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ORIGINAL PAPER

The Plackett–Burman Design for Evaluating the Production of Polyunsaturated Fatty Acids by *Physcomitrella patens*

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Abstract Identification of the parameters that had significant effects on polyunsaturated fatty acids (PUFAs) and biomass production by the moss *Physcomitrella patens* was performed using nine culture variables (temperature, agitation speed, pH, sucrose, di-ammonium tartrate, $CaCl_2 \cdot 2H_2O$, $MgSO_4 \cdot 7H_2O$, KH_2PO_4 and KNO_3) with the statistical design technique of Plackett–Burman. Statistical analysis revealed that two physical variables (pH and temperature) had significant effects on the production of both biomass and PUFAs (linoleic acid, LA; γ-linolenic acid, GLA; α-linolenic acid, ALA; eicosadienoic acid, EDA; di-homo-γ-linolenic acid, DHGLA; arachidonic acid, ARA; eicosapentaenoic acid, EPA). Three nutritional variables (sucrose, $CaCl_2$ and $MgSO_4$) had an influence only on the production of some of the PUFAs. Of the two levels used in this study, higher concentrations of sucrose had a positive effect on LA, ARA and EPA production, whereas higher concentrations of metal ions ($CaCl_2$ and $MgSO_4$) had a negative effect only on ARA and EPA production. After adjustment by multiple linear regression, it can be concluded that pH, temperature, sucrose, $CaCl_2$ and $MgSO_4$ were the most statistically significant parameters for the growth of *P. patens* and for PUFA production by this moss.

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Introduction

Polyunsaturated fatty acids (PUFAs) are essential requirements in human nutrition due to humans' inability to perform the desaturase-catalyzed formation of linoleic acid (LA) from oleic acid (OA). Unsaturated fatty acids in microorganisms are elongated or further desaturated to physiologically active substances such as prostaglandins or leukotrienes [1, 2]. Currently, the major commercial sources of food products containing PUFAs, or of partially purified extracts, or pure individual PUFAs are marine fish, seed plants and certain mammals [3]. However, another major source of commercial PUFA products is obtained by a fermentation process using microalgae [4, 5]. Based on the increasing demand of PUFAs in human nutrition and medicine, numerous studies to discover new potential sources of these compounds have been conducted.

Some PUFAs are presently obtained commercially from selected seed plants. Plant seeds such as nuts are rich sources of fatty acids. Higher plants generally synthesize up to C_{18} PUFAs including LA, γ-linolenic acid (GLA) and α-linolenic acid (ALA), while the long-chain ($\geq C_{20}$) PUFAs such as arachidonic acid (ARA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) usually come from marine fish [3, 6, 7] and microalgae [4, 5]. Although fish oil is a major and traditional source for the fatty acids, due to shrinking fish populations, the unpleasant odor of fish oils, contamination with heavy metals and the presence of cholesterol, this source is not ideal for the production of dietary supplements, despite the fact that modern processing methods have alleviated some of these disadvantages.

It is worth noting that the PUFAs in marine fish have their origins in marine organisms. Marine phytoplankton are rich in PUFAs; marine zooplankton retain the fatty acids ingested in their phytoplankton diet, but they also produce a range of fatty acids that are not present in the phytoplankton. These fatty acids are then further transmitted into the fish food chain by zooplanktonivorous fish [8]. This knowledge has led to the search for alternate sources of PUFAs in microalgae, lower fungi and other microorganisms [9]. The moss *Physcomitrella patens* (Funariales, Bryophyta), which is a lower plant, is also capable of producing several PUFAs including LA, GLA, ALA, eicosadienoic acid (EDA), di-homo-γ-linolenic acid (DHGLA), ARA and EPA [10, 11]. The ability to synthesize fatty acids up to C_{20} by bryophytes makes such lower plants a better choice for investigating them as a versatile source of PUFAs. However, since PUFA production in plants is strongly influenced by climate conditions [6], plant cell culture offers an attractive approach for the stable production of PUFAs. *P. patens* can be easily grown under axenic conditions in a simple medium of inorganic salts, with light and carbon dioxide as the only energy and carbon sources. Hormones and complex additives are not needed for cultivation [12]. A further interesting feature of *P. patens* is its incredible regeneration capacity. Moreover, liquid cultures of *P. patens* consist of differentiated plants, preventing genetic variations and thus providing stable conditions for any production processes performed in the moss systems [13]. Therefore, cell suspension cultures of *P. patens* are regarded as the most suitable cultures for the synthesis of PUFAs, and these cultures could be an alternative viable source for the production and commercialization of PUFAs. To date, maximization of the yield of PUFAs in *P. patens* using systematic growth enhancement and optimization of key physiological and environmental factors have not been conducted.

One of the strategies to obtain maximum production of PUFAs is to optimize the cultivation conditions. Process optimization may involve the study of many biochemical and physical parameters, including media formulation and culture parameters. The classical method of changing one medium variable at a time, in order to optimize performance, is impractical. The need for an efficient methods for screening a large number of variables has led to the adoption of statistical experimental design techniques. The methodology based on the Plackett–Burman design [14] provides an efficient way of screening a large number of variables, and identifying those that are most important. Such statistical methods have been applied to the optimization of bacterial cultures [15] and animal cell cultures [16], but very rarely to moss cultures.

The objectives of the present study were to investigate the factors affecting cell growth, and to identify the key factors that have an effect on production of PUFAs in shake-flask cultures of *P. patens*, using the Plackett–Burman statistical approach under photomixotrophic growth conditions.

Experimental Procedures

Materials

All chemicals used were reagent grade from Sigma. Fatty acids were purchased from Nu-Chek-Prep (Elysian, MN, USA).

Plant Material and Growth Conditions

The Gransden strain of *P. patens* [17] as received from Prof. Ralph S. Quatrano (Washington University, St. Louis, USA) was initially subjected to mechanical disruption by homogenization of the protonemata, followed by sub-culture on a solid BCD medium (for composition of BCD media, see reference [18]). The medium contained 5 mM of di-ammonium tartrate and 30 g/l of sucrose at pH 6.5, and the cells were cultured in a growth room at 25 °C under continuous light provided by fluorescent tubes [18].

Starter Inoculum Preparation

Protonemata tissue (14-day-old, 1 g) was blended with 100 ml of modified liquid BCD basal medium formulated by the Plackett–Burman design with a homogenizer (Ystral® X10/25, Germany) at a speed of 3,000 rpm for 2 min yielding 1.0% (w/v) starter inoculum. The starter inoculum was diluted 1:10 for all subsequent experiments.

Selection of Significant Variables by Plackett–Burman Design

For the selection of the most important variables that result in the production of high levels of biomass and PUFAs in *P. patens*, a total of 11 (k) variables (Table 1), including 6 nutritional (sucrose, di-ammonium tartrate, $CaCl_2{\cdot}2H_2O$, $MgSO_4{\cdot}7H_2O$, KH_2PO_4, KNO_3), three physical parameters (temperature, agitation speed, pH) and two dummy (or unassigned) variables were studied in 12 (N or $k + 1$) experiments (runs) via the Plackett–Burman design [14]. Each variable was tested at two levels, high (+) and low (−) (e.g. high pH and low pH). The high levels represented the concentrations of BCD macronutrients and culture conditions routinely used for *P. patens* cultivation [14].

Table 1 Culture variables studied for biomass and PUFA production by *P. patens* using the Plackett–Burman statistical design technique

Code	Variables (unit)	High level (+)	Low level (−)
X_1	Temperature (°C)	25	10
X_2	Agitation speed (rpm)	150	100
X_3	Dummy 1	–	–
X_4	pH	7.0	5.0
X_5	Sucrose (g/l)	30	3
X_6	Di-ammonium tartrate (g/l)	0.92	0.092
X_7	$CaCl_2{\cdot}2H_2O$ (g/l)	1.47	0.147
X_8	$MgSO_4{\cdot}7H_2O$ (g/l)	0.25	0.025
X_9	KH_2PO_4 (g/l)	0.25	0.025
X_{10}	KNO_3 (g/l)	1.01	0.101
X_{11}	Dummy 2	–	–

The statistical software package Design Expert® 7.1.5 (Stat Ease Inc., Minneapolis, USA) was used to generate the Plackett–Burman experimental design.

The cultivation of the sample tissues was in a 250-ml Erlenmeyer flask containing 100 ml of modified liquid BCD basal medium formulated by Plackett–Burman, using an orbital shaker (Heidolph® Unimax 2010, Schwabach, Germany), in a growth room at 25 °C under continuous light provided by fluorescent tubes [18] for 14 days. After cultivation, the dry cell weight (DCW) and production of PUFAs in the cells were estimated as described later in the text. All the experiments were performed in triplicate on two separate occasions and the responses are reported as the mean of these responses. The technique of Plackett–Burman is based on the first-order polynomial model:

$$Y = \beta_0 + \sum \beta_i X_i \qquad (i = 1, \ldots \ldots, k)$$

where Y is the estimated response (production of biomass, or of each of the PUFAs), β_0 is model intercept, β_i is the regression coefficient, X_i is the level of the independent variable, k is number of variables. The effects of each variable were determined by following standard equation:

$$E_{(X_i)} = \frac{2\left[\sum R_i^+ - \sum R_i^-\right]}{N}$$

where $E_{(X_i)}$ is the effect of the tested variable. R_i^+ and R_i^- are responses (production of biomass, or of each of the PUFAs) when variables were at high and low levels, respectively. N is total number of experiments or runs ($N = 12$). Experimental error was estimated by calculating the variance among the dummy variables as follows:

$$V_{\text{eff}} = \frac{\sum (E_{\text{d}})^2}{n}$$

where V_{eff} is the variance of the effect of high/low levels of variable, E_{d} is the effect of high/low levels of dummy variable and n is the number of dummy variables. The standard error (SE) of the high/low levels of variable is the square root of the variance of an effect, and the significance level (p value) of each effect of high/low levels of variable was determined using the Student's t test:

$$t_{(X_i)} = \frac{E_{(X_i)}}{\text{SE}}$$

where $E_{(X_i)}$ is the effect of the variable X_i.

The variables at or above the 85% confidence level ($p < 0.15$) were considered to have significant effects on responses (production of biomass, or of each of the PUFAs).

Dry Cell Weight Determination (Biomass)

For DCW determination, cell samples were harvested from the shake-flasks by filtration through a sieve (100 µm) and washed twice with 100 ml of distilled water. The fresh cells were then freeze dried overnight to a constant weight.

Fatty Acid Analysis (PUFA Production)

Total fatty acids from dried cell samples (50 mg) were transmethylated with 1 ml of 2.5% sulfuric acid in methanol at 85 °C for 30 min. Fatty acid methyl esters (FAMEs) were then extracted in 1 ml of heptane, the organic layer evaporated to dryness by nitrogen gas, and the residue dissolved with 50 µl of heptane before gas chromatography (GC) [11]. GC analysis of FAMEs was conducted using an HP 6890 Series gas chromatograph equipped with 0.25 mm × 30 m × 0.25 µM HP-INNO-Wax capillary column and a flame ionization detector with helium as the carrier gas. A 1-µl sample of each extract was injected onto the GC column using the injector in the split mode. The initial column temperature was 185 °C (0.50 min) and was increased at a rate of 8 °C min^{-1} to 230 °C (5.62 min); this temperature was then maintained for 6.50 min. FAMEs were identified by comparison with the retention times of authentic standards (Nu-Chek Prep, Elysian, MN, USA). The amounts of fatty acids were estimated from peak areas compared with calibration standards.

Results and Discussion

Effect of Culture Variables on Biomass

Table 1 shows the independent variables studied and the respective high and low levels used in the study. Table 2 shows the biomass response from the Plackett–Burman experimental design for 12 trials with two levels of each

Table 2 Plackett–Burman design matrix for evaluating variables influencing production of biomass (g/l) by *P. patens*

Run	Variables											Biomass (g/l)[a]
	X_1	X_2	(X_3)	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	(X_{11})	
1	+	+	−	+	+	+	−	−	−	+	−	1.62
2	−	+	+	−	+	+	+	−	−	−	+	0.12
3	+	−	+	+	−	+	+	+	−	−	−	1.50
4	−	+	−	+	+	−	+	+	+	−	−	0.62
5	−	−	+	−	+	+	−	+	+	+	−	0.28
6	−	−	−	+	−	+	+	−	+	+	+	0.74
7	+	−	−	−	+	−	+	+	−	+	+	0.41
8	+	+	−	−	−	+	−	+	+	−	+	0.24
9	+	+	+	−	−	−	+	−	+	+	−	0.30
10	−	+	+	+	−	−	−	+	−	+	+	0.38
11	+	−	+	+	+	−	−	−	+	−	+	3.62
12	−	−	−	−	−	−	−	−	−	−	−	0.23

+, high level; −, low level

The two variables X_3 and X_{11} are designed as "dummy variables"

[a] Represents mean biomass yield (g/l) based on three separate experiments

variable. The variables X_1–X_{11} represent the nine physical and chemical parameters included, with two dummy (or unassigned) variables (X_3 and X_{11}). Table 3 shows the results of Plackett–Burman experiments with respect to the biomass after statistical analysis using the Design Expert® software.

The variables were screened at a confidence level of 85% on the basis of their effects. Where the variables showed a significance at or above the 85% confidence level, and if their effect was negative, this was an indication that the variables were effective in biomass production, but the amount required was lower than the indicated at low (−) level in the Plackett–Burman experiment. If the effect was positive, a higher level than the indicated high (+) level was required during further optimization studies.

Table 3 Statistical analysis of Plackett–Burman design showing calculated regression coefficient, *t* value and confidence level of each variable on biomass production

Variables	Biomass production		
	Coefficient	*t* value	Confidence (%)
Temperature	0.443	2.975	**90.3**
Agitation speed	−0.292	−1.957	81.1
pH	0.575	3.858	**93.9**
Sucrose	0.273	1.834	79.2
Di-ammonium tartrate	0.088	−0.593	38.7
$CaCl_2{\cdot}2H_2O$	−0.223	−1.498	72.7
$MgSO_4{\cdot}7H_2O$	−0.267	−1.789	78.5
KH_2PO_4	0.128	0.861	52.0
KNO_3	−0.217	−1.454	71.7

The bold values indicate the significance at or above the 85% confidence level

Among the investigated factors in our study, the confidence level for some variables (agitation speed, sucrose, di-ammonium tartrate, $CaCl_2{\cdot}2H_2O$, $MgSO_4{\cdot}7H_2O$, KH_2PO_4 and KNO_3) were below 85% for biomass production, and were considered insignificant. Only two physical variables, temperature and pH, showed confidence levels above 85% and were considered to be significant (Table 3).

The media pH was the most significant factor (93.9% confidence level) over the range tested on biomass response (Table 3). The DCW of *P. patens* at a neutral pH (7.0) was considerably higher than at the lower pH (5.0) (Table 2). Because the media pH is a significant factor that influences the physiology of a microorganism by affecting nutrient solubility and uptake, enzyme activity, cell membrane morphology, by-product formation and oxidative–reductive reactions [19], and therefore it is a parameter that clearly influences microorganism growth. In this experiment, altering the nutrient medium to an acidic pH probably affected to cell wall composition and led to a suppression of the growth and development. However, it is important to note that the effect of pH on cell growth is species dependent. For example, the diatom *Phaedactylum tricornutum* shows good growth within the pH range from 6.4 to 8.4 [20]. Jiang and Chen [21] also found that a neutral pH (7.2) was the best in terms of specific growth rate, and dried cell weight concentration for *Crypthecodinium cohnii* culture. While in the culture of another diatom, *Isochrysis galbana*, the specific growth rate of cells decreased with decreasing the pH from pH 8.0 to 6.0 [22]. Similarly, a higher pH (8.0–8.5) was favorable for the increase of biomass for *Mortierella alpina* I_{49}-N_{18} culture [23].

Temperature is another significant parameter influencing cell growth (90.3% confidence level; Table 3). Cell growth was greatly inhibited when the temperature decreased to 10 °C, whereas the biomass reached a maximum when cultivated at the higher temperature (25 °C; Table 2). It was reported that a temperature of about 22–27 °C is optimal for growth of *P. patens*, whereas development is slower at 15 °C, and this cool temperature is necessary to induce gamete production [24]. The rate at which plant material grows in vitro usually declines as temperatures are reduced below the optimum [25]. However, the effects of temperature on microorganism growth might also be species specific and might be dependent on the habitat of the original isolate. For example, *Phaedactylum tricornutum* and *Isochrysis galbana* grow well at relatively low temperatures (around 20 °C); the growth was greatly inhibited when the temperature exceeded 25 °C [20, 22]. In contrast, the marine alga, *Crypthecodinium cohnii* can tolerate a temperature of up to 31 °C [26].

Overall the data suggests that the production of biomass by *P. patens* can be increased by manipulating the two physical parameters, temperature (X_1) and pH (X_4), and this is confirmed by the significance level (Table 3). Therefore the reduced polynomial equation may be written as Eq. 1:

For biomass:

$$Y_{biomass(g/l)} = 0.838 + 0.443X_1 + 0.575X_4 \quad (1)$$

Effect of Culture Variables on the Production of PUFAs

Tables 4 and 5 show the production of PUFAs (mg/l) and PUFA yields (mg/g DCW) from the Plackett–Burman experimental design for 12 trials. Table 6 shows the statistical parameters for PUFA production in *P. patens* obtained after the analysis of data using the Design-Expert® software. The pH of the media showed a higher than 85% confidence level for production of all PUFAs (LA, GLA, ALA, EDA, DHGLA, ARA and EPA) by *P. patens*. The pH had a positive effect on the production of every PUFA. This positive correlation that exists between pH and production of PUFAs implies that higher pH is more effective in increasing production in the experimental limits chosen.

The production of PUFAs by *P. patens* under neutral condition (pH 7.0) was considerably higher than at low pH (5.0) (Tables 4, 5), and this observation is similar to that for biomass production. However, the effect of pH on production of PUFAs depends on the species of microorganism used. For example, the yield of EPA by the diatom *Phaedactylum tricornutum* reaches its maximum when the pH is 7.6 [20]. Jiang and Chen [21] also found that a neutral pH (7.2) was optimum in terms of degree of fatty acid unsaturation, and the proportion of ω-3 PUFAs produced by *Crypthecodinium cohnii*. Whereas the percentageof EPA in the total lipids in the culture of another diatom, *Isochrysis galbana*, increased with decreasing the pH from pH 8.0 to 6.0 [22]. Yuan et al. [23] reported that higher pH values (8.0–8.5) were favorable for the production of ARA in lipids and in media by *Mortierella alpina* I_{49}-N_{18}. However, Nuutila et al. [27] reported that lowering the pH to 5.0 caused the ARA concentrations to increase, whereas the optimum pH for EPA production was 7.6 in the cultivation of the red alga *Porphyridium cruentum*.

The data from our study suggests that PUFA production from *P. patens* cultivations is affected by the pH of the medium, with pH 7.0 affording considerably higher PUFA production than a pH of 5.0.

Temperature is another parameter that significantly influences production of most PUFAs (except ALA and DHGLA), as confirmed by a confidence level above 85% in Table 6. Table 4 shows that the production of all PUFAs (yield per unit culture volume) reaches a maximum at the higher temperature (25 °C), as was the case for biomass production (Table 2). On the other hand, when considering the yield of PUFAs (mg/g DCW), most yields (except LA and ARA) were highest at the lower temperature (10 °C) as shown in Table 5. In addition, the proportion of ω-3 (ALA and EPA) to ω-6 PUFAs (LA, GLA, EDA, DHGLA, and

Table 4 Plackett–Burman design matrix for evaluating variables influencing production of each PUFA (mg/l) by *P. patens*

Run	Variables											PUFA production (mg/l)[a]							Total PUFAs
	X_1	X_2	(X_3)	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	(X_{11})	LA	GLA	ALA	EDA	DHGLA	ARA	EPA	
1	+	+	−	+	+	+	−	−	−	+	−	9.09	1.51	5.52	0.39	1.26	12.54	1.03	31.34
2	−	+	+	−	+	+	+	−	−	−	+	0.16	0.05	0.05	0.03	0.08	0.16	0.01	0.54
3	+	−	+	+	−	+	+	+	−	−	−	3.76	0.89	2.17	0.25	0.99	3.62	0.46	12.14
4	−	+	−	+	+	−	+	+	+	−	−	1.43	0.48	2.94	0.10	0.47	3.47	0.40	9.29
5	−	−	+	−	+	+	−	+	+	+	−	0.48	0.29	2.54	0.05	0.42	1.18	0.32	5.28
6	−	−	−	+	−	+	+	−	+	+	+	0.57	0.23	2.14	0.04	0.17	1.44	0.26	4.85
7	+	−	−	−	+	−	+	+	−	+	+	0.85	0.14	0.47	0.07	0.34	0.29	0.16	2.32
8	+	+	−	−	−	+	−	+	+	−	+	0.35	0.09	0.29	0.07	0.25	0.27	0.13	1.45
9	+	+	+	−	−	−	+	−	+	+	−	0.86	0.24	1.13	0.04	0.17	1.65	0.26	4.35
10	−	+	+	+	−	−	−	+	−	+	+	0.79	0.29	2.60	0.13	1.20	1.46	0.30	6.77
11	+	−	+	+	+	−	−	−	+	−	+	19.75	3.55	17.64	0.81	2.30	29.38	1.68	75.11
12	−	−	−	−	−	−	−	−	−	−	−	0.37	0.18	1.54	0.04	0.43	0.75	0.21	3.52

+, higher level; −, lower level

The two variables X_3 and X_{11} are designed as "dummy variables"

LA linoleic acid, *GLA* γ-linolenic acid, *ALA* α-linolenic acid, *EDA* eicosadienoic acid, *DHGLA* di-homo-γ-linolenic acid, *ARA* arachidonic acid, *EPA* eicosapentaenoic acid

[a] Represents the mean PUFA production (mg/l) based on three separate experiments

Table 5 Plackett–Burman design matrix for evaluating variables influencing yield of each PUFA (mg/g DCW) by *P. patens*

Run	Variables											PUFA yield (mg/g DCW)[a]							Total PUFAs
	X_1	X_2	(X_3)	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	(X_{11})	LA	GLA	ALA	EDA	DHGLA	ARA	EPA	
1	+	+	−	+	+	+	−	−	−	+	−	5.61	0.93	3.41	0.24	0.78	7.74	0.64	19.35
2	−	+	+	−	+	+	+	−	−	−	+	1.33	0.42	0.42	0.25	0.67	1.33	0.08	4.50
3	+	−	+	+	−	+	+	+	−	−	−	2.51	0.59	1.45	0.16	0.66	2.42	0.31	8.10
4	−	+	−	+	+	−	+	+	+	−	−	1.31	0.77	4.74	0.15	0.76	5.60	0.65	13.98
5	−	−	+	−	+	+	−	+	+	+	−	1.72	1.04	9.07	0.17	1.48	4.23	1.15	18.86
6	−	−	−	+	−	+	+	−	+	+	+	0.77	0.31	2.89	0.06	0.23	1.95	0.36	6.57
7	+	−	−	−	+	−	+	+	−	+	+	2.07	0.35	1.15	0.18	0.82	0.71	0.39	5.67
8	+	+	−	−	−	+	−	+	+	−	+	1.47	0.38	1.23	0.30	1.04	1.14	0.53	6.09
9	+	+	+	−	−	−	+	−	+	+	−	2.88	0.79	3.76	0.13	0.55	5.50	0.86	14.47
10	−	+	+	+	−	−	−	+	−	+	+	2.08	0.76	6.83	0.34	3.16	3.85	0.79	17.81
11	+	−	+	+	+	−	−	−	+	−	+	5.46	0.98	4.87	0.22	0.64	8.12	0.46	20.75
12	−	−	−	−	−	−	−	−	−	−	−	1.59	0.78	6.81	0.19	1.86	3.26	0.93	15.42

+, higher level; −, lower level

The two variables X_3 and X_{11} are designed as "dummy variables"

LA linoleic acid, *GLA* γ-linolenic acid, *ALA* α-linolenic acid, *EDA* eicosadienoic acid, *DHGLA* di-homo-γ-linolenic acid, *ARA* arachidonic acid, *EPA* eicosapentaenoic acid

[a] Represents the mean PUFA yield (mg/g DCW) based on three separate experiments

ARA) was also highest at the lower temperature (10 °C). This observation is similar to *M. alpina* which accumulated a considerable amount of EPA when it was grown at a low temperature (12 °C). Whereas at temperatures above 20 °C, this particular species produced ARA with a very high yield [28]. While the highest biomass yield and ARA concentration of *M. alpina* I_{49}-N_{18} were obtained at a higher temperature (30 °C), the ARA yield in the lipid reached a maximum at a lower temperature (25 °C) [23]. Therefore we can conclude that a lower temperature favors the yield of PUFAs by the moss *P. patens*, the data being similar to that reported for other moss cultures, *Bryum bicolor* [29] and red alga, *Porphyridium cruentum* [30]. Although *P. patens* is normally distributed in temperate zones [31], cold acclimatization could increase its PUFA production yields because of the stabilization of the lipid phase at low temperatures [32, 33]. Furthermore, a low temperature apparently leads to an increased availability of intracellular molecular oxygen, which facilitates the oxygen-dependent enzymes in the desaturation and elongation of PUFAs [34, 35]. In this work, we observed that a higher temperature (25 °C) was favorable for increasing biomass, but a lower temperature (10 °C) was more suitable for higher yields of PUFAs. Therefore, the higher temperature could be used in the primary stage for biomass production, while the lower temperature might be applied at a later stage for optimum production of PUFAs. The two-stage cultivation for PUFA production has been reported by Yuan et al. [23] the higher temperature was favorable for an increase in biomass and lipids, but the lower temperature was more suitable for the accumulation of ARA in *Mortierella alpina* I_{49}-N_{18}.

Besides those two significant physical variables (pH and temperature), we also observed that three nutritional variables significantly influenced production of some PUFAs at a confidence level above 85% as shown in Table 6. Sucrose, in the tested range, had a significant effect on LA, ARA, and EPA production by *P. patens*, while $CaCl_2$ and $MgSO_4$ only influenced the production of ARA and EPA.

Sucrose, the carbon source used in the medium, had a positive effect on LA, ARA and EPA production, indicating that higher concentrations are more suitable for increasing LA, ARA and EPA production. Whereas the concentration levels of $CaCl_2$ and $MgSO_4$ (which act as a source of Ca^{2+} and Mg^{2+}ions, respectively) in the medium, were found to influence ARA and EPA production with negative effects, which signify their effectiveness at lower concentrations in the experimental range.

$CaCl_2$ and $MgSO_4$ are known to play important roles in various enzyme reactions. For example, in the synthesis of lipids and PUFAs, acetyl-CoA carboxylase catalyzing the initial step of fatty acid synthesis requires bivalent ions as cofactors [35]. One reason for these phenomena is that acetyl-CoA carboxylase, which catalyzes the conversion of acetyl-CoA into malonyl-CoA, requires bivalent metal ions as the cofactors [36]. Added minerals may act as cofactors of this enzyme system, which catalyzes the initial step of fatty acid synthesis [34]. However, the concentrations of the ions in the medium needs to be at appropriate levels for maximizing yields.

Table 6 Statistical analysis of Plackett–Burman design showing calculated regression coefficient, *t* value and confidence level of each variable on LA, GLA, ALA, EDA, DHGLA, ARA and EPA production

Variables	LA production			GLA production			ALA production		
	Coefficient	*t* value	Confidence (%)	Coefficient	*t* value	Confidence (%)	Coefficient	*t* value	Confidence (%)
Temperature	2.572	2.979	**90.3**	0.408	2.488	**86.9**	1.282	1.441	71.4
Agitation speed	−1.092	−1.265	66.7	−0.218	−1.330	68.5	−1.167	−1.312	68.0
pH	2.693	3.120	**91.1**	0.497	3.026	**90.6**	2.247	2.526	**87.3**
Sucrose	2.088	2.419	**86.3**	0.342	2.081	82.7	1.605	1.805	78.7
Di-ammonium tartrate	−0.803	−0.931	55.0	−0.152	−0.924	54.7	−1.137	−1.278	67.0
$CaCl_2.2H_2O$	−1.933	−2.239	84.6	−0.323	−1.970	81.2	−1.772	−1.992	81.5
$MgSO_4.7H_2O$	−1.928	−2.234	84.5	−0.298	−1.817	78.9	−1.420	−1.597	74.9
KH_2PO_4	0.702	−0.813	49.8	0.152	0.924	54.7	1.192	1.340	68.8
KNO_3	−1.098	−1.272	66.9	−0.212	−1.289	67.4	−0.855	−0.961	56.2

	EDA production			DHGLA production		
	Coefficient	*t* value	Confidence (%)	Coefficient	*t* value	Confidence (%)
Temperature	0.103	2.649	**88.2**	0.212	1.549	73.9
Agitation speed	−0.042	−1.068	60.3	−0.102	−0.744	46.6
pH	0.118	3.033	**90.6**	0.392	2.866	**89.7**
Sucrose	0.072	1.880	79.9	0.138	1.012	58.2
Di-ammonium tartrate	−0.030	−0.769	47.8	−0.145	−1.061	60.0
$CaCl_2.2H_2O$	−0.080	−2.050	82.3	−0.303	−2.220	84.3
$MgSO_4.7H_2O$	−0.057	−1.452	71.6	−0.062	−0.451	30.4
KH_2PO_4	0.017	0.427	28.6	−0.043	−0.317	21.9
KNO_3	−0.048	−1.239	65.9	−0.080	−0.585	38.2

	ARA production			EPA production		
	Coefficient	*t* value	Confidence (%)	Coefficient	*t* value	Confidence (%)
Temperature	3.274	2.634	**88.1**	0.185	3.687	**93.4**
Agitation speed	−1.426	−1.147	63.0	−0.080	−1.594	74.8
pH	3.968	3.191	**91.4**	0.253	5.048	**96.3**
Sucrose	3.153	2.536	**87.9**	0.165	3.288	**91.9**
Di-ammonium tartrate	−1.482	−1.192	66.5	−0.067	−1.329	68.5
$CaCl_2.2H_2O$	−2.912	−2.343	**85.6**	−0.177	−3.521	**92.8**
$MgSO_4.7H_2O$	−2.969	−2.388	**86.0**	−0.140	−2.790	**89.2**
KH_2PO_4	1.547	1.245	66.1	0.073	1.461	71.9
KNO_3	−1.591	−1.280	67.1	−0.047	−0.930	54.9

The bold values indicate the significance at or above the 85% confidence level

LA: linoleic acid; *GLA* γ-linolenic acid; *ALA* α-linolenic acid; *EDA*: eicosadienoic acid; *DHGLA* di-homo-γ-linolenic acid; *ARA*: arachidonic acid; *EPA*: eicosapentaenoic acid

Chiou et al. [37] found that ferrous (Fe^{2+}) ions increased the production of ARA and EPA in cultures of the bryophyte, *Marchantia polymorpha*, whereas there was no notable change in PUFA production by additional Mg^{2+}, Mn^{2+} and Cu^{2+}. Enhancement of PUFA yield by Fe^{2+} resulted in an increase of intracellular lipid content, rather than selective enhancement of certain fatty acids [37]. On the other hand Sajbidor et al. [38] studied the influence of Ca^{2+}, Mg^{2+}, Mn^{2+} and Fe^{2+} on ARA production in the culture of *Mortierella* sp. and found that a low concentration (2 mg/l) of Mn^{2+} was beneficial for ARA production, whereas a higher concentration repressed lipid accumulation. However, these three chemical parameters still influenced LA production [38].

Therefore, production of each PUFA by *P. patens* is better explained using the physical and nutritional variables, temperature (X_1), pH (X_4), sucrose (X_5),

$CaCl_2{\cdot}2H_2O$ (X_7) and $MgSO_4{\cdot}7H_2O$ (X_8), as confirmed by the significance levels (Table 6). The reduced polynomial equations may be written as Eqs. 2–8:

For LA production:

$$Y_{\mathrm{LA\,(mg/l)}} = 3.205 + 2.572X_1 + 2.693X_4 + 2.088X_5 \quad (2)$$

For GLA production:

$$Y_{\mathrm{GLA\,(mg/l)}} = 0.662 + 0.408X_1 + 0.497X_4 \quad (3)$$

For ALA production:

$$Y_{\mathrm{ALA\,(mg/l)}} = 3.255 + 2.247X_4 \quad (4)$$

For EDA production:

$$Y_{\mathrm{EDA\,(mg/l)}} = 0.168 + 0.103X_1 + 0.118X_4 \quad (5)$$

For DHGLA production:

$$Y_{\mathrm{DHGLA\,(mg/l)}} = 0.673 + 0.392X_4 \quad (6)$$

For ARA production:

$$Y_{\mathrm{ARA\,(mg/l)}} = 4.684 + 3.274X_1 + 3.968X_4 + 3.153X_5 - 2.912X_7 - 2.969X_8 \quad (7)$$

For EPA production:

$$Y_{\mathrm{EPA\,(mg/l)}} = 0.435 + 0.185X_1 + 0.253X_4 + 0.165X_5 - 0.177X_7 - 0.140X_8 \quad (8)$$

Conclusions

In this study, statistical culture variable optimization was first carried out in the moss *P. patens* to evaluate biomass and production of PUFAs. The Plackett–Burman design demonstrated that pH and temperature have significant effects on biomass and PUFA production in *P. patens*. The data also revealed that sucrose is the carbon source, and that its concentration influences the production of C_{20} eicosanoid precursors ARA and EPA, and C_{18} essential fatty acid LA. In addition, Ca^{2+} and Mg^{2+} ions are essential for ARA and EPA production.

Although our current preliminary study on *P. patens* has not reached the stage where we can isolate oil, the stability of this will need to be carefully assessed in the future. An interesting scope for further research would be to determine the optimal levels of the selected variables by a central composite design and to use the optimized medium for a kinetic study of biomass production and the synthesis of individual PUFAs in *P. patens*.

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