Research Article

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

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ARTICLE INFO

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):

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

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

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

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
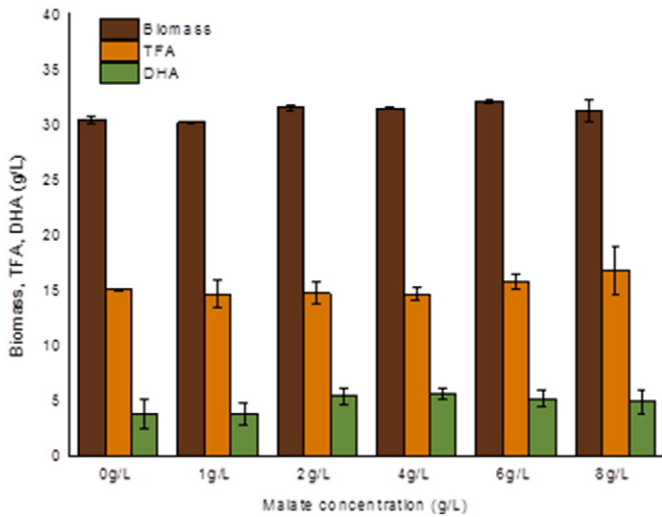
Fig. 1. Biomass, total fatty acids (TFAs) and DHA concentration in shaker cultures at different added malate concentrations during the rapid lipid accumulation stage.

density and high productivity of total fatty acids and DHA. In accordance with the above experimental results, fed-batch fermentation with 4 g malate/L added to the fermentation system

at 24 h benefited the biosynthesis of fatty acids and increased DHA production in subsequent experiments.

Fig. 2a shows changes in malate concentration with time during cultivation of *Schizochytrium* sp. B4D1 in fed-batch cultures with malate (4 g/L) added at 24 h. During the observation of the malic acid concentration, the control group maintained a constant malate concentration at about 1.53 g/L, which indicates that malate can be produced during fatty acid synthesis in the fermentation process. Meanwhile, malate concentration in the experimental group decreased from 5.97 to 3.23 g/L, with a consumption rate of 0.028 g/L/h. Time profiles of the two fermentations are shown in Fig. 2b, c. The results indicated that malate played a vital role in fatty acid and DHA production. As shown in Fig. 2b, c, the glucose consumption rate in the experimental group was slightly reduced, decreased from 80 to nearly 0 g/L during the first 36 h, as compared with the control group (40 h). After this period, the glucose feed was started and manually controlled to maintain a residual glucose concentration between 5 and 20 g/L. Over a period of 120 h fermentation, a final biomass concentration of 107.91 g/L dry biomass was achieved in the experimental group, which showed a slight increase compared with the control group (93.53 g/L). A similar trend occurred in total lipid. Final product levels of 86.32 g/L and 65.37 g/L was achieved, respectively. Furthermore, a relatively high final DHA concentration of up to 26.2 g/L was obtained with the addition of 4 g malate/L, 35% higher than the group without malic acid addition
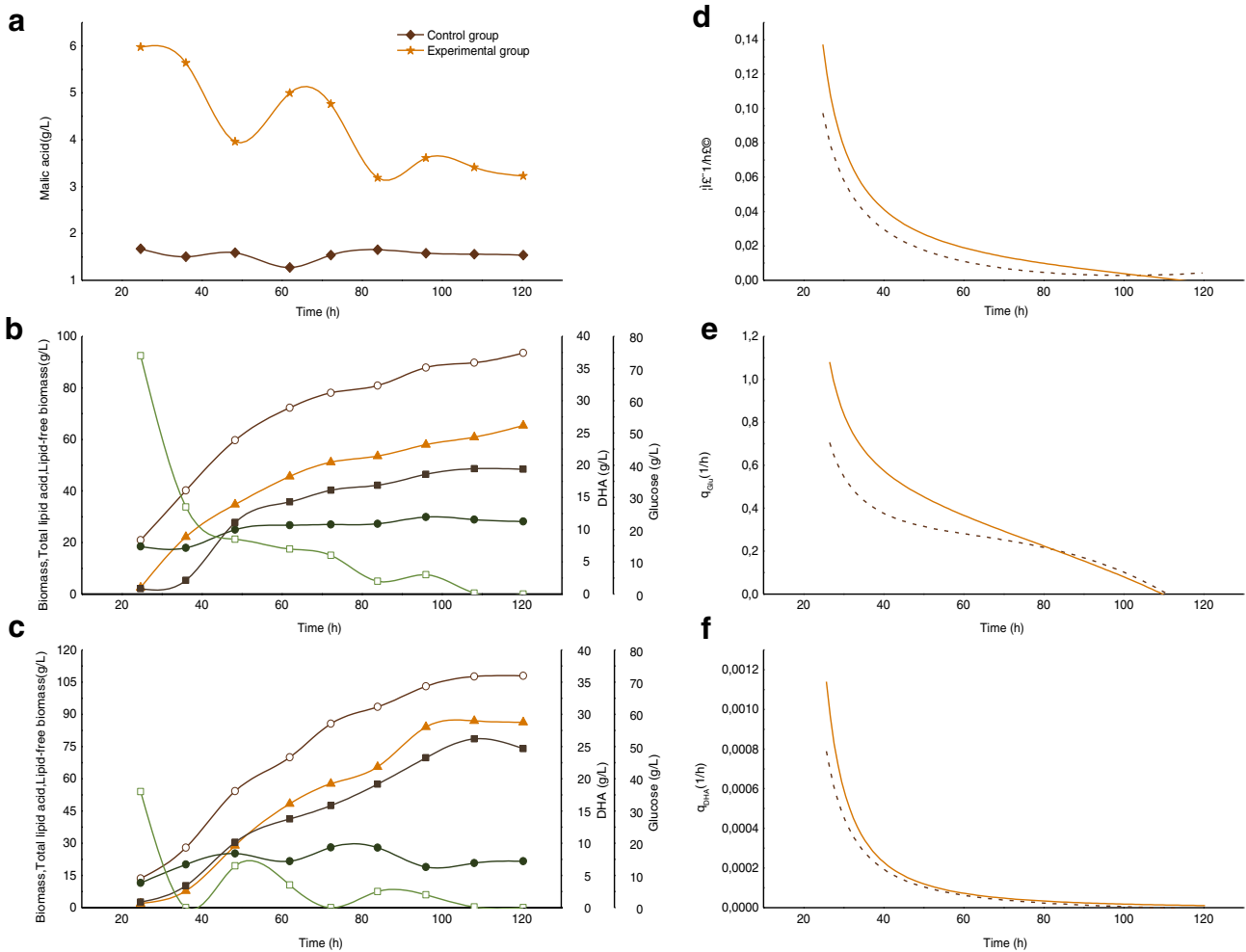

Fig. 2. a–c: Time profiles of malate, biomass, glucose, total fatty acids, lipid-free biomass, DHA for *Schizochytrium* sp. B4D1 cultivation when adding malate at 24 h. (a) Malate concentration; (b) group without malate addition (control group); (c) group with 4 g malate/L addition at 24 h (experimental group). The symbols represent biomass (○), glucose (□), total fatty acids (▲), DHA (■), and lipid-free biomass (●). d–f: Comparison of the kinetic parameters in DHA fermentation by *Schizochytrium* sp. B4D1. Control group (dashed line); experimental group (solid line). (d) Specific cell growth rate (μ); (e) specific glucose consumption rate (q_{Glu}); and (f) DHA formation rate (q_{DHA}).

(19.4 g/L). The lipid-free biomass of the both groups remained constant at 20 ± 2 g/L during the most of the fermentation time, indicating that the use of malate resulted in improved conversion of glucose to target products, but did not increase the overall use of glucose.

To analyze the kinetics of the fed-batch fermentations, three parameters, μ, q_{Glu} and q_{DHA}, as shown in Fig. 2d–f, were calculated based on the data in Fig. 2b–c. All three parameters showed similar tendencies, reducing gradually throughout the fermentation. Compared with the control group, μ and q_{Glu} were higher at the point of addition of malate at the beginning of fermentation (about 24 h). This showed that the addition of malate improved cell growth and glucose consumption during the first 84 h. However, after 84 h, the two groups had similar q_{Glu} values. Combining this result with the analysis of q_{DHA}, there was an increasingly smaller gap between the two groups caused by the reduced DHA accumulation rate in the experimental group after 48 h. It could be concluded that the higher malate concentration was beneficial for high DHA accumulation during the latter stage of cultivation. Based on the analysis of the three parameters and the superior performance induced by malate on DHA production of *Schizochytrium* sp. B4D1 relative to the control group, a feeding strategy was proposed to further optimize the fed-batch fermentation process and improve the induction effects of malate addition.

3.3. DHA production in a fed-batch fermentation using a malate feeding strategy

According to the above experimental results, addition of malate to the cultivation environment at 24 h benefited the biosynthesis of fatty acids and increased DHA production. However, later on during the fermentation, the induced effect decreased, through consumption of malate. To determine if DHA production would be enhanced by stabilizing the malate concentration during the lipid accumulation phase, a strategy of feeding malate at a rate of 0.028 g/L/h after 36 h of fed-batch fermentation, following the addition at 24 h, was carried out. This feeding strategy was used in the expectation that the entire system was exposed to conditions whereby the malate was appropriately supplied. The malate feed rate was kept constant (consistent with the consumption rate) throughout the run, such that the malate concentration in the growth medium was at inducing but non-inhibitory levels (4–6 g/L).

The time course of this strategy for DHA production is shown in Fig. 3. Addition of malate (4 g/L) to the fed-batch culture led to an immediate increase in malate concentration in the growth medium, reaching 5.15 g/L at 24 h. After entering the feeding stage, the malate concentration gradually increased and achieved a maximum of 6.74 g/L at 120 h. It was observed that by using the constant-concentration feeding strategy, the maximum concentration of DHA reached 30.7 g/L with a productivity of 284 mg/L/h. These values were both higher than those obtained through the single-addition process (26.2 g/L DHA and 242 mg/L/h DHA productivity after 4 g/L malate added at 24 h) and those without malate addition (19.4 g/L of DHA concentration). In particular, by using this feeding strategy, the final DHA yield from glucose reached 0.103 g DHA/g glucose, which was 7.3% higher than the best result obtained by the single-addition strategy and up to 158.5% of the value of 0.065 g DHA/g glucose achieved by the process without malate addition. It can be concluded that this constant-concentration malate feeding strategy could not only considerably improve DHA concentration in the broth and the DHA yield from glucose but also increase DHA productivity.

3.4. Determination of enzyme kinetics

During the fermentation runs, the activities of the two key enzymes involved in NAPDH production for fatty acid biosynthesis
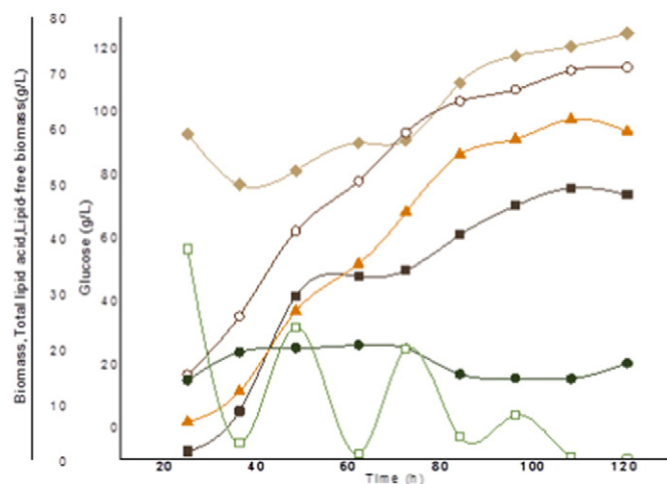

Fig. 3. Time profiles of malate, biomass, glucose, total fatty acids, lipid-free biomass, and DHA concentrations for *Schizochytrium* sp. B4D1 cultivation using the constant-concentration malate feeding strategy. The symbols represent: malate (♦), biomass (○), glucose (□), total fatty acids (▲), DHA (■), and lipid-free biomass (●).

(ME and glucose·6-phosphate dehydrogenase, G6PDH) were measured every 12 h.

Fig. 4 shows the enzyme activity differences among the single-addition group, the two-stage feeding strategy group and the group without malate addition throughout the whole fermentation run. The two key enzyme activities of the malate-addition groups were commonly higher than the control group. The highest ME and G6PDH activities in the single-addition group were up to 81 and 195 U/mg protein, respectively, which were increases of 134.2% for ME and 74.1% for G6PDH, compared with the control group. Furthermore, the constant-concentration feeding strategy group achieved an even higher level of ME and NADPH than the single-addition group, which remained at a high and constant value of 63 ± 3 U/mg for most of the cultivation for ME, and was increased compared with the single-addition group by 13.8% for G6PDH.

The phenomena described above are consistent with Palmieri and co-workers' belief [16] that an efficient citrate efflux system (citrate/malate cycle) exists in the mitochondrial membrane for the export of citrate (in exchange for malate), which therefore accumulates in the mitochondria. Citrate enters the cytosol and is cleaved by ATP:citrate lyase (ACL) to give acetyl-CoA and oxaloacetate. The acetyl-CoA is
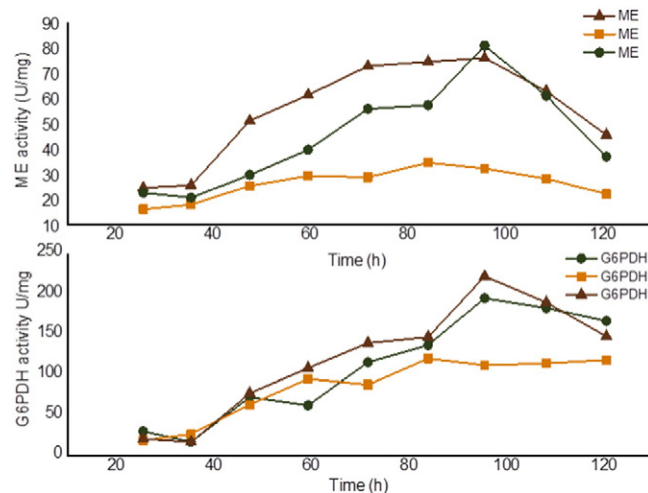

Fig. 4. Time course of G6PDH and ME activity with different methods of adding malate. The symbols represent: group without malate addition (■); single-addition group (●); constant-concentration group (▲).
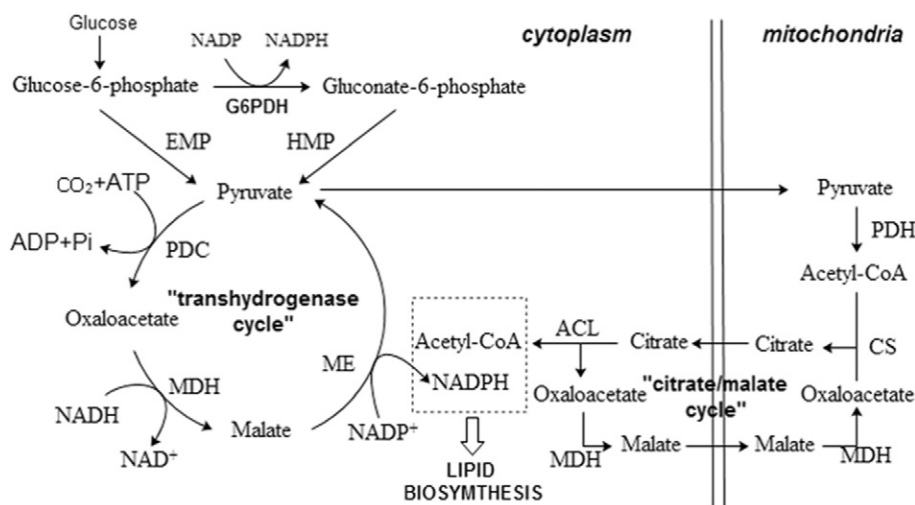
Fig. 5. The mechanisms for the citrate/malate and the cytosolic transhydrogenase cycles in providing acetyl-CoA and NADPH for fatty acid synthesis. PDC: pyruvate decarboxylase, MDH: malate dehydrogenase, ME: malic enzyme, PDH: pyruvate dehydrogenase, CS: citrate synthase, ACL: ATP: citrate lyase, Net reaction for NADPH production (the transhydrogenase cycle): $NADH + NADP^+ + ATP \rightarrow NAD^+ + NADPH + ADP^+ + Pi$ (adapted from [8,10]).

then used for fatty acid biosynthesis as the basic unit of the fatty acid carbon skeleton [17]. The addition of malate may promote the citrate efflux system [9], which then enhances both fatty acid synthesis and substrate consumption.

As shown in Fig. 5, a higher G6PDH activity would strengthen the hexose monophosphate pathway (HMP) and thus produce more NADPH. While a higher ME activity would produce more NADPH through the "transhydrogenase cycle" system, one study proposed that malate could induce a structural change in the malic enzyme, from the dimer to the more active tetramer or octamer forms [18], enhancing the NADPH generating reaction (Malate + NADP + → Pyruvate + CO_2 + NADPH). Therefore, the level of NADPH for DHA synthesis in the malate-addition group would be higher than the group without malate addition. Furthermore, a sufficient high malate concentration during lipid synthesis was obtained by using the constant-concentration feeding strategy, causing a relative high DHA accumulation in the constant-concentration group.

4. Conclusion

Optimization of DHA production by *Schizochytrium* sp. B4D1 was achieved by a constant-concentration malate feeding strategy, which enhances NADPH supply during the oil accumulation stage by improving the malic enzyme activity. The maximum DHA production yield reached 30.7 g/L, with the productivity of 284 mg L^{-1} h^{-1}. The idea of using an inductive factor can be used as reference for producing other products and scaled-up to industrial processes and combined with other optimization methods to simultaneously achieve high product concentrations, yields and productivities.

Financial support

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