Kasetsart J. (Nat. Sci.) 41 : 130 - 135 (2007)

Expression of Fatty Acid Desaturase Enzymes from Cyanobacterium *Spirulina platensis* in Yeast *Saccharomyces cerevisiae*

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ABSTRACT

Spirulina sp. is the cyanobacterium that has an ability to produce γ-linolenic acid or GLA at high level (1-1.5% of dry weight) which could be used as a new source of GLA production. The biosynthesis of GLA in *Spirulina* sp. involved with three enzymes, Δ^9, Δ^{12} and Δ^6 desaturase which encoded by gene *desC*, *desA* and *desD*, respectively. The desaturase genes from *Spirulina* that involve in GLA biosynthesis from cyanobacteria were studied by gene cloning and transforming into yeast, *Saccharomyces cerevisiae*. Gas Chromatography has been used to follow the activity of desaturase enzymes in yeast cells. The results indicated that both Δ^{12} and Δ^6 desaturase genes from *Spirulina platenesis* can functionally co-expressed in the heterologous yeast system. The final product, GLA has been detected under the condition described here.

Key words: desaturase, fatty acid desaturation, *Spirulina platensis*

INTRODUCTION

Spirulina is a cyanobacterium belongs to the group of blue-green algae with an ability to perform oxygenic photosynthesis activity. This cyanobacterium is widely used as human health food and animal feed. It contains a high level of proteins, vitamins and unsaturated fatty acid especially γ-linolenic acid (GLA: C18:3$^{\Delta 9,\ 12,\ 6}$) (Deshnium *et al*., 2000).

GLA has been reported as a precursor in prostaglandin biosynthesis, which plays a role in variety of important processes in human health and diseases. For example, it can prevent the development of atherosclerosis and coronary heart disease by lowering the level of plasma cholesterol and triglycerides. Therefore, *Spirulina* has been considered as a new source for GLA production.

Unsaturated fatty acid biosynthesis is achieved by a process called desaturation. Fatty acid desaturases are enzymes that convert a single bond between two carbon atoms (C-C) to a double bond (C=C) in a fatty acyl chain. The distribution of fatty acid desaturases is almost universal. The enzymes have been found in most organisms examined, with the exception of some bacteria such as *Escherichia coli*. In the case of *S. platensis*, three desaturase genes; *desC*, *desA* and *desD* are responsible for the introduction of three double bonds into fatty acids that have been esterified to

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Received date : 02/11/06 Accepted date : 04/12/06

glycerolipids (Murata *et al*., 1996). The three double bonds are introduced at the Δ^9, Δ^{12} and Δ^6 positions of stearic acid (C18:0), oleic acid (C18:$1^{\Delta 9}$) and linoleic acid (C18:$2^{\Delta 9,\ 12}$), respectively.

There are three types of fatty acid desaturases: acyl-CoA, acyl-ACP and acyl-lipid desaturases (Murata and Wada, 1995). In plants and cyanobacteria, most desaturation reactions are catalyzed by acyl-lipid desaturases, which introduce unsaturated bonds into fatty acids that are in a lipid-bound form. Acyl-ACP desaturases are present in the plastids of plant cells and introduce the first double bond into fatty acids that are bound to acyl carrier protein (ACP). Acyl-CoA desaturases are present in animals, yeast and fungal cells, and they introduce unsaturated bonds into fatty acids that are bound to coenzymeA (CoA). The desaturation of fatty acids is an oxidation reaction and requires two electrons in addition to one molecule of oxygen (Shanklin and Cahoon, 1998). In the case of acyl-ACP desaturases in plant plastid and acyl-lipid desaturases in cyanobacteria and plants, the electron donor is ferredoxin, whereas in the case of the acyl-lipid desaturases in plant cytoplasm and the acyl-CoA desaturases of animals and fungi, the donor is cytochrome b_5, either in the form of cytochrome b_5 fusion domain or in free form.

The three desaturase genes from *S. platensis* had been successfully expressed and characterized in *E. coli* (Hongsthong *et al*., 2004). However, the *desD* gene can not function in the *in vivo* system in *E. coli*, although it can function when ferredoxin is externally provided *in vitro* (Hongsthong *et al*., 2004). The *desD* gene was further expressed successfully for the first time in yeast, *Saccharomyces cerevisiae* (Kurdrid *et al*., 2005). However, the *desA* gene from *S. platensis* has not been expressed and characterized in yeast cells. It is necessary to explore ever details of the mechanism of fatty acid biosyntheis and desaturation. Therefore, in this short report we describe the functional expression of *S. platensis desA* together with *desD* genes. The function was determined using *Saccharomyces cerevisiae* as a heterologous host.

MATERIALS AND METHODS

Construction of yeast expression plasmids

The *S. platensis desA* coding sequences were generated by PCR. Primers used were pYAYF (5'-CGC GGA TCC GAT GAC CCT ATC AAT T-3') and pYAYR (5'-CGC GAA TTC CTA AAC TCT TTT TGT-3'). *Bam*HI and *Eco*RI sites were used for the restriction enzyme site for forward and reverse primers, respectively. The PCR products flanked with *Bam*HI and *Eco*RI were digested and cloned into pYES2 (Invitrogen, NV Leek, The Netherlands) which digested with the same restriction enzymes. The recombinant plasmid was designed as pY*desA* plasmid. The *desD* gene in pYES2 plasmids (pY*desD*) were constructed by Kurdrid *et al*. (2005)

S. cerevisiae strain DBY746 (*α, his 3-Δ1, leu 2-3, leu 2-112, ura3-52, trp 1289*), was used as a host for the expression of the *desA* and *desD* gene of *S. platensis* C1. The medium for growing the *S. cerevisiae* strain DBY746 was YPD (1%(w/v) bacto-yeast extract. 2%(w/v) bacto-peptone, 2%(w/v) dextrose) or synthetic minimal medium (SD, 0.67%(w/v) bacto-yeast nitrogen base (without amino acids), 2%(w/v) dextrose) supplemented with appropriate amino acids (Ausubel *et al*., 1993). The cells were incubated at 30°C, and shaken at 200 rpm until the optical density at 600 nm reached 0.5. The cells were stored indefinitely in 15% (v/v) glycerol at -80°C and for up to 6 months at 4°C on YPD slants.

Expression of the *desA* and *desD* gene in *Saccharomyces cerevisiae*

The transformation of the yeast cells *S. cerevisiae* strain DBY746 was conducted using lithium acetate (Gieta, 1992). A single colony of

S. cerevisiae strain DBY746 was subsequently inoculated into 5 ml of YPD and incubated overnight at 30°C, with a constant shaking at 200 rpm. The transformant plates were incubated at 30°C for 3 days on. Only cells contain pYES2 plasmid or recombinant plasmid will survive on uracil deficient SD medium. The transformants were then screened using PCR for the desaturase gene coding sequence.

Yeast culture conditions

The transformants of the *desA* (pY*desA*) and *desAD* (pY*desA* + pY*desD*) genes were grown in SD medium (Ausubel *et al*., 1993) with and without 200 μM of oleic acid (OA, C18:1$^{\Delta 9}$; Sigma) in the form of sodium salt at 30°C until the optical density at 600 nm reached 0.5. The cells were then centrifuged and transferred into SD medium containing 2% (w/v) of galactose, and grown for 24 hours at 25°C. The cells were then harvested by centrifugation at 9,000 rpm at a temperature of 4°C for 5 minutes, and washed twice with distilled water. The wet cells were then freeze dried for 3 hours and used for the fatty acid analysis.

Fatty acid analysis

Two ml of 5% (v/v) HCl in methanol and an internal standard (Heptadecanoic acid: $C_{17}H_{33}COOH$; Sigma) was added at a concentration of 10 mg/ml to the dry cells. Subsequently, the mixture was stirred using a magnetic bar and incubated at 85°C in the dark for 90 minutes. The samples were then cool down to room temperature, mixed with 1 ml of distilled water and 1 ml of hexane with 0.01% (w/v) of Butylated hydroxytoluene (BHT). The mixture was centrifuged at 4,000 rpm for 5 minutes to allow complete separation. The hexane phase was filtered through Na_2SO_4 into a new vial (Sato and Murata, 1988).

GC analysis of the transmethylated fatty acids was performed using a fused silica capillary column (OMEGAWAX TH 250; Supelco, Pennsylvania, USA), with film thickness of 30 m $\times$ 0.25 μm. The column temperature was 205°C, injector temperature 250°C, the detector temperature 260°C, the flow rate 2.0 ml/min and the linear velocity was 48 cm/sec with a split ratio of 1:50. The areas of chromatographic peaks were used to calculate the relative quantities of fatty acid methyl esters.

RESULTS

pYES2, pY*desD*, and pY*desA* were extracted from *E. coli* DH5α, and subsequently transformed into *S. cerevisiae* strain DBY746. *S. cerevisiae* strain DBY746 was grown in YPD medium to performed transformation experiment. The uracil deficient SD medium was used for selection of the transformants. The function of transformed genes was observed by expression in *S. cerevisiae* in a medium supplemented with and without the Δ^{12}-desaturase substrate, oleic acid (C18:1$^{\Delta 9}$). The expressions of both control (pYES2) and recombinant plasmids were induced by an addition of 2% (w/v) galactose.

The fatty acid methyl esters (FAME) from the cultures transformed with pYES2 and the recombinant plasmids were analyzed by gas chromatography. The control experiment was yeast cells (DBY746) transformed with pYES2 only as yeast cells with pYES2 can not grow in medium containing OA. The fatty acid profiles in Figure 1 showed the GC analysis of yeast cells transformed with pYES2 (control), as well as the recombinant plasmids (pY*desA* and pY*desA* + pY*desD*) grown in the presence of substrate (50 μM OA).

Figure 2 showed the GC profile of yeast cells in the same order as shown in Figure 1, however, without exogenous added substrate.

Analysis of the fatty acid profile in yeast lipid fraction indicated that the exogenous oleic acid was incorporated into lipids of transformed yeast cells. A novel fatty acid peak with an identical

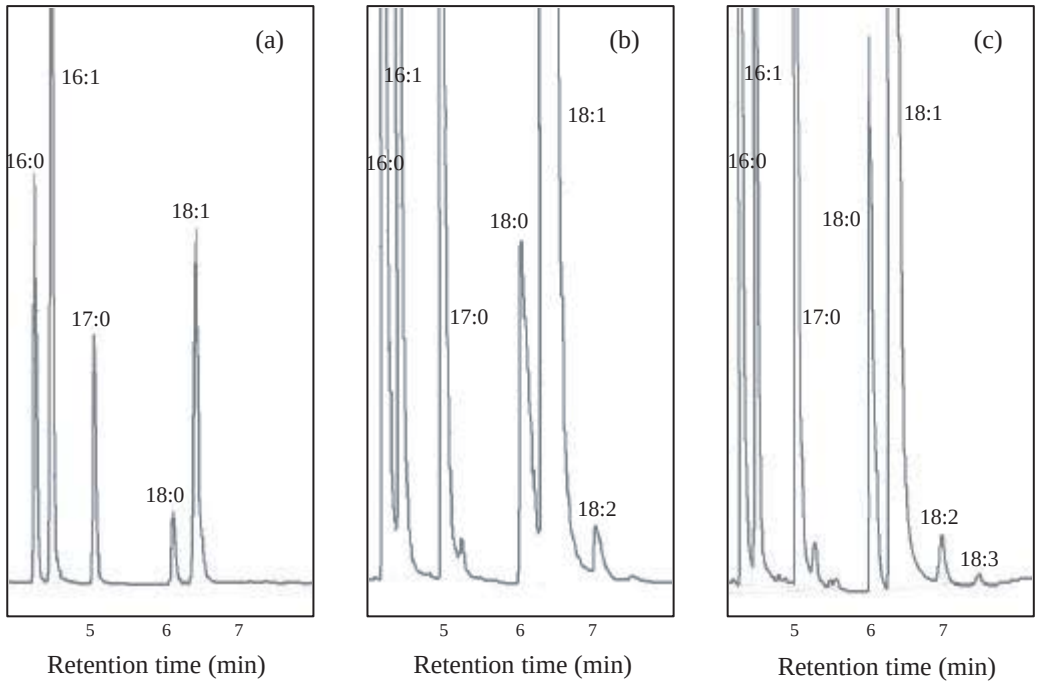

Figure 1 GC analysis profile of total fatty acid from (a) *S. cerevisiae* harbouring pYES2. (b) pY*desA* and (c) pY*desA* and pY*desD* in SD medium with 200 μM of oleic acid (C18:1).

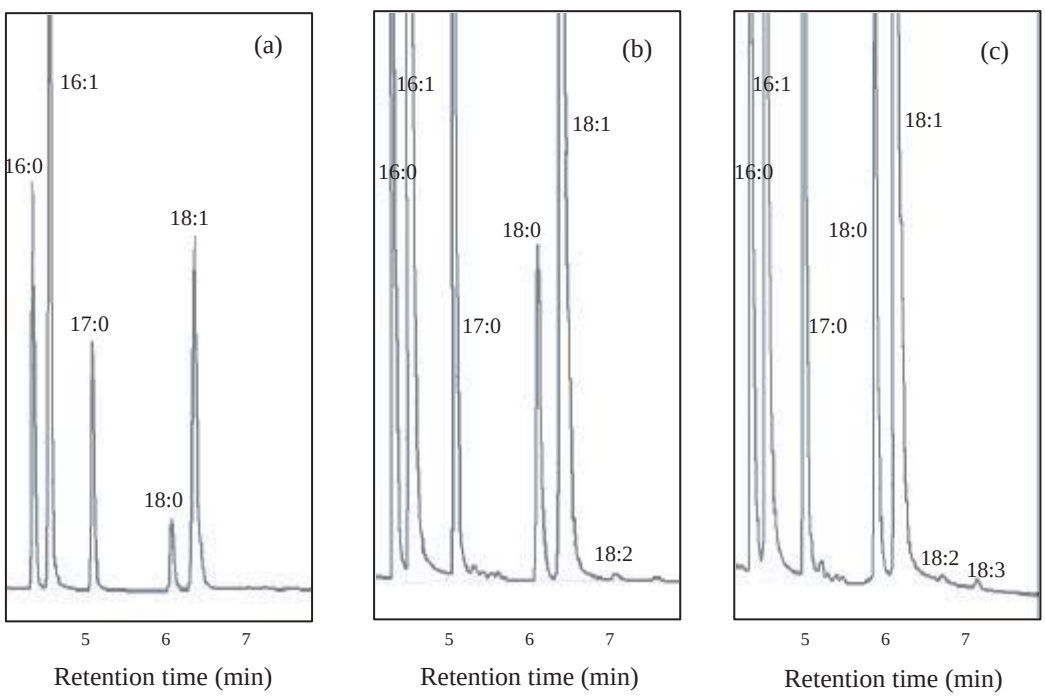

Figure 2 GC analysis profile of total fatty acid from (a) *S. cerevisiae* harbouring pYES2. (b) pY*desA* and (c) pY*desA* and pY*desD* in SD medium.

retention time to that of the authentic linoleic acid (LA, C18:$2^{\Delta 9, 12}$) was observed in cells carrying pY*desA* and both pY*desA* and pY*desD* in both conditions, with and without exogenous OA. Interestingly, a fatty acid peak with an identical retention time to the GLA (C18:$3^{\Delta 9, 12, 6}$) was identified in only in cells carrying both plasmids, pY*desA* and pY*desD* either exogenous OA were present or absent (Figure 1(c) and Figure 2(c)). These results indicated a successfully used of internal substrate, OA (C18:$1^{\Delta 9}$) in yeast cells by desaturase genes from *S. platensis*. The amounts of C18:$1^{\Delta 9}$, C18:$2^{\Delta 9, 12}$ and C18:$3^{\Delta 9, 12, 6}$ were estimated by comparing their peak areas with that of the internal standard, C17:0, on the chromatogram (Table 1). The activity of each desaturases was calculated from the increasing amount of the reaction product in the particular transformant compared to that of the control, which carried the expression vector without inserted gene. The biosynthesis of GLA from the endogenous oleic acid in *S. cerevisiae* would require the simultaneous expression of Δ^{12}- and Δ^{6}-desaturases. The co-expression of the two desaturase genes under the control of GAL1 promoters in pYES2 vector in yeast cells resulted in the synthesis of GLA.

DISCUSSION

The results showed here indicated that both Δ^{12}- and Δ^{6}-desaturase enzyme from *S. platensis* can express in *S. cerevisiae*, and that it can synthesize the final reaction product, GLA, *in vivo*. In the absence of exogenous oleic acid as substrate, these recombinant yeast cells could converted the endogenous oleic acid to linoleic acid and convert to GLA in the presence of both pY*desA* and pY*desD* (Table 1). This finding suggests that the *Spirulina* desaturases are able to use an electron donor in yeast cells for the desaturation reaction pathway for GLA production. This electron donor is more likely to be cytochrome b_5, which is present in the yeast cells. The hypothesis still remain to be proved by further test on detection of GLA production by cytochrome b_5- *Spirulina* desaturase protein expressed in *S. cerevisiae*, comparing with the *Spirulina* desaturase protein expressed without cytochrome b_5 in *S. cerevisiae* cells.

The Δ^{12}-desaturase enzyme is responsible for the synthesis of LA from oleic acid, acts on both *sn*-position of phosphatidylcholine (PC), and this activity is not restricted to only PC while the *sn*-2 position of PC is the major site for Δ^{6}-desaturation in yeast cells (Domergue *et al*., 2003). Only a small proportion is incorporated into lipids, the form of substrate in which the cyanobacterial desaturase can react which could explain the low GLA yield obtained from the expression of *Spirulina*-Δ^{6}-desaturase in yeast in the presence of LA (Hongsthong *et al*., 2004). This reason could also explain the low level of both LA and GLA production yield obtained in this study.

In summary, this study has shown the functional expression of *S. platensis* Δ^{12}- and Δ^{6}-

Table 1 % Fatty acid composition in total lipids from yeasts harbouring pYES2, pY*desA*, and pY*desA* + pY*desD* with and without exogenous added oleic acid (C18:$1^{\Delta 9}$)

Transformants	Fatty acid composition (mol%)				
	C16:0	C18:0	C18:$1^{\Delta 9}$	C18:$2^{\Delta 9, 12}$	C18:$3^{\Delta 9, 12, 6}$
pYES2	35.92	9.33	54.74	-	-
pY*desA* with exogenous OA	18.30	3.99	77.25	0.44	-
pY*desA*+pY*desD* with exogenous OA	16.94	4.17	78.35	0.48	0.074
pY*desA*	33.47	10.58	55.77	0.16	-
pY*desA*+pY*desD*	33.08	11.40	55.21	0.11	0.176

desaturation in yeast cells. This study can be done further on the regulation mechanism of the desaturation process and improvements in the GLA production of *S. platensis*, using yeast *S. cerevisiae* as the model for studying.

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