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ORIGINAL PAPER

Improvement in the docosahexaenoic acid production of *Schizochytrium* sp. S056 by replacement of sea salt

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Abstract *Schizochytrium* is a marine microalga that requires high concentrations of sea salt for growth, although problems arise with significant amounts of chloride ions in the culture medium, which corrodes the fermenters. In this work, we evaluated that cell growth and docosahexaenoic acid (DHA) production can be improved when using 1 % (w/v) sodium sulfate instead of 2 % (w/v) sea salt in the culture medium for *Schizochytrium* sp. S056. In practice, the use of sodium sulfate as the sodium salt led to chloride ion levels in the medium that can be completely removed, thus avoiding fermenter corrosion during *Schizochytrium* sp. S056 growth, reducing cost and increasing DHA production, and simplifying the disposal of fermentation wastewater. Additionally, we demonstrated that the osmolality of growth media did not play a crucial role in the production of DHA. These findings may be significantly important to companies involved in production of PUFAs by marine microbes.

Keywords Marine microalgae · *Schizochytrium* · Docosahexaenoic acid · Sea salt · Sodium sulfate · Osmolality

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Introduction

Omega-3 polyunsaturated fatty acids (ω-3 PUFAs), particularly docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), are of significant commercial interest because they have been recently recognized as important dietary compounds in preventing and treating heart disease, high blood pressure, inflammation, and certain forms of cancer [1–3]. Clear clinical and animal evidence began to accumulate that indicated that DHA (22:6, n-3) is particularly important, given its role in the development of neural and retinal functions, and could improve memory and eyesight in babies [4–7]. Although traditional commercial sources of DHA are fish oils, such as sardine, and salmon, to name a few [8], the considerable amount of saturated fatty acids in fish oils complicates the purification process and increases production cost [9]. It is important to note that DHA from fish oil has an undesirable fishy smell and contains inevitable contamination by marine pollution (such as nuclear radioisotopes, dioxins, polychlorinated biphenyls and heavy metals including mercury compounds) which is taken up by the fish and concentrated in the liver and other organs. These properties make fish oil an unsuitable source of DHA [10–12]. Over the past 20 years, DHA production by microorganisms has been sought as a replacement for fish oil and has been successfully developed [13–15]. Among numerous microalgae strains, *Schizochytrium* is noteworthy and often considered a satisfactory alternative to fish oil due to advantages such as its fast growth rate and high productivity [16–18].

Schizochytrium are heterotrophic marine thraustochytrids that produce about 35–40 % DHA in their total fatty acids [19, 20]. DHA-containing oil from *Schizochytrium* sp. (thraustochytrid) has been in commercial production for nearly two decades by Martek Biosciences

Corporation, which is now part of the Royal DSM (Dutch State Mines) [14]. As a marine microalgae, *Schizochytrium* requires high concentrations of sea salt for growth [21–23], resulting in a significant amount of chloride ion in the culture medium. Chloride during growth for DHA production can result in corrosion of the vessel [24–26], which makes it unsuitable for large-scale commercial operation. Therefore, it is highly desirable to solve the corrosive effects of chloride ions on fermentation equipment. Unagul et al. reported that by replacing artificial sea salts with magnesium sulfate and sodium chloride, the concentration of chloride was reduced more than tenfold, but the media still required supplementation [27]; Shabala et al. suggested that thraustochytrid can be grown in low-salt (1 mM NaCl) culture media, but mannitol and sucrose were still required for osmotic adjustment, and this modified culture media including chloride ion, mannitol, and sucrose is not suitable for large-scale fermentation [28].

The aim of this work was to avoid the corrosive effects of chloride ions on fermentation equipment and increase DHA production in *Schizochytrium* sp. S056. We found a novel process for growing *Schizochytrium*, which includes the growth of the microalga in a culture medium with non-chloride containing sodium salts, specifically sodium sulfate. The final results show that the microalga biomass grown was significantly improved after sea salt was replaced by sodium sulfate in the culture medium.

Materials and methods

Microorganism

Schizochytrium sp. S056 (CCTCC M 2013459) was used in this study; it was originally isolated from seawater and deposited at the China Center for Type Culture Collection (CCTCC, Wuhan, China, http://www.cctcc.org).

Culture conditions

Schizochytrium sp. S056 was inoculated into flasks (250 mL) containing 50 mL seed culture medium at 25 °C and cultivated for 48 h. The seed culture (4 %, v/v) was then transferred to flasks (250 mL) containing 50 mL fermentation medium at 25 °C and incubated under reciprocal shaking (200 rpm) for 6 days. The seed culture medium contained (%, w/v): glucose (5), proteose peptone (0.2), yeast extract (0.5), KH_2PO_4 (0.15), $MgSO_4{\cdot}7H_2O$ (0.05), sea salts (2). The fermentation culture medium contained (%, w/v): glucose (8), $NaNO_3$ (0.3), yeast extract (1.5), KH_2PO_4 (0.15), $MgSO_4{\cdot}7H_2O$ (0.05), sea salts (2). The sea salt used was artificial sea salt (NaCl ≥99 %), purchased from Haifu Biotech Co., Ltd, Guangdong, China.

Osmolality measurement of the fermentation broth

The osmolality of the growth medium was measured by BS-100 osmotic pressure tester (Shanghai, China) in milliosmoles/L (mOsm/L).

Determination of dry cell weight

Culture samples (50 mL) were centrifuged (8000*g*, 10 min, 4 °C) after growing at 25 °C accompanying rotational shaking (200 rpm) for 6 days and biomass was washed three times in demineralized water. Biomass was dried at 80 °C in a vacuum oven for 12 h, and then weighed.

Lipid analysis

Lipids were extracted according to the method described by Zarnowski and Suzuki [29]. Fatty acids were methylated according to Indarti et al. [30] and Ren et al. [31] with some modifications. 1 mL of KOH 1 mol/L in methanol was added into 80 μL of lipids in a 10 mL test tube with a stopper. The mixture was then heated in a water bath for 30 min at 65 °C, then cooled to room temperature and 2 mL BF3-ether: CH_3OH (3:7) was added into the system and was allowed to react for 5 min at 65 °C. The system was then cooled to room temperature and 1 mL n-hexane was added. Finally, 1 mL saturated NaCl solution was added into the mixture. After shaking vigorously, the solution allowed to stand for 10 min, and then separate the upper layer of *n*-hexane which contains fatty acid methyl esters (FAMEs). The FAMEs were analyzed by gas chromatography–mass spectrometer described previously [32] with some modifications. The GC (Agilent 5975C) equipped with FID (flame ionization detector) through a capillary column (HP-FFAP 30 m × 0.32 mm × 0.25 μm) using nitrogen as carrier gas (2 mL/min). The temperature of oven was initially maintained at 150 °C, but then later increased to 220 °C at a ramping of 10 °C/min for 15 min. The injector and detector temperature were maintained at 250 °C with a split ratio of 1:10, with 1 μL sample was injected to the gas chromatograph. The composition of individual FAMEs were quantified by internal standard method and nonadecanoic acid (C19:0) (Sigma Co., USA) was used as the internal standard.

Results

Effects of sea salt concentration on *Schizochytrium* sp. S056 cells growth

The effects of sea salt concentration on biomass and lipids content of the biomass and DHA yield were examined for

artificial sea salt in the range of 0–2.5 % (w/v). As shown in Fig. 1, the highest biomass and DHA yield (34.76 and 6.61 g/L, respectively) were obtained when sea salt concentration was 2 % (w/v). Little change in DHA yield was observed in the range 0–1.5 % (w/v), and the strain was able to grow in medium without sea salt. However, when the sea salt concentration increased from 2 % (w/v) to 2.5 % (w/v), the biomass and DHA yield were decreased from 34.76 to 33.13 g/L and 6.61 to 6.27 g/L, respectively. Similar results were obtained by other groups, such as Yokochi et al. [19], who found that the strain SR21 can grow in medium with a sea water concentration in the range of 0–200 % (the salt concentration in the medium, compared with the sea water), and the optimum salinity varied from 50 to 200 % relative to the concentration in sea water. Zhu et al. [20] found that *S. Limacinum* grows better with salinity at 1.8–3.6 % (w/v); growth was inhibited when salinity decreased from 0.9 % (w/v) to 0. Thus, 2 % (w/v) sea salt was chosen to obtain the best fermentation results.

Effects of substitution of non-chloride sodium salts for sea salt on *Schizochytrium* sp. S056 cells growth with isotonic environment

Different non-chloride sodium salts were chosen as a substitute for sea salt to maintain a consistent isotonic environment (2 % (w/v) sea salt in the culture medium), and we examined their effect on the growth, lipid content and DHA yield of *Schizochytrium* sp. S056. Since this strain was isolated from seawater, high levels of sea salt in the culture medium may play a significant role in cell growth and lipid synthesis [33]. *Schizochytrium* also uses sea salt for osmotic adjustment [34, 35]. Shabala et al. [36] proposed that sodium was the major contributor in the process of osmotic adjustment and Raghukumar [33] indicated that thraustochytrids have an obligate requirement for sodium ions.

Thus, according to their theory, we must find a non-chloride sodium salt that can replace sea salt under an isotonic environment. Non-chloride sodium salts that have been tested as potential substitutes for sea salt include sodium carbonate, sodium bicarbonate, sodium sulfate and sodium nitrate. We adjusted the concentration of non-chloride sodium salts in the culture medium to maintain a consistent isotonic environment (2 % (w/v) sea salt in the culture medium) (Table 1). In parallel, mannose was used to adjust the osmolality of the growth medium without sodium salts as a control group. The initial pH of the medium was adjusted to 6.0–7.0 with 5 M KOH or 5 M HNO_3. The results were shown in Fig. 2, under equivalent osmotic pressure: when 5 % (w/v) sodium sulfate was compared with 2 % (w/v) sea salt, biomass was remained stable (33.74 and 34.76 g/L, respectively) and DHA yield goes down slightly from 6.61 to 5.96 g/L. *Schizochytrium* sp. S056 grew under other non-chloride sodium salts, with growth levels and DHA yield almost half that of sea salt. When 12 % (w/v) mannose was used to adjust isotonic environment, cell growth levels were much lower when compared to 2 % (w/v) sea salt, while the DHA yield was approximately 30 % relative to sea salt. These results demonstrated that without sodium salts, the desired DHA yield cannot be obtained even after maintenance of osmolality by mannose. However, Fig. 2 indicated that sodium sulfate may be a viable sodium salt for productive cultivation of *Schizochytrium* sp. S056.

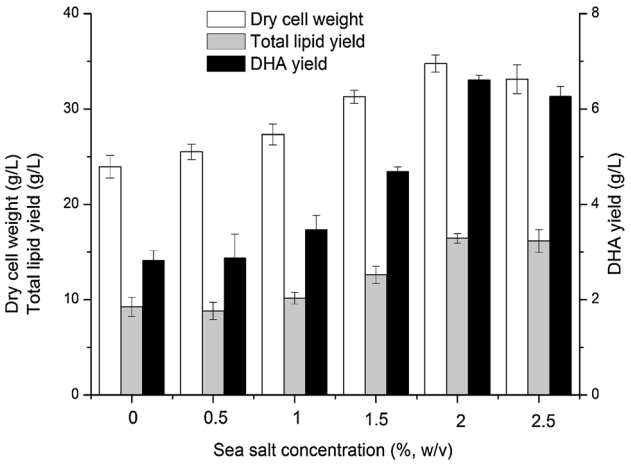

Fig. 1 Effects of sea salt concentration on the amount of biomass, lipids and DHA in *Schizochytrium* sp. S056 cells. Data were mean ± SD of three experimental replicates

Effects of sodium sulfate concentration on media osmolality and *Schizochytrium* sp. S056 cells growth

The effects of sodium sulfate concentration over a range of 0–5 % (w/v) on media osmolality and cell growth was examined (Table 2). When compared to 2 % (w/v) sea salt, sodium sulfate at 5 % (w/v) caused the salinity of the medium increased by 150 %. This results in increased difficulty in fermentation sewage disposal and the increased amount of sodium salt in the culture medium may lead to an increased cost for raw materials. As shown in Table 2, there were slight fluctuations in dry cell weight when sodium sulfate concentration was varied. The optimum sodium sulfate concentration was 1 % (w/v), resulting in dry cell weight and DHA yield of 38.93 and 7.95 g/L, respectively, which is higher than sea salt. *Schizochytrium* sp. S056 also showed a wide tolerance of media osmolality: initial media osmolality was reduced from 1378 to 724 mOsm/L when the concentration of sodium sulfate was reduced from 5 (w/v) to 0 % (w/v). Thus,

Table 1 Salts concentration and media osmolality

Sodium salt	Concentration (%, w/v)	Media osmolality (mOsm/L)
Sea salt	2	1346 ± 84
Sodium carbonate	4	1351 ± 27
Sodium bicarbonate	3.5	1335 ± 31
Sodium sulfate	5	1378 ± 44
Sodium nitrate	3.5	1342 ± 63
Mannose	12	1327 ± 56

Data were mean ± SD of three experimental replicates

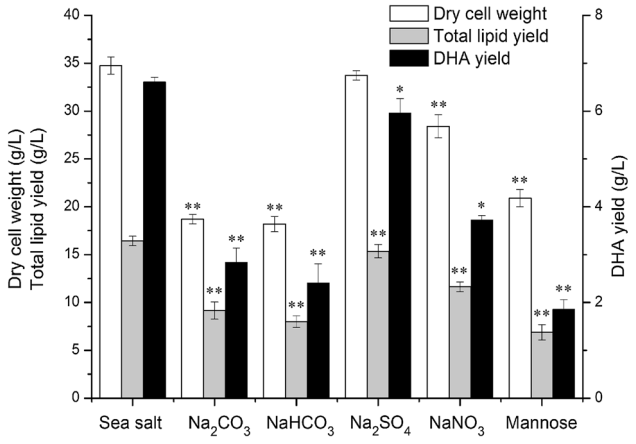

Fig. 2 Effects of sodium salts and mannose under the same osmotic pressure on the amount of biomass, lipids and DHA in *Schizochytrium* sp. S056 cells. Concentration of the salts (w/v): sea salt 2 %, sodium carbonate 4 %, sodium bicarbonate 3.5 %, sodium sulfate 5 %, sodium nitrate 3.5 %, mannose 12 %. Data were mean ± SD of three experimental replicates. The statistical significance between sea salt (2 %) and the others was presented by *t* test, $*P < 0.05$, $**P < 0.01$

Schizochytrium sp. S056 could be grown under non-chloride conditions to yield higher DHA production.

Effect of sodium sulfate (1 %) substituted for sea salt (2 %) on the fatty acid profile of *Schizochytrium* sp. S056

We further investigated the effect of 1 % (w/v) sodium sulfate, substituted for 2 % (w/v) sea salt, on the fatty acid profile (Table 3). According to Table 3, the proportion of DHA in total fatty acids increased to 44.36 % at 1 % (w/v) sodium sulfate from 40.23 % at 2 % (w/v) sea salt. The proportion of tetradecanoic acid (C14:0) and docosapentaenoic acid (DPA) increased from 2.91 to 3.74 % and 7.25 to 8.64 %, respectively. Pentadecanoic acid (C15:0) and palmitic acid (C16:0) decreased from 4.16 to 3.23 % and 38.73 to 33.87 %, respectively, when the salts changed from sea salt to sodium sulfate, while the other fatty acids were found at the same concentrations in 2 % (w/v) sea salt and 1 % (w/v) sodium sulfate. These results suggested the proportion of DHA increased after the changed in growth media to sodium sulfate. Thus, sodium sulfate was found to be superior to sea salt.

Effect of the osmolality changes between sodium sulfate (1 %) and sea salt (2 %) during the fermentation process of *Schizochytrium* sp. S056

The osmolality of growth media gradually decreased during the fermentation process due to the consumption of nutrients (carbon and nitrogen sources, data not shown) (Fig. 3). As shown in Fig. 3, the osmolality of growth

Table 2 Effect of sodium sulfate concentration on cell growth, lipid and DHA production of *Schizochytrium* sp. S056

Sodium sulfate concentration (%, w/v)	Media osmolality (mOsm/L)	Dry cell weight (g/L)	Total lipid content (g/L)	DHA yield (g/L)
0	724 ± 41	23.94 ± 0.57**	9.25 ± 0.63**	2.83 ± 0.24**
0.5	798 ± 38	36.41 ± 0.91**	16.51 ± 0.27	6.45 ± 0.21
1	857 ± 65	38.93 ± 0.78**	17.93 ± 0.72**	7.95 ± 0.35*
1.5	914 ± 44	37.22 ± 0.52**	17.88 ± 0.43**	7.21 ± 0.26
2	986 ± 67	37.19 ± 1.84**	17.54 ± 0.74**	7.02 ± 1.03*
2.5	1034 ± 32	36.21 ± 0.77**	16.77 ± 0.57**	6.58 ± 0.78
3	1108 ± 72	35.63 ± 1.06**	16.82 ± 1.05	6.64 ± 0.62
3.5	1164 ± 68	34.85 ± 0.45	15.96 ± 0.73**	6.27 ± 0.84**
4	1237 ± 71	33.81 ± 0.33*	15.61 ± 1.02*	6.11 ± 0.38
4.5	1301 ± 85	34.44 ± 0.67	15.43 ± 0.89**	5.94 ± 0.29
5	1378 ± 44	33.74 ± 0.41	15.36 ± 0.76**	5.96 ± 0.36*
Sea salt (2)	1346 ± 84	34.76 ± 1.14	16.43 ± 0.65	6.61 ± 0.92

Data were mean ± S.D. of three experimental replicates. Statistical significance between sodium sulfate and sea salt (2 %) was presented by *t* test

* $P < 0.05$, ** $P < 0.01$

Table 3 Fatty acid profile of *Schizochytrium* sp. S056 grown in two kinds of salts

Salt (w/v)	Fatty acid content (% total fatty acids)							
	C14:0	C15:0	C16:0	C17:0	C18:0	EPA (*n*-3)	DPA (*n*-6)	DHA (*n*-3)
Sea salt (2 %)	2.91 ± 0.08	4.16 ± 0.11	38.73 ± 0.37	1.11 ± 0.15	1.42 ± 0.07	0.67 ± 0.04	7.25 ± 0.18	40.23 ± 0.46
Sodium sulfate (1 %)	3.74 ± 0.13**	3.23 ± 0.28**	33.87 ± 0.45**	0.82 ± 0.05*	1.47 ± 0.17	0.88 ± 0.02*	8.64 ± 0.22**	44.36 ± 0.53**

The concentration of sea salt and sodium sulfate were 2 % (w/v) and 1 % (w/v), respectively

Data were mean ± SD of three experimental replicates. The statistical significance between sea salt (2 %) and sodium sulfate (1 %) was presented by *t* test

DHA docosahexaenoic acid, *DPA* docosapentaenoic acid, *EPA* eicosapentaenoic acid

* $P < 0.05$, ** $P < 0.01$

media decreased from beginning to end of fermentation as follows: 1346–758 mOsm/L for 2 % (w/v) sea salt and 857–341 mOsm/L for 1 % (w/v) sodium sulfate, and the curve changes of osmolality of the growth media remained essentially the same for both salts. These results suggested that *Schizochytrium* sp. S056 can adapt to a wide range of osmolality [37].

Discussion

Schizochytrium sp., which contains a high content of PUFAs, was cultivated for the production of DHA in batch culture [14]. Being marine organisms, *Schizochytrium* sp. requires a high concentration of sea salts for growth [35]. This study indicated that content at 2 % (w/v) of sea salt was the optimum concentration for growth of

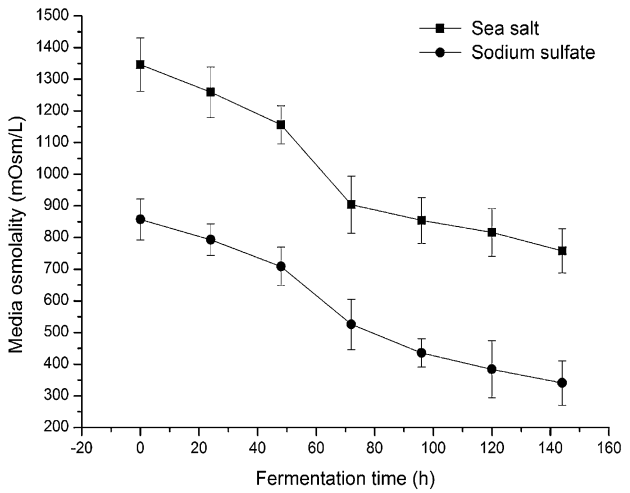

Fig. 3 The osmolality of growth media changed during the fermentation period. The concentration of sea salt and sodium sulfate was 2 % (w/v) and 1 % (w/v), respectively. Data were mean ± SD of three experimental replicates

Schizochytrium sp. S056. In this study, we found that biomass of *Schizochytrium* sp. S056 decreased when sea salt concentration was reduced, consistent with results from numerous previous studies [19–23]. However, high levels of sea salts generate chloride ions in culture medium that can corrode the fermenter, which is not desirable for large-scale commercial production.

It was reported that *Schizochytrium* sp. use sea salt for osmotic adjustment, and that sodium was the major contributor to osmotic adjustment and cell growth [33, 36]. Thus, in this follow-up research, we measured the osmotic pressure of culture medium, which contained sea salt. A series of non-chloride sodium salts were subsequently chosen as substitutes for sea salt under an isotonic environment achieved by adjusting concentrations. We demonstrated that 5 % (w/v) sodium sulfate can replace sea salt without affecting the biomass of *Schizochytrium* sp. S056, but when 5 % (w/v) sodium sulfate was compared with 2 % (w/v) sea salt, we found a 150 % increase in the salt concentration of the culture medium. This increased salt concentration resulted in more difficult disposal of fermentation sewage as well as increasing the amount of sodium sulfate in culture medium; both of these effects will lead to increase production costs. When mannose was used to adjust the osmolality of culture medium, the biomass and total lipid content of the cell was significantly decreased, indicating that *Schizochytrium* sp. S056 may need sodium salts to maintain metabolism and growth, in addition to maintaining osmolality [33]. This conclusion is different from Shabala et al. who suggest that the requirement of sodium salts for cell growth is due to its role in microorganism osmotic adjustment, and not involvement in cell metabolism [28, 36].

To make the fermentation wastewater easier to process and save on the cost of raw materials, the optimum sodium sulfate concentration in culture medium must be studied. We found that the biomass of *Schizochytrium* sp. S056 increased from 34.76 to 38.93 g/L and the DHA yield

increased by 20.27 % when 2 % (w/v) sea salt was replaced by 1 % (w/v) sodium sulfate (Table 2). The osmolality of culture media decreased from 1346 to 857 mOsm/L and the DHA content in total fatty acids increased from 40.23 to 44.36 % when sodium salts were changed from sea salt (2 %, w/v) to sodium sulfate (1 %, w/v) (Table 2). The results presented here indicate that, although the osmolality declines sharply, sodium sulfate was more suitable for the cultivation of *Schizochytrium* sp. S056, when compared to sea salt. Our results also demonstrate that the osmolality of culture media was not important for efficient growth and DHA production in *Schizochytrium* sp. S056 (Fig. 3), and cell biomass and DHA production appear to potentially benefit from growth in low-osmolality media which complemented with sodium sulfate [38]. It was also previously reported that lowering salt concentrations to the minimum required quantity can maximize biomass and DHA production [39, 40]. There phenomenon indicated that low-osmolality media may be beneficial to the growth and biosynthesis of DHA of *Schizochytrium* sp. S056 [41].

In conclusion, our results indicated that cell growth and DHA production can be improved by using 1 % (w/v) sodium sulfate instead of 2 % (w/v) sea salt in the culture medium for *Schizochytrium* sp. S056. We also were able to demonstrate that the osmolality of growth media did not play a crucial role in the production of DHA. In practice, the use of sodium sulfate as the sodium salt led to chloride ion levels in the medium that can be completely removed, thus avoiding fermenter corrosion during *Schizochytrium* sp. S056 growth, reducing cost and increasing DHA production, and simplifying the disposal of fermentation wastewater. These findings are thought to be quite important to companies involved in production of PUFAs by marine microbes.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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