1 **Adaptive evolution of *Schizochytrium* sp. by continuous high oxygen**
2 **stimulations to enhance docosahexaenoic acid synthesis**

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23 Abstract

24 Adaptive laboratory evolution (ALE) is an effective method in changing the strain
25 characteristics. Here, ALE with high oxygen as a selection pressure was applied to
26 improve the production capacity of *Schizochytrium* sp. Results showed that cell dry
27 weight (CDW) of endpoint strain was 32.4% higher than that of starting strain. But
28 slight lipid accumulation impairment was observed. These major performance
29 changes were accompanied with enhanced isocitrate dehydrogenase enzyme activity
30 and reduced ATP: citrate lyase enzyme activity. And a serious decrease of 62.6% in
31 $S_{DHA\ 140rpm \rightarrow 170rpm}$ was observed in the endpoint strain. To further study the
32 docosahexaenoic acid (DHA) production ability of evolved strain, fed-batch strategy
33 was applied and 84.34g/L of cell dry weight and 26.40g/L of DHA yield were
34 observed. In addition, endpoint strain produced greatly less squalene than starting
35 strain. This work demonstrated that ALE may be a promising tool in modifying
36 microalga strains.

37 **Key words:** *Schizochytrium* sp; docosahexaenoic acid; adaptive evolution; enzyme
38 activity

39 **1 Introduction:**

40 *Schizochytrium* sp. belongs to a kind of marine fungi and is famous for producing
41 docosahexaenoic acid (DHA, 22:6n-3). DHA, a long-chain polyunsaturated fatty acid,
42 is essential for human health, and it has attracted particular attention as essential for
43 neurological development of infants (Ji et al., 2015). In addition, DHA plays an
44 important role in alleviating cardiovascular diseases, hypertension, diabetes and
45 neuro-psychiatric disorders (Ursin, 2003) . In the past, the main resource of DHA
46 derived from fish oil. However, it is predicted that fish resources will decrease in the
47 near future, creating the need to establish a sustainable source of DHA (Cohen et al.,
48 1995). Recently, *Schizochytrium* sp. is believed to be a highly promising alternative for
49 commercial DHA production.

50 Oxygen plays a vital role in cell proliferation and lipid accumulation of
51 *Schizochytrium* sp. (Jakobsen et al. 2008). In recent years, various fermentation
52 strategies for *Schizochytrium* were performed by controlling oxygen supply for the
53 optimization of DHA production. Chi et al. (2009a) applied a two-stage oxygen
54 control method for *A. limacinum* SR21 and achieved 37.9g/L of cell dry weight and
55 6.56g/L of DHA yield. Ren et al. (2010) developed a stepwise aeration control
56 strategy for *Schizochytrium* sp. M209059, which reached to 71g/L of CDW and
57 15.76g/L of DHA yield. These previous works indicated that a contradiction existed in
58 DHA production. High oxygen supply was beneficial to shorten the fermentation
59 period and obtain high cell dry weight, but high DHA percentage in total fatty acids
60 could only be obtained under low oxygen supply condition (Bailey et al., 2003; Chi et al.,

2009b) . Although those previous studies have proposed several regulation strategies and both biomass and DHA production have been improved. But, the time point of fermentation control is difficult to determine accurately, which leads to the instability of DHA production. If there is a strain which have higher DHA productivity ability in a relative high oxygen condition, the production process would be more easily controlled.

As we known, engineered strains after genetic modification have the potential to improve cell dry weight and DHA yield. However, transgenic technology is difficult to be applied in the food industry (Menrad et al. 2011). Microorganisms have ability to adapt themselves rapidly to different environments. This property has been gradually developed and utilized in varied microorganisms by conducting adaptive laboratory evolution (ALE) experiments to improve the activity or tolerance of strains (Lu et al., 2012). The selective pressure is very important to the success of ALE, and the pressures can be divided in two main categories: nutrient stress and environmental stress (Nam et al., 2011). Nutrient stress was often used to enhance the utilization rate of some low cost substrates. For example, *Rhodococcus opacus* can only use glucose and xylose to produce lipid. Kazuhiko et al. (2015) constructed a *Rhodococcus opacus* strain capable of high-cell-density cultivation at high glycerol concentrations by ALE with medium containing 100g/L glycerol. Similarly to *Rhodococcus opacus*, nutrient adaptation was also extensively studied in *Saccharomyces cerevisiae*. Early studies in glucose-limited chemostats revealed that glucose-limited selection led to increased biomass yield and a decreased fermentative

capacity (Ferea et al., 1999; Jansen et al., 2005). Environmental stresses mainly include temperature, osmotic stress, oxygenation, organic solvent tolerance and so on (Dragosits et al., 2013). In the modest yeast *Saccharomyces cerevisiae*, adaptation towards hyperosmotic conditions improved glycerol formation and reduced ethanol yield (Tilloy et al., 2015). Li et al. (2015) also successfully improved high CO₂ tolerance of *Chlorella* sp by ALE. Considering *Schizochytrium* sp. is extremely sensitive to oxygen supply, and a slight difference of oxygen supply could result in large difference in DHA yield a high oxygen adaption strategy would be applied in this study to change the oxygen sensitivity of *Schizochytrium* sp. In addition, PUFAs has strong oxidation resistance (Richard et al., 2008), and the high oxygen stimulus might induce cell to produce more polyunsaturated fatty acids (PUFAs) to protect themselves from oxidative injury.

In this work, the ALE experiment was sustained for 40 cycles to obtain endpoint strain under continuous high oxygen condition. Four key enzymes activities, oxygen sensitivity, cell dry weight and DHA yield were used to compare the difference between endpoint strain and starting strain. In addition, a fed-batch cultivation strategy was also carried out in 5L bioreactor to further study the DHA production ability of endpoint strain. The aim of this study was to provide a new method from the perspective of ALE to improve the productivity of the strain, which also might be used in other oil-producing microalga.

2. Materials and methods

2.1 Microorganism and culture conditions

105 *Schizochytrium* sp. HX-308(CCTCC M 209059), isolated from seawater and stored
106 in China Center for Type Culture Collection (CCTCC) (Huang et al., 2009), was used
107 in this study as the starting strain. The strain was preserved in 20%(v/v) glycerol at
108 -80°C.

109 The seed culture medium and conditions of propagation were the same as those
110 used in our previous study (Ren et al., 2010). After three generations of cultivation,
111 the seed culture (1% v/v) was then transferred to a 500-ml shaking flasks with 100
112 medium cultured at 28°C under agitation at 170 rpm and the resulting cell suspension
113 was used to inoculate for fermentation. The fermentation medium was the same as our
114 previous study.

115 ***2.2 Adaptive laboratory evolution experiments***

116 ALE was based on a long-term serial transfer procedure using high agitation rate as
117 stress inducer. The agitation of adaptive evolution was set at 230rpm compared with
118 170rpm for original culture. In addition to agitation, the culture medium of evolution
119 strain was the same as ancestral strain. ALE was conducted repeatedly in a 24h cycle.
120 Four each new cycle, the seed culture (1%v/v) was transferred to a fresh medium.
121 Every 10 cycles, the evolved strain (10%v/v) was incubated to ferment in a 500-ml
122 shaking flasks with 100mL medium cultured at 28°C under agitation at 170 rpm until
123 exhausting initial glucose for 120 g/L. For each 10 cycles, ALE strains were named
124 ALE10, ALE20 and ALE30, receptivity. And ALE 40 cycles strain was the endpoint
125 strain. The fermentation medium was the same as our previous study (Ren et al.,
126 2010). All fermentation experiments were performed in triplicate. Such serial transfers

127 were repeated for 40 cycles and 1-mL samples of the evolving population were taken
128 and stored at -80°C in 20% glycerol for subsequent analysis every 10 cycles.

129 ***2.3 Cell dry weight, total lipids, specific growth rate and oxygen sensitivity***

130 Cell dry weight was determined gravimetrically by filtering 10-mL of samples
131 using centrifuge for 5 min at 4500g. Then dry the sample for 12 hours at 60°C by
132 transferring cells to the filter paper which has dried and weighted. During the drying
133 process, the weight was recorded repeatedly until the mass kept stable. The specific
134 growth rate (μ) was calculated by the equation, $\mu = (\ln X_2 - \ln X_1) / (t_2 - t_1)$, where X_1 and
135 X_2 are the cell dry weight concentrations (g/L) at time t_1 and t_2 , respectively (Liu et al.
136 2015).

137 The measurements of total lipids (TLs), extracellular glucose, glutamate
138 weredetermined as our previous study (Qu et al., 2011). To investigate the oxygen
139 sensitivity of *Schizochytrium* sp., the metabolic properties of starting strain and
140 endpoint strain at different rotate speed (140rpm, 170rpm and 200rpm) were
141 compared in 500-mL shaking flasks. The oxygen sensitivity to CDW (S_{CDW}) was
142 calculated by using the equation $S_{CDW} = (X_2 - X_1) / (r_2 - r_1)$, where X_1 and X_2 are the cell
143 dry weight concentrations (g/L) at rotate speed r_1 and r_2 , respectively. The oxygen
144 sensitivity to DHA (S_{DHA}) was calculated by using the equation $S_{DHA} = (Y_2 - Y_1) /$
145 $(r_2 - r_1)$, where Y_1 and Y_2 are the DHA percentage in TFAs (%) at rotate speed r_1 and
146 r_2 , respectively.

147 ***2.4 Fatty acid, squalene, degree of unsaturation analysis and oxygen transfer***

148 ***coefficient detection***

149 Fatty acid methyl esters (FAMES) preparation were performed as previously
150 described products. FAMES were prepared from 0.2g of dried cells and analyzed by
151 gas system (GC-2010, Shimadzu, Japan), equipped with a capillary column (DB-23,
152 60m *0.22 mm) and flame ionization detector (FID). Nitrogen was used as the carrier
153 gas. The injector was maintained at 250 °C with an inject volume of 1 l μL. The
154 column was raised from 100 °C to 200 °C at 25 °C/min and then increased to 230 °C
155 at 4 °C/min, keeping this temperature for 9 min. The FID detector temperature was set
156 at 280 °C. FAMES and squalene was identified through comparison with related
157 external standards (Sigma, USA). The quantities of individual FAMES and squalene
158 were estimated from the peak areas on the chromatogram using nonadecanoic acid
159 (C19:0) as the internal standard. The degree of unsaturation was calculated based on
160 the following formula, degree of unsaturation = 1 (% of monoene) + 2 (% of diene) +
161 3 (% of triene) + 4 (% of tetraene)+5 (% of pentaene) +6(% of hexaene) (Peng et al.
162 2010). The sulfite method (Na₂SO₃ method) is proposed for the measurement of
163 oxygen transfer coefficients (kLa) (Qu et al., 2010).

164 ***2.5 Preparation of cell extracts and Enzyme assay***

165 The microorganism was harvested by centrifugation, washed with ice-cold distilled
166 water and then by a washing buffer [400 mM Tris–HCl buffer, pH 7.4, containing
167 20%(w/v) glycerol, 1 mM dithioerythritol (DTT)]. Finally, the microorganism is
168 suspended in clean washing buffer with 0.5 mM phenyl-methane-sulfonyl fluoride
169 (PMSF). After being disrupted by ultrasonic disrupter for 15 min, the material was
170 centrifuged (120,000 rpm for 15 min at 4 C) and the supernatant was used

171 immediately for the determination of enzyme activity. Protein was determined using
172 the Bradford method with bovine serum albumin (BSA) as a standard. Total protein
173 content of crude enzyme was calculated by an empirically determined calibration
174 equation (protein content = $0.1833 \text{ OD}_{595\text{nm}} - 0.0033$).

175 Malic enzyme (ME) and glucose-6-phosphate dehydrogenase (G6PDH) activities
176 were determined spectrophotometrically by monitoring the rate of NADPH formation
177 at 28 °C and 25 °C, respectively. ATP:citrate lyase (ACL) enzyme activity was
178 detected according to the method of Takeda et al. (1969). Isocitrate dehydrogenase
179 (ICDH) enzyme activity was assayed as described by Wynn et al. (1997). A unit of
180 enzyme activity (U) was defined as the formation of the quantitative amount of
181 NADPH/min, which was equivalent to an increase in $\text{OD}_{340\text{nm}}$ of 0.001/min. Specific
182 activity (U/mg protein) was defined as the units of activity/mg of protein.

183 3. Results and discussion

184 3.1 Growth properties variation of *Schizochytrium* sp. during ALE

185 230rpm was chosen as a high oxygen stimulus condition in this study, and the
186 original *Schizochytrium* sp. strain was cultivated at this condition for 40 cycles. High
187 shaking speed resulted in high volumetric oxygen-transfer coefficient (kLa) value in
188 ALE, which forced more oxygen to be dissolved in the fermentation broth. Samples
189 collected each 10 cycles were analyzed to monitor the glucose and MSG consumption
190 (Fig.1 A), dry cell weight (CDW) and DHA production (Fig.1 B) of each evolution
191 experiment, which was recognized as first indicators of the success or failure of ALE.
192 As the extension of the evolving time, due to the natural accumulation of mutations,

193 ALE increased the consumption of glucose and MSG. Cell dry weight of endpoint
194 strain reached 45.2 g/L, which was 31% higher than that of starting strain, thus giving
195 the specific growth rate of 0.159. However, there was a slight decrease in DHA
196 percentage in total fatty acids (TFAs) (Table 1). This might attributed to the
197 polyketide sythases (PKS) pathway, which do not consume vast oxygen, and was
198 distinct from the fatty acid synthase pathway (FAS) (Song et al., 2013). This also
199 ascribed to the fact that the high oxygen has no advantage on DHA production in
200 *Schizochytrium* sp (Huang et al., 2012).

201 Moreover, a significant difference was observed in squalene content between
202 staring strain and evolved strains (Table 1). Squalene content downgraded to 0% after
203 30 cycles adaptation. Squalene is an unsaponifiable matter containing six isoprene
204 units that provide the backbone for the biosynthesis of cholesterol, bile acids and
205 steroid (Kohn et al. 1995). In this work, the reduced squalene might be consumed by
206 organisms, or converted to other compounds, or oxidized by oxygen. The period of
207 evolution was determined by adaptation conditions and microbial species, which in
208 the range of weeks to years (Dragosits et al., 2012) . Essentially, kLa directly
209 determined availability of oxygen in culture medium (Qu et al., 2010).The volumetric
210 oxygen-transfer coefficient (kLa) at 230rpm was 152.7S^{-1} , which was about twice
211 than that of 71.2S^{-1} at 170rpm. Therefore, during the ALE process, 230rpm exceed the
212 reasonable oxygen supply range and create an environmental stress to *Schizochytrium*
213 sp. Cultivated in this condition, cells were bound to accelerate the intracellular
214 metabolism. And *Schizochytrium* sp. might only require 40 cycles to produce a special

215 phenotype.

216 ***3.2 Fermentation characteristics changes of ALE strains***

217 To investigate the fermentation characteristics changes, four ALE strains (ALE10,
218 ALE20, ALE30 and endpoint strain) were cultured at 170rpm. The time courses of
219 substrate consumption (including glucose and MSG) by different ALE strains were
220 shown in Fig.2 A and B, respectively. Compared with the starting strain, ALE strains
221 significantly enhanced the glucose and MSG consumption rates, leading to a
222 shortening fermentation period of 36h in endpoint strain. CDW of endpoint strain
223 reached 62.15 g/L, which was 32.4% higher than that of starting strain. As shown in
224 Table 2, the increase of CDW productivity was most obvious in endpoint strain, which
225 was 76.53% higher than that of starting strain. No significantly difference in specific
226 growth rate was observed before 20 cycles. However, further increasing the evolved
227 cycles to 40, specific growth rate severely increased to 0.115, indicating that high
228 oxygen adaptation is effective to improve the growth of *Schizochytrium* sp. In contrast,
229 high oxygen adaptation exhibited adverse effect on lipid accumulation. Total lipids
230 (TLs) of endpoint strain was only 16.97g/L, which decreased by 15.6% than that of
231 starting strain (Table 2). On the one hand, these results may be caused by the changes
232 of key enzymes activities related to cell growth and lipid accumulation. In addition to
233 achieve objective phenotype at the endpoint of evolution, ALE usually could
234 accompany with the phenomenon of trade-offs (Dragosits et al., 2012). For example,
235 *E. coil* increased ethanol tolerance comes at the cost of decreased resistance to acidic
236 conditions (Goodarzi et al., 2010). Yeast cells were evolved for efficient galactose

utilization subsequently showed trade-offs when grown on glucose as carbon source (Hong et al., 2011). Similarly, in *Schizochytrium* sp., long term batch cultivation on high oxygen condition led to a rapid decay of non-used metabolic functions, including lipid production on rational oxygen conditions.

C14:0, C16:0, DPA and DHA were the main fatty acids in *Schizochytrium* sp., and DHA percentage in TFAs was about 40%. DHA percentage in TFAs of endpoint strain increased to 52.52%, compared with 49.21% in starting strain. It indicated that high oxygen adaptation has advantage in improving DHA percentage in total fatty acids. In addition, squalene has a slight decrease in evolved strains. Higher DHA content and lower SFA content with evolved strains might be ascribed to the oxygen sensitivity change of *Schizochytrium* sp. Moreover, endpoint strain also got the highest PUFAs/SFAs of 4.35 (Table 2). Meanwhile, the unsaturation degree of TFAs was increased by ALE, which was 9.5% higher than that of starting strain. 230rpm imposed cells into high oxidative conditions. As we all know, PUFAs was a famous kind of antioxidants (Richard et al., 2008). Then, cells might product more PUFAs to protect themselves from oxidative injury.

3.3 Key enzymes activities of starting strain and endpoint strain

In order to investigate whether the increase of CDW and decrease of total lipids (TLs) were related to the shift of metabolic pathway, the activities of four key enzymes, malic enzyme (ME), glucose-6-phosphate dehydrogenase (G6PDH), isocitrate dehydrogenase (ICDH) and ATP:citrate lyase (ACL) activities were detected every 8h during the fermentation of starting and endpoint strain. All the assays were

259 carried out in duplicate and the average values were shown in Figure.3.

260 ME and G6PDH activities were used to reflect NADPH supply information and it
261 was well known that the supply of reducing power in the form of NADPH plays an
262 important role in fatty acid biosynthesis and accumulation (Ren et al., 2009)
263 (Figure.4). As shown in Figure.3 A, regardless of starting strain or endpoint strain, the
264 activity of ME increased as the culture time went and reached its maximum of 157
265 U/g protein at 32h and 210 U/g protein at 40h, respectively. The change trend of
266 G6PDH activity was similar to those of ME, but the difference was that the time point
267 of the maximum value of ME activity was behind the time point of highest G6PDH
268 activity (Figure.3 B). This result was in good agreement with our previous study (Lian
269 et al., 2010). It illustrated that G6PDH was the main source of NADPH for early lipid
270 accumulation stage, and ME solve the insufficient NADPH supply at the later lipid
271 accumulation stage.

272 Interestingly, higher level of ICDH activity and lower level of ACL activity were
273 observed in endpoint strain (Fig.3 C and D). Endpoint strain exhibited an overall
274 above of ICDH activity in comparison to the starting strain, e.g, 232 U/mg protein at
275 32h, which showed an increase of 48.7% than starting strain (Figure.3 C). This
276 implied that citric acid might crack more isocitric acid to participate in the Krebs
277 Cycle, which provided more ATP for cell growth. This might explained the highest
278 CDW in endpoint strain.

279 According to the metabolic pathway of DHA biosynthesis in *Schizochytrium* sp.,
280 the formation of acetyl-CoA in oleaginous microorganism mainly depends on the

281 presence of ACL. In starting strain, ACL activity was 45 U/mg protein when first
282 detected at 8h and reached a maximum of 327 U/mg protein at 32h, followed by a
283 slight decline (Figure.3 D). Here, ACL activity showed a peak value in lipid
284 accumulation stage, which was in accordance with the highest demand for acetyl-CoA
285 to accumulate lipids (Wynn et al., 1997). But endpoint strain inhibited ACL activity.
286 The ACL activity was only 207 U/mg protein at 32h, which was 36.7% less than the
287 value of the starting strain. Lower ACL activity might result in the deficient supply of
288 acetyl-CoA to participate lipid accumulation, which was not conducive to lipid
289 production. Key enzymes involved in cell growth and lipid accumulation and
290 consumption in oleaginous microorganisms have been discussed previously
291 (Papanikolaou et al., 2004; Ratledge, 2004). Chang et al. (2013b) has studied key
292 enzymes (ME, ACL) changes in batch cultures on glycerol with stepwise dissolved
293 oxygen strategy. It has been demonstrated that ACL and ME played vital roles in the
294 generation of cytosolic acetyl-CoA and NADPH, respectively. Continuous supplies of
295 them are the prerequisites for oleaginous microorganisms to accumulate abundant
296 lipids. In endpoint strain, ACL enzyme activity was lower than the value of starting
297 strain, which might be the key reason for lower lipid production.

298 ***3.4 Oxygen sensitivity differences between starting and endpoint strain***

299 To examine the effect of high oxygen adaptation on the sensitivity of
300 *Schizochytrium* sp. to oxygen, the metabolic properties of starting strain and endpoint
301 strain at different rotate speed (140rpm, 170rpm and 200rpm) were compared in
302 500-mL shaking flasks. As shown in Table 3, with increasing rotate speed, CDW of

303 two different strains all increased and fermentation period all decreased. Moreover,
304 CDW of endpoint strain reached 62.23g/L at 170rpm, which was higher than the
305 value of starting strain even at 200rpm. S_{CDW} indicates the potential oxygen sensitivity
306 to cell dry weight of strain. Endpoint strain obtained 0.384 of $S_{CDW\ 140rpm \rightarrow 170rpm}$,
307 which was approximately 1.5 times than the value of starting strain. This indicated
308 that ALE could improve growth performance by enhanced oxygen sensitivity to CDW
309 of strain. However, the same result was not observed in $S_{CDW\ 170rpm \rightarrow 200rpm}$ of endpoint
310 strain (Table 3), which might attribute to the limit of initial glucose supply. As we
311 known, high oxygen supply is favorable for cell proliferation but cannot facilitate
312 DHA production. In this study, DHA percentages in TFAs of endpoint strain were all
313 higher than that of starting strain at different rotate speed. DHA percentage in TFAs at
314 170rpm reached 52.52% in endpoint strain, amazingly, which was essentially the
315 same with 52.70% of starting strain at 140rpm (Table 3). Interestingly, when rotate
316 speed increased from 140rpm to 170rpm, DHA percentage in TFAs of starting strain
317 reduced by 3.46% (from 52.7% to 49.24%), while the value of evolved strain only
318 reduced by 0.76%. Though the changes that DHA percentage in TFAs when rotate
319 speed increased from 170rpm to 200rpm were different from above, DHA percentage
320 in TFAs of endpoint strain at 200rpm increased 7.8% than starting strain at 200rpm
321 and only decreased 2.05% than starting strain at 170rpm. A serious decrease (62.6%)
322 in $S_{DHA\ 140rpm \rightarrow 170rpm}$ and a 5.3% decrease in $S_{DHA\ 170rpm \rightarrow 200rpm}$ were observed in the
323 endpoint strain (Table 3). These findings indicate that 40 cycles high oxygen
324 adaptation pressurize cells decreased the oxygen sensitivity of *Schizochytrium* sp. to

325 DHA, so endpoint strain also can obtain higher DHA content in a higher oxygen
326 supply. Overall, endpoint strain could combine with the advantages of high DHA
327 percentage in total fatty acids and high CDW under a relative higher oxygen supply
328 condition at the same time.

329 ***3.5 Fed-batch fermentation comparison between starting and endpoint strain***

330 To resolve the lower lipid accumulation impairment and further study the ability of
331 the evolved strain to produce DHA, endpoint strain to fed-batch was further applied in
332 5L fermenter. As shown in Fig.4 A, no difference in glucose consumption was
333 observed between endpoint strain and starting strain, which indicated that ALE did
334 not affect substrate consumption in fed-batch. During the initial stage(<84h), although
335 CDW apparently increased in both starting strain and endpoint strain, but CDW of
336 endpoint strain obviously surpassed that of the starting strain (Fig.4 B), leading to an
337 increase in CDW by 22.96% than the control culture at 84h. This indicated that high
338 oxygen adaptation was conducive to cell proliferation. During the later stage (>84h),
339 the biomass increased till 120h and then reached a plateau stage (Fig.4 B). Until the
340 end of fermentation, CDW of endpoint strain reached 84.34g/L, which was 19%
341 higher than that of starting strain (Table 4).

342 High oxygen adaptation exhibited significant effect on not only total lipids (TLs)
343 yield but also DHA yield. As shown in Fig.4 B, at 48h, TLs of endpoint strain was
344 16.67% lower than that of starting strain, which was accordance with the lipid
345 accumulation impairment before. But lipid yield was improved after first glucose
346 feeding at 48h. Moreover, after 60h, with the increase of CDW, endpoint strain

347 exhibited stronger ability on lipids accumulation than starting strain. In the stage of
348 60-96h, lipid accumulation showed a rapid increase of both strains. Final TLs of
349 endpoint strain was 54.32g/L, which was 24.07% higher than that of starting strain
350 (Table 4). These results indicated that fed-batch can make up the deficiency of lower
351 lipid yield at the early lipid accumulation stage of endpoint strain.

352 DHA percentage in TFAs of endpoint strain continued to rise and finally reached
353 48.61 %, which was 8.7 % higher than that of starting strain (Fig.4 C). This might be
354 explained by the fact that the oxygen sensitivity of endpoint strain has been weakened
355 compared to starting strain, which would trigger little oxygen participate in anaerobic
356 PKS pathway. Compared with the control group, the adaptive groups achieved much
357 higher DHA yield. The highest DHA yield of 26.40g/L was obtained at 120h in
358 endpoint strain, which was 34.83 % higher than that of the control (Fig.4 C). These
359 results clearly indicated that strain by ALE can promote to produce lipids and DHA
360 effectively.

361 As shown in Fig.4 D, two different strains had the same tendency that the squalene
362 content in TLs decreased as the culture time extension. This trend was also observed
363 in the cells of *Thraustochytrium* sp and *Saccharomyces cerevisiae* (Bhattacharjee et
364 al., 2001; Dessi et al., 2002). However, squalene content of endpoint strain was all
365 lower than that of starting strain. Finally squalene content reached 0.79g in 100g lipids,
366 which was 63.08% lower than starting strain. The results implied that continuous high
367 oxygen stimulation was beneficial to reduce squalene production.

368 4 Conclusions

369 In this study, continuous high oxygen stimulations and ALE were explored on the
370 growth and DHA production in *Schizochytrium* sp. Adapted strain produced higher
371 cell dry weight and lower lipid accumulation compared with staring strain. These
372 major performance changes were accompanied with enhanced isocitrate
373 dehydrogenase (ICDH) enzyme activity, reduced ATP: citrate lyase (ACL) enzyme
374 activity and weaken oxygen sensitivity to DHA. ALE combined fed-batch
375 substantially increased total lipid production and DHA yield. In addition, ALE was
376 also beneficial to reduce squalene production. Therefore, ALE may be an effective
377 strategy in further developing *Schizochytrium* sp. strain for industrial applications.

378

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498 **Figure Captions**

499 Fig 1 Glucose consumption, MSG consumption, dry cell weight (CDW) and DHA
500 content (%TFAs) of different evolved cycles during adaptive laboratory evolution.

501 Fig 2 Time course of glucose (A) and MSG (B) consumption of starting strain, ALE10,
502 ALE20, ALE30 and endpoint strain.

503 Fig 3 Changes of different key enzyme activities of starting and endpoint strain. ME
504 activity (A), G6PDH activity (B), ICDH activity (C) and ACL activity (D). Each
505 datum is the mean value of three identical samples.

506 Fig 4 Comparisons between starting strain and endpoint strain for glucose
507 consumption (A), CDW (B), TLs (C), DHA percentage in TFAs and DHA yield (D) in
508 5L bioreactor.

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511 Fig 1 Glucose consumption, MSG consumption, dry cell weight (CDW) and DHA

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Fig 2 Time course of glucose (A) and MSG (B) consumption of starting strain, ALE10, ALE20, ALE30 and endpoint strain.

Fig 3 Changes of different key enzyme activities of starting and endpoint strain. ME activity (A), G6PDH activity (B), ICDH activity (C) and ACL activity (D). Each datum is the mean value of three identical samples.

Fig 4 Comparisons between starting strain and endpoint strain for glucose consumption (A), CDW (B), TLs (C), DHA percentage in TFAs and DHA yield (D) in 5L bioreactor.

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555 Table1 Fatty acid composition (%TFAs) of different evolved strains

Parameters	Strains				
	Starting strain	ALE10 strain	ALE20 strain	ALE30 strain	Endpoint strain
C14:0	3.14 ± 0.03	5.14 ± 0.04	4.18 ± 0.02	4.31 ± 0.02	5.01 ± 0.03
C16:0	21.91 ± 0.07	24.53 ± 0.06	22.38 ± 0.07	23.17 ± 0.04	24.76 ± 0.05
DPA	15.34 ± 0.05	15.98 ± 0.03	16.76 ± 0.02	16.45 ± 0.04	15.78 ± 0.03
DHA	52.24 ± 1.2	49.49 ± 0.8	51.59 ± 1.0	50.50 ± 0.9	49.78 ± 1.1
Squalene	1.39 ± 0.01	0.8 ± 0.01	0.2 ± 0.01	0 ± 0.00	0 ± 0.00

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558 Table 2 Fermentation parameters and fatty acid profiles of different evolved strains in
559 shake flask culture.

Parameters	Strains				
	Starting strain	ALE10 strain	ALE20 strain	ALE30 strain	Endpoint strain
CDW(g/L)	46.94±1.6	51.75±1.8	52.24±1.9	58.14±2.1	62.15±1.9
TLs concentration (g/L)	20.12±0.9	19.89±0.8	18.19±0.9	18.01±0.7	16.97±0.6
DHA yield (g/L)	9.90±0.9	9.76±0.5	9.46±0.4	9.31±0.5	8.88±0.3
TLs in CDW (w/w, %)	38.60	31.53	34.82	35.23	30.98
Fermentation period (h)	48	48	45	42	36
CDW productivity (g/L/h)	0.98±0.01	1.08±0.01	1.16±0.02	1.38±0.01	1.73±0.02
The specific growth rate	0.080	0.082	0.088	0.097	0.115
C14:0	4.77±0.03	3.8±0.02	3.60±0.02	3.58±0.03	3.00±0.02
C16:0	18.11±0.3	17.8±0.4	13.87±0.3	15.32±0.4	14.11±0.3
DPA	18.82±0.2	21.8±0.4	21.33±0.3	20.48±0.3	22.09±0.4
DHA	49.21±0.8	49.1±0.9	52.01±1.1	51.71±0.9	52.52±0.7
Squalene	4.67±0.02	2.1±0.01	3.04±0.01	4.37±0.02	3.78±0.01
Degree of unsaturation	3.89	4.04	4.19	4.13	4.26
PUFAs/SFAs	2.94	3.23	4.17	3.85	4.35

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568 Table 3 Effect of different rotate speed on biomass, lipid production and DHA content

569 (%TFAs) in starting strain and endpoint strain.

	Starting strain			Endpoint strain		
	140rpm	170rpm	200rpm	140rpm	170rpm	200rpm
CDW (g/L)	42.02±0.9	46.72±1.1	51.13±0.1	50.72±0.8	62.23±1.2	65.16±1.3
TLs (g/L)	10.06±0.2	20.21±0.3	21.08±0.4	10.15±0.4	16.97±0.3	15.23±0.2
DHA (%TFAs)	52.70±0.3	49.24±0.3	44.72±0.5	53.82±0.1	52.52±0.2	48.23±0.4
Fermentation time (h)	72	48	36	61	36	61
S_{CDW} 140rpm→170rpm	0.157			0.384		
S_{CDW} 170rpm→200rpm	0.147			0.098		
S_{DHA} 140rpm→170rpm	-0.115			-0.043		
S_{DHA} 170rpm→200rpm	-0.151			-0.143		

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572 Table 4 Fermentation parameters at 120h in 5L bioreactor.

	Starting strain	Endpoint strain	Increase (%)
CDW (g/L)	70.81 ± 1.5	84.34 ± 2.1	19.10
TLs concentration (g/L)	43.78 ± 1.1	54.32 ± 1.2	24.07
TLs in CDW (w/w)	0.62	0.64	3.22
DHA content (%TFAs)	44.72 ± 0.8	48.61 ± 0.6	8.70
DHA yield (g/L)	19.58 ± 0.1	26.40 ± 0.1	34.83
DHA productively (mg/L/h)	163.17 ± 1.3	220.04 ± 2.3	34.85
Squalene content (g/100g lipids)	2.14 ± 0.23	0.79 ± 0.12	-63.08

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