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Research article

Alanine mother liquor as a nitrogen source for docosahexaenoic acid production by *Schizochytrium* sp. B4D1

Jian Xu [a,b,1], Yujing Zhu [a,1], Hanchen Li [c], Limei Chen [b], Wuxi Chen [b], Min Cui [b], Lina Han [d], Wenbo Hou [d], Demao Li [b,*]

[a] College of Animal and Veterinary Science, Shenyang Agriculture University, Shenyang 110866, China
[b] Tianjin Key Laboratory for Industrial Biological Systems and Bioprocess Engineering, Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences, Tianjin 300308, China
[c] Hebei Normal University of Science & Technology, Qinhuangdao, 066004, China
[d] Zhucheng Bureau of Animal Husbandry and Veterinary Administration, Weifang 262200, China

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ABSTRACT

Alanine mother liquor, a type of industrial waste from alanine fermentation, was used as a nitrogen source to produce docosahexaenoic acid (DHA) by *Schizochytrium* sp. B4D1. The results indicated that yeast extract could trigger the utilization of the alanine mother liquor. Additionally, the alanine can be quenched during the culture, which aids in DHA accumulation. The medium components were optimized via response surface methodology as follows: 99.98-g/L glucose, 0.05-g/L yeast extract and a 183.17 dilution factor of the alanine mother liquid (v/v, with an alanine content of 0.72 g/L) and 17.98% inoculum concentration (v/v). Finally, in a 50-mL shake-flask fermentation, the DHA yield was 2.29 g/L.
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1. Introduction

Docosahexaenoic acid (DHA), a type of long-chain polyunsaturated fatty acid [1], plays an important role in maintaining human health [2] and preventing various diseases [2]. Furthermore, DHA is essential for the proper function of the nervous system and for visual functions [3] because DHA is an integral component in the brain [4] and retina [5, 6]. For these reasons, DHA has garnered an increasing amount of attention from scientists [7]. The traditional commercial approach to DHA extraction is from tissues of marine fishes [8]; however, this method is not environmentally friendly, prone to heavy metal contamination and restricted to non-vegetarian diets. Many marine microorganisms, such as *Schizochytrium* [9] and *Thraustochytrium* [10], have been substitute sources of DHA production due to their high growth rates and capacity for DHA yield [11] and due to their qualities of high production, non-contamination and vegetarian sources. However, the high price, related to the source shortage and the intricacy of the extraction process, prevents full acceptance of *Schizochytrium* as a source of DHA. Thus, DHA production demands a reduction in cost. A viable alternative is the use of industrial waste to replace expensive culture media. Using of industrial waste lowers the costs of the medium, results in safe production and reduces the level of pollution of these wastes.

The main two components in the culture medium are carbon and nitrogen. Prior studies have shown that biodiesel-waste glycerol [12], *Shochu* distillery wastewater [13], and soybean meal hydrolysate from the food industry [14] can be used as the carbon source for DHA production. According to these studies, many more alternative carbon sources have been utilized than the nitrogen source. However, in terms of production costs, the nitrogen source is much more expensive than the carbon source [15]. Therefore, further studies should be conducted to search for a proper substitute for the nitrogen medium.

Alanine, a non-essential amino acid, has been widely used in the chemical industry, for medical treatments and in other fields. In the industrial production process of alanine according to Qinghuangdao Huaheng Biotechnology Co., Ltd., an alanine production company,

* Corresponding author.
E-mail address: li_dm@tib.cas.cn (D. Li).
[1] These authors contributed equally to this work.
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after the fermentation of *E. coli* and the crystallization processes, substantial amounts of alanine were obtained [16]. Alanine is deposited via the processes of decolorization, compression and other steps, and the remaining fluid is the so-called alanine mother liquor. In addition to high concentrations of alanine, the alanine mother liquor also contains Maillard Reaction Products (MRPs), organic acid, glucose and other components. Given the great demand for alanine, tons of alanine mother liquor is produced. The treatment of this industrial waste costs an enormous amount of money annually. Currently, the preferred disposal method of the hazardous waste is to use it as chemical fertilizer, which does not fully dispose of it.

In the present study, alanine mother liquor was used as the nitrogen source in the production of docosahexaenoic acid, which dramatically reduces the production cost of nitrogen to nearly zero. Yeast extract was used as the growth factor, which regulates metabolism and cell proliferation [17], to trigger the utilization of alanine. Utilization of alanine mother liquor not only renders commercial DHA production much more economically competitive but also positively addresses the problem of industrial waste.

2. Materials and methods

2.1. Microorganism, media and materials

Schizochytrium sp. B4D1 (CGMCC No. 8313) was used in this study and was stored in 20% (v/v) glycerol at −80°C. Active cultures for inoculating the seed cultures were prepared on solid medium which contains glucose (30 g/L), yeast extract (4 g/L) and agar powder (2 g/L) dissolved in artificial sea water. The seed culture was grown in 250-mL flasks containing 50 mL of either greater yeast extract medium (hereafter called MYE medium, composed of glucose (30 g/L) and yeast extract (4 g/L) dissolved in artificial sea water) or lesser yeast extract medium (hereafter called LYE medium, composed of glucose (30 g/L) and yeast extract (2 g/L) dissolved in artificial sea water) at 26°C for 2 d. Further cultures were also conducted in 250-mL flasks containing 50-mL medium at 26°C. All the fermentations were performed in triplicate on a shaker incubator at 180 rpm.

Alanine mother liquor, provided by Qinghuangdao Huaheng Biotechnology Co., Ltd., contains 131.87-g/L alanine, and the percentage of main elements were as follows: C 17.39%, H 9.59%, N 6.48%, O 58.18%, P 7.07%, S 0.41%. The total nitrogen (%) of yeast extract, beef extract peptone and soy peptone were 10.6, 14.21 and 9.1, respectively. All the other chemicals were of analytical grade, unless otherwise specified.

2.2. Methods

2.2.1. Isolation of the growth factors sources

Liquid seed from MYE medium (10%) and single colony from solid medium were inoculated into initial culture medium separately, cultured for two days to compare OD_{600} of medium. The composition of initial culture medium was glucose (30 g/L) and dilution multiple of alanine mother liquid (100, v/v) dissolved in artificial sea water. Different types of possible growth factors sources, including yeast extract (Oxoid, Basing-Stoke, UK), soy peptone (SCRC, Shanghai, China), beef extract peptone (AOBOX, Beijing, China), inorganic trace elements, and urea (SCRC, Shanghai, China), were added into the culture medium to select optimal growth factor sources. The concentration of the possible growth factors source was 2 g/L. The remaining component in the culture medium was glucose (30 g/L) dissolved in artificial sea water. Seeds were cultured in LYE medium for two days, and the inoculum concentration was 10% (v/v).

2.2.2. Effects of alanine mother liquor concentration on DHA production

To obtain higher DHA productivity per unit volume of medium, additional shake-flask cultures were developed to obtain the optimum concentration of alanine mother liquor. The various ranges of alanine mother liquor concentrations are listed in Table 1. In addition to the alanine mother liquor, the composition of the culture medium was glucose (80 g/L) dissolved in artificial sea water. Seeds were cultured in MYE medium; the inoculum concentration was 10% (v/v).

2.2.3. Effects of glucose concentration on DHA production

Studies were performed to determine the optimal density of glucose, concentrations tested were 60, 80, 100, and 120 g/L. In addition to glucose, the composition of the culture medium included a dilution factor of the alanine mother liquid (150, v/v) dissolved in artificial sea water. The total cell biomass and DHA content were examined after glucose was exhausted. Seeds were cultured in MYE medium; the inoculum concentration was 10% (v/v).

2.2.4. Effects of yeast extract concentration on DHA production

To make sure yeast extract in seed medium can be exhausted so that make no influence to the further fermentation, same quantity of single colony was inoculated into MYE medium and LYE medium to compare the utilization of yeast extract. OD_{600} and concentration of total nitrogen in MYE medium and LYE medium should be measured to draw growth curve and nitrogen consumption curve. To investigate the optimal concentration of yeast extract, concentrations were tested among 0, 0.2, 0.3, 0.4, 0.5, 0.6, and 0.7 g/L. In addition to the yeast extract, the composition of the culture medium was glucose (80 g/L) and a dilution factor of the alanine mother liquid (150, v/v) dissolved in artificial sea water. Seeds were cultured in LYE medium for inoculating continuous runs (10%, v/v).

2.2.5. Effects of inoculum concentration on DHA production

The components of alanine mother liquor are complex, and many of these components may be disadvantageous for the growth of *Schizochytrium* sp. B4D1. Furthermore, DHA production is an intracellular process. To obtain a higher yield of DHA, substantially more biomass is required. Increasing the inoculum concentration is the simplest and most direct method to achieve a higher biomass.

To investigate the optimal inoculum concentration, concentrations were tested among 10%, 12%, 14%, 16%, 18%, and 20% (v/v). The composition of the culture medium was glucose (80 g/L), a dilution factor of the alanine mother liquid (150, v/v), yeast extract (0.3 g/L), and artificial sea water. Seeds were cultured in LYE medium.

2.2.6. Response surface methodology design

Based on the results of the above experiments concerning the approximate conditions for DHA yield, namely, the amounts of glucose, alanine mother liquid, yeast extract and inoculum, the response surface methodology was applied to optimize the interaction of the different factors. Response surface methodology was used to identify and optimize the nutrients that have a significant effect on DHA yield [18]. This was tested at three levels and included 7 center points. The actual response is shown in Table 2, which shows the design and the results regarding the studied variables of glucose concentration (X_1), the dilution factor of the alanine mother liquid (X_2), the yeast extract concentration (X_3) and the inoculum concentration (X_4). Seeds were cultured in LYE medium.

To examine the results of the predicted responses and the fatty acid components, further experiments (hereafter called Mb) were performed based on the results of response surface methodology (hereafter called Ma). The components of Ma and Mb are as follows:

Ma: glucose (80 g/L), dilution factor of the alanine mother liquid (190.22, v/v), yeast extract (0.05 g/L), artificial sea water, inoculated from seed culture medium with 17.98% (v/v).

Table 1
Concentrations of alanine mother liquor tested.

Runs	Dilution of alanine mother liquid (v/v)	Concentration of alanine (g/L)
1	50	2.64
2	100	1.32
3	150	0.88
4	200	0.66
5	250	0.53
6	300	0.44
7	350	0.38
8	400	0.33

Mb: glucose (80 g/L), alanine (0.72 g/L), yeast extract (0.05 g/L), artificial sea water, inoculated from seed culture medium with 17.98% (v/v).

2.3. Analysis methods

2.3.1. Biomass determination

Cell suspensions (20 mL) were centrifuged at 10,000 rpm and 4°C for 10 min and then washed twice with distilled water and dried in a vacuum freezing dryer (FD-1C-50, BJBYK Co, Beijing, China) at −52°C for 24 h to obtain the biomass powder. The biomass powder was then weighed to obtain the dry cell weight.

2.3.2. Analysis of glucose

Glucose was analyzed using SBA-40C biosensor equipment (Biological Institute of Shandong Academy of Science, Shandong, China).

2.3.3. Composition determination of alanine mother liquor

Element of P in alanine mother liquor was measured by Plasma atomic emission spectrometer (PerkinElmer Inc., USA). Element of C, H, N, O, S in alanine mother liquor were measured by Elementar vario EL cube (Elementar Analysensysteme GmbH, Germany). Total N in alanine mother liquor was measured by Multi N/C 2100S (Analytikjena Co., Ltd., Germany).

2.3.4. Analysis of lipid weight and fatty acid composition

Biomass powder of 0.1 g was suspended in 1 mL of 50% hydrochloric acid and heated at 75°C for 2 h to break the cell wall. *n*-Hexane (5 mL) was added to the sample, and the mixture was shaken for 12 h to extract fatty acids. The supernatant was collected and dried at 60°C under the protection of nitrogen. Furthermore, 1 mL of a 0.5-M KOH-methanol solution was used to dissolve the dried sample, and the sample was heated at 60°C for 15 min; then, the lipids were esterified in 2 mL of a 14% BF_3-methanol solution and heated at 60°C for 2 min. After the sample was extracted with 1 mL of *n*-hexane, the fatty acid content was analyzed with a gas chromatograph (GC) (GC-20, Shimadzu, Japan) equipped with a flame ionization detector (FID) and a sp-2560 column (100 m ∗ 0.25 mm ∗ 0.20 μm, Supelco, USA). Nitrogen was used as the carrier gas. The initial column

Table 2
Central composite design and results.

Runs	Coded variables				Real variables				DHA yield (g/L)
	X1	X2	X3	X4	X1(g/L)	X2(v/v)	X3(g/L)	X4(%)	
1	0	0	0	0	80	150	0.05	10	1.11
2	0	0	0	−1	80	150	0.3	14	1.32
3	1	1	0	1	100	250	0.3	14	1.08
4	−1	−1	−1	0	60	50	0.3	14	0.53
5	0	1	0	0	80	250	0.05	14	1.00
6	0	−1	0	1	80	50	0.05	14	0.41
7	1	0	1	0	100	150	0.3	10	1.21
8	1	0	0	−1	100	150	0.55	14	1.48
9	0	0	1	0	80	150	0.3	14	1.18
10	0	−1	0	0	80	50	0.3	10	0.26
11	0	1	1	−1	80	250	0.3	18	1.50
12	1	0	0	0	100	150	0.05	14	1.54
13	1	−1	0	0	100	50	0.3	14	0.42
14	1	0	0	−1	100	150	0.3	18	1.15
15	−1	0	0	1	60	150	0.55	14	1.44
16	−1	0	0	1	60	150	0.3	10	1.74
17	0	1	1	1	80	250	0.55	14	1.19
18	−1	1	−1	1	60	250	0.3	14	0.90
19	0	0	1	0	80	150	0.55	10	2.25
20	0	0	0	0	80	150	0.3	14	1.03
21	0	0	−1	0	80	150	0.3	14	1.40
22	0	1	0	0	80	250	0.3	10	1.01
23	−1	0	0	−1	60	150	0.3	18	1.32
24	0	−1	0	1	80	50	0.3	18	0.35
25	0	0	−1	0	80	150	0.55	18	1.23
26	0	0	0	0	80	150	0.05	18	2.12
27	0	−1	0	1	80	50	0.55	14	0.73
28	0	0	1	0	80	150	0.3	14	1.20
29	−1	0	0	−1	60	150	0.05	14	1.54

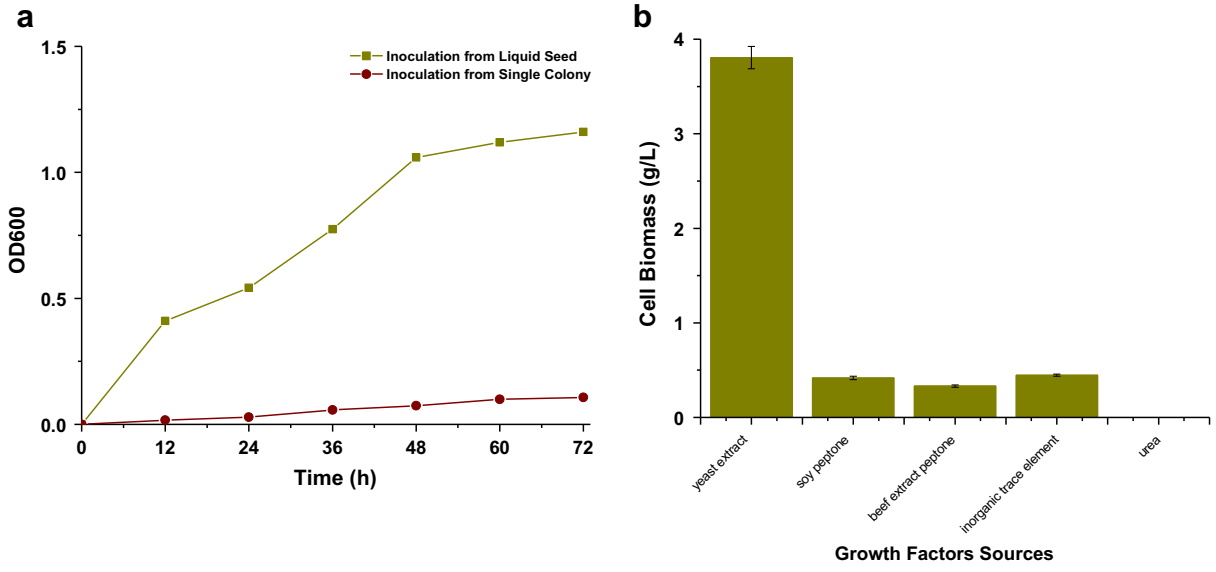

Fig. 1. Effect of inoculum sources and growth factors sources on cell biomass. (a) Effect of different inoculum sources on growth. (b) Effect of different growth factors sources on growth.

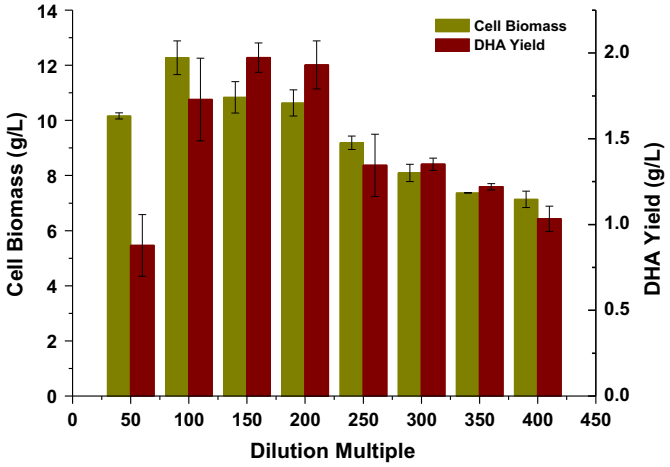

Fig. 2. Effect of alanine mother liquor concentration on cell biomass and DHA yield.

temperature was set at 180°C and was increased at 30°C/min until the temperature reached 240°C, which was maintained for 18 min. The temperature of the FID was set at 250°C. The flow rates of helium, hydrogen and air were 63.7, 40 and 400 mL/min, respectively [19]. The amount of fatty acid methyl esters (FAMEs) was measured based on a comparison with the peak areas of methyl nonadecanoate (Sigma Co., St. Louis, Mo, USA) [19]. Data were analyzed by the software of Origin 8.5; average data±standard deviation of each parallel groups were shown in the results.

2.3.5. Analysis of the alanine concentration

Sample cell suspensions were centrifuged at 10,000 rpm for 10 min, and then, the supernatant was collected. Analysis of the concentration of alanine was performed using high-performance liquid chromatography (HPLC) (HPLC-20AD, Shimadzu, Japan) equipped with a UV detector and a C-8 column (4.6 mm*150 mm*5 μm, Agilent, USA). Mobile phase A consisted of 0.05-M CH_3COONa-methanol. Mobile phase B consisted of methanol. These two mobile phases were mixed at a ratio of 7:3 at a flow rate of 1 mL/min. The UV detector wavelength was set at 334 nm. The temperature of the column was maintained at 30°C. Data were analyzed by the software of Origin 8.5; average data± standard deviation of each parallel group were shown in the results.

2.3.6. Analysis of the response surface

Design-expert (V8.0.6) software was used for the response surface methodology. Prior to the central composite design, the optimal conditions for DHA yield, namely, the concentrations of glucose, alanine mother liquor, yeast extract, and inoculums, were determined by changing one factor while keeping the other factors constant. The model proposed for the response was explained by [Equation 1]:

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum \sum_{i<j=1}^{3} \beta_{ij} X_i X_j \quad (1)$$

where Y is the predicted response variable; β_0, β_i, β_{ii} and β_{ij} represent the intercept, linear, quadratic and interaction terms, respectively, and X_i and X_j are independent variables [20]. The fitted parameters were tested for four factors at three levels, 1 (high), 0 (medium), and −1 (low), and were expressed using surface and contour plots to visualize the relationship between the response and experimental levels, thus determining the optimum conditions [21]. The actual responses are shown in Table 2.

3. Results and discussion

3.1. Isolation of the growth factor source

As shown in Fig. 1, much more biomass can be obtained in initial culture medium with the inoculation of liquid seed from MYE medium rather than single colony from solid medium. Compared OD_{600} at 72 h, inoculation from liquid seed is equal to 10 times inoculation from single colony. Reasons lies in not only much more biomass was inoculated into initial culture medium from liquid seed, but also the composition in liquid seed that contains many growth factors trigger the growth of *Schizochytrium* sp. B4D1. Base on the ingredients of MYE medium, the growth factor is yeast extract [22] for it is produced from the yeast cells with the mainly components of amino acids, peptides, vitamins, nucleotides, minerals and other soluble components of yeast cells [23] which are essential for the growth of *Schizochytrium* sp. B4D1. Therefore, it is necessary to search for optimal growth factors source. The effects of different possible growth factors sources are shown in Fig. 1. The highest production of cell biomass was acquired with the addition of yeast extract. The results indicated that yeast extract can supply optimal growth factors,

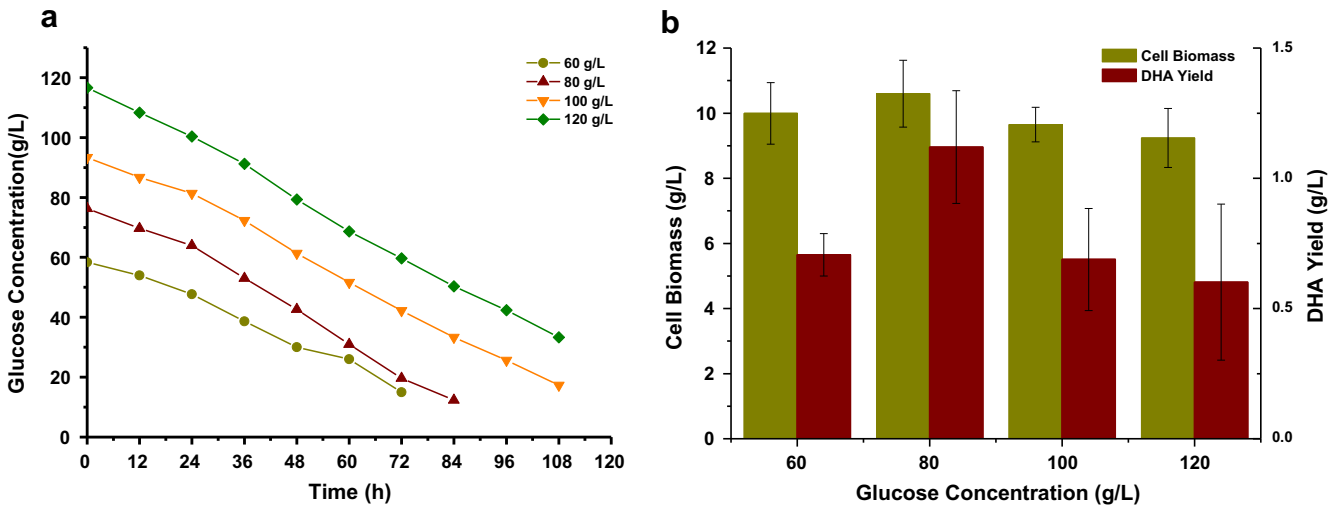

Fig. 3. Glucose consumption and effects on cell biomass and DHA yield. (a) Consumption of glucose by *Schizochytrium* sp. B4D1 with different initial concentration used at the beginning. (b) Effects of glucose on DHA yield and cell biomass of *Schizochytrium* sp. B4D1.

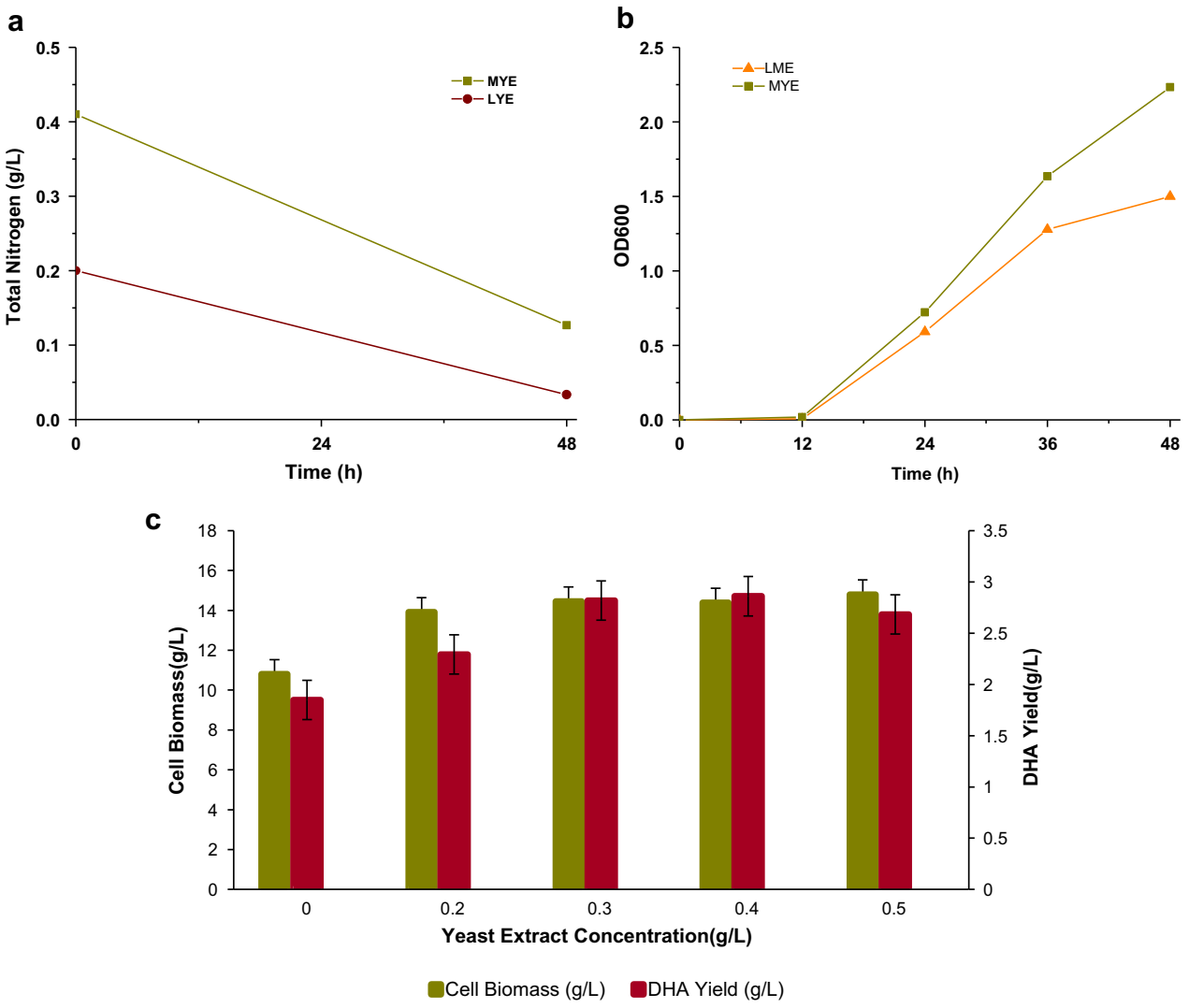

Fig. 4. Consumption curve of total nitrogen in MYE and LYE medium and effect of yeast extract on biomass in seed culture and DHA yield in fermentation. (a) Consumption of total nitrogen in MYE and LYE medium. (b) Cell biomass in MYE and LYE medium. (c) Effect of yeast extract concentration on cell biomass and DHA yield.

which did not initially exist in the medium and can be used once it passes through the cell walls and protoplasm [24]. Compared with animal or plant extracts and inorganic trace elements, yeast extract, a type of microorganism extract, contains many more growth factors, such as vitamins, amino acids and nucleotides, which better suit the growth of microorganisms. Additionally, amino acids contained in yeast extract can maintain the balance of amino acids, wherein stationary amino acids can be utilized at a faster rate, which is beneficial for the growth of *Schizochytrium* sp. B4D1. No growth was observed with urea because no growth factors were presented. Beef extract and soy peptones were mainly used as the only nitrogen source, and other factors, such as protein and vitamins, were minimal. Therefore, yeast extract was verified as the optimal growth factor source to promote the growth of *Schizochytrium* sp. B4D1.

3.2. Effects of alanine mother liquor concentration on DHA production

The nitrogen source was directly linked to the intracellular metabolic profiles, which play important roles in affecting the production of fermentation [24]. The effect of alanine mother liquor concentration is shown in Fig. 2. Among the tests using different concentrations of alanine mother liquor, the highest cell biomass was achieved at the dilution multiple of 100 (v/v; $P < 0.01$), whereas the highest DHA production was achieved at the dilution multiple of 150 (v/v; $P < 0.05$).

Alanine mother liquor, a kind of industry waste in alanine production was used as nitrogen source in DHA production can reduce

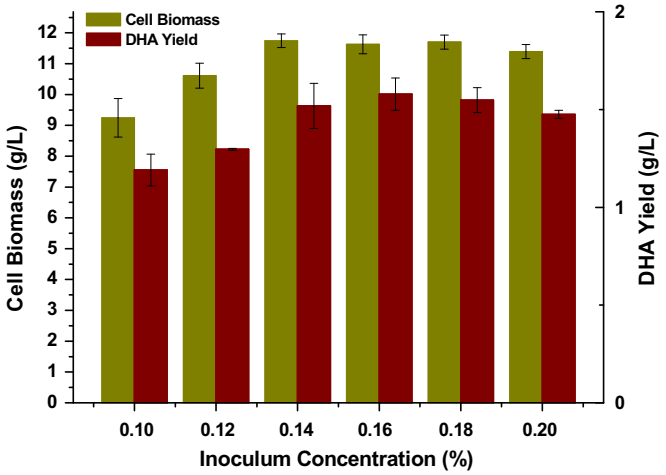

Fig. 5. Effect of inoculum concentration on cell biomass and DHA yield.

Table 3
Analysis of variance for the full quadratic model.

Source	Sum of squares	Degrees of freedom	Mean squares	F-value	Probe > F
Model	5.91	14	0.42	10.79	<0.0001
Residual	0.55	14	0.039		
Lack of fit	0.47	10	0.047	2.37	0.2099
Pure error	0.079	4	0.020		
Total	6.46	28			

Table 4
Model coefficient estimated by liner regression.

Independent variables	Sum of squares	Degree of freedom	Mean square	F-value	Probe>F
X_1	0.0299	1	0.0299	0.7643	0.3967
X_2	1.3185	1	1.3185	33.7234	<0.0001
X_3	0.0307	1	0.0307	0.7855	0.3304
X_4	0.0007	1	0.0007	0.0168	0.8985
X_1X_2	0.0195	1	0.0195	0.4987	0.4916
X_1X_3	0.0003	1	0.0003	0.0057	0.9270
X_1X_4	0.0332	1	0.0332	0.8490	0.3724
X_2X_3	0.0044	1	0.0044	0.1136	0.7411
X_2X_4	0.0421	1	0.0421	9.0776	0.3168
X_3X_4	1.0297	1	1.0297	26.3374	0.0002
X_1^2	0.0071	14	0.0071	0.1807	0.6773
X_2^2	2.2424	10	2.2424	57.5560	<0.0001
X_3^2	0.3896	4	0.3896	9.7639	0.0070
X_4^2	0.1381	28	0.1381	3.5311	0.0812

the cost on nitrogen source. What's more, it can also save the cost on degrade alanine mother liquor. So utilization of alanine mother liquor not only renders commercial DHA production much more economically competitive but also positively addresses the problem of industrial waste.

Table 5
Canonical analysis of the response surface.

Factor	Coded	Uncoded
Glucose concentration (X1)	0.997	99.95 g/L
Dilution factor of alanine mother liquid (X_2)	0.332	183.17 (v/v)
Yeast extract concentration (X_3)	−0.999	0.05 g/L
Inoculum concentration (X_4)	0.996	17.98%
Stationary point	Maximum	
Predicted value		2.26 g/L
Observed value		2.29 g/L

3.3. Effects of glucose concentration on DHA production

Glucose has been proven to be the optimal carbon source in *Schizochytrium* culture [25,26], therefore, glucose is used and studies were performed to determine the optimal density of glucose. The concentration of glucose in the culture medium was measured every 12 h (Fig. 3). The effect of glucose is shown in Fig. 3. According to Fig. 3, the fermentation process in the shake flasks should be terminated when glucose is nearly exhausted at the initial concentration of 60 g/L and 80 g/L at 72 h and 82 h, respectively. Otherwise, *Schizochytrium sp.* B4D1 would utilize the lipids reserved in their own cells [25]. However, at the initial concentrations of 100 g/L and 120 g/L, the fermentation processes in the shake flasks should be terminated at 108 h (initial time of the steady phase), even though glucose has not been exhausted. The results were measured in terms of biomass and DHA production. At the initial concentration of 80 g/L, the highest DHA yield was achieved ($P < 0.05$). It can be concluded that the optimal concentration of glucose is 80 g/L, and the optimal culture period is 3 d.

3.4. Effects of yeast extract concentration on DHA production

Yeast extract has been proved the source of growth factors that is an essential trigger for the growth of *Schizochytrium* sp. B4D1 with alanine

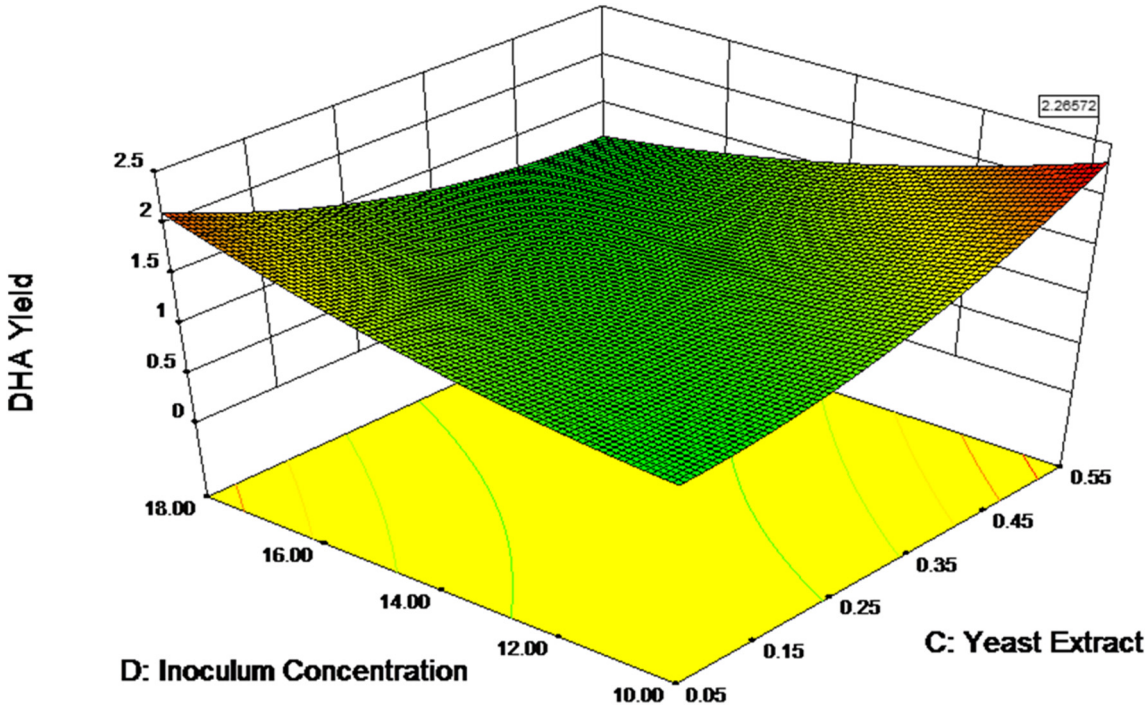

Fig. 6. Response surface plot showing the effect of all the variables on DHA production.

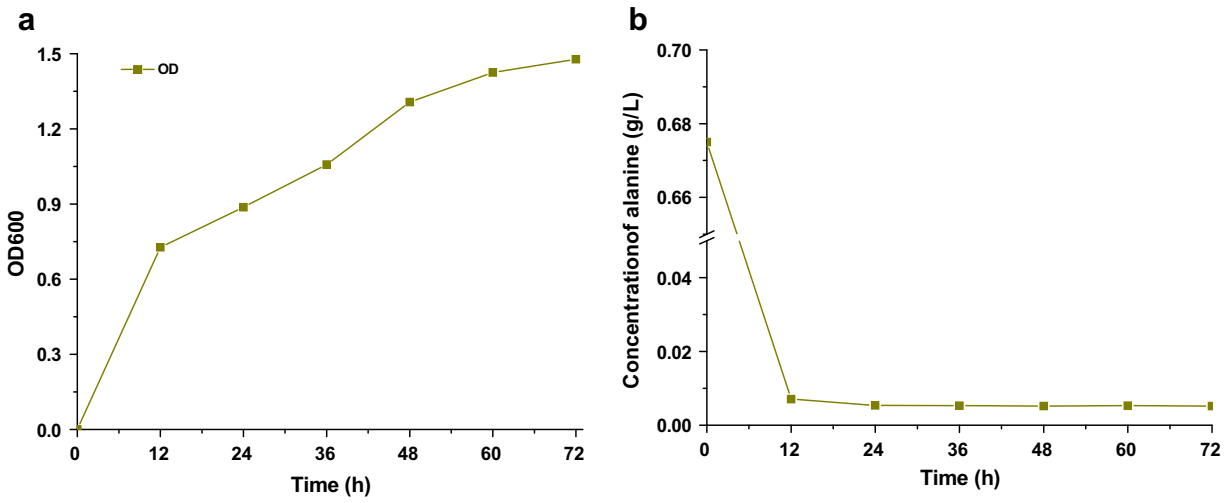

Fig. 7. Growth curve and consumption curve of alanine in optimal culture medium. (a) Cell biomass in optimal culture medium. (b) Consumption curve of alanine in optimal culture medium.

mother liquid as the nitrogen source. To make sure yeast extract in seed medium can be exhausted so that make no influence to the further fermentation, same quantity of single colony was inoculated into MYE medium and LYE medium to compare the utilization of yeast extract. As shown in Fig. 4, at 48 h, concentration of total nitrogen in LYE medium is 0.03 g/L; it's nearly to be extracted; we have reason to believe that the growth factors contained in the yeast extract are also nearly to be exhausted. So the LYE medium can make sure that the growth factors can be exhausted so that they make no influence to further culture. However, as shown in Fig. 4, compared OD_{600} of the two medium, MYE medium is better than LYE medium; LYE medium can reduce the influence on further fermentation, but it's also reduce inoculum concentration, then reduce DHA yield. The effects of different concentrations of total nitrogen are shown in Fig. 4. According to the DHA yield, the optimal concentration of yeast extract is 0.3 g/L ($P < 0.05$). This small amount of yeast extract was not used as the nitrogen source, but as growth factors to trigger the utilization of the alanine mother liquor, which is confirmed by the alanine assumption curve in Fig. 4.

3.5. Effects of inoculum concentration on DHA production

The inoculum concentration, which has a substantial and direct effect on the lag phase and other factors, is an important factor for biomass production and DHA yield. The effect of the inoculum concentration is shown in Fig. 5. The highest biomass and DHA yield were achieved at the inoculum concentration of 14% ($P < 0.05$). This is the simplest and most economical method to increase biomass and DHA yield. Thus, the optimal inoculum concentration is 14%.

3.6. Response surface methodology optimization

A central composite rotatable design with four variables was applied to design the response surface methodology [21], and the DHA yields are shown in Table 2. The analysis of variance for the second-order response surface models is shown in Table 3. The $F_{0.05}$ value for the model ($P < 0.0001$) was significant, therefore indicating that the model was highly significant. The $F_{0.05}$ value for the lack of fit (0.2099) was not significant, therefore indicating that the fitted model was appropriate for the description of the response surface [20].

The statistical analysis results of the independent variables are shown in Table 4. The probability value was used to check the significance of each variable. It can be concluded that X_2 was the most important factor affecting the DHA yield ($P < 0.01$). Additionally, X_3X_4, X_2^2 and X_3^2 had significant effects on the DHA yield ($P < 0.05$).

The 3D response surface plots, which represent two different variables while maintaining the third variable at the zero level, suggesting the direction of the well-defined optimal variables [18], are shown in Fig. 6. Furthermore, the maximum and minimum values or a saddle point can be easily obtained from the contour plots of the different variables. The model of DHA yield was as follows:

$$\begin{aligned}\text{DHA Yield} = {} & 1.23 - 0.050 * X_1 + 0.33 * X_2 + 0.051 * X_3 + 0.07 * X_4 \\ & + 0.070 * X_1 * X_2 + 0.09 * X_1 * X_3 + 0.091 * X_1 \\ & * X_4 - 0.033 * X_2 * X_3 + 0.10 * X_2 * X_4 - 0.51 * X_3 * X_4 \\ & + 0.033 * {X_1}^2 - 0.59 * {X_2}^2 + 0.25 * {X_3}^2 + 0.15 * {X_4}^2\end{aligned}$$

According to the response surface results, critical values for the four variables were determined and are listed in Table 5; the predicted maximum of the DHA yield is 2.26 g/L. Independent experiments were carried out to examine the adequacy of the model. The results are listed in Table 5. The results of the experiments were very close to the predicted optimum; therefore, the model is reasonable and reproducible.

Experiments were carried out under the condition of Mb to examine the response surface result with the condition of Ma. The concentration of alanine and OD_{600} was measured every 12 h, and the results are shown in Fig. 7. Alanine was exhausted at 12 h so that no nitrogen source existed in the stationary phase, which is beneficial for DHA production [27]. It is also indicative that the yeast extract in the culture medium was not used as a nitrogen source but as a trigger for the utilization of the alanine mother liquor. Experiments were also performed to compare the components of oil and DHA yield. The results showed that there is no difference in the components of oil. Complex components in the alanine mother liquor cannot affect the synthesis of fatty acids intracellularly; therefore, via the yeast extract trigger, the alanine mother liquor can be used for DHA production.

4. Conclusions

Yeast extract was proven to act as a trigger for alanine mother liquor utilization of *Schizochytrium* sp. B4D1 for DHA production. Thus, the alanine mother liquor can be fully utilized to produce this highly valuable product, rendering commercial DHA production much more economically competitive. However, the DHA yield is lower than that for yeast extract or soy peptone; thus, further studies should be

performed to improve the DHA yield. Additional inexpensive raw materials from industry should be utilized as alternative culture media to reuse industrial waste and to render the production of DHA much more economical competitive.

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Conflict of interest statement

The authors declare that they have no competing interest.

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