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Enhanced Fermentation Process for Production of High Docosahexaenoic Acid Content by *Schizochytrium* sp. GCD2032

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Abstract: There is significant demand for high-purity DHA in the pharmaceutical industry. Traditionally, this high-purity DHA is extracted from raw materials with relatively low DHA content (10–20%), such as fish oil. Recently, through electroporation-induced mutation, a high-DHA-content strain of *Schizochytrium* sp. GCD2032 was isolated. To further enhance its DHA production, optimizations were conducted on the culture medium and fermentation conditions (in shaking flasks), as well as different nitrogen source concentrations (in a 5 L fermenter) for biomass, fatty acid content, and DHA content (as a percentage of total fatty acids). In a 5 L fermenter, *Schizochytrium* sp. GCD2032 achieved a biomass of 50 g/L, with fatty acid content of 55.71% and DHA content of 61.29%. Notably, the DHA content reached an impressive 341.45 mg/g of dry weight. This strain consistently produces high levels of fatty acids and DHA, demonstrating significant potential for pharmaceutical applications.

Keywords: *Schizochytrium* sp. GCD2032; fermentation optimization; nitrogen source; fatty acids; DHA

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1. Introduction

Docosahexaenoic acid (DHA) is a member of the omega-3 series of polyunsaturated fatty acids (PUFAs) and is abundantly found in the human nervous system, retina, and breast milk [1]. It plays critical roles in human physiology, including promoting brain development, protecting vision, and exerting anti-cancer and anti-inflammatory effects [2–4]. Furthermore, DHA is beneficial in the prevention and treatment of cardiovascular diseases, Alzheimer's disease, and immune disorders [5]. Recent research indicates that PUFAs like Arachidonic acid (ARA) and DHA can inactivate the severe acute respiratory syndrome coronavirus, enhancing human resilience and recovery from viral infections. They may also improve the structure of gut microbiota [6,7]. Consequently, DHA is increasingly recognized and applied in pharmaceuticals. High-purity polyunsaturated fatty acids (>95%) are essential for synthesizing specialized pro-resolving mediators (SPMs) such as protectins, resolvins, and maresins used in clinical settings [8]. However, the current primary source of high-purity DHA is primarily extracted from raw materials with low content, such as fish oil. The preparation of raw materials with high DHA content remains a significant factor limiting production costs.

In recent years, *Schizochytrium* has gained significant value as an ideal industrial strain for DHA production due to its high DHA content, rapid growth rate, safety certification, and ease of cultivation [9–13]. Currently, industrial strains can achieve DHA content as high as 50–55%. Some studies have attempted to further increase DHA content. For instance,

Wang et al. successfully engineered a strain of *Schizochytrium* with DHA content comprising 61% of total fatty acids, reaching 331 mg/g [14]. Chen et al. developed a high-voltage electroporation-induced mutagenesis method and identified a strain named *Schizochytrium* sp. GCD2032 with 3.51%, 95.51%, and 71.67% higher fatty acid content, DHA content, and DHA yield than those of the wild strain, although its fermentation process has not yet been optimized [10].

The yield and quality of DHA are influenced not only by the strain itself but also by the fermentation process. For example, variations in carbon/nitrogen (C/N) source supply and cultivation temperature can lead to coordinated changes in DHA content, lipid content, and microbial biomass [15]. A high C/N ratio favours lipid accumulation, but excessively high ratios can decrease DHA content within the lipids. During nitrogen starvation, decreased AMP levels in the mitochondrial matrix inhibit the conversion of isocitrate to α-ketoglutarate, leading to citrate accumulation. Once citrate reaches a critical concentration, it is converted by ATP-citrate lyase into acetyl-CoA and oxaloacetate, facilitating lipid accumulation through reverse β-oxidation [16]. Researchers have conducted extensive studies on fermentation optimization [17–19], but only a few reports have achieved DHA content exceeding 60%. However, these instances often come at the cost of lower lipid content, resulting in reduced conversion efficiency [20].

In this study, the strain *Schizochytrium* sp. GCD2032 with a high DHA production capacity, which was screened out in the previous study, was used [10]. Initially, the stability of *Schizochytrium* sp. GCD2032 was assessed, and following the establishment of a stable culture, optimization of the fermentation medium and conditions was performed in shaking flasks. Subsequently, scale-up fermentation was conducted in a 5 L fermenter, with particular attention given to the impact of different nitrogen source compositions and concentrations on DHA production (Figure 1). The study aimed to investigate the synergistic changes among various components of *Schizochytrium* sp. GCD2032 during fermentation, providing technical support for future industrial-scale production of high DHA content.

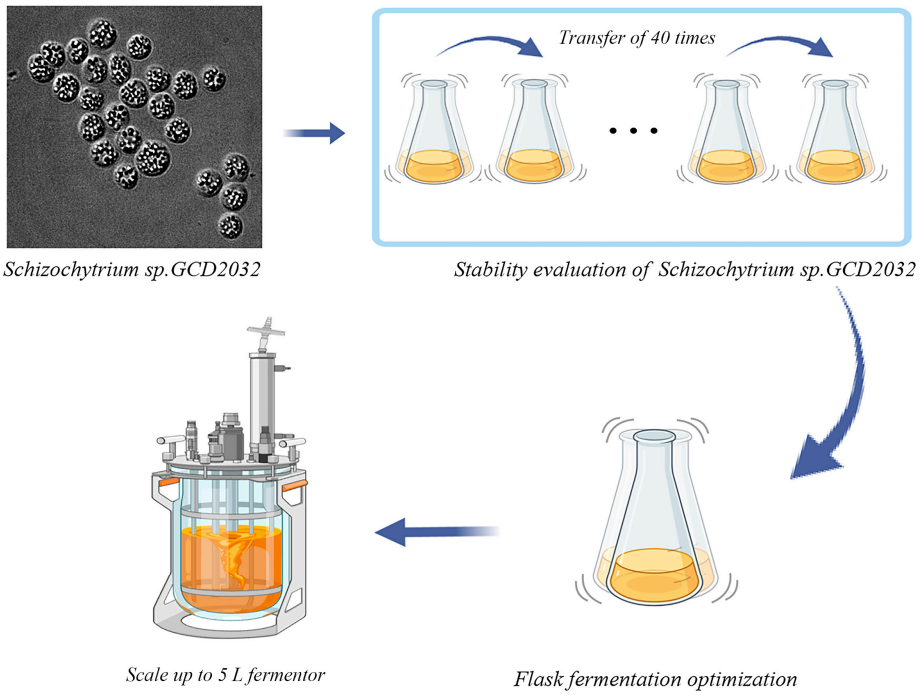

Figure 1. The main research process.

2. Materials and Methods

2.1. Strain and Medium

Schizochytrium sp. GCD2032, a mutant strain screened by our laboratory, is stored in a −80 °C ultra-low temperature freezer [10]. The seed liquid medium and basic fermentation medium were prepared according to references in the literature [10,21]. The seed medium contained glucose (30 g/L; AR, Fufeng Biotechnology Co., Qingdao, China), yeast extract (2 g/L; AR, OXOID, Cheshire, UK), soya peptone (2 g/L; AR, OXOID), and artificial seawater salt (15 g/L; AR, Tianjin Damao Chemical Reagent Factory, Tianjin, China). The original fermentation medium was comprised of glucose (80 g/L), yeast extract (4 g/L), soya peptone (4 g/L), artificial seawater salt (15 g/L), $MgCl_2 \cdot 6H_2O$ (6.4 g/L; AR, Shanghai McLean Biochemical Technology Co., Shanghai, China), $CaCl_2 \cdot 2H_2O$ (1 g/L; AR, Tianjin Damao Chemical Reagent Factory), $MgSO_4 \cdot 7H_2O$ (5 g/L; AR, Shanghai McLean Biochemical Technology Co.), and KCl (2 g/L; AR, Shanghai McLean Biochemical Technology Co.). Both media were sterilized for 20 min at 115 °C before inoculation [21].

2.2. Experimental Methods

2.2.1. Preparation of Seeds

Schizochytrium sp. GCD2032, retrieved from the −80 °C ultra-low temperature freezer (Thermo 900), was streaked onto glycerol tubes after thawing. A 100 µL aliquot was spread onto seed medium plates (including 18 g/L agar) and incubated at 28 °C for 48 h. Single colonies were picked and transferred to 50 mL seed liquid medium in 250 mL Erlenmeyer flasks. The cultures were shaken at 180 rpm for 19–25 h until an OD 600 of 0.6 was reached. This was the seed ready for inoculation.

2.2.2. Strain Stability Evaluation

The seed culture (10% v/v) was transferred to 50 mL of fermentation medium in 250 mL Erlenmeyer flasks and incubated at 180 rpm and 28 °C. After fermentation in the 250 mL flask for 96 h, 5 mL of the fermentation broth was transferred to a new flask with the same medium for the same amount of time. This process was repeated 40 times and the biomass, fatty acid content, and DHA content were determined every 10 cycles during fermentation.

2.2.3. Single-Factor Fermentation Optimization of *Schizochytrium* sp. GCD2032

Various parameters were investigated in shake flasks to optimize DHA production by *Schizochytrium* sp. GCD2032. Factors examined included glucose concentration, nitrogen source and concentration, and inoculum volume in flasks (250 mL) with 50 mL liquid medium (natural pH, 2% inoculum, 28 °C, 180 rpm) after 96 h of incubation.

Carbon Source

Glucose, xylose, fructose, sucrose, glycerol, and lactose were tested for the effects of different carbon sources. The fermentation medium was modified from the original fermentation medium with a different carbon source at 80 g/L. After fermentation, residual glucose, biomass, fatty acid content, and DHA content were determined.

Glucose Concentration

Different glucose concentrations (60 g/L, 70 g/L, 80 g/L, 90 g/L, 100 g/L) were tested for the effects of glucose concentration. The fermentation medium was modified from the original fermentation medium with different concentrations of glucose. After fermentation, residual glucose, biomass, fatty acid content, and DHA content were determined.

Nitrogen Source

Yeast extract, corn steep powder, peptone, tryptone, ammonium sulfate, ammonium chloride, and ammonium nitrate at 5 g/L were tested for the effects of different nitrogen sources. The fermentation medium was modified from the original fermentation medium

with different nitrogen sources. After fermentation, residual glucose, biomass, fatty acid content, and DHA content were determined.

Yeast Extract Concentration

Different concentrations of yeast extract (3 g/L, 5 g/L, 7 g/L, 9 g/L, 11 g/L) were tested for the effects of yeast extract concentration. The fermentation medium was modified from the original fermentation medium with different concentrations of yeast extract. After fermentation, residual glucose, biomass, fatty acid content, and DHA content were determined.

Inoculation Volume

Single-factor experiments were conducted using inoculation volumes of 2%, 4%, 6%, 8%, and 10% for fermentation. The fermentation medium was the original fermentation medium with 80 g/L of glucose and 5 g/L of yeast extract. When preparing the fermentation medium, a certain volume of seed liquid was reserved according to its volume to maintain the total volume at 50 mL. After fermentation, residual glucose, biomass, fatty acid content, and DHA content were determined.

Effects of Different Concentrations of Yeast Extract in a 5 L Stirred Tank Fermenter

Fermentation was scaled up to 5 L in a glass fermenter (Shanghai Baoxing Biological Equipment Engineering Co., Shanghai, China) with single-blade agitation technology and jacketed for temperature control. The optimized medium in 2.2.3 named 5-N, the medium modified from 5-N by fed-batch of glucose named 5-F, the media modified from 5-F using 10 g/L, 15 g/L, 20 g/L, and 25 g/L of yeast extract named 10-F, 15-F, 20-F, and 25-F were used to study the effects on biomass, fatty acid content, and DHA content.

The seed was prepared in a 250 mL flask with 50 mL working volume in the seed medium and was inoculated into the 5 L fermenter when the OD reached 0.6. Fed-batch fermentation was carried out in the 5 L fermenter at 300 rpm rotation speed, 1 vvm aeration rate, 28 °C, and pH 6.0. The initial volume in the 5 L fermenter was set at 3.15 L. Before inoculation, 0.35 L of 400 g/L glucose solution was added to the fermenter to a final concentration of 33 g/L. Subsequently, when the residual glucose was less than 20 g/L after 36 h, 400 g/L of glucose solution was continuously added to the fermenter to maintain its glucose concentration at between 20 and 30 g/L. During the fermentation process, 2 M sodium hydroxide solution and 1 M sulfuric acid solution were used to adjust the pH value, and samples were collected every 12 h to determine the biomass, fatty acid content, and DHA content. The fermentation was carried out for 142 h and the down-tanking was carried out when the biomass did not increase any more or when the growth of the biomass slowed down.

2.3. Analytical Methods

2.3.1. Glucose Concentration and Glucose Consumption Rate Determination

The glucose concentration was determined by a biosensor (SBA-40D, Shandong Academy of Sciences, Jinan, China) according to Guo et al. as follows: Absorb 2 mL fermentation liquid into a 2 mL centrifuge tube, centrifuge at 8000 r/min for 5 min [22], and run the supernatant through the water filter membrane to filter impurities. The filtered liquid was diluted 100 times with deionized water (the glucose concentration was stable in the range of 0.05–1 g/L) and loaded into 2 mL centrifuge tubes for determination (M0, g/L). The glucose curve was calibrated by its program.

$$\text{Glucose Concentration (g/L)} = \text{M0} \times 100 \quad (1)$$

The glucose consumption rate was defined as the glucose consumed (g/L) per hour, calculated as shown in Equation (2).

$$\text{Glucose consumption rate (g/L/h)} = \text{glucose consumed (g/L)/time used (h)} \quad (2)$$

2.3.2. Determination of the Biomass

Biomass represents the total dry weight of *Schizochytrium* sp. GCD2032 in a given volume (g/L). The method from Keskin et al. was used to determine the biomass [23]. At the end of cultivation, centrifuged cell pellets from a given volume (V, L) were washed three times with deionized water. Subsequently, the centrifuged samples were frozen and dried in a freeze dryer (F-D1A-50+, Beijing Boyikang Co., Ltd., Tianjin, China) until a constant weight was achieved (M1, g). The calculation was performed using Equation (3).

$$\text{Biomass (g/L)} = M1/V \quad (3)$$

2.3.3. Fatty Acid Extraction and Quantification

According to Zhao et al., the following improved methods were used for fatty acid extraction [24]. 0.2 g mass of biomass powder was weighed to perform lipid extraction. An aqua distillate/hydrochloric acid mixture of 10 mL (1:1 v/v) was added to the powder and placed into 75 °C water baths for 2 h after mixing. After the sample was cooled, 10 mL n-hexane was added and mixed; this was repeated three times. Supernatants were collected and evaporated in a nitrogen purging instrument (Hangzhou Aosheng Instrument Co., Hangzhou, China) to get a constant weight (M2, g). The fatty acid content was defined as the ratio of the total amount of fatty acids to the dry weight of the cells required to extract the fatty acid, which was quantified using Equation (4).

$$\text{Fatty acid content (\%)} = (M2/0.2) \times 100 \quad (4)$$

2.3.4. Fatty Acid Composition and DHA Content Analysis

The total fatty acid composition was determined using an improved method from our previous study [12]. Fatty acid methyl esters (FAMEs) were prepared by a modified standard method as follows: 1 mL potassium hydroxide/methanol (0.5 M) was added to extracted fatty acids 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 complex was added as a catalyst for a 2 min reaction at 60 °C for transesterification. After transesterification, 2 mL of saturated sodium chloride solution 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 anhydrous sodium sulfate was added and gas chromatography (GC) analysis was performed [25].

A gas chromatograph (Agilent 7200 Q-TOF GC/MS) was used for qualitative analysis of all fatty acid fractions in the lipids in *Schizochytrium* sp. GCD2032. The fatty acid fractions of *Schizochytrium* sp. GCD2032 were identified by referring to the peak times of Supelco's 37 fatty acid methyl ester fractions (crm47885) or fatty acid methyl ester standards such as DHA methyl ester [26].

A Shimadzu GC2010 (GC-2010, Shimadzu, Japan) equipped with a hydrogen flame ionization detector (FID) and a Supelco SP-2560 gas-phase capillary column (100 m × 0.25 mm × 0.20 μm, Supelco, Bellefonte, PA, USA) was selected for the gas chromatographic analysis. The parameters were as follows: injection volume: 1 μL; shunt ratio: 30:1; carrier gas: nitrogen; heating procedure: inlet temperature of 250 °C, detector temperature of 260 °C; column chamber procedure: firstly, keep the column at 150 °C for 5 min, then increase it to 180 °C at 8 °C/min, keep it at 180 °C for 5 min, and then increase it to 240 °C at the same temperature increase rate, and finally keep it at 240 °C for 16 min. Samples were injected in split mode during sample collection. DHA content (%) is calculated as the percentage of the DHA peak area to the total peak area of all eluted fatty acids in the determined samples.

2.3.5. DHA Conversion Rate Analysis

The DHA conversion rate is the ratio of DHA production to the glucose used, which is indicated in Equation (5).

$$\text{DHA conversion rate (g/g)} = [\text{Biomass (g/L)} \times \text{Fatty acid content (\%)} \times \text{DHA content (\%)}]/\text{Glucose consumed (g/L)} \quad (5)$$

2.4. Data Statistics and Analysis

The above experiments were repeated three times and are presented as mean ± standard deviation. All data were analyzed using SPSS 20.0 for *t*-tests to determine significant differences in experimental data, with * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

3. Results

3.1. Stability Assessment of Schizochytrium sp. GCD2032 Fermentation

Schizochytrium sp. GCD2032 is a mutant isolated from an accidental electroporation treatment [10], which needs further evaluation for stable fatty acid production capacity. To evaluate the stability of *Schizochytrium* sp. GCD2032, continuous fermentation over 40 generations was conducted, and biomass, fatty acid content, and DHA content were determined at the 10th, 20th, 30th, and 40th cycles of fermentation (Figure 2A). The results showed that the biomass, fatty acid content, and DHA content of the 10th fermentation cycle strain were 22.9 g/L, 70.24%, and 61.22%, respectively, which were comparable to the original strain [10]. (This also means that there were no differences from the 1st to the 10th generations). However, from the 20th generation of fermentation to the 40th generation of fermentation, the biomass, fatty acid content, and DHA content decreased compared to the 10th generation of fermentation. The biomasses of the 20th, 30th, and 40th generation fermentation strains were 16.35%, 15.66%, and 16.88% lower than that of the 10th generation, while at the same time, the fatty acid content and DHA content were 14.41% and 15.67%, 15.30% and 13.07%, and 13.31% and 13.66% lower, respectively. Notably, there was no significant difference in biomass, fatty acid content, and DHA content of the strain in the 20th, 30th, and 40th fermentation cycles, indicating that it was stable after the 20th consecutive fermentation.

Fermentation stability refers to the ability of a given microbial population to maintain desired morphological and biosynthetic characteristics in qualitative and quantitative terms. The reduced stability of fungi during continuous cultivation is a well-known phenomenon; however, reports in the literature are rather rare [27]. The loss of production capacity of a microorganism may be caused by genetic or epigenetic factors, and factors such as genetic mutations, culture conditions, stress, or ageing may play important roles [28]. For example, scholars have developed a protocol for inducing denaturation in *Trichoderma reesei*, a technique that can be used to calculate the extent of strain denaturation and to compare the denaturing behaviours of different evolved strains. Research has found that *T. reesei* QM6a shows no degeneration and *T. reesei* Rut-C30 exhibits minimal degeneration. This is followed by *T. reesei* Iogen-M4 with approximately 10% degeneration and *T. reesei* Iogen-M10 with nearly 100% degeneration [27]. This variability may enable fungi to better adapt to changing environments; however, it leads to a loss of production capacity in an industrial strain. Thus, it may take some time for *Schizochytrium* sp. GCD2032 to recover after mutations in the genome that lead to loss of biomass and reduced fatty acid content and DHA content.

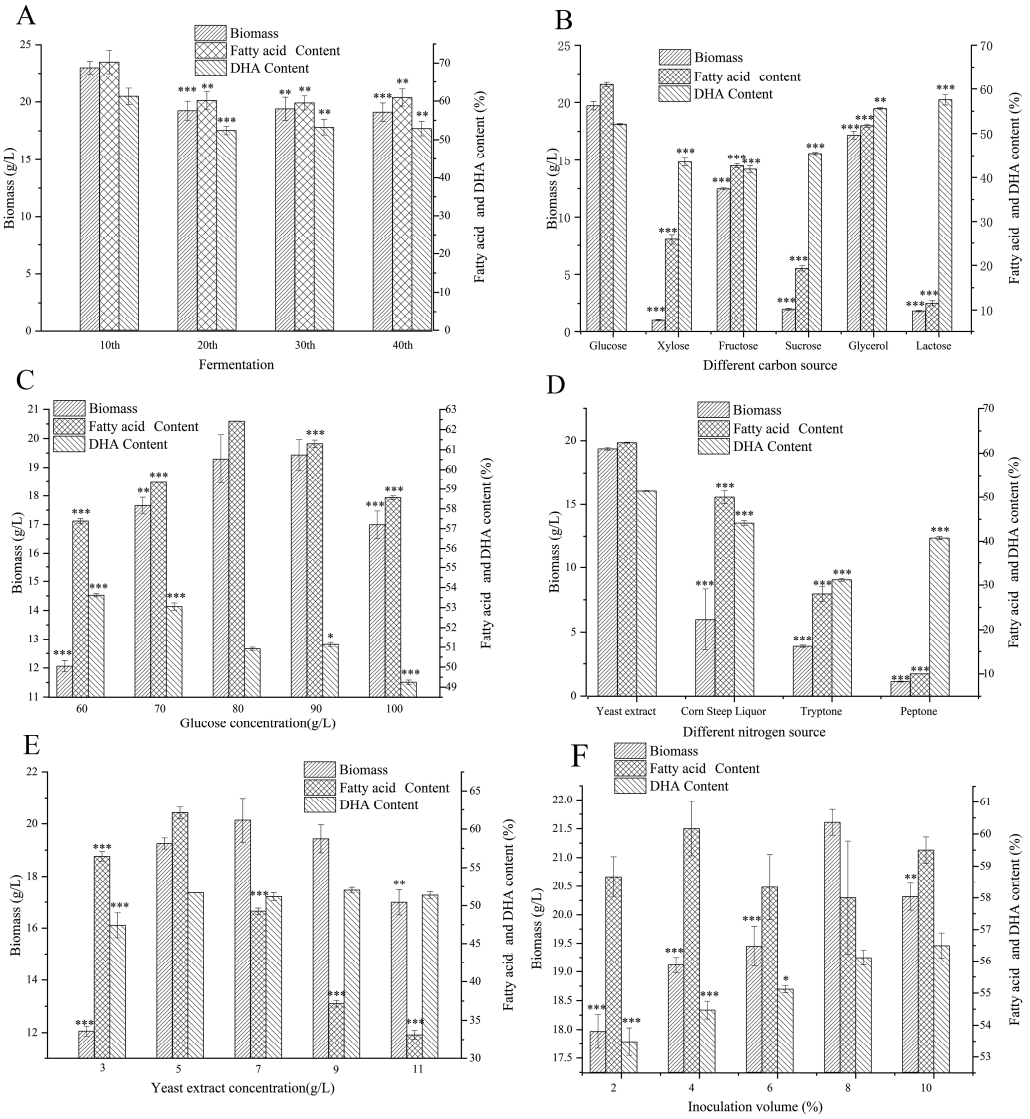

Figure 2. Fermentation characteristics of *Schizochytrium* sp. GCD2032. (**A**) Comparison of the biomass, fatty acid content, and DHA content at the 10th, 20th, 30th, and 40th cycles of fermentation of *Schizochytrium* sp. GCD2032. (**B**) Effects of different carbon sources on fermentation biomass and fatty acid and DHA contents. (**C**) Effects of glucose concentrations on fermentation biomass and fatty acid and DHA contents. (**D**) Effects of different nitrogen sources on fermentation biomass and fatty acid and DHA contents. (**E**) Effects of yeast extract concentrations on fermentation biomass and fatty acid and DHA contents. (**F**) Effects of inoculum volume on fermentation biomass and fatty acid and DHA contents. The statistical significances of the final results were analyzed by *t*-test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3.2. Shake Flask Fermentation Optimization of Schizochytrium sp. GCD2032

To optimize the fermentation conditions of *Schizochytrium* sp. GCD2032, the effects of different carbon sources, carbon concentrations, nitrogen sources, nitrogen concentrations, and inoculum volumes on biomass, fatty acid content, and DHA content were investigated in flasks. The results are shown in Figure 2B–F. *Schizochytrium* sp. GCD2032 demonstrated efficient utilization of glucose, fructose, and glycerol; the highest biomass of 19.71 g/L was observed when glucose was used as a carbon source and the highest DHA content of 55.57% was observed when glycerol was used as a carbon source. In contrast, cultivation with xylose, sucrose, and lactose limited its growth, resulting in generally lower biomass, fatty acids, and DHA production, with minimal utilization (Figure 2B). Biomass and fatty acid content

increased with glucose concentrations ranging from 60 to 80 g/L; however, when the glucose concentration was over 80 g/L, both biomass and fatty acid content decreased. Meanwhile, as glucose concentration increased, DHA content gradually decreased (Figure 2C).

Organic nitrogen sources are more suitable for the growth of *Schizochytrium* sp. GCD2032 compared to inorganic nitrogen sources. *Schizochytrium* sp. GCD2032 grew poorly (biomass less than 1 g/L) when an inorganic nitrogen source (such as NH_4SO_4, $NaNO_3$, etc.,) was used. In a medium where yeast extract was used as the nitrogen source, 69.21%, 80.11%, and 94.21% higher dry weight, 19.86%, 55.11%, and 83.97% higher fatty acids, and 14.09%, 39.34%, and 20.67% higher DHA content were obtained compared to fermentation performed in media with corn steep, tryptone, or peptone as nitrogen sources (Figure 2D). The concentration of yeast extract is one of the important factors affecting DHA content; as yeast extract concentration increases, biomass increases. However, excessive yeast extract hinders the rapid accumulation of fatty acids; therefore, when the yeast extract concentration is 5 g/L, the biomass, fatty acid content, and DHA content are optimal (Figure 2E).

Inoculum volume is also an important parameter. A too-low inoculum volume can lead to a low initial density of microorganisms in the culture medium, thereby affecting their growth rate and metabolic efficiency. Conversely, an excessively high inoculum volume results in a high initial cell density, leading to rapid *Schizochytrium* sp. GCD2032 growth that can cause insufficient oxygen levels in the fermentation broth, which is unfavourable for cellular division metabolism. Optimal inoculum volume ensures proportional substrate consumption and fermentation time, thereby improving nutrient substrate utilization efficiency. In the range from 2% to 8%, the biomass of *Schizochytrium* sp. GCD2032 increased as the inoculum volume increased. When the inoculum reached the maximum value of 8%, the biomass was 21.61 g/L and did not increase further. Subsequently, as the inoculum continued to increase, the biomass of the strain decreased. However, there was no significant effect on the fatty acid content, whereas the DHA content gradually increased to 56.49% with increasing inoculum volume. Taking everything into consideration, an inoculum volume of 8% was chosen as appropriate (Figure 2F).

Research has shown that the addition of citric acid and malic acid can enhance DHA content [13,19]. However, during single-factor fermentation optimization, the influence of citric acid and malic acid on DHA content was examined, and the results indicated that their addition did not affect the biomass, fatty acid content, and DHA content (data not published).

3.3. Scale up in 5 L Fermenters

The effects of different yeast extract concentrations under batch and fed-batch fermentation conditions on fermentation biomass, fatty acid content, and DHA content were investigated (Figure 3). Results of fermentation in 5 L fermenters using optimized shake flask media showed biomass, fatty acid content, and DHA content comparable to shake flask results, maybe indicating glucose limitations for *Schizochytrium* sp. GCD2032 in the 5 L fermenter despite its better oxygen availability (Figure 3A). During fed-batch fermentation with increasing yeast extract concentrations, the highest biomass and DHA content were achieved after 132 h, reaching peak values with 20 g/L of yeast extract before declining. Total fatty acid content varied minimally between 5 and 15 g/L of yeast extract as the nitrogen source, decreasing gradually as yeast extract concentration increased from 20 to 25 g/L, where 25 g/L of yeast extract resulted in only 25% fatty acid content. Glucose uptake and the rate of DHA conversion by *Schizochytrium* sp. GCD2032 also increased gradually with increasing yeast extract concentration, reaching maximum levels at 20 g/L of yeast extract, indicating that 20 g/L of yeast extract was optimal for biomass and fatty acid production in the 5 L fermenters, with a DHA conversion rate of 0.17 g/g (Figure 3B).

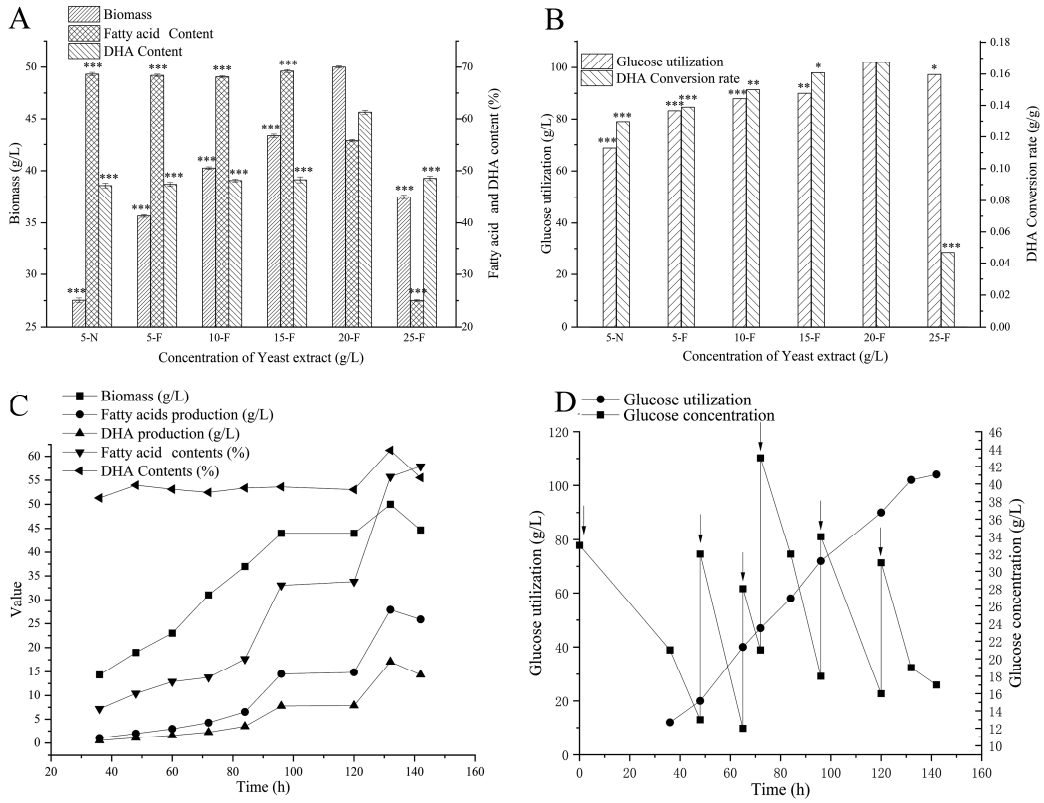

Figure 3. Fermentation of *Schizochytrium* sp. GCD2032 in a stirred tank fermenter. (**A**) Effects of yeast extract concentrations on biomass and fatty acid and DHA contents; (**B**) effects of yeast extract concentrations on glucose utilization and DHA conversion rate; (**C**) fermentation curve of *Schizochytrium* GCD2032 with 20 g/L of yeast extract as nitrogen source (the data and the units are all shown in the legend); and (**D**) glucose utilization and glucose-fed curve of GCD2032 with 20 g/L of yeast extract as nitrogen source. The statistical significances of the final results were analyzed by *t*-test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 3C displays the variations in biomass, fatty acid production and content, and DHA production and content, with feeding glucose as the carbon source (Figure 3D) and 20 g/L of yeast extract as the nitrogen source. The results show that as fermentation progresses, biomass, fatty acid production, fatty acid content, and DHA content reach their peaks at 132 h, with the maximum DHA content reaching 61.29%, followed by a decline; meanwhile, fatty acid content continues to increase. Glucose consumption increases linearly throughout the fermentation process, reaching its highest point at 132 h, after which its rate gradually slows down (Figure 3D).

Nitrogen deficiency is a major trigger for initiating fatty acid synthesis [29]. Compared with flask fermentation, a 5 L fermenter requires better oxygenation and space, thereby improving the efficiency of microorganism growth and fatty acid accumulation. Therefore, in fed-batch fermentation, if the optimal nitrogen source concentration from shake flasks is used in the 5 L fermenter, the nitrogen source concentration will limit the fermentation yield, and increasing the nitrogen source concentration is necessary to improve the fatty acid yield. Therefore, in theory, excessively high nitrogen source concentrations may delay the initiation of fatty acid accumulation, resulting in high biomass but insufficient fatty acid accumulation. Conversely, too low nitrogen source concentrations may halt cell division and initiate fatty acid accumulation without accumulating enough cells, resulting in high fatty acid content per cell but overall low fatty acid production.

Table 1 indicates different DHA contents from various studies. Not all studies provide exact DHA content data; some derive it from the ratio of DHA production to fatty acid production. According to Table 1, the DHA content reported in this study for *Schizochytrium*

sp. GCD2032 is relatively high compared to others in the literature. Only one study reported a DHA content of 62.4% [19], achieved at a scale of 50 L with a fatty acid content of 38.00%. In contrast, this study achieved a fatty acid content of 55.71% while maintaining a high DHA content of 61.29%. This difference may be attributed to variations in strains and fermenter conditions.

Table 1. Comparison of DHA contents in different studies.

Strains	Working Volume/Reactor Volume	Biomass	Fatty Acid Content	DHA Content
A recombinant strain of OPKSABC-PPT originated from *Schizochytrium* sp. ATCC20888 [30]	-	-	-	40.60%
Schizochytrium sp. HX-308 (CCTCC M209059) overexpressing the diacylglycerol acyltransferase (ScDGAT2C) gene [19]	100 mL/250 mL	-	-	50.10%
Schizochytrium sp. ATCC 20888 with 5 mg/L proanthocyanidins [31]	50 mL/250 mL	36.7 g/L	55.31%	48.30%
Aurantiochytrium sp. 6–2 [32]	1 L/2 L	-	-	28.80%
Thraustochytrium sp. [33]	100 mL/250 mL	9.88 g/L	66.50%	24.80%
Schizochytrium sp. co-overexpressed PPTase and ω-3 FAD. [34]	-/5 L	-	-	55.70%
Schizochytrium sp. with seawater and fermentation wastewater as fermentation broth [20]	30 L/50 L	195.8 g/L	38%	62.40%
The synergistic effect of chemical regulators is applied to *Schizochytrium* sp. [35]	1.5 L/2 L	2.04 ± 1.12 g/L/d	49.02%	38.60%
Schizochytrium sp. GCD2032	3.5 L/5 L	50 g/L	55.71%	61.29% (this study)

Note: "-" indicates no data in the reference.

Currently, the extraction and purification of DHA from microalgae still face challenges, including high energy consumption during cell drying and fatty acid extraction, making large-scale production difficult. This issue mainly arises from the low DHA content. Therefore, if a *Schizochytrium* species with high DHA content can be constructed, for example, *Schizochytrium* sp. GCD2032, it would significantly reduce the cost of fatty acid extraction and achieve energy and resource savings. In the future, further exploration of gene mutation sites in *Schizochytrium* sp. GCD2032 through technologies like genome sequencing should be conducted to elucidate the mechanisms and regulatory pathways behind its high DHA content. Additionally, genome editing techniques can be used to create new cell factories that are both high in DHA content and production. Concurrently, efficient extraction and purification technologies for DHA should be developed, laying a solid foundation for the development of DHA-based pharmaceuticals.

4. Conclusions

The pharmaceutical and food industries have a huge demand for high-purity DHA products, while traditional methods only extract it from fish oil, whose low concentration leads to higher product costs. This study observed that the mutant strain *Schizochytrium* sp. GCD2032 initially showed a decrease in DHA content after 10 cycles of fermentation but stabilized in its ability to produce fatty acids and DHA after 40 transfer cycles. Using this stable strain, optimal fermentation conditions were established through the optimization of shake flask media and fed-batch fermentation with glucose addition and varied nitrogen source concentrations in a 5 L fermenter. After optimization, the DHA content increased by 16.06%, DHA production increased by 179.24%, and the final DHA content reached 341.45 mg/g cell dry weight. These optimizations resulted in the highest fatty acid fermentation outcomes, providing a robust foundation for future high-purity DHA production.

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