Inositol enhances lipid production by *Schizochytrium limacinum* SR21 using defatted silkworm pupae hydrolysate

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Abstract: In the present study, the inositol was evaluated for its effect on lipid and docosahexaenoic acid (DHA) accumulation in *Schizochytrium limacinum* SR21. To investigate effects of inositol on lipid and DHA yield during the whole fermentation process, the time courses (120 h) of lipid and DHA yields by *S. limacinum* SR21. It was found that lipid content and DHA yield of *S. limacinum* SR21 increased by 13.64% and 20.82%, respectively, with 120 mg/L of inositol being added to the medium at 48 h. However, the content of total polyunsaturated fatty acids in the cells increased with the addition of inositol, especially the content of DHA contents were majorly induced. To understand the biochemistry behind these improvements, lipogenic enzyme assays were used and revealed that enzyme activity were increased with inositol addition. Thus, inositol can be used as a new enhancer to improve accumulation of lipid and DHA yield.
Keywords: Defatted silkworm pupae hydrolysate, lipid production, *Schizochytrium limacinum*, Inositol, Docosahexaenoic acid (DHA).

Introduction

Docosahexaenoic acid (DHA) which belongs to the omega-3 group, is important for various physiological functions in the human body [1-3]. In the visual and nervous system tissues, DHA is an particularly important component of the cellular membranes, and it plays a significant role in brain and vision proper development in infants and young children [4, 5].

For too long, marine fish oil has been a primary commercial source of DHA, but environmental pollution and the fishing restrictions and some other problems directly influence the DHA's quantity and quality. Single cell oils (SCOs) avoid these disadvantages of fish oils, and their production may readily be scaled up via biotechnological methods. Algae-derived DHA has thus become the subject of intensive research, and *Schizochytrium sp*. has been commercially utilized to produce DHA by fermentation.

To improve the viability of producing DHA from single-celled microorganisms, some of the efficient approach are required. Some of enhancers of microalgal growth and lipid production have been identified. Thereinto, phytohormones and their derivatives play important role in many metabolic pathways [6]. Gibberellin and 6-benzylaminopurine has proven to be enhancer of the lipid and DHA biosynthesis in *Aurantiochytrium sp.* at metabolic level [7]. Moreover, the addition of gibberellin caused 14.4%, 43.6% and 79.1% increases in biomass, lipid and DHA production respectively contrast to the control group (without gibberellin). Similarly, the lipid and DHA yields were significantly enhanced by 6-BAP treatment, during 120 h fermentation [8]. Besides, Taoka et al. have reported supplementation of 1% tween-80 in the medium induced an increase of 2 fold biomass and 1.15 fold lipid content in *Thraustochytrium aureum* ATCC 34304 [9]. In addition, Y. Zhang et al. found that the 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 [10]. In *Schizochytrium sp.* strains, some polylols, such as myo-inositol were also reported to make a great contribution to responses to environmental stress. Our previous studies have extensively investigated and discussed the effect of fermentation conditions of Defatted silkworm pupae hydrolysate on lipid yields [11,12], to pursue a high productivity and low cost bio-product, it is vital to find a new enhancer to improve cell growth and accumulation of lipid and DHA in large-scale cultivation of *Aurantiochytrium sp.*.

In this study, inositol as a DHA enhencer was introduced to fermentation system to enhance DHA yield of *S. limacinum* SR21, and the effect of inositol on growth, lipid and DHA production of *S. limacinum* SR21 was investigated. Epifluorescence micrograph of *S. limacinum* SR21 stained with nile red in after 5-day cultivation were detected. Furthermore, the lipogenic enzyme assays in *S. limacinum* SR21 under the inositol-treated and the control groups at different time points were analyzed and compared. As a preliminary experiment, we cultivated *S. limacinum* SR21 in a medium with inositol-treated. Until now, there has been almost no study of the use of inositol

for enhancing DHA and lipid production in *S. limacinum* SR21. The aim of this study is to obtain basic knowledge regarding the effect of inositol on growth, lipid accumulation, fatty acid composition and especially DHA production in *S. limacinum* SR21.

Materials and methods

Strain and Cultivations

S. limacinum SR21 (ATCC MYA-1381) was procured from the American type culture collection (ATCC) and it was maintained on a medium consisted of 5 g L^{-1} glucose, 1 g L−1 yeast extract, 1 g L^{-1} tryptone , 20 g L^{-1} agar and 30 g L^{-1} artificial sea salt. The algal inoculum medium comprised: 20 g L^{-1} glucose, 4 g L^{-1} yeast extract, 4 g L^{-1} tryptone and 40 g L^{-1} artificial sea salt. The inoculum medium was cultured with rotary shaking (160 rpm) at 25 °C for 24 h and was inoculated at 10% (v/v). For the components of fermentation medium consists of 100 g/L glucose as C-source and 3.33 g/L, 5 g·L−1 yeast extract and 5 g·L^{-1} tryptone as the N-source and 40 g L−1 artificial sea salt. The shake flask was cultured in an orbital shaker at 25◦C and 160 rpm for 5 days. The batch cultivation was performed in a New Brunswick, BioFlo/CelliGen 115 reactor of total volume 3 L, and temperature was controlled at 25 ◦C, DO at 20%, aeration rate at1.25 VVM, pH at 6.5 and working volume 1.5 L.

S. limacinum SR21 growth and inositol treatment

The effects of different initial inositol concentrations on *S. limacinum* SR21 growth and DHA productivity. For inositol treatment, different concentrations of inositol (0-500 mg/mL) were added to the media before cultivations and the media without inositol addition were as control.

The effects of different inositol feeding time on algae growth and DHA productivity. Inositol was supplied during cultivation phase to different media respectively at the 5 different incubation times (when fermentation time reached 0 h, 24 h, 48 h, 72 h, 96 h and 120 h).

Three parallel samples of samples of the control and the inositol-treated groups were taken every 24 h until the total fermentation time reached 120 h, and biomass, lipid content, fatty acid profile and metabolic profile were analyzed as described below.

Analysis of culture supernatant

The concentrations of glucose in the fermentation broth were measured simultaneously by a bioanalyzer (SBA-40C, Institute of Biology, Shandong Academy of Sciences, China) [13].

Determination of biomass

The biomass was expressed as the cell dry weight (DCW) after centrifuging the cell suspension at 7000 g (the cell were washed twice by pure water) and then overnight drying at freeze drier（Lyoquest-55 Teltsar）.

Lipid extraction and Analysis of fatty acids

1 g of freeze-dried cells was added to 10 mL 20% HCl solution using ultrasonic waves for 30 min, and then centrifuged at 6000 rpm and 4 C for 10 min. The process was repeated 2-3 times, then the supernatants were collected and solvents were evaporated and dried to constant weight in a vacuum oven. Subsequently the TFA (total fatty acid) was measured on the DCW basis.

A two-step methylation process was applied for the determination of fatty acid (FA) composition in *S. limacinum* SR21[14].The FAMEs content was determined [15] via capillary GC on a DB-5, 30 m×0.25 mm id, 0.25-μm capillary column (Model 6820N; Agilent) installed on an Agilent Technologies gas chromatograph equipped with a Hewlett Packard 3396 Series II integrator and 7673 controller, a flame ionization detector, QA/QC NDS (Networked Data System) cerity system and split injection (Agilent Technologies Inc., Santa Clara, CA). The initial oven temperature was 80 °C, which was held for 1 min and subsequently increased to 200 °C at a rate of 20 °C/min, then increased to 250 °C at a rate of 1.5 °C/min. The column head pressure was 280 kPa. The injector was set to 250 °C and the detector to 280 °C. The split ratio was 50:1; the carrier gas is nitrogen with a flow rate of 1 mL/min. FAs were identified by comparing their retention times with the FAMEs standards described previously. GC–MS analysis was performed using an Agilent 6890 gas chromatograph with a 5973 MS detector equipped with 60 m×0.25 mm i.d. 0.25-lm/MS capillary column (DB-WAX). The following temperature ramp was used: injector at 250 °C, oven initially at 200 °C, which was held for 1 min and then heated to 230 °C (1.5 °C /min, then held for 10 min). The characterization and identification of FAMEs from microalgae oil was completed in scan mode. The selected ion monitoring mode was from 35 to 320 m/z.

Enzyme assays and protein estimation

Five key lipogenic enzymes were assayed during the lipogenic phase of culture (respectively at 48 h, 72 h, 96 h and 120 h). ATP-citrate lyase (ACL), lipase, glucose-6-phosphate dehydrogenase (G-6-PDH) and NADP-malic enzyme (NADP-ME) were assayed. Enzyme activity was assayed using a UV spectrophotometer (Jasco model B-530, Tokyo, Japan) at 25 °C. All assays were carried out with at least two concentrations of cell-free extract.

Protein concentration was estimated from the supernatant using the Bradford method with fat-free BSA as the standard. All enzyme and protein assays were done in three replicates and the standard error (SE) calculated using Microsoft excel software [16].

Microstructure Epifluorescence micrograph of cells of S. limacinum SR21

Epifluorescence micrograph of cells of *S. limacinum* SR21 stained with nile red for detection of total cellular lipids (fluorescing yellow) on days five of cultivation.

Statistical analysis

All cultivations were carried out in duplicate. All the determinations were carried out in at least duplicate and the final values were expressed as mean ± standard deviation. Analysis of variation (ANOVA) of single factor was conducted to analyze the variance of these results with significance declared at $P<0.05$ using Origin Pro 8.0.

Results and discussion

Effects of inositol on growth, lipid and DHA production

In this study, *S. limacinum* SR21 was growth in culture medium with different concentration of inositol ranging from 0 to 200 mg/L when inoculation. Fig.1 shows adding inositol showed no significant impact at any of the six concentrations on cell growth and biomass, but the lipid and DHA accumulation of *S. limacinum* SR21 was significantly enhanced by the addition of inositol to the culture medium. As shown in Fig.1 a, production of DHA increased when the concentration added increased from 0 to 120 mg inositol /L. The maximum of growth, lipid and DHA production (38.53 g/L, 56.03% biomass and 7.99 g/L respectively) were obtained when 120 mg/L of inositol was added to the medium, and the supplementation of inositol caused 7.58% and 13.17% increases in lipid and DHA production respectively in comparison with medium without inositol. This result shows that the inositol can enhance the lipid accumulation of *S. limacinum* SR21 and increased the DHA yield. Further, in order to explore the effects of different feeding time on *S. limacinum* SR21, an experiment on addition time of inositol was carried out. In the study, 120 g inositol /L was added to the fermentation medium at different point of time (0, 24, 48, 72, 96 h after inoculation), and Fig.1 b shows changes in biomass, lipid and DHA products of *S. limacinum* SR21. Significant differences were observed among the groups. Production of DHA increased when inositol added from 0 to 48 h. The maximum DHA yield (8.53 g/L) of was achieved with inositol added when cultured for 48 hours. while adding inositol after 48 h did not result in further increase in DHA accumulation. It was found that lipid content and DHA yield of *S. limacinum* SR21 increased by 13.64% and 20.82%, respectively, with 120 mg/L of inositol being added to the medium at 48 h.

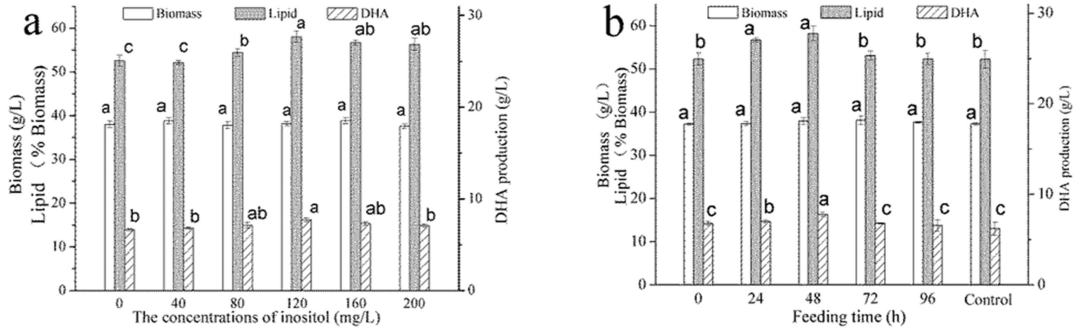

Fig. 1. Effects of different concentrations and feeding of inositol time on biomass, lipid content and DHA yield in *S. limacinum* SR21. (a: different concentrations of inositol; b: different feeding time of inositol). All data are means of three replicates; vertical bars represent error bars with the value equal to the standard error of the mean.

Impact of inositol on fatty acid composition

Fatty acid composition of lipid are both major contributors to the cellular composition of microalgae that are affected by various factors such as nutrients, salinity and temperature; and its composition and productivity have been used as potential indicators for edible oil production. As observed in Table 1, for our *S. limacinum* SR21, in the

composition of fatty acids the major FA detected were myristic acid (C14:0), palmitic acid (C16:0), docosapentenoic acid (C22:5) and DHA(C22:6), and C16:0 and C22:6 were the major forms under all experimental conditions. Results from the experiments show that the Fatty acid composition of *Schizochytrium sp.*SR21 varied between the experimental groups (with addition of inositol) and the control group (without inositol). Moreover, the accumulation of palmitic acid (C16:0) and myristic acid (C14:0) in inositol treated groups were less than the control groups, while the opposite results were detected in the accumulation of DPA (C22:5) and DHA (C22:6). In the fatty acid profile, the percent of the total polyunsaturated fatty acids significantly decreased in the cells cultured with inositol in adding inositol group, and especially in the groups that inositol was added to the medium at 48 h. The palmitic acid (C16:0) and myristic acid (C14:0) fatty acids were majorly decreased under the influence of inositol, compared to the control. However, the content of total polyunsaturated fatty acids in the cells significantly increased with the addition of inositol, especially the content of DHA contents were majorly induced.

Table1 Effect of inositol on fatty acid profiles and contents of produced lipids, and UFAs/SFAs of cultured *S. limacinum* SR21

Fatty acid（%）	Different treatment		
	Control	0 h	48 h
C12:0	0.27±0.16	0.21±0.14	0.25±0.13
C14:0	7.66±0.57	6.97±0.63	6.63±0.12
C15:0	3.35±0.08	3.29±0.31	3.01±0.15
C16:0	44.48±3.10	42.85±0.40	40.54±1.12
C17:0	0.58±0.04	0.58±0.01	0.56±0.03
C18:0	0.26±0.16	0.28±0.10	0.37±0.00
C18:1	0.79±0.09	0.84±0.03	0.79±0.03
C18:3	0.21±0.07	0.19±0.10	0.08±0.02
C20:5 (EPA)	1.23±1.82	1.32±2.06	3.62±0.77
C22:5 (DPA)	6.45±0.24	6.78±0.55	6.75±0.22
C22:6 (DHA)	35.20±0.68	37.02±2.87	37.32±1.27
UFAs	43.67±2.13	46.00±1.17	48.50±1.35
SFAs	56.33±2.13	54.00±1.17a	51.50±1.35
UFAs/ SFAs	0.78	0.85	0.94

[1]UFA-Unsaturated fatty acids. In *S. limacinum* SR21, main UFAs include C18:1, C18:3, C20:4, C20:5, C22:5 and C22:6; SFA-Saturated fatty acids. In *S. limacinum* SR21, main SFAs include C14:0, C15:0, C16:0, C17:0 and C18:0.

It is known that in *Schizochytrium*, PUFAs are synthesized by polyketide synthases (PKS) or fatty acid synthases (FAS), and polyketide pathway has been reported to synthesize fatty acids. DHA is synthesized without desaturation/elongation steps in PKS pathway. In our study, compared with the control group, the content of Fatty acids significantly increased with the supplementation of inositol. It is a possibility that inositol stimulates the FAS or PKS which resulted in increasing the lipid content. However, Table.2 showed that the content of palmitic acid (C16:0) decreased and DHA (C22:6) significantly increased with the supplementation of inositol. The result may indicate the activation of PKS or FAS, and resulted in increasing the total lipid content. Thus, it is the first time to report that inositol could enhance polyunsaturated fatty acid content in *Schizochytrium*.

Table 2 The biomass and DHA yield by the strains *Schizochytrium* and *Aurantiochytrium* at flash levels.

Strain	Additive	Amount of enhanser (mg/L)	Culture conditions	Fold increase of lipid or DHA (%)	Biomass (g/L)	DHA yield (g/L)	Refs
Aurantiochytrium sp. YLH70	gibberellin	4		43.6	21.39	5.39	[2]
*Aurantiochytrium sp.*YLH70	6-benzylamin opurine	3		25.9	20	4.6	[3]
Schizochytrium sp. B4D1	malate		fed-batch	47	107.91	30.7	[4]
Thraustochytrium aureum ATCC34304	Tween-80	1%	flasks	31.1	3.4		[1]
*Schizochytrium sp.*SR21	inositol		batch		38.67	8.63	This study

Time course of lipid production using inositol addition

To investigate effects of inositol on biomass, lipid and DHA yield during the whole fermentation process, the time courses (120 h) of lipid and DHA yields by *S. limacinum* SR21 were shown in Fig. 2, with 120 mg/L of inositol being added to the medium at 0 h. As shown in Fig. 2b and c, addition led to an immediate increase and the lipid and DHA yields with inositol addition were obviously higher than those from the control groups(without inositol). However, we didn't find that inositol having little impact on biomass of *S. limacinum* SR21 in Fig. 2a. Thus, it is the first time to report that inositol promotes the fatty acids and lipid accumulation in marine DHA-producing *Schizochytrium sp.*. Thus, inositol can promotes the lipid accumulation in marine DHA-producing *S. limacinum* SR21.

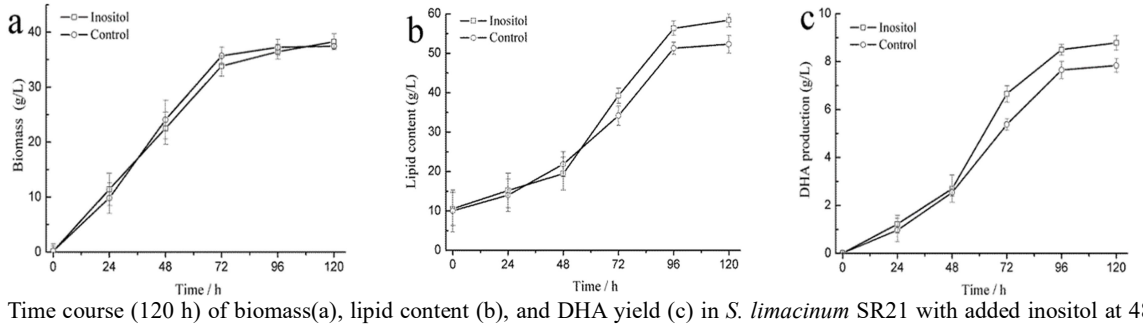

Fig. 2. Time course (120 h) of biomass(a), lipid content (b), and DHA yield (c) in *S. limacinum* SR21 with added inositol at 48 and without inositol addition. All data are means of three replicates; vertical bars represent error bars with the value equal to the standard error of the mean.

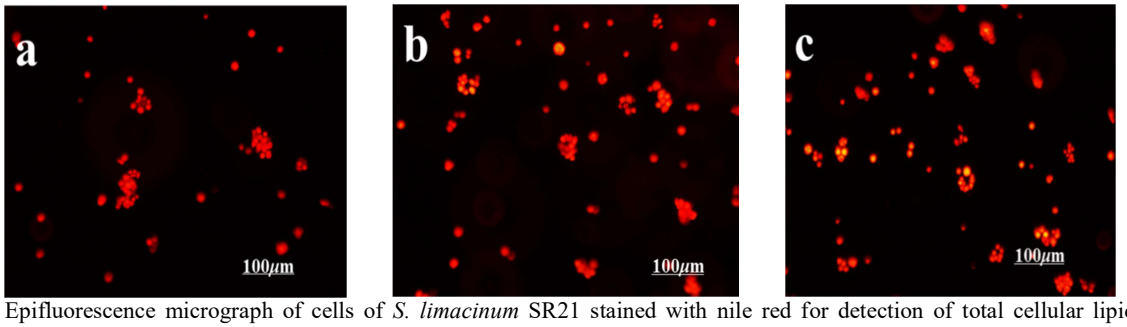

Fig. 3. Epifluorescence micrograph of cells of *S. limacinum* SR21 stained with nile red for detection of total cellular lipids (fluorescing yellow) on days five of cultivation. The strain was cultivated on (a) medium without inositol; (b) the strain was cultivated on medium with inositol being added before the culture; (c) the strain was cultivated on medium with inositol being supplemented at 48 h. Culture conditions: initial glucose concentration 120 g/L; DO was 20% of saturation; incubation temperature 26 °C.

In Fig. 3, the accumulated lipids within the *S. limacinum* SR21 cells are depicted in the form of lipid droplets, after staining with Nile Red. Epifluorescence micrograph of cells of *S. limacinum* SR21 stained with nile red for detection of total cellular lipids (fluorescing yellow) on days five of cultivation. The strain was cultivated on (a) medium without inositol; (b) the strain was cultivated on medium with inositol being added before the culture; (c) the strain was cultivated on medium with inositol being supplemented at 48 h. During our study, the *S. limacinum* SR21 cells apparently were found be more intense lipid staining dyes with the supplementation of inositol than those from the control groups(without inositol) at the end of days 5 of the growth, and the cells which was cultivated on medium with inositol being supplemented at 48 h had most intense lipid staining dyes. For microorganisms, it is known It is known that intensity of lipid staining dyes stained with nile red is proportional to lipid content of cells. Thus, it is the first time to report that inositol could enhance polyunsaturated fatty acid content in *Schizochytrium*. Therefore, this further confirmed inositol could enhance lipid accumulation in *S. limacinum* SR21.

Determination of enzyme kinetics

To investigate the mechanism of inositol enhancing lipid and DHA production by *S. limacinum* SR21，four key enzymes of lipogenesis under different treatments were analyzed in various phases of of cultivation. Pyruvate carboxylase (PC), glucose-6-phosphate dehydrogenase (G6PDH), NADP-malic enzyme (NADP-ME), Isocitrate dehydrogenase mitochondrial (ICDHm) and ATP-citrate lyase (ACL). As illustrated in Fig. 5, the enzyme activity differences among the 48th hours addition group, the addition at the beginning of fermentation group and the group

without inositol addition during the whole fermentation process. The Fig. 5 shows cell enzymes ACL, PC, G6PDH and NADP-ME activity raised at early stage, and then lowered and the four key enzyme activities of the inositol addition groups were commonly higher than the control group.

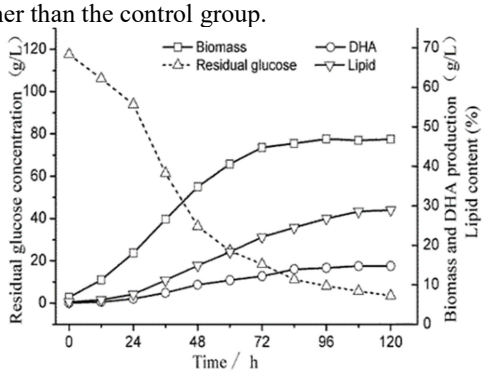

Fig. 4. Change of *S. limacinum* SR21. residual sugar, DHA yield, lipid content in batch fermentation. Culture conditions: initial glucose concentration 120 g/L; inositol concentration 120 mg/L, DO was 20% of saturation; incubation temperature 26 °C.

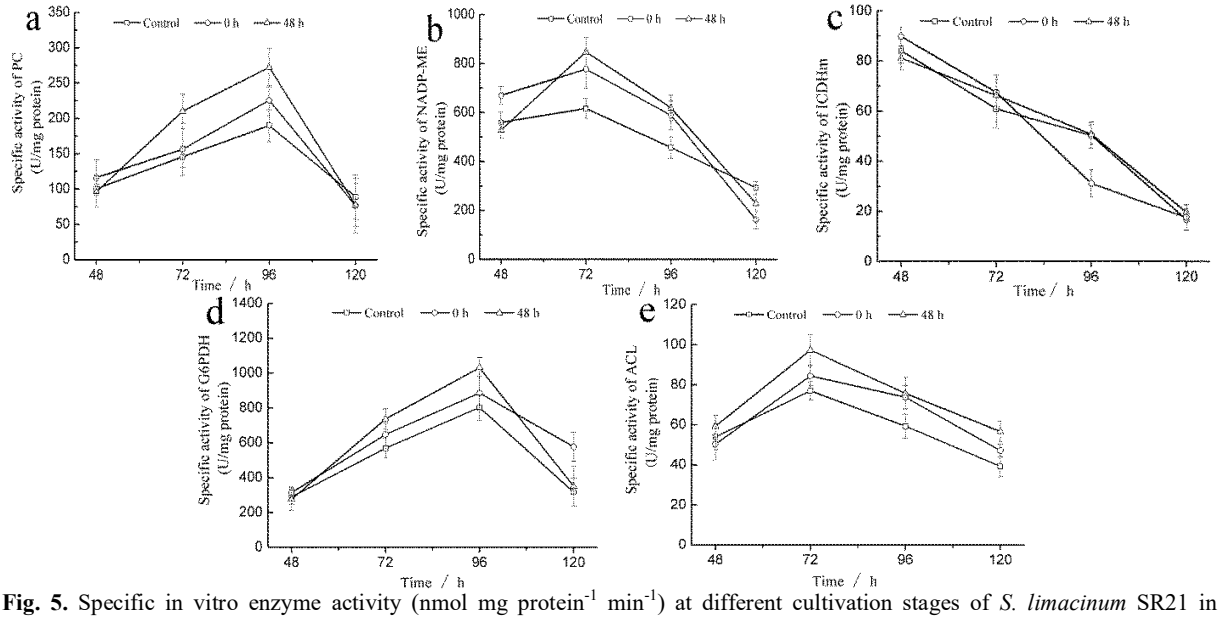

Fig. 5. Specific in vitro enzyme activity (nmol mg protein^{-1} min^{-1}) at different cultivation stages of *S. limacinum* SR21 in different treatments: respectively without inositol (white bar), with inositol being added before the culture(gray bar) and with inositol being supplemented at 48 h (slash-filled bar). in shake flask.(a) PC: pyruvate carboxylase; (b) NADH-ME: NADP-malic enzyme; (c) ICDHm: Isocitrate dehydrogenase mitochondrial; (d) G6PDH: Glucose-6-phosphate dehydrogenase; (e) ACL: ATP-citrate lyase.

In the lipid producing microorganisms, pyruvate carboxylase (PC) is considered in the OAA as acetyl CoA and NADPH played a role in the process of synthesis of intermediate cycle [17]. And During the lipogenic phase, PC showed increased trends, suggesting that PC is also an important lipogenic enzyme for *S. limacinum* SR21.

In the process of eukaryotic microorganism lipid synthesis, for NADPH supply, the main enzymes involved are NADP-ME and the enzymes of the HMP pathway (such as glucose 6-phosphate dehydrogenase (G6PDH) and 6-phosphate glucose acid dehydrogenase (6PGDH)). In the process of cultivating 48 to 120 h, G6PDH vitality of *S. limacinum* SR21 showed tendency of increase in about 48 to 96 h. It is also illustrate that the HMP is a major source of NADPH for lipid synthesis. And after adding inositol, the activity of G6PDH always significantly higher than the control group, suggests that adding a certain amount of inositol increased in G6PDH. NADP-ME activity showed decreased after the first increased trend in Fig. 5b, and the highest ME activity in the addition at 48 h group were up to 847.68 (U/mg prot), and in the period of lipid synthesis (48 to 96 h), ME activity of adding inositol group showed higher than control group, particularly within 72 to 96 h. After cultured 72 hours, however, NADP-ME activity decreased rapidly during lipogenesis in (Fig. 5b). This suggested that enzyme activity decreased under after N-depletion, even in the present of glucose, a similar trend has been observed in other research [18].

The activity of ICDHm in all medium was decreased constantly during 48~96 h. It is a key enzyme in the tricarboxylic acid cycle system. There was no significant difference between the control group and the experimental group.

ATP-citrate lyase (ACL) is considered to be a key limiting enzyme for lipid synthesis in oleaginous microorganisms [19]. In this study, the specific activity trends of ACL and NADP-ME were very similar, and the highest activity reached 100.29 (U/mg protein). ACL of *Y. lipolytica* was ca. 80 nmol mg protein^{-1} min^{-1} [20]. The high specific activity of ACL would imply superior Acetyl CoA provision and thus higher fatty acid productivity of SR21in comparison with other oleaginous microorganisms.

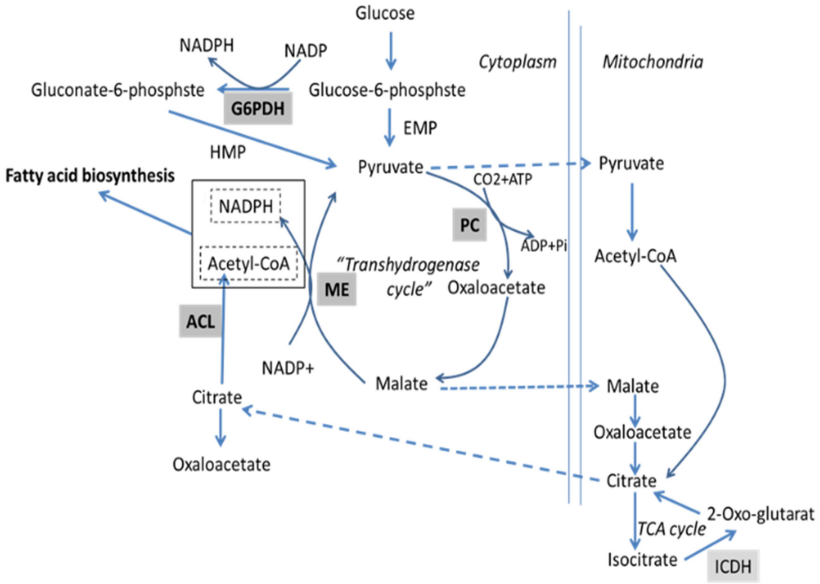

Fig. 6. Metabolic pathway of fatty acid biosynthesis and the related key enzymes in *S. limacinum* SR21

As shown in Fig. 6, a higher ME activity would produce more NADPH through the "transhydrogenase cycle" system, and a higher G6PDH activity would strengthen the hexose monophosphate pathway (HMP) and thus produce more NADPH. Similarly, a higher ACL activity would produce more acetyl CoA. Therefore, the level of NADPH and acetyl CoA for DHA synthesis in the inositol -addition group would be higher than the group without malate addition. Overall, the results showed that the specific activities of most of the lipogenic enzymes were significantly increased under inositol-treated conditions. This information might also be useful for further DHA production enhancement.

Conclusions

Optimization of DHA production by *S. limacinum* SR21 was achieved by inositol feeding strategy, which enhances lipid accumulation during the oil accumulation stage by improving the lipogenic enzymes. The maximum DHA production yield reached 8.53 g/L, with the productivity of 71.08 mg L^{-1} h^{-1}. It was found that 120 mg/mL of inositol increased DHA yield of *S. limacinum* SR21 by 20.82%. 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.

Acknowledgments

This study was financially supported by the Key Research and Development Program (Modern Agriculture) of Jiangsu Province (BE2017322), the Six Talent Peaks Project of Jiangsu Province (2015-NY-018), the Qing Lan Project of Jiangsu Province (2014), the Shen Lan Young scholars program of Jiangsu University of Science and Technology (2015), and the China Agriculture Research System (CARS-18- ZJ0305).

The authors have declared no conflicts of interest.

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