Industrial Production of Dihomo-γ-linolenic Acid by a Δ5 Desaturase-defective Mutant of *Mortierella alpina* 1S-4 Fungus[1]

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ABSTRACT: Dihomo-γ-linolenic acid (DGLA)-containing oil (triacylglycerol) was produced by the fungus *Mortierella alpina* S14, which is a Δ5 desaturase-defective mutant of the arachidonic acid-producing strain 1S-4. Using soy flour as the nitrogen source, S14 produced 8.1 g DGLA/L of culture medium in a 50-L jar fermenter. Shifting the cultivation temperature from 26 to 28°C resulted in reduction of the percentage of DGLA in total fatty acids. Under optimal conditions in a 10-kL industrial fermenter, DGLA production reached 7.0 g/L (percentage of DGLA, 43.9%) at day 12 of cultivation. The other fatty acids were palmitic (18.2%), stearic (7.9%), oleic (7.5%), linoleic (4.4%), γ-linolenic (3.2%), arachidonic (0.4%), and lignoceric (7.7%) acids.

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KEY WORDS: Arachidonic acid, Δ5 desaturase, DGLA, dihomo-γ-linolenic acid, *Mortierella,* sesamin.

Dihomo-γ-linolenic acid (8,11,14-eicosatrienoic acid, DGLA) is a precursor for arachidonic acid (AA) biosynthesis. In animals, DGLA is converted to the prostaglandin (PG) 1 group, which has many unique biological activities. DGLA and DGLA-containing oil (triacylglycerol) were reported to exhibit many activities, such as anti-inflammatory (1), antithrombotic (2), antihypertensive (3), and antiallergic (4) activities. Recently, γ-linolenic acid (GLA)-containing oils, such as evening primrose oil and borage oil, have been used to treat allergy symptoms, and their efficacy was proven (5,6). These effects of GLA are thought to result from DGLA and PG 1 group, which are synthesized from GLA in the human body. Since DGLA and PG 1 group are the main factors responsible for many efficacies, and sometimes GLA is not efficiently converted to DGLA in the human body (7), DGLA-containing oil is expected to be a new and much better functional oil. However, there is no commercial source of DGLA to date.

We have established a method for the microbial production of AA-containing oil by the fungus *Mortierella alpina* 1S-4 in a 10-kL industrial fermenter. This AA-containing oil is used as a component of infant milk and functional foods (8). By modifying this process, we have developed two methods for DGLA production, based on blocking the Δ5 desaturation activity that catalyzes the conversion of DGLA to AA. One involves the addition of a Δ5 desaturase inhibitor, such as lignan compounds from sesame seed oil (9,10) or curcumin from turmeric spice (11), to the culture medium. The other involves the cultivation of Δ5 desaturase-defective mutants, Mut44 (12,13) or S14 (14), instead of the AA-producing 1S-4 strain. The latter is a better method, because DGLA productivity is higher and the addition of a Δ5 desaturase inhibitor is not necessary.

Here we optimized the culture conditions for DGLA production by a Δ5 desaturase-defective mutant S14 in a 10-kL industrial fermenter. This process will enable the commercial production of DGLA-containing oil.

MATERIALS AND METHODS

Microorganism and culture conditions. Mortierella alpina S14 (14), a Δ5 desaturase-defective mutant derived from *M. alpina* 1S-4, was used in this study. For the jar fermenter study, an inoculum was prepared in a 500-mL flask containing 100 mL medium with 2% glucose and 1% yeast extract, with shaking for 3 d at 28°C. The main culture was grown in a 50-L jar fermenter (Mitsuwa Bio Systems Co., Ltd., Osaka, Japan) containing 25 L of medium. Basal medium contained 3.1% soy flour, 2% glucose, 0.1% soybean oil, 0.3% KH_2PO_4, 0.1% Na_2SO_4, 0.05% $CaCl_2{\cdot}2H_2O$, and 0.05% $MgCl_2{\cdot}6H_2O$, pH 6.0. The agitation speed was changed during cultivation to maintain the dissolved oxygen (DO) concentration at 10–15 ppm. DO concentration was monitored with a DO electrode (Ingold, Urdorf, Switzerland). The cultivation temperature is indicated in the text. The other conditions were as follows: inoculation rate, 0.5%; aeration rate, 25 L/min; and headspace pressure, 200 kPa.

For the industrial study, an inoculum was prepared in a

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50-L jar fermenter containing 25 L of medium with 2% glucose, 1% yeast extract, and 0.1% soybean oil, followed by cultivation for 2 d at 28°C. The main culture was carried out in a 10-kL fermenter (Kansai Chemical Engineering Co., Hyogo, Japan), with a working volume of 4 kL. The medium contained 3.1% soy flour, 2% glucose, 0.1% soybean oil, 0.3% KH_2PO_4, 0.1% Na_2SO_4, 0.05% $CaCl_2 \cdot 2H_2O$, and 0.05% $MgCl_2 \cdot 6H_2O$, pH 6.0. The agitation speed was controlled in the range 27–90 rpm to maintain DO concentration. The other conditions were as follows: temperature, 26°C; inoculation rate, 0.5%; aeration rate, 240 m^3/min; and headspace pressure, 200 kPa.

Analytical methods. Cell growth was expressed as dry cell weight, after suction filtration, washing with distilled water, and drying at 105°C for 2 h.

For analyzing fatty acids in total cellular lipids, dry cells were transmethylated in methanolic HCl, and then the fatty acid methyl esters were extracted and quantified by gas–liquid chromatography as described (15).

RESULTS AND DISCUSSION

Fermentation in 50-L jar. In the previous study with a 5-L jar fermenter, *M. alpina* S14 produced 2.4 g DGLA/L of culture medium with 1% yeast extract as the nitrogen source (14). Here we used soy flour as the nitrogen source, because the parent strain *M. alpina* 1S-4 produced a large amount of AA (10.9 g/L) using soy flour (8). *Mortierella alpina* S14 was cultivated at 26°C for 7 d in a 50-L jar fermenter containing 25 L medium with 3.1% soy flour and 2% glucose (Fig. 1). Dry cell weight reached 44.5 g/L of culture medium on day 7 of cultivation, followed by an increase in DGLA production. At day 7 of cultivation, DGLA production reached 8.1 g/L of culture medium (183 mg/g dry cells). The percentage of DGLA in the total fatty acids increased also and was 43.1% at day 7 of cultivation. Therefore, soy flour is suitable for DGLA production by S14 as well as AA production by 1S-4.

Effect of cultivation temperature. Desaturation reactions are activated at low temperature (16). Since Δ5 desaturation is not related to DGLA production, the cultivation temperature may be shifted to higher temperature. The cultivation temperature was shifted from 26 to 28°C at day 2 of cultivation (Table 1). Although dry cell weight and production of total fatty acids were similar between cultivations at 26 and 28°C, the percentage of DGLA was higher for cultivation at 26°C (43.1%) than for that at 28°C (39.8%). On the other hand, the percentage of linoleic acid was lower for cultivation at 26°C (5.6%) than for that at 28°C (9.0%). This result suggests that the cultivation temperature has a great effect on the biosynthesis of DGLA, even if the difference is only 2°C. Δ6 desaturation from linoleic acid to GLA seems to be most significantly affected.

Industrial fermentation, 10-kL scale. DGLA production was carried out in a 10-kL industrial fermenter using soy flour as the nitrogen source. The cultivation of *M. alpina* S14 at 26°C for 12 d gave 7.0 g of DGLA/L of culture medium (167 mg/g dry cells) (Fig. 2). The percentage of DGLA in total fatty acids increased to 40.0% at day 6 of cultivation, and reached 43.9% at day 12 of cultivation. The fatty acid composition of the mycelial lipids at day 12 of cultivation was as follows: palmitic acid (18.2%), stearic acid (7.9%), oleic acid (7.5%), linoleic acid (4.4%), GLA (3.2%), DGLA (43.9%), AA (0.4%), lignoceric acid (7.7%) and others (6.8%).

We have developed various methods for microbial production of DGLA, as shown in Table 2. First, DGLA was obtained as a by-product of AA production by strain 1S-4.

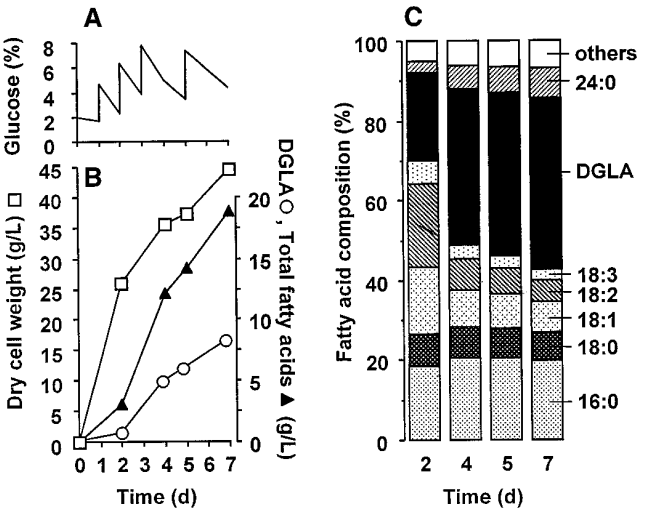

FIG. 1. Time course of dihomo-γ-linolenic acid (DGLA) production by *Mortierella alpina* S14 cultivated in 50-L jar fermenter. (A) Glucose concentration in the culture medium; (B) dry cell weight (□), production of total fatty acids (▲), and DGLA production (○); and (C) fatty acid composition. Abbreviations: 16:0, palmitic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 18:3, γ-linolenic acid; and 24:0, lignoceric acid.

TABLE 1
Effect of Temperature Shift from 26 to 28¡C

	Temperature (°C)	
	26[a]	26→28[b]
Dry cell weight (g/L)	44.5	46.8
Total fatty acids (g/L)	18.9	18.9
DGLA (g/L)	8.1	7.5
Fatty acid composition (%)[c]		
16:0	19.7	19.5
18:0	7.2	7.2
18:1	7.5	8.6
18:2	5.6	9.0
18:3	2.8	2.7
20:3	43.1	39.8
24:0	7.3	7.0
Others	6.8	6.2

[a]*Mortierella alpina* S14 was cultivated in a 50-L jar fermenter at 26°C for 7 d.
[b]*Mortierella alpina* S14 was cultivated in a 50-L jar fermenter at 26°C for 2 d and then at 28°C for a further 5 d.
[c] 16:0, palmitic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 18:3, γ-linolenic acid; 20:3, dihomo-γ-linolenic acid (DGLA); 24:0, lignoceric acid.

TABLE 2
Comparison of DGLA Production by *Mortierella alpina* Resulting from Various Methods

Strain	Blocking of Δ5 desaturase	DCW (g/L)	DGLA (g/L)	DGLA (%)	AA (g/L)	AA (%)	Scale	Reference
1S-4	None	50.6	0.9	3.8	10.9	45.0	10-kL	8
1S-4	Curcumin	12.1	0.3	10.5	0.6	19.1	10-mL	11
1S-4	Sesame oil	20.3	2.2	23.1	1.1	11.2	50-L	9
1S-4	Sesamin etc.	26.0	2.6	26.8	1.4	14.5	50-L	Unpublished[b]
Mut44	Δ5 DS defect	26.0	3.2	23.4	0.5	3.7	10-L	13
Mut44	Δ5 DS defect + sesamin etc.	28.4	2.1	16.1	0.2	1.3	10-L	19
S14	Δ5 DS defect	17.1	2.4	43.3	0.1	1.5	5-L	14
S14	Δ5 DS defect	41.7	7.0	43.9	0.1	0.4	10-kL	This study

[a]Volume of fermenter or flask.
[b]Unpublished data (Akimoto, K., H. Kawashima, and S. Shimizu). Abbreviations: AA, arachidonic acid; DCW, dry cell weight; DS, desaturase. For other abbreviations, see Table 1.

DGLA productivity and the percentage of DGLA were very low (8,17). Then, we found sesamin, its related lignans (9,10), and curcumin (11,18) to be potent Δ5 desaturase-specific inhibitors, and added them to the culture medium to obtain DGLA-enriched oil. Under optimal conditions, when a mixture of sesamin and episesamin was added at the start of cultivation and at days 1 and 3 thereafter (75 mg/L × 3), *M. alpina* 1S-4 produced 2.6 g of DGLA/L of culture medium in a 50-L jar fermenter (unpublished data, Akimoto, K., H. Kawashima, and S. Shimizu). The percentage of DGLA in total fatty acids was 26.8%, while that of AA was only 14.5%. In using this method, oil containing a large amount of DGLA and a small amount of AA was obtained for the first time.

Furthermore, we obtained two Δ5 desaturase-defective mutants, Mut44 (12,13) and S14 (14). These mutants produced a large amount of DGLA without sesamin addition. For the present study, we used the S14 strain, which has little Δ5 desaturase activity and higher productivity than Mut44, and optimized the culture conditions in a 10-kL fermenter. Through this study, the industrial production of DGLA has been enabled, as well as AA production. Microbial AA-containing oil is already in use as a component of infant milk and functional foods. In the future, DGLA-containing oil consisting of more than 40% DGLA and less than 1% AA will be applied to various functional foods and medicines for human health.

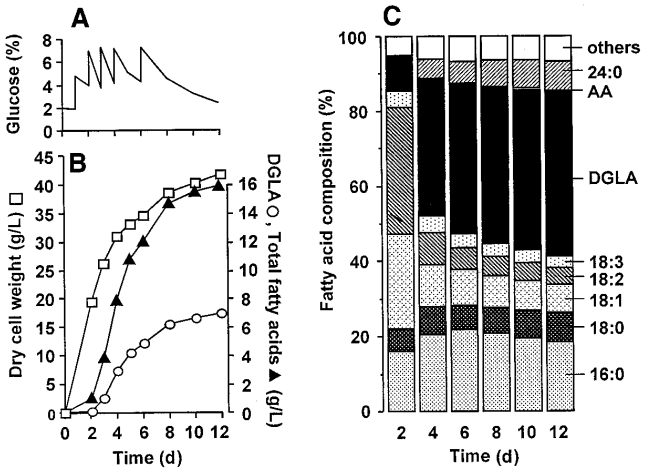

FIG. 2. Time course of DGLA production by *M. alpina* S14 cultivated in 10-kL jar fermenter. (A) Glucose concentration in the culture medium; (B) dry cell weight (□), production of total fatty acids (▲), and DGLA production (○); and (C) fatty acid composition. AA, arachidonic acid; other abbreviations are shown in Figure 1.

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