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Research article

Selection of *Schizochytrium limacinum* mutants based on butanol tolerance

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ABSTRACT

Background: Mutation breeding is one of the most important routes to achieving high docosahexaenoic acid (DHA) productivity using *Schizochytrium*. However, few selection strategies have been reported that aim to generate a high DHA content in *Schizochytrium* lipids.
Results: First, culture temperature altered the butanol tolerance of *Schizochytrium limacinum* B4D1. Second, *S. limacinum* E8 was obtained by selecting mutants with high butanol tolerance. This mutant exhibited a 17.97% lower proportion of DHA than the parent strain *S. limacinum* B4D1. Third, a negative selection strategy was designed in which *S. limacinum* F6, a mutant with poor butanol tolerance, was obtained. The proportion of DHA in *S. limacinum* F6 was 11.22% higher than that of parent strain *S. limacinum* B4D1. Finally, the performances of *S. limacinum* B4D1, E8 and F6 were compared. These three strains had different fatty acid profiles, but there was no statistical difference in their biomasses and lipid yields.
Conclusion: It was feasible to identified the relative DHA content of *S. limacinum* mutants based on their butanol tolerance.

1. Introduction

Docosahexaenoic acid (DHA, C22:6 n-3) is a kind of polyunsaturated fatty acids (PUFAs), and it is beneficial to human health [1,2]. Increased intake of DHA has a positive effect on cardiovascular diseases, arthritis, hypertension and neuropsychiatric disorders [3]. In addition, DHA is essential for the visual and cognitive development of infants [4]. Marine fish oil is traditionally the primary source of DHA. However, fish oil has many drawbacks such as scarcity and poor taste [5]. At present, *Schizochytrium* is often considered as an alternative source for DHA-enriched oil [6].

Schizochytrium is a type of fast-growing oleaginous fungus and it has a simple lipid composition [7]. To achieve a high DHA content in *Schizochytrium* biomass, considerable efforts have been devoted to developing new fermentation strategies. These strategies mainly fall into two areas of focus: increasing lipid content in biomass and increasing DHA content in total fatty acids (TFAs). Regarding the former strategy, the supplementation of compounds such as ethanol, ammonium acetate and malic acid, has been shown to be effective in promoting the accumulation of lipids [8,9,10]. With respect to the latter strategy, the proportion of DHA in TFAs was increased when low temperature, oxygen limitation or the addition of pentanoic acid was applied during fermentation [11,12,13].

The improvement of strains by mutagenesis is also important in achieving high DHA productivity. Strategies have been designed to quickly select for mutants with a high lipid content in *Schizochytrium*. For instance, mutants with a high lipid content could be identified when indoacetic acid or Sudan black B was used during mutant selection [14, 15]. By comparison, it was difficult to identify mutants with a high DHA content in TFAs because there was no simple and localized protocol to determine the content of DHA and other PUFAs in cells [16].

The selection of mutants with a high DHA content was also impeded by limited knowledge of DHA biosynthesis in *Schizochytrium*. The fatty acid synthase (FAS) pathway is incomplete in *Schizochytrium*. It produces saturated fatty acids (SFAs), but it could not convert SFAs into PUFAs (Fig. 1) [17]. PUFAs, including DHA, are synthesized *de nove* via a polyketide synthase (PKS) pathway in *Schizochytrium*, and they are essential for the normal cell functions of *Schizochytrium* [7,17,18]. Therefore, mutants may have an enhanced PKS pathway and high DHA content if they are able to tolerate high levels of PKS inhibitor in medium. In other words, it is possible to select *Schizochytrium* mutants with a high DHA content by using selective plates supplemented with PKS inhibitor. However, the PKS pathway in *Schizochytrium* is unique and the biosynthesis of PUFAs could not be inhibited by iodoacetamide, an effective PKS inhibitor [19]. As a result, the selection of mutants with a high DHA content is usually laborious and time-consuming

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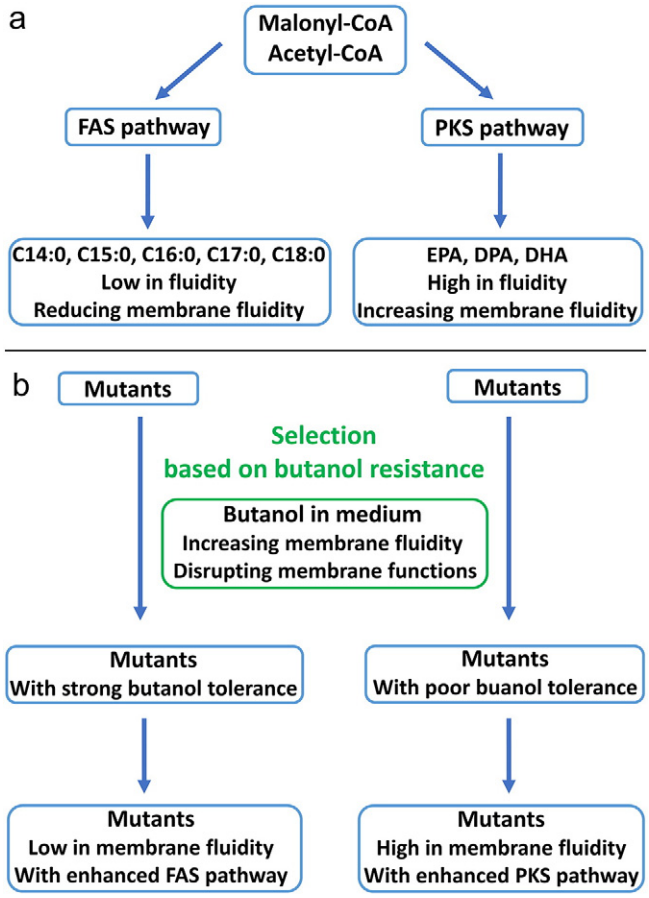

Fig. 1. The biosynthesis of fatty acids in *Schizochytrium* (a) and selection strategies designed based on butanol tolerance (b).

because lipid extraction, fatty acid methylation and gas chromatography (GC) analyses are all necessarily involved in this process [16].

It was reported that the fatty acid profiles of *Clostridium* strains were correlated to their butanol tolerance [20]. Therefore, butanol tolerance of *Schizochytrium* was studied in this work. Moreover, two selection strategies were designed based on butanol tolerance and the performances of selected mutants and the original strain were compared. The results of this study will provide new insights into strain improvement strategies for *Schizochytrium*.

2. Materials and methods

2.1. Microorganism

Schizochytrium limacinum B4D1 was stored in the China General Microbiological Culture Collection Center (CGMCC No. 8313).

2.2. Agar plates

Three types of agar plates were used in this study. Plates A (original agar plates) contained 30 g/L glucose, 15 g/L artificial sea salt, 15 g/L agar, 2 g/L yeast extract (OXOID Ltd), and 2 g/L soybean peptone. Plates B and C were the same recipe as plates A but contained 6 and 8 g/L butanol, respectively.

2.3. Positive selection

S. limacinum B4D1 cells grown to logarithmic phase were suspended in 50% sterile artificial seawater and transferred to plates C using the spread plate method. These plates were then exposed to UV light with the exposure time adjusted to give a survival rate of approximately 5%. After UV mutagenesis, plates were incubated in the dark at 25°C until colonies appeared. Then, large mutant colonies were selected (Fig. 2).

2.4. Negative selection

The mutagenesis for the negative selection had similar procedures as above, except *S. limacinum* B4D1 cells being plated on plates A before mutagenesis. Resulting colonies were replica plated onto new plates A and B. These plates were incubated at 25°C until colonies appeared. Mutants exhibiting no growth on plates B were selected for further analyses (Fig. 3).

2.5. Liquid medium and fermentation conditions

Liquid medium was used when parental strain and mutants were compared and was identical to that in a previously published study [19]. The culturing of cells was performed in 250 mL flasks with 50 mL of medium with the shaker set to 25°C and 180 rpm. The fermentation medium was inoculated with 10% (*v*/*v*) of seed that had been cultured for 48 h. Once the glucose was totally consumed, the fermentation was stopped to prevent lipid turnover [21].

2.6. Analytical methods

To determine the dry cell weight (DCW), 40 mL of cell suspension was centrifuged at 8000 rpm for 10 min and was washed twice with distilled water. The cell pellets were then freeze-dried at -50°C until a constant weight was reached.

The total lipids were extracted by the acid-heating method [21]. Approximately 0.3 g of lyophilized biomass was incubated in 50% HCl at 75°C for 120 min. The total lipids in the mixture were extracted with 10 mL of hexane overnight. Then, the organic phase was collected and hexane was removed under N_2, after which the total lipids were weighed.

The fatty acids in the total lipids were saponified with 3 mL of 0.5 M KOH-methanol solution at 60°C for 15 min and then were methylated with 6 mL of 14% (*w*/*w*) BF_3/methanol (ANPEL) at 60°C for 2 min. After the mixture was cooled to room temperature, 2 mL of hexane was added to extract the fatty acid methyl esters (FAMEs). Then, 1 mL

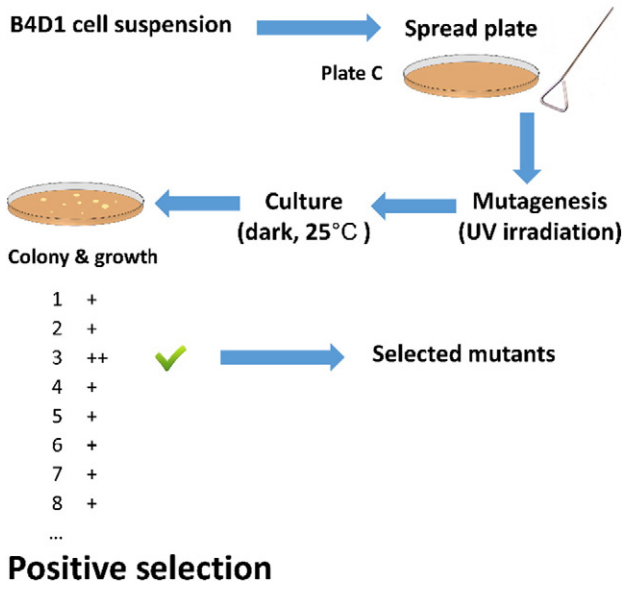

Fig. 2. Selection of butanol-tolerant mutants. ++ growth, + impaired growth.

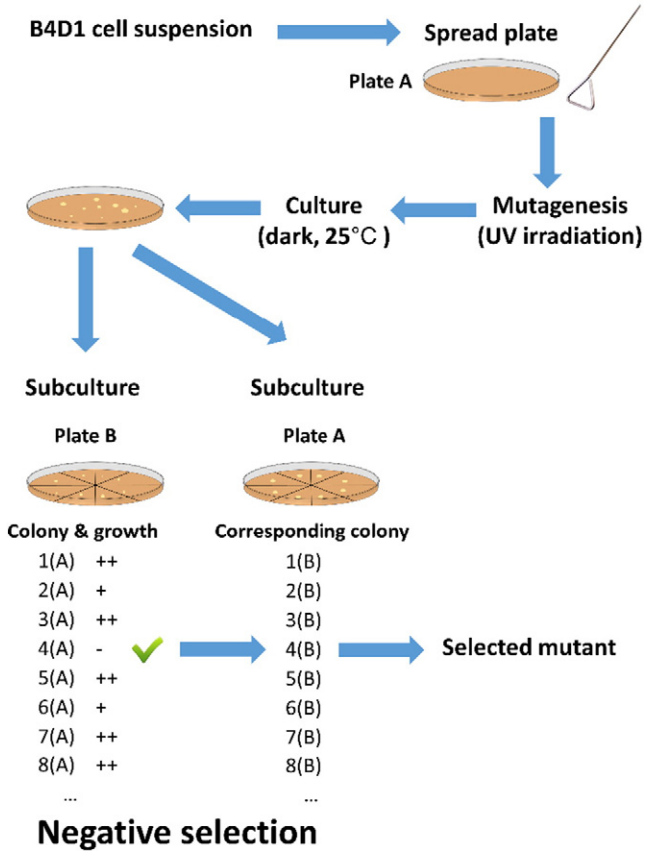

Fig. 3. Selection of mutants with poor butanol tolerance. ++ growth, + impaired growth, - no observed growth.

of saturated sodium chloride solution was added to promote the separation of the mixture into two layers. The upper layer was then collected and dried with anhydrous Na_2SO_4.

The FAMEs sample was analyzed using a GC (Shimadzu GC 2010) equipped with an SP-2560 capillary column (100 m × 0.25 mm id, 20 pm film thickness) and an FID detector. Nitrogen was used as the carrier gas. The GC oven was initially maintained at 150°C for 5 min, then increased to 180°C at 3°C/min, where it was maintained for 5 min, and then increased to 240°C at 8°C/min, where it was maintained for 16 min. In order to identify FAMEs, Supelco® 37 Component FAME Mix (Sigma) and methyl all-cis-4,7,10,13,16-docosapentaenoate (Sigma) were used as standards [22].

2.7. Statistical analysis

All experiments were replicated in triplicate, and data were presented as the mean values ± standard deviation of the means, with the exception of the data of maximal survival concentration. SPSS software (SPSS Inc., version 17.0) was used for statistical analyses. The biomass, lipid yield and DHA yield data were evaluated via one-way analysis of variance (ANOVA), followed by Turkey's test. In other experiments, significant differences were determined using Student's *t*-test. Differences at $P < 0.05$ were considered significant.

3. Results and discussion

3.1. Butanol tolerance of S. limacinum B4D1

Previous studies show that butanol can partition into membranes of *Clostridium*, increasing the membrane fluidity and disrupting membrane functions because butanol was very high in fluidity compared with membrane lipids [23]. One evidence was the correlation between culture temperature and butanol resistance of *Clostridium* [24], knowing that temperature can greatly change membrane fluidity [13]. Therefore, the butanol tolerance of *S. limacinum* B4D1 was also examined at different temperature.

S. limacinum B4D1 cells were not able to survive in liquid medium containing more than 8.0 g/L butanol at 25°C (Table 1). However, when the culture was incubated at 15°C, *S. limacinum* B4D1 was able to tolerate 8.5 g/L butanol. By contrast, *S. limacinum* B4D1 hardly grew in medium supplemented with more than 4 g/L butanol when the culture was incubated at 35°C.

Both high temperature and butanol can increase membrane fluidity [13]. This explains the reduction of the maximal survival concentration of butanol (from 8 to 4 g/L) when the culture temperature was raised from 25°C to 35°C. Conversely, membrane fluidity was reduced when the culture temperature was decreased to 15°C. As a result, *S. limacinum* B4D1 was able to tolerate more than 8.0 g/L of butanol in liquid medium.

3.2. Selection of butanol-tolerant mutants

The fatty acid composition of microorganisms plays an important role in maintaining membrane fluidity because SFAs in membrane reduce membrane fluidity whereas PUFAs in membrane increase membrane fluidity [20]. Our previous study shows that *S. limacinum* B4D1 synthesizes less PUFAs and more SFAs in response to the addition of butanol [25]. Thus, this section designed a positive selection strategy, and attempted to obtain *S. limacinum* mutants with lower DHA contents by selecting butanol-tolerant mutants (Fig. 1b).

After mutagenesis, 8 surviving mutants on plates C were selected and cultured in liquid media. Then, their DHA contents in TFAs were determined. As shown in Fig. 4, the DHA content in TFAs was 29.31% in *S. limacinum* B4D1, which was higher than that of all the selected mutants ($P < 0.05$). Among the 8 mutants, *S. limacinum* E2 had the highest DHA content (27.72%), and *S. limacinum* E8 had the lowest DHA content (24.06%). The proportion of DHA in other mutants varied from 24.87% to 26.95%. *S. limacinum* mutant E8 was stored for further analysis.

3.3. Selection of mutants with poor butanol tolerance

As demonstrated in the previous section that butanol-tolerant mutants tended to have low DHA contents, this section designed a negative selection strategy and attempted to obtain *S. limacinum* strains with high DHA contents by picking mutants having poor butanol tolerance.

8 mutants that were unable to survive on agar plates B (with 6 g/L butanol) were selected and cultured in liquid media (Fig. 3). Then, their DHA contents in TFAs were analyzed (Fig. 5). Among the 8 mutants, *S. limacinum* F6 had the highest DHA content in TFAs (36.60%), and it was stored for further analysis. Only one mutant, *S. limacinum* F42, had a similar DHA content as the parental strain *S. limacinum* B4D1 ($P > 0.05$). The proportion of DHA in other mutants varied from 30.02% to 32.26%. That is, most mutants had a higher DHA content than *S. limacinum* B4D1, demonstrating the feasibility of this negative selection strategy.

It was notable that the changes in the DHA content between the parent strain and selected mutants were relatively moderate in the

Table 1
Maximal butanol concentrations in which *S. limacinum* B4D1 could survive at different culture temperatures.

	Culture temperature		
	15 °C	25°C	35°C
Maximal butanol concentration	8.5 g/L	8.0 g/L	4.0 g/L

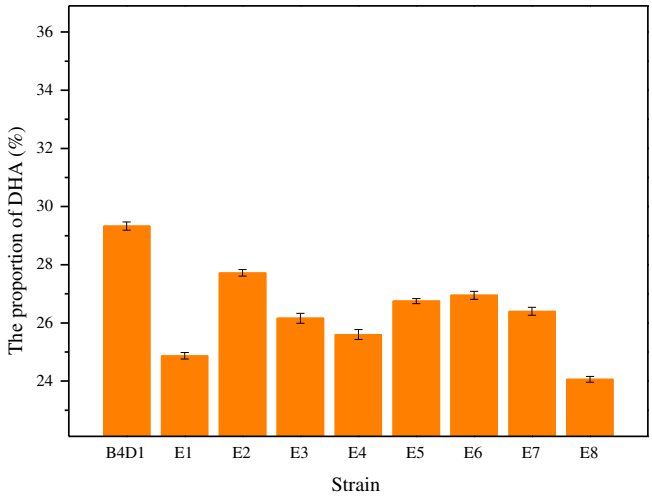

Fig. 4. DHA contents of the parent strain and mutants with strong butanol tolerance.

negative selection compared to the positive selection mutants. This shows that the positive selection was more effective than the negative selection.

To our knowledge, few fast methods have been reported that can select strains with a high PUFA content in lipids. To some extent, the selection strategies described in this work provide a simple protocol to detect the relative PUFA content in cell. This is not only beneficial for the commercial utilization of *Schizochytrium*, but also helpful for the improvement of other oleaginous microorganisms.

3.4. Performances of S. limacinum B4D1, E8 and F6

The performances of *S. limacinum* B4D1, E8 and F6 were compared after they were cultured in liquid medium. In addition to DHA, *S. limacinum* B4D1 could also accumulate two other PUFAs - docosapentenoic acid (DPA) and eicosapentaenoic acid (EPA). The fatty acid profile showed that the proportions of DHA, DPA and EPA were all decreased in mutant *S. limacinum* E8 and increased in mutant *S. limacinum* F6 compared with *S. limacinum* B4D1 ($P < 0.05$) (Table 2). Accordingly, the proportion of SFAs (C14:0, C15:0, C16:0, C17:0 and C18:0) was highest in *S. limacinum* E8 and lowest in mutant *S. limacinum* F6. For the DHA yield, it was 18.9% lower in *S. limacinum* E8 and 11.2% higher in *S. limacinum* F6 compared with *S. limacinum* B4D1 (3.86 g/L) (Fig. 6). However, there was no significant difference between the biomasses and lipid yields of *S. limacinum* B4D1, E8 and F6 ($P > 0.05$), which were approximately 25.20 g/L and 16.10 g/L, respectively. These results show the specificity of the two selection strategies, as *S. limacinum* B4D1, E8 and F6 were only different in the DHA yield.

Table 2
Fatty acid composition of *S. limacinum* B4D1 and F6.

	Fatty acid composition (%)		
	B4D1	E8	F6
C14:0	4.79 ± 0.22	5.29 ± 0.12	4.27 ± 0.07
C15:0	2.77 ± 0.03	2.99 ± 0.09	2.37 ± 0.03
C16:0	52.98 ± 0.44	58.38 ± 0.08	50.06 ± 0.10
C17:0	0.81 ± 0.04	0.87 ± 0.10	0.66 ± 0.03
C18:0	1.11 ± 0.05	1.24 ± 0.03	1.03 ± 0.00
EPA	0.28 ± 0.01	0.22 ± 0.01	0.31 ± 0.00
DPA	5.92 ± 0.12	4.85 ± 0.11	6.51 ± 0.12
DHA	29.33 ± 0.14	24.06 ± 0.10	32.62 ± 0.08
Others	2.00 ± 0.21	2.09 ± 0.04	2.16 ± 0.05
SFAs	62.47 ± 0.31	68.78 ± 0.17	58.39 ± 0.06
PUFAs	35.53 ± 0.25	29.13 ± 0.21	39.45 ± 0.05

What's more, these three strains are ideal experimental models for comparative omics (e.g., comparative transcriptomics), since such analyses on them may be helpful in revealing metabolic traits of *S. limacinum* regarding DHA accumulation. By contrast, previously reported mutants and their parental strains had large differences in biomass and lipid content [14,15].

Under typical culture conditions, *S. limacinum* B4D1 cells were full of lipid bodies (Fig. 7) and its lipid content (nearly 65%) was higher than most previously reported *Schizochytrium* strains [12,26,27]. In addition, this strain was able to achieve more than 100 g/L of DCW in fed-batch fermentation [9]. However, the proportion of DHA in TFAs was relatively low in *S. limacinum* B4D1. Therefore, low DHA content in *S. limacinum* B4D1 lipids was the bottle neck in achieving high DHA yield and the negative selection strategy designed in this study was the suitable solution to this problem.

Although a high DHA content is desired in DHA production, *S. limacinum* mutants with low DHA contents, such as *S. limacinum* E8, may also have practical use. Autotrophic microalgae are considered as good candidates for biofuels, but microalgae biofuel production is limited by low growth rate and lipid yield [28]. As an alternative, *Schizochytrium* may provide a promising way to overcome these bottlenecks [29]. However, most reported *Schizochytrium* strains are rich in DHA, which makes their lipids susceptible to oxidation during

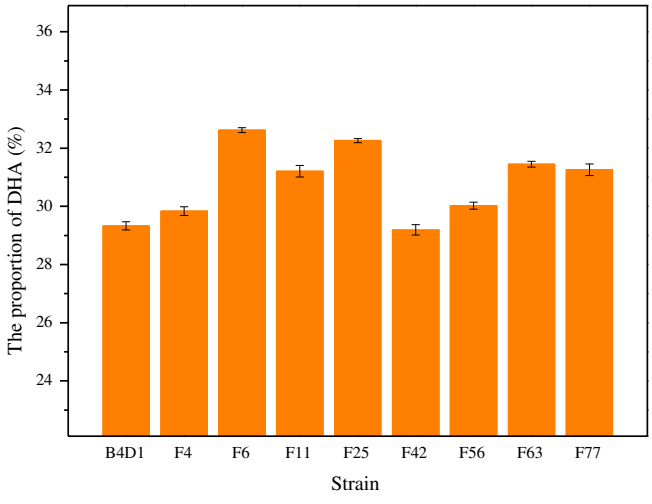

Fig. 5. DHA contents of the parent strain and mutants with poor butanol tolerance.

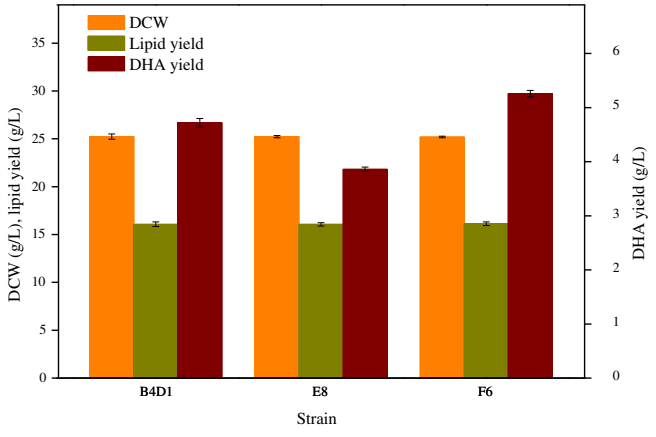

Fig. 6. The DCW, lipid and DHA yield of *S. limacinum* B4D1, E8 and F6.

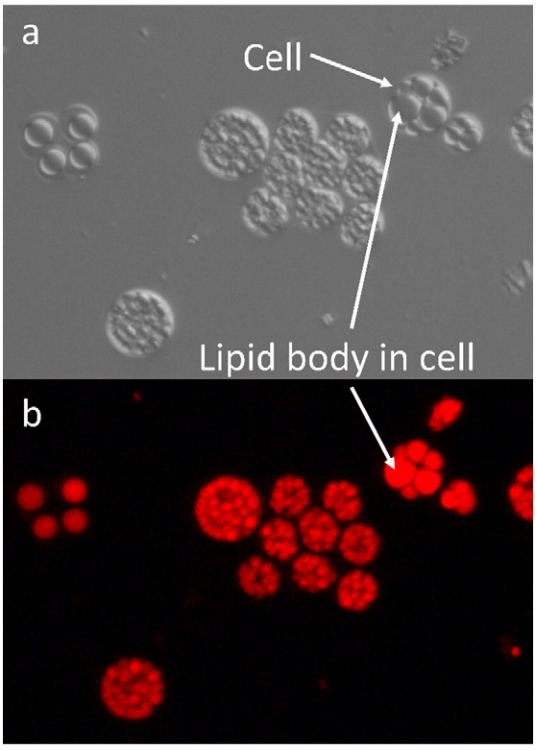

Fig. 7. Photomicrographs of *S. limacinum* B4D1 cells (a) and lipid bodies detected through Nile Red staining method (b).

storage and reduces their usefulness in biodiesel production [30,31,32]. Therefore, reducing the DHA content is beneficial for improving the quality of biodiesel. Due to an overemphasis on valuable DHA, previous studies mainly focused on improving, rather than reducing the DHA content. For this reason, mutant *S. limacinum* E8, as well as the corresponding selection strategy, provides insights into the production of biodiesel.

4. Conclusions

In conclusion, this study designed two selection strategies based on butanol tolerance. By selecting butanol-tolerant mutants, *S. limacinum* E8, a mutant with low DHA content, was obtained. By selecting mutants with poor butanol tolerance, *S. limacinum* F6 was obtained and it had a high DHA content. The comparison of *S. limacinum* B4D1, E8 and F6 showed that these three strains had significant differences in DHA yield, but no difference in biomass and lipid yield, demonstrating the specificity of the two selection strategies.

Conflict of interest

The authors declare that they have no competing interest.

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