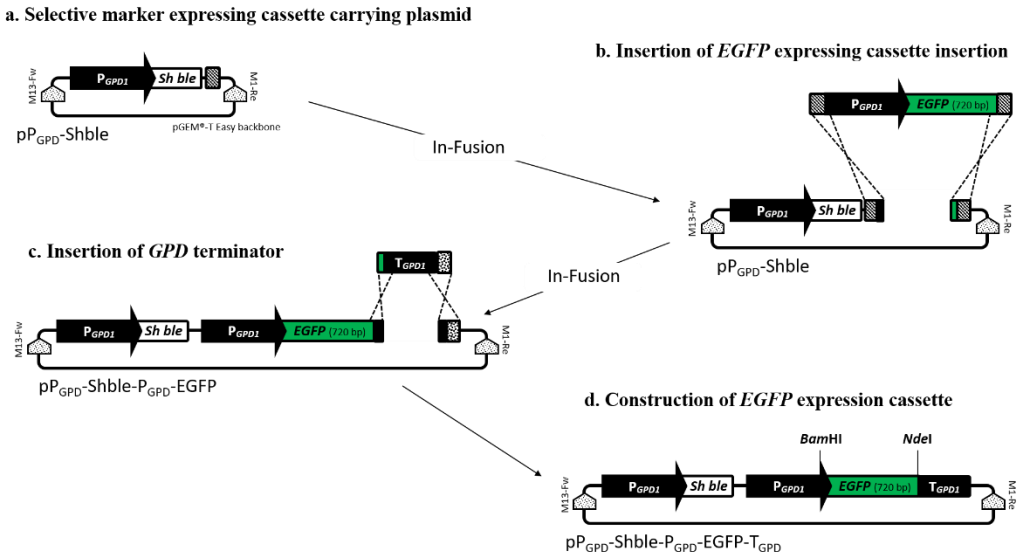


Fig. 5.2-b: The construction of *EGFP* gene expression cassette.

The *EGFP* gene was cloned under the control of *GPD1* promoter, and a *GPD1* terminator was inserted after the gene. P: promoter; T: terminator.

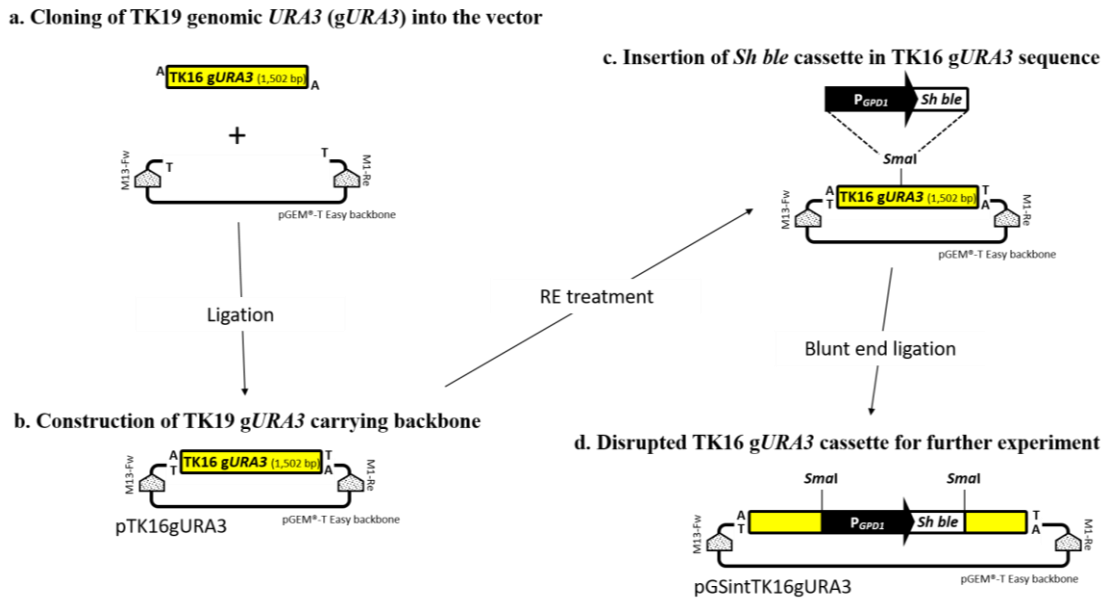


Fig. 5.2-c: The construction of TK16 genomic *URA3* gene disruption cassette.

The TK16 genomic *URA3* gene was cloned into a pGEM®-T easy vector by TA-cloning. A *Sh ble* cassette was inserted into the *Sma*I digested TK16 genomic *URA3* gene carrying plasmid (pGSintTK16Gura3) by blunt end ligation. P: promoter.

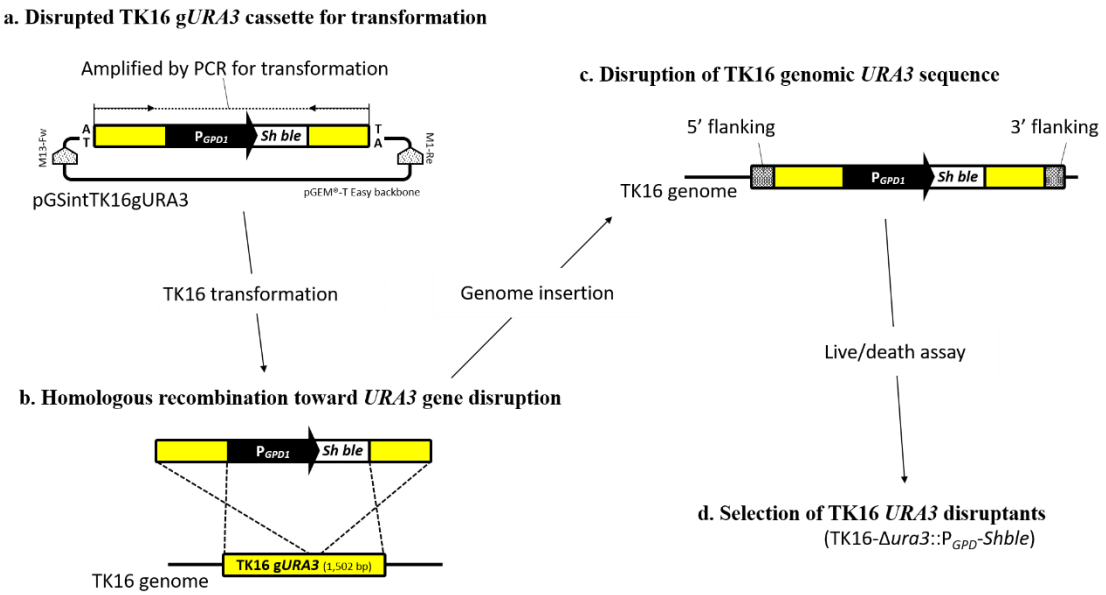


Fig. 5.2-d: The construction of TK16 genomic *URA3* gene disruption cassette toward the disruption of TK16 endogenous *URA3* gene.

The disrupted TK16 genomic *URA3* gene was used for transformation toward obtaining TK16 *URA3* diruptants (Δ*ura3*). P: promoter.

The fragment of *ScOLE1* gene for constructing the *R. toruloides* expression cassette was amplified by PCR using KOD-Plus-Neo DNA polymerase (Toyobo, Osaka, Japan) with the primers ScOLE1-BamHI-F and ScOLE1-NdeI-R, and the CDS and genomic sequence of *RtΔ9FAD* gene from *R. toruloides* NP11 were also amplified with the primer pair NP11OLE1-BamHI-F and NP11OLE1-NdeI-R for cassette construction (**Table 2**).

The prepared fragments were then used for plasmid construction to obtain the expression cassette and resulted in plasmids that we named pP_{GPD}-Shble-P_{GPD}-ScOLE1-T_{GPD}, pP_{GPD}-Shble-P_{GPD}-RtΔ9FAD-T_{GPD} and pP_{GPD}-Shble-P_{GPD}-RtgΔ9FAD-T_{GPD} individually (**Fig. 5.2-e**). Likewise, for the complementary experiment in *S. cerevisiae* BY4389, *ScOLE1* gene, CDS and the genomic sequence of *RtΔ9FAD* gene were amplified with the primer pairs ScOLE1-BamHI-F and ScOLE1-XbaI-R, and NP11OLE1-BamHI-F and NP11OLE1-XbaI-R respectively (**Table 2**) by PCR using KOD-Plus-Neo DNA polymerase. They were then cloned into pYES2 vector under the control of *GAL1* promoter to obtain plasmids that we named pYES2-ScOLE1, pYES2-RtΔ9FAD and pYES2-RtgΔ9FAD individually (**Fig. 5.2-f**).

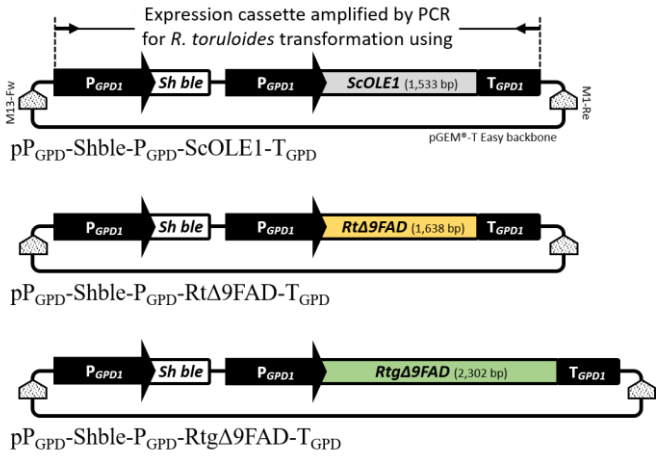


Fig. 5.2-e: Constructions of Δ*9FAD* gene expression cassette.

Constructs of the expression cassette for *R. toruloides* transformation. P: promoter. T: terminator. M13-Fw, Re: M13 primer site on the vector.

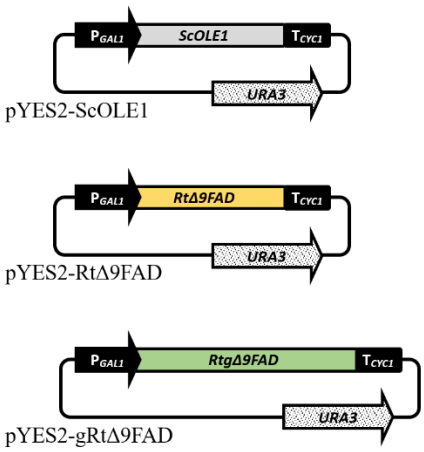


Fig. 5.2-f: Constructions of Δ*9FAD* gene expression cassette.
Plasmids for the *S. cerevisiae* strain transformation of the complementary test. P: promoter. T: terminator. M13-Fw, Re: M13 primer site on the vector.

5.3 Preparation of carrier DNA

Carrier DNA was prepared as described previously (Schiestl and Gietz, 1989). Salmon sperm DNA (Sigma-Aldrich, St. Louis, MO) was immersed in TE buffer containing 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA at a concentration of 10 μg/μL and incubated on ice overnight. After overnight immersion the salmon sperm DNA was dissolved as a transparent mixture with high viscosity. The dissolved salmon sperm DNA was sonicated by a probe sonicator (UR-20P; TOMY, Tokyo) at power level 7 for 10 min or 20 min for the larger or smaller size preparation, respectively. The resulting DNA had average sizes around 10 kb or 7 kb respectively as determined by ethidium bromide-staining agarose gel electrophoresis.

5.4 Transformation of yeast

The transformation of *R. toruloides* stains was conducted and modified by a lithium acetate-based method that has been developed and applied in *R. toruloides* strains or *Rhodosporidiobolus fluvialis* (Polburee *et al*., 2018). Briefly, yeast cells were

precultured for 16 h at 28°C in 5 mL of YM broth, and then inoculated into 25 mL of YM broth and cultivated at 28°C with agitation for 16 h. The grown yeast culture (OD_{600nm} = 3.0) was harvested and resuspended in 100 μL of transformation mixture (35% PEG-4000, 100 mM lithium acetate, 10 mM Tris-HCl at pH 4.9, and 1 mM EDTA) with a linear DNA fragment and 10% DMSO (*v/v*). The transformation mixture was incubated without agitation at 28°C for 3 h and treated by heat shock, then recovered by the addition of YM broth. Finally, the cells were harvested and spread onto a YM agar plate containing 150 μg/mL of Zeocin (Invitrogen, Massachusetts, USA) and incubated at 28°C to obtain colonies (**Figs. 2.2.2-b, c**).

The *S. cerevisiae* BY4389 was transformed by a lithium acetate-based method (Ito *et al*., 1983; Kawai *et al*., 2010). All resulting transformants were named as shown in **Table 3**. The foreign gene insertion was verified by PCR with the colony and extracted genome. The primer pair used for confirming insertion of *ScOLE1* gene was ScOLE1-BamHI-F and ScOLE1-NdeI-R, and the inserted *Rt*NP11 Δ*9FAD* gene was amplified by NP11OLE1-BamHI-F and NP11OLE1-NdeI-R (**Table 2**).

5.5 Stability of transformants

To assess the stability of the foreign genes inserted in the yeast genome, the transformants were cultured on a YM agar plate without Zeocin for ten generations. The transformants were then transferred onto a YM agar plate containing 150 μg/mL Zeocin for selection. Finally, the existence of the foreign genes was verified by colony PCR and Southern blot.

5.6 Yeast colony PCR

To confirm the gene (*Sh ble*, *EGFP* expression cassette) introduction and assess the stability of gene (*Sh ble* cassette) integration in *R. toruloides* DMKU3-TK16 genome,

transformants were cultured on YM agar plate without Zeocin for ten generations. Transformants were then transferred onto solid YM medium plate containing 150 μg/mL Zeocin for transformants growing. Finally, the existence of the foreign gene insertion was verified by colony PCR using KOD FX Neo DNA polymerase (Toyobo).

5.7 Genomic DNA preparation for gene cloning

First, inoculate *R. toruloides* ATCC10657 colony in test tube with 5 mL YM broth medium then cultivate with shaking at 28°C for saturated culture. Collected cells were carried out genomic DNA extraction as manual described in kit Dr. GenTLE® (from Yeast) High Recovery (TAKARA, Shiga, Japan). Extracted *R. toruloides* ATCC 10657 genomic DNA was used as template for *GPD1* promoter cloning by PCR using KOD-Plus-Neo DNA polymerase (Toyobo).

5.8 Genomic DNA preparation for Southern blot analysis

Wild-type TK16 and TK16 *Sh ble* transformants which had been selected by colony PCR as *Sh ble* gene containing colonies were inoculated in 5 mL YM medium and scaled up in 20 mL YM medium by 24 h cultivation to reach saturated culture. TK16 transformant culture was then collected and pelleted by centrifugation at 4,000 g for 5 min at 4°C. After centrifugation, supernatant was discarded and the pelleted cell was washed by suspension in 1 mL PBS, then collected cells by centrifugation at 4,000 g for 5 min at 4°C. Again, after washing and collecting cells, discarded supernatant then resuspended cells in 1 mL sorbitol solution (1 M sorbitol, 100 mM sodium citrate, 10 mM EDTA and 0.4 mg/mL Zymolase at pH = 5.8) and incubated the cells at 37°C for 1 h with occasionally inverting. After preparation of TK16 spheroplast, 400 μL lysis solution (10 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, 2% Triton X-100 and 1% SDS

at pH = 8) was added and cell was heated by 70˚C for 30 min then chilled on ice immediately, followed by adding 400 μL phenol/chloroform mixture and vortex for 30 sec. Afterwards, centrifuged the mixture with 15,000 rpm for 10 min at 4˚C then transferred the upper phase of mixture into new 1.5 mL microfuge tube with equal volume of isopropanol. Pelleted DNA was washed by 70% ethanol and dissolved in TE buffer with suitable volume. RNase B (10 μg/mL) was added to digest extracted RNA then repeated phenol/chloroform extraction to prevent RNA contamination during Southern blot analysis.

5.9 Southern blot

Ten micrograms of extracted genomic DNA from *Sh ble* transformants of TK16 were digested by *Bam*HI–*Hind*III, and electrophoresed on 0.8 % agarose gel. DNA was then transferred and fixed onto positively charged nylon membrane (Roche, Basel, Switzerland). Blot was hybridized with DIG labeled-fragment of *Sh ble* CDS as probe, developed by chemiluminescence (DIG High Prime DNA Labeling and Detection Starter Kit II; Roche), exposed to RX-U Medical X-ray film (Fujifilm, Tokyo), and visualized with an FPM100 medical processor (Fujifilm).

5.10 Immuno blot

Transformants carrying the EGFP expression cassette was cultivated at 28°C for 16 h in YM broth containing 150 μg/mL of Zeocin. The grown cells were harvested, resuspended in 200 μL of alkaline extraction buffer containing 0.1 M NaOH, 50 mM EDTA, 2% SDS and 2-mercaptoethanol, boiled for 5 min, and neutralized by addition of 5 μL of 4 M acetic acid. After addition of BPB, the crude protein extracts were separated by 12% SDS-PAGE and electro-blotted onto a polyvinylidene difluoride membrane (Millipore, Burlington, Massachusetts). Immuno detection analysis was

conducted using rabbit anti-GFP IgG antiserum (MBP) as the primary antibody and horseradish peroxidase-conjugated donkey anti-rabbit whole antibody (GE Healthcare, Chicago, Illinois) as the secondary antibody. EGFP-specific signals were developed by enhanced chemiluminescence (Immobilin Western; Millipore), exposed to RX-U Medical X-ray film (Fujifilm), and visualized with an FPM100 medical processor (Fujifilm).

5.11 Lipid staining

Sudan IV (Wako, Osaka, Japan) stock solution was prepared at the concentration of 2 mg/mL in isopropanol and stored protected from light for later experimental use. Transformants were precultured in 5 mL of YM broth, and the preculture was further cultured in 25 mL of YM broth for 2 days. The grown cells were harvested and then resuspended in 25 mL of nitrogen-limited broth. After a 4-day culture, 1 mL of cells was collected from the nitrogen-limited medium, washed with 1X phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4), and resuspended in 200 μL of Sudan IV staining mixture (Sudan IV stock solution : 1X PBS : DMSO = 10 : 9 : 1).

The samples were incubated in the dark at room temperature for 30 min, and the cells collected by centrifugation were then washed twice with 1X PBS. The washed cell pellet was resuspended in 100 μL of 10% formaldehyde aqueous solution for a 30-min sample fixation. The fixed cells were collected and washed with 1X PBS again and then resuspended in 200 μL of 1X PBS buffer. Four microliters of the cells were then placed on a glass slide for further microscopy observation. Microscopy images were obtained with an Axioskop 2 microscope (Zeiss, Oberkochen, Germany).

5.12 Lipid sample preparation and analysis by gas chromatography

Transformants were precultured in 5 mL of YM broth, and the preculture was cultivated in 25 mL of YM broth with shaking at 140 rpm for 2 days. The grown cells were harvested and resuspended in 25 mL of nitrogen-limited broth and cultured for ≥4 days. Grown yeast cells were then harvested, and total cellular lipids were extracted and used for the preparation of fatty acid methyl esters (FAMEs) by transmethylation by described modified methods (Bligh and Dyer, 1959). The resulted FAMEs samples were analyzed by gas chromatograph (GC 353B, GL Sciences, Tokyo) equipped with a TC-70 capillary column (0.25 mm ID × 60 m, film thickness 0.25 μm; GL Sciences) and a flame ionization detector (FID) method. We used a commercially available FAMEs mix standard (Supelco®, F.A.M.E. Mix C8-C24, CRM18918, Sigma-Aldrich, St. Louis, MO) and methyl heptadecanoate (C17; H0566, TCI, Tokyo) as the internal standard for the further FAME identification and quantification.

For the GC analysis, 1 μL of hexane recovered sample was applied for the injection. Helium was used as the carrier gas with the constant pressure at 20 kPa. The column temperature for analyzing was set to start at 120ºC (2 min) then increased by 20ºC/min up to 160ºC (2 min), 6ºC/min up to 190ºC (1 min), and by 20ºC/min until the temperature reached the final stage at 220ºC (2 min). The temperature of the injector and the detector was 250ºC. The quantitative analysis of the results was performed using the ImageJ program to determine the detected response signal, and all presented values are the means of three independent quantifications (Breuer *et al*., 2013).

5.13 Green fluorescence observation

EGFP-expressing transformants were grown for 24 h in YM broth containing 150 μg/mL Zeocin, and fluorescence images were observed with a Leica TCS SP5 confocal microscope (Zeiss).

5. 14 Real-time PCR analysis

The RNA of the target transformants were extracted by the phenol/chloroform method (Schmitt *et al*., 1990; Collart and Oliviero, 2001) and applied for cDNA synthesis by a commercial kit (PrimeScript™ RT reagent Kit Perfect Real Time, TAKARA). The real-time PCR was performed by a SYBR® Green-based system (THUNDERBIRD® SYBR® qPCR Mix, Toyobo) with the comparative C_T ($\Delta\Delta C_T$) method for the relative expression analysis of *Rt*Δ*9FAD* gene. *URA3* gene was used as an endogenous control to normalize and the wild-type group was set as the reference for the NP11 transformants.

In the *R. toruloides* strain TK16, the *URA3* gene was used as an endogenous control and the *Rt*Δ*9FAD* group was set as the reference for the analysis of the expression level in the transformants. The NP11 *URA3* gene (XM_016415986) was amplified with the primer pair RtNP11-URA3qPCR-F and RtNP11-URA3qPCR-R as the endogenous control, and the TK16 *URA3* was amplified with the primer pair RtTK16-URA3qPCR-F and RtTK16-URA3qPCR-R for the same purpose. The expression of target *Rt*Δ*9FAD* gene was defined with the primer pair RtNP11-OLE1qPCR-F and RtNP11-OLE1qPCR-R in all target samples (**Table 2**).

Primer name	Sequence (5'-3')	Feature
ScOLE1-BamHI-F	GTT TTG GAT CCA TGC CAA CTT CTG GAA CTA C	Plasmid construction
ScOLE1-NdeI-R	GTT TTC ATA TGT TAA AAG AAC TTA CCA GTT TCG	Plasmid construction
ScOLE1-XbaI-R	GTT TTT CTA GAT TAA AAG AAC TTA CCA GTT TCG	Plasmid construction
NP11OLE1-BamHI-F	GGA TCC ATG ACT GCC TCT TCG GCA C	Plasmid construction
NP11OLE1-NdeI-R	CAT ATG TTA CGC CTT GAC CTT CAG	Plasmid construction
NP11OLE1-XbaI-R	GTT TTC TAG ATT ACG CCT TGA CCT TCA G	Plasmid construction
RtNP11-URA3qPCR-F	ACC AAC CTC TGC GTT TCA GTC	Real-time PCR
RtNP11-URA3qPCR-R	CCT CCC AAA TCA GAA AAT CG	Real-time PCR
RtNP11-OLE1qPCR-F	TCA CCC CGA CTA CAC TCA GA	Real-time PCR
RtNP11-OLE1qPCR-R	GGT GGA TGT TCT TCC AGG TG	Real-time PCR
RtTK16-URA3qPCR-F	ACG CAA TAA TGC TTG TGC AG	Real-time PCR
RtTK16-URA3qPCR-R	AGC GAT CTC TCT CCC TCT CC	Real-time PCR

Table 2 Primers used in the present study.

Strain	Genotype/feature	Source
BY4389	*Saccharomyces cerevisiae* strain NBRP ID BY4389 (*MATa*, *ole1*Δ*::LEU2*, *ura3-52*, *his4*)	NBRP-Yeast collection
BY4389+Vector	BY4389/ pYES2 empty vector	This study
BY4389+*ScOLE1*	BY4389/ pYES2-ScOLE1 plasmid	This study
BY4389+*Rt*Δ*9FAD*	BY4389/ pYES2-RtΔ9FAD plasmid	This study
BY4389+g*Rt*Δ*9FAD*	BY4389/ pYES2-RtgΔ9FAD plasmid	This study
TK16	*Rhodosporidium toruloides* strain DMKU3-TK16	(44)
TK16+*ScOLE1*	TK16/ P_{GPD}-*Shble*-P_{GPD}-*ScOLE1*-T_{GPD}	This study
TK16+*Rt*Δ*9FAD*	TK16/ P_{GPD}-*Shble*-P_{GPD}-*CDS *Rt*Δ*9FAD*-T_{GPD}	This study
TK16+g*Rt*Δ*9FAD*	TK16/ P_{GPD}-*Shble*-P_{GPD}-genomic *Rt*Δ*9FAD*-T_{GPD}	This study
NP11	*Rhodosporidium toruloides* strain NP11	ATCC collection
NP11+*ScOLE1*	NP11/ P_{GPD}-*Shble*-P_{GPD}-*ScOLE1*-T_{GPD}	This study
NP11+*Rt*Δ*9FAD*	NP11/ P_{GPD}-*Shble*-P_{GPD}-*CDS *Rt*Δ*9FAD* -T_{GPD}	This study
NP11+g*Rt*Δ*9FAD*	NP11/ P_{GPD}-*Shble*-P_{GPD}-genomic *Rt*Δ*9FAD*-T_{GPD}	This study

*CDS: coding sequence

Table 3 Strains used in the present study.

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7. RESEARCH ACHIEVEMENTS

7.1 Publications:

Present study

1. **Tsai, YY., Ohashi, T., Kanazawa, T., Polburee, P., Misaki, R., Limtong, S., Fujiyama, K.:** Development of a sufficient and effective procedure for transformation of an oleaginous yeast, *Rhodosporidium toruloides* DMKU3-TK16. Curr. Genet., **63**, 359-371 (2017).
2. **Tsai, YY., Ohashi, T., Wu, CC., Bataa, D., Misaki, R., Limtong, S., Fujiyama, K.:** Delta-9 fatty acid desaturase overexpression enhanced oleic acid content in *Rhodosporidium toruloides* for preferable yeast lipid production. J. Biosci. Bioeng., doi: 10.1016/j.jbiosc.2018.09.005. (2018)

Related studies

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Now this is not the end.

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But it is, perhaps, the end of the beginning. (Winston Churchill)

Tsai, 2018 Sep.