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Influence of nitrogen sources on growth of thraustochytrids in waste water from the demineralization of cheese whey

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Abstract: An experimental design was ued to optimize the growth of two thraustochytrids, (*Schizochytrium limacinum* PA-968 and *Japonochytrium marinum* AN-4), on different nitrogen sources (yeast extract, corn steep liquor, ammonium sulphate) supplemented into saline waste water from the demineralization of cheese whey. Yeast extract was found to be the most suitable complex nutrient source. Nitrogen limitation was found to increase the lipid content in shake flask cultures of thraustochytrids by 12.7–22.4% w/w. The maximum total lipid content (79.1% w/w) and docosahexaenoic acid productivity (0.465 g/l per day) were achieved by *J. marinum* AN-4 in shake flask cultures. Fed-batch cultures of *J. marinum* AN-4, under conditions of nitrogen limitation, yielded biomass with a lower lipid content (72.1% wt.) but higher docosahexaenoic acid productivity (1.43 g/l per day). These results provide proof of concept that fed-batch cultivation of thraustochytrids, combined with nitrogen limitation, can be an appropriate strategy for the productive use of saline waste water from the dairy industry.

Keywords: docosahexaenoic acid; *Japonochytrium marinum*; nitrogen limitation; saline waste water; *Schizochytrium limacinum*

Increasing production of demineralized whey poses a significant problem in the elimination of saline waste water (Diblikova *et al.* 2013) although this water may be suitable as the basis for a medium that would support the cost effective cultivation of thraustochytrids (Humhal *et al.* 2017).

Thraustochytrids are obligatory marine, non-photosynthetic, microorganisms of the order *Thrastochytriales* (Tsui *et al.* 2009). Thraustochytrids are able to accumulate large amounts of triacylglycerols with a high proportion of docosahexaenoic acid (DHA), and high producing strains can grow to a high cell density and accumulate lipids to levels of 50–70% w/w with a DHA content of 30–70% w/w of total fatty acids (Raghukumar 2008; Chang *et al.* 2013; Li *et al.* 2015). Other features of thrautochytrids include the production of extracellular polysaccharides (Gupta *et al.* 2012), carotenoids and enzymes (Raghukumar 2008).

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Thraustochytrids can utilize a wide range of carbon and energy sources, but from the efficiency-versus-cost point of view, the two major substrates are glucose and glycerol (Honda *et al.* 1998, Wu *et al.* 2005). For cultivation where high biomass productivity is required, a medium rich in carbon (glucose or glycerol) and nitrogen (*e.g.* yeast extract, monosodium glutamate, peptone) is required (Chi *et al.* 2009; Ethier *et al.* 2011; Chang *et al.* 2013, Luy and Rusing, 2014). Information regarding increased lipid accumulation by thraustochytrids in response to stress are controversial (Aasen *et al.* 2016).

An experimental design to optimize growth of two thraustochytrids on different nitrogen sources was followed. Subsequently, we studied the effect of nitrogen limitation on the accumulation of lipids by *Schizochytrium limacinum* and *Japonochytrium marinum*. The best results from shake flask cultures were verified in a laboratory scale bioreactor.

MATERIAL AND METHODS

Microorganisms. Strains *Schizochytrium limacinum* PA-968 and *Japonochytrium marinum* AN-4 were deposited in the culture collection of the Department of Biochemistry and Microbiology, University of Chemistry and Technology Prague. These strains were maintained in ATCC790 By + medium (Humhal *et al.* 2017).

Media. Saline waste water (SWW) was produced by electrodialysis of cheese whey (EWDU 6xEDR-II/250-0.8; MEGA a.s., Czech Republic) in a dairy company (Moravia Lacto a.s., Czech Republic). The SWW was pre-treated as described in Humhal *et al.* (2017).

The chemical composition of pre-treated SWW without added glycerol or nitrogen was (in g/kg): ash (11.1 ± 0.8), lactose (4 ± 1.8), Cl^- (2.6 ± 0.45), P (0.3 ± 0.1), SO_4^{2-} (0.5 ± 0.15), Ca^{2+} (0.23 ± 0.06), Mg^{2+} (0.11 ± 0.01), K^+ (3.41 ± 0.5), Na^+ (2.00 ± 0.4), NO^{3-} (0.3 ± 0.05), proteins (2.2 ± 0.2), NH_4^+ (0.17 ± 0.06), conductivity (1756 ± 155 mS/cm). The analysis of SWW was carried out by EMPLA AG s.r.o. (Czech Republic).

Shake flask and fermenter cultures. *S. limacinum* and *J. marinum* were cultivated in waste saline medium (WSM) – 20 g/l glycerol and 2.9 g/l yeast extract (YE); until stationary phase and then used to inoculate sterile WSM at 10% v/v.

Shake flask cultures were grown in 500 ml Erlenmeyer flasks with 200 ml of medium on a rotary shaker (PSU-20i; Biosan, Latvia) at 130 rpm and 23°C for 120 hours. WSM consisted of pre-treated SWW, glycerol (50 g/l) and variable amounts of nitrogen (Sigma-Aldrich, Czech Republic) (Table 1). The nitrogen contents of corn steep liquor (CSL) (16.9 g N/kg) and YE (106 g N/kg) were analysed using the Kjeldahl method. In shake flask cultures the initial pH was 7.3 and during the cultivation it did not decrease below 6.5.

In the case of experiments under nitrogen limitation, the shake flask cultures (20 g/l glycerol, 1.6 g/l YE, 23°C, inoculum 10% v/v) after 88 h of cultivation were diluted – 1:1 with SWW, pre-treated with glycerol (40 g/l) and shaken for an additional 144 hours.

Fermenter cultures were carried out in 3.6 l bioreactors (Labfors 4; Infors HT, Switzerland) with a 2 l working volume. Cultivations were carried out using the following parameters: pH was maintained at 7.3 with addition of 0.25 mol/l NaOH, agitation speed 150 rpm (2 Rushton impellers, 6 blades, diameter 46 mm), temperature 23°C and airflow rate 4 l/min. WSM consisted of pre-treated SWW, glycerol and YE (4.0 g/l). During fed-batch culture, the feed added after 90 h of cultivation contained pre-treated SWW and glycerol (100 g/l). It was added in amount to achieve 40 g/l glycerol in fermenter.

Experimental data were statistically evaluated using the t-test. All statements of significance were based on a probability of $P < 0.05$.

Analyses. Biomass dry cell weight, glycerol concentration, cellular lipid content (LC) and fatty acids in lipid extracts were determined according to Humhal *et al.* (2017). Elemental analysis of biomass was carried out using a vario EL Cube (Elementar, UK).

Experimental design and data analysis. Response surface methodology (RSM) was used to study the effect of different nitrogen sources on biomass yield (X) of thraustochytdrids. Concentrations of YE (A), CSL (B) and $(NH_4)_2SO_4$ (C) were varied over three levels (Table 1) based on Humhal *et al.* (2017), where the optimum YE supplementation of WSM was found to be 3.6 g/l. The variables were optimized using a small central composite design (alpha face centered) with a total of 12 experiments (Table 2). Response variables were maximum final biomass yield (Equation 1) and volumetric biomass productivity (Equation 2):

$$X = X_{final} - X_{initial} \quad (1)$$

$$P_X = (X_{final} - X_{initial})/t \quad (2)$$

where: t – cultivation time to achieve X_{final}; $X_{initial}$ – initial biomass concentration; X_{final} – final biomass concentration

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Experiments were performed in triplicate. Experimental design and regression analyses were performed using Design Expert software (version 9.0.4.1, Stat-Ease Inc., USA).

RESULTS AND DISCUSSION

Optimization of nitrogen source. Waste saline medium (WSM) consists of pre-treated saline waste water (SWW), glycerol and a source of nitrogen (Table 1). The maximum final biomass yield (X) for *S. limacinum* (12.95 g/l) was obtained in run 4 (Table 2). In the case of *J. marinum,* the highest biomass yield (11.73 g/l) was obtained in run 11 (Table 2). The differences between maximum X of *S. limacinum* (12.95 g/l) and other cultivations of the same strain were statistically significant ($P < 0.05$) starting from cultivation with X equal to 9.25 g/l (run 6, Table 2). The same boundary for a statistically significant difference in the case of *J. marinum* was between maximum X = 11.73 g/l and X = 7.55 g/l from run 1. Reduced X of thraustochytrids was observed in run 8 without added nitrogen and in the absence of YE (run 10). Reduced growth was also observed in run 3 at a high concentration of CSL combined with a low concentration of the remaining two nitrogen sources (Table 2).

Table 1. Independent variables (g/l), their coded levels and actual values

Independent variables	Actual levels of coded variables		
	–1	0	+1
Yeast extract (A)	0	2	4
Corn steep liquor (B)	0	2.5	5
$(NH_4)_2SO_4$ (C)	0	2	4

The most suitable N source for both maximum X and biomass productivity (P_X) was YE. However, since $(NH_4)_2SO_4$ is more than 10 × cheaper than YE (http://www.alibaba.com/Chemicals_p8), its partial replacement may be economically beneficial. YE was also found to be the most suitable complex N source in other studies carried out on *Schizochytrium* sp. (Gupta *et al.* 2012; Jiang *et al.* 2017). Optimization of N sources for *Japonochytrium marinum* has not yet been carried out. Other potential N sources such as food grade peptones were not studied since they are not cheaper than YE.

Corn steep liquor, which is used as an organic nitrogen source for the preparation of thraustochytrid medium (Luy & Rusing 2014; Ling *et al.* 2015), was not a suitable N source in this work. Although an inhibitory effect of CSL has been described for diarrhea-causing organisms (Abdus-Salaam *et al.* 2014) and fungi (Chinta *et al.* 2014), this has not been reported for thraustochytrids.

The results of ANOVA analysis for models of biomass growth (X) of *S. limacinum* and *J. marinum*

Table 2. Central composite design matrix and response values as a result of variations in concentrations in WSM medium

Run	Design matrix variables			Response (g/l)			
				S. limacinum		*J. marinum*	
	A	B	C	X	P_X	X	P_X
1	0	0	0	8.92 ± 0.74	1.78	7.55 ± 0.92	1.51
2	0	0	0	8.64 ± 0.99	1.72	7.09 ± 0.77	1.42
3	0	+1	0	4.55 ± 0.67	0.91	3.68 ± 0.80	0.74
4	0	–1	0	12.95 ± 1.44	2.59	9.9 ± 0.89	1.98
5	+1	+1	–1	11.25 ± 0.70	2.25	10.2 ± 1.12	2.04
6	0	0	–1	9.25 ± 0.73	1.85	7.45 ± 0.67	1.49
7	–1	+1	+1	7.53 ± 0.95	1.5	7.08 ± 0.62	1.42
8	–1	–1	–1	2.31 ± 0.44	0.46	2.45 ± 0.34	0.49
9	0	0	+1	10.82 ± 1.20	2.16	11.15 ± 1.39	2.23
10	–1	0	0	4.28 ± 0.49	0.86	3.71 ± 0.57	0.74
11	+1	0	+1	11.45 ± 0.68	2.29	11.73 ± 1.39	2.34
12	+1	0	0	11.75 ± 0.99	2.35	11.35 ± 0.93	2.27

X – biomass yield; P_x – productivity (g/l per day); A – yeast extract; B – corn steep liquor; C – $(NH_4)_2SO_4$

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had high F (80.22 and 57.99) and low P ($P < 0.01$), which indicated that the models were highly significant (Table 3). All parameters showed that the developed model was able to correctly predict X. Multiple regression analysis of the experimental data demonstrated the highest significance ($P < 0.05$) of linear (A, B), interactive (A × C) and quadratic (A^2) terms in predicting X of *S. limacinum*. The same analysis for X of *J. marinum* identified the statistically most significant terms as linear (A, B, C), interactive (A × C) and quadratic (C^2) (Table 3).

The application of RSM resulted in quadratic regression equations for final biomass growth (X). The 3D response surface plots are graphical representations of the regression equations, visualizing the relationship between the independent and response variables (Figure 1). Considering biomass growth (X) of the strain *S. limacinum,* it can be seen that X mostly increased with concentrations of YE and $(NH_4)_2SO_4$ (Figure 1A, C and E). The exception was medium with high concentrations of both these N sources, where a decline in X was observed (Figure 1C). The positive effect of YE and $(NH_4)_2SO_4$ on X was disrupted by high concentrations of CSL (Figure 1A and E). The negative effect of CSL was more pronounced in medium containing YE than $(NH_4)_2SO_4$. According to the model, the highest X of *S. limacinum* (22 g/l) can be achieved when YE is used as the only N-source. When using only $(NH_4)_2SO_4$, the X of *S. limacinum* was predicted to be 37% lower. The maximum P_X predicted under optimum conditions was 4.36 g/l per day. A negative effect of non-organic N on biomass growth was also observed by Jiang *et al.* (2017).

The effect of different N sources on biomass growth (X) of the strain *J. marinum* was very similar to that of *S. limacinum* (Figure 1B, D and F). However, the highest X of *J. marinum* (17.3 g/l) predicted by the model, when using only YE as the N-source, was significantly lower than that of *S. limacinum* (22 g/l). When using only $(NH_4)_2SO_4$, the X of *J. marinum* was predicted to be 27% lower. The maximum P_X of *J. marinum* predicted under optimum conditions was 3.46 g/l per day. CSL used in this work was not a suitable N-source for *S. limacinum* nor *J. marinum*. The 3D surface plots, where the third linear variable was kept at coded levels 0 or +1 (data not shown), showed the same effects of tested N-sources on both *S. limacinum* and *J. marinum*. Consequently, YE at maximum tested concentration (4 g/l) was identified as the optimum N-source for both *S. limacinum* and *J. marinum*.

For both *S. limacinum* (4.36 g/l per day) and *J. marinum* (3.46 g/l per day), maximum biomass productivities (P_X) predicted by the RSM model for shake flask cultures were rather high when compared with the literature. For instance, the P_X of *S. limacinum* strains grown on pure glycerol were 4.51 g/l per day (Chang *et al.* 2013), 2.41 g/l per day (Chi *et al.* 2007) and 1.92 g/l per day (Pyle *et al.* 2008). The biomass productivity (P_X)

Table 3. ANOVA for response surface quadratic models of biomass growth in WSM medium

Source	*Schizochytrium limacinum* PA-968					*Japonochytrium marinum* AN-4				
	SSQ	df	MSQ	F	P (Pro > F)	SSQ	df	MSQ	F	P (Pro > F)
Model	124.66	9	13.85	80.22	0.0124	112.43	9	12.49	57.99	0.0171
A	27.93	1	27.93	161.73	0.0061	29.18	1	29.18	135.48	0.0073
B	35.38	1	35.38	204.90	0.0048	19.35	1	19.35	89.85	0.0109
C	1.22	1	1.22	7.08	0.1170	6.84	1	6.84	31.75	0.0301
A × B	0.49	1	0.49	2.82	0.2353	0.13	1	0.13	0.60	0.5202
A × C	40.19	1	40.19	232.76	0.0043	20.12	1	20.12	93.38	0.0105
B × C	0.41	1	0.41	2.37	0.2636	0.70	1	0.70	3.23	0.2141
A^2	3.58	1	3.58	20.72	0.0450	0.14	1	0.14	0.63	0.5095
B^2	0.53	1	0.53	3.05	0.2230	2.39	1	2.39	11.10	0.0795
C^2	1.70	1	1.70	9.86	0.0882	5.87	1	5.87	27.24	0.0348
R^2	0.9972	–	–	–	–	0.9962	–	–	–	–
Adj. R^2	0.9848	–	–	–	–	0.9790	–	–	–	–

SSQ – sum of squares; df – degree of freedom; MSQ – mean square; A, B, and C – linear terms; A × B, A × C, and B × C – interactive terms; A^2, B^2, and C^2 – quadratic terms

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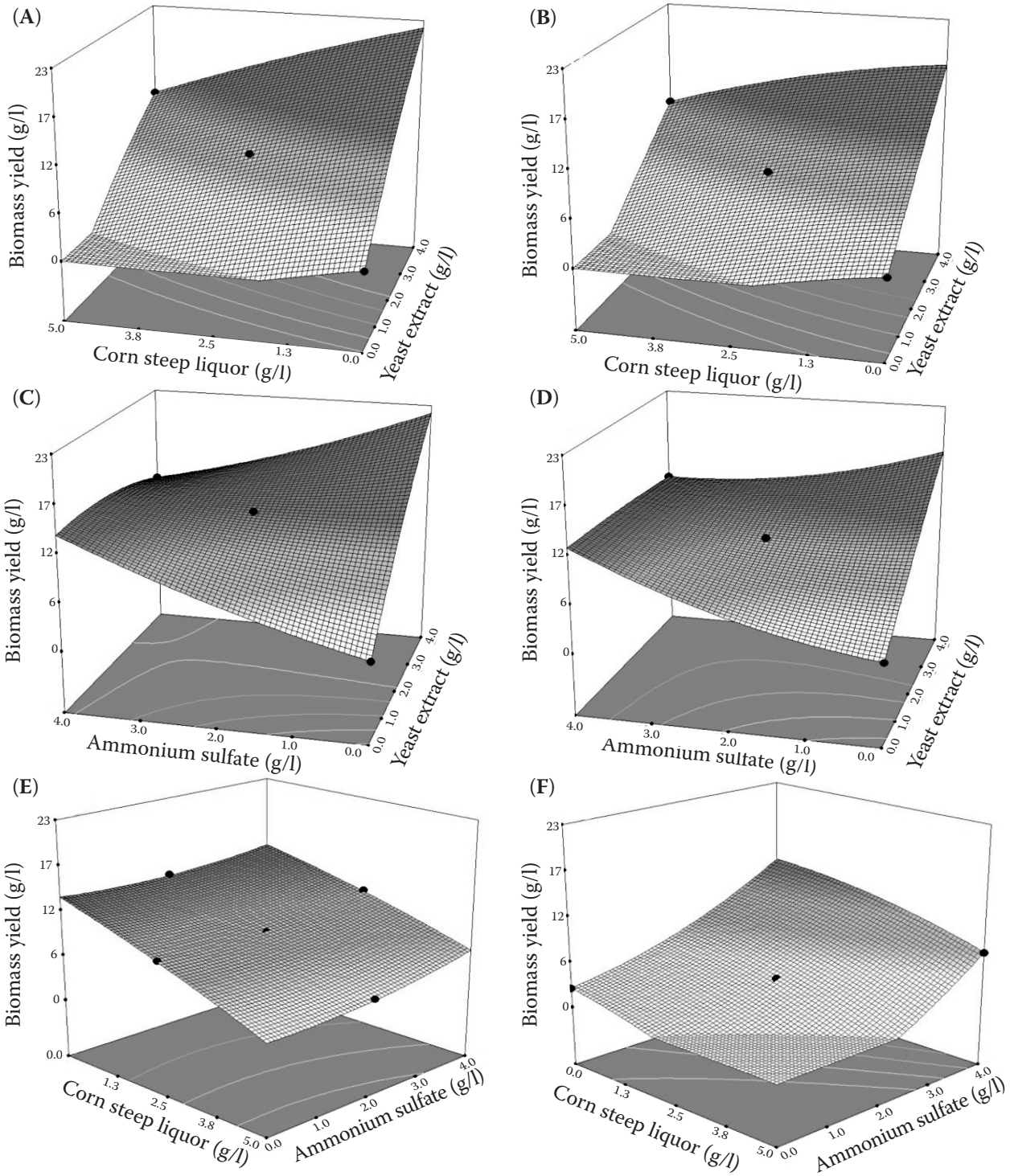

Figure 1. Response surfaces showing the effect of yeast extract and corn steep liquor (**A**) and (**B**), yeast extract and (NH4)2SO4 (**C**) and (**D**), corn steep liquor and (NH4)2SO4 (**E**) and (**jh**) on the biomass growth (*X*) of *S. limacinum* (**A**), (**C**), and (**E**) and *J. marinum* (**B**), (**D**), and (**F**), when the remaining variable was held at level –1

of *J. marinum* observed in fed-batch fermenter cultures (4.48 g/l per day) were somewhat higher than those obtained by *S. limacinum* SR21 (3.06 g/l per day in batch regime) grown on crude glycerol (Ethier *et al.* 2011). The P_X was also higher than that obtained for *S. limacinum* (3.71 g/l per day) and *J. marinum* (3.27 g/l per day) in previous work with pure glycerol in WSM medium (Humhal *et al.* 2017). This can be ascribed to differences in the compositions of SWW between the two studies.

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Effect of nitrogen limitation on lipid accumulation. Oleaginous microalgae, fungi and yeasts typically start accumulating storage lipids at N (or other nutrient) limitation and excess of C or energy (Ratledge 2004; Prochazkova *et al.* 2014). In contrast, thraustochytrids are reported to accumulate storage lipids during either their growth period (Ganuza & Izquierdo 2007) or when exposed to N and/or O_2 limitation (Jakobsen *et al.* 2008; Ling *et al.* 2015).

Improved accumulation of lipids in thraustochytrids was attempted by N-limitation in shake flask cultures. The biomass of both *S. limacinum* and *J. marinum* from shake flask cultures contained 2.8 ± 0.6% w/w of nitrogen. At this biomass composition, the amount of YE (4 g/l) as a nitrogen source in WSM was sufficient for growth of 15.14 g/l biomass. This is in good agreement (validation of previous data) with X from the first stage of shake flask (Table 4) and fed-batch cultures (16.4 g/l) (Figure 2). Consequently, the second stage of shake flask and fed-batch cultures was carried out under nitrogen limitation.

The total lipid (TL) content in shake flask cultures of *S. limacinum* and *J. marinum* increased in response to N-limitation, by differences of 12.7 and 22.4% (w/w), which was statistically significant (P (0.0003–0.009) (Table 4). The lipid productivity (P_L) and DHA productivity (P_{DHA}) in shake flask cultures were higher for *J. marinum* (Table 4). Application of N-limitation in shake flask cultures yielded results that were comparable with an increase in TL of 19.7% in shake flask cultures of *S. limacinum* (Ling *et al.* 2015).

The proportion of C22:6*n*-3 (DHA) in total fatty acids of *S. limacinum* and *J. marinum* grown in shake flask cultures after N-limitation was 48.2 ± 3.8 and 48.4 ± 2.9% (w/w), respectively. The second most abundant fatty acid in thraustochytrid biomass was C16:0. The two fatty acids C16:0 and C22:6*n*-3 (DHA) accounted for at least 70% of the total fatty acid content.

The effect of N-limitation on lipid content was verified on *J. marinum* in a laboratory scale bioreactor by a fed-batch culture (Figure 2). The X in fed-batch fermenter culture during the first growth phase (16.4 g/l) was achieved in 88 h, which corresponded to a P_X of 4.48 g/l per day. Total lipids in a fermenter culture of *J. marinum* increased in response to N-limitation, by 13.8% (w/w), which was statistically significant (P = 0.001). Biomass of *J. marinum* grown in fermenter culture after N-limitation contained 47.9 ± 3.6% of C22:6*n*-3 (DHA) in total fatty acids.

In the study of Humhal *et al.* (2017) on cultivation of thraustochytrids in SWW no N-limitation was used. Consequently, the lipid (P_L) and DHL (P_{DHA}) productivities were lower compared to this work. In this work, P_L and P_{DHA} (Table 4) values were either comparable (Chi *et al.* 2009; Ethier *et al.* 2011) or lower (Qu *et al.* 2013) than in the literature. The improvement in P_L and P_{DHA} achieved by culturing *J. marinum* in an aerated

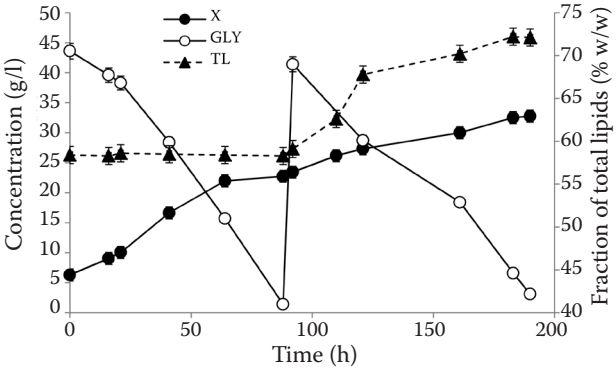

Figure 2. Fed-batch cultivation of *Japonochytrium marinum* AN-4 in a bioreactor

X – biomass concentration; GLY – glycerol concentration; TL – weight fraction of total lipids in biomass

Table 4. Effects of N-limitation on biomass and lipid production in waste saline medium (WSM) in shake flask and fermenter cultures

Strain	X_1	X_2	TL_1	TL_2	ΔTL	P_L	P_{DHA}
	(g/l)		(% w/w)			(g/l per day)	
S. limacinum[a]	6.5 ± 0.9	12.95 ± 0.8	46.3 ± 3.5	58.9 ± 2.9	12.7	0.79	0.38
J. marinum[a]	6.0 ± 1.5	11.8 ± 0.3	56.7 ± 1.3	79.1 ± 2.7	22.4	0.965	0.465
J. marinum[b]	22.3 ± 0.75	32.76 ± 0.6	58.3 ± 2.1	72.1 ± 1.9	13.8	2.98	1.43

[a]shake flask cultures; [b]fermenter culture; X_1 – biomass growth before N-limitation; X_2 – biomass growth after N-limitation; TL_1 – weight fraction of total lipids in biomass before N-limitation; TL_2 – weight fraction of total lipids in biomass after N-limitation; ΔTL – difference in weight fractions of total lipids in biomass before and after N-limitation; P_L – lipid productivity; P_{DHA} – DHA productivity

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and mechanically agitated bioreactor can be ascribed to higher P_X (3.34 g/l per day) as compared to a shake flask culture (0.91 g/l per day).

CONCLUSIONS

A sustainable economy demands the productive utilization of by-products and waste. This study confirmed that SWW from the demineralization of cheese whey, in combination with YE as a complex source of nutrients, is a suitable medium for the cultivation of thrausochytrids. Nitrogen limitation was confirmed to have positive effects on lipid and DHA contents in the biomass of *S. limacinum* and *J. marinum*.

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