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Improvement of culture conditions for cell biomass and fatty acid production by marine thraustochytrid F24-2

Violeta Ugalde[1,2] · Roberto E. Armenta[1,2] · Azadeh Kermanshahi-pour[1] · Zhiyong Sun[2] · Kevin T. Berryman[2] · Marianne S. Brooks[1]

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Abstract Identification of fermentation parameters affecting biomass and total fatty acid (TFA) production by thraustochytrid F24-2 was conducted using a Plackett-Burman experimental design. Factors influencing biomass accumulation were initial pH, sea salts, and glucose concentration. Additionally, temperature, initial pH, soy peptone, glucose, and sea salt concentration affected TFA production. Docosapentaenoic acid (n-6 DPA), palmitic acid (C16:0), and docosahexaenoic acid (DHA) were the predominant fatty acids produced. The best biomass (10.71 ± 0.04 g L^{-1}), TFA (2.11 ± 0.07 g L^{-1}), DHA (0.92 ± 0.04 g L^{-1}), and C16:0 (0.65 ± 0.02 g L^{-1}) concentrations were obtained at 25 °C with a medium adjusted to pH 7 and containing (per liter): 20 g glucose, 20 g soy peptone, 18 g sea salts, 0.2 g ammonium sulfate, 3 μg vitamin B_{12}, 3 μg biotin, and 0.6 mg thiamine hydrochloride. Under these conditions, DHA production increased 18.5% in comparison to a complex medium previously used to grow thraustochytrid *Thraustochytrium aureum* ATCC 34304.

Keywords Thraustochytrid · Oily biomass · Culture condition screening · Plackett-Burman · DHA · Palmitic acid

✉ Marianne S. Brooks
Su-Ling.Brooks@dal.ca

[1] Department of Process Engineering and Applied Science, Dalhousie University, Halifax, NS B3H 4R2, Canada

[2] Mara Renewables Corporation, 101A Research Drive, Dartmouth, NS B2Y 4T6, Canada

Introduction

Thraustochytrids are a group of osmoheterotrophic fungoid protists (Raghukumar et al. 2000) that play an important role in the detrital food web of diverse marine environments comprising of mangrove leaves, algae, oceanic waters, and sediments. These protists have industrial importance due to their ability to produce omega-3 (ω-3) long chain polyunsaturated fatty acids (LC-PUFA) that are associated with health benefits (Armenta and Valentine 2013; Singh et al. 2014), such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). DHA is a major structural lipid that can be found as a constituent of phospholipids or triacylglycerols, or as a free fatty acid in animals (Marchan et al. 2017). In addition to DHA, the oil from several thraustochytrids, such as *Aurantiochytrium* sp., has been shown to contain high levels of palmitic acid, which can be used for biodiesel production (Kim et al. 2013).

Numerous organisms including bacteria, yeast, filamentous fungi, and microalgae have been reported to produce different types of fatty acids (Gupta et al. 2012). In general, fungal strains such as *Mortierella alpina* typically produce low quantities of lipids (e.g., total lipid content of 26% of biomass) and require long cultivation periods of more than 2 weeks (Gupta et al. 2012), making them unsuitable for commercial production. And, although 29 fungal strains representing different *Mortierella* species have been shown to be capable of synthesizing EPA, the final concentrations of this important fatty acid were low amongst the strains tested (0.03–2.8 mg EPA/g of dry biomass) (Jacobs et al. 2009). Despite the reports of several bacterial strains being capable of producing DHA and EPA, bacteria are also considered unsuitable for the production of PUFAs, due to their low lipid accumulation (2–5%). In addition, the presence and characteristics of some undesirable lipids make the bacteria less attractive as PUFA producers,

and there have been no reports of the use of bacterial lipids for animal consumption (Gupta et al. 2012). Amongst the heterotrophic marine microalgae, thraustochytrids and the dinoflagellate *Crypthecodinium cohnii* have been identified as prolific producers of DHA (Gupta et al. 2012; Mendes et al. 2009). These microorganisms, particularly the thraustochytrids, may provide one of the best alternatives to fish oil for omega-3 fatty acid production (Miller et al. 2007), as they have been shown to produce twice the amount obtained from bacterial and fungal cultures per dry weight of biomass (Gupta et al. 2012).

Currently, the major global source of omega-3 LC-PUFA, and particularly DHA, is fish oil sourced from wild fisheries. However, there are growing concerns regarding the sustainable supply of fish oil as the growing worldwide population and the expanding aquaculture industry put pressure on the health of ocean fish stocks (Miller et al. 2007; Raghukumar 2008; Lee Chang et al. 2012). These factors, in addition to issues associated with odor, possible heavy metal contamination, and the presence of unfavorable PUFAs in fish oil, have led to the exploration of other sources for DHA production (Raghukumar 2008). Thraustochytrids are an alternate renewable source of omega-3 LC-PUFA-rich oil for industries such as the feed, food, and nutraceutical industries (Lee Chang et al. 2012). As they are heterotrophic microorganisms, thraustochytrids can be cultivated to achieve high biomass concentrations within fermenters, which is advantageous for commercial production (Raghukumar 2008). However, bioprocess engineering strategies should be employed in order to screen and optimize the nutritional and environmental conditions for growing thraustochytrids to achieve higher productivity and minimize process costs (Song et al. 2007; Gupta et al. 2012).

One of the statistical designs that can be used for screening independent variables is Plackett-Burman design. The main advantage of the Plackett-Burman design is that relatively few experiments can be used to identify the important factors in comparison to the conventional one-factor-at-time technique. In the case of the fermentation process factors used in a Plackett-Burman design, either nutritional components or environmental conditions can be tested (Song et al. 2007; Lu et al. 2011; Manikan et al. 2014). Recent studies have demonstrated that thraustochytrids can be induced to produce higher levels of biomass (Arafiles et al. 2011) and overproduce specific fatty acids by manipulating the fermentation culture medium (Jakobsen et al. 2008; Rosa et al. 2010). Although some papers have reported the screening and optimization of culture conditions affecting biomass and fatty acid production by different thraustochytrids (Song et al. 2007; Rosa et al. 2010; Arafiles et al. 2011; Manikan et al. 2014), these conditions cannot be extrapolated to other thraustochytrid strains. Therefore, the objective of this work was to evaluate the potential of thraustochytrid strain F24-2 to produce oily biomass rich in value-added fatty acids. This was done using a Plackett-Burman experimental design on the medium composition and culture conditions, to identify factors with a significant influence on biomass and total fatty acids (TFA) and improve the overall product levels.

Materials and methods

Microorganism and chemicals

Thraustochytrid strain F24-2 was obtained as a gift culture from Dr. A. J. Windust of the National Research Council Canada (NRC) and was isolated from *Spartina alterniflora* (saltmarsh cordgrass) by pollen baiting. Cordgrass samples were collected from salt marsh habitats along the eastern Atlantic shore and the Bay of Fundy, Nova Scotia, Canada (Cui et al. 2012). The molecular identification of this strain indicates that it belongs to the family Thraustochytridae. Prior analyses of sequence alignments and phylogenetic information revealed that it is most closely associated with *Thraustochytrium* sp. (ATCC26185). Morphologically, F24-2 colonies appear pale orange in color and butyrous after 4 days of growth on basal medium plates incubated at 25 °C.

Instant Ocean® sea salts were from Aquarium Systems (USA); yeast extract and soy peptone were from TekniScience Inc. (Canada). The rest of the chemicals were analytical grade or higher purity and were purchased from Fisher Scientific (Waltham, USA) and from Sigma-Aldrich (USA).

Activation and maintenance of thraustochytrid F24-2

Briefly, sample vials of pure F24-2 cultures were thawed in a water bath at 35 °C, until all ice crystals were melted (approximately 2–3 min). The entire contents of the vials were transferred under aseptic conditions to Erlenmeyer flasks containing 15 mL of sterile basal medium composed of the following components: 20 g L^{-1} glucose, 9 g L^{-1} sea salt, 20 g L^{-1} soy peptone, and 5 g L^{-1} yeast extract. Flasks were incubated for 24 h in the dark at 28 °C without shaking on an incubator Model 1545 (Sheldon Manufacturing Inc., USA); after which, the flasks were taken out of the dark and incubation continued under shaking (200 rpm) and at 25 °C on a New Brunswick Scientific Innova 4430 orbital shaker (USA) until glucose and nitrogen were depleted. Cultures were monitored by microscopic examination following initiation and any cultures showing contamination were discarded. Once growth was stabilized, cultures were maintained on agar slants, where approximately half of the surface area was overlaid with sterile artificial sea water (ASW, 35 g L^{-1} sea salts) containing 1% (*v*/*v*) penicillin-streptomycin (PS). Agar slants were prepared with medium components (10 g L^{-1} glucose, 5 g L^{-1} soy

peptone, 5 g L^{-1} yeast extract, and 10 g L^{-1} agar) dissolved in ASW and then sterilized at 121 °C for 20 min; after which, 1% (*v*/*v*) PS solution was added before solidification. Slants were subcultured monthly by aseptically transferring 1 mL of the ASW containing pure F24-2 culture to a fresh agar slant and then adding the overlay solution of 1% (*v*/*v*) PS in sterile ASW.

Seed culture preparation

Seed culture for inoculation into the fermentation test media was made from pure F24-2 cultures kept on ASW agar slants containing 1% (*v*/*v*) PS overlaid with sterile ASW containing 1% (*v*/*v*) PS. Then, 1 mL aliquots of the axenic F24-2 culture were used to inoculate 150-mL baffled Erlenmeyer flasks with 30 mL of sterile basal medium, where the composition of the basal medium consisted of (per liter) the following: 20 g glucose, 9 g sea salts, 20 g soy peptone, and 5 g yeast extract. Seed flasks were incubated at 25 °C and 200 rpm for 4 days on a New Brunswick Scientific Innova 4430 orbital shaker. Cultures were monitored by microscopic examination and any cultures showing contamination were discarded. Once growth was stabilized, seeds were adjusted with sterile fresh basal medium to an OD_{600} of 1.5 (approx. 10^7 cells) and were used to inoculate all fermentation test medium flasks.

Fermentation conditions

Five-milliliter aliquots of seed cultures were taken under aseptic conditions and added to 95 mL of sterile test medium in 500 mL Erlenmeyer flasks. Test medium composition and culture conditions for these flasks were evaluated using a Plackett-Burman experimental design, where the following variables were tested at a high (+ 1) and low (− 1) level: glucose, soy peptone, sea salts, ammonium sulfate, vitamin B stock solution (0.01 g L^{-1} vitamin B_{12}, 0.01 g L^{-1} biotin, and 2 g L^{-1} thiamine hydrochloride), initial pH, and temperature (Tables 1 and 2). After 4 days of fermentation at 200 rpm on an orbital shaker, 40 mL samples of broth were taken from each flask and cells were harvested by centrifugation at 4150 rpm at 2 °C for 20 min using a Thermo Scientific Sorvall Legend RT+ centrifuge (Germany). The pellet was rinsed with distilled water to remove the salts and residual substrate, and then re-centrifuged (Chaung et al. 2012). Pellets were frozen at − 80 °C, freeze-dried, and stored at − 20 °C prior to biomass and fatty acid analysis. The improved culture conditions found for growing thraustochytrid F24-2 after applying the Plackett-Burman design were compared to a complex medium developed to cultivate *Thraustochytrium aureum* ATCC 34304. Complex medium ingredients and their concentrations were reported elsewhere (Min et al. 2012).

Biomass determination

Freeze-dried cell pellets were weighed in pre-weighed conical centrifuge tubes to determine cell biomass of F24-2 cultures and reported as dry weight of cells per unit volume of medium (g L^{-1}) (Chaung et al. 2012).

Fatty acid methyl ester (FAME) preparation

A direct one step transesterification method (Lewis et al. 2000) modified elsewhere (Armenta et al. 2009) was conducted to prepare FAMEs from freeze-dried biomass. Briefly, 30–50 mg of dry biomass was weighed into a glass vial followed by the addition of 950 μL of C23:0 internal standard solution (1 mg mL^{-1}) to prepare FAMEs from freeze-dried biomass. Aliquots of toluene (6 mL) and 12.5% (*v*/*v*) acetyl chloride in methanol solution (6 mL) were added to each tube. Tubes were capped, vortexed, and placed in an agitated hot water bath for 2 h at 80 °C. Samples were then cooled to room temperature, followed by the addition of 10 mL of 6% (*w*/*v*) sodium carbonate solution to each sample and centrifugation at 1100 rpm for 15 min. The organic phase was recovered and 1 g of anhydrous sodium sulfate was added to remove traces of water. One milliliter of the organic phase from each sample was then passed through silica cartridges (previously conditioned with toluene) using vacuum filtration to remove interferences. Filtrated samples were collected in test tubes and then transferred to 2-mL glass vials for gas chromatography (GC) analysis.

GC FAME analysis

FAME analyses were performed on an Agilent 6890 series gas chromatograph equipped with autosampler, split injection, and flame ionization detector (FID) (USA). The FID was set at 275 °C with an inlet temperature of 250 °C. Injections of 1 μL of sample were used with a 10:1 split having helium as a gas carrier. The column was a Mandel Scientific FAMEWAX (30 m × 0.32 mm × 0.25 μm film thickness) (Canada) and was held at 190 °C for 1 min after injection, then temperature increased at a rate of 5 °C min^{-1} to a final temperature of 240 °C and held for 1 min (Armenta et al. 2009). Fatty acids (mg g^{-1}) were measured in duplicate.

Statistical design and analysis

The Plackett-Burman experimental design (Plackett and Burman 1946) is a fraction of a two-level factorial design. It allows the investigation of *n*-1 variables in at least *n* experiments and is based on the first-order model (Eq. 1):

$$Y = \beta_0 + \sum \beta_i x_i \quad (1)$$

Table 1 Independent variables and their levels used in the Plackett-Burman design

Variables	Coded variable x_i	Coded level and value	
		− 1	+ 1
Glucose (g L^{-1})	x_1	5	20
Soy peptone (g L^{-1})	x_2	2	20
Sea salt (g L^{-1})	x_3	9	18
Ammonium sulfate (g L^{-1})	x_4	0.2	2
Vitamin B stock solution (mL L^{-1})[a]	x_5	1	3
Initial pH	x_6	4	7
Temperature (°C)	x_7	25	31

[a] Vitamin B stock solution contains vitamin B_{12}, biotin, and thiamine hydrochloride in the following concentrations (g L^{-1}): 0.01, 0.01, and 2, respectively

where Y is the predicted response, β_0, β_i are constant coefficients; and x_i is the independent variable estimate or factor (Soliman et al. 2005). This model assumes no interaction amongst factors, x_i, in the range of variables under consideration (Zeng et al. 2011).

In this work, a Plackett-Burman design was used to explore the significance of seven culture variables on biomass and fatty acid production. The main effect (contrast coefficient) of each culture variable was calculated as the difference between the averages of the response values obtained for the high-level (+ 1) and low-level (− 1) conditions (Ortiz et al. 2017). This contrast coefficient provides the evaluation for the effect of each factor, which can be positive or negative. A culture variable (factor) having a positive effect means that the difference between the response at the high level (+ 1) of a culture variable and the response at the low level (− 1) of the same variable is positive. Conversely, a culture variable having a negative effect means that the difference between the response at the high level (+ 1) of a culture variable and the response at the low level (− 1) of the same variable is negative; this happens because the high level (+ 1) of a culture variable scored a lower response than the low level (− 1) of the same culture variable. Finally, a large contrast coefficient, positive or negative, indicates a factor of large impact, and a contrast coefficient close to zero implies a factor with little or no effect (Zeng et al. 2011).

A total number of 12 experimental runs were carried out in duplicate, following the combination of culture variables shown in Table 2. The Plackett-Burman design matrix was generated using the statistical software Statgraphics Centurion XVI version 16.0.07 (USA). The same software was used to carry out multi-factor analysis of variance (multi-factor ANOVA) of the data for biomass and fatty acid production. Culture parameters showing a p value less than

Table 2 Plackett-Burman experimental design matrix with observed values of biomass, cellular TFA yield, and TFA concentration

Run	Coded variable							Process variable							Response variables		
	x_1	x_2	x_3	x_4	x_5	x_6	x_7	x_1	x_2	x_3	x_4	x_5	x_6	x_7	Biomass (g L^{-1})	Cellular TFA yield (g g^{-1})	TFA concentration (g L^{-1})
1	+ 1	− 1	+ 1	− 1	− 1	− 1	+ 1	20	2	18	0.2	1	4	31	1.75 ± 0.01	0.32 ± 0.00	0.56 ± 0.01
2	+ 1	+ 1	− 1	+ 1	− 1	− 1	− 1	20	20	9	2	1	4	25	0.09 ± 0.00	ND	ND
3	− 1	+ 1	+ 1	− 1	+ 1	− 1	− 1	5	20	18	0.2	3	4	25	0.02 ± 0.01	ND	ND
4	+ 1	− 1	+ 1	+ 1	− 1	+ 1	− 1	20	2	18	2	1	7	25	3.64 ± 0.23	0.42 ± 0.03	1.54 ± 0.19
5	+ 1	+ 1	− 1	+ 1	+ 1	− 1	+ 1	20	20	9	2	3	4	31	0.21 ± 0.01	0.21 ± 0.02	0.04 ± 0.01
6	+ 1	+ 1	+ 1	− 1	+ 1	+ 1	− 1	20	20	18	0.2	3	7	25	10.71 ± 0.04	0.20 ± 0.01	2.11 ± 0.07
7	− 1	+ 1	+ 1	+ 1	− 1	+ 1	+ 1	5	20	18	2	1	7	31	2.50 ± 0.13	0.17 ± 0.00	0.42 ± 0.03
8	− 1	− 1	+ 1	+ 1	+ 1	− 1	+ 1	5	2	18	2	3	4	31	0.76 ± 0.04	0.25 ± 0.00	0.19 ± 0.01
9	− 1	− 1	− 1	+ 1	+ 1	+ 1	− 1	5	2	9	2	3	7	25	0.11 ± 0.00	0.06 ± 0.00	0.01 ± 0.00
10	+ 1	− 1	− 1	− 1	+ 1	+ 1	+ 1	20	2	9	0.2	3	7	31	1.58 ± 0.06	0.26 ± 0.00	0.41 ± 0.01
11	− 1	+ 1	− 1	− 1	− 1	+ 1	+ 1	5	20	9	0.2	1	7	31	2.80 ± 0.01	0.17 ± 0.01	0.47 ± 0.01
12	− 1	− 1	− 1	− 1	− 1	− 1	− 1	5	2	9	0.2	1	4	25	0.04 ± 0.01	ND	ND

x_1 glucose (g L^{-1}), x_2 soy peptone (g L^{-1}), x_3 sea salts (g L^{-1}), x_4 ammonium sulfate (g L^{-1}), x_5 vitamin B stock solution (mL L^{-1}), x_6 initial pH, x_7 temperature (°C), ND not detectable. Biomass accumulation for these experimental runs was not enough to obtain TFA by gas chromatography (GC) analysis

0.05 ($p < 0.05$) were considered to be significant for the response variables.

Results and discussion

The Plackett-Burman design was used to (1) identify the effect of seven culture parameters on biomass production and TFA accumulation by the thraustochytrid strain F24-2 and (2) improve liquid culture conditions of this microorganism to increase cell biomass and TFA production. As shown in Table 1, five variables represented ingredients in the liquid medium (x_1–x_5), and the remaining two variables represented culture conditions for microorganism growth (x_6–x_7). Under the different culture conditions tested, biomass and total fatty acid contents varied markedly, as discussed in the following sections. Other studies on thraustochytrid strains have also reported the influence of extrinsic factors (e.g., salinity, concentration of substrates, initial pH, and temperature), on the biomass, fatty acid composition, TFA content, and quality of the oil (Burja et al. 2006; Arafiles et al. 2011; Chaung et al. 2012). However, our approach is suitable for determining the medium composition and fermentation parameters that result in a high productivity of biomass and TFA, and that can influence the fatty acid profile of the lipids of F24-2.

Biomass

The main effects of the culture parameters on biomass production are shown in Fig. 1. The Pareto chart of standardized main effects shows that the most important parameters influencing biomass production by F24-2 were initial pH in the medium, followed by sea salts and glucose concentrations.

Our results confirmed that different nutritional conditions promoted varying levels of biomass. Analysis of variance (ANOVA) showed that these three parameters had a positive statistically significant effect ($p < 0.05$) on biomass accumulation (Fig. 1), which meant that when the initial pH, sea salts, and glucose concentrations were tested at a high (+ 1) level 7, 18 and 20 g L^{-1}, respectively (Table 1), biomass production increased. On the other hand, ANOVA for biomass showed that the concentration of ammonium sulfate in the medium was close to the 5% significance level used to analyze the data. As seen in Fig. 1, the standardized estimated effect of ammonium sulfate was negative, indicating that less biomass accumulated in the flasks when a high concentration of ammonium sulfate was added to the medium. Similarly, Ren et al. (2014) reported that *Schizochytrium* sp. biomass decreased from 40.15 to 33.66 g L^{-1} when the added ammonium sulfate concentration in the medium increased from 1.5 to 3.5 g L^{-1}. Ammonium sulfate decreases the pH of the broth affecting F24-2 cell proliferation, and may also have some toxicity affecting cell division (Willard 1984).

The maximum biomass concentration (10.71 ± 0.04 g L^{-1}) was obtained when fermentations were conducted under conditions for run 6 (Table 2). This enhanced production of biomass could be attributed to a high salinity, high glucose content, neutral pH, and low ammonium sulfate concentration in the medium. The initial pH of the medium has been reported to affect cell membrane functions, the uptake of nutrients and product biosynthesis of microorganisms (Kim et al. 2005), and would have a profound influence on both cell growth and metabolite production (Zhu et al. 2008). In this work, the maximum accumulation of biomass was obtained when the initial pH of the medium was neutral, which is in accordance with a previous study where the thraustochytrid, *Schizochytrium limacinum*, produced the greatest biomass and DHA yield after cultivation in a medium with a pH of 7.0 (Wu et al. 2005). Carbon sources such as glucose and glycerol generally play dominant roles in fermentation because these nutrients are directly linked with the production of biomass and metabolites (Zhu et al. 2008). Our results are consistent with those of other studies, which indicate that biomass production of thraustochytrids is expected to increase proportionally with the organic carbon concentration (Lowrey et al. 2016). The high salinity level required for growth of thraustochytrid F24-2 was expected, since it was originally isolated from a salt marsh habitat, and the ions in seawater are thought to be essential for normal thraustochytrid cell growth and development (Shabala et al. 2009). In general, the biomass production of F24-2 during run 6 agrees with previous reports on thraustochytrid cultivation, where a significant accumulation of biomass was observed at a cultivation pH between 5 and 8 and temperatures between 25 and 28 °C (Arafiles et al. 2011). In a previous study, *Schizochytrium* sp. SW1 was found to accumulate up to 29.2 g L^{-1} biomass at a flask level, when grown in an improved medium obtained from a factorial experimental design (Manikan et al. 2014). The concentration of sea salts was the most significant factor for biomass production by *Schizochytrium* sp. SW1, and the presence of sea salts at high levels was associated with the promotion of cell growth (Manikan et al. 2014). Our results agree with this finding. In another study, *Aurantiochytrium* sp. 4W-1b produced 20.1 g L^{-1} biomass when cultivated in flasks using a basal liquid medium with 60 g L^{-1} glucose (Nakazawa et al. 2012). In our study, thraustochytrid F24-2 produced 10.7 g L^{-1} biomass with 20 g L^{-1} glucose in the initial liquid medium. As discussed previously, a high concentration of glucose is one of the factors that promote F24-2 cell division, and it may be possible to raise biomass production above 10 g L^{-1} by increasing the glucose level further. Under the best culture conditions for biomass production (run 6, Table 2), thraustochytrid F24-2 accumulated more biomass compared to other thraustochytrids, including *T. aureum* ATTC 34304, *Thraustochytrium* sp. 20892, and *Thraustochytrium* sp.

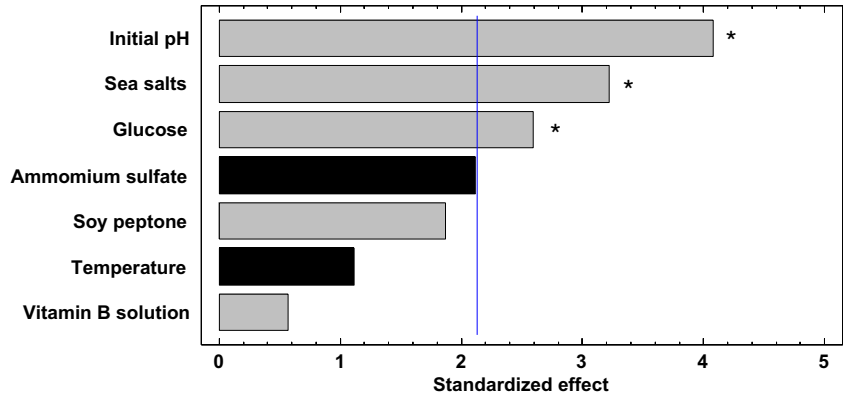

Fig. 1 Standardized Pareto chart for biomass production: positive estimated effects (gray bars); negative estimated effects (black bars). *Factors going beyond the vertical line are statistically significant at 5% level ($p < 0.05$)

KK17-3 which produced 5.7, 6.1, and 7.1 g L^{-1}, respectively (Gupta et al. 2012).

Cellular TFA yield

ANOVA of cellular TFA yield data showed that temperature, pH, glucose, and sea salt concentration had a statistically significant positive effect ($p < 0.05$) on TFA yield; whereas, soy peptone concentration had a statistically significant negative effect ($p < 0.05$) on TFA yield (Fig. 2).

Growth of thraustochytrid F24-2 in the medium, containing 20 g L^{-1} glucose, 18 g L^{-1} sea salts, 2 g L^{-1} soy peptone, neutral pH, and a temperature of incubation of 25 °C, yielded 0.42 g TFA per g of dry weight cells (run 4, Table 2). Under proper culture conditions, a liquid medium having an excess of organic nitrogen favors biomass production by thraustochytrids. Conversely, to promote lipid production, thraustochytrids require cultivation in a growth medium with an excess of carbon substrate and a limiting amount of other nutrients such as nitrogen (Ratledge 2004), as nitrogen limitation is responsible for lipid accumulation in the cell. The ability of thraustochytrid F24-2 to accumulate different levels of cellular TFA, depending on the cultivation conditions, likely relates to its ability to adapt to the changing conditions found in its natural habitat. Thraustochytrids are able to thrive in estuarine habitats, where the physicochemical parameters are in constant flux due to the nature of the aquatic environment (Arafiles et al. 2011).

The effect of culture conditions on cellular TFA yield by thraustochytrid F24-2 using a Plackett-Burman experimental design showed that a high concentration of sea salts in the liquid medium had a significant effect on TFA yield. Salinity enhances biomass production by several thraustochytrids; however, a previous study reported that it is unclear if these ions are directly involved in cell metabolism (Huang et al. 2001). Our results showed that a liquid medium containing a high concentration of sea salts stimulated the production of TFA, indicating that salt ions could be involved in the activation of cellular mechanisms that regulate the fatty acid synthesis in thraustochytrid-like microorganisms.

TFA concentration

As shown in the standardized Pareto chart (Fig. 3), initial pH, sea salts, and glucose concentration in the medium were the most important variables influencing TFA concentration. These variables showed a positive statistically significant effect ($p < 0.05$) on TFA concentration (Fig. 3). Biomass and TFA concentration were significantly influenced by the same independent variables. Also, when growing F24-2 in a medium with a neutral pH, high salinity, and high glucose concentration, biomass and TFA concentration increased. The best TFA concentrations resulted from run 6 (2.11 ± 0.07 g L^{-1}) and run 4 (1.54 ± 0.19 g L^{-1}) (Table 2).

Production of value-added fatty acids

Thraustochytrid F24-2 produced 32 and 26% of its total biomass as lipids (runs 1 and 10, respectively) when the initial carbon/nitrogen (C/N) ratio in the medium was 9.1 (Fig. 4). When this strain was cultured under the best conditions for biomass accumulation (run 6, C/N = 1), cells only accumulated up to 20% of their total biomass dry weight as lipids. By contrast, F24-2 cells accumulated 42% of lipids when the initial C/N ratio in the medium was 5.

Literature indicates that lipid accumulation in oleaginous organisms requires a high C/N ratio, and under nitrogen depletion, these microbes cannot multiply but continue to assimilate C as lipids (Raghukumar 2008). Our results confirmed that C/N ratio is an important factor regarding TFA content in thraustochytrid cells. However, the highest C/N ratio (C/N = 9.1) does not always guarantee a high production of cellular TFA because cellular TFA production also depends on other environmental conditions such as pH, temperature, and salt concentration, as shown in Fig. 2.

The main fatty acids synthesized by thraustochytrid F24-2 were n-6 DPA, C16:0, and DHA. In this study, DHA, C16:0,

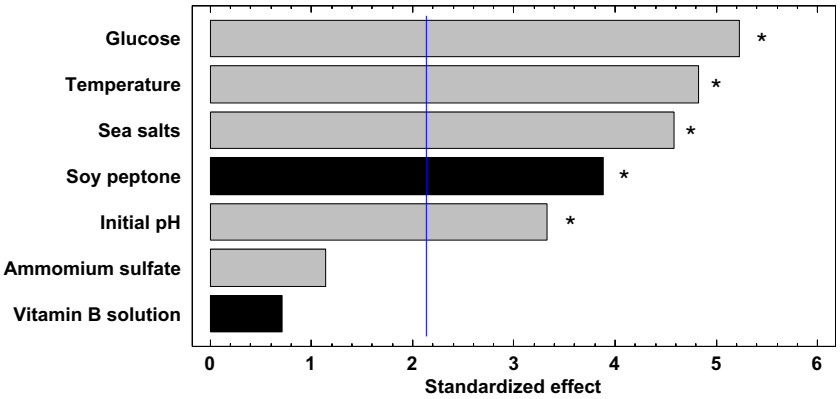

Fig. 2 Standardized Pareto chart for cellular TFA yield: positive estimated effects (gray bars); negative estimated effects (black bars). *Factors going beyond the vertical line are statistically significant at 5% level ($p < 0.05$)

and n-6 DPA content varied significantly amongst the culture conditions tested for thraustochytrid F24-2, and more DHA, C16:0, and n-6 DPA were produced when grown under the culture conditions in runs 1 and 4 (Fig. 5).

As shown in Table 2, these experiments were carried out using a medium with a low concentration of soy peptone, setting the conditions for a C/N ratio of 9.1 and 5 in runs 1 and 4, respectively (Fig. 4). Concentrations of DHA and C16:0 in run 4 were similar: 0.46 ± 0.04 and 0.45 ± 0.05 g L^{-1}, respectively. F24-2 strain was also grown in a complex medium (run 13). The composition of this complex medium was used to cultivate *Thraustochytrium aureum* ATCC 34304 and was reported elsewhere (Min et al. 2012). The same trend was found in the case of complex medium (run 13, Fig. 5), which allowed F24-2 to produce 0.77 ± 0.05 g L^{-1} C16:0 and 0.75 ± 0.01 g L^{-1} DHA. However, 0.65 g L^{-1} C16:0 and the highest DHA concentration of 0.92 ± 0.04 g L^{-1} were found in run 6 (Table 2). The high overall concentration of DHA in run 6 can be attributed to the maximum production of biomass observed under these culture conditions (Fig. 5).

Amongst the fatty acids found in the lipids from different thraustochytrid strains, DHA and C16:0 are compounds with significant commercial value for nutritional and biodiesel purposes, respectively. In the study by Johnson and Wen (2009), heterotrophic growth of thraustochytrid *S. limacinum* on crude glycerol was focused on DHA production, and a total fatty acid (TFA) content of around 50% (dry basis) was achieved with 30% of the TFA as DHA, with the remaining TFA (mainly, C14:0, C16:0, and C22:5) of interest as a potential source of biodiesel fuel. Kim et al. (2013) also reported that in addition to DHA, microalgal oil from thraustochytrid *Aurantiochytrium* sp. was rich in palmitic acid (C16:0), which could be used for biodiesel production, due to its high cetane number, low iodine content, and high oxidation stability. The biodiesel cetane number is dependent on the feedstock, with high cetane numbers associated with longer fatty acid carbon chains and a greater degree of saturation, and higher cetane numbers observed for esters of saturated fatty acids such as palmitic (C16:0) and stearic (C18:0) acids (Ramos et al. 2009; Knothe et al. 1998).

Cultivation conditions in runs 1, 4, and 10, which correspond to a high C/N, led to the highest production of the major fatty acids (Fig. 5). Additionally, in runs 1 and 4, in which sea salts concentration was higher compared to run 10, a significantly higher production of TFA was observed (Fig. 5). Thraustochytrid F24-2 synthesized more DHA when it grew in a medium containing a low concentration of organic nitrogen (soy peptone) and a high concentration of glucose. This result was consistent with earlier studies that suggested that

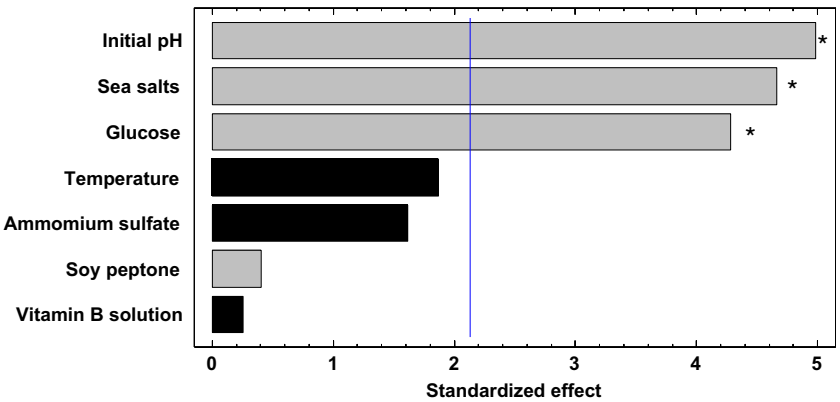

Fig. 3 Standardized Pareto chart for TFA concentration: positive estimated effects (gray bars); negative estimated effects (black bars). *Factors going beyond the vertical line are statistically significant at 5% level ($p < 0.05$)

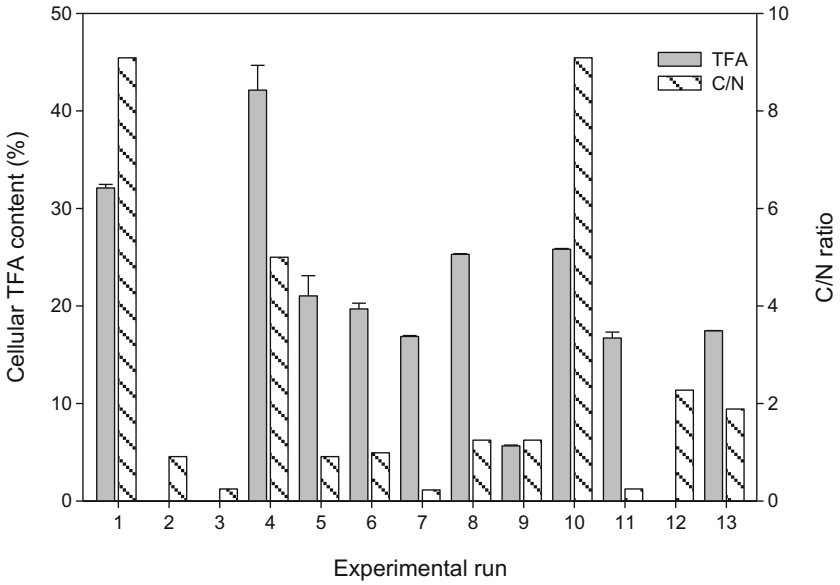

Fig. 4 Cellular TFA content of F24-2 under different culture conditions having distinct carbon to nitrogen (C/N) ratios. Values represent mean ± standard deviation (n = 2). Experimental runs 1–12 used culture conditions from the Plackett-Burman design matrix; Run 13 used a complex medium reported elsewhere (Min et al. 2012)

triacylglycerides and DHA are overproduced in thraustochytrids that are subject to adverse environmental and nutritional conditions (Raghukumar et al. 2000).

Fatty acid profile

Different fatty acid profiles were obtained under the culture conditions tested using the combinations of independent variables generated with the Plackett-Burman design matrix. Amongst the culture conditions evaluated, F24-2 produced C16:0, DHA, and n-6 DPA in a range between 15.6–58.3%, 17.0–45.1% and 9.3–23.2% of TFA, respectively (Fig. 6).

In another study, thraustochytrid *S. limacinum* SR21 was found to contain up to 7.2% of n-6 DPA in total lipids (Raghukumar 2008), which is lower than the content of n-6 DPA (ranging from 9.3 to 23.2%) produced by thraustochytrid F24-2 in this work. Myristic acid (C14:0) was found in runs 4, 5, and 6, while stearic acid (C18:0) was only produced under culture conditions tested in runs 1, 5, and 8. Palmitoleic acid (C16:1) was found in run 4 at 5.9%. Heptadecanoic acid (C17:0) and EPA were found in run 9 at 7.4 and 6.9%, respectively. Other fatty acids such as vaccenic acid (C18:1) was only found in runs 1 (3.3%) and 4 (14.5%). Fatty acids at less than 3% of TFA were considered minor fatty acids. Fatty acid profiles in runs 2, 3, and 12 could not be determined using the method described in this work due to the insufficient biomass produced in these experiments. Our results support evidence that environmental and nutritional conditions can affect specific fatty acid biosynthesis and their content in thraustochytrid-like microorganisms (Kermanshahi-Pour

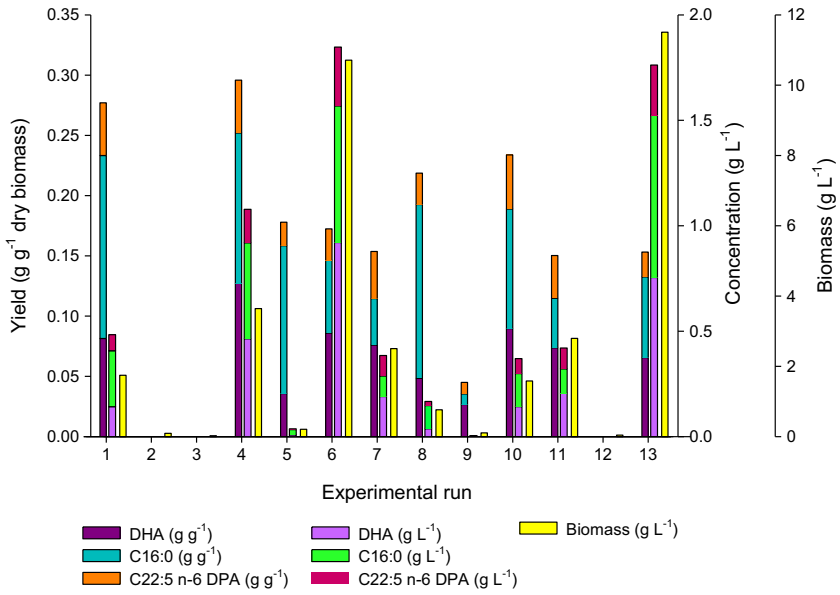

Fig. 5 DHA, palmitic acid (C16:0), and docosapentaenoic acid (C22:5 n-6 DPA) yields and concentrations of DHA, C16:0, n-6 DPA, and biomass produced by F24-2 under different culture conditions. Values represent mean (n = 2). Experimental runs 1–12 used culture conditions from the Plackett-Burman design matrix; Run 13 used a complex medium reported elsewhere (Min et al. 2012)

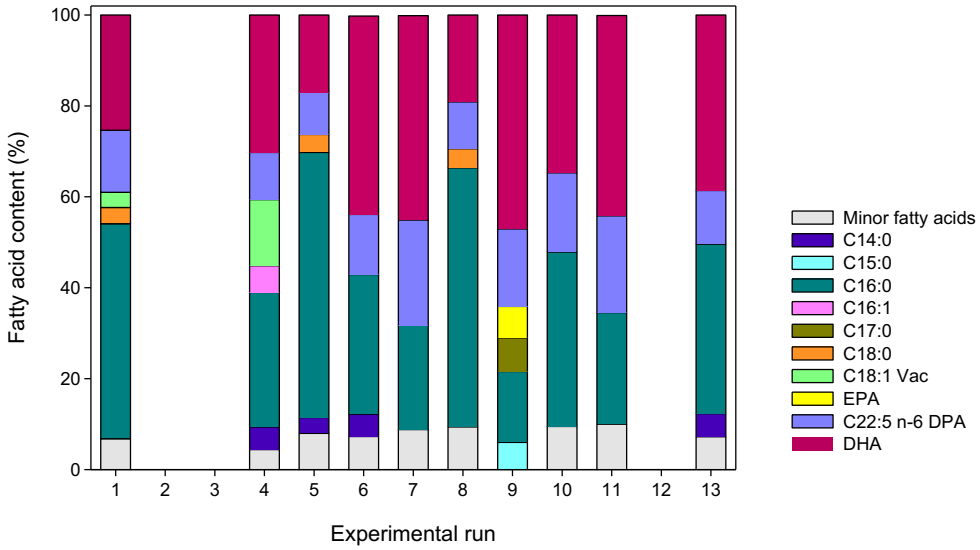

Fig. 6 Fatty acid profile of the F24-2 cells obtained under different culture conditions. Values represent mean ($n = 2$). Experimental runs 1–12 used culture conditions from the Plackett-Burman design matrix; Run 13 used a complex medium reported elsewhere (Min et al. 2012)

et al. 2013). For instance, as shown in run 9 of the Plackett-Burman design matrix (Table 2), adverse culture conditions such as a liquid medium with low concentration of glucose, soy peptone, and sea salts stimulated the synthesis of EPA by thraustochytrid F24-2. It is hypothesized that the biosynthesis of EPA and other PUFAs by thraustochytrids is to provide antioxidants to protect cells when subjected to oxidative stress during starvation periods (Mukherjee et al. 2004).

When F24-2 grew in the complex medium, the same fatty acid profile was observed as in run 6. However, DHA content was 13% higher in run 6 (43.6% of its TFA) than in the complex medium (38.6% of TFA). Conversely, C16:0 concentration was 17.7% lower in run 6 (30.7% of TFA) than in the complex medium (37.3% of TFA). In a previous work, *Schizochytrium* sp. produced 22.5% of its TFA as DHA (Jain et al. 2007), while *Aurantiochytrium* sp. KRS101 accumulated more than 30% of its TFA as C16:0 when cultured in a medium using 20 g L^{-1} glucose and 2 g L^{-1} yeast extract (Kim et al. 2013). When F24-2 was cultured under run 6 conditions (Table 2), this microorganism accumulated more DHA than *Schizochytrium* sp., and a concentration of C16:0 similar that produced by *Aurantiochytrium* sp. KRS101.

Conclusions

This work has shown that the medium ingredients and culture conditions for enhanced biomass production by thraustochytrid F24-2 were different from those that stimulated the accumulation of total fatty acids inside the cells. However, by applying a Plackett-Burman design to improve culture conditions increases in biomass concentration, TFA, C16:0, and DHA yields were achieved. It was also found that a high C/N influenced the synthesis of TFA in thraustochytrid F24-2 cells; however, having a medium with the highest C/N ratio did not produce the maximum cellular TFA content. This was because TFA production in F24-2 cells was significantly affected by salinity and physicochemical factors such as pH in the liquid medium and temperature of incubation of the cultures. The culture conditions tested within the Plackett-Burman design also influenced the production of different fatty acid profiles, in which the predominant fatty acids were C16:0, DHA, and n-6 DPA. With the best cultivation conditions obtained through the Plackett-Burman design, the DHA content and the overall yield of DHA were respectively 13 and 18.5% higher than in the complex medium, although the fatty acid profile was the same. The present work can serve as a scientific experimental benchmark for testing other similar microorganisms to assess their suitability for producing lipids containing value-added fatty acids. Finally, future optimization experiments could be conducted with thraustochytrid F24-2, to further improve and scale-up the production of both cell biomass and lipids rich in specific fatty acids such as DHA and C16:0.

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