Preliminary Investigation: Stearidonic Acid Production by Genetically Modified *Saccharomyces cerreviseae* Using Linseed Oil as A Fatty Acid Source

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ABSTRACT

Stearidonic acid (SDA); 18:4(n-3), an ω-3 Polyunsaturated Fatty Acid (PUFA), could be produced by Genetically Modified *Saccharomyces cerreviseae* (GMY) using Linseed Oil (LO) as fatty acid source. In nature, *S. cerrevisae* accumulates only very small amount lipid and contains until mono unsaturated fatty acids. But this GMY strain beside has capability to accumulate lipid, it also contains Δ6 desaturase gene which leads to convert α-Linolenic Acid (ALA); 18:3(n-3) to be SDA. Gas Chromatograph analysis of transmethylated LO sample showed that main component of fatty acid was ALA (about 57%), therefore LO can be used as a cheap source for other PUFA production. In order to provide ALA as a source for the yeast, enzymatic hydrolysis of LO using lipase was done. The lipase from *Rhizomucor miehei* (L4277-SIGMA) showed the highest activity among 5 lipases when 1 g/L LO in the medium for yeast (containing 6.7g/L yeast nitrogen base, 20 g/L glucose and 2.5g/L tergitol NP-40) was hydrolyzed. For SDA production, 1g/L LO in medium was aseptically hydrolyzed by lipase in 50 unit/mL for 18 hours at 30 ^{o}C and 140 rpm. The pre-culture of GMY (1% V/V) was then inoculated into the treated medium and 2.59 g/L dried cells were obtained after 5 days cultivation. ALA was accumulated in the cells at 0.06g/L (11% of total ALA in LO), and only 25% (0.015g/L) of accumulated ALA were converted to SDA. These results suggested that LO can be used as a source for PUFA production. In order to improve the productivity of SDA using GMY, hydrolysis of LO as well as cultivation condition and genetically improvement of the yeast must be highly considerated.

Keywords: PUFA, Stearidonic acid, Linseed oil, *Saccharomyces cerreviseae*

INTRODUCTION

Long chain polyunsaturated fatty acids, PUFAs of the omega-3 series, such as EPA (eicosapentaenoic acid) and DHA (docosahexaenoic acid) have gained much attention due to their advantages for the use in nutraceuticals, pharmaceuticals, treating such conditions as hypertension and diabetes (Kogteva *et al.* 1998, Kato *et al.* 1997, Less & Karel 1990). PUFAs are extracted mostly from fish oil (Stredansky *et al.* 2000, Kumon *et al.* 2005) that originally produced by single cell microorganisms mainly by marine microorganisms, e.g. several algae (Cohen 1990, Shiran *et al.* 1996, Stredanska *et al.* 1993), marine bacteria (Ringo *et al.* 1992), and some fungi such as *Phytium, Phytophtora* and the Zygomycetes genus *Mortierella* (Gandhi & Weete 1991, Shimizu *et al.* 1989, Stredanska *et al.* 1993, Stinson *et al.* 1991).

PUFAs are fatty acids of 18 carbons or more in length with two or more methylene-interrupted double bonds in the cis position. PUFAs can be grouped into two main families, ω6 (or n-6) and ω3 (or n-3) families, depending on the position of the first double bond proximate to the methyl end of fatty acids (Huang *et al.* 2004).

The ω6- and ω3-PUFAs are derived from linoleic acid (LA, C18:2n-6) and α-linolenic acid (ALA, C18:3n-3) respectively. Humans are incapable of synthesizing these two fatty acids due to lack of the Δ12 and Δ15-desaturases (Huang *et al.* 2004). However, humans can metabolize these two fatty acids obtained from the diet to form longer and more unsaturated PUFAs through a series of desaturation and elongation steps (Figure 1) (Kumon *et al.* 2005). In mammals, PUFAs are important structural components that modulate membrane fluidity and permeability (Uauy *et al.* 1996). For example, docosahexaenoic acid (DHA, C22:6n-3), a long-chain n-3 PUFA, and arachidonic acid (ARA, C20:4n-6), a long-chain n-6 PUFA, are found in high proportions in neuronal tissues such as brain and retina, and testis (Bourre *et al.* 1992; Retterstol *et al.* 2000). PUFAs also serve as precursors for a number of biologically active molecules, such as eicosanoids, growth regulators and hormones (Jump 2002). Thus, PUFAs have profound effects on human health (Simopoulos 2002).

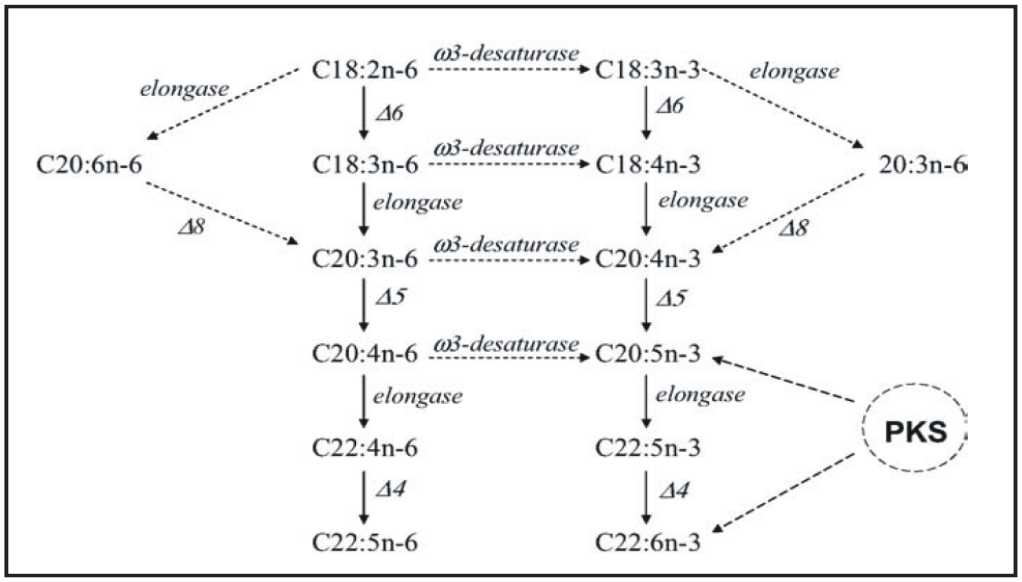

Figure 1. Long chain PUFAs biosynthesis pathway, adapted from Huang *et al*. 2004.

Recently, the productions of fish oils fluctuate year by year and limited resources are now under the over-fishing (Sargent & Tacon 1999). Furthermore, the cost of production of the above mentioned commercial oils is very high. For example, the increasing cost involved in processing, refining, and stabilizing the fish oils, and the decreased yields have continuously driven up the cost of fish oils (Sargent & Tacon 1999). The recent finding of toxic chemicals and heavy metals (Ratledge 2004) in fish oil has further raised the safety concern on consumption of fish oil (Guallar *et al.* 2002). To anticipate and resolve this problem, other resources must be explored and investigated for the constant and cheap production of PUFAs. One option is to modify the vegetable oils to produce PUFAs using microorganism.

Biological material production using *S. cerevisiae* has been well studied so far. However, this yeast can originally accumulate only less lipid and contain only up to mono-unsaturated fatty acids. Recently, genetically modified *S. cerevisiae* which can accumulate much lipids has been developed in this group. This GMY can accumulate free fatty acids from the broth into the cell. Moreover, this group has already developed the genetically modified *S. cerevisiae* which contains enzymes for the biosynthesis of higher PUFAs from ALA. For example, the development of GMY strain, beside has capability to accumulate the lipid, also contains Δ6 desaturase gene which leads to convert α-Linolenic Acid (ALA) 18:3(n-3) to stearidonic acid (18:4(n-3)) (SDA). Thus, this study is a trial for the use of natural lipid (vegetable oil) as a source of fatty acid to produce PUFAs and is set to the target on SDA production by GMY. Natural lipid source that selected for this purpose is linseed oil. General idea of this research can be seen in Figure 2. This research can adopted as one way for an improvement natural oil quality.

METHODS

Lipid extraction and transmethylation

Sample solution 1mL was extracted with mixture containing chloroform:methanol (2mL:2mL) and 1 mL Na-Phosphate buffer 10mM pH 7.2, then shake well for 5 minutes. The chloroform phase at bottom layer which contained lipid was collected and evaporated by exposure to nitrogen. The extracted lipid was mixed with 1mL of 10% methanolic HCl and 0.5 mL dichloromethane containing heptadecanoic acid (Sigma, St. Louis, MO, USA) as an internal standard. The mixture was transmethylated at 60^{o}C for 3 hours. After cooling down in tap water, 2 mL saturated NaCl and 1 mL hexane were added, and then followed by shaking and centrifugation at 2000rpm for 2 minutes.

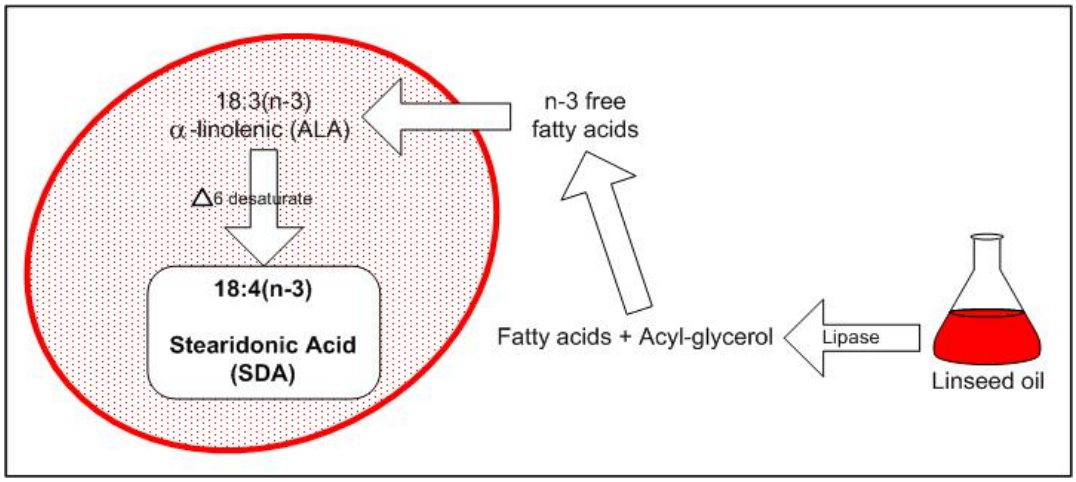

Figure 2. Living cell of the GMY containing Δ6 desaturase gene was expected can accumulate fatty acids ALA from LO hydrolysis and converted to SDA.

The resultant transmethylated samples were then applied to a Gas Chromatograph (GC-2010; Shimadzu, Kyoto, Japan), equipped with an auto-injector AO20i and a TC-70 capillary column (GL Science, Tokyo, Japan) under temperature programming 180-220 °C, at 4 °C/min increments.

Medium for cultivation

SD medium (6.7 g/L Yeast Nitrogen base without amino acids (Difco) and Glucose 20g) was used with 2.5g/L tergitol NP40. For GMY cultivation, histidin 2g/L and methionin 2g/L were added.

Lipase selection

In this research lipases from porcine pancreas (L3126-SIGMA), *Mucor miehei* (L9031-SIGMA), *Rhizomucor miehei* (L4277-SIGMA), Porcine Pancreas (20552-02 Nacalai) and Lipase D-Amano (Amano) were bought for the selection. The enzyme selection was based on the capability of lipases to hydrolyzed LO in medium for the GMY growth. The hydrolysis was conducted in a series of time incubation at 30^{o}C and shaking at 140rpm using rotary shaker. After hydrolysis, the free fatty acids released were determined by Non-Esterified Fatty Acids (NEFA)-C kit (Wako) and oleic acid was used as standard.

The GMY strain and its maintenance

The GMY strain was developed and provided by Lipid Engineering Research Group, Institute for Biological Resources and Functions, National National Institute of Advanced Industrial Science and Technology (AIST), Japan. In order to maintained GMY strain, 10mL slant agar with SD medium containing histidin 2g/L and methionin 2g/L was inoculated with GMY and grown at 30 ^{o}C. After 2 days cultivation, the strain was stored at 4 ^{o}C. This maintenance was done once a month by transferring to a new slant agar.

Pre-culture the GMY strain

Pre-culture was conducted by inoculating one loop of GMY into 5 mL SD medium in test tube containing histidin 2g/L and methionin 2g/L. Pre-culture was conducted at 30 ^{o}C and 140 rpm rotary shaking for 2 days.

Lipase sterilization and storing

Sterile lipase solution was prepared by filtration using membrane filter 0.45micron Low Protein Biding Durapore (PVDF) SLHV 013-Millipore. Sterilized lipase solution was stored at -4 ^{o}C.

Analysis of SDA production

For the SDA production, 5 mL medium containing 1 g/L LO was hydrolyzed with 50 unit/mL lipase for overnight. After hydrolysis, pre-culture of GMY was inoculated by (1% V/V) followed by cultivation at 30^{o}C and 140 rpm rotary shaking. For this experiment, the medium without lipase (but with LO 1g/L) and the medium (without lipase and LO) were used as control. After 5 days cultivation, the cells from 4mL culture were collected from the broth by centrifugation at 3000 rpm for 10 minutes. The cells were dried for 3 hours at 105 ^{o}C and weighed for determination of growth. The dried cells were directly transmethylation and the fatty acids content were analyzed using GC. For analysis of remaining lipid (as free fatty acids and acylglycerols) in the broth, the liquid / liquid extraction using methanol and hexane was employed. Lipids extracted from 1 mL of broth was evaporated and suspended with 0.5mL borate buffer 50mM (pH 10.5). The mixture was then mixed with 1.5mL methanol and 3mL hexane. The mixture was shaken well and centrifuged at 3000 rpm for 5 min. Hexane phase which contained acylglycerol was withdrawn from methanol phase using

micropipette. After separation, hexane and methanol phases were evaporated using nitrogen. The evaporated samples were transmethylated and applied to GC with the same condition as described previously. Furthermore hydrolyzation of LO (%), accumulated lipid (g/L), cells dry weight (g/L), lipid content, SDA content in the cell, conversion ratio (%) and accumulation ratio (%) were calculated.

RESULTS AND DISCUSSION

Linseed oil

Analysis using gas chromatograph (Figure 3) showed that the main component of linseed oil (LO) was α-linolenic acid (ALA) 57% while the other components were oleic acid (20%), linoleic acid (14%) and less than 10% were palmitic acid and stearic acid respectively (Table 1).

According to the composition of LO, it seems LO can be a good candidate source for others PUFA production. However, since ALA exists as a form of triglycerides in LO, hence this matter would not be utilized or converted to other PUFAs by the GMY directly. To release the ALA from LO as free fatty acids, hydrolysis can be employed.

Table 1. Fatty Acids composition of LO

Fatty acid	Composition (%)
Palmitic acid (16:0)	6
Stearic acid (18:0)	3
Oleic acid (18:1)	20
Linoleic acid (18:2)	14
α-Linolenic acid (18:3(n-3))	57

In this research, hydrolysis was conducted enzymatically using lipase under the culture condition of genetically modified *S. cerrevisae* (GMY) directly. It was expected that during GMY cultivation using medium containing ALA, the GMY could utilize and accumulate ALA to their cells. This condition has possibility that GMY cells convert the accumulated ALA to other PUFAs.

Selection of lipase

For the hydrolysis of LO, SD medium containing 1g/L LO, 2.5g/L tergitol NP40 and lipase with the total volume 5mL was used. All was conducted at sterile condition.

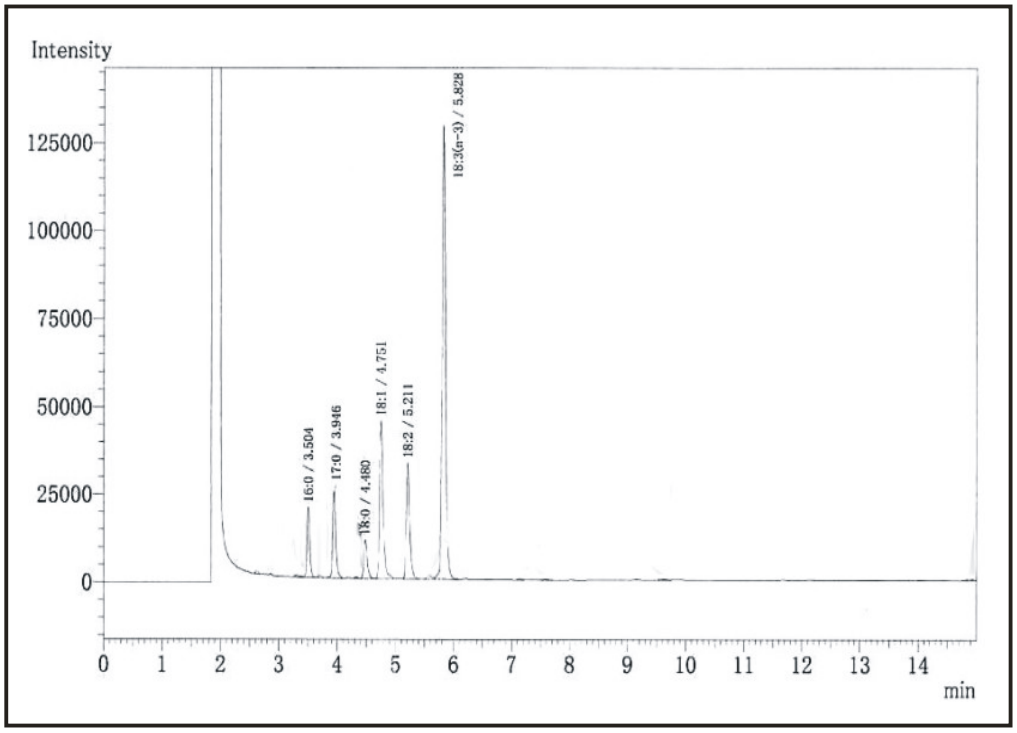

Figure 3. Profile of gas chromatograph analysis of LO. Heptadecanoic acid (17:0) was used as an internal standard.

Table 2 shows the comparison of hydrolysis of LO with various lipases by the determination of free fatty acids released from the hydrolysis using NEFA-C kit. At 23 hours hydrolysis, lipase from *R. miehei* at concentration 10 unit/mL in medium, released 0.229 g/L of free fatty acids, 2 - 4 times much bigger than other lipases with 50 unit/mL. Other than *R. miehei* lipase showed nearly no release of free fatty acids after 23 hours incubation with 10 unit/mL lipases (data not shown). At the same time, when the lipase from *R. miehei* was added at 50 unit/mL in medium, this enzyme released 0.412 g/L free fatty acids, 4 to 8 times fold more than other lipases. Base on this capability to hydrolyze LO, the lipase from *R. miehei* was selected. GC analysis of produced free fatty acids showed that the highest of free fatty acids was ALA with the percentage 45 - 50% (data not shown). This occurrence also proved that hydrolysis LO using the lipase from *R. miehei* can produce an ALA-rich mixture of free fatty acids. Thus, according to this situation, LO hydrolysis by *R. miehei* has a possibility to be an ALA source for GMY to produce other n-3 PUFAs.

Hydrolysis of LO by *R. miehei* lipase

In order to see the capability of lipase *R. miehei*, LO hydrolysis was tested in various concentration of lipase in medium containing 1 g/L LO. The incubation was done under the same temperature and shaking, and was sampled in a series period of time (Figure 4). Then, free fatty acid extracted from 1 mL sample was fractioned from acylglycerols by liquid/liquid extraction using hexane and methanol. The free fatty acids (in the methanol phase) was transmethylated then injected to GC. Analysis showed that for the medium with lipase concentration 200 and 250 unit/mL produced free fatty acids of 0.42 and 0.38 g/L after 18 hours. However, the medium with lipase concentration 25, 50 and 100 unit/mL exhibited the maximum free fatty acids productions after 31 hours incubation with free fatty acids in a range of 0.23 - 0.37g/L.

SDA production using LO

For the SDA production, 1g/L LO in medium containing 2.5g/L tergitol NP40 was aseptically hydrolyzed by the lipase in 50 unit/mL within 18 hours (~1day) at 30^{o}C in the 140 rpm shaker. Fully grown (2d) pre-culture of GMY was then inoculated (1% V/V) into the treated medium followed by 5 days cultivation 30^{o}C in the 140 rpm rotary shaker.

According the data from Table 3, SDA was produced by GMY in the medium with LO and lipase. However, the cultivation by using the medium without lipase or without LO and lipase did not produce SDA. It was shown GMY accumulated ALA from LO and some of ALA were converted to SDA.

Table 2. Comparison of hydrolysis of LO with various lipases

Lipase	Unit/mL applied in medium[1)]	Free fatty acid produced (g/L) in a series time (h) hydrolysis			
		0	4	23	47
Porcine pancreas (L3126-SIGMA)	50	0.019	0.031	0.065	NA
Mucor miehei (L9031-SIGMA)	50	0.019	0.027	0.107	NA
Porcine Pancreas (20552-02 Nacalai)	50	0.019	0.026	0.076	0.072
D "Amano" (Amano)	50	0.019	0.063	0.100	NA[2)]
Rhizomucor miehei (L4277-SIGMA)	10	0.019	0.050	0.229	0.278
Rhizomucor miehei (L4277-SIGMA)	50	0.019	0.081	0.412	0.467
Without enzyme	-	0.019	0.027	0.049	0.048

Notes:
[1)] 5 mL SD medium contain 1 g/L Linseed oil and 2.5 g/L tergitol-NP40
[2)] NA : Not analyzed

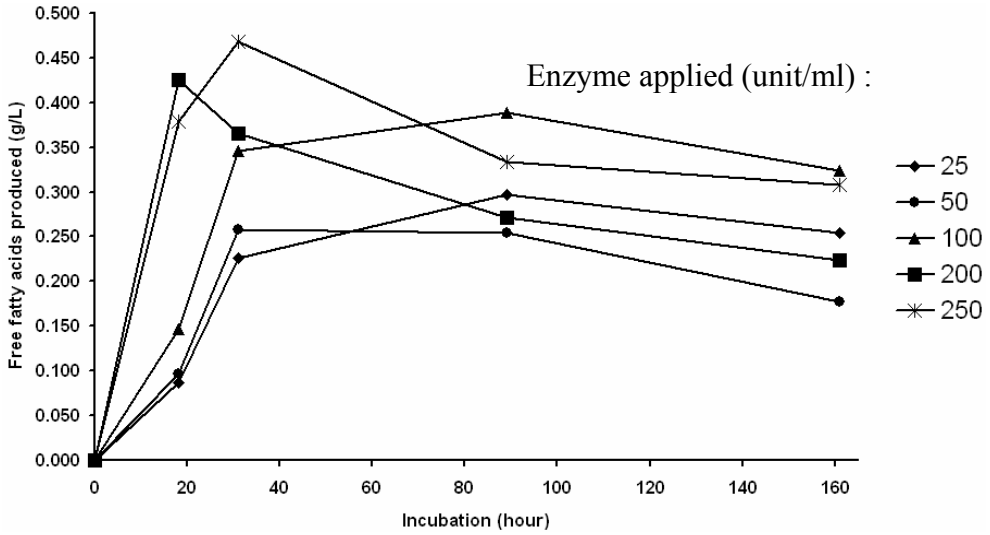

Figure 4. Effect of lipase concentration on hydrolysis of 1 g/L LO in SD medium containing 2.5 g/L tergitol NP40.

Table 3 showed that from 2.59 g/L cells of GMY could accumulate total lipid 0.147 g/L. Thus, lipid content was about 6%. Accumulation ratio of ALA calculated from accumulated SDA and ALA in the cells comparing with initial ALA content in medium was about 11%. Furthermore, conversion ratio ALA to SDA which was calculated from the percentage of SDA comparing with the total of SDA and ALA in the cells, was about 25%. 0.0155g/L SDA was produced by GMY during 5 days cultivation in the SD medium containing 1g/L LO. Fatty acids contents in the medium were measured after the fractionation of free fatty acids and acylglycerols. Table 4 showed this fractination results.

There were no ALA found in the medium as a free fatty acids, but little amount of free fatty acids less than 18:2 remain (0.04g/L). The medium with LO and glucose or the medium containing glucose only, free fatty acids was not detected. It is suggested that free fatty acids were obtained from the hydrolysis of LO and most of them were uptaken by GMY. By rough calculation, 0.11 g/L (=0.79-0.68) lipids were hydrolyzed by the lipase and 0.054 g/l (=0.153-0.061) lipids were additionally accumulated into the cells.

Table 3. ALA accumulation and SDA production using LO and GMY

Medium			Accumulated lipid (TL) (g/L)	Dry cell weight (DCW) (g/L)	Lipid content (TL / DCW)	ALA in the cell (g/L) (A)	SDA in the cell (g/L) (S)	SDA content in the cell (g/g) (S/ DCW)	SDA Conversion ratio (%) (S/(A+S))	Accumulation ratio of ALA (%)
Glucose (g/L)	LO (g/L)	Lipase (unit/mL)								
20	1	50	0.153	2.59	0.059	0.047	0.015	0.006	24.2	11.04
20	1	-	0.097	1.89	0.051	0	0	0	0	0
20	-	-	0.099	2.04	0.049	0	0	0	0	0

Table 4. Fatty acid content in the supernatant of the medium*

Medium		Phase	Fatty acids composition g/L			Total lipid (g/L)
			Less than 18:2	ALA	SDA	
Initial medium with LO		acylglycerols	0.46	0.57	0.00	1.03
		free fatty acids	0.00	0.00	0.00	0.00
5 days cultivation	LO + Glucose + Lipase	acylglycerols	0.30	0.38	0.00	0.68
		free fatty acids	0.04	0.00	0.00	0.04
	LO + Glucose	acylglycerols	0.36	0.43	0.00	0.79
		free fatty acids	0.00	0.00	0.00	0.00
	Glucose	acylglycerols	0.00	0.00	0.00	0.00
		free fatty acids	0.00	0.00	0.00	0.00

Notes:
* : Supernatant by centrifugation at 3000rpm for 10 min was measure

CONCLUSION

ALA-rich LO can be used as fatty acids source by GMY for SDA production has been shown. In this research, GMY accumulated 11 % of total ALA in LO and about 25% of accumulated ALA was converted to SDA after 5 days cultivation. Future, in order to improve the productivity of SDA using GMY, hydrolysis of LO as well as cultivation condition and genetically improvement of the yeast must be highly considerated.

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