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

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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,

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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).