تولید دوکوزاهگزانوئیک‌اسید توسط سویهٔ بومی *آئورانتیوکیتریوم* در تخمیر خوراک‌دهی‌شده

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چکیده

مقدمه: دوکوزاهگزانوئیک‌اسید، اسیدچرب غیراشباع با چندین پیوند دوگانه است که نقش مهمی در جلوگیری از بیماری‌های قلبی و عروقی دارد. آغازیان ترائوستوکیترید خصوصا سویه‌های *آئورانتیوکیتریوم* یکی از تولیدکننده‌های اصلی این اسیدچرب امگا ۳ هستند. افزایش بیومس، روغن و محتویات دوکوزاهگزانوئیک‌اسید در سویهٔ *آئورانتیوکیتریوم* ازطریق خوراک‌دهی گلوکز یا گلیسرول در سیستم تخمیری ۳ لیتری از اهداف اصلی تحقیق است.

مواد و روش‌ها: سویهٔ بومی *آئورانتیوکیتریوم* qe-4 (KR091914.1) برای تولید دوکوزاهگزانوئیک استفاده شد. گلیسرول و گلوکز به‌عنوان منبع کربن در محیط کشت استفاده شدند. رشد سلولی، تولید روغن و دوکوزاهگزانوئیک‌اسید در سویهٔ *آئورانتیوکیتریوم* در شرایط کشت خوراک‌دهی‌شده در فرمانتور ۳ لیتری مطالعه شدند.

نتایج: نتایج نشان دادند نسبت کربن به نیتروژن برابر با ۱/۵ و محیط کشت شامل ۳۰ گرم بر لیتر گلیسرول، ۱۰ گرم بر لیتر پپتون و ۱۰ گرم بر لیتر عصارهٔ مخمر، سویهٔ بومی *آئورانتیوکیتریوم* به‌ترتیب ۳۰/۲، ۸/۸ و ۱/۷ گرم بر لیتر بیومس، روغن و دوکوزاهگزانوئیک‌اسید تولید کرده است. همچنین، این سویه در نسبت کربن به نیتروژن برابر با ۲ و محیط کشت شامل ۴۰ گرم بر لیتر گلوکز، ۲۰ گرم بر لیتر سدیم گلوتامات و ۶ گرم بر لیتر عصارهٔ مخمر به‌ترتیب ۲۷/۶، ۱۲/۵ و ۱/۴۵ گرم بر لیتر بیومس، روغن و دوکوزاهگزانوئیک‌اسید تولید کرده است. میزان اکسیژن محلول کمتر از ۳ درصد اشباع برای تولید دوکوزاهگزانوئیک‌اسید مناسب بود.

بحث و نتیجه‌گیری: در طول فرایند تخمیر، میزان اکسیژن محلول و نسبت کربن به نیتروژن، فاکتورهای حیاتی‌اند که بر تولید دوکوزاهگزانوئیک‌اسید تأثیر می‌گذارند. این نتایج نشان دادند سویهٔ بومی *آئورانتیوکیتریوم* در حضور گلیسرول نسبت به گلوکز، کارآیی بهتری در تولید دوکوزاهگزانوئیک‌اسید دارد.

واژه‌های کلیدی: *آئورانتیوکیتریوم*، دوکوزاهگزانوئیک‌اسید، اکسیژن محلول، کشت خوراک‌دهی‌شده، تخمیر

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Production of Docosahexaenoic Acid by Marine *Aurantiochytrium* sp. in Fed-batch Fermentation

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Abstract

Introduction: Docosahexaenoic acid (a polyunsaturated fatty acid) plays an important role in the prevention of arteriosclerosis and cardiovascular diseases. Thraustochytrid protists, specifically *Aurantiochytrium* strains, are among the main microbial producers of omega-3 fatty acid. The aim of the present study was to enhance biomass, oil, and docosahexaenoic acid (DHA) content of *Aurantiochytrium* sp. by glucose or glycerol feeding in a 3L-fermentation system.

Materials and Methods: A native strain of *Aurantiochytrium* sp. qe-4 (KR091914.1) was used for DHA production. Glycerol and glucose were used as a carbon source in a defined medium. Cell growth, lipid, and DHA accumulation in *Aurantiochytrium* sp. were studied under fed-batch cultivation by using accurate feeding techniques in a 3 L fermentor.

Results: The results of the present study indicated that at C/N ratio of 1.5 and medium composition of 30 $g.L^{-1}$ glycerol, 10 $g.L^{-1}$ peptone, and 10 $g.L^{-1}$ yeast extract, the native strain of *Aurantiochytrium* produced 30.2 $g.L^{-1}$ biomass, 8.8 $g.L^{-1}$ lipid, and 1.7 $g.L^{-1}$ DHA. Also at C/N ratio of 2 and medium composition of 40 $g.L^{-1}$ glucose, 20 $g.L^{-1}$ mono-sodium glutamate (MSG) and 6 $g.L^{-1}$ yeast extract, 27.6 $g.L^{-1}$ biomass, 12.5 $g.L^{-1}$ lipid, and 1.45 $g.L^{-1}$ DHA were produced. It was also shown that low dissolved oxygen less than 3% of saturation was efficient for DHA production.

Discussion and conclusion: During the fermentation process, dissolved oxygen values and carbon-to-nitrogen ratios are critical factors that influence growth and DHA synthesis in *Aurantiochytrium*. These results showed that the native strain of *Aurantiochytrium* had a better performance to produce DHA in a medium containing glycerol than glucose as the sole carbon source.

Key words: *Aurantiochytrium* sp., Docosahexaenoic Acid, Dissolved Oxygen, Fed-batch Culture, Fermentation.

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Introduction

Docosahexaenoic acid (DHA) is an essential polyunsaturated fatty acid (PUFA) with a wide range of applications in the food and pharmaceutical industries (1). DHA contains 6 double bonds or unsaturated C=C linkages in its long chain backbone structure (2) and prevents the development of a wide variety of diseases such as cardiovascular failures and several types of cancer and tumor (3-7). Also, DHA is essential for the normal growth of infants' central nervous system and visual sight and hence is secreted in human breast milk (8). Fish oil is the world's traditional source of DHA. However using fish-derived oil is not achieved in the food industry yet, because of its fishy taste and smell, and high sensitivity to oxidation during food processing (9). Another drawback of using fish oil in infant formula is the presence of eicosapentaenoic acid (EPA), which is not recommended in milk formulation (10). Therefore, alternative and sustainable sources of DHA are under research and investigation. *Aurantiochytrium* is a marine thraustochytrid with the ability to grow on the culture media containing various carbon sources. This strain is known for the production of a high amount of DHA (3). Production of this microbial oil is feasible to scale up through biotechnological processes (11). Cultivation of *Aurantiochytrium* at high cell densities and on a large scale requires proper oxygen transferring between culture broth and gas phase. Also, it has been shown that the production of biomass and DHA is mainly affected by the ratio of carbon to nitrogen (C/N) and dissolved oxygen (DO) (2, 12-15). Also, the effect of a wide range of raw materials (16-18), physicochemical parameters (13, 19, 20), and fermentation conditions were studied on the production of DHA by microbial strains (14, 21-24). Furthermore, batch, fed-batch, and continuous cultures were investigated (12, 25, 26). Barclay (1992) studied the production of DHA in 14 L bioreactor at 48 hours using glucose, low salinity, and chloride level, and ammonium sulfate as the sole nitrogen source (27). The results showed that 65-70 $g.L^{-1}$ biomass and 8 $g.L^{-1}$ DHA were produced with the productivity of 0.1 $g.L^{-1}.h^{-1}$ (28). Also, fed-batch cultivation was used with low dissolved oxygen and ammonium as nitrogen sources and results showed 170-210 $g.L^{-1}$ biomass with 409 $mg.L^{-1}.h^{-1}$ DHA productivity (29). In this research, a strain of *Aurantiochytrium* was used to produce oil rich in DHA in a fed-batch cultivation fermentor. Growth and oil production at a small scale (250 mL flasks) were studied in previous studies (30). The aims of the present study were to investigate the production of biomass, oil, and DHA in a bench-scale fermentor. So, experiments were conducted in a 3L fermentor in order to assess the effects of medium composition and various C/N ratios on biomass, oil, and DHA production. Glycerol and glucose were studied separately as the sole carbon source. Finally, the findings of this research paved the way for the large-scale production of oil rich in DHA as a sustainable alternative for fish oil.

Materials and Methods

Fed-batch Cultivation in the Fermentor: *Aurantiochytrium* sp. qe-4 (KR091914.1) had been isolated and identified earlier from the mangrove forests of Qeshm in the south of Iran (N2642 E5540) (30). The strain was cultured in the medium containing 30 $g.L^{-1}$ glycerol or glucose, 5 $g.L^{-1}$ yeast extract (YE), 3 $g.L^{-1}$ peptones, 0.3 $g.L^{-1}$ penicillin/streptomycin, and 60% v/v natural seawater (30). A 3 L Bench-Top fermentor FS-01-A was used for fed-batch cultivation of *Aurantiochytrium* strain. Before inoculation, saturation was

controlled by agitation speed (250-300 rpm) and sparging of 0.5-1.5 volume of air per volume of medium per minute (vvm) at 28 °C and pH 7±0.2 (acid and base: 2 N NaOH and 2 N HCl). Two media (I and II) were used to investigate the production of DHA by *Aurantiochytrium* in 2 L fermentation medium in the fermentor. The medium I contained 30 $g.L^{-1}$ glycerol, 10 $g.L^{-1}$ YE, 10 $g.L^{-1}$ peptones, (C/N: 1.5), 100% natural seawater. Fresh seed culture was used as inoculum (10% v/v). Also, medium II contained 40 $g.L^{-1}$ glucose, 6 $g.L^{-1}$ YE; 20 $g.L^{-1}$ monosodium glutamate (C/N: 2), 100% natural seawater, and a 10% v/v seed culture. Cell suspensions were taken at time intervals to measure cell dry weight (CDW), residual glycerol and glucose, oil, and DHA concentration. Medium I and II were prepared only with glycerol and glucose as the sole carbon source.

Controlling of Parameters during Fermentation: The aeration in the medium I was controlled at 1 vvm and 0.5 vvm at the growth phase (0-168 h) and lipid production phase (168-240 h), respectively. Moreover, aeration in culture with glucose as the sole carbon source was controlled at 1.5 vvm and 1 vvm at the growth phase (0-190 h) and lipid accumulation phase (190-337 h), respectively. In both cultures, the agitation speed, pH, and temperature were kept at 300 rpm, 7, and 28 °C, respectively. Fermentation data were monitored through equipment readouts. During the fermentation, the formation of foam was notobserved, so no surfactant and antifoam were used.

Determination of Cell Growth and Biomass: CDW was measured by the sampling of 10 mL suspension culture and centrifuged at 5000 rpm for 10 min. Then, the removed supernatant and pellets were dried at 110 °C for 48 h (31).

Glycerol Measurement: The concentration of glycerol was determined in the culture supernatants in time intervals. Briefly, two reagents including reagent I (periodate reagent: 18 $mg.mL^{-1}$ sodium periodate dissolved in 10% v/v acetic acid/distilled water (DW)) and reagent II (acetylacetone reagent: 1% v/v acetylacetone in isopropyl alcohol) were prepared and used. The calibration curve (glycerol 50-200 $mg.L^{-1}$) was determined in a 96-well microplate. Forty µL of the culture supernatant was added into each well and then 40 µL of the reagent I was added, mixed, and incubated for 10 min. Then, reagent II (125 µL) was added to the wells and mixed and incubated for 25 min. The absorption was assessed at 410 nm by a microplate reader. Residual glycerol was determined in the culture supernatant according to the standard curve (32).

Glucose Measurement: The dinitrosalicylic acid (DNS) method was used for the determination of glucose. Briefly, 1 mL sugar solution was prepared in DW and mixed with 4 mL of the DNS reagent. Solutions were incubated in a boiling water bath for 5 min and then transferred to the ice for rapid cooling. Then, tubes were brought to room temperature and absorbance was determined at 540 nm using spectrophotometer CARY 50 (33).

Lipid Extraction: One-hundred mg of CDW were weighted in screw cap tubes and then 2 mL DW was added. Samples were ultrasonicated for 5 min and chloroform (2.5 mL) and methanol (5 mL) were added. Ultrasonication and homogenization were done for 5 min and 2.5 mL chloroform and 2.5 mL of DW were added and shaked for 30 s. Then, suspensions were centrifuged (4000 rpm for 15 min) to separate both organic and inorganic phases. The bottom layer (organic phase) was separated and the solvent was evaporated. Finally, extracted oils were determined gravimetrically (30).

Measurement of DHA: The DHA content was analyzed by the gas chromatography (GC) method. Three mL of methanolic sulfuric acid 4% (v/v) was added to a certain amount of extracted lipid in screw cap tubes and vortexed for 10 s. Then, tubes were incubated in a water bath (90 min at 80 °C). After that, tubes were cooled and 1 mL of DW was added and vortexed for 10 s. Finally, 1 mL of hexane was used for the extraction of fatty acid methyl esters (FAMEs). The organic layer was recovered and Na_2SO_4 was added to the tubes for the elimination of remained H_2O. Agilent 6890 along with a flame ionization detector (FID) and DB-23 (30 m × 0.32 mm, 0.25 μm; Agilent Technologies) capillary column were used for GC analysis. The carrier gas was nitrogen. Then, 0.5 μL of each FAMEs was injected under splitless injection mode. The temperature of the injector and detector was set at 300 °C. The temperature program of the column was set as 50 °C; 2 min, 10 °C.min^{-1} to 180 °C; 5 min, 5 °C.min^{-1} to 240 °C; 7 min. C19:0-FAME (Sigma) was used as an internal standard and mixed PUFA-standard (Sigma) was used for determining the retention time of each FAME peak (30). Finally, Biomass yield ($Y_{x/s}$), oil yield ($Y_{p/s}$), and DHA yield ($Y_{DHA/s}$) coefficients were assessed and determined (22).

Results

Aurantiochytrium Growth during fed-batch Fermentation

Medium I: Feeding strategies are important in fed-batch culture systems to achieve high productivity of intracellular lipids. As shown in figure 1A**,** biomass, oil, and glycerol consumption were monitored during the cultivation of *Aurantiochytrium.* After 114 h of cultivation, 10 g.L^{-1} glycerol, 3.3 g.L^{-1} YE, 3.3 g.L^{-1} peptones (C/N: 1.5) were fed to the fermentor. This feeding was for the growth and multiplication of cells. Glycerol was consumed and reached below 6 g.L^{-1} after the first feeding. With the consumption of 26 g.L^{-1} glycerol in 186 hours of cultivation, biomass increased to 18 g.L^{-1}. The concentration of glycerol was kept between 5-25 g.L^{-1} during the fermentation process. The second feeding was used for oil accumulation and contained only 17 g.L^{-1} glycerol. This feeding was done at 186 h of cultivation. After this feeding, oil increased to 8.8 g.L^{-1}. and final biomass, total oil, and DHA content reached 30.2 g.L^{-1}, 8.8 g.L^{-1}, and 22.08% (of total fatty acids) in medium I, respectively. The maximum productivity of DHA was 7.15 mg.L^{-1}.h^{-1}.

When glycerol solution was fed to the culture medium at 114 h and 186 h of cultivation, the DO level decreasedwhile the oxygen consumption rate and cell growth increased. The time course of DO concentration during the fermentation is shown in Figure 1B. Analysis of DO level at the end of the process showed that DO less than about 3% of saturation was favored for the production of DHA. A stirrer and intensive mixing are required to reduce viscosity and provide aerobic conditions for DHA production during fermentation. In this fermentation, agitation speed of 300 rpm did not destroy cells structure and was favorable for mixing culture medium.

Medium II: A significant increase in biomass was observed between 106-290 h. During this period, biomass increased from 6.4 g.L^{-1} to 27.33 g.L^{-1}. Also, the rate of glucose consumption was high, and glucose was added and fed every 20 h. A certain amount of concentrated glucose solution was added to the culture medium when the residual glucose decreased below 20 g.L^{-1}. After 340 h, the biomass and total lipid reached 27.6 g.L^{-1} and 12.5 g.L^{-1}, respectively. As shown in Figure 2A, feedings 1 and 2 were contained 20 g.L^{-1} glucose. Third feeding contained 10 g.L^{-1}

glucose, 20 $g.L^{-1}$ MSG and 6 $g.L^{-1}$ YE at 105 h of cultivation. When this feeding was added to the culture medium, the DO level dropped quickly and then agitation increased to 300 rpm (Fig. 2B). This variation in agitation was done by the fermentor because it was set up between 250-300 rpm and agitation can fluctuate in this range to increase or decrease DO during feeding. As shown in Figure 2B, agitation decreased to 250 between 50-100 h of incubation. During this period, several feedings were done and cells were growing. They required more oxygen for their metabolisms, which was provided to them by increasing and decreasing levels of DO and agitation, respectively. These results showed that glucose feeding without a nitrogen source was not suitable for cell growth during the early fermentation period. Table 1 shows various parameters which were obtained from the medium I and II.

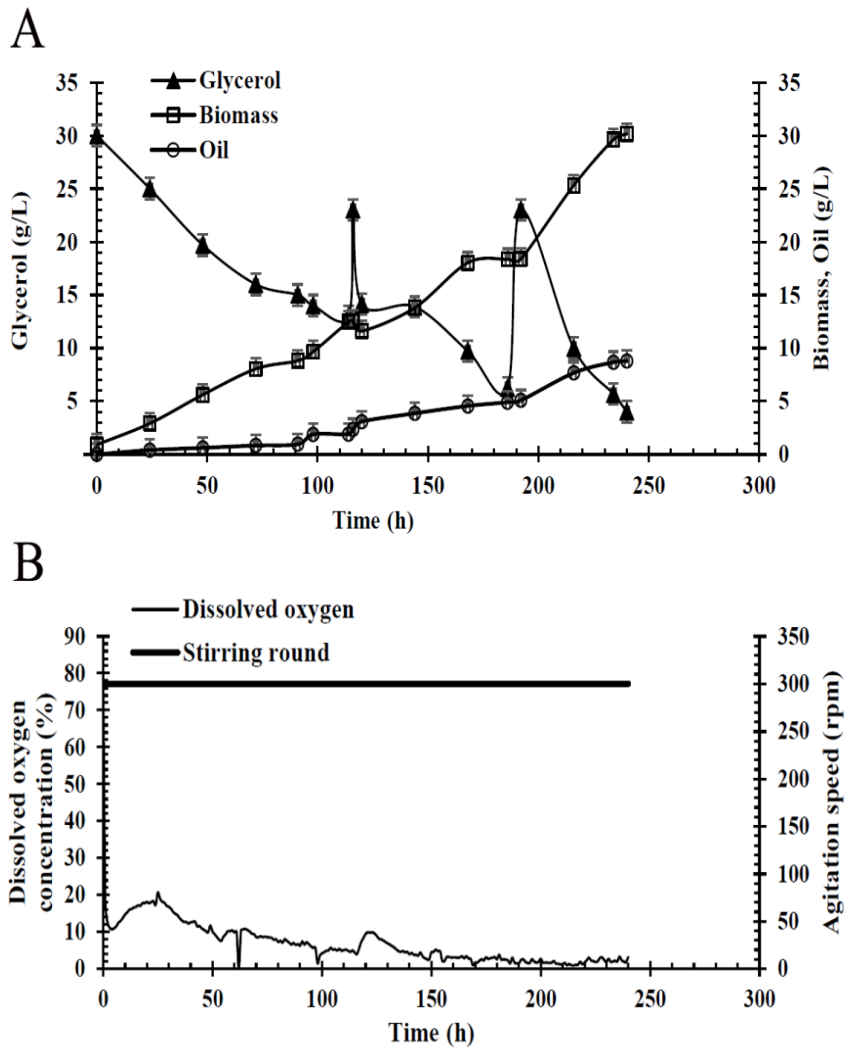

Fig. 1- A) Biomass, oil production, and glycerol consuming during fed-batch cultivation of *Aurantiochytrium* in medium I. B) DO variation in fermentation process at 300 rpm agitation speed

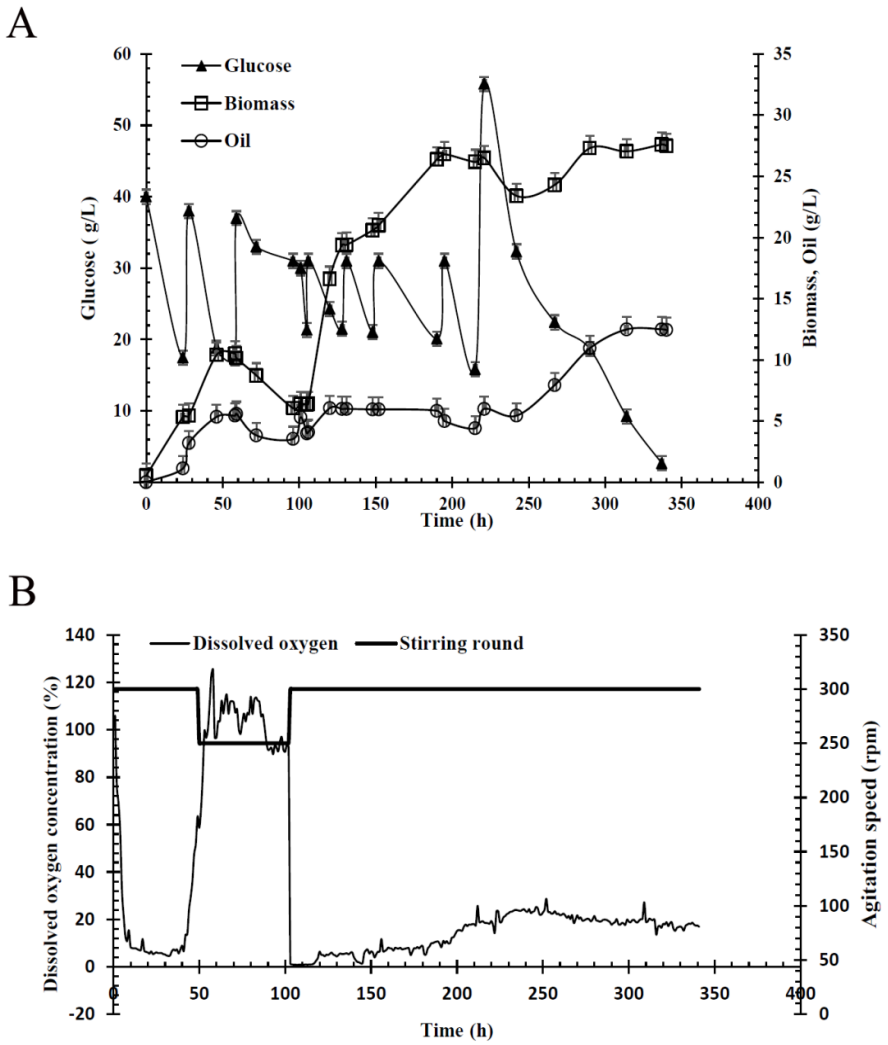

Fig. 2- A) Biomass, oil production, and glucose consumption during fed-batch cultivation of *Aurantiochytrium* with glucose as a carbon source. B) Profile of DO concentrations in the fed-batch cultivation of *Aurantiochytrium*

Table 1- Several Parameters Obtained in DHA Production by *Aurantiochytrium*

Parameters	Medium	
	I	II
Biomass ($g.L^{-1}$)	30.15	27.33
Total lipids ($g.L^{-1}$)	7.77	10.95
Lipid (% CDW)	25.77	40.07
DHA/TFA(%)	22.08	13.27
DHA/total lipids (%)	22.14	13.24
DHA content ($g.L^{-1}$)	1.72	1.45
Fermentation time (h)	240	290
DHA productivity ($mg.L^{-1}.h^{-1}$)	7.15	5.01

TFA: Total Fatty Acids

DHA Analysis: Figures 3A and 3B show a GC chromatogram of methyl esterified fatty acids produced by *Aurantiochytrium* after 240 of cultivation in medium I. Lipids were extracted, esterified, and prepared for analysis. Tables 2 and 3 show the fatty acid composition of the medium I and II. It was observed that the DHA content and productivity reached 1.72 $g.L^{-1}$ and 7.15 $mg.L^{-1}.h^{-1}$ in the medium I, respectively.

DHA content and productivity were 18.63% and 42.71% higher than the results obtained from medium II. Overall, these two parameters were improved by using glycerol as the sole carbon source in the fermentation medium.

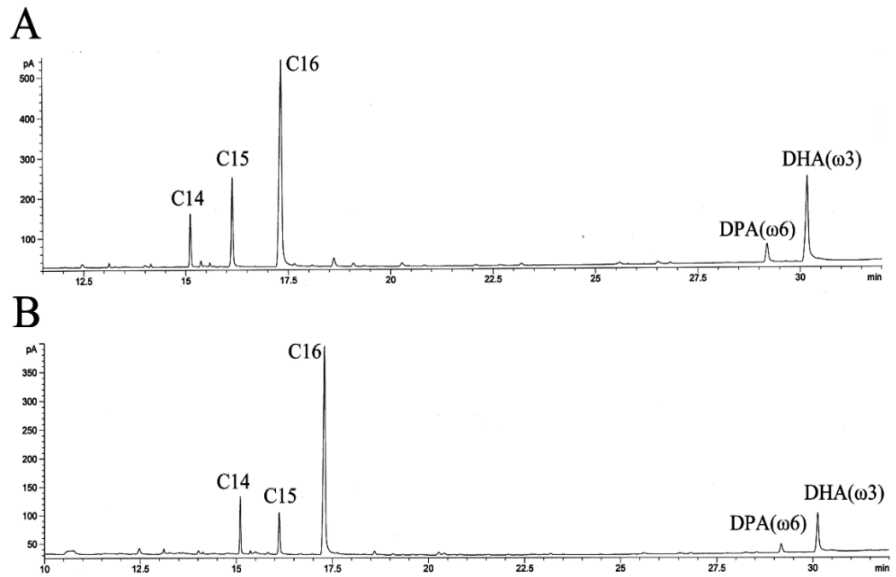

Fig. 3- A) GC chromatogram of methyl esterified fatty acid produced by *Aurantiochytrium* after 240 h of cultivation in medium I. B) GC chromatogram of methyl esterified fatty acid produced by *Aurantiochytrium* after 290 of cultivation in medium II

Table 2- Fatty Acid Composition (% of Total Fatty Acids) in Time Intervals in Medium I

Fermentation Time (h)	C14:0	C15:0	C16:0	C16:1	C18:0	C18:1	DPA	DHA
98	3.14	34.21	20.78	0.3	3	0.78	4.71	23.6
144	7.02	1.14	7.34	1.11	0.3	6.07	1.1	8.19
234	3.49	13.07	47.57	0.3	1.76	0.2	3.93	21.56
240	6.54	13.48	50.87	0.3	1.74	0.003	4.31	22.08

Table 3- Fatty Acid Composition (% of Total Fatty Acids) in Time Intervals in Medium II

Fermentation Time (h)	C14:0	C15:0	C16:0	C16:1	C18:0	C18:1	DPA	DHA
128	4.28	1.36	16.77	0.7	0.6	1.85	2.55	2.98
267	10.84	9.03	59.6	0.3	0.2	0.4	1.27	4.82
290	9.66	8.32	62.94	0.3	0.6	0.4	2.66	13.27

Determination of Kinetic Parameters: Kinetic parameters obtained from the medium I and II are shown in Table 4. The parameters include biomass yield coefficient ($Y_{x/s}$), oil yield coefficient ($Y_{p/s}$), and DHA yield coefficient ($Y_{DHA/s}$). $Y_{x/s}$, $Y_{p/s}$, and $Y_{DHA/S}$ were significantly higher in medium I in comparison to medium II.

Table 4- Kinetic Parameters at Different Fermentation Stages

	Medium I			Medium II	
	Stage1 (0-98 h)	Stage2 (98-144 h)	Stage3 (144-240 h)	Stage 1 (0-128 h)	Stage 2 (128-290h)
Yx/s (%)	60.38	41.36	61.34	28	14.62
Yp/s (%)	11.75	19.82	14.68	8.66	6.77
YDHA/s (%)	2.78	1.16	5.21	0.26	1.74

Microscopic Observation of Cells during Fermentation: *Aurantiochytrium* sp. showed a spherical morphology with a diameter ranging from 4 µm to 20 µm. Development of lipid body was detected by Nile red staining and fluorescence microscopy. Other pictures were taken by optical microscopy (Axioplan2 Imaging) (Fig. 4**)**. In medium I, zoospores were observed during 24-144 h and then transformed into some sporangia through binary cell division. After the addition of feeding at 186 h, cells accumulated intracellular oil bodies (Fig. 4A**)**. As shown in Figure 4B, in medium II, cell growth and division stopped at 96h, due to the lack of enough nitrogen source. After the simultaneous addition of MSG and glucose at 120 h, cell growth and division increased. The positive effect of nitrogen with carbon source on cell growth was observed during the fermentation period (128-190 h). After 215 h of cultivation, the cells were saturated with intracellular oil droplets.

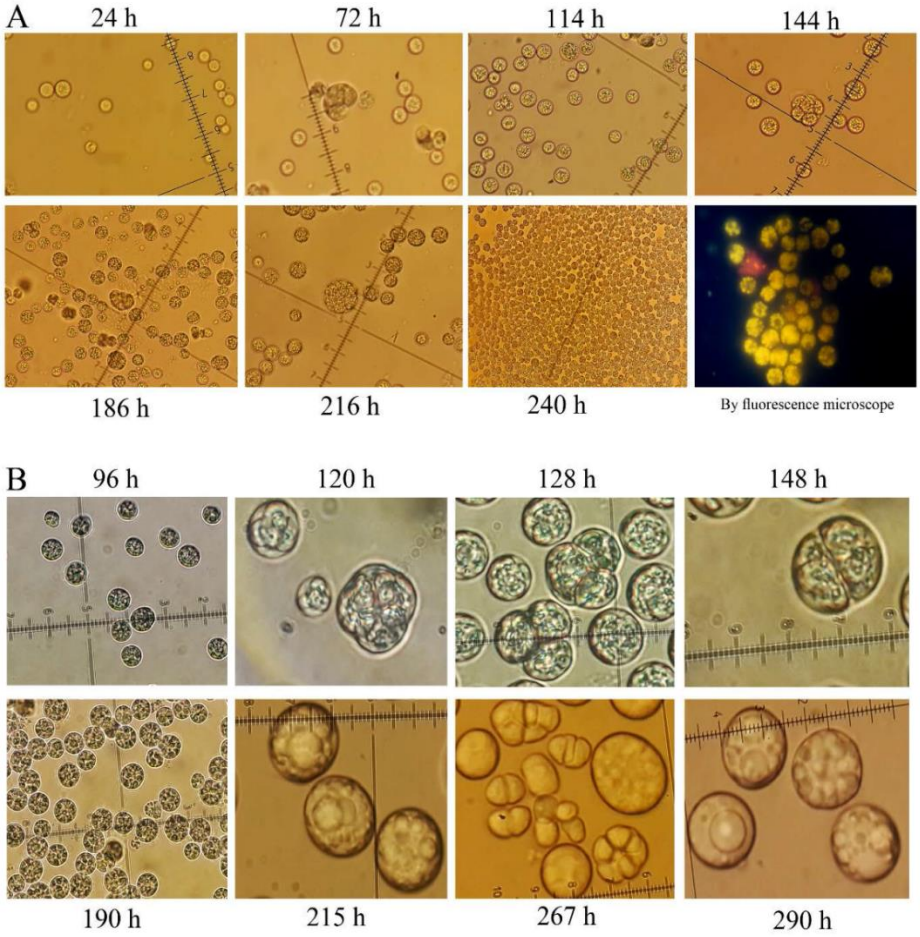

Fig. 4- The cell morphology of *Aurantiochytrium sp*. during fermentation in 3 L fermentor. (A) Medium I and magnification of 40 (B) Medium II and magnification of 100

Discussion and Conclusion

In the present study, the authors investigated the effects of different carbon sources and DO levels on the cell growth, lipid, and DHA production in *Aurantiochytrium sp.* at 3 L fermentor. Significant DHA productivity was achieved in the medium containing glycerol compared with the medium with glucose as the sole carbon source (7.15 and 5.01 $mg.L^{-1}.h^{-1}$,

respectively). The results of the study showed the cell growth increased to a high amount in the fermentor (30.15 $g.L^{-1}$) in comparison to the shake flask (10.32 $g.L^{-1}$) in the previous study (30). Janthanomsuk et al. (2015) obtained a high cell density of *Aurantiochytrium* B072 and high productivity of DHA by the fed-batch pH auxostat fermentation process. They showed that glucose-limited feeding increased the percentage of DHA per total fatty acids up to 37% (w/w) (34). The bioreactor provides more dissolved oxygen for the growing of *Aurantiochytrium* cells than shake flasks, so vegetative cells consume the most carbon and nitrogen sources for growth and multiplication. Moreover, we used a strategy for the simultaneous feeding of MSG and glucose to improve DHA productivity. Trovao et al. (2020) used two media (I and II) containing monosodium glutamate/glucose and corn steep powder/glycerol, respectively. Their results showed that medium I and II increased DHA content 1.7 and 3 folds, respectively (35). Ren et al. investigated DHA production in a 1500 L bioreactor with glucose and MSG in fed-batch culture. They achieved 71 $g.L^{-1}$ biomass, 35.75 $g.L^{-1}$ lipid, and 48.95% DHA (% of total fatty acids) (22).

There are two important factors that effectively increase lipid accumulation in oleaginous microorganisms: 1- the continuous generation of acetyl-CoA, a precursor for fatty acid synthase (FAS); and 2- proper amount of NADPH as the essential reductant in fatty acid biosynthesis (36, 37). ATP: citrate lyase (ACL) enzyme facilitates the formation of acetyl-CoA in the cytosol of oleaginous *Aurantiochytrium* sp. and malic enzyme (ME) and glucose-6-phosphate dehydrogenase (G6PDH) are responsible for generating NADPH for the biosynthesis of fatty acids (38). Therefore, the limitation of nitrogen sources in the fermentation medium increases the cytosolic level of both acetyl-CoA and NADPH and consequently leads to the accumulation of lipid granules by *Aurantiochytrium* cells (2).

Furthermore, the addition of MSG can activate acetyl-CoA carboxylase (10) and glucose-6-phosphate dehydrogenase (39), hence it increases enough supplement of acetyl-CoA and NADPH for the biosynthesis of lipid and DHA. Ye et al. (2020) used the fed bath fermentation strategy of mixed carbon sources including glucose and glycerol. The results showed that 52.2 $g.L^{-1}$ biomass and 100.7 $mg.L^{-1}.h^{-1}$ DHA were produced. Their results revealed that simultaneous feeding of glucose and glycerol upregulates putative genes in fatty acid synthase (FAS) and polyketide synthase (PKS) pathways (40).

In the lipid accumulation phase, low oxygen amount is needed for DHA production. Chi et al showed that a high level of DO (50% of saturation) during the lipid accumulation phase has a declining effect on lipid content (41). In addition, Kim et al. (2013) showed that the nitrogen source is responsible for the cell growth and multiplication during the early stage of fermentation. Also, their results showed that a carbon source is required during the entire process of cultivation for cell growth and particularly lipid accumulation (42). Jakobsen (2008) showed that a low level of DO in the fermentation medium was favored for the production of DHA (14). Also, Bailey et al. (2003) reported that a high DO level (40%) led to the accumulation of 18.2% (w/w) lipids in CDW. On the other hand, a low DO level (less than 3% of saturation) stimulated the production of DHA up to 24.4% of total fatty acids (29). In conclusion, a low level of oxygen is more effective for lipids accumulation by *Aurantiochytrium sp.* Also, it is crucial for long-chain PUFAs production via the O_2-independent-PUFA-synthase pathway (43). This work proved and paved the way to use native *Aurantiochytrium* strain for the production of DHA at the industrial scale.

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