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ORIGINAL PAPER

# Simple, effective protein extraction method and proteomics analysis from polyunsaturated fatty acids-producing micro-organisms

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**Abstract** Polyunsaturated fatty acids (PUFAs) are valuable ingredients in the food and pharmaceutical products due to their beneficial influence on human health. Most studies paid attention on the production of PUFAs from oleaginous micro-organisms but seldom on the comparative proteomics of cells. In the study, three methods (i.e., cold shock, acetone precipitation and ethanol precipitation) for lipid removal from crude protein extracts were applied in different PUFAs-producing micro-organisms. Among the selective strains, *Schizochytrium* was used as an oleaginous strain with high lipid of 60.3 (w/w %) in biomass. The *Mortierella alpina* and *Cunninghamella echinulata* were chosen as the low-lipid-content strains with 25.8 (w/w %) and 21.8 (w/w %) of lipid in biomass, respectively. The cold shock resulted as the most effective method for lipid removed, thus obtained higher protein amount for *Schizochytrium*. Moreover, from the comparative proteomics for the three PUFAs-producing strains, it showed more significant proteins of up or down-regulation were explored under cold shock treatment. Therefore, the essential proteins (i.e., polyunsaturated fatty acid synthase) and regulating proteins were observed. In conclusion, this study provides a valuable and practical approach for analysis of high PUFAs-producing strains at the proteomics level, and would further accelerate the understanding of the metabolic flux in oleaginous micro-organisms.

**Keywords** Polyunsaturated fatty acids · Oleaginous micro-organisms · Protein extraction · Cold shock · Comparative proteomics

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## Introduction

Polyunsaturated fatty acids (PUFAs) are valuable additives for food and pharmaceutical products due to their beneficial influence on human health [1]. Common examples include docosahexaenoic acid (DHA), arachidonic acid (ARA) and γ-linolenic acid (GLA) [2]. DHA presents beneficial physiological functions for humans in anti-aging and preventing chronic diseases [3]; ARA is important as a natural constituent of biological membranes and a precursor for numerous eicosanoids [4, 5]; GLA is the key precursor for the biosynthesis of several prostaglandins that additionally presents anti-inflammatory and anti-cancer effects [6, 7]. Therefore, the interest in PUFAs has attracted the attention for many researchers recently.

Fish oil as the conventional raw source of PUFAs is a limited resource due to high demand in PUFAs and also has variable composition and quality [1–4]. Therefore, PUFAs-producing micro-organisms are currently under investigation as they are promising and alternative sources. On the other hand, as the derived biofuels, PUFAs also led to increasing studies to explore the mechanism for governing the prominent ability of lipid accumulation by

PUFAs-producing micro-organisms [7–9]. Metabolic investigations have suggested that nitrogen limitation could enhance the amount of acetyl-CoA and NADPH, which were the necessary precursors for lipid accumulation [10]. The key enzymes involved in lipid accumulation and consumption in the oleaginous micro-organisms have been previously discussed [4, 7]. In addition, fatty acid (FA) composition shifts in lipid turnover have also been investigated in the various PUFAs-producing micro-organisms [7, 11, 12]. Furthermore, numerous researches have been conducted on increasing the lipid content in PUFAs-producing micro-organisms [2, 5, 6]. However, till now, only few studies have been reported to explore the mechanism of high lipid accumulation in PUFAs-producing strains at the proteomics level, which may be interrupted due to the lack of an effective approach to extract proteins from oleaginous strains.

Conventionally, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) combined with Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF-MS/MS) is considered as a method widely applied for protein analysis in comparative proteomics due to its higher efficiency and repeatability compared to traditional two-dimensional gel electrophoresis (2-DE) [13, 14]. For practical analysis, protein samples should have relatively high protein concentration and be free from other disturbing factors, such as salts, lipids, ionic detergents, nucleic acids and pigments, which would interfere with protein analysis in electrophoresis [15, 16]. Protein analysis in PUFAs-producing micro-organisms was highly challenged by high lipids content as it can form complexes with proteins or detergents, which consequently reduced the protein enrichment/separation efficacy [17]. When protein extracts from lipid producers were analyzed by electrophoresis, the resolution of proteins was poor because of the interference of lipid contaminants [18]. Although it had reported that lipid contaminants could be partly removed by organic solvents during protein extraction from lipid-accumulating oleaginous yeast or oilseed [18, 19], no literature available showed a simple and effective protocol for lipids removal and protein extraction from PUFAs-producing micro-organisms prior to electrophoresis.

The objective of this study is to develop an effective method for protein extraction and lipid removal from PUFAs-producing micro-organisms Three PUFAs-producing micro-organisms, *Schizochytrium* as a source of DHA [20], *Mortierella alpina* of producing ARA [4, 21], and *Cunninghamella echinulata* of accumulating GLA [6, 22], were employed. Three cell-disruption techniques combined with three methods for lipid removal were compared to identify the most reliable approach for extracting proteins as well as removing lipids from micro-organisms. Ultimately, SDS-PAGE combined with MALDI-TOF-MS/MS was applied to examine the differential proteins expressed at different growth phases.

## Materials and methods

### Micro-organisms and culture conditions

The *Schizochytrium* sp. LU310 strain was isolated from a mangrove forest and preserved in 20 % (v/v) glycerol at −80 °C. *M. alpina* and *C. echinulata* were obtained from Xiamen University, China, and stored at 8 °C on potato dextrose agar (PDA). All cultures were performed in 1000 mL flasks. Cultures of *Schizochytrium* sp. LU310 in broth medium were generated as previously described [23]. The fermentation broth contained 120 g/L glucose and 5 g/L corn steep powder (Sigma, USA), which were dissolved in artificial sea water. The artificial sea water contained (g/L): $Na_2SO_4$ 10; Monosodium glutamate 10; $MgSO_4$, 2; $(NH_4)_2SO_4$ 1; $KH_2PO_4$ 1; KCl 0.2; $CaCl_2{\cdot}2H_2O$ 0.1 and the trace elements (mg/L): $FeSO_4{\cdot}7H_2O$ 10; $MnCl_2{\cdot}4H_2O$ 3; $ZnSO_4{\cdot}7H_2O$ 3; $NiSO_4{\cdot}6H_2O$ 2; $CuSO_4{\cdot}5H_2O$ 2; $CoCl_2{\cdot}6H_2O$ 0.04; $Na_2MoO_4{\cdot}2H_2O$ 0.04. Vitamin solution was filter-sterilized (0.22 μm) and contained (mg/L): thiamine 9.5; cyanocobalamin 0.15. Batch cultures of *M. alpina* were grown at 28 °C, 150 rpm for 168 h with 200 mL of medium containing (g/L) glucose 50, yeast extract 8, monosodium glutamate 1, $KH_2PO_4$ 2, $MgSO_4{\cdot}7H_2O$ 0.1, $ZnSO_4{\cdot}7H_2O$ 0.1, and trace element solution (1 mL/L) and vitamin solution (2 mL/L) at pH 6. The trace element solution contained (mg/L) $Ca^1/_2$ Pantothenate 3.2, $MnCl_2{\cdot}4H_2O$ 0.8, $H_3BO_3$ 0.5, $FeCl_3{\cdot}6H_2O$ 0.2, $NiSO_4{\cdot}6H_2O$ 0.05, $CoCl_4{\cdot}6H_2O$ 0.005 and $CuSO_4{\cdot}5H_2O$ 0.002. The vitamin solution consisted of (mg/L) thiamine 9.5 and cyanocobalamin 0.15. A batch culture of *C. echinulata* was also grown at 28 °C, 150 rpm for 9 days with 200 mL of medium containing (g/L) glucose 90, tryptone 9, $KH_2PO_4$ 3, $NaNO_3$ 2, yeast extract 1.8 and $MgSO_4$ 0.5 at pH 6.5. Yeast extract was purchased from OXOID Ltd., UK. If not specifically mentioned, all of the other chemicals were provided by Sinopharm Chemical Reagent Co. Ltd., China.

### Dry cell weight, total lipid and fatty acid analysis

The dry cell weight (DCW) and DHA, total lipids (TLs) and fatty acid compositions were determined as in our previous study [23]. 3 mL fermentation broth was mixed with 4 mL HCl (12 N) and incubated in water bath at 65 °C for 45 min. TLs were extracted from the mixture by three times with 5 mL n-hexane, and then the collected extracts were purified in a rotary vacuum evaporator at 60 °C. Fatty acid methyl esters (FAMEs) were prepared by

the modified methods [23] as follows: 5 mL 0.5 M KOH–methanol was added to a tube containing TLs. The tubes were heated in a water bath at 65 °C for 10 min, after then 5 mL 30 % $BF_3$-ether was added. The tubes were then heated in a water bath at 65 °C for 30 min again, and then 5 mL hexane was added when the tubes cooled down to room temperature. Two internal standards: Arachidic acid methyl (4 g/L, Sigma, USA) and heptadecanoic acid methyl (4 g/L, Sigma, USA) were used for ARA and GLA, respectively. They were placed in the tubes and mixed through a vortex for 1 min, and then allowed to settle for separation of two phases after adding 1 mL saturated sodium chloride solution for preventing emulsification. The upper phase containing FAMEs was applied to a gas chromatograph (Agilent GC 7890, USA) equipped with a 100 m × 0.25 mm capillary column (SP$^{TM}$-2560, USA). The column was increased from 140 to 240 °C at 3 °C per min and then maintained at 240 °C for 30 min. The temperatures of the injector and detector were both set at 260 °C. Nitrogen was used as the carrier gas at 20 cm/s. Peaks were identified using the authentic standards of fatty acid methyl esters (Sigma, USA). Fatty acids were quantified from the peak areas relative to the peak of the internal standard. The 3, 5-dinitrosalicylic acid (DNS) method was used to evaluate the glucose content [24].

### Protein sample preparation and protein analysis

All experiments were carried out on ice to avoid the denaturation of proteins during the cell lysis and protein extraction. The cells were collected by centrifugation at 8000 × *g*, 4 °C for 5 min. The cell pellets were washed twice with cold water, and the supernatant was discarded. The pellets were re-suspended in deionized water, and then crushed by a homogenizer at 15 kpsi (Constant system, One Shot Model, UK) for one and two cycles or by liquid nitrogen grinding. After centrifugation at 8000 × *g* for 15 min at 4 °C, the aqueous phase was collected as the crude sample. Before electrophoresis analysis using SDS-PAGE, lipid contaminants in crude protein sample were removed by three different methods (i.e., cold shock, acetone precipitation and ethanol precipitation) according as demonstrated below. The crude sample was maintained overnight at room temperature (20 °C), and then centrifuged to collect the supernatant as the control. The cold shock consisted of keeping the crude sample overnight at −20 °C and then collecting the aqueous phase by centrifugation. The first step of the acetone or ethanol precipitation was to treat the crude sample with cold 100 % acetone or cold 80 % ethanol at ratio of 1:2 (v/v) for overnight at 4 °C. The pellets were collected by centrifugation at 8000 × *g* for 1 min and dissolved in water. The protein concentration of was determined using the Bradford Protein Assay (Bio-Rad) [25]. For standard sample preparation, SDS-PAGE and MALDI-TOF-MS/MS analysis for three independent batches of each sample were performed as previously described [13]. A Quantity One (Bio-Rad) analysis was used to detect the expressed proteins after SDS-PAGE analysis. The proteins that were subjected to MS analysis were selected through a two-way hierarchical clustering methodology using the software PermutMatrix as previously described [26].

## Results and discussion

### Biomass and lipid accumulation during batch fermentation

The time course of cell growth and lipid accumulation for the three PUFAs-producing micro-organisms is depicted in Fig. 1. The lipid accumulation was monitored in three different phases, i.e., log phase (*Schizochytrium* at 24 h, *M. alpina* at 48 h and *C. echinulata* at 3 days); exponential phase (*Schizochytrium* at 72 h, *M. alpina* at 120 h and *C. echinulata* at 5 days) and decrease phase (*Schizochytrium* at 120 h, *M. alpina* at 168 h and *C. echinulata* at 9 days). The lipid accumulation in *Schizochytrium* (Fig. 1a) increased with the increasing of cell mass, and the highest concentration of 30.6 g/L lipid content was achieved at 84 h. Moreover, the DHA production was closely in parallel with the lipid accumulation, and reached 13.6 g/L at 84 h. As shown in Fig. 1b, the DCW rapidly increased with time and reached the maximum of 24.5 g/L at 96 h for *M. alpina*. The lipid and ARA yield reached the maximum of 6.7 and 1.3 g/L, respectively, at 144 h. As show in Fig. 1c, the cell growth was colligated with the lipid production in *C. echinulata*. The biomass and lipid content were decreased after 6 days, while the GLA concentration was increased linearly and reached the maximum of 0.59 g/L after 9 days. The Table 1 presented the biomass and lipid production of the three strains during the log, exponential and late phases. The highest lipid contents of *Schizochytrium*, *M. alpina* and *C. echinulata* were 60.3, 27.7 and 21.8 %, respectively. The lipid content of our strains was lower than that of previously published in the literature [5, 6, 11, 22, 23], possibly due to the fact that the cells were cultured in shake flasks under the fed-batch mode, which was used for the laboratory-scale experiments.

The fatty acid profiles for DHA, ARA and GLA that were harvested at different stages are presented in Table 2. The highest DHA, ARA and GLA contents in total lipids (w/w) were 44 % at 84 h, 16.3 % at 168 h and 15 % at 9 days, respectively. It supposed that the decrease in DHA proportion and caused by the insufficient NADPH supply [3, 4, 20]. The ARA biosynthesis in *M. alpina* was

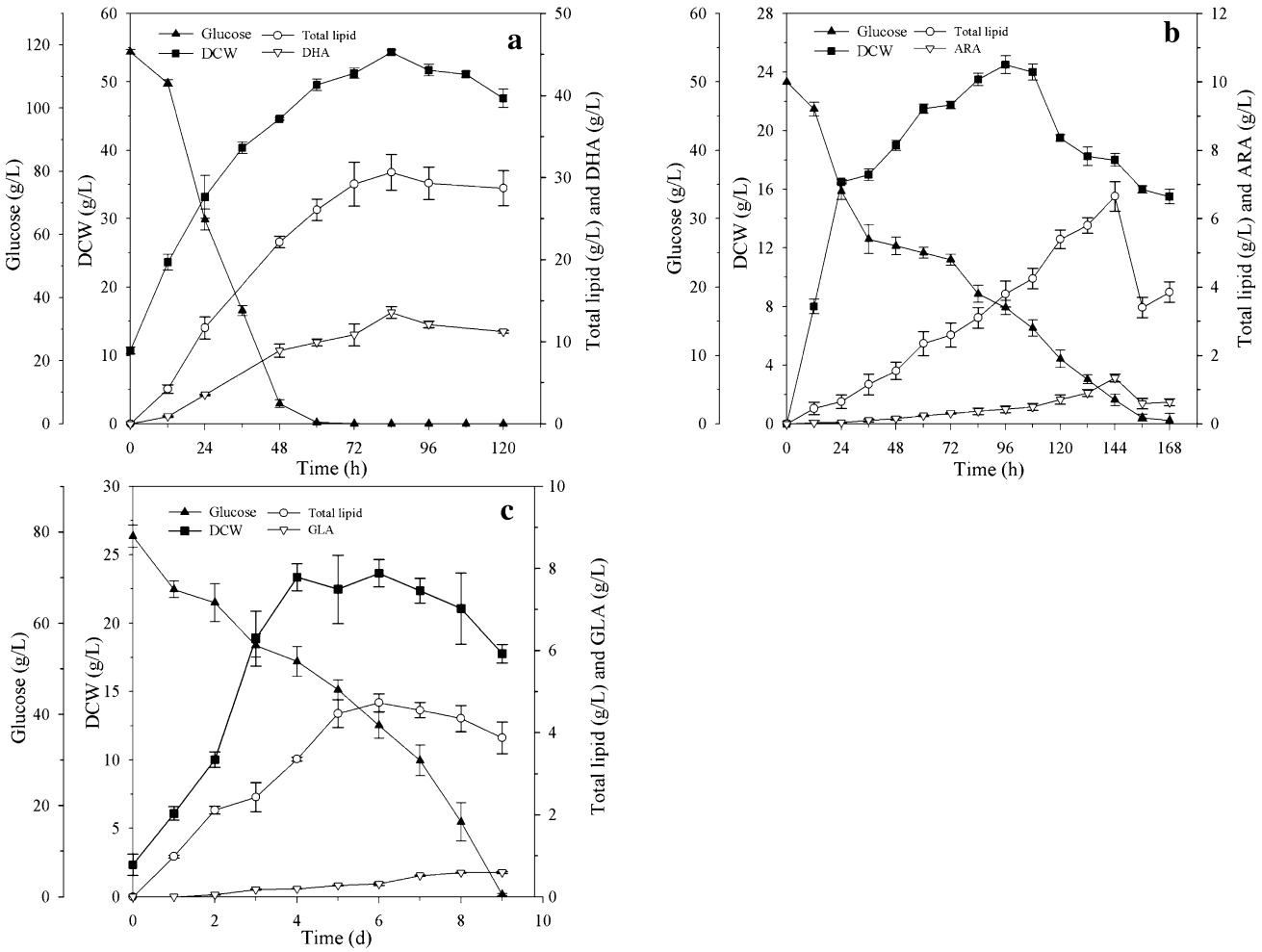


**Fig. 1** Profiles of cell growth, glucose consumption, and fatty acid production in three independent batch cultures at 28 °C with 1000 mL baffled flasks; **a** DHA production by *Schizochytrium* sp. LU310 at 200 rpm, **b** ARA production by *M. alpina* at 150 rpm, **c** GLA production by *C. echinulata* at 150 rpm. Glucose (*filled triangle*), DCW (*filled square*), Total lipid (*open circle*), and DHA, ARA or GLA (*open inverted triangle*). The data shown are the average of two replicates (±SD standard deviation)

stimulated in the medium in the absence of residual glucose [27]. On the other hand, the fatty acid analysis of *C. echinulata* revealed that the conditions favoring for GLA synthesis were inappropriate for lipid accumulation and vice versa. The biosynthesis of GLA in the mycelium seemed proportional to lipid-free biomass synthesis [7, 22]. Therefore, lipid accumulation in the exponential phase was chosen for the protein extraction as it was the most applicable and effective time for evaluating the lipid effect of PUFAs-producing micro-organisms.

## Effectiveness of cell-disruption method on protein preparation

Cell disruption is the crucial process for protein release and separation. Conventional cell disruption applied for fungal mycelial micro-organisms usually pulverized cells in a mortar and pestle with liquid nitrogen (sometimes with the help of glass beads), or crushed cells with a homogenizer [13, 19]. Both methods were applied in the present work. The visual images of sample preparation from *Schizochytrium* by homogenizer are presented as step 1 and step 2 in Fig. 2. The homogenate was divided into three layers after centrifugation, i.e., a lipid pad typically floated in the top layer, aqueous phase was in the middle and cell pellet was in the bottom (step 3, 4 in Fig. 2). The aqueous was then treated with the cold shock (step 6 in Fig. 2), acetone precipitation (step 7 in Fig. 2) and ethanol precipitation (step 8 in Fig. 2). After protein concentration estimated by Biorad assay (Fig. 3a–c), the effective methods contributing to proteins release were shown in the order of homogenizer for two cycles, homogenizer for one cycles and liquid nitrogen grinding, which was consistent with previous studies [28]. On the other hand, the crude extracts for *M. alpina* and *C.*

**Table 1** DCW, lipid content, and DHA, GLA, ARA percentage harvested at the three different stages of lipid accumulation in 1000 mL baffled flasks

| Time | DCW (g/L) | Lipid in biomass (%, w/w) | DHA, ARA or GLA in biomass (%, w/w) | DHA, ARA or GLA in total lipid (%, w/w) |
|---|---|---|---|---|
| *Schizochytrium sp. LU310 for DHA* | | | | |
| 24 h | 33.2 ± 3.2 | 35.2 ± 2.8 | 10.6 ± 1.7 | 30.2 ± 2.2 |
| 72 h | 51.2 ± 0.7 | 57.0 ± 1.7 | 21.2 ± 0.4 | 37.2 ± 0.9 |
| 120 h | 47.6 ± 1.3 | 60.3 ± 1.1 | 23.6 ± 0.8 | 39.1 ± 1.4 |
| *Mortierella alpine for ARA* | | | | |
| 48 h | 19.0 ± 0.4 | 8.2 ± 1.6 | 0.008 ± 0.003 | 10.1 ± 0.4 |
| 120 h | 19.5 ± 0.2 | 27.7 ± 1.3 | 0.036 ± 0.010 | 13.2 ± 0.5 |
| 168 h | 15.5 ± 0.5 | 25.8 ± 0.6 | 0.040 ± 0.008 | 16.3 ± 0.2 |
| *Cunninghamella echinulate for GLA* | | | | |
| 3 days | 18.9 ± 2.0 | 12.9 ± 1.4 | 0.009 ± 0.001 | 7.3 ± 0.7 |
| 5 days | 22.5 ± 2.5 | 19.9 ± 2.0 | 0.012 ± 0.008 | 6.2 ± 0.6 |
| 9 days | 17.8 ± 0.7 | 21.8 ± 0.2 | 0.033 ± 0.003 | 15.3 ± 0.3 |

**Table 2** Fatty acid profiles of lipids harvested at the three different stages of lipid accumulation of DHA by *Schizochytrium* sp. LU310, ARA by *M. alpine*, and GLA by *C. echinulata*

| Time | FA compositions (wt % of total lipid) | | | | | |
|---|---|---|---|---|---|---|
| | C14:0 | C16:0 | C18:0 | C22:5 | DHA | Others |
| *Schizochytrium sp. LU310 for DHA* | | | | | | |
| 24 h | 4.4 ± 0.3 | 35.4 ± 1.5 | 1.2 ± 0.2 | 3.8. ± 0.1 | 30.2 ± 2.2 | 25.0 ± 0.8 |
| 72 h | 3.3 ± 0.0 | 30.2 ± 0.8 | 1.6 ± 0.2 | 5.8. ± 0.0 | 37.2 ± 0.9 | 20.1 ± 1.2 |
| 120 h | 4.2 ± 0.4 | 35.6 ± 0.1 | 1.3 ± 0.1 | 4.4. ± 0.3 | 39.1 ± 1.4 | 17.3 ± 0.4 |
| | C16:0 | C18:0 | C18:1 | C18:2 | ARA | Others |
| *Mortierella alpine for ARA* | | | | | | |
| 48 h | 12.2 ± 0.8 | 25.5 ± 0.3 | 22.6 ± 0.4 | 7.8 ± 0.1 | 10.1 ± 0.4 | 21.8 ± 0.2 |
| 120 h | 24.8 ± 0.2 | 19.0 ± 0.9 | 15.1 ± 0.7 | 10.6 ± 0.6 | 13.2 ± 0.5 | 17.3 ± 0.1 |
| 168 h | 26.8 ± 0.7 | 12.6 ± 0.7 | 9.4 ± 0.8 | 6.8 ± 0.8 | 16.3 ± 0.2 | 28.0 ± 0.6 |
| | C16:0 | C18:0 | C18:1 | C18:2 | GLA | Others |
| *Cunninghamella echinulate for GLA* | | | | | | |
| 3 days | 24.7 ± 0.9 | 6.3 ± 0.6 | 48.1 ± 0.2 | 9.5 ± 1.0 | 7.3 ± 0.7 | 4.1 ± 0.3 |
| 5 days | 21.7 ± 0.7 | 6.0 ± 0.8 | 46.8 ± 0.3 | 10.9 ± 0.5 | 6.2 ± 0.6 | 8.4 ± 0.8 |
| 9 days | 14.0 ± 1.7 | 5.7 ± 1.0 | 40.4 ± 1.2 | 13.0 ± 1.2 | 15.3 ± 0.3 | 11.6 ± 1.0 |

*echinulata* were presented in a much clear pattern (Fig. 4b, c) caused by they are the low-lipid strains.

Liquid nitrogen grinding was poorly repeatable and uncontrollable, while homogenizing could generate stronger shear forces, thus producing a finer powder which is favorable for cell disruption and proteins relinquish. That's why the highest protein quantity was obtained with a homogenizer at 15 kpsi for two cycles (Fig. 3, lane 10). Therefore, homogenizer at 15 kpsi for two cycles was the optimal method for cell disruption. Notably, the crude protein from *Schizochytrium* failed in separation of electrophoresis by the homogenizer or liquid nitrogen treatment without cold shock. This was caused by the relatively high lipid content accumulated in *Schizochytrium*, which formed protein-lipid complexes, consequently inducing distortion and smearing in the gel pattern. More detailed interpretation of result and discussions is presented in the next section.

## Comparison of lipid removal methods from crude sample prior to proteomic analysis

When we look insight to the comparison between cold shock, acetone precipitation and ethanol precipitation, the sample from *Schizochytrium* processing by cold shock

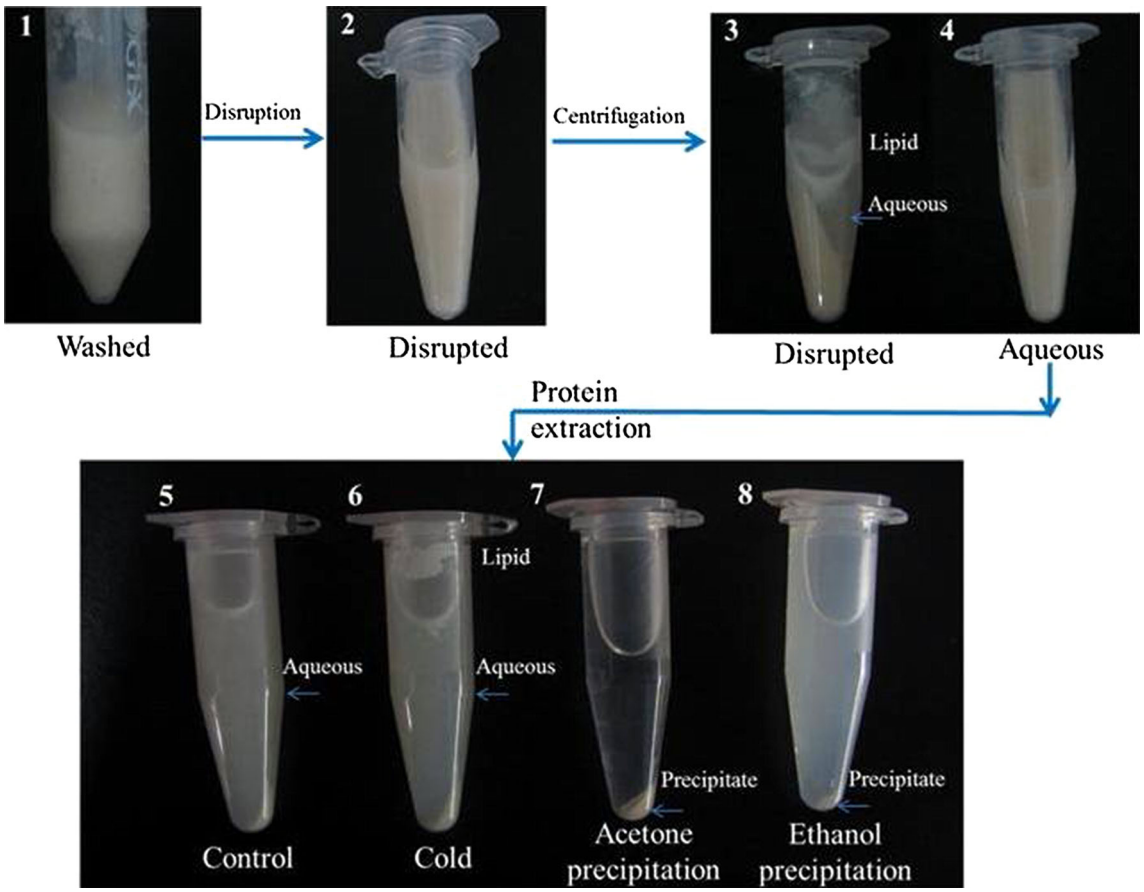


**Fig. 2** Visual image of sample preparation from *Schizochytrium* sp. LU310 by a homogenizer prior to proteomic analysis; *Step 1* washed cells; *Step 2* disrupted sample before centrifugation; *Step 3* disrupted sample after centrifugation (8000 × *g* for 15 min at 4 °C); *Step 4* aqueous phase obtained from *step 3*; *Step 5* sample stored overnight at room temperature after centrifugation; *Step 6* sample stored overnight at −20 °C after centrifugation; *Step 7* sample centrifuged after acetone precipitation; *Step 8* sample centrifuged after 80 % ethanol precipitation. The centrifugation in *Steps 5–8* was operated at 8000 × *g* for 15 min at 4 °C. The *arrows* (*Steps 3*, *5*, *6*, *7* and *8*) were the samples that were used for electrophoresis

would remove much more lipids after centrifugation (step 6 in Fig. 2). The same phenomenon occurred in *M. alpina* and *C. echinulate*. However, only few lipid pads floated on the top layer as they were low-lipid content strains (data not shown). The samples after acetone or alcohol precipitation presented only two layers, suggesting that there were still some lipids in the aqueous phase. Only the cold shock treatment resulted in proteins free from the lipids. The electrophoresis pattern of the proteins extracted with different conditions is presented in Fig. 4. It also proofed that samples with high resolution only occurred in cold shock treatment. For plant membrane proteins, best quality of electrophoresis was obtained when they were extracted with phenol and precipitated with ammonium acetate in methanol, causing a clear background staining, no streaking and entire resolving proteins [29]. In addition, the effectiveness of TCA precipitation was found not affected by the presence of detergents, lipids, salt, buffers, and beta-mercaptoethanol [30]. Moreover, acetone/chloroform methods were applied to lipid-rich tissues (such as oil seeds [18], oleaginous yeasts [19] ) and protein pellets were easily resolubilized in SDS buffer or 2-D rehydration buffer and were almost free of lipid contaminants. Although aforementioned methods could remove lipids to some extent, they used very expensive reagents or incurred some chemicals hence influencing the subsequent downstream processes and enzyme activity. After lipid removal by cold shock (our protocol), an improvement in protein separation was observed in the 1-D gel with a lower background and sharper bands (Fig. 4a, lanes 2, 6 and 10). The cold shock as a new protocol was very effective in removing the lipid contaminants from proteins. The lipid-protein complex formed during acetone precipitation was insoluble in the aqueous solution, consequently poorly adapted to SDS-PAGE. On the other hand, protein loss was observed during ethanol precipitation, probably due to the ethanol-wash step, the protein concentrations were much lower compared to those recovered with other treatments (Fig. 3a–c).

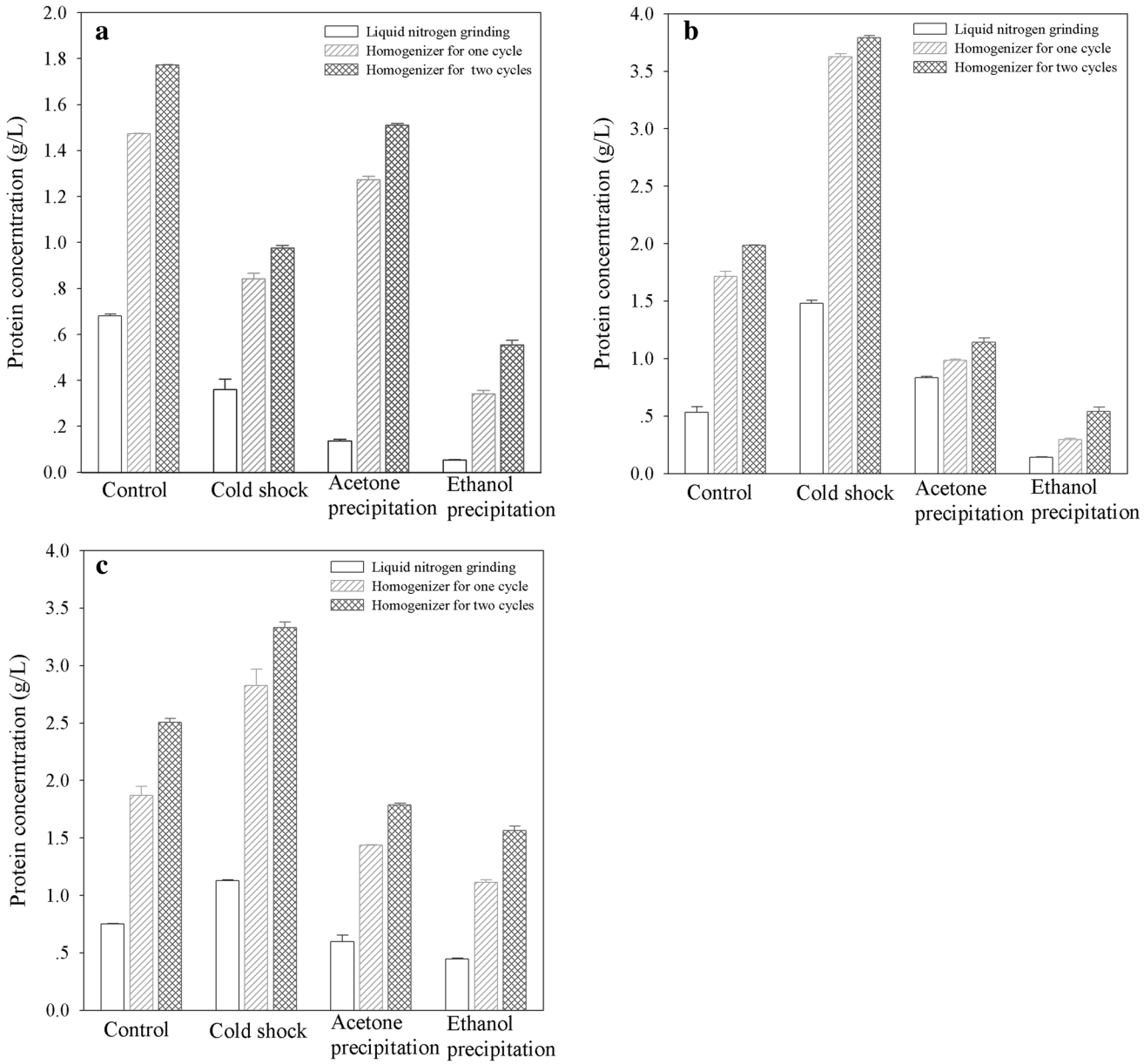


**Fig. 3** Protein concentration of *Schizochytrium* sp. LU310, *M. alpina and C. echinulata* from different separation approaches. **a** cells collected from *Schizochytrium* at 72 h; **b** cells collected from *M. alpina* at 120 h; **c** cells collected from *C. echinulata* at 5 days. Symbols for liquid nitrogen grinding (*open bar charts*), homogenizer at 15 kpsi for one cycle (*sparse bar charts*), homogenizer at 15 kpsi for two cycles (*dense bar charts*)

A lower background and clear bands were observed for all samples from two lower PUFAs-producing strains (*M. alpina* and *C. echinulata*) (Fig. 4b, c). After cell disruption, most of the lipids were effectively removed. The crude sample had lower lipid content, thus less influencing the electrophoresis. Moreover, the protein concentrations in cold shock were higher than those recovered by the other treatments (Fig. 3b, c). Thus, the application of cold shock permitted a clear visualization of more proteins than ethanol or acetone precipitation.

The effectiveness of the cold shock method was compared to other lipid removal techniques applied in protein extraction (Table 3). In the case of lipid-rich tissues, excess lipids could be removed with acetone and chloroform [18, 19]. Thiourea/urea combined with TCA was the efficient and reliable method for 2D separation of soybean seed proteins and subsequent identification by mass spectrometry [30]. Alternatively, Joo et al. compared the use of CHAPS and subsequent centrifugation to sample boiling in SDS and dialysis [31]. Moreover, a new

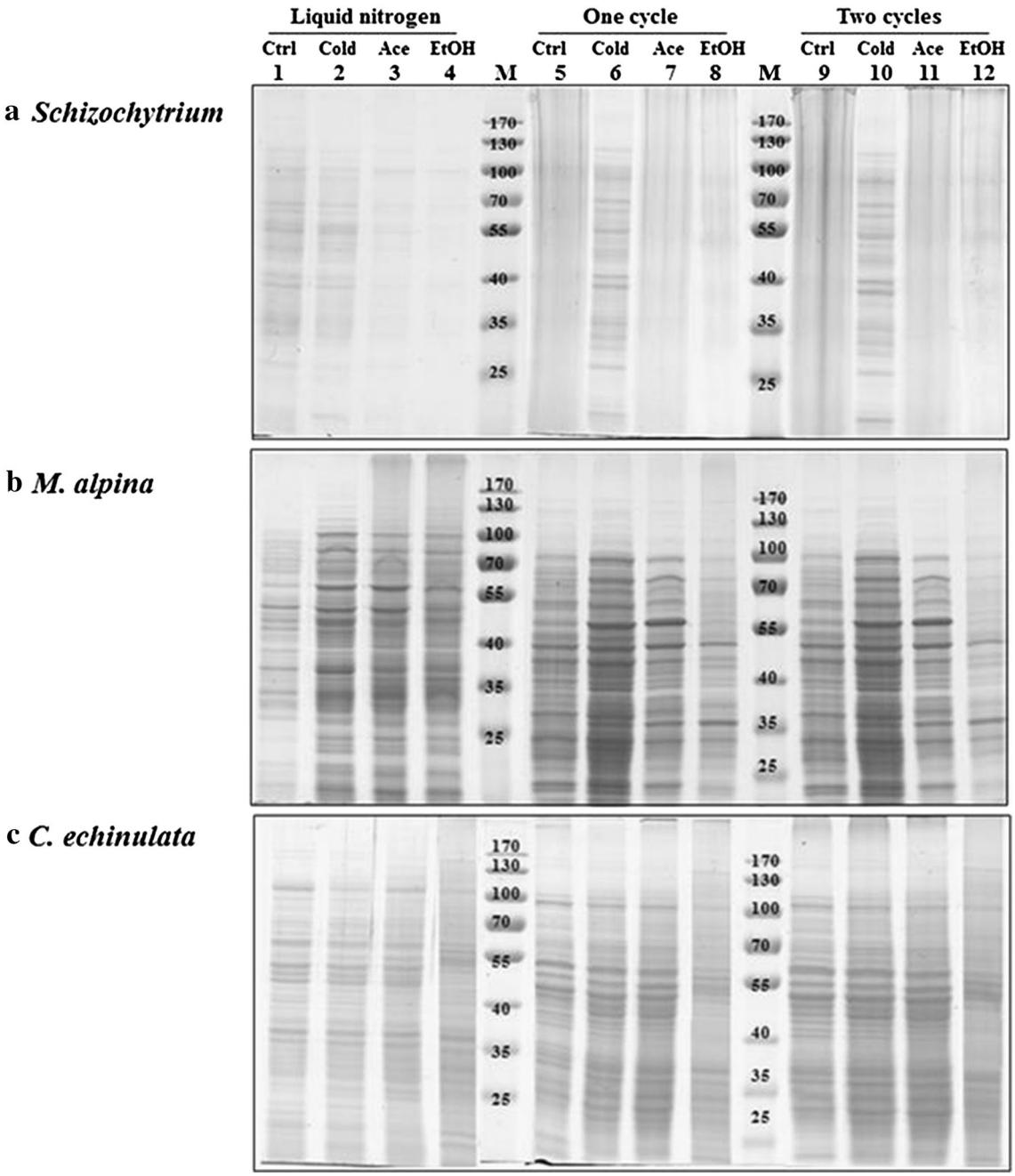


**Fig. 4** SDS-PAGE profiles of intracellular proteins from different designs in Fig. 2 and followed by Coomassie blue R-250 staining. *Lanes 1–4* liquid nitrogen grinding; *lanes 5–8* homogenizer at 15 kpsi for one cycle; *lanes 9–12* homogenizer at 15 kpsi for two cycles. Lane *M* protein markers in kDa is 5 L of Fermentas prestained protein ladder$^{TM}$. The proteins were extracted and subjected to 10 % (w/v) SDS-PAGE. **a** Cells collected from *Schizochytrium* at 72 h; **b** cells collected from *M. alpina* at 120 h; **c** cells collected from *C. echinulata* at 5 days

method was introduced for delipidation of human serum lipoproteins involving the use of a reversed-phase C18 solid-phase extraction cartridge [32]. Unlike those protein extractions, cold shock approach does not use any expensive reagent or chemicals which may influence the subsequent downstream processes and enzyme activity. Therefore, cold shock is suitable for microorganism with high oil content that is easy to operate, economic and more environmental friendly. The optimal approach (i.e. cold shock after homogenizer two cycles) provides the production of higher and more reproducible protein yield than others. Moreover, it allows an easiest dilapidation of the lipoproteins for subsequent mass spectrometric analysis.

**Table 3** Removal of lipid contaminants by different methods from lipid-protein extract prior to electrophoresis

| Material | Protocol | Note | References |
|---|---|---|---|
| Castor seeds | Acetone and chloroform | Used in lipid-rich tissues and a single micro tube | [18] |
| Oleaginous yeasts | Acetone and chloroform | Used in lipid-cumulating oleaginous yeast | [19] |
| Soybean seeds | Thiourea/urea and TCA methods | Used in soybean cotyledons, cultured root tissues, tobacco flowers, and tobacco leaves | [30] |
| Body fluids | A combined centrifugal filter device and a sample treating buffer including CHAPS | Used in body fluids (plasma, amniotic fluid, urine, and tear) | [31] |
| Human serum | A reversed-phase C18 solid-phase extraction cartridge | Used for the delipidation of human serum lipoproteins | [32] |
| Oleaginous strain | Cold shock | Used in the micro-organisms of high lipid content | This study |

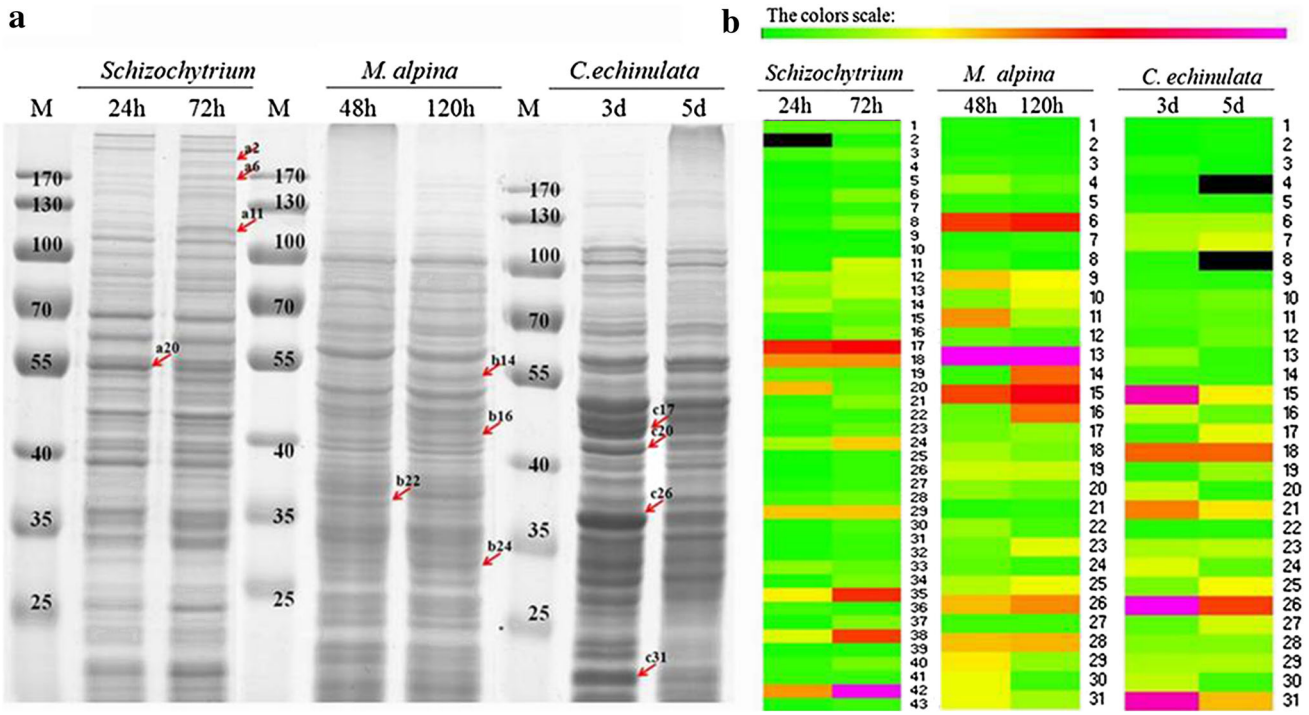


**Fig. 5** **a** SDS-PAGE analysis of the whole-cell proteins of *Schizochytrium* sp. LU310, *M. alpina* and *C. echinulata* at different times. The *arrows* indicate the different proteins between time-course comparative proteomics experiments that were sent for analysis by MALDI-TOF-MS/MS. **b** the clustering analysis was performed with PermutMatrix software

## Changes in protein profiles among different lipid accumulation stages

To sustain the practicability of the presented separation method, proteins expression patterns were further assessed at the log and exponential lipid accumulation phases of the three strains. Figure 5a shows the SDS-PAGE results of the total intracellular proteins, and Fig. 5b represents the cluster analysis. It can be seen from Fig. 5a, some differential proteins were expressed during the log and exponential lipid accumulation phases. Thus, selected proteins as confirmed by the cluster analysis were further analyzed by MALDI-TOF-MS/MS and the results are shown in Table 4.

The identified proteins in *Schizochytrium* strain had three different fatty acid synthases (a2, a6 and a20) and one heat shock protein 90 (a11). Among these proteins, a2 (type I fatty acid synthase) and a6 (polyunsaturated fatty acid synthase subunit C) were up-regulated at 72 h, especially for the protein score of the type I fatty acid synthase, which was quite high and reliable. The type I fatty acid synthase was a type of fatty acid synthase complex involved in the fatty acid biosynthetic process. The polyunsaturated fatty acid synthase subunit had the nitronate monooxygenase activity and the transferase activity, which were prosperous for lipid synthesis [33]. At 48 h of the log phase of lipid and DHA accumulation, more fatty

**Table 4** Mascot protein identification of differential protein produced by *Schizochytrium* sp. LU310, *M. alpina and C. echinulata* at different time

| Sample code | Protein name | Strain species | Protein score | Accession no. | Mw (Da) pI |
|---|---|---|---|---|---|
| a2 | Type I fatty acid synthase | *Schizochytrium* sp. ATCC 20888 | 117 | gil116489746 | 446856.2 5.34 |
| a6 | Polyunsaturated fatty acid synthase subunit C | *Schizochytrium* sp. ATCC 20888 | 21 | gil158518693 | 166475.7 6.32 |
| a11 | Heat shock protein 90 | *Thraustochytrium aureum* | 77 | gil223029705 | 42056.2 4.96 |
| a20 | Polyunsaturated fatty acid synthase subunit A | *Schizochytrium* sp. ATCC 20888 | 14 | gil158518689 | 308907.2 4.9299 |
| b14 | Delta-5 fatty acid desaturase | *Umbelopsis isabellina* | 18 | gil281314595 | 5855.2 9.41 |
| b16 | Orotidine-5′-phosphate decarboxylase | *Mortierella alpina* | 13 | gil50355629 | 29338 6.24 |
| b22 | 6-pyruvoyltetrahydropterin synthase | *Mortierella alpina* | 15 | gil344049920 | 16633.4 6.49 |
| b24 | Diacylglycerol acyltransferase | *Neurospora crassa* | 18 | gil38567182 | 67323.8 8.91 |
| c17 | NADPH-dependent cytochrome P450 oxidoreductase, partial | *Cunninghamella echinulata* | 13 | gil9622870 | 71856.7 5.09 |
| c20 | 6-phosphogluconate dehydrogenase | *Cunninghamella elegans* | 9 | gil3152297 | 53411.1 5.84 |
| c26 | Malic enzyme | *Cunninghamella echinulata* | 13 | gil304366186 | 61324.5 6.35 |
| c31 | Delta-6-desaturase | *Cunninghamella binariae* | 13 | gil74422653 | 52617.2 6.55 |

acids were synthesized and more fatty acid synthases (a2 and a6) were expressed (Fig. 5a). Hauvermalea et al. [34] reported that DHA accumulation in *Schizochytrium* sp. was synthesized by a multi-subunit PUFA synthase and the type I fatty acid synthase. The heat shock protein 90, which was up-regulated in the log and exponential phase of lipid accumulation, would be explained by the fact that cells were grown in a high-glucose and low-nitrogen medium. The environment was considered restrictive, thus triggering an up-regulation of stress-response-related proteins [19]. Some proteins related to lipid synthesis were also identified in *M. alpina* and *C. echinulata* (Table 4), whereas the protein scores were quite low. Due to incomplete genome sequences for *Schizochytrium* and *C. echinulata* and the restrictive condition of the protein information for *M. alpina*, progress in proteomics is limited, and resulting in the low protein score.

## Conclusions

The study successfully established a simple and efficient protocol for protein separation aimed at removing lipids from PUFAs-producing micro-organisms prior to electrophoresis. It also provided an overall reflection at the proteomics level, hence contributing to a better understanding of excessive lipid accumulation in oleaginous micro-organisms. By applying cold shock approach to protein separation, it may accelerate the understanding of metabolic flux for higher lipid accumulation in oil-producing micro-organisms.

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**Compliance with ethical standards**

**Conflict of interests** The authors declare that they have no conflict of interests.

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