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Effect of culture conditions on docosahexaenoic acid production by *Schizochytrium* sp. S31

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Abstract

The effect of culture conditions, pH, carbon sources and nitrogen sources on the production of docosahexaenoic acid, the fatty-acid profiles and the secreted acids in the broth by the oleaginous microorganism, *Schizochytrium* sp. S31, were studied at 30 °C with orbital shaking. Docosahexaenoic acid was the main omega-3 polyunsaturated fatty acid produced. The monosaccharides, glucose and fructose and yeast extract were suitable carbon and nitrogen sources, respectively. The fatty acids in the organism and the secreted acids in the broth by the organism were changed dynamically during cultivation. *Schizochytrium* sp. S31 could accumulate lipid up to 40% of biomass and 13% (w/w) DHA content of lipid at pH 7.0. The more productive culture conditions for docosahexaenoic acid production by *Schizochytrium* sp. S31 were an initial pH of 7.0 with 20 g L^{-1} glucose and 0.4% (w/v) yeast extract in 4-day incubation.
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1. Introduction

Polyunsaturated fatty acids (PUFAs), one type of fatty acids, are important for human physiology. This is especially true for the long-chain ω-3 PUFAs, eicosapentaenoic acid (EPA; 20:5) and docosahexaenoic acid (DHA; 22:6), which have been scientifically recognized in human nutrition and for the prevention of diseases. DHA is an important compound incorporated into the developing brain, particularly in newborn infants. The balance of PUFAs in the body is believed to help maintain the normal functioning of the organs. They can prevent abnormalities in the immune system, the cardiovascular system, the inflammatory system, the endocrine system and the nervous system [1,2]. A high content of DHA in the brain and retina is important for proper nervous system and visual function [3]. Recently, some articles have reported that long-chain ω-3 polyunsaturated fatty acids can act as breast and colon cancer chemopreventive agents [4,5].

Mammals lack the enzymes needed to synthesize long-chain ω-3 PUFAs, such as EPA and DHA and therefore, these must be supplied in the diet. Owing to the medical and nutritional importance of PUFAs, the demand for naturally occurring fats and oils containing these fatty acids has increased dramatically. Concentrated marine fish oil is the main source of PUFAs. But there are a number of problems with fish oil including a highly saturated fatty-acid content, odor, taste, stability and a complicated purification process. Alternatively, some oleaginous microorganisms have the ability to synthesize long-chain PUFAs. Certain marine bacteria, phototrophic and heterotropic algae, fungi and mosses are abundant in various PUFAs and these have been used as the raw material for preparation of ω-3 PUFAs [6,7]. The fungi, *Thraustochytrium* and *Entomophthora* spp., can accumulate relatively large amounts of DHA [8]. The marine microalgae, *Isochrysis galbana* [9] and *Nannochloropsis* sp. [10], have been proposed for commercial production of EPA. Chlorophyll containing callus cells of

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Marchantia polymorpha are able to produce PUFAs, including EPA [11].

Some species of the order Thraustochytriale, namely *Thraustochytrium aureum* [12], *Thraustochytrium roseum* [13] and *Thraustochytrium* sp. ATCC 20892 [14] produce significant quantities of DHA. *Schizochytrium* sp. S31 belongs to the order Thraustochytriale and is a spherical unicellular microorganism. Unicellular microorganisms have been favored for study as they are much easier to cultivate than filamentous moulds. In this study, the influence of medium composition on cell growth, the production of lipids and PUFAs and the secretion of acids into the broth have been investigated in *Schizochytrium* sp. S31.

2. Materials and methods

2.1. Organism and cultivation medium

The strain *Schizochytrium* sp. S31 (ATCC 20888) was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) through the Culture Collection & Research Center (CCRC, Food Industry Research and Development Institute, Taiwan, ROC). The strain was maintained on nutrient agar slants and subcultured every 2 months. The basal medium was modified from Bajpai et al. [15] and consisted of NaCl, 25 g L^{-1}; KH_2PO_4, 1 g L^{-1}; $MgSO_4{\cdot}7H_2O$, 5 g L^{-1}; $CaCl_2$, 0.2 g L^{-1} and KCl, 1.0 g L^{-1}. The trace mineral solution contained per liter, Na_2EDTA, 6.0 g; $FeCl_3{\cdot}6H_2O$, 0.29 g; H_3BO_3, 6.84 g; $MnCl_2{\cdot}4H_2O$, 0.86 g; $ZnCl_2$, 0.06 g; $CoCl_2{\cdot}6H_2O$, 0.026 g and $CuSO_4{\cdot}5H_2O$, 0.002 g [16].

2.2. Cultivation conditions

Cultures were grown in 250 mL Erlenmeyer flasks with 50 mL of basal medium and the experimental carbon and nitrogen sources. The initial pH of the culture was controlled with buffer (potassium dihydrogen phosphate–sodium hydroxide). The inoculum was prepared in an Erlenmeyer flask containing basal medium with 2% (w/v) glucose and 0.4% (w/v) yeast extract for 48 h, which the organism was at exponential growth phase. Cultures were inoculated with this at a rate of 5% (v/v) and incubated on an orbital shaker at 150 rpm at 30 °C. Results in this study were average values based on the simultaneous cultivation of three replicates.

2.3. Biomass, glucose, secreted acids and nitrogen measurements

The dry weight of biomass was determined by centrifuging the cell suspension, washing twice with 1% (w/v) NaCl solution, once with distilled water and drying at 100 °C for 15 h. The concentrations of initial glucose, residual glucose and secreted acids in the broth were analyzed by high-performance liquid chromatography (Jasco, Model: PU-980) using an Aminex HPX-87H 300 mm × 7.8 mm column. An aliquot of sample was treated with acetonitrile and centrifuged at 12,000 rpm for 5 min to remove the cells and impurities. The mobile phase was 5 mM sulphuric acid, run at 0.6 mL min^{-1}. Two different detectors were used for measurements: a refractive index (RI) detector for glucose and an ultraviolet-visible (UV–vis) detector for secreted acids at a wavelength of 210 nm. Calibration curves of glucose, malic acid, citric acid, pyruvic acid, succinic acid and fumaric acid were established to calculate the concentration of the initial and residual glucose and secreted acids in the broth. Nitrogen content in the yeast extract and residual nitrogen in the medium was determined by micro-Kjeldhal method.

2.4. Lipid extraction, methylation and determination

An aliquot of cell suspension was harvested by centrifugation, washed twice with 1% (w/v) NaCl, once with distilled water and dried by vacuum oven at 40 °C. Lipid in the dried cells was extracted with chloroform:-methanol mix (2:1, v/v) for 2 h and re-extracted with the same mix two–three times until the extract was colorless. The extract was vaporized at room temperature and dried at 70 °C for 20 min. Tricosanoic acid ($C_{23}H_{46}O_2$) was used as the internal standard for fatty-acids analysis by gas chromatography. Extracted lipid was saponified by methanolic base (2N NaOH in methanol), methylized by methanolic acid (2.5N HCl in methanol) and extracted by *n*-hexane. Fatty acid methyl esters were analyzed by gas chromatography (Hitachi, model: 263-50) using a GP 10% SP-2330 column (Supelco, USA). The GC analytical conditions were carrier gas: N_2, gas rate: 20 mL min^{-1}; oven condition, initial temperature: 190 °C, temperature rate: 4 °C min^{-1}, final temperature: 240 °C; temperature of injection port and flame ionization detector: 240 °C.

3. Results

3.1. Biomass, lipid and DHA production

The experiment was performed in 1 L flasks with 200 mL of basal medium, 20 g L^{-1} (2%, w/v) glucose and 4 g L^{-1} (0.4%, w/v) yeast extract. An aliquot of 20 mL of cell suspension was taken from the culture for analysis every day for 9 days. The time course of biomass and lipid production by *Schizochytrium* sp. S31 is illustrated in Fig. 1a and increased to a maximum at 4–5 days, then decreased. Glucose was completely depleted on day 5 that the biomass, lipid and DHA production decreased. The results were similar to those for *Thraustochytrium* sp. ATCC 20892 [14].

The fatty-acid profiles changed during cultivation are shown in Fig. 1b. DHA production had a maximum of

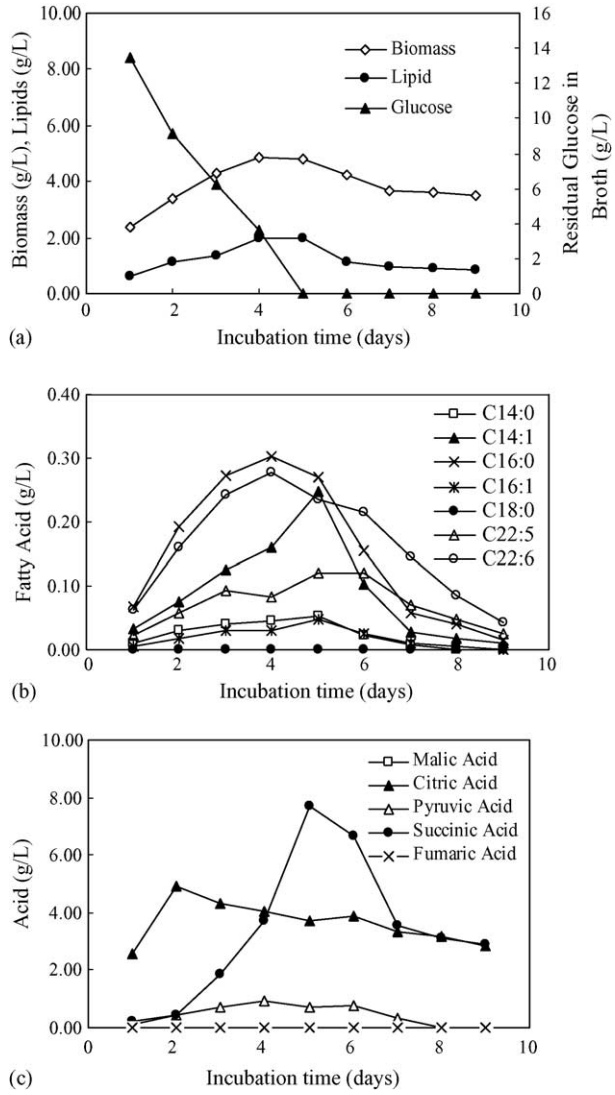

Fig. 1. Time course of (a) biomass, lipid production and glucose concentration in the broth, (b) fatty-acid yields and (c) secreted acids in the broth by *Schizochytrium* sp. S31.

288.6 mg L^{-1} and 59.3 mg g^{-1} of biomass on day 4. After the carbon source (glucose) was consumed completely, almost all the fatty-acid yields began to decrease. A low amount of C18 fatty acids was produced by *Schizochytrium* sp. S31.

Schizochytrium sp. S31 secreted some intermediate metabolic acids into the broth, as seen in Fig. 1c. Succinic acid was secreted at significant levels into the broth on day 5.

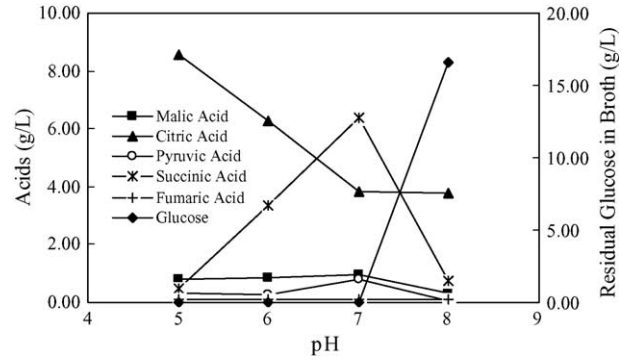

Fig. 2. Effect of initial culture pH on secreted acids in the broth by *Schizochytrium* sp. S31.

Citric acid and pyruvic acid were also present in the broth and their concentration changed during cultivation. There were only trace amounts of malic acid and fumaric acid in the broth.

3.2. Effect of initial culture pH

When the initial culture pH was varied between pH 5 and 8, maximum biomass (6.01 g L^{-1}) and DHA yield (314.2 mg L^{-1}) were observed at pH 7.0 (Table 1). The strain did not grow well at an initial culture pH of 8.0. *Schizochytrium* sp. S31 could accumulate lipid up to 40% of biomass and 13% (w/w) DHA content of lipid at pH 7.0. The secreted intermediate metabolic acids and residual glucose in the broth are shown in Fig. 2. A lower initial culture pH produced a higher concentration of secreted citric acid in the broth, but resulted in a reduction in the succinic acid secreted. Secreted pyruvic acid in the broth was about 0.8 g L^{-1} at pH 7.0. The amount of malic acid secreted in all the different pH media was the same (1 g L^{-1}), except at pH 8.0.

3.3. Effect of medium composition

The influence of varying carbon sources in the broth was illustrated in Table 2. The monosaccharides, glucose and fructose were suitable for the cell growth. Glucose provided the most effective carbon source for biomass, lipid and DHA productions. The disaccharides, lactose, maltose, sucrose and the polysaccharide, soluble starch, could all be used by *Schizochytrium* sp. S31 for cell growth and lipid production, but were not as good for DHA production.

Table 1
Effect of initial culture pH on biomass, lipid and DHA production

Initial pH	Biomass (g L^{-1})	Lipid (g L^{-1})	Lipid in biomass (%, w/w)	DHA in biomass (%, w/w)	DHA in lipid (%, w/w)	DHA yield (g L^{-1})
5	3.72	1.44	38.80	4.52	11.65	0.168
6	4.97	1.88	37.71	4.13	10.97	0.206
7	6.01	2.38	39.59	5.23	13.22	0.314
8	Trace	Trace	Trace	Trace	Trace	Trace

Table 2
Effect of carbon sources on biomass, lipid and DHA production

Carbon sources	Biomass (g L^{-1})	Lipids (g L^{-1})	Lipid in biomass (%, w/w)	DHA in biomass (%, w/w)	DHA in lipid (%, w/w)	DHA yield (g L^{-1})
Monosaccharides						
Glucose	5.510	2.437	44.23	5.60	12.65	0.308
Fructose	5.245	1.765	33.65	4.79	14.24	0.251
Disaccharides						
Lactose	2.785	0.050	1.80	Trace	Trace	Trace
Maltose	3.090	0.015	0.48	Trace	Trace	Trace
Sucrose	3.045	0.153	5.01	Trace	Trace	Trace
Polysaccharide						
Soluble starch	3.425	0.105	3.07	Trace	Trace	Trace

Table 3
Effect of nitrogen sources on biomass, lipid and DHA production

Nitrogen sources	Biomass (g L^{-1})	Lipids (g L^{-1})	Lipids in biomass (%, w/w)	DHA in biomass (%, w/w)	DHA in lipids (%, w/w)	DHA in yield (g L^{-1})
Complex nitrogen						
Tryptone	2.133	0.505	23.68	5.48	32.52	0.178
Peptone	5.220	1.755	33.62	1.61	4.92	0.084
Yeast extract	6.108	2.063	33.77	5.26	15.19	0.314
Defined nitrogen						
Urea	2.725	1.183	43.39	7.13	16.48	0.194
Monosodium glutamate	6.585	2.195	33.33	9.94	26.58	0.294
Sodium nitrate	7.459	2.403	32.21	2.14	6.86	0.163
Ammonium chloride	6.230	1.223	19.62	7.78	36.03	0.270

Complex medium was added 0.4% (w/v) and defined medium was added 0.1% nitrogen (w/v).

Seven different nitrogen sources were tested (Table 3). Among the three complex nitrogen sources, yeast extract was the most effective for biomass, lipid and DHA production. Among the defined nitrogen sources, monosodium glutamate and ammonium chloride had the same potential for DHA production.

The effect of glucose concentration was studied in basal medium supplemented with 0.4% (w/v) yeast extract (Table 4). Increasing the initial glucose concentration in the medium increased the biomass production. Lipid and DHA production increased up to 20 g L^{-1} glucose (Fig. 3a). The concentration of C16:0 fatty acid remained constant when the glucose content was above 20 g L^{-1}. The other fatty acids were only synthesized in small amounts. The concentration of secreted succinic acid in the broth was maximal at 20 g glucose L^{-1} (Fig. 3b). Citric acid was higher at a low glucose concentration and decreased on reaching 40 g glucose L^{-1}. Pyruvic acid remained almost constant at different glucose concentrations. Above 40 g glucose L^{-1}, malic acid secretion increased.

The effect of yeast extract concentration on *Schizochytrium* sp. S31 is illustrated in Table 5. Increasing the concentration of yeast extract increased the biomass and lipid production and a maximum was obtained at 0.4% (w/v) yeast extract. Medium with 0.4% (w/v) of yeast extract had the highest DHA production.

4. Discussion

Schizochytrium sp. S31 is a spherical single-cell organism and growth and metabolites production are influenced by cultivation conditions. The DHA yield and

Table 4
Effect of glucose content in the medium on biomass, lipid and DHA production

Glucose (g L^{-1})	Biomass (g L^{-1})	Lipid (g L^{-1})	Lipid in biomass (%, w/w)	DHA in biomass (%, w/w)	DHA in lipid (%, w/w)
5	2.75	0.37	13.38	0.80	5.99
10	3.86	1.65	42.80	3.14	7.35
20	5.46	2.43	44.48	5.82	13.09
40	5.86	2.21	37.63	5.13	13.64
60	6.56	2.24	34.10	4.43	12.98
80	6.46	2.20	34.06	4.36	12.80

Table 5
Effect of yeast extract concentration on biomass, lipid and DHA production

Yeast extract (%, w/v)	Biomass (g L^{-1})	Lipid (g L^{-1})	Lipid in biomass (%, w/w)	DHA in biomass (%, w/w)	DHA in lipid (%, w/w)	DHA yield (g L^{-1})
0.1	5.060	1.260	24.90	3.51	14.10	0.178
0.2	5.553	2.035	36.65	4.02	10.97	0.223
0.4	5.900	2.042	34.61	5.57	16.08	0.328
0.6	4.823	1.307	27.10	5.08	18.75	0.245
0.8	4.963	1.418	28.57	3.34	11.69	0.166

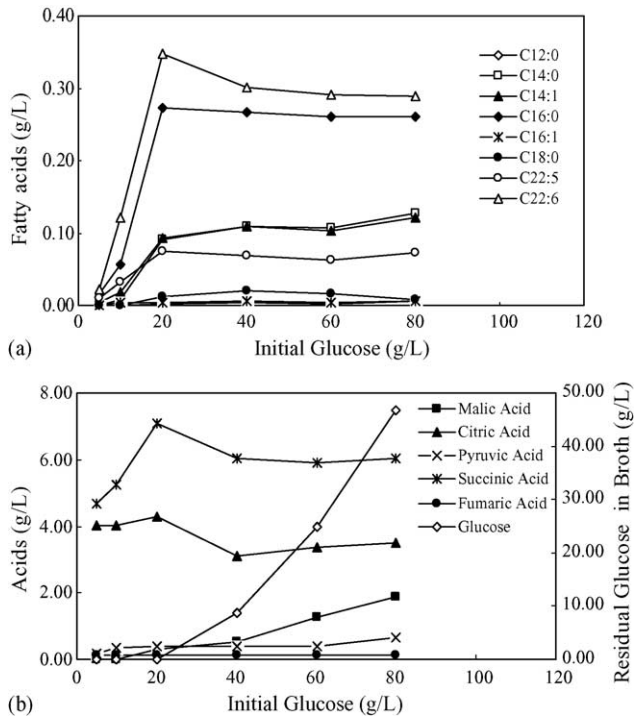

Fig. 3. Effect of glucose concentration on (a) the fatty-acid profiles and (b) the secreted acids in the broth by *Schizochytrium* sp. S31.

the predominant fatty acid produced were about 300 mg L^{-1} and C16:0 fatty acid, respectively. There were lower yields of the various C18 fatty acids by *Schizochytrium* sp. S31 than by *Thraustochytrium* sp. ATCC 20892 [14] and by *T. roseum* [13]. The same strain was studied by Singh et al. and had a lower DHA yield (<100 mg L^{-1}) under pH 7 and 25 °C after 5 days orbital shaking at 200 rpm [14]. The different results illustrated that varying the cultivation conditions profoundly affects cell growth and fatty-acid production by these microorganisms.

Glucose supplied in the medium was not the only available carbon source for *Schizochytrium* sp. S31 during cultivation. When the microorganisms had exhausted all its supply of carbon in the medium, they began to consume their own reserves of lipids in the cell [17] as well as the metabolic acids secreted in the broth. The accumulated lipids and fatty acids within oleaginous microorganisms are, therefore, dynamic storage materials and are produced in times of plenty when carbon is sufficient and utilized in times of starvation when carbon is deficient.

The initial glucose concentration in the medium affected the growth of the organism, the fatty-acid synthesis and the secreted acids in the broth. The microorganism synthesized fatty acids and secreted acids into the broth when the glucose concentration was more than 40 g L^{-1} in the medium. To obtain higher DHA production and avoid wasting carbon source, the most suitable amount of added carbon source must be investigated before production of the desired product.

At the lower initial pHs used for cultivation, pH 5.0 and 6.0, the microorganism consumed the carbon source completely in an identical manner to cultivation at pH 7.0. The production of lipid and DHA by *Schizochytrium* sp. S31 incubated at initial pH 5.0 and 6.0 was low. These indicated that some carbon source was used to maintain their physiological condition at the less than optimal pH instead of cell growth, lipid and fatty-acid production. An initial pH of 8.0 for cultivation was too basic for growth and there was a high residual glucose, 16 g L^{-1}, in the medium after 4 days cultivation.

The results presented in this study illustrated that the organism's growth, the lipid profile and the fatty-acid profile of *Schizochytrium* sp. S31 can be enhanced by adjusting cultivation time, initial pH and medium composition. The dynamic interactions among the growth, fatty-acid synthesis and nutrient limitation result in significant variations in the fatty-acid profile and DHA production. The more productive culture conditions for docosahexaenoic acid production by *Schizochytrium* sp. S31 in this work was an initial pH of 7.0 with 20 g L^{-1} glucose and 0.4% (w/v) yeast extract in 4-day incubation.

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