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Research Article

Effect of malate on docosahexaenoic acid production from *Schizochytrium* sp. B4D1

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ABSTRACT

Background: Malate involves in the citrate/malate and transhydrogenase cycles to provide precursors for docosahexaenoic acid (DHA) synthesis. The optimal strategy was investigated for increasing DHA production in *Schizochytrium* species during fermentation.
Results: DHA production increased by 47% and reached 5.51 g/L when 4 g malate/L was added during the rapid lipid accumulation stage in shake-flasks culture. Inducing effects of malate was further investigated through the analysis of three kinetic parameters, including specific cell growth rate (μ), specific glucose consumption rate (q_{Glu}) and DHA formation rate (q_{DHA}). DHA concentration was enhanced through a novel fed-batch strategy to a maximum value of 30.7 g/L, giving a yield of 0.103 g DHA/g glucose and a productivity of 284 mg L^{-1} h^{-1}.
Conclusion: A novel malate feeding strategy was developed that enhanced DHA yield and productivity of Schizochytrium species which may offer a desirable method for industrial applications.

1. Introduction

Long-chain ω-3 polyunsaturated fatty acids (PUFAs), such as docosahexaenoic acid (DHA; C22:6), have been reported as effective in human nutrition and the prevention of conditions such as heart disease, high blood pressure and mood disorders [1,2,3]. In recent years, production of DHA by microbial fermentation has attracted considerable attention [4]. Certain marine bacteria, algae and fungi are abundant in DHA content thus have been explored as potential source of DHA [5]. Among these various organisms, *Schizochytrium* sp. has been considered as a cell factory with great potential for commercial application, because it offers the advantages of fast growth, high productivity and a consistent product quality [6]. However, the availability of this DHA source is limited by high production costs, especially compared with current fish oil prices [7]. Therefore, it is necessary to improve DHA productivity in *Schizochytrium* sp. fermentation to decrease the costs of DHA production.

Enhanced DHA production could be achieved by nicotinamide adenine dinucleotide phosphate (NADPH) regulation, which is controlled by the activity of the malic enzyme (ME, which acts as the sole source of NADPH for fatty acid synthase) during the lipid accumulation phase in *Schizochytrium* sp. [8]. Malate is a major participator in the citrate/malate cycle and the transhydrogenase cycle in fatty acid synthesis [9,10]. Ren et al. [6] found that the DHA content of total fatty acids increased from 35 to 60% through addition of 4 g malic acid/L during the rapid lipid accumulation stage. However, the dynamics of the DHA inducing effect during malate consumption and optical malate feeding strategies have not been studied in fed-batch fermentation for industrial applications.

In this work, malate as a DHA precursor was introduced to a fermentation system during the fast lipid accumulation stage to enhance NADPH supply. Furthermore, a fed-batch feeding strategy based on kinetic analysis was implemented during fermentation to increase NADPH supply by enhancing the effects of malate on the key enzymatic activity. This simple approach to feed cultures continuously with an inductive factor at an appropriate stage of fermentation has the potential to decrease production costs and could provide insights for other fermentation systems.

2. Materials and methods

2.1. Microorganism and culture media

Schizochytrium sp. B4D1 used in this study was preserved in 20% (v/v) glycerol at -80°C in our lab. The main culture medium comprised (g L^{-1}): artificial seawater salt 20, glucose 80, yeast extract 4, peptone 4, $MgCl_2$ 3, $CaCl_2$ 2, H_2O 1, $MgSO_4 \times 7H_2O$ 5, KH_2PO_4 4, and KCl 2, all media were sterilized at 115°C for 20 min.

2.2. Fermentations in shake-flasks

Inoculum *Schizochytrium* sp. B4D1 solution and medium were inoculated at a 1:10 (v/v) ratio; 50 mL culture volume in a 250-mL

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flask; culture temperature, 25°C; rotational speed, 170 rpm; incubation time, 84 h.

2.3. Fed-batch fermentations

Fed-batch fermentation was carried out in a 5-L fermenter with a broth volume of 3-L (60%, v/v) under the following conditions: temperature, 26°C; the dissolved oxygen (DO) was controlled above 30%, then limited to 5% after 72 h by varying agitation speed and air flow; pH, 6 measured by an Ingold pH probe and maintained at that level by the addition of 4 M NaOH and 4 M H_2SO_4. Dissolved oxygen was measured using an Ingold sterilizable polarographic electrode. Glucose feed and malate feed were supplied during the fed-batch phase by calibrated peristaltic pumps with speed controllers. The glucose concentration was maintained at 5 to 20 g L^{-1} by feeding glucose during the fermentation.

2.4. Measurement of dry cell weight

The cells were collected by centrifugation, washed with distilled water, placed on filter paper, and then dried in a freeze drier (FD-1C-50, BJBYK Co, Beijing China) until the sample weight became constant.

2.5. Lipid extraction

Amounts of 0.1–0.2 g of biomass powder were weighed to perform oil extraction. An aqua distillate/hydrochloric acid mixture of 2 mL (1:1 v/v) was added to the powder and placed into 70–80°C water baths for 2 h after mixing. After the samples were cooled, 5 mL of n-hexane was added and shaken for 10 h. Supernatants were collected and evaporated in a Pressure Blowing Concentrator (MD200, Beijing China). Measurements of total lipid content were derived from the analysis of these samples.

2.6. Fatty acid analysis

Fatty acid methyl esters (FAMEs) were prepared by a modified standard method as follows: 1 mL of 0.5 M potassium hydroxide/methanol was added to extracted oil samples. Samples were heated at 60°C in water baths for 15 min to saponify. After the samples were cooled, 2 mL of 14% boron trifluoride/methanol was added as a catalyst for a 2-min reaction at 60°C for transesterification. After transesterification, 1 mL of saturated saline was added to prevent emulsification. Then, 2 mL of chromatographically pure n-hexane was added, and the transesterified FAMEs were extracted into the n-hexane layer. Finally, 0.5 g of anhydrous sodium sulfate was added and gas chromatography (GC) analysis was performed.

GC (GC-2010, Shimadzu, Japan) analysis conditions were as follows: an sp-2560 column (100 m × 0.25 mm × 0.20 μm, Supelco, USA) was applied; ovens were initially set at 180°C and increased at an average of 30°C/min until the temperature was maintained at 240°C for 18 min. Injector and detector (flame ionization detector, FID) temperatures were set at 250 and 260°C, respectively. Helium flow rates were 63.7 mL min^{-1}, hydrogen flowrates were 40 mL min^{-1}, and air flow rates were 400 mL min^{-1}. Sample injection volumes were 1 μL. Fatty acid standards (Sigma Co., St. Louis, MO, USA) were used to compare the fatty acid peaks in samples with standard individual peak areas, and the amount of individual FAMEs in samples were determined [11].

2.7. Enzyme assay

ME and G6PDH activities were determined spectrophotometrically by monitoring the rate of NADPH formation at 28 and 25°C [12,13]. One unit of enzyme activity was defined as the amount of enzyme that liberated 1 μmol of G6PDH per minute from *Schizochytrium* sp. B4D1 cells under the assay conditions, which was estimated based on the absorbance at 340 nm every 30 s. The control used was the inactive enzyme which was boiling at 100°C for 10 min.

Protein concentrations were determined using a trace protein concentration determination kit via the Bradford method.

2.8. Malate determination

Malate from fermentation broth was determined using HPLC with a refractive index detector (Shimadzu, Kyoto, Japan). All samples were diluted and passed through a 0.45 μm filter before HPLC analysis. The malate was separated in an Aminex HPX-87 H column (Bio-Rad Laboratories, Carlsbad, CA, USA) running at a flow rate of 0.6 ml/min at 50°C, with 0.5 mM H_2SO_4 as eluent [14].

2.9. Glucose analysis

Glucose concentration was determined using an SBA-4 biological sensor analyzer (Biological Institute of Shandong Academy of Science, Shandong, China).

2.10. Calculation of the kinetic parameters

The specific cell growth rate (μ, h^{-1}) and specific glucose consumption rate (q_{Glu}, h^{-1}) were estimated from experimental or fitted data of cell growth (x, g/L), and DHA production rate (q_{DHA}, h^{-1}) using [Equation 1], [Equation 2] and [Equation 3] respectively [15]. The fitted data were obtained by interpolating experimental data for cell growth, glucose consumption amount or DHA production yields at definite times (dt = 0.1 h) using the cubic spline interpolation approximation method in Origin software (Version 8.5, OriginLab Corp., Northampton, MA, USA):

$$\mu = \frac{1}{x}\frac{dx}{dt} = \frac{1}{x}\lim_{\Delta t \to 0}\frac{\Delta x}{\Delta t} \quad \text{[Equation 1]}$$

$$q_{Glu} = \frac{1}{x}\frac{d_{Glu}}{dt} = \frac{1}{x}\lim_{\Delta t \to 0}\frac{\Delta Glu}{\Delta t} \quad \text{[Equation 2]}$$

$$q_{DHA} = \frac{1}{x}\frac{d_{DHA}}{dt} = \frac{1}{x}\lim_{\Delta t \to 0}\frac{\Delta DHA}{\Delta t}. \quad \text{[Equation 3]}$$

3. Results and discussion

3.1. Malate effects on DHA products in shake-flasks culture

Production of biomass, total lipid, and DHA were studied in shake-flask cultures of *Schizochytrium* sp. B4D1. The malate addition period was based on earlier studies on *Schizochytrium* sp. [8].

Fig. 1 shows changes in biomass, lipid and DHA products of *Schizochytrium* sp. B4D1 during batch cultivation with different malate concentrations (1, 2, 4, 6, 8 g/L) added during the rapid lipid accumulation stage. As shown in Fig. 1, adding malate showed no significant impact at any of the five concentrations on cell growth and total fatty acid products, but DHA production was markedly improved. This observation is consistent with the study of Ren et al. [8] on *Schizochytrium* sp. HX-308. Production of DHA increased when the concentration increased from 0 to 4 g malate/L. The maximum DHA content (40%) of total fatty acids was achieved when 4 g malate/L was added, while higher concentrations did not result in further increase in DHA accumulation. Consequently, the final DHA increased by 47% and reached 5.51 g/L.

3.2. Induced effects of malate in fed-batch fermentation

The most important issues affecting batch fermentation include the pH and dissolved oxygen concentration in the fermentation broth. Fed-batch fermentation is preferable for reaching high cell