J. Microbiol. Biotechnol. (2010), **20**(11), 1555–1562
doi: 10.4014/jmb.1003.03004
First published online 30 September 2010

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Fermentation Process Development of Recombinant *Hansenula polymorpha* for Gamma-Linolenic Acid Production

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Received: March 1, 2010 / Revised: August 11, 2010 / Accepted: August 12, 2010

Development of the strain and the fermentation process of *Hansenula polymorpha* was implemented for the production of γ-linolenic acid (GLA, C18:3$\Delta^{6,9,12}$), an *n*-6 polyunsaturated fatty acid (PUFA) that has been reported to possess a number of health benefits. The mutated Δ^6-desaturase (S213A) gene of *Mucor rouxii* was expressed in *H. polymorpha* under the control of the methanol oxidase (*MOX*) promoter. Without the utilization of methanol, a high-cell-density culture of the yeast recombinant carrying the Δ^6-desaturase gene was then achieved by fed-batch fermentation under glycerol-limited conditions. As a result, high levels of the Δ^6-desaturated products, octadecadienoic acid (C18:2$\Delta^{6,9}$), GLA, and stearidonic acid (C18:4$\Delta^{6,9,12,15}$), were accumulated under the derepression conditions. The GLA production was also optimized by adjusting the specific growth rate. The results show that the specific growth rate affected both the lipid content and the fatty acid composition of the GLA-producing recombinant. Among the various specific growth rates tested, the highest GLA concentration of 697 mg/l was obtained in the culture with a specific growth rate of 0.08 /h. Interestingly, the fatty acid profile of the yeast recombinant bearing the *Mucor* Δ^6-desaturase gene was similar to that of blackcurrant oil, with both containing similar proportions of *n*-3 and *n*-6 essential fatty acids.

Keywords: γ-Linolenic acid, Δ^6-desaturase, high-cell-density cultivation, *Hansenula polymorpha*, specific growth rate

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The significance of two major classes of *n*-3 and *n*-6 polyunsaturated fatty acids (PUFAs) for human health has already been widely recognized. In addition to their structural role in biological membranes, PUFAs serve as precursors for the synthesis of both bioactive compounds called eicosanoids and a variety of lipid derivatives that regulate the proper functions of living cells [9]. Furthermore, the specific roles of individual PUFAs in a variety of biological processes have also been elucidated, resulting in a recommended optimal intake for certain PUFAs and their application for particular purposes [29]. Among *n*-6 PUFAs, γ-linolenic acid (GLA, C18:3$\Delta^{6,9,12}$) has a variety of therapeutic uses in the treatment and alleviation of several diseases, such as premenstrual syndrome, diabetic neuropathy, rheumatoid arthritis, and some cancers [5, 7]. Oils rich in GLA are commercially supplied from seed oil plants, such as evening primrose, borage, and blackcurrant. In addition, various microorganisms, particularly Mucorales fungi, have also been considered as promising alternatives for GLA production [3]. However, the cost of microbial oil production by fermentation technology is high compared with the market price for the oil when using traditional plant oils. Thus, to address this issue, numerous strategies have been investigated to either improve the GLA yield or reduce the cost of the production process.

Over the past 20 years, advances in recombinant DNA technology, metabolic engineering, and lipid biochemistry have facilitated the manipulation of the fatty acid profiles in organisms of interest using heterologous expression. Thus, genes coding Δ^6-desaturases, which catalyze the conversion of linoleic acid (C18:2$\Delta^{9,12}$) to GLA, have already been identified from diverse organisms [15, 26], and several biochemical studies on the enzyme characteristics have also been reported [10]. In addition, attempts have

been made to improve the GLA production in plant, yeast, and fungal strains by transforming these organisms with Δ^6-desaturase genes [15, 33, 36], while considerable attention has been directed toward the production of high-value fatty acids by the engineering of additional relevant metabolic pathways [20]. Although a genome-wide study has been thought to be an effective strategy to unveil the fatty acid metabolism in several model organisms [17, 21], it has been documented that the existence of metabolic bottlenecks in the fatty acid flux between different substrate pools can restrict the accumulation of nutritionally important fatty acids in oilseed [1]. A similar finding has also been reported in engineered yeasts [12]. Thus, to overcome this problem, a combination of metabolic engineering, biotransformation, and bioprocess engineering approaches has been suggested as a potential strategy to improve the fatty acid content in engineered yeasts [14].

The methylotrophic yeast *Hansenula polymorpha* has several advantages for biotechnological applications, including a GRAS (generally recognized as safe) status, high-cell-density growth, and an efficient expression system [30, 34]. This yeast strain also offers a potential host system for synthesizing novel fatty acids based on endogenous fatty acids. Similar to plant seed oils, it contains a high level of linoleic acid (40–60% of total fatty acids), which is a substrate for GLA biosynthesis [24]. Genetic modification of the fatty acid biosynthesis in *H. polymorpha* was already performed by introducing the native Δ^6-desaturase of *Mucor rouxii*, and a shake-flask study of the engineered strain demonstrated the possibility of GLA production in this yeast, from which a high yield of GLA (up to 10% of total fatty acids) was derived when using methanol [2% (v/v)] as the sole carbon source [15]. Recently, the *M. rouxii* Δ^6-desaturase was functionally characterized by site-directed mutagenesis studies in *Saccharomyces cerevisiae* [19], and it was found that replacing a serine with the alanine residue (S213A) of the *M. rouxii* Δ^6-desaturase increased the enzymatic activity, where the yeast bearing the mutated Δ^6-desaturase (S213A) contained a higher level of GLA than the yeast strain transformed with the native Δ^6-desaturase of *M. rouxii*.

Nonetheless, although many strategies have been explored to develop efficient fermentation processes for producing biologically active compounds in microorganisms, in addition to considering the features of the desired metabolite, an appropriate fermentation strategy also depends on the growth behavior and physiology of the host cells. As such, substrate feeding strategies have been regarded as an effective fed-batch fermentation approach for increasing product formation. Yet, although several parameters can be applied to control the substrate feeding mode, the specific growth rate is perhaps the most important parameter, since most of the biochemical reactions for product formation are either directly or indirectly associated with cell growth [28]. Accordingly, this study developed a fermentation process for the production of GLA in an *H. polymorpha* recombinant harboring the mutated Δ^6-desaturase (S213A) gene of *M. rouxii*. Glycerol-limited fed-batch fermentation was used to establish a high-cell-density culture of the engineered yeast. The effect of the specific growth rate on the lipid and GLA contents was also studied. This is the first report on the optimization of GLA production by the high-cell-density fermentation of *H. polymorpha* in a laboratory-scale bioreactor.

Materials and Methods

Strain and Media

H. polymorpha strain KYC625 (*leu1-1 ade11-1*) was kindly provided by Dr. Y. Kaneko (Osaka University) and used as the recipient for the expression of the mutated Δ^6-desaturase gene of *M. rouxii*. The strain was routinely grown in a YPD medium that consisted of 10 g/l yeast extract, 20 g/l bacto-peptone, and 20 g/l glucose at 30°C. The *Escherichia coli* strain, DH5α, was used for the plasmid propagation and grown at 37°C in a Luria–Bertani medium supplemented with 50 μg/ml of kanamycin when necessary.

Plasmid Construction and Yeast Transformation

The mutated Δ^6-desaturase cDNA (S213A) of *M. rouxii* was amplified by a PCR using AccuPrime Pfx DNA polymerase (Invitrogen, Carlsbad, USA) and the pMG281 plasmid as the template [18]. Two specific primers were designed based on the nucleotide sequences of the *M. rouxii* Δ^6-desaturase. The sequences of the sense and antisense primers were 5'-ACGCGTCGAC ATGAGCAGCGACGTAGGAG-3' (KL-SALD6) and 5'-TCCCCCG GGTTAGAGCATTTTTTTGCTGA-3' (KL-SMAD6), respectively (in which the underlined letters indicate the *Sal*I and *Sma*I sites, respectively). The PCR fragment was subcloned into the yeast expression vector pHIPX4, which contained the methanol oxidase (*MOX*) promoter, kanamycin resistance gene, and *LEU2* selectable marker [13]. The resulting plasmid carrying the S213A Δ^6-desaturase was then sequenced and designated as pML194. Thereafter, pML194 and pHIPX4 (empty vector) were transformed into *H. polymorpha* using the lithium acetate method [31]. The recombinant yeast cells were selected by growing them at 30°C on an SD agar consisting of a 6.7 g/l bacto-yeast nitrogen base without amino acids, 20 g/l glucose, 20 g/l bacto-agar, and 20 mg/l adenine sulfate. Under the control of the *MOX* promoter, induction of the gene expression was performed by cultivating the transformants in a minimal medium containing methanol (6.7 g/l bacto-yeast nitrogen base without amino acids, 20 ml/l methanol, and 20 mg/l adenine sulfate) at 30°C until the cultures reached the logarithmic phase. The cells were then harvested and used for a fatty acid analysis. The pML194 recombinant, which was capable of GLA synthesis, was then subcultured for more than 20 generations and used to optimize the GLA production by fed-batch fermentation.

Fed-Batch Fermentation of pML194 Yeast Transformant

A primary inoculum was prepared by picking a single colony of the *H. polymorpha* recombinant carrying pML194 in 5 ml of SD broth

supplemented with 20 mg/l of adenine sulfate. The culture was then incubated at 30°C with shaking at 200 rpm for 18–24 h. Thereafter, the overnight culture was transferred into a 250-ml shake-flask containing 100 ml of a glucose basal salt (Glu-BS) medium supplemented with adenine sulfate. One liter of the Glu-BS medium consisted of 26.7 ml 85% H_3PO_4, 0.93 g $CaSO_4$, 18.2 g K_2SO_4, 14.9 g $MgSO_4 \cdot 7H_2O$, 4.13 g KOH, 40.0 g glucose, and 4.35 ml PTM1 trace salts. The PTM1 trace salts contained 6.0 g $CuSO_4 \cdot 5H_2O$, 0.08 g KI, 3.0 g $MnSO_4 \cdot H_2O$, 0.2 g $Na_2MoO_4 \cdot 2H_2O$, 0.02 g H_3BO_3, 20.0 g $ZnCl_2$, 65 g $FeSO_4 \cdot 7H_2O$, 0.5 g $CoCl_2 \cdot 6H_2O$, 5.0 ml H_2SO_4, and 0.2 g biotin in 1 l of distilled water. The culture was allowed to grow for a further 36–48 h.

The fed-batch fermentation was carried out in a 2-l stirred tank bioreactor (Biostat B fermentor, B. Braun Biotech International, Germany). One liter of the glycerol basal salt (GBS) medium was used in the fermentation, where the medium contained 26.7 ml 85% H_3PO_4, 0.93 g $CaSO_4$, 18.2 g K_2SO_4, 14.9 g $MgSO_4 \cdot 7H_2O$, 4.13 g KOH, 40.0 g glycerol, and 4.35 ml PTM1 trace salts. The fermentation parameters were automatically controlled under the following conditions: temperature 30°C, aeration 1–4 l/min, and pH 5.0. A 25% ammonium hydroxide solution was used to adjust the pH of the culture broth. The dissolved oxygen (DO) was maintained above 20% saturation by controlling either the agitation rate or the supply of oxygen gas. A two-phase fermentation method was employed, where the initial batch-phase fermentation, which was continued until the glycerol was completely consumed, as indicated by the DO signal, was followed by glycerol-limited fed-batch fermentation. A glycerol feed (GF) medium, containing 670 g/l glycerol and 12 ml/l PTM1 trace salts, was added to the bioreactor according to the exponential feeding rate. The feeding rate was programmed based on the desired specific growth rate (μ) following a predetermined exponential feeding model [4] using the following formula:

$$F(t) = \frac{\mu V(t_0) X(t_0) \exp[\mu(t-t_0)]}{S_0 Y_{X/S}}$$

where F is the glycerol feeding rate (l/h), μ is the desired specific growth rate (/h), V is the fermentation volume (l), X is the biomass (g/l), S_0 is the glycerol concentration in the feed medium (g/l), $Y_{X/S}$ is the biomass yield on glycerol (g/g), which is assumed to be constant with a value of 0.50 g/g, and t and t_0 are the cultivation time and cultivation time at the start of the fed-batch phase (h), respectively. In this study, five different specific growth rates were used: 0.04, 0.06, 0.08, 0.12, and 0.16 /h.

Biomass Measurement

The biomass was monitored by either measuring the optical density (OD_{600}) or dry cell weight (DCW) of the culture. To determine the DCW, the cells were harvested by centrifugation. The cell pellet was then washed twice with distilled water and dried at 80°C to derive a constant weight.

Glycerol Measurement

The glycerol concentration in the fermentation broth was measured by high-performance liquid chromatography, HPLC (Shimadzu Ltd., Tokyo, Japan) using an Aminex HPX-87H (Bio-Rad Laboratories, USA) column and refractive index detector (RID). An H_2SO_4 buffer (3 mmol/l) was used as the mobile phase at an isocratic velocity of 0.6 ml/min. Glycerol identification was performed by comparing the retention time with a glycerol standard and the concentration quantified using the profile of known glycerol concentrations.

Fatty Acid Analysis

The total fatty acids were extracted using the direct transmethylation method, as previously described [16]. Heneicosanoic acid (C21:0) was used as the internal standard. The fatty acid methyl esters (FAMEs) were analyzed by gas chromatography (Shimadzu Ltd., Tokyo, Japan) using an OMEGAWAX column (30 m×0.25 mm) and flame ionization detector. The temperatures of the injector, column, and detector were kept at 250, 205, and 260°C, respectively. Helium was used as the carrier gas. The chromatographic peaks of individual fatty acids were identified by comparing their retention times with those of standard FAMEs (Sigma). The areas of the chromatographic peaks were used to calculate the relative amounts of the fatty acids.

Data Analysis

All the experiments were performed in triplicate to ensure reproducible data. The experimental data were statistically analyzed using the Statistical Package for the Social Sciences (SPSS15.0; SPSS Inc.).

RESULTS

Construction of Recombinant Strains of *H. polymorpha* and Analysis of Their Fatty Acid Compositions

A 1,404-bp fragment of the mutated Δ^6-desaturase (S213A) gene of *M. rouxii* was obtained from a PCR using the primers KL-SALD6 and KL-SMAD6. The recombinant plasmid pML194 was constructed by subcloning the amplified fragment downstream of the *MOX* promoter. After transforming pML194 and pHIPX4 into *H. polymorpha*, the fatty acid composition of the yeast transformants was analyzed. Using methanol [2% (v/v)] as the inducer, the Δ^6-desaturated products, octadecadienoic acid (C18:2$\Delta^{6,9}$), GLA, and stearidonic acid (SDA, C18:4$\Delta^{6,9,12,15}$) derived from the conversion of endogenous substrates, oleic acid (C18:1Δ^9), linoleic acid, and α-

Table 1. Fatty acid composition of *H. polymorpha* recombinants carrying pHIPX4 (empty vector) and pML194 grown in minimal medium containing 2% (v/v) methanol.

Culture	Fatty acid composition in total fatty acids (%)									%TFA/DCW (w/w)
	C16:0	C16:1Δ^9	C18:0	C18:1Δ^9	C18:2$\Delta^{6,9}$	C18:2$\Delta^{9,12}$	GLA	ALA	SDA	
pHIPX4	16.0±0.6	1.9±0.1	3.6±0.2	25.2±0.5	0.0±0.0	43.1±1.0	0.0±0.0	10.2±0.4	0.0±0.0	4.1±0.0
pML194	14.3±1.4	1.3±0.3	4.5±0.2	26.3±0.2	3.9±0.0	27.9±0.6	11.9±0.5	7.6±0.4	2.3±0.0	4.0±0.2

linolenic acid (C18:3$\Delta^{9,12,15}$, ALA), respectively, were only observed in the transformant carrying pML194 and not in the control (pHIPX4), as shown in Table 1. This result is consistent with a previous report on the substrate utilization of the *M. rouxii* Δ^6-desaturase [18, 19]. Obviously, GLA represented a major proportion of the Δ^6-desaturated products. After subculturing for more than 20 generations to derive mitotic stability, the pML194 recombinant accumulated high amounts of Δ^6-desaturated fatty acids, including C18:2$\Delta^{6,9}$, GLA, and SDA (Table 1).

Development of High-Cell-Density Fed-Batch Fermentation of GLA-Producing Recombinant

To obtain a high-cell-density of the *H. polymorpha* recombinant carrying the mutated gene of the fungal Δ^6-desaturase (pML194), fed-batch fermentation was performed using glycerol-limited conditions. According to the growth kinetics, the maximum specific growth rate (μ_{max}) of the pML194 recombinant during cultivation using glycerol as the sole carbon source was 0.18–0.21 /h. A predetermined exponential feeding model was used to control the desired specific growth rate at 0.16 /h during the fed-batch phase. During the batch phase, a biomass of 20 g/l was obtained after cultivation for 36 h. When feeding a GF medium into the culture based on the predetermined exponential feeding model, the biomass exponentially increased and reached 80 g/l after 47 h of cultivation, which matched well with the predetermined biomass concentration (R^2>0.9500), as shown in Fig. 1. In addition, the residual glycerol concentration was close to zero during the fed-batch phase, indicating that the μ had been set with reasonable accuracy. The fatty acid analysis showed that the proportion of all Δ^6-desaurated products, 18:2$\Delta^{6,9}$, GLA, and SDA of the pML194 recombinant increased with the cell growth in the batch phase (Table 2). However, a slight decrease in the proportion of GLA and SDA was observed after feeding the medium into the culture for 6–9 h, whereas the content of intracellular total fatty acid continued to increase with the increasing biomass.

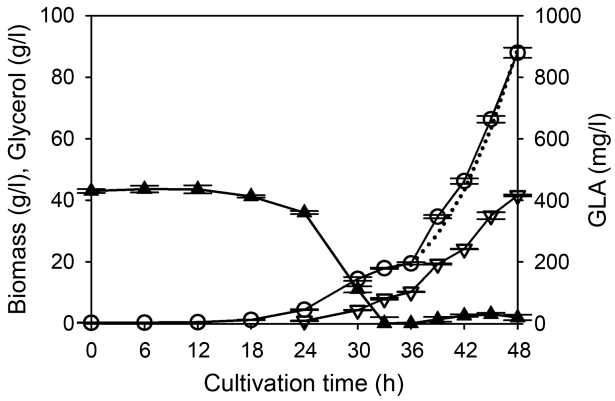

Fig. 1. Fed-batch fermentation of *H. polymorpha* recombinant carrying pML194 with specific growth rate of 0.16 /h.
The glycerol concentration (-▲-), biomass (-○-), and GLA content (-▽-) were determined during the cultivation. The dotted line indicates the predetermined biomass.

Influence of Specific Growth Rate on GLA Content in pML194 Recombinant

The effect of various specific growth rates on the fatty acid composition and GLA level in the *H. polymorpha* recombinant carrying pML194 was investigated. Similar to the fed-batch fermentation using a specific growth rate of 0.16 /h, the biomass of the cultures using other specific growth rates (0.04, 0.06, 0.08, and 0.12 /h) was also close to the predicted model (Fig. 2). Moreover, a growth-dependent change in the fatty acid profile was observed in the cultures using different specific growth rates. The contents of the Δ^6-desaturated fatty acids (C18:2$\Delta^{6,9}$, GLA, and SDA) and total fatty acids in the biomass gradually increased during the cell growth (data not shown). Notwithstanding, differences in both the GLA proportion of TFA and the TFA content in the biomass were found in the cultures. In the high-cell-density phase (80 g/l), the culture with a specific growth rate of 0.08 /h showed the maximal amounts of GLA and TFA, which accounted for 14.0% of the TFA and 6.1% of the dry cell weight, when

Table 2. Fatty acid composition of *H. polymorpha* recombinant carrying pML194 during fed-batch fermentation with specific growth rate of 0.16 /h.

Cultivation time (h)	Fatty acid composition in total fatty acids (%)									%TFA/DCW (w/w)
	C16:0	C16:1Δ^9	C18:0	C 18:1Δ^9	C 18:2$\Delta^{6,9}$	C 18:2$\Delta^{9,12}$	GLA	ALA	SDA	
Batch phase										
12	18.3±0.1	1.6±0.1	8.1±0.1	18.6±0.1	0.0±0.0	41.0±0.1	0.0±0.0	12.4±0.0	0.0±0.0	1.9±0.0
24	15.9±0.1	1.4±0.0	10.6±0.0	17.7±0.1	0.6±0.1	35.7±0.0	7.6±0.0	8.9±0.0	1.6±0.0	2.6±0.0
36	13.7±0.0	1.2±0.0	5.0±0.0	13.9±0.0	0.5±0.1	41.3±0.0	9.1±0.0	13.0±0.0	2.4±0.0	4.7±0.0
Fed-batch phase										
39	14.7±0.3	1.4±0.0	7.5±0.0	20.2±0.1	1.0±0.0	31.8±0.1	11.1±0.0	9.6±0.0	2.7±0.0	5.0±0.1
42	14.4±0.1	1.6±0.0	7.2±0.0	21.6±0.0	1.0±0.0	33.2±0.0	10.4±0.0	8.3±0.1	2.4±0.0	5.0±0.0
45	14.0±0.1	1.6±0.0	6.4±0.0	20.1±0.0	0.7±0.0	37.8±0.0	9.5±0.2	8.2±0.1	1.8±0.0	5.6±0.1
48	14.0±0.4	1.3±0.0	7.4±0.1	19.5±0.1	0.6±0.0	39.7±0.1	8.8±0.1	7.4±0.0	1.4±0.0	5.4±0.1

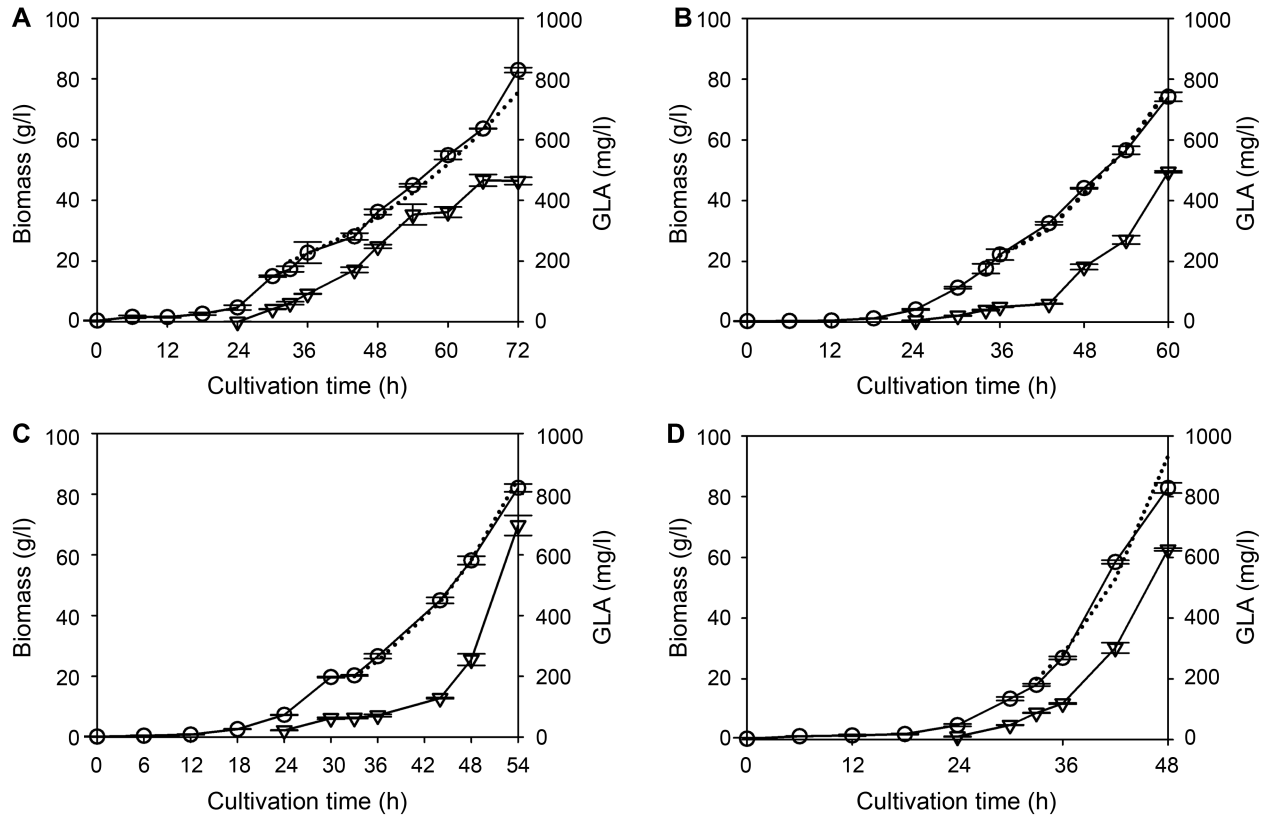

Fig. 2. Fed-batch fermentation of *H. polymorpha* recombinant carrying pML194 with different specific growth rates: 0.04 (**A**), 0.06 (**B**), 0.08 (**C**), and 0.12 (**D**) /h.
The biomass (-○-) and GLA content (-▽-) were determined during the cultivation. The dotted line indicates the predetermined biomass.

compared with the cultures grown at other specific growth rates (Table 3 and Fig. 3A). Therefore, the highest GLA concentration of 697 mg/l was obtained under such conditions (Fig. 3B). As shown in Table 3 and Fig. 3, the culture grown at the high growth rate (0.16 /h) contained a low amount of GLA when compared with the other cultures, owing to a low proportion of GLA in the TFA (8.8%). Therefore, these results suggest that the specific growth rate affected both the fatty acid composition and the lipid content, as expressed by the amount of total fatty acids in the cell dry weight of the *H. polymorpha* recombinant carrying the *M. rouxii* Δ^6-desaturase gene.

DISCUSSION

A methylotrophic yeast able to produce high-value GLA was successfully engineered using the mutated Δ^6-desaturase gene (S213A) of *M. rouxii*. In addition, the GLA content in the *H. polymorpha* recombinant (pML194) was shown to be affected by the cultivation conditions. Based on the function of the *MOX* promoter, the expression of recombinant proteins is generally driven by repression/derepression or methanol-induced mechanisms [8, 11]. However, for the yeast strain in this study, glycerol-limited conditions (derepression) can be used to induce the

Table 3. Fatty acid composition of *H. polymorpha* recombinant carrying pML194 after fed-batch fermentation with different specific growth rates.

μ (/h)	Fatty acid composition in total fatty acids (%)								
	C16:0	C16:1Δ^9	C18:0	C18:1Δ^9	C18:2$\Delta^{6,9}$	C18:2$\Delta^{9,12}$	GLA	ALA	SDA
0.04	13.6±0.2	1.1±0.0	9.4±0.0	25.2±0.0	1.8±0.0	32.9±0.0	12.2±0.1	2.9±0.0	0.9±0.1
0.06	14.1±0.2	1.1±0.1	11.3±0.2	24.3±0.6	1.5±0.2	31.2±0.4	12.6±0.1	2.9±0.1	1.0±0.0
0.08	13.9±0.5	1.4±0.1	6.1±0.1	21.7±0.5	1.4±0.0	35.3±0.9	14.0±0.1	4.9±0.1	1.4±0.1
0.12	14.5±0.0	1.3±0.0	7.3±0.0	20.2±0.0	1.7±0.0	33.8±0.0	13.5±0.0	5.9±0.1	2.0±0.0
0.16	14.0±0.4	1.3±0.0	7.4±0.1	19.5±0.1	0.6±0.0	39.7±0.1	8.8±0.1	7.4±0.0	1.4±0.0

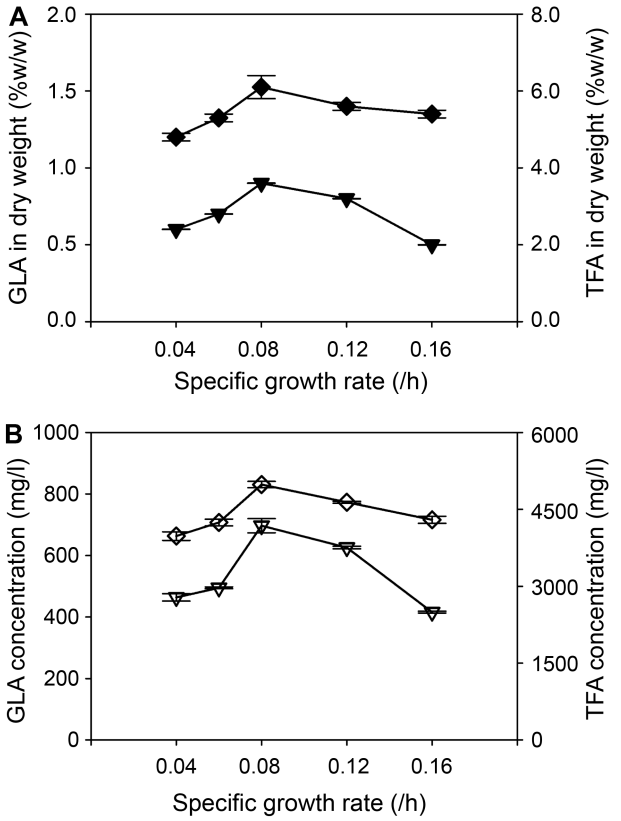

Fig. 3. GLA and TFA production of *H. polymorpha* recombinant carrying pML194 cultivated under glycerol-limited conditions with different specific growth rates.
The GLA and TFA are represented by the inversed triangle and the diamond, respectively.

expression of heterologous genes, without utilizing methanol. Moreover, glycerol can be used as the sole carbon source for *H. polymorpha* growth and it is largely supplied as a by-product from the biodiesel production process. Thus, a substrate feeding strategy was implemented in this study using a derepression mechanism during the fed-batch fermentation of the pML194 recombinant yeast strain. With glycerol as the sole carbon source, a high-cell-density culture (about 80 g/l) was achieved when controlling the exponential feeding rate at different specific growth rates. The establishment of derepression conditions, as indicated by a decrease in the glycerol concentration in the culture broth, was also found to lead to a significant increase in the GLA proportion during the growth in the batch phase (Table 2 and Fig. 1). A previous study also reported the occurrence of heterologous gene expression in *H. polymorpha* under the *MOX* promoter with a restrictively low glycerol concentration [6]. Thus, a glycerol-limited feeding mode not only promotes a high-cell-density culture, but can also be used to maintain the level of GLA in the culture.

It is noteworthy that the GLA and TFA production in the *H. polymorpha* recombinant carrying pML194 depended on the specific growth rate, which is a key parameter for controlling the glycerol feeding rate during the fed-batch phase. The relationship between the specific growth rate and the product (GLA) content in the pML194 recombinant yeast (Fig. 3) was also similar to the production of other recombinant biologically active compounds in methylotrophic yeast systems using alcohol oxidase promoters at different specific growth rates [25, 27, 35]. With the exception of the culture that used a 0.16 /h specific growth rate, there was not much difference in the fatty acid composition of the cultures that used different specific growth rates (Table 3). Thus, the GLA content was indirectly related to the specific growth rate, which was largely dependent on the TFA content in the cells grown under such conditions. One could also conclude that the specific growth rate greatly influenced the lipid biosynthetic process in the *H. polymorpha*. Although *H. polymorpha* is a non-oleaginous yeast, the pML194 strain accumulated a higher intracellular TFA content after prolonged cultivation in a batch-phase culture; this accumulation was also seen in a previous study of oleaginous fungi [23]. In a continuous culture of oleaginous microorganisms, lipid accumulation requires a slow growth rate (μ=0.05 /h) to allow the cells to assimilate the carbon source [22, 32]. In this study, the sufficiently low growth rate for optimizing the GLA production by the fed-batch fermentation of the pML194 recombinant was found to be 0.08 /h, which enhanced both the proportion of GLA in the TFA as well as the content of TFA. It is also interesting to note that the fatty acid profile and composition of the *H. polymorpha* recombinant carrying the fungal Δ^6-desaturase were similar to those of blackcurrant seed oil, containing similar proportions of *n*-3 (ALA and SDA) and *n*-6 (LA and GLA) PUFAs [2].

In conclusion, this study showed that *H. polymorpha* is able to synthesize the essential fatty acids, GLA and SDA, based on the overexpression of a fungal Δ^6-desaturase that possesses a high enzymatic activity. When combining the expression control of the *MOX* promoter and a glycerol-limited feeding mode, high-cell-density cultivation of the *H. polymorpha* recombinant was derived that enhanced the GLA content by optimizing the specific growth rate of the fed-batch fermentation.

Acknowledgments

The authors are grateful to Assoc. Prof. Dr. Supapon Cheevadhanarak, Dr. Chanan Phonprapai, and Dr. Theppanya Charoenrat for their useful comments on this work. Bhimabol Khongto was partially supported by the King Mongkut's Brilliant Student Scholarship from King Mongkut's University of Technology Thonburi. This work

was supported by a grant from National Research University, Thailand.

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