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Increase of Docosahexaenoic Acid Production by *Schizochytrium* sp. Through Mutagenesis and Enzyme Assay

Min Lian · He Huang · Lujing Ren · Xiaojun Ji · Jingyao Zhu · Lijing Jin

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Abstract The present study focused on improving docosahexaenoic acid (DHA) production by *Schizochytrium* sp. through *N*-methyl-*N*-nitro-*N*-nitrisiguanidine treatment coupled with ultraviolet radiation based on the metabolic pathway analysis. The activity of glucose-6-phosphate dehydrogenase of the mutant was higher than the parent strain, which indicated that the hexose monophosphate pathway of the mutant was strengthened, and more NADPH was thus produced. Also, the activities of malic enzyme and ATP–citrate lyase in the cell extract of the mutant were higher than the parent strain, which indicated that the screening method increased NADPH and acetyl–CoA supply in vivo effectively. Finally, in the batch culturing of the mutant, 34.84% higher lipid was accumulated with the cell dry weight at the same level compared with the parent strain. Moreover, the DHA percentage of the total fatty acids up to 56.22% was achieved using the mutant, which was 38.88% higher than the parent strain. When the cultures were maintained under appropriate conditions, the final DHA yield was 0.20 and 0.11 g/g dry biomass, for the mutant and parent, respectively.

Keywords Docosahexaenoic acid · *Schizochytrium* sp. · Mutagenesis · Enzyme activity

Introduction

Docosahexaenoic acid (DHA, C22:6), a primary ω-3 structural fatty acid, exists in most of the highly active human neural and retina tissues [1]. It is believed that DHA plays important roles in human health. Lack of DHA may cause cardiovascular disease, cancer, diabetes, and neuropsychiatric disorders. The conventional source of DHA is fish oil [2]. However, it is believed that marine fish do not synthesize C20:5(n-3), eicosapentaenoic

M. Lian · H. Huang (✉) · L. Ren · X. Ji · J. Zhu · L. Jin
State Key Laboratory of Materials-Oriented Chemical Engineering,
College of Biotechnology and Pharmaceutical Engineering, Nanjing University of Technology,
No. 5 Xinmofan Road, Nanjing 210009, People's Republic of China
e-mail: biotech@njut.edu.cn

acid (EPA), and C22:6(n-3), DHA, themselves, but instead obtain those compounds as such from their feed [3].

At present, DHA production by microalgae such as *Crypthecodinium cohnii*, *Schizochytrium* sp., etc. isolated from the ocean has attracted increasing attention. Although the industrial production of DHA has been achieved at very large scale, some methods were for attempts to improve DHA synthesis via intrinsic parameters [4] or extrinsic parameters [5]. However, some common characteristics of the strains, such as degeneration, weak adaptability, and low production, still hinder the large-scale production [6]. As a kind of simple and effective breeding strategy, mutagenesis is widely used for selecting various kinds of high-producing strains, Meireles [4] increased the yields of EPA and DHA by the microalga following mutagenesis, and it had also been shown to improve the EPA content of microalgae *Phaeodactylum tricornutum* via ultraviolet (UV) mutant [7].

There were two kinds of fatty acid synthesis pathway existed in the species of thraustochytrids. One is named typical fatty acid synthesis pathway, comprising a series of alternating desaturation and elongation steps of short-chain saturated fatty acids existed in *Thraustochytrium* sp. [8]. The other one is found to be homologous to the polyketide synthase system and usually existed in *Schizochytrium* sp. [9, 10]. However, no matter which kind of pathway by which fatty acid was synthesized, two key factors, NADPH and acetyl–CoA, were important for achieving efficient lipid accumulation in microorganisms [11, 12].

In the present study, a mutant of the microalgae *Schizochytrium* sp., named HX-308M, was obtained through enforcing the supply of two key necessary factors in fatty acid biosynthesis by *N*-methyl-*N*-nitro-*N*-nitrisiguanidine (NTG) treatment coupled with UV radiation based on metabolic pathway analysis. The difference of the key enzyme activities between the mutant and the parent strain was investigated to reveal the high DHA accumulation mechanism of the mutant.

Materials and Methods

Strain, Medium, and Culture Conditions

The parent strain used in this work was *Schizochytrium* sp. China Center for Type Culture Collection (CCTCC) M209059, isolated from seawater in our previous work and stored in CCTCC. The strain was maintained in the medium containing 40 g/l glucose and 0.4 g/l yeast extract in artificial seawater. This medium also contained trace elements in a prepared solution (2 ml/l) and vitamin solution (2 ml/l). All the medium components were separately sterilized (121 °C, 30 min). The trace element solution contained as follows (gram per liter): Na_2EDTA 6, $FeSO_4$ 0.29, $MnCl_2{\cdot}4H_2O$ 0.86, $ZnSO_4$ 0.8, $CoCl_2{\cdot}6H_2O$ 0.01, $Na_2MoO_4{\cdot}2H_2O$ 0.01, $NiSO_4{\cdot}6H_2O$ 0.06, and $CuSO_4{\cdot}5H_2O$ 0.6. Vitamin solution was filter sterilized (0.22 μm) and contained (milligram per liter): thiamine 50, biotin 1.0, and cyanocobalamin 10 [12]. Cells were inoculated into 50 ml of medium in 250-ml shaking flasks and incubated at 25 °C at 170 rpm. Subcultured cells were used as inoculums for future studies with 5% (*v*/*v*).

Mutagenesis and Mutants Selection

The fresh cells were inoculated into medium and cultured for 20 h at 25 °C on a rotary shaker. 0.5 ml culture was mixed with 19.5 ml 0.2 M phosphate buffer solution and agitated with six glass beads. The cell suspension was obtained by filtration through six

layers of sterile gauze. Three different concentrations of NTG, 1, 1.5, and 2 mg/ml, were dispensed with 0.2 M phosphate buffer solution. One milliliter cell suspension was then added to 1 ml of each NTG solution. Three treatment lengths, 20, 30, and 40 min, gave a result of 1% to 10% survival rate. For the UV radiation, approximately 0.1 ml cells suspension was added in 1 ml of buffer solution to make the cell suspension with a final density of 10^4 cells per milliliter. The sample was exposed to UV light (30 W, 30 cm) for 3, 6, and 9 min, respectively, and then diluted and inoculated to the selective media plates. The plates were incubated for 2 days at 25 °C. The mutagenesis procedure was repeated when it was necessary to make sure that all the plates had over two colonies.

The survival mutants of the NTG–UV-coupled treatment were then cultured in plate media at 25 °C. The iodoacetate acid- and malonic acid-resistant mutants were obtained via incubating the strains on the plates including 0.06 g/l iodoacetate acid and 0.1 g/l malonic acid [13, 14].

Analytical Methods

Cell dry weight (CDW) was determined by firstly centrifuging 10 ml cell suspension at 6,000 rpm for 5 min after washing twice with 0.2 M phosphate buffer solution. Secondly, the cell pellet was resuspended and transferred to a filter paper, and then dried at 80 °C until the weight was constant. The methods of lipid extraction and fatty acid analysis were as indicated in our previous study [12]. Glucose was analyzed by a biosensor equipped with glucose oxidase electrode (SBA-40C, Institute of Biology, Shandong Academy of Sciences, China).

Preparation of Cell-Free Extracts and Measurement of the Enzymatic Activities

The cells were harvested by filtration and washed with distilled H_2O. Samples of the culture filtrate were retained for separate analysis (stored at −20 °C if necessary). Biomass was suspended in 50 mM KH_2PO_4 buffer (pH 7.4) containing 20% (*w*/*v*) glycerol, 1 mM EDTA, and 2 mM DL-dithiothreitol and disrupted by ultrasonic disrupter for 10 min on the ice. The resulting homogenate was centrifuged at 8,000 rpm for 5 min at 4 °C; the supernatant was used to determine the enzyme activities. Protein concentration was determined according to Bradford assay, with bovine serum albumin as a standard [15].

All the enzyme activities were determined by using continuous spectrophotometric assay to test the oxidation or reduction of NADPH at 340 nm at 25 °C. Malic enzyme (EC 1.1.1.40, ME) was assayed with malate as substrate as described by Hsu and Lardy [16]. The activities of ATP–citrate lyase (EC 4.1.3.8, ACL) and glucose-6-phosphate dehydrogenase (EC 1.1.1.49, G6PDH) were measured with the methods described by Takeda et al. [17] and Langdon [18], respectively. One unit of enzyme activity (U) was defined as the formation of the quantities amount of NADPH per minute, and specific activity was defined as the unit of enzyme activity per milligram of protein (U/mg protein).

Results and Discussion

Metabolic Pathway Analysis of DHA Production in *Schizochytrium* sp.

The key to fatty acid accumulation is sufficient supplement of NADPH and continuous supplement of acetyl–CoA [19–21]. Two additional NADPH forming metabolic cycles

were known to occur [22]. One was the hexose monophosphate pathway (HMP), which consisted of the action of G6PDH; the other was the citrate cleavage pathway, which consisted of the sequential action of ACL and ME. And in citrate cleavage pathway, more acetyl–CoA would be produced. Indoacetic acid could interact with the sulfydryl belonging to phosphoglyceraldehyde dehydrogenase [13]. As shown in Fig. 1, this interaction could inhibit the Embden–Meyerhof pathway (EMP). In consequence, the HMP of this resistant strain could become more effective. Also, as an inhibitor of respiration, malonic acid could weaken the tricarboxylic acid cycle (TCA). As shown in Fig. 2 [23], it was obvious that citric acid would be accumulated and transferred from mitochondrion to cytoplasm, as a result of inhibition of the TCA. Therefore, more acetyl–CoA and NADPH, which were prerequisite to fatty acid production, could be obtained from the accumulated citric acid in cytoplasm via the catalysis by ACL and ME. Therefore, based on the previous metabolic pathway analysis, NADPH and acetyl–CoA supply could be enhanced through selecting indoacetic acid- and malonic acid-resistant mutants, respectively.

It had been suggested that the function of malic enzyme in fatty acid biosynthesis was to supply NADPH for fatty acid synthase and desaturase [24, 25]. Acetyl–CoA was too large to pass through biological membranes, therefore eukaryotic cells possessed transport mechanisms to translocate this molecule between cellular compartments. In yeast, ACL and carnitine acetyltransferase had been found to be important for this transport [26]. G6PDH was the key enzyme of HMP, which generated more NADPH than the EMP. Therefore, enhanced G6PDH activity could improve the concentration of NADPH supplying energy for fatty acid production.

UV–NTG Survival and Mutant Selection

The length of the UV–NTG treatment was a crucial factor for mutagenesis. A survival rate was between 1% and 15% after UV treatment and was between 1.5% and 20% after NTG treatment. Through fermentation comparison, the optimal UV–NTG treatment condition was UV for 3 min, and 1.5 mg/ml NTG for 30 min, the survival rate was 2.5%.

The iodoacetate acid and malonic acid could inhibit EMP and respiration, respectively. Some strains could not grow or grow slowly on the plate adding iodoacetate acid and malonic acid. Under this mutant condition, some larger colonies were selected for following

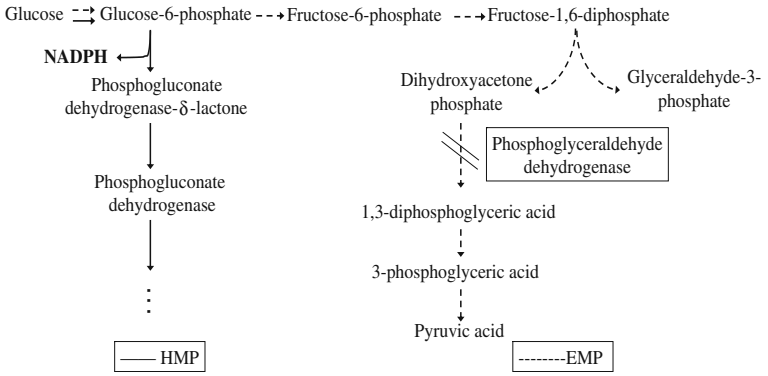

Fig. 1 The hexose monophosphate pathway (*HMP*) and Embden–Meyerhof pathway (*EMP*) in *Schizochytrium* sp.

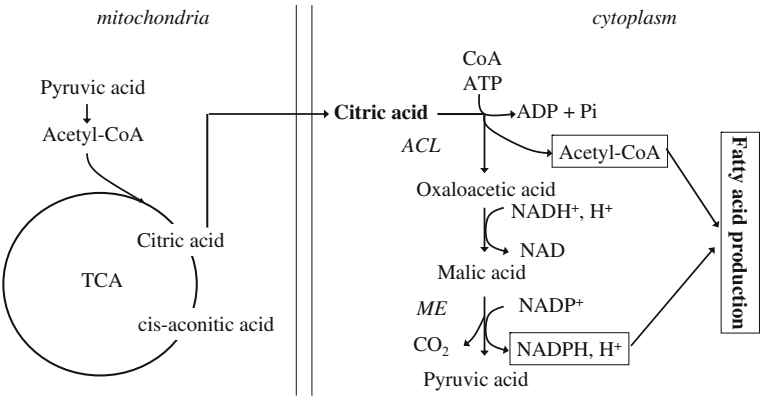

Fig. 2 Citric acid transport system in *Schizochytrium* sp.

selection. And a mutant named *Schizochytrium* sp. HX-308M was obtained at the following fermentation comparison.

Growth and Metabolite Production by the Parent and the Mutant Strain

The fermentation parameters of the mutant and the parent strain were shown in Table 1. Through parallel batch fermentation, the mutant showed a 34.84% higher lipid accumulation than the parent strain with a similar cell dry weight. Moreover, the DHA content up to 56.22%, 38.88% higher than the parent strain, was achieved. The final DHA yields, when the cultures were maintained under appropriate conditions, were 0.20 and 0.11 g/g dry biomass, for the mutant and parent, respectively.

Analysis of Key Enzyme Activities Involved in DHA Formation

During the fermentation, the activities of the three key enzymes involved in DHA formation (G6PDH, ME, and ACL) were measured every 8 h. Zink and Katz [24, 27] reported that the main function of ME was in the metabolism of pyruvate while some other research showed evidence that ME was also a major source of NADPH for de novo lipid biosynthesis and

Table 1 The fermentation performance of *Schizochytrium* sp. HX-308M and CCTCC M209059.

	CDW (g/l)	Fatty acid (g/l)	DHA content (% total fatty acid)	DHA (g g^{-1} dry weight)
HX-308M	45.24±0.40	17.83±0.30	58.25±0.20	0.23±0.15
	40.16±0.35	13.48±0.25	54.75±0.25	0.18±0.18
	42.88±0.28	15.44±0.17	55.66±0.34	0.20±0.21
CCTCC M209059	45.44±0.25	13.41±0.28	40.41±0.40	0.12±0.45
	44.38±0.42	12.95±0.35	42.10±0.25	0.12±0.21
	40.72±0.50	8.31±0.19	38.93±0.40	0.08±0.15

desaturation [26]. Wynn and Ratledge [28] reported the importance of ME in lipid metabolism of *Aspergillus nidulans* and hypothesized that this enzyme was important in the generation of NADPH for lipid biosynthesis in oleaginous organisms. Ratledge et al. [29] reported that the ACL activity was at its highest activity when the physiological demand for acetyl–CoA was highest. ACL activity varied in response to demand for acetyl–CoA.

Figure 3 showed the enzyme activity differences between the mutant and the parent strain in the whole fermentation. The three key enzyme activities of the mutant were commonly higher than the parent strain. The highest G6PDH, ME, and ACL activity of the mutant was up to 3,944.4, 4,755.6, and 4,344.4 U/mg protein, respectively, which increased 27.6% for G6PDH, 152.3% for ME, and 200% for ACL compared with the corresponding value of the parent strain. Also, as shown in Fig. 3, the time when each enzyme activity reached the maximum was ahead about 15 h.

As shown in Figs. 1 and 2, higher G6PDH activity would strengthen the HMP and thus produce more NADPH. While higher ACL and ME activity would produce more NADPH and acetyl–CoA through citric acid transport system. Therefore, the level of NADPH and acetyl–CoA for DHA synthesis in the mutant would be higher than the parent strain. And this caused relative higher DHA accumulation in the mutant.

Conclusions

A mutant of *Schizochytrium* sp. which was resistant to iodoacetate acid and malonic acid known as inhibitors of the EMP pathway and respiration, respectively, was isolated. The activity of G6PDH of the mutant was higher than the parent strain, which indicated that the HMP pathway of the mutant was strengthened, and more NADPH was thus produced. Also, the activities of ME and ACL in the cell extract of the mutant were higher than the parent strain; this indicated that the screening method could increase the concentration of NADPH and acetyl–CoA in vivo effectively. The activities of the three key enzymes also increased, 27.6% for G6PDH, 152.3% for ME, and 200% for ACL. Finally, in the batch fermentation, the mutant showed a 34.84% higher lipid accumulation than the parent strain with a similar cell dry weight. Moreover, the DHA percent of total fatty acids up to 56.22% was achieved using the mutant.

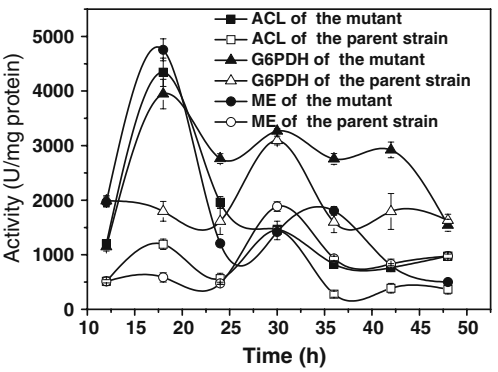

Fig. 3 Time course of glucose-6-phosphate dehydrogenase (*G6PDH*), malic enzyme (*ME*), and ATP–citrate lyase (*ACL*) activities of the mutant and the parent strain

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