Polyunsaturated fatty acids-enriched lipid from reduced sugar alcohol mannitol by marine yeast *Rhodosporidiobolus fluvialis* Y2

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Abstract

Brown macroalgae is a promising marine biomass for the production of bioethanol and biodiesel fuels. Here we investigate the biochemical processes used by marine oleaginous yeast for assimilating the major carbohydrate found in brown macroalgae. Briefly, yeast *Rhodosporidiobolus fluvialis* strain Y2 was isolated from seawater and grown in minimal medium containing reduced sugar alcohol mannitol as the sole carbon source with a salinity comparable to seawater. Conditions limiting nitrogen were used to facilitate lipid synthesis. *R. fluvialis* Y2 yielded 55.1% (w/w) and 39.1% (w/w) of lipids, per dry cell weight, from mannitol in the absence and presence of salinity, respectively. Furthermore, mannitol, as a sugar source, led to an increase in the composition of polyunsaturated fatty acids, linoleic acid (C18:2) and linolenic acid (C18:3), compared to glucose. This suggests that oxidation of mannitol leads to the activation of NADH-dependent fatty acid desaturases in *R. fluvialis* Y2. Such fatty acid composition may contribute to the cold-flow properties of biodiesel fuels. Our results identified a salt-tolerant oleaginous yeast species with unique metabolic traits, demonstrating a key role as a decomposer in the global carbon cycle through marine ecosystems. This is the first study on mannitol-induced synthesis of lipids enriched with polyunsaturated fatty acids by marine yeast.

Keywords

Marine yeast, *Rhodosporidiobolus fluvialis*, Lipid synthesis, Polyunsaturated fatty acids, Mannitol, Salt tolerance

1. Introduction

Marine ecosystems play a significant role in the global carbon cycle by sequestrating and redistributing of carbon dioxide (CO_2) [1]. Marine vegetation habitats, such as mangroves, salt marshes, seagrasses and algae, serve as primary producers in marine ecosystems and form carbon sinks for long-term storage of organic carbon [1–3]. Importantly, macroalgae stores large amounts of carbon and contributes to climate change mitigation [2,3]. Diverse marine microorganisms act as decomposers of organic compounds in the marine ecosystems [4]. Understanding how marine microorganisms utilize organic components of macroalgae may contribute to efficient bioconversion of indigestible marine biomass [5].

Biofuels from terrestrial biomasses have been proposed as a sustainable, renewable energy alternative to fossil fuels [6]. More recently, marine brown macroalgae has attracted attention as a promising raw material for biofuel production, due to a higher growth rate and lower lignin content, compared to terrestrial plants [7–9]. Alginate, a

polysaccharide composed of guluronic acid and mannuronic acid, is a major component of brown macroalgae, and accounts for 20-30% of the dry biomass [9]. Mannitol, a reduced sugar alcohol derived from mannose, occupies 10-20% of the dry biomass [9]. Although utilization of the above carbohydrates by microorganisms has been reported [5,10–14], industrial biofuel production from brown macroalgae has not been fully established; due in-part to the low assimilability of alginate and mannitol.

In the terrestrial biomass, the budding yeast *Saccharomyces cerevisiae* and the oleaginous yeast *Yarrowia lipolytica* are the representative species for biofuel production, due to their well-characterized physiology, availability of genetic tools and high productivity of ethanol or lipids [15,16]. In our previous work, an engineered *S. cerevisiae* strain MK6286 was constructed for bioethanol production using brown macroalgae carbohydrates [17,18]. In this strain, the genes required for the utilization of alginate and mannitol were artificially overexpressed and growth was enhanced through adaptive evolution. In contrast, this study focused on marine yeast species that inherently utilize marine biomass. Salt-tolerant marine yeast may enable the use of seawater as natural and cost-effective medium for sustainable biofuel production.

2. Materials and Methods

2.1. Yeast isolation and identification

To isolate yeast assimilating components of brown macroalgae from marine environments, microbes in 100 µl of seawater from Okinawa Prefecture, Japan, were aerobically cultured at 30℃ in 10 ml of enrichment medium [0.5% (w/v) sodium alginate, 0.1% (w/v) $(NH_4)_2SO_4$, 0.1% (w/v) KH_2PO_4, 0.1% (w/v) Na_2HPO_4 and 0.01% (w/v) $MgSO_4 \cdot 7H_2O$] for 2-4 d. The cultures were spread on solidified enrichment medium and incubated at 30℃ for an additional 2-4 d. Single colonies of yeast were isolated by repeatedly streaking the culture on the isolation medium [5% (w/v) glucose, 0.5% (w/v) peptone, 0.2% (w/v) yeast extract, 0.4% (w/v) K_2HPO_4, 0.2% (w/v) KH_2PO_4, 0.02% (w/v) $MgSO_4 \cdot 7H_2O$]. Identification of the isolated yeast clone was conducted at the Techno Suruga Laboratory Co., Ltd. (Shizuoka, Japan), using sequencing analysis of the D1/D2 region of 26S rDNA and phylogenetic analysis. The DNA sequence has been deposited in the DDBJ/EMBL/GenBank database under the Accession Number LC530805.

2.2. Scanning electron microscope (SEM) analysis

For protein fixation, 700 µl of 4% (w/v) formaldehyde was added to yeast cells and

incubated at room temperature for 1 h. Cells were washed with sterilized water three times, immersed in 1 ml of 1% (w/v) osmium oxide for lipid fixation, and incubated at room temperature for 1 h. After this, samples were washed with sterilized water three times and dehydrated sequentially with 50%, 70%, 90%, and 100% (v/v) ethanol, followed by immersion in 1 ml of *t*-butyl alcohol and drying. Samples were coated with Pt-Pd layer under Ar gas and observed with SEM (HITACHI FE-SEM SU8230, Tokyo, Japan), using 1.5 kV electron beams.

2.3. Mannitol assimilation test

The isolated marine yeast strain, the bioengineered mannitol-assimilating *S. cerevisiae* strain MK6286 [17,18], and a *Y. lipolytica* strain NBRC 1195, purchased from NITE Biological Resource Center (NBRC) (Chiba, Japan), were used in this study. The yeast strains were aerobically precultured at 30℃ in YNB medium [0.67% (w/v) yeast nitrogen base w/o amino acids (Becton, Dickinson and Company, New Jersey, USA)] with 2% (w/v) glucose for 2 d. The inoculum was diluted in 1 ml of YNB medium with or without 2% (w/v) mannitol, to an optical density at 600 nm (OD_{600}) of 0.05, and aerobically cultured at 30℃. To test salt tolerance, 3% (w/v) NaCl was supplemented to the culture medium. The culture turbidity (OD_{600}) was measured using a plate reader

(Tecan Infinite 200PRO, Männedorf, Switzerland).

2.4. Lipid synthesis test

Yeast cells were aerobically precultured at 30℃ in 20 ml of nitrogen-limited medium [0.17% (w/v) yeast nitrogen base w/o amino acids and ammonium sulfate (Becton, Dickinson and Company) supplemented with 0.055% (w/v) $(NH_4)_2SO_4$] with 3% (w/v) glucose or mannitol for 2 d. The inoculum was diluted in 100 ml of nitrogen-limited medium with 7% (w/v) glucose or mannitol to an OD_{600} of 0.01 and aerobically cultivated at 30℃ for 3 d. Aliquots (10 ml) of the culture solution were collected for lipid analysis.

2.5. Nile Red staining

Yeast cells were washed twice with 1 ml of 50 mM Tris-HCl (pH 7.5) and resuspended in 990 μl of the same buffer. The suspension was mixed with 10 μl of 0.1 mg/ml Nile Red (Sigma-Aldrich, Missouri, USA) in ethanol and incubated for 20 min at room temperature. Immediately after staining, yeast cells were observed under a fluorescence microscope (Olympus BX51, Tokyo, Japan).

2.6. Lipid analysis

Intracellular lipids were extracted according to the method described by Bourque *et al*. [19]. Preparation of fatty acid methyl esters from lipids after 48 h post inoculation to nitrogen-limited medium was conducted following the method described by Ichihara *et al.* [20]. Fatty acid methyl esters were detected with gas chromatography equipped with frame ionization detector (GC/FID) (Shimadzu GC-2014, Kyoto, Japan), using a helium carrier gas and a capillary column Rtx-Wax (30 m×0.53 mm×1 μm, Restek, Pennsylvania, USA). The injection temperature was set to 250℃; the column temperature was set to 50℃ for 1 min, then raised to 200℃ at 25℃/min, and finally raised to 230℃ at 3℃/min, and held for 10 min; the detector temperature was set to 280℃. Fatty acid methyl esters were identified and quantified using AOCS No. 6 (RM-6) (Sigma-Aldrich).

3. Results

3.1. Isolation of a mannitol-assimilating marine yeast

To identify yeast species that effectively utilize marine biomass, marine yeast cells inhabiting marine environments were explored. As a result, mixed colonies of bacteria and yeasts were grown on solidified enrichment medium. A yeast clone was isolated by repeatedly streaking the culture. Differential interference contrast (DIC) and

SEM analysis revealed that the yeast cells actively proliferated by budding (Fig. 1A). Based on sequencing analysis of the D1/D2 region of 26S rDNA, the isolated strain (hereafter, referred to as Y2) was identified as being an oleaginous yeast *Rhodosporidiobolus fluvialis*. The *R. fluvialis* Y2 grew with mannitol as the sole carbon source amongst the carbohydrates in brown macroalgae although no growth on alginate occured (data not shown). Thus, mannitol assimilation of mannitol-assimilating *S. cerevisiae* MK6286 (constructed in our previous studies [17,18]; Fig. 1B), representative oleaginous yeast *Y. lipolytica* NBRC 1195 (Fig. 1C), and *R. fluvialis* Y2 (Fig. 1D) were compared. Although *S. cerevisiae* MK6286 and *Y. lipolytica* NBRC 1195 showed no growth without a carbon source, the growth was markedly enhanced by mannitol. Similarly, *R. fluvialis* Y2 exhibited a mannitol-dependent growth phenotype, indicating that mannitol was assimilated by *R. fluviais* Y2 as the sole carbon source. No significant inhibition in the growth of *S. cerevisiae* MK6286 and *R. fluvialis* Y2 was observed in the presence of 3% (w/v) NaCl while the growth of *Y. lipilytica* NBRC 1195 was severely inhibited by salt (Fig. 1B-D). Both *S. cerevisiae* MK6286 and *R. fluvialis* Y2 were tolerant to salinity conditions that were comparable to seawater. This suggests that marine-derived *R. fluvialis* Y2 has the potential to assimilate mannitol derived from marine brown macroalgae.

3.2. Lipid synthesis from mannitol by R. fluvialis Y2

In a previous report, intracellular lipid accumulation occurs in *R. fluvialis* DMKU-RK253 [21]. To test lipid synthesis from mannitol, *R. fluvialis* Y2 cells were cultivated in nitrogen-limited medium with mannitol and intracellular lipids were observed by Nile Red staining. While no clear signals were observed in the log phase (6 h after inoculation), uniformed intracellular staining and dot-like lipid bodies were detected in the stationary phase (72 h) (Fig. 2A). Similar signals were observed in the presence of 3% (w/v) NaCl in the stationary phase (Fig. 2B).

Since *R. fluvialis* Y2 was found to synthesize lipids from mannitol, lipid synthesis by *S. cerevisiae* MK6286 and *R. fluvialis* Y2 was compared during the growth in nitrogen-limited medium. Lipid contents of *S. cerevisiae* MK6286 were less than 20.0% (w/w) throughout the cultivation periods (Fig. 3A-D). Neither carbon sources nor NaCl severely impacted lipid synthesis. *R. fluvialis* Y2 exhibited a significantly larger maximum value of lipid content, compared to *S. cerevisiae* MK6286, in all conditions (one-sided t-test, $p < 0.05$). In the absence of NaCl, lipid content increased to 20.0% (w/w) in 72 h, when glucose was used as the carbon source (Fig. 3E). Lipid accumulation from mannitol peaked at 48 h [55.1% (w/w); Fig. 3F]. In the presence of 3% (w/v) NaCl, *R. fluvialis* Y2 yielded high contents of lipids from both glucose [29.2% (w/w) at 72 h; Fig.

3G] and mannitol [39.1% (w/w) at 48 h; Fig. 3H]. These data suggest that *R. fluvialis* Y2 acts as a decomposer in marine ecosystems and effectively converts mannitol into lipids.

3.3. Fatty acid composition of lipids synthesized by R. fluviais Y2

Fatty acid composition of lipids, at 48 h after inoculation to nitrogen-limited medium, was analyzed by GC/FID. Lipids synthesized by *S. cerevisiae* MK6286 were only weakly influenced by carbon sources or NaCl (Fig. 4A). Palmitic acid (C16:0), palmitoleic acid (C16:1), and oleic acid (C18:1) were the major constituents whereas linoleic acid (C18:2) and linolenic acid (C18:3) were undetectable in all samples (Fig. 4A). In contrast, lipids synthesized from glucose by *R. fluvialis* Y2 contained palmitic acid (C16:0) and oleic acid (C18:1) as the major components (Fig. 4B). Lipids synthesized from mannitol contained lower levels of oleic acid (C18:1) and higher levels of linoleic acid (C18:2) and linolenic acid (C18:3), both of which were formed by desaturation of oleic acid. Thus, lipids synthesized from mannitol by *R. fluviais* Y2 are characterized by high contents of polyunsaturated fatty acids.

4. Discussion

Marine brown macroalgae and their components are gaining attention as

renewable resources for lipid synthesis. However, the biochemical conversion of individual marine biomass components into fatty acids needs to be further elucidated. Xu *et al.* proposed a stepwise process using the oleaginous yeast *Cryptococcus curvatus* for production of biodiesel fuels from mixed carbon sources, primarily mannitol and volatile fatty acids extracted from brown macroalgae; this yielded up to 48.3% (w/w) of lipids, per dry cell weight [22]. In our study, the marine oleaginous yeast *R. fluvialis* Y2 exhibited a high lipid content of 55.1% (w/w) when pure mannitol was used as the sole carbon source (Fig. 3F). Because *R. fluvialis* Y2 cells accumulate more lipids in the presence of mannitol than in glucose, mannitol is one of the most suitable substrates for yeast-based lipid synthesis, among brown macroalgae-derived carbon sources. Additionally, salt tolerance of *R. fluvialis* Y2 may contribute to direct utilization of seawater for cost-effective biofuel production with minimal environmental impact.

R. fluvialis Y2 assimilates mannitol to synthesize lipids enriched with polyunsaturated fatty acids. Since unsaturated fatty acids contribute to cold-flow properties [23], lipids synthesized from mannitol by *R. fluvialis* Y2 may be suitable for biodiesel fuels in cold climates. But what is the physiological significance of polyunsaturated fatty acid synthesis from mannitol? Many animals, including those living in marine environments, have difficulty in utilizing mannitol directly as a carbon source

and require linoleic acid and linolenic acid as essential fatty acids [24,25]. Thus, *R. fluvialis* Y2 may play an important role as a marine decomposer, by converting the major component of brown macroalgae into nutrients for other organisms.

This study is the first to report on the mannitol-induced biosynthesis of polyunsaturated fatty acids. Although linoleic acid is synthesized, to some extent by *C. curvatus*, the substrates used in this process contain various carbon sources from brown macroalgae [22]. Notably, Fig. 4A shows that *S. cerevisiae* MK6286 yielded no detectable levels of polyunsaturated fatty acids. This is consistent with the fact that the model eukaryotic microorganism *S. cerevisiae* lacks Δ^{12} and ω3-fatty acid desaturases [26,27]. In contrast, polyunsaturated fatty acids were detected in *R. fluvialis* Y2 (Fig. 4B). Mannitol-derived lipids contained higher levels of polyunsaturated fatty acids than glucose-derived lipids, suggesting mannitol-dependent activation of fatty acid desaturases. Yeast fatty acid desaturases require NADH as an electric donor for the activity [28]. Some bacteria convert mannitol to fructose by mannitol-2-dehydrogenase using NAD^+ as a coenzyme [13]; oxidation of reduced sugar alcohol to sugar may lead to consumption of NAD^+. Together, desaturation of fatty acids likely replenishes NAD^+ for mannitol assimilation to maintain NAD^+/NADH redox balance.

In conclusion, our results propose a major key role of *R. fluvialis* Y2 in the global

carbon cycle through marine ecosystems. The finding of this marine yeast synthesizing polyunsaturated fatty acids will help elucidate regulation of lipid biosynthesis in response to extracellular carbon source and intracellular redox status.

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Figure legends

Fig. 1. Isolation of a mannitol-assimilating yeast *R. fluviais* Y2.

(A) DIC (upper) and SEM (lower) images of *R. fluvialis* Y2 cells. Bar, 10 μm (upper) or 1 μm (lower). (B-D) Mannitol assimilation test. *S. cerevisiae* MK6286 (B), *Y. lipolytica* NBRC 1195 (C), and *R. fluvialis* Y2 (D) were cultivated in the 1 ml of YNB medium with (triangles) or without (squares) mannitol. Yeast cells were cultivated in the absence (open symbols) or presence (closed symbols) of 3% (w/v) NaCl. Data given as means ± SD from two or three independent experiments.

Fig. 2. Nile Red staining of *R. fluvialis* Y2 cells during lipid synthesis.

R. fluvialis Y2 was cultivated in 100 ml of nitrogen-limited medium with 7% (w/v) mannitol in the absence (A) or presence (B) of 3% (w/v) NaCl. Log-phase (6 h, left) and stationary-phase (72 h, right) cells were stained with Nile Red. Upper panels, DIC images; lower panels, fluorescence microscopy images. Bar, 10 μm.

Fig. 3. Lipid synthesis in nitrogen-limited medium.

Lipid synthesis by *S. cerevisiae* MK6286 (A-D) or *R. fluvialis* Y2 (E-H) from glucose (A,

C, E, G) or mannitol (B, D, F, H). Yeast cells were cultivated in the absence (A, B, E, F) or presence (C, D, G, H) of 3% (w/v) NaCl. Squares, dry cell weight (DCW); circles, lipid yield; triangles, lipid content. Data given as mean ± SD from three independent experiments.

Fig. 4. Fatty acid composition of lipids.

Fatty acid composition of lipids synthesized by *S. cerevisiae* MK6286 (A) or *R. fluvialis* Y2 (B) at 48 h after inoculation to the below nitrogen-limited media. White, nitrogen-limited medium with 7% (w/v) glucose without NaCl; light gray, nitrogen-limited medium with 7% (w/v) glucose and 3% (w/v) NaCl; dark gray, nitrogen-limited medium with 7% (w/v) mannitol without NaCl; black, nitrogen-limited medium with 7% (w/v) mannitol and 3% (w/v) NaCl. C14:0, myristic acid; C16:0, palmitic acid; C16:1, palmitoleic acid; C18:0, stearic acid; C18:1, oleic acid; C18:2, linoleic acid; C18:3, linolenic acid. Composition of fatty acid methyl esters was calculated assuming that the total amount of detected fatty acid methyl esters was 100%. n.d.: not detected. Bars with different letters are significantly different according to a two-sided t-test with Bonferroni correction ($p < 0.05$). Data given as mean ± SD from three independent experiments.

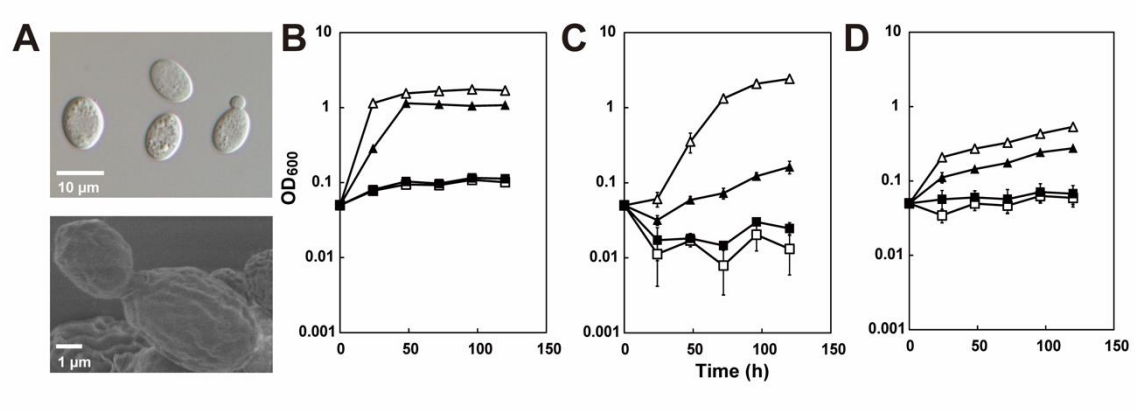

Fig. 1

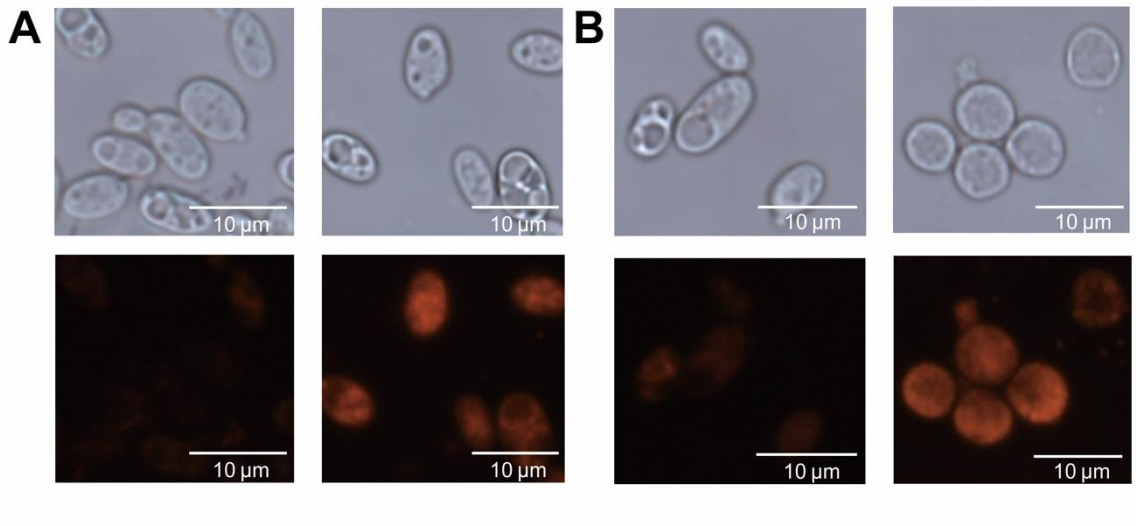

Fig. 2

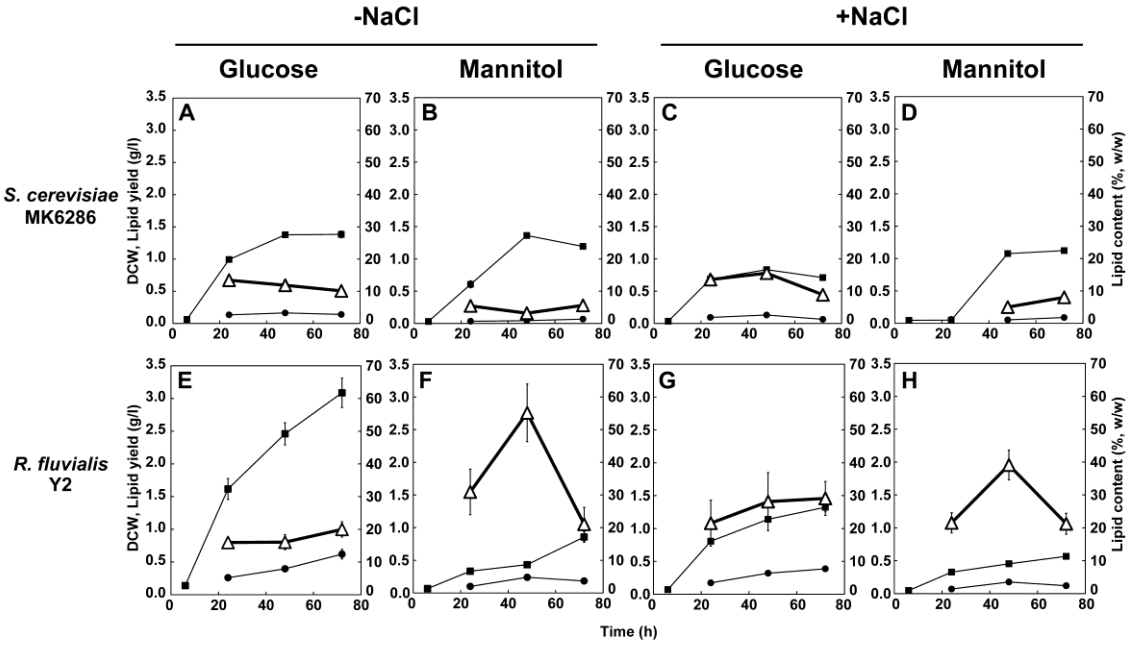
-NaCl
+NaCl
Glucose
Mannitol
Glucose
Mannitol
S. cerevisiae
MK6286
R. fluvialis
Y2
A
B
C
D
E
F
G
H
DCW, Lipid yield (g/l)
Lipid content (%, w/w)
Time (h)

Fig. 3

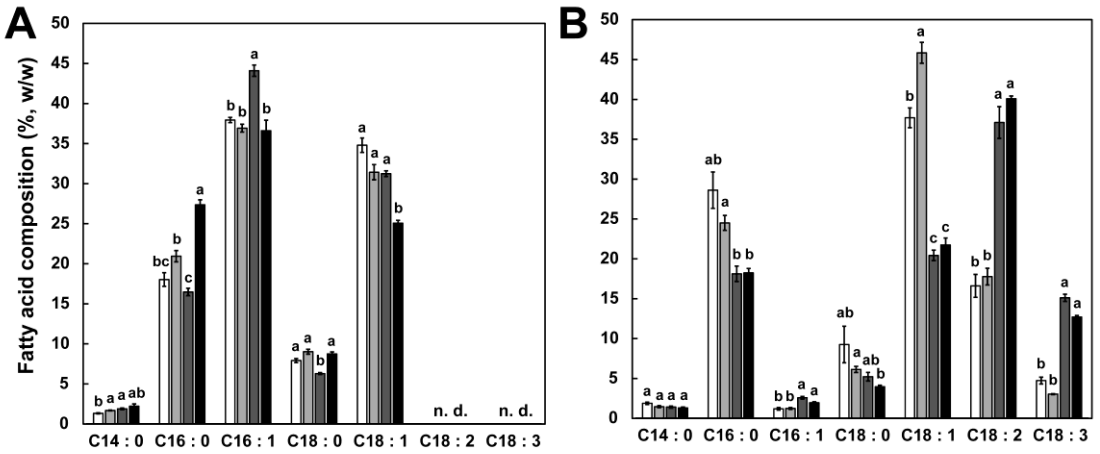
A
B
Fatty acid composition (%, w/w)
C14 : 0
C16 : 0
C16 : 1
C18 : 0
C18 : 1
C18 : 2
C18 : 3
n. d.
n. d.

Fig. 4