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Development of a Temperature Shift Strategy for Efficient Docosahexaenoic Acid Production by a Marine Fungoid Protist, *Schizochytrium* sp. HX-308

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Abstract Temperature was one of the important environmental factors affecting the biosynthesis of docosahexaenoic acid (DHA; C22:6, ω−3). Generally, a low temperature would slow the strain growth, but promote the accumulation of unsaturated fatty acids. According to this information, the effects of temperature and different two-stage temperature shifting strategies on fatty acid production and DHA content of the marine fungoid protist, *Schizochytrium* sp. HX-308, were investigated in this study. Finally, the highest DHA percentage was up to 51.98% (per total fatty acids) with the DHA production of 6.05% (per dry cell weight), which was obtained with the method of shifting temperature from 30 °C for 32 h to 20 °C for 12 h.

Keywords *Schizochytrium* sp. · Docosahexaenoic acid · Temperature shift

Introduction

Docosahexaenoic acid (DHA; C22:6, ω−3), one of the primary structural polyunsaturated fatty acids (PUFAs), exists in most of the highly active human neural and retina tissues [1]. It is believed that DHA plays important roles in human health and thus it is widely used as a nutraceutical in the food and feed market. The marine fungoid protists such as the species of *Schizochytrium* [2] and the marine microalgae such as *Crypthecodinium cohnii* [3] have been shown to be excellent DHA producers. The industrial production of DHA has been achieved to a very large scale. However, the improvement of the bioprocess and the reduction of cost are still crucial for the expansion of the DHA market.

The marine fungoid protest, *Schizochytrium* sp., a kind of heterotrophic marine thraustochytrid, is able to accumulate about 35–40% of their total fatty acids (TFAs) as

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DHA with a short culture period [4, 5]. To achieve efficient DHA production by *Schizochytrium* sp., a number of studies were focused on improving PUFA synthesis by optimizing extrinsic parameters. The strategies such as limiting essential nutrient levels (carbon and nitrogen concentration), controlling dissolved oxygen tension, pH, salinity, and culturing at a fixed low temperature were applied to induce lipid accumulation [6–10].

Medium composition, incubation temperature, pH, salinity, culture age, seawater concentration, impeller speed, and fermentor shape all could influence DHA production [11, 12]. And temperature is thought to be one of the most important environmental factors that affect all aspects of the growth and the fatty acid composition of most microorganisms. It also could affect the enzymatic reactions, cell membrane transport system, and some other cellular characters [4, 13–15]. A low-temperature growth condition will lead to a spontaneous response of strain, which aims at maintaining proper membrane lipid fluidity and functions. This reaction will increase the proportion of unsaturated fatty acids, especially PUFAs [16]. However, low temperature would limit the strain growth and thus decrease the biomass formation.

The purpose of this study was to investigate the effect of temperature on DHA fermentation by *Schizochytrium* sp. and to develop some proper temperature control strategies for efficient DHA production.

Materials and Methods

Strain, Medium, and Culture Conditions

Schizochytrium sp. HX-308 (CCTCC M 209059) was isolated from seawater in our previous work and stored in the China Center for Type Culture Collection. The culture medium and conditions were as indicated in our previous study [17].

Cells were inoculated into 50 mL medium in 250-mL shaking flasks and incubated at 25 °C at 170 rpm. Sub-cultured cells were used as inoculums for future studies with 5% (v/v).

Analytical Methods

Biomass was estimated as dry cell weight (DCW). Sample was determined by firstly centrifuging 10 mL of cell suspension at 6,000 rpm for 5 min after washing twice with 0.2 M phosphate buffer solution. Secondly, the cell pellet was transferred to a filter paper and then dried at 80 °C until the weight was constant. The methods of lipid extraction and fatty acid methyl esters (FAMEs) preparation were as indicated in our previous study [17]. The FAMEs were analyzed by a gas chromatograph (GC-2010; Shimadzu, Japan) which was equipped with a capillary column (DB-23, 60 m×0.25 mm×0.25 μm). Nitrogen was used as the carrier gas. The injector was maintained at 250 °C, with an inject volume of 1 μL. The column was raised from 100 to 200 °C at 25 °C/min, and then increased to 230 °C at 4 °C/min, keeping at this temperature for 9 min. Fatty acids were identified by comparison to related external standards (Sigma, USA). The quantities of individual FAMEs were estimated from the peak areas on the chromatogram using nonadecanoic acid (C19:0) as the internal standard. Glucose was analyzed by a biosensor equipped with glucose oxidase electrode (SBA-40C; Institute of Biology, Shandong Academy of Sciences, China).

Determination of Specific Growth Rate and Production Rate

Specific growth rate and production rate were determined by plotting the natural logarithm of DCW and lipid weight against time, respectively.

Results

Effects of Temperature on Cell Growth, Substrate Consumption and Fatty Acid Accumulation

It has been reported that the proper culture temperature for *Schizochytrium* ranged from 16 to 37 °C. According to the study of Song et al. [18], the optimal temperature for *Schizochytrium* was 27 °C. In this study, *Schizochytrium* sp. HX-308 was subsequently cultured at five different temperatures of 15 °C, 20 °C, 25 °C, 30 °C, and 35 °C until the glucose was exhausted.

The fermentation period of *Schizochytrium* sp. HX-308 would be elongated when it was carried out at 15 °C. In addition, cell growth was inhibited at 35 °C or even higher temperature (data not shown). So this study would focus on the temperature ranging from 20 °C to 30 °C. The effects of temperature on DCW, residual glucose concentration, lipid concentration, and DHA production are shown in Fig. 1. The biomass increased from 62.20 g/L at 20 °C to 84.40 g/L at 30 °C, the lipid concentration also increased significantly from 6.167 g/L at 20 °C to 13.42 g/L at 30 °C, and the DHA production increased from 3.16 g/L at 20 °C to 4 g/L at 30 °C. With the increasing of temperature, cell growth rate and glucose consumption rate both increased. The values of two kinetic parameters related to cell growth and product formation of DHA fermentation using *Schizochytrium* sp. HX-308 at different temperatures are shown in Table 1. It was obvious that the maximal specific growth rate (μ_m) and the maximal specific production rate (q_{pm}) of *Schizochytrium* sp. HX-308 increased with the increasing of the temperature. The μ_m was 0.179 h^{-1}, which was obtained at 20 h with the cells cultivated at 30 °C, while the q_{pm} of 0.026 h^{-1} was obtained at 32 h at the same culture temperature.

Effect of Temperature on Fatty Acid Composition

Table 2 shows the changes of the main fatty acid composition when the cells were cultivated at different temperatures (20 °C, 25 °C, 30 °C). Palmitic acid (C16:0), as the major saturated fatty acid, increased from 15.56% to 21.64% when the temperature increased from 20 °C to 30 °C. At 20 °C, the proportion of unsaturated fatty acids, including DHA, docosapentaenoic acid (DPA; C22:5, ω−6), and eicosapentaenoic acid (EPA; C20:5, ω−3), was 61.28%, which increased by 1.25 times when temperature was shifted to 30 °C. The highest DHA percentage of TFAs was 51.28% at 20 °C, indicating that the synthesis of unsaturated fatty acids was higher at lower temperature, which was similar to the situation observed in other eukaryotic microalgae such as *Cyanobacteria* [19].

Effect of Temperature Shift on DHA Production

In order to improve the DHA accumulation, two kinds of two-stage experiments were thus designed based on the effect of temperature on fermentation characteristics. In the first experiment, cultures were incubated at 30 °C for 20 h (first stage), then shifted to 20 °C and

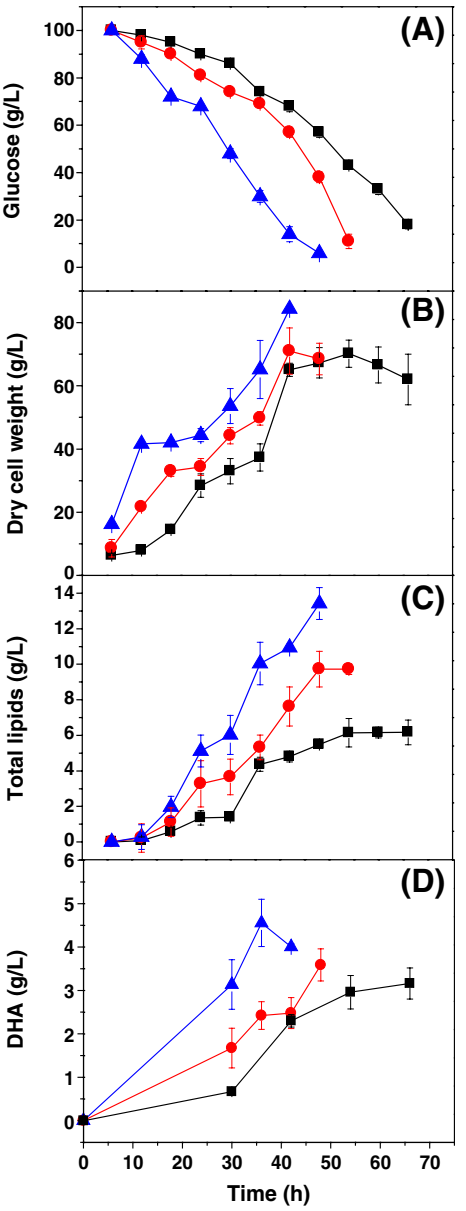

Fig. 1 Time course of DHA fermentation using *Schizochytrium* sp. HX-308 at different temperatures: glucose (**a**), dry cell weight (**b**), total lipids (**c**), and DHA (**d**). *Filled square* 20 °C, *filled circle* 25 °C, *filled triangle* 30 °C

Table 1 Kinetic parameters of DHA fermentation using *Schizochytrium* sp. HX-308 at different temperatures

Parameters	20 °C	25 °C	30 °C
Max specific growth rate, μ_m (h^{-1})	0.095	0.169	0.179
Max specific production rate, q_{pm} (h^{-1})	0.009	0.014	0.026

Table 2 The fatty acid composition (% total fatty acids) of *Schizochytrium* sp. HX-308 cultured at different temperatures

Fatty acid	20 °C				25 °C				30 °C		
	30 h (%)	42 h (%)	54 h (%)	66 h (%)	30 h (%)	36 h (%)	42 h (%)	48 h (%)	30 h (%)	36 h (%)	42 h (%)
C14:0	7.38	9.31	10.32	15.14	10.34	10.05	21.83	18.36	5.83	9.99	5.53
C16:0	17.95	21.06	16.65	15.56	25.26	22.61	23.88	20.17	22.84	23.44	21.64
C18:0	0.91	0.63	–	3.58	1.75	–	3.04	2.19	1.03	1.55	–
C20:5, EPA, ω−3	2.35	2.16	1.07	3.42	–	–	–	1.38	1.06	1.55	–
C22:5, DPA, ω−6	6.89	7.87	7.45	6.58	11.43	10.05	8.04	7.72	16.07	12.50	12.32
C22:6, DHA, ω−3	48.05	47.98	48.22	51.28	45.72	45.50	32.53	36.91	52.02	45.38	36.62

– content ≤0.3%, *C14:0* myristic acid, *C16:0* palmitic acid, *C18:0* stearic acid, *EPA* eicosapentaenoic acid, *DPA* docosapentaenoic acid, *DHA* docosahexaenoic acid

25 °C until the glucose was exhausted, respectively. In the second experiment, cultures were incubated at 30 °C for 32 h, and then shifted to lower temperature. DHA accumulation under different temperature control strategy is shown in Table 3. Although the highest DHA production (%DCW) was 6.10% when the culture shifted from 30 °C (32 h) to 25 °C, the DHA production (%TFAs) was low, only 33.94%. In contrast, in the other temperature shift strategy, the optimal DHA production (%TFAs) was 51.98% and DHA production (% DCW) was up to 6.05% at the same time.

Discussion

Several previous researches suggested that a high temperature within allowed extent could result in a higher growth rate for fungi [20, 21] and alga [22, 23]. And temperature was thought to play an important role in regulating cell growth and fatty acid accumulation. In the present study, the fermentation period of DHA production using *Schizochytrium* sp. HX-308 was about 42 h at 30 °C, 48 h at 25 °C, and 66 h at 20 °C, respectively (Fig. 1).

Table 3 DHA accumulation in *Schizochytrium* sp. HX-308 cultured at different temperatures

	20 °C			25 °C			30 °C
	Shift from 30 °C (20 h) to 20 °C	Shift from 30 °C (32 h) to 20 °C	Constant	Shift from 30 °C (20 h) to 25 °C	Shift from 30 °C (32 h) to 25 °C	Constant	Constant
Total fermentation time (h)	56	52	66	46	44	48	42
DHA production (%TFAs)	51.55	51.98	51.28	33.94	37.38	36.91	36.62
DHA production (%DCW)	5.97	6.05	5.1	6.1	5.94	5.21	5.82

%TFAs per total fatty acids, *%DCW* per dry cell weight

This could be explained by the fact that lower temperatures reduced the activity of enzymes involved in the glycolysis and tricarboxylic acid cycle, thus slowing the catabolism rate [24]. Generally, fatty acid composition of microorganisms could exhibit considerable variations with various conditions. Lower temperature usually resulted in more PUFAs such as DHA accumulation [25]. The strain responded to the culture temperature by increasing the degree of fatty acid unsaturation at lower temperatures. Increased unsaturation of fatty acids at low temperature in cells might be a response to maintain membrane fluidity and function, allowing the cell to acclimate to low temperature stress, as reported in some *Cyanobacterium* [26]. The level of unsaturated fatty acids at low temperatures might be associated with the activity of membrane-bound desaturase, which is a kind of stress-responsive enzyme, and its expression levels are highly sensitive to temperature changes. Moreover, the result of real-time quantitative PCR showed that the expression of genes involved in PUFAs biosynthetic pathway was higher in lower culture temperature compared to higher temperature (data not shown). Increased gene expression could more or less account for the higher productivity of PUFAs.

As an intracellular product, lipid weight was associated with biomass. Although lower temperature was the major regulating factor for the increased biosynthesis of PUFAs [27], low temperature also limited the biomass formation. In order to increase the DHA production, temperature shifting from high to low had been designed. In the constant temperature control experiments, it showed that the specific growth rate of *Schizochytrium* sp. HX-308 reached the maximum when it was cultured for about 20 h, while the specific production rate reached the maximum when it was cultured for about 32 h. Therefore, to determine the optimal time for temperature shifting, two time sets (20 and 32 h) were thus chosen to shift the temperature respectively. And the results proved that 32 h was a proper time set.

In the present study, the DHA accumulation was expressed as the percentage over the TFAs and the DCW. DHA production (%TFAs) means the percentage of DHA in the TFAs and it indicates the final lipid product quality, while the DHA production (%DCW) means the net DHA accumulation. These two parameters were both important in industrial production. Here, by using a temperature shifting strategy, the optimal results of 51.98% and 6.05% were obtained, respectively, which had a competitive edge over the traditionally used constant temperature control strategy. In addition, as shown in Table 2, DHA production (%TFAs) could achieve 52% when the cells were cultured at constant 30 °C for 30 h; this might indicate that there was no necessity of applying a temperature shift strategy in the DHA production process as there was no difference between the constant and two-stage control strategy. However, during the cell growing process, the fatty acid was still accumulating; that is to say, we should not only consider DHA percentage in the TFAs but also the DCW and the TFAs, as the net DHA accumulation was the objective. In fact, the DHA percentage in the TFAs changed when culturing at constant temperature (30 °C); it got smaller with the time (Table 2). This indicated the possibility of using a temperature shift strategy otherwise.

In conclusion, this study provided a detailed understanding of the effects of temperature on the fermentation characteristics of *Schizochytrium* sp. HX-308, and a temperature shifting strategy aimed at increasing DHA production was developed. The highest DHA production (%TFAs) was up to 51.98%, and its production (%DCW) was 6.05%, which was obtained with the method of shifting temperature from 30 °C for 32 h to 20 °C for 12 h. Culturing the marine fungoid protist at its optimal temperature for the highest biomass density and then shifting it to a lower temperature could assure a higher DHA yield. At the same time, the two-stage culture strategy proposed based on the fermentation characteristics developed in this paper could be applied to the other similar single-cell oil production processes.

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