รายงานวิจัยฉบับสมบูรณ์

โครงการ

การศึกษาการสร้าง C22 polyunsaturated fatty acids (PUFAs) ในมอส *Physcomitrella patens* โดยยีนที่ควบคุมเอนไซม์ Δ^5-elongase จากสาหร่าย *Pavlova* sp.

Biosynthesis of C22 polyunsaturated fatty acids (PUFAs) in the moss *Physcomitrella patens* by the algae *Pavlova* sp. Δ^5-elongase

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ชื่อโครงการ : การศึกษาการสร้าง C_{22}-polyunsaturated fatty acids (PUFAs) ในมอส *Physcomitrella patens* โดยยีนที่ควบคุมเอนไซม์ Δ^5-elongase จากสาหร่าย *Pavlova* sp.

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บทคัดย่อ

การศึกษาปัจจัยต่างๆ ที่มีผลต่อการเจริญและการผลิตกรดไขมันไม่อิ่มตัวสูงในมอส *Physcomitrella patens* โดยใช้วิธีทางสถิติ Plackett-Burman พบว่ามีปัจจัยทางกายภาพ 2 ปัจจัยคือ พีเอช และอุณหภูมิ ที่มีผลต่อการเจริญและการผลิตกรดไขมันไม่อิ่มตัวสูงหลายชนิด ได้แก่ กรดไลโนเลอิก แกมม่า-ไลโนเลนิก แอลฟ่า-ไลโนเลนิก อีโคซะไดอีโนอิก ได-โฮโม-แกมม่า-ไลโนเลนิก อะแรคซิโดนิก และอีโคซะเพนตะอีโนอิก) อย่างมีนัยสำคัญทางสถิติ ส่วนปัจจัยทางเคมี 3 ปัจจัยคือ ซูโครส แคลเซียมคลอไรด์ และแมกนีเซียมซัลเฟตส่งผลต่อการผลิตกรดไขมันเพียงบางชนิดเท่านั้น โดยการใช้น้ำตาลซูโครสความเข้มข้นสูงมีแนวโน้มเพิ่มการผลิตกรดไขมันไลโนเลอิก กรดอะแรคซิโดนิก และกรดอีโคซะเพนตะอีโนอิก แต่การเพิ่มความเข้มข้นของแร่ธาตุแคลเซียมและแมกนีเซียม กลับมีผลลดการสร้างกรดอะแรคซิโดนิกและอีโคซะเพนตะอีโนอิก

กรดอะดรินิกเป็นกรดไขมันไม่อิ่มตัวสูงกลุ่มโอเมก้า-6 ที่มีศักยภาพในการนำไปใช้เป็นยา แต่ในสภาวะธรรมชาติของมอส *P. patens* ที่ไม่พบกรดไขมันดังกล่าว ปัจจุบันมีการศึกษากระบวนการชีวสังเคราะห์ของน้ำมันจากพืช ตลอดจนการศึกษาทางด้านพันธุวิศวกรรมกันอย่างแพร่หลาย ดังนั้นการปรับเปลี่ยนวิถีชีวสังเคราะห์ของกรดไขมันเพื่อให้พืชผลิตน้ำมันชนิดใหม่จึงเป็นทางเลือกหนึ่งที่น่าสนใจ การศึกษานี้จึงได้นำยีน Δ^5-elongase จากสาหร่าย *Pavlova* sp ที่เกี่ยวข้องการการสังเคราะห์กรดไขมันไม่อิ่มตัวสูงที่มีความยาว 22 คาร์บอน มาศึกษาในมอสเพื่อให้มอสสามารถผลิตกรดอะดรินิก พบว่ายีนดังกล่าวสามารถแสดงออกในมอสได้ภายใต้การควบคุมของ 35S promoter จำนวน 2 ชุดซ้ำกัน โดยทำให้เนื้อเยื่อมอสผลิตกรดอะดรินิกได้เท่ากับ 0.42 มิลลิกรัมต่อลิตร จากสารตั้งต้นกรดอะแรคซิโดนิกที่มีอยู่ตามธรรมชาติในเนื่อเยื่อมอส และเมื่อทำให้ปรับสภาวะในการเพาะเลี้ยงโดยวิธี response surface methodology (RSM) พบว่ามอสสามารถผลิตกรดอะดรินิกได้เพิ่มขึ้นอย่างมีนัยสำคัญทางสถิติได้เท่ากับ 4.51 มิลลิกรัมต่อลิตร งานวิจัยนี้นับเป็นการศึกษาแรกเกี่ยวกับการแสดงออกของเอนไซม์ในการสร้างกรดไขมันไม่อิ่มตัวสูงในพืชชั้นต่ำที่ไม่มีเมล็ดโดยไม่มีการเติมสารตั้งต้นของกรดไขมันแต่อย่างใด

คำหลัก : มอสฟิสโคมิทราลา พาเทนส์ · สาหร่ายแพบโลวา · กรดไขมันไม่อิ่มตัวสูง · เดลต้าไฟ-อีลองเกส · กรดอะดรินิก

Abstract

Project Code : MRG5080203

Project Title : Biosynthesis of C_{22}-polyunsaturated fatty acids (PUFAs) in the moss *Physcomitrella patens* by the algae *Pavlova* sp. Δ^5-elongase

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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, $CaCl_2.2H_2O$, $MgSO_4.7H_2O$, 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.

Adrenic acid (ADA), an ω-6 PUFA) naturally absent in *P. patens*, has attracted much interest due to its pharmaceutical potential. Exploiting the wealth of information currently available on *in planta* oil biosynthesis, and coupling this information with the tool of genetic engineering, it is now feasible to deliberately alter fatty acid biosynthetic pathways to generate unique oils in commodity crops. In this study, a Δ^5-elongase gene from the algae *Pavlova* sp. related to the biosynthesis of C_{22}-PUFAs was targeted to enable production of ADA in *P. patens*. Heterologous expression of this gene was under the control of a tandemly duplicate 35S promoter. It was established that ADA (0.42 mg/l) was synthesized in *P. patens* from endogenous ARA via the expressed *Pavlova* sp. Δ^5-elongase in the moss. In an attempt to maximize ADA production, medium optimization was effected by the response surface methodology (RSM), resulting in a significant elevation of ADA (4.51 mg/l) production under optimum conditions. To the best of our knowledge, this is the first study describing the expression of a PUFA synthesizing enzyme in non-seed lower plant without supplying the exogenous fatty acid.

Keywords : *Physcomitrella patens* · *Pavlova* sp. · Polyunsaturated fatty acids · Δ^5-elongase · Adrenic acid

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Executive summary

Polyunsaturated fatty acids (PUFAs) are essential requirements in human nutrition due to human's inability to perform desaturase-catalyzed formation of linoleic acid (LA) from oleic acid (OA) (Fig. 1). Unsaturated fatty acids in microorganisms are elongated or further desaturated to physiologically active substances such as prostaglandins or leukotrienes. The human brain contains a high proportion of PUFAs derived from the two main essential fatty acids, docosahexaenoic acid (DHA, 22:6 $\Delta^{4,7,10,13,16,19}$) and arachidonic acid (ARA, 20:4 $\Delta^{5,8,11,14}$). PUFAs are known to be essential constituents of the central nervous system. Adrenic acid (ADA, 22:4 $\Delta^{7,10,13,16}$), an ω-6 PUFA, is the third most abundant fatty acid in the brain and it is particularly enriched in myelin lipid. While the specific functions of ADA are not yet clear, it is suggested to play an important role in myelination in neural tissues, its abnormality being implicated in the pathogenesis of Alzheimer's disease, and evidence from *in vitro* studies indicates that it serves as a substrate for dihomoeicosanoid formation tissues. In addition, ADA is an abundant fatty acid in the vasculature that causes endothelium-dependent relaxation in bovine coronary arteries. ADA has also been found to significantly enhance tissue factor (TF) activity of thrombin-stimulated endothelial cells, and it is therefore a potential prothrombotic agent.

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. 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.

The moss *Physcomitrella patens* (Funariales, Bryophyta), which is a lower plant, is also capable of producing several PUFAs including LA, γ-linolenic acid (GLA), α-linolenic acid (ALA), eicosadienoic acid (EDA), di-homo-γ-linolenic acid (DHGLA), ARA and eicosapentaenoic acid (EPA). 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. The cell culture of *P. patens* containing a range of high quality PUFAs is therefore

considered a potential source of these important fatty acids and could in future be a viable alternative over standard fish sources, provided yields can be increased, and production costs are not too high.

In this study, statistical culture variable optimization was firstly carried out in the moss *P. patens* to evaluate biomass and production of PUFAs. Plackett-Burman design demonstrated the effects of pH and temperature to be significant on biomass and PUFA productions 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.

In addition, the moss *P. patens* contains high proportions of ARA and some EPA. ARA plays an important role as a precursor for ADA production, which involves the enzyme Δ^5-elongase. However, *P. patens* lacks this C_{22}-PUFA synthesizing enzyme, with the result that the algae accumulates large amounts of DHA in the cells. Accumulation of this C_{22}-PUFA indicates high activities of very long-chain PUFA synthesizing enzymes in the algae. Previously gene encoding for Δ^5-elongase was isolated from the algae *Pavlova* sp. The study with *P. patens* therefore provided further evidence of biotechnological applications involving engineering of new plant oils to provide an economical alternative to naturally occurring oil. Successful application of such techniques should help the increasing demands of the chemical, pharmaceutical and nutraceutical industry for such PUFAs.

The experiments described here demonstrate successful production of ADA in *P. patens* by expression of *Pavlova* sp. Δ^5-elongase under the control of a tandemly duplicated CaMV 35S promoter. Furthermore, significantly higher levels of ADA (4.51 mg/l) can be achieved by statistical nutrient optimization via RSM. Therefore the cell cultures of *P. patens* containing a high quantity of ADA could be considered as a potential alternative source of this important fatty acid. Nutritionally enhanced food oils derived from agriculture biotechnology offer great promise in providing a sustainable and plentiful supply of high value fatty acids.

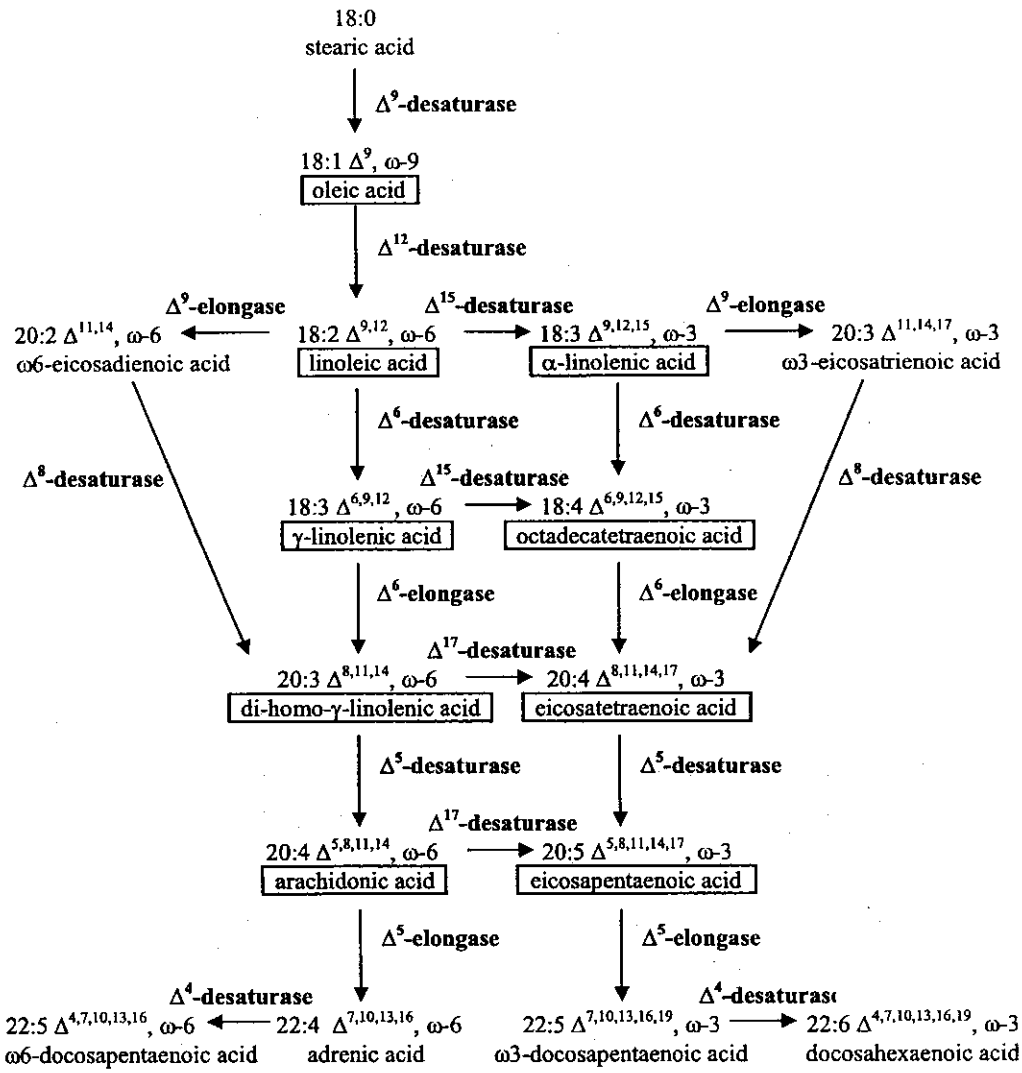

Fig. 1. Biosynthesis pathway of long chain PUFAs in lower eukaryotes (Pereira et al., 2003). The common pathway for synthesis of ω-6 and ω-3 long chain PUFAs is shown in bold arrows. The boxes indicate the fatty acids detected in the moss P. patens (Kaewsuwan et al., 2006).

Objectives

1. To study the factors affecting PUFA production by the moss *P. patens*
2. To clone the Δ^5-elongase gene from the marine algae *Pavlova* sp. CCMP459
3. To function express of the marine algae *Pavlova* sp. CCMP459 Δ^5-elongase gene in the moss *P. patens*
4. To obtain the stable transgenic *P. patens* producing C_{22}-PUFA

Part I : Factors affecting polyunsaturated fatty acid (PUFA) by the moss *Physcomitrella patens*

Materials and Methods

1. Materials

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

2. Plant material and growth conditions

The Gransden strain of *P. patens* (Ashton and Cove, 1977) was initially subjected to mechanical disruption by homogenization of the protonemata, followed by sub-culture on solid BCD medium (Knight et al., 2002). 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^{o}C under continuous light provided by fluorescent tubes (Knight et al., 2002). .

3. Starter inoculum preparation

Protonemata tissue (14-day-old, 1 g) was blended with 100 ml of modified liquid BCD basal medium formulated by Plackett-Burman design with a homogenizer yielding 1.0% (w/v) starter inoculum. The starter inoculum was diluted 1:10 for all subsequent experiments.

4. 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 eleven (k) variables (Table 1), including six nutritional (sucrose, di-ammomiun tartrate, $CaCl_2.2H_2O$, $MgSO_4.7H_2O$, KH_2PO_4, KNO_3), three physical parameters (temperature, agitation speed, pH) and two dummy (or unassigned) variables were studied in twelve (N or k+1) experiments (runs) *via* Plackett-Burman design (Plackett and Burman, 1946). 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 (Plackett and Burman, 1946).

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

Table 1

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

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

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, in a growth room at 25°C under continuous light provided by fluorescent tubes (Knight et al., 2002) 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:

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

where $E_{(Xi)}$ 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:

$$V_{eff} = \frac{\sum (E_d)^2}{n}$$

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 (S.E.) 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:

$$t_{(Xi)} = \frac{E_{(Xi)}}{S.E.}$$

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

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

5. 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.

6. 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) (Kaewsuwan et al., 2006). GC analysis of FAMEs was conducted using an HP 6890 Series gas chromatograph equipped with 0.25 mm × 30 m × 0.25 μM HP-INNOWax capillary column and a flame ionization detection with helium as carrier gas. A 1 μl 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

1. 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 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 the confidence level of 85% on the basis of their effects. Where the variables showed significance at or above 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.

Among the investigated factors in our study, the confidence level for some variables (agitation speed, sucrose, di-ammonium tartrate, $CaCl_2.2H_2O$, $MgSO_4.7H_2O$, 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* in 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 (ornwick et al., 19969), and therefore it is a parameter that clearly influences microorganism growth. In this experiment, altering the nutrient medium to acidic pH probably affected to cell wall composition and led to suppress 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 (Yongmanitchai and Ward, 1991). Jiang and Chen (2000) also found that neutral pH (7.2) was the best in terms of specific growth rate, and dried cell weight concentration for *Crypthecodinium cohnii* culture (Jian and Chen, 2000). While in the culture of another diatom, *Isochrysis galbana*, the specific growth rate of cells decreased with decreasing pH from pH 8.0 to 6.0 (Molina et al., 1992). Similary, higher pH (8.0-8.5) was favorable for the increase of biomass for *Mortierella alpina* I_{49}-N_{18} culture ((Yuan et al., 2002).

Table 2

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

Run	Variables											Biomass[a]
	X_1	X_2	(X_3)	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	(X_{11})	(g/l)
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.

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
$CaCl_2.2H_2O$	-0.223	-1.498	72.7
$MgSO_4.7H_2O$	-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.

Temperature is another significant parameter influencing cell growth (90.3% confidence level; Table3). Cell growth was greatly inhibited when temperature decreased to 10°C, whereas biomass reached maximum when cultivated at the higher temperature (25°C; Table 2). It was reported that a temperature of about 22 to 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 (Cove, 2000). The rate at which plant material grows *in vitro* usually declines as temperatures are reduced below the optimum (George, 1993). 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 temperature exceeded 25°C (Molina et al., 1992; Yongmanitchai and Ward, 1991). In contrast, the marine alga, *Crypthecodinium cohnii* could tolerate a temperature up to 31°C (Tuttle and Loeblich, 1975).

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:

$$Y_{\text{biomass (g/l)}} = 0.838 + 0.443X_1 + 0.575X_4 \qquad [1]$$

2. 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 above 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 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 and 5), and this observation is similar to that for biomass production. However, the effect of pH on production of PUFAs depends on species of microorganism used. For example, the yield of EPA by the diatom *Phaedactylum tricornutum* reaches the maximum when the pH is 7.6 (Yongmanitchai and Ward, 1991). Jiang and Chen (2000) also found that neutral pH (7.2) was optimum in terms of degree of fatty acid unsaturation, and proportion of ω-3 PUFAs produced by *Crypthecodinium cohnii* (Jiang and Chen, 2000).

Whereas the percentage EPA in the total lipids in culture of another diatom, *Isochrysis galbana*, increased with decreasing pH from pH 8.0 to 6.0 (Molina et al., 1992). Yuan et al. (2002) reported that higher pH values (8.0-8.5) were favorable for production of ARA in lipids and in media by *Mortierella alpina* I_{49}-N_{18} (Yuan et al., 2002). However, Nuutila et al. (1997) 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 red alga *Porphyridium cruentum* (Nuutila et al., 1997).

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 considerable 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 above 85% confidence level in Table 6. Table 4 shows that 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 ARA) was also highest at the lower temperature (10°C). This observation is similar to *M. alpina* that accumulated considerable amount of EPA when it was grown at low temperature (12°C). Whereas at temperature above 20°C, this particular species produced ARA at very high yield (Shimizu et al., 1988). While the highest biomass yield and ARA concentration of *M. alpina* I_{49}-N_{18} were obtained at higher temperature (30°C), ARA yield in the lipid reached maximum at lower temperature (25°C) (Yuan et al., 2002). Therefore we can conclude that lower temperature favors the yield of PUFAs by the moss *P. patens*, the data being similar to that reported for other moss cultures, *Bryum biocolor* (Al-Hasan et al., 1989) and red alga, *Porphyridium cruentum* (Lee and Tan, 1988). Although *P. patens* is normally distributed in temperate zones (Smith, 2004), cold acclimatization could induce its PUFA production yields because of the stabilization of the lipid phase at low temperature (Nichols et al., 1995; Richmond, 1986). Furthermore, 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 (Higashiyama et al., 1999: Singh and Ward, 1997). In this work, we observed that higher temperature ($25^{o}C$) was favorable for increasing biomass, but lower temperature ($10^{o}C$) 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 the late stage for optimum production of PUFAs. The two-stage cultivation for PUFA production was reported which higher temperature was favorable for the increase of biomass and lipids, but lower temperature was more suitable for accumulation of ARA in *Mortierella alpina* I_{49}-N_{18} (Yuan et al., 2002).

Besides those two significant physical variables (pH and temperature), we also observed that three nutritional variables significantly influenced production of some PUFAs at above 85% confidence level 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 production of ARA and EPA.

Sucrose, the carbon source used in the medium, had a positive effect towards 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.

Table 4

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

Run	Variables											PUFA production[b] (mg/l)							
	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	Total PUFAs
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".

[b] represents mean PUFA production (mg/l) based on three separate experiments.

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

Table 5

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

Run	Variables											PUFA yield[c] (mg/g DCW)							
	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	Total PUFAs
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".

[c] represents mean PUFA yield (mg/g DCW) based on three separate experiments.

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

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

Variables	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

Variables	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

$CaCl_2$ and $MgSO_4$ are known to play important roles in various enzyme reactions. For example, in the synthesis of lipid and PUFAs, acetyl-CoA carboxylase catalyzing the initial step of fatty acid synthesis requires bivalent ions as cofactors (Singh and Ward, 1997). 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 (Guchhait et al., 1974). Minerals added may act as cofactors of this enzyme system, which catalyzes the initial step of fatty acid synthesis (Higashiyama et al., 1999). However, the concentrations of the ions in the medium needs to be at appropriate levels for maximizing yields.

Chiou et al. (2001) found that ferrous (Fe^{2+}) ion increased the production of ARA and EPA in cultures of the bryophyte, *Marchantia polymorpha*, whereas there was no remarkable change in PUFA production by additional Mg^{2+}, Mn^{2+} and Cu^{2+}. Enhancement of PUFA yield by Fe^{2+} resulted by an increase of intracellular lipid content, rather than selective enhancement of certain fatty acids (Chiou et al., 2001). On the otherhand Sajbidor et al 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 (Sajbidor et al., 1992).

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), $CaCl_2.2H_2O$ (X_7) and $MgSO_4.7H_2O$ (X_8), as confirmed by the significance levels (Table 6). The reduced polynomial equations may be written as Eqs. [2-8]:

For LA production:

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

For GLA production:

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

For ALA production:

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

For EDA production:

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

For DHGLA production:

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

For ARA production:

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

For EPA production:

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

Conclusions

In this study, statistical culture variable optimization was firstly carried out in the moss *P. patens* to evaluate biomass and production of PUFAs. Plackett-Burman design demonstrated the effects of pH and temperature to be significant 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 state 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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Part II : High level production of a C_{22}- polyunsaturated fatty acids (PUFAs), adrenic acid, in *Physcomitrella patens* using the algae *Pavlova* sp. Δ^5-elongase gene

Materials and Methods

1. Materials

Restriction enzymes, polymerases, and DNA-modifying enzymes were obtained from Takara Bio (Shiga, Japan) unless indicated otherwise. All other chemicals used were reagent grade from Sigma (St. Louis, MO, USA). Cloning vectors and plant gateway destination vectors were obtained from Invitrogen (Carlsbad, CA, USA) and Arabidopsis Biological Resource Center (ABRC) (Columbus, OH, USA), respectively. Fatty acids were purchased from Nu-Check-Prep (Elysian, MN, USA).

2. Strains and Culture Conditions

Pavlova sp. CCMP459 was obtained from Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP, West Boothbay Harbor, ME, USA). The Gransden strain of *P. patens* (Ashton and Cove, 1977) supplied by Prof. Ralph S. Quatrano (Washington University, St. Louis, USA) was used throughout these studies. Protonemata (14-day-old) were grown in liquid BCD basal medium to which di-ammonium tartrate was added to 5 mM, and cultured at 25°C under continuous light provided by fluorescent tubes (Knight et al., 2002).

3. Isolation and cloning of the marine algae *Pavlova* sp. CCMP459 Δ^5-elongase gene

3.1 RNA extraction

Approximately 20 mg fresh weight of the *Pavlova* sp. CCMP 459 was ground to a fine powder under liquid nitrogen using a pre-cooled mortar and pestle. Total RNA was extracted from *Pavlova* sp. CCMP 459 using the RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions manufacturer's instructions.

3.2 cDNA synthesis

Five µl of total RNA was reversed transcribed to cDNA with the SuperScript™ III Reverse Transcriptase (Invitrogen). One µl of RACE 32 primer and 10 mM dNTPs were added to 5 µl of total RNA in a 14 µl volume reaction, incubated at 65°C for 5 min and placed on ice. The cDNA synthesis mix containing 4 µl of 5x first strand buffer, 1 µl of 0.1 M DTT and 15 units/µl SuperScript™ III Reverse Transcriptase were then added to the previous reaction on ice. After incubation at 4°C for 10 min, followed by 60 min at 50°C, the reaction was terminated by incubating at 75°C for 15 min. Finally, the cDNA diluted with TE buffer in a total volumn of 100 µl was then used as a template for PCR amplification with primers.

3.3 PCR-based cloning

Primers were synthesized based on NCBI sequence data (Pereira et al., 2004). The forward primer was PavELO5-FOR, 5'-ATG ATG TTG GCC GCA G-3' and the reverse primer was PavELO5-REV, 5'-TTA CTC CGC CTT GAC CG-3'. The PCRs were carried out in a total volume of 50 µl containing 2 µl of 10 µM each primers, 10x PCR buffer and 10 mM dNTPs, 1 µl of cDNA as a template, 1.5 µl of 50 mM $MgCl_2$, and 0.5 µl of 5 U/µl *Taq* DNA Polymerase (Invitrogen). After initial denaturation at 94°C for 4 min, amplification was performed in 35 cycles of 1 min at 94°C, 0.5 min at 49°C and 2.5 min at 72°C, followed by a final extension at 72°C for another 10 min.

Amplification products were fractionated on 1.0% agrarose gels and aliquot 1 µl was directly ligated into pCR®2.1-TOPO® vector (Invitrogen) (Fig. 1) in a total volume of 6 µl at room temperature for 60 min. Three µl of the ligation reaction was then transformed into One Shot®TOP10 Chemically Competent *E. coli* cells (Invitrogen) and cultured on solid LB medium containing 100 µg/ml ampicillin, surface spread by 50 µl of 100 mM IPTG and 50 mg/ml X-Gal.

Plasmids DNA were purified from transformed *E. coli* culture by High-Speed Plasmid Mini Kit (Geneaid). The resulting plasmid were verified by digestion with *Eco*RI for 60 min and analyzed by 1.0%

agarose gel electrophoresis and 2 μl of interested plasmid DNA was sequenced with M13 Forward and Reverse by using an automated sequenced, resulting in the plasmid named PsELO5.

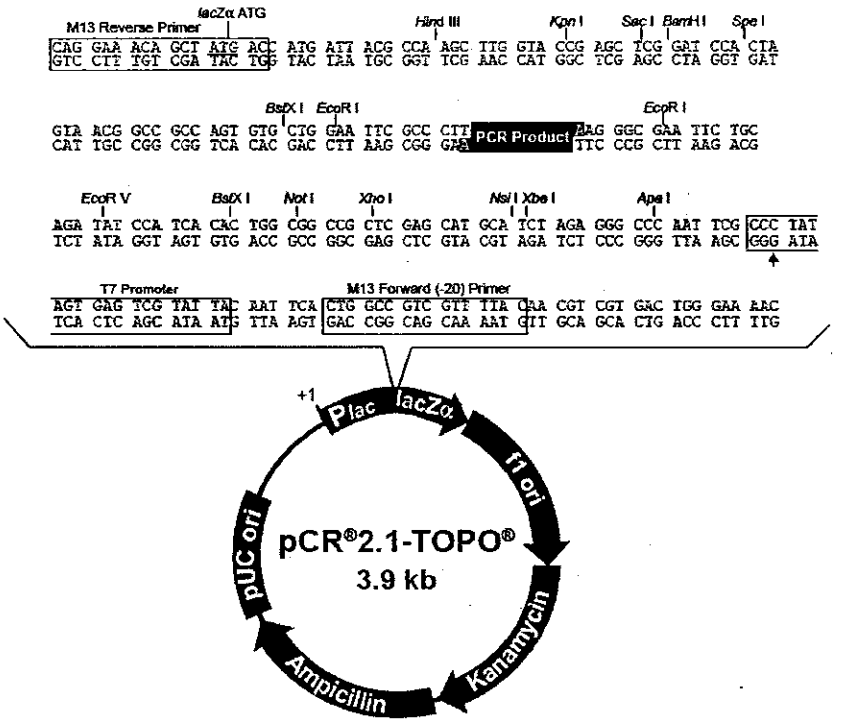

Fig. 1. Map of cloning vector pCR®2.1-TOPO® (Invitrogen).

4. Functional analysis of the marine algae *Pavlova* sp. CCMP459 Δ^5-elongase gene in the moss *P. patens*

4.1 Construction of the entry vector

The primers with PavELO5-FOR, 5'-ATG ATG TTG GCC GCA G-3' and PavELO5-REV, 5'-TTA CTC CGC CTT GAC CG-3' were used for PCR amplification of the plasmid PsELO5 with *Taq* DNA Polymerase (Invitrogen).

PCR was carried out in a total volume of 50 μl. Each reaction contains 2 μl of 10 μM of each primers and 10xPCR buffer, 2 μl of 10 mM dNTPs, 1.5 μl of 50 mM $MgCl_2$, 1 μl of 1/50 diluted plasmid PsELO5 as a template, and 0.5 μl of 5 U/μl *Taq* DNA Polymerase. The thermocycling conditions were as follows; initial

denaturation for 4 min at 94°C, followed by 28 cycles of 1 min at 94°C, 0.5 min at 49°C, 2.5 min at 72°C, and terminated by 10 min final extension at 72°C.

One µl of amplification product was I directly ligated into 1 µl of pCR®8/GW/TOPO® vector (Invitrogen) (Fig. 2) in a total volumn of 6 µl at room temperature for 60 min and transformed into One Shot®TOP10 Chemically Competent *E. coli* cells (Invitrogen). The transformants were cultured on LB medium containing 100 µg/ml spectinomycin. Plasmids DNA were purified from transformed *E. coli* culture by High-Speed Plasmid Mini Kit (Geneaid).

The resulting plasmids were verified by digestion with *Eco*RI for 60 min and analyzed by 1.0% agarose gel electrophoresis and 2 µl of interested plasmid was sequenced with M13 forward and reverse primers, yielding the plasmid *att*L1-PsELO5-*att*L2. The resulting plasmid had PsELO5 sequence flanked by *att*L recombination sequences was then recombined with *att*R sites of destination vectors using the Gateway® LR Clonase™ II Enzyme Mix (Invitrogen).

4.2 Construction of the over-expression vector

This reaction transferred 150 ng (2 µl) of PsELO5 sequence into 100 ng (3 µl) of desired destination vectors (pMDC32 (ABRC) (Fig. 3) in a total volume of 10 µl solution containing 3 µl of TE buffer (pH 8.0), and 2 ul of LR Clonase™ II Enzyme Mix (Invitrogen) at 4°C for 2 days. The mixtures were further incubated with 1 µl of Proteinase K at 37°C for 10 min. An aliquot of 3 µl was introduced into One Shot® TOP10 Chemically Competent *E. coli* cells (Invitrogen). The transformants were cultured on LB medium containing 50 µg/ml kanamycin. Plasmids DNA were further purified from transformed *E. coli* cultures by High-Speed Plasmid Mini Kid (Geneaid). The resulting plasmids were verified by digestion with *Eco*RI for 60 min and analyzed by 1.0% agarose gel electrophoresis, resulting in the over-expression vectors named pMDC32-PsELO5.

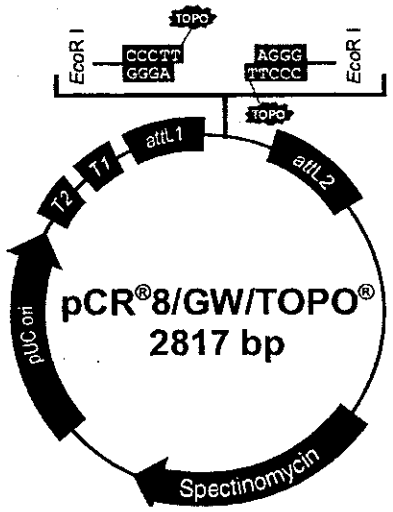

Fig. 2. Map of Gateway cloning vector pCR8®/GW/TOPO® (Invitrogen).

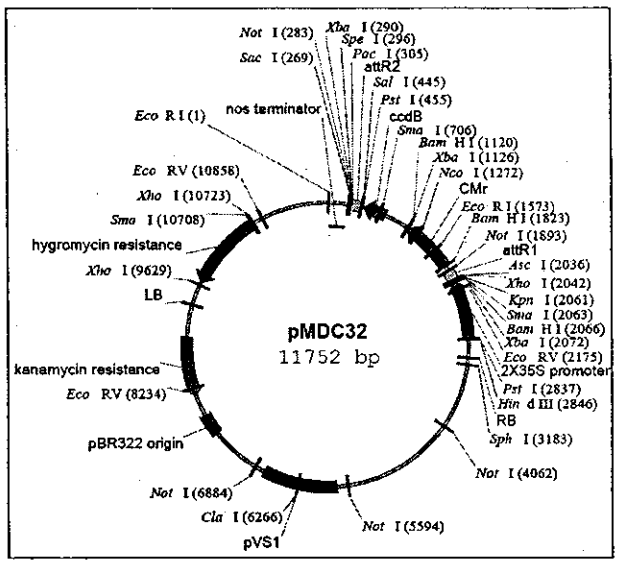

Fig. 3. Map of Gateway plant expression vectors, pMDC32 (ABRC).

4.3 Protoplast transformation and regeneration

4.3.1 Protoplast isolation

Protoplasts were isolated from 2 plates of 14-day-old protonemal wild-type cultures by digestion with 20 ml of 0.5% Driselase enzyme suspension dissolved in 8% mannitol for 60 min. The digested moss material was successively passed through sieve with a mesh and carefully washed in 20 ml of 8%

mannitol twice. Subsequently, the protoplast pellet was resuspended in 10 ml of CaPW and estimated protoplast density using a haemocytometer.

4.3.2 Polyethylene-glycol (PEG)-mediated transformation of protoplast and *P. patens* regeneration for transgenic plant expressions

The over-expression vectors (pMDC32-PsELO5) driven by a tandemly duplicated 35S promoter and nos terminator with hygromycin resistance (Hyg^r) selection cassette was digested with *Hind*III at 37°C for 5 h. The linear DNA fragments were precipitated before transformation.

Fiveteen micrograms of each linearized DNA were transformed into $5x10^5$ protoplasts of *P. patens* by PEG-method (Schaefer *et al.*, 1991). After protoplast transformation, the regenerating protoplasts were grown on protoplast regeneration medium for 2 weeks and then they were transferred to the solid BCD medium containing hygromycin (25 µg/ml) and further cultivated for 2 weeks, followed by 2 weeks release period on medium without antibiotic and a second selective medium for a further 2 weeks. Strongly growing plants that still survived on this selection were defined as stable transformants.

4.4 Phenotype analysis of *P. patens* by GC

Total fatty acids extracted from dried cell samples (50 mg) were transmethylated with methanol containing 2.5% H_2SO_4 in methanol at 85°C for 30 min. Fatty acid methyl esters (FAMEs) were then extracted in heptane, the organic layer was evaporated to dryness by nitrogen gas, and the residue was dissolved with heptane before gas chromatography (GC) (Kaewsuwan *et al.*, 2006). GC analysis of FAMEs was conducted using an HP 6890N Series gas chromatograph equipped with 0.25 mm x 30 m x 0.25 µm HP-INNOWax capillary columm and a flame ionization detector with helium as carrier gas. The initial column temperature was 185°C (0.5 min) and was ramped at a rate of 8°C min^{-1} to 230°C (5.62 min), followed by maintaining at 230°C for 6.50 min. FAMEs were identified by comparison with 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. The corresponding fatty acids were further characterized by gas chromatography-mass

chromatography (GC-MS) using the HP 6890N Series operating at an ionization voltage of 70 eV with a scan range of 50-500 Da.

5. Molecular analysis of *P. patens* by PCR and Southern blotting

5.1 Genomic DNA extraction for PCR analysis

Approximately 50 mg fresh weight of protonemal wild-type and transgenic moss tissues were frozen using liquid nitrogen and pulverized in a microcentrifuge tube. The powdered tissues were blended with 250 µl of shortly extraction buffer twice, incubated on ice for 5 min, and centrifuged at 13,000 rpm for 5 min. A volume of 350 µl of supernatant was mixed with 350 µl of isopropanol by vortex and centrifuged at 13,000 rpm for an additional 15 min. Genomic DNA pellet was washed with 250 µl of 70% ethanol, followed by resuspended dried genomic DNA with 400 µl TE buffer before PCR analysis.

5.2 PCR analysis

For this purpose, after genomic DNA of wild type and transgenic plants was extracted, DNA integration events were confirmed by PCR experiments with primers PavELO5-FOR and PavELO5-REV derived from start and stop regions of PsELO5, respectively and with primers A (5'-ATG AAA AAG CCT GAA CTA CCG-3') and B (5'-CTA TTT CTT TGC CCT CGG A-3') derived from the hygromycin resistance (Hyg^r) coding region of the selection cassette.

The PCRs were carried out in a total volume of 50 µl containing 2 µl of 10 µM each primers, 10x PCR buffer and 10 mM dNTPs, 1 µl of genomic DNA as a template, 1.5 µl of 50 mM $MgCl_2$, and 0.5 µl of 5 U/µl Taq DNA Polymerase (Invitrogen). After initial denaturation at 94°C for 4 min, amplification was performed in 35 cycles of 1 min at 94°C, 0.5 min at 50°C and 2.5 min at 72°C, followed by a final extension at 72°C for another 10 min and amplification products were fractionated on 1.0% agarose gel electrophoresis.

5.3 Genomic DNA extraction for Southern blotting

Genomic DNA was extracted from approximately 1 g fresh weight of 14-day-old protonemal wild-type and transgenic lines using the Nucleon™ PhytoPure™ Genomic DNA Extraction Kit (Amersham Biosciences). DNA was recovered by ethanol precipitation and dissolved in 250 µl of TE buffer.

5.4 Southern blotting

One microgram aliquots of genomic DNA from wild-type and transgenic lines were completely digested with *Eco*RV and *Eco*RI for 6 h, separated on a 0.6% agarose gel electrophoresis, and then blotted onto a Biodyne B positively charged 0.45 nylon membrane (Pall Corporation) in 10x saline sodium citrate (SSC) for 4 h. To improve transfer of the larger DNA fragments, the gel was soaked in 0.25 M HCl for 10 min to partially depurinate the DNA, followed by denatured twice with 0.5 M NaOH/1.5 M HCl for 15 min and neutralized twice with 0.5 M Tris base/1.5 NaCl pH 7.0 for an additional 15 min. After prehybridization with DIG Easy Hyb (Roche) at 40°C for 45 min, the membrane was probed with *PsELO5* coding region of Δ^5-elongase. In addition, the membrane was also probed with the Hyg^r coding region of hygromycin resistance cassette. The probes were labeled with DIG by PCR Dig Probe Synthesis Kit (Roche). Hybridization was performed overnight at 40°C in DIG Easy Hyb (Roche). The membrane was washed twice with 2x SSC, 0.1% sodium dodecyl sulfate (SDS) at room temperature for 5 min and then wash twice with 0.1x SSC, 0.1% SDS at 65°C for 30 min. Detection was accomplished with a chemi-luminescent substrate (CSPD, Roche) according to the manufacturer's instructions.

6. Optimization of ADA production using response surface methodology (RSM)

The optimization of medium constituents to improve C_{22}-PUFA (ADA) production in transgenic *P. patens* (C6) was carried out based on BCD medium (Knight et al., 2002) using RSM (Rao et al., 2000). The effects of sucrose (A), KNO_3 (B) and glutamate (C) were studied by central composite design (CCD) method with five settings (-2, -1, 0, +1, +2) of each three factor levels (Table 1). The relationship of the independent

variables and the responses (biomass or ADA production) was calculated by the second-order polynomial equation:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} Xi^2 + \sum_{i} \sum_{j} \beta_{ij} X_i X_j \quad (1)$$

Y is the predicted response, β_0 is the model constant, β_i is the linear coefficient, β_{ii} is the quadratic coefficient, and β_{ij} is the interaction coefficient.

The analysis of variance (ANOVA) for the experimental data and the model coefficients were calculated using the software, Design-Expert® v. 7.1.5. (Stat Ease Inc., MN, USA). In addition, two-dimension contour plots were constructed for visual observation of the trend of maximum responses and the interactive effects of the significant variables on the responses.

Table 1

Experimental results of the central composite experimental design used to study the effect of sucrose (A), KNO_3 (B) and glutamate (C) concentrations on biomass production of the transgenic line (C6).

Runs	Sucrose (A) (g/l)	KNO_3 (B) (g/l)	Glutamate (C) (g/l)	Biomass production[a] (g/l)	ADA production[a] (mg/l)
1	40 (-1)	0.6 (-1)	1.0 (-1)	15.50	1.88
2	80 (1)	0.6 (-1)	1.0 (-1)	14.73	2.02
3	40 (-1)	1.0 (1)	1.0 (-1)	15.63	2.04
4	80 (1)	1.0 (1)	1.0 (-1)	16.10	1.78
5	40 (-1)	0.6 (-1)	2.0 (1)	15.30	3.62
6	80 (1)	0.6 (-1)	2.0 (1)	12.67	1.01
7	40 (-1)	1.0 (1)	2.0 (1)	16.90	3.98
8	80 (1)	1.0 (1)	2.0 (1)	17.50	1.61
9	20 (-2)	0.8 (0)	1.5 (0)	14.77	2.77
10	100 (2)	0.8 (0)	1.5 (0)	12.47	1.01
11	60 (0)	0.4 (-2)	1.5 (0)	13.11	2.06
12	60 (0)	1.2 (2)	1.5 (0)	16.57	1.93
13	60 (0)	0.8 (0)	0.5 (-2)	15.53	2.03
14	60 (0)	0.8 (0)	2.5 (2)	13.97	1.86
15	60 (0)	0.8 (0)	1.5 (0)	16.80	3.05
16	60 (0)	0.8 (0)	1.5 (0)	16.93	3.00
17	60 (0)	0.8 (0)	1.5 (0)	16.63	3.00
18	60 (0)	0.8 (0)	1.5 (0)	16.60	3.09
19	60 (0)	0.8 (0)	1.5 (0)	16.80	3.08
20	60 (0)	0.8 (0)	1.5 (0)	16.87	3.04

[a] The values given in the table are the means of three dependent experiments. ADA, adrenic acid.

Results and Discussion

1. Cloning of the marine algae *Pavlova* sp. Δ^5-elongase gene

The *Pavlova* sp. has received attention because of its ability to produce several very-long chain PUFAs, including ARA (20:4 $\Delta^{5,8,11,14}$), EPA (20:5 $\Delta^{5,8,11,14,17}$) and DHA (22:6 $\Delta^{4,7,10,13,16,19}$) (Pereira et al., 2004). We were therefore interested in the production of C_{22}-PUFAs substrate for further ω-6 DPA and DHA production. To identify a gene coding for Δ^5-elongase involved C_{22}-PUFA biosynthesis, cDNA from reverse-transcribed mRNA from tissue of *Pavlova* sp. was amplified by PCR method. An amplification product containing the expected length (approximately 834 bp indicated by arrow) (Fig. 4) was cloned into pCR®2.1-TOPO® vector (Invitrogen) (Fig. 1) and verified by digested with *EcoR*I. After screening the five plasmid DNA (P1-5), two of those (P1-2) were released the approximately 800 bp sized gene of interest (I) from the approximately 4,000 bp sized of vector (V) (Fig. 5). Clone No. P2 was selected for sequencing both directions with M13 Forward and Reverse primers, yielding the plasmid PsELO5.

The open reading frame of the PsELO5 cDNA contains 834 bp and encodes a protein of 277 amino acids with a calculated molecular mass of 31.8 kDa (Fig. 6) Comparative sequence analysis with other Δ^5-elongases revealed that PsELO5 shared sequence homology with other algae *Ostreococcus tauri* (NCBI accession No. AAV67798) and *Thalassiorira pseudomonas* (NCBI accession No. AAV67800) (39% identity) that recognize C_{20} PUFA substrates (Meyer et al., 2004). Similar to other PUFA elongases, this protein contains two conserved motifs, a histidine box and a tyrosine box as well as 17 invariant amino acid residues that are conserved in the ELO family protein (Leonard et al., 2004). Hydropathy analysis revealed that PsELO5 is highly hydrophobic and is predicted to contain six transmembrane domains (Pereira *et al.*, 2004).

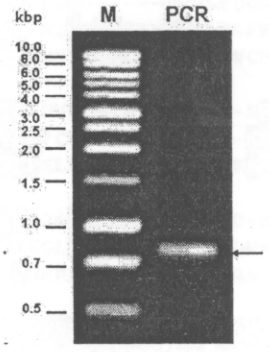

Fig. 4. PCR amplification product from cDNA of *Pavlova* sp. with PavELO5-FOR and PavELO5-REV primers analyzed on 1% agarose gel electrophoresis. The DNA sizes in kbp are indicated on the left. Arrow indicated the expected size.

M : 1 kbp DNA Ladder (Promega) PCR : PCR amplification product

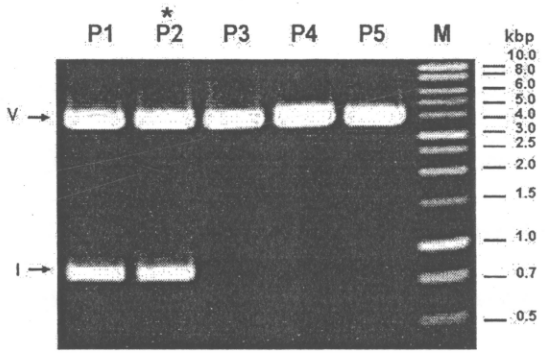

Fig. 5. Plasmid DNA isolated from *E. coli* cultures and digested with *EcoR*I. The DNA sizes in kbp are indicated on the left.

M : 1 kbp DNA Ladder (Promega) PCR : PCR amplification product

P1-5 : Plasmid DNA from clone No. 1-5

V : Approximately 4,000 bp sized pCR®2.1-TOPO vector (Invitrogen)

I : Approximately 800 bp sized gene of interested

* : Sequenced clone

Fig. 6. Comparison of the amino acid sequence of PsELO5 with other Δ^5-C_{20} PUFA elongases. Identical or conserved residues are shaded. The conserved histidine and tyrosine boxes are boxed. The 17 amino acid residues that are highly conserved in the ELO family are marked with asterisks. GenBank Accession Numbers of the sequences are AAV33630 (PsELO5), AAV67798 (OtELO5) and AAV67800 (TpELO5).

2. Functional analysis of the marine algae *Pavlova* sp. CCMP459 Δ^5-elongase gene in the moss *P. patens*

2.1 Construction of the entry vector

In this approach, PCR was used to amplify the plasmid PsELO5 with specific primers, PavELO-FOR and Pav-ELO5-REV. Fig. 7 shows the expected DNA size of approximately 800 bp. This amplification product was facilitated directional incorporation into pCR8®/GW/TOPO® entry vector (Invitrogen) (Fig. 2). After

digestion with *EcoR*I and sequencing, the resulting recombination plasmid from clone No. P4 had the PsELO5 (I) and approximately 3,000 bp (Fig. 7) and flanked by *att*L recombination sequences.

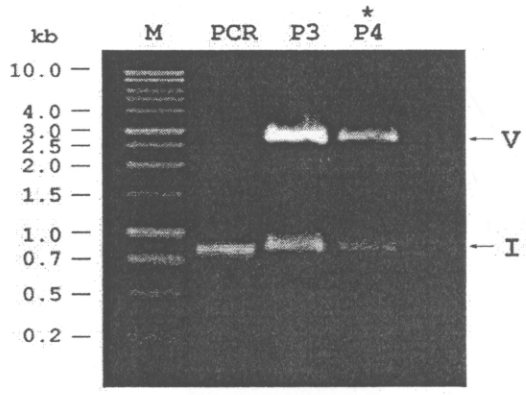

Fig. 7. Plasmids DNA isolated from *E. coli* cultures and digested with *EcoR*I. The DNA sizes in kbp are indicated on the left.

M : 1 kbp DNA Ladder (Promega) PCR : PCR amplification product

P3-4 : Plasmid DNA from clone No. 3 and 4

V : Approximately 3,000 bp sized pCR8/GW/TOPO vector (Invitrogen)

I : Approximately 800 bp sized gene of interested

* : Sequenced clone

2.2 Construction of the over-expression vector

Gateway-compatible plant destination vectors, pMDC32 and pMDC43 (ABRC) were used for protein over-expression of PsELO5 in *P. patens*. The recombination plasmid from clone No. P4 (section 2.1) was then separately recombined with *att*R sites of desired destination vectors (pMDC32) (Fig. 3) and verify by digested with *EcoR*I. After screening the plasmids DNA (P4.1, P4.2), both were released the approximately 800 bp sized gene of interest (I) from the approximately 10,000 and 300 bp sized of vector (V) (Fig. 8),

resulting in the over-expression constructs named pMDC32-PsELO5 5 containing a gene of *Pavlova* sp. Δ^5-elongase driven by a tandemly duplicated 35S promoter and nos terminator with hygromycin resistance (Hyg^r) selection cassette (Fig. 9), and finally transformed into moss protoplasts.

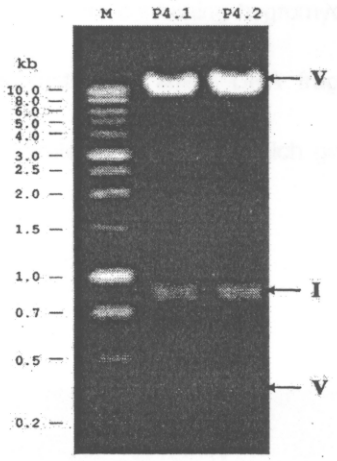

Fig. 8. Plasmids DNA isolated from *E. coli* cultures and digested with *EcoR*I. The DNA sizes in kbp are indicated on the left and right.

M : 1 kbp DNA Ladder (Promega)

P4.1,4.2 : Plasmid DNA from clone No. 4.1 and 4.2

V : Approximately 10,000 and 300 bp sized pMDC32 vector

I : Approximately 800 bp sized gene of interested

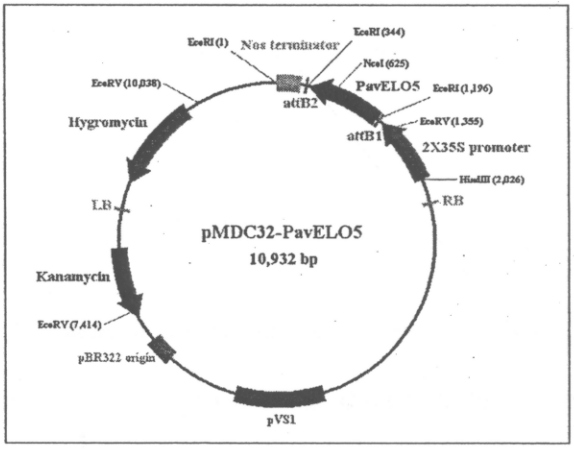

Fig. 9. Map of the over-expression vectors, pMDC32-PsELO5.

2.3 Protoplast and regeneration

After protoplasts transformation, strongly growing plants that still survived on the selection medium were defined as stable transformants. Wild type cannot grow on a selection medium whereas only stable transformants can survive on solid BCD medium containing hygromycin (Fig. 10). In the current study, PEG-mediated direct gene transfer of protoplasts with linear DNA fragment was utilized. The survival rates obtained were approximately 35% regenerated protoplasts, which gave 76% stable transformants from 122 picked regenerants on selective media.

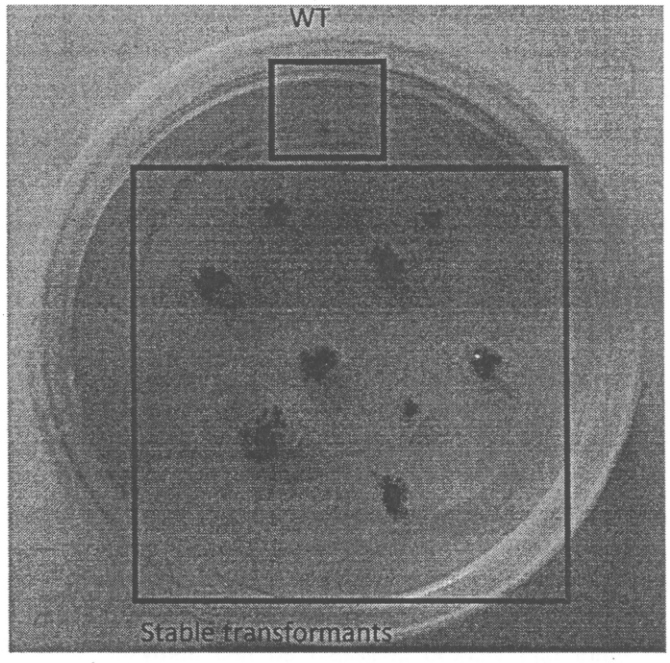

Fig. 10. Stable transgenic and wide-type *P. patens* grew on solid BCD medium containing 25 μg/ml hygromycin.

2.4 Phenotype analysis of *P. patens* by GC

To produce ADA in *P. patens*, it is necessary to express the Δ^5-elongase gene. Generally, various promoters are used to facilitate gene expression in plants. The CaMV 35S promoter is highly active in most of the transgenic plants. However, a tandemly duplicated CaMV 35S promoter showed a 6-fold higher

expression level of activity in *P. patens* than the normal CaMV 35S promoter (Horstmann et al., 2004). In the present study, *P. patens* was transformed with the construct that contains PsELO5 cDNA (Pereira et al., 2004) under the control of a tandemly duplicated CaMV 35S promoter. All stable transgenic lines (92 lines) from picked regenerants were screened by GC. Some of them produced ADA in the range of 0.04-0.42 mg/l. Independent transgenic plants were obtained with a trace of an additional peak compared with the control wild type (Fig. 11). The retention time of the additional peak (RT = 14.78 min) was identical to that of the methyl ester of authentic ADA. This compound, when investigated by GC-MS, displayed a molecular ion of 346 m/z, which is the expected molecular ion for methyl ADA (Fig. 12). Table 2 shows PUFA production from the six highest ADA production lines (C1-6). These results indicated that expression of the transgene PsELO5, under the control of a tandemly duplicated CaMV 35S promoter, was therefore successful. The highest production of ADA (0.42 mg/l) from the available endogenous ARA substrate was with the transgenic line C6. Although a low level of expression was obtained, other stronger promoters might be applied for higher level gene expression in *P. patens*, including complete rice actin (*Act*1) (Horstmann et al., 2004), wheat early-methionine labled (EM) (Knight et al., 1995), and maize ubiquitin promoters (Bezanilla et al., 2003). This study also confirmed that PsELO5 is specific only for C_{20} PUFA, without any activity toward C_{16} and C_{18} fatty acids, which is in agreement with the previous report (Pereira et al., 2004). However, ω-3 docosapentaenoic acid (DPA, 22:5 $\Delta^{7,10,13,16,19}$) was not detected with these transgenic lines; the absence of an ω-3 product is likely explained by the low level of substrate EPA naturally present in *P. patens*. Based on the specificity to substrate fatty acids, ELO-like enzymes can be classified broadly into three groups: one is specific to saturated and monounsaturated fatty acid (SFA and MUFA), another to PUFA of fixed chain-length ("single-step"), and the other is to PUFA with variable chain-lengths ("multi-step") (Meyer et al., 2004). PsELO5 showed specificity only to C_{20} PUFA (Pereira et al., 2004); this specificity also seen with algal Δ^5-elongases from *O. tauri*, *T. pseudonana* (Meyer et al., 2004), and liverwort *Marchantia polymorpha* (Kajikawa et al., 2006), and thus PsELO5 belongs to the second group (PUFA "single step").

The transformants were morphologically indistinguishable from the wild type plant. They gave rise to normal filamentous growth and gametophore formation.

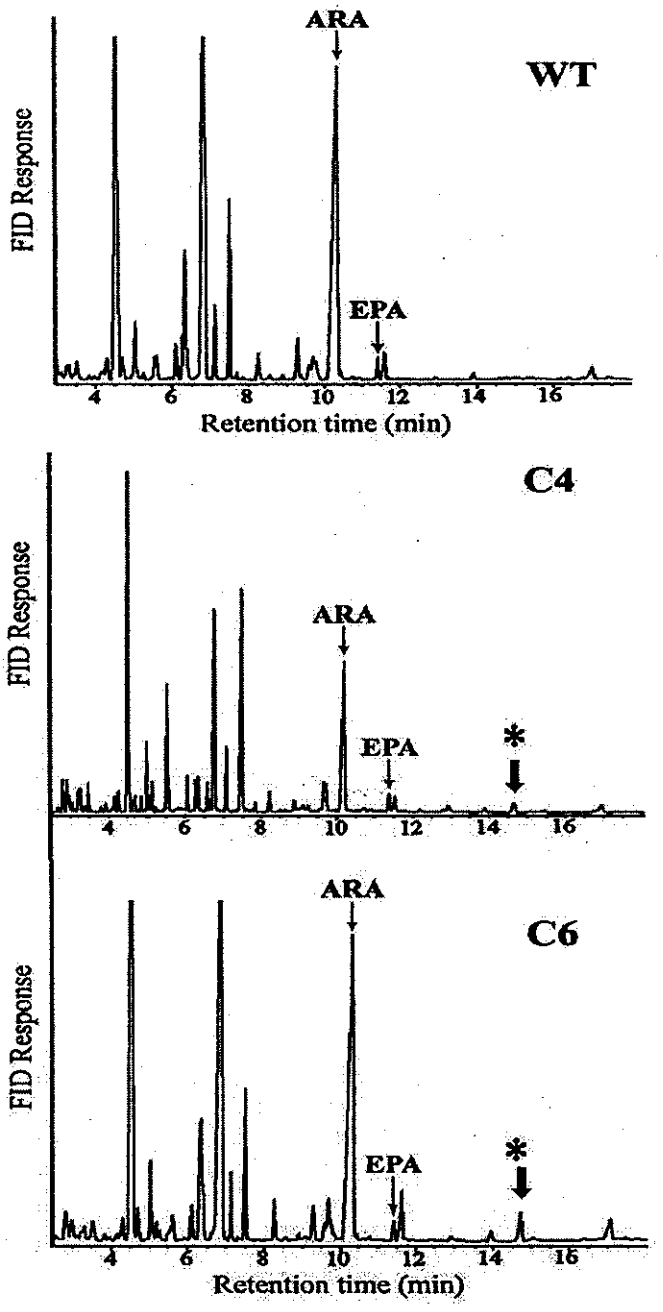

Fig. 11. Fatty acid profiles of *P. patens* wild type (WT) and the transgenic lines (C4 and C6). The FAMEs of the total lipids were analyzed by GC. The chromatograms WT, C4, and C6 show the FAMEs of the protonemata grown in liquid BCD medium for 14 days. ARA, archidonic acid; EPA, eicosapentaenoic acid. The additional peak which corresponds to the retention time of ADA is indicated by asterisk.

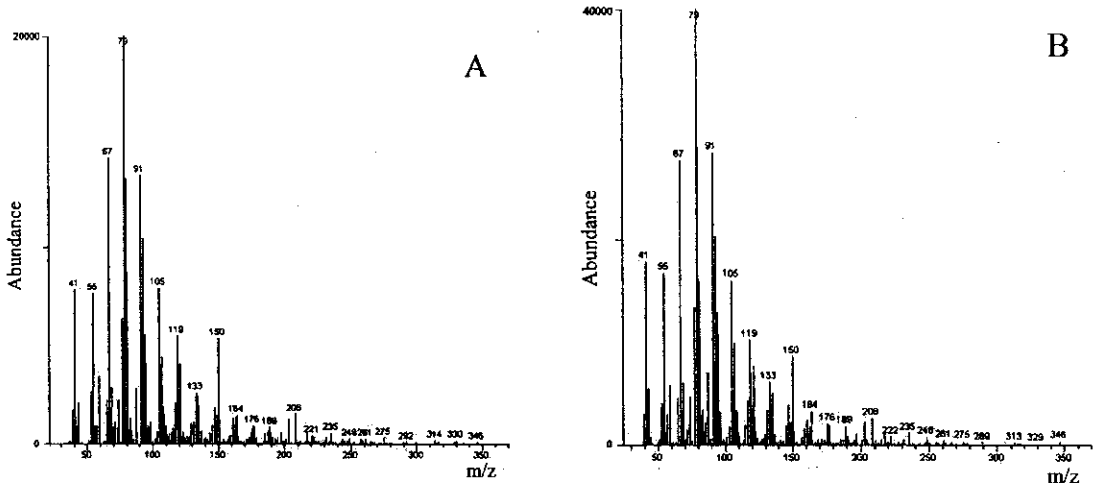

Fig. 12. GC mass spectrometric analysis of the methyl ester derivative of the novel peak identified in transgenic line (C6). A comparison is shown of the mass spectra of the novel peak (A) and the methyl ester of authentic ADA standard (B).

Table 2

Biomass and polyunsaturated fatty acid (PUFA) production from the wild type (WT) and transgenic *P. patens* grown in liquid BCD medium for 14 days.

Transgenic line No.	Biomass (g/L)	PUFA production (mg/l)							
		LA	GLA	ALA	EDA	DHGLA	ARA	EPA	ADA
1	4.07	27.14	3.81	16.21	0.41	5.45	16.52	0.47	0.17
2	3.83	24.67	2.88	12.12	0.45	1.61	17.32	0.48	0.15
3	3.77	21.17	2.98	10.88	0.41	1.61	12.63	0.58	0.15
4	5.17	22.76	3.45	15.57	0.53	2.13	20.28	0.96	0.22
5	4.40	33.65	3.95	18.56	0.66	7.02	18.20	0.61	0.20
6	4.83	41.33	2.88	8.67	2.05	1.45	37.25	1.22	0.42
WT	5.53	45.58	2.96	9.71	2.50	1.27	42.89	1.29	0.00

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; ADA, adrenic acid.

3. Molecular Analysis of *P. patens* by PCR and Southern blotting

The specific integration of the transformed DNA into *P. patens* was analyzed by PCR using genomic DNA from the six transgenic lines (C1-6) and the wild type (WT). PCR, with the primer pair PavELO5-FOR /Pav-ELO5-REV amplified a fragment of 0.8 kilobase pairs (kbp), which corresponded to PsELO5 cDNA. With the primer pair A/B, a fragment of 1.0 kbp corresponded to hygromycin resistance (Hyg^r) coding region from each of the six transgenic lines (C1-6), whereas the wild type (WT) gave negative results (Fig. 13). The length of bands suggested successful DNA integration, and indicated that PsELO5 and hygromycin resistance (Hyg^r) genes were transferred into the *P. patens* genome. However, a low number of copies of a transgene in a plant chromosome has a much lower incidence of instability (Jones, 2005). In addition, stably transformed transgenic *P. patens* usually have multiple numbers of integrated transgenes in the genome (Kamisugi et al., 2006).

To provide further evidence for DNA integration, the genomic DNA of the highest ADA production line (C6) and the wild type was analyzed by Southern blot hybridization with DIG-labled PsELO5 (ELO5) and hygromycin resistance (Hyg^r) probes. Blotting of DNA from the transgenic line digested with *Eco*RV generated strong signals of 2.2 and 2.6 kbp when hybridized with PsELO5 (ELO5) and hygromycin resistance (Hyg^r) probes (Fig. 14A), respectively, consistent with the sizes of fragment of relevant gene cassette used for transformation. Similarly, digestion with *Eco*RI produced high intensity bands of 0.9 and 9.7 kbp, respectively, which are the sizes of transgene fragments. These results confirmed that the C6 transgenic line has multiple copies of the expression construct inserted at *P. patens* genomic locus, but no insertion at ectopic sites.

This study has therefore described efficient gene modification in *P. patens*, and provided further evidence of the value of transforming PUFA synthesizing enzyme in plants to produce novel oils, including soybean, mustard and Arabidopsis (Hong et al., 2002; Fraser et al., 2004; Chen et al., 2006).

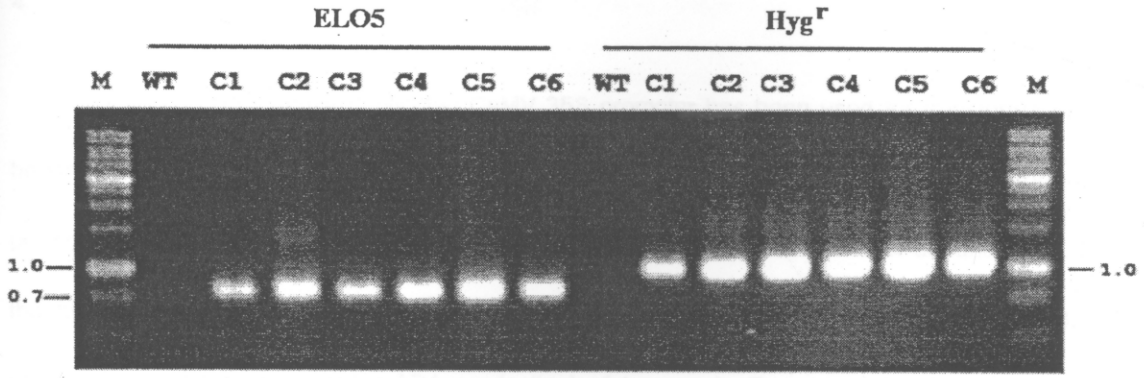

Fig. 13. Verification of PsELO5 (ELO5) and hygromycin resistance (Hyg^r) genes by PCR amplification of *P. patens* wild type (WT) and transgenic lines (C1-6). The DNA sizes in kbp are indicated on the left and right.

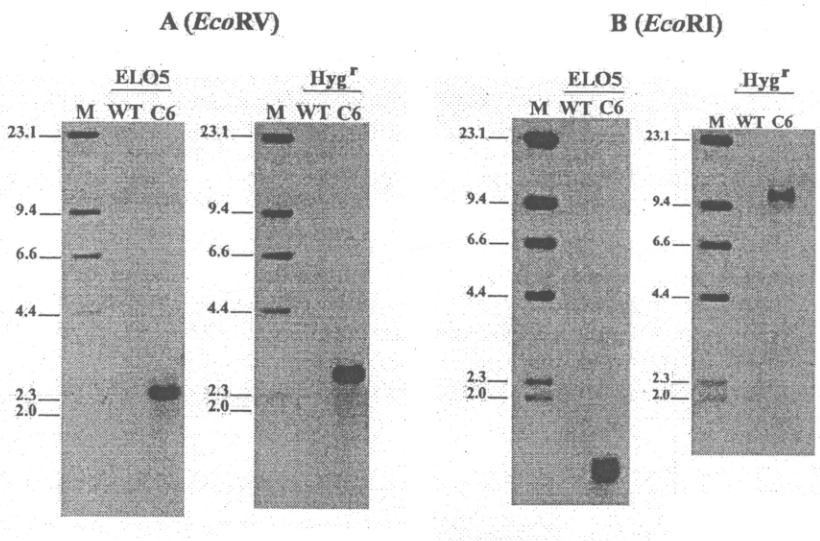

Fig. 14. Southern blotting of *P. patens* wild type (WT) and the transgenic line (C6). Genomic DNA (1 μg) from wild type and transgenic line (C6) was digested with *Eco*RV (A) and *Eco*RI (B) and hybridized with PsELO5 (ELO5) and hygromycon resistance (Hyg^r) probes. DNA sizes in kbp are indicated on the left.

4. Optimization of ADA production using RSM

Although a tandemly duplicated CaMV 35S promoter has been used for transformation, it might not be strong enough for gene expression (Zeidler et al., 1996; Horstmann et al., 2004). Apart from using stronger promoters, optimization of culture medium is a useful alternative tool for product enhancement. In this study, the highest production line (C6) was further optimized by the RSM experiments (Rao et al., 2000). The observed responses (biomass or ADA production) of transgenic *P. patens* (C6) for studying the effects of sucrose (A), KNO_3 (B) and glutamate (C) are summarized in Table 2. Tables 3 and 4 show the regression coefficient of each variable in terms of linear, quadratic and interaction along with *p*-value for biomass and ADA production, respectively. Coefficients with lower *p*-values ($p<0.05$) are more significant. The coefficient estimates and the corresponding *p*-values suggest that among test variables used in the study, sucrose (A), sucrose^2 (A^2), $KNO_3{}^2$ (B^2), glutamate^2 (C^2) were significant for both biomass and ADA production, whereas B (KNO_3) was significant only for biomass production. In addition, interactions between sucrose (A) and KNO_3 (B); and KNO_3 (B) and glutamate C) were also significant for biomass production while interaction between sucrose (A) and glutamate (C) was significant for ADA production. Other interactions were found to be insignificant.

The corresponding second-order regression equation provided levels of biomass and ADA production can be predicted by Eq. (2) and (3), respectively.

$$Y_{biomass(g/l)} = 16.87 - 0.43A + 0.93B - 0.17C - 0.74A^2 - 0.43B^2 - 0.46C^2 + 0.56AB - 0.22AC + 0.62BC \quad (2)$$

$$Y_{ADA(mg/l)} = 3.05 - 0.54A + 0.04B + 0.14C - 0.29A^2 - 0.26B^2 - 0.27C^2 + 0.02AB - 0.61AC + 0.13BC \quad (3)$$

The fit of the model for biomass and ADA production expressed by the coefficients of regression R^2 which were found to be 0.9299 and 0.9370, respectively, indicating that the second-order polynomial model Eq. (2) and (3) could explain 92.99 and 93.70% of the total variation, respectively. Isoresponse contour plots were generated to provide a better understanding of the main, as well as the interaction effects of two factors, while maintaining another factor constant at the central point level. Figures 15A-C depict the

significant interaction between two variables by keeping the other variables at their middle levels for biomass and ADA production. The maximum predicted value is indicated by the surface confined in the smallest ellipse in the contour diagram (Tanyildizi et al., 2005). The response surface having circular contour plot indicates no interaction whereas, an elliptical or saddle nature of the contour plot indicates significant interaction between the corresponding variables. It was evident from our plots that the higher concentration of glutamate (C), the lower concentration of sucrose (A) and the middle concentration of KNO_3 (B) were responsible for the enhancement of ADA production (Fig. 15C). The presence of organic carbon source is necessary for cell growth, however high sugar concentration results in an increase of osmotic pressure that inhibits cell growth and reduces PUFA productivity (Chiou et al., 2001). In addition, higher glutamate supplementation promoted ADA production, which is probably caused by acceleration of its substrate ARA metabolism (Lan et al., 2002; Yu et al., 2003).

The analysis of the regression equation and plots suggests that the optimum concentrations of the three variables would be, sucrose 22.06 g/l, KNO_3 1.00 g/l and glutamate 2.35 g/l; these conditions would lead to the production of 4.48 mg/l of ADA. The predicted production efficiency was verified by performing an experiment with the optimized concentrations in basal medium and the recovery of ADA was around 4.51 mg/l, which was very close to the predicted value, and 11 times higher than non-optimization experiment (0.42 mg/l).

Table 3

Analysis of variance (ANOVA) for response surface quadratic model of biomass production of the transgenic line (C6).

Factor[b]	Coefficient Estimate	S.E.[c]	SS[d]	DF[e]	*F*-value	*Prob* > *F* *P*-value
Model	16.87	0.22	40.58	9	14.74	0.0001
A	-0.43	0.14	3.00	1	9.82	0.0106
B	0.93	0.14	13.78	1	45.04	< 0.0001
C	-0.17	0.14	0.47	1	1.53	0.2449
A^2	-0.74	0.11	13.74	1	44.90	< 0.0001
B^2	-0.43	0.11	4.73	1	15.46	0.0028
C^2	-0.46	0.11	5.22	1	17.08	0.0020
AB	0.56	0.20	2.49	1	8.15	0.0171
AC	-0.22	0.20	0.38	1	1.23	0.2938
BC	0.62	0.20	3.04	1	9.94	0.0103

[b] A, sucrose; B, KNO_3; C, glutamate. [c] Standard error.

[d] Sum of square. [e] Degree of freedom.

Table 4

Analysis of variance (ANOVA) for response surface quadratic model of ADA production of the transgenic line (C6).

Factor[b]	Coefficient Estimate	S.E.[c]	SS[d]	DF[e]	*F*-value	*Prob* > *F* *P*-value
Model	3.05	0.11	12.00	9	16.52	< 0.0001
A	-0.54	0.071	4.64	1	57.47	< 0.0001
B	0.04	0.071	0.03	1	0.31	0.5905
C	0.14	0.071	0.29	1	3.64	0.0853
A^2	-0.29	0.057	2.06	1	25.49	0.0005
B^2	-0.26	0.057	1.69	1	21.00	0.0010
C^2	-0.27	0.057	1.86	1	23.09	0.0007
AB	-0.02	0.10	0.00	1	0.03	0.8568
AC	-0.61	0.10	2.97	1	36.77	0.0001
BC	0.13	0.10	0.14	1	1.69	0.2221

[b] A, sucrose; B, KNO_3; C, glutamate. [c] Standard error.

[d] Sum of square. [e] Degree of freedom.

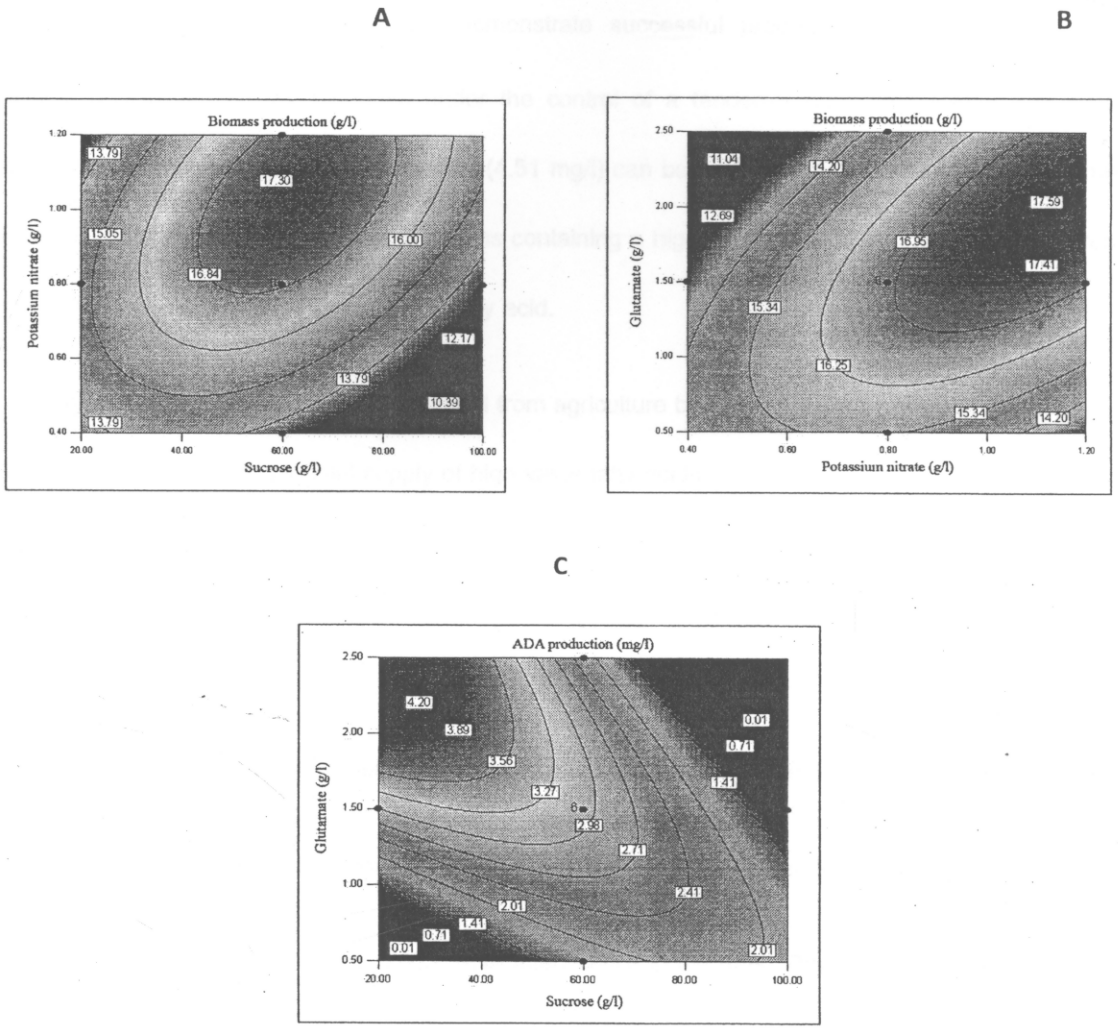

Fig. 15. Contour plots showing the effects of sucrose and KNO_3 (A), KNO_3 and glutamate (B) on biomass production; sucrose and glutamate (C) on ADA production of the transgenic line (C6). ADA, adrenic acid.

Conclusions

The experiments described here demonstrate successful production of ADA in *P. patens* by expression of *Pavlova* sp. Δ^5-elongase under the control of a tandemly duplicated CaMV 35S promoter. Furthermore, significantly higher levels of ADA (4.51 mg/l) can be achieved by statistical nutrient optimization via RSM. Therefore the cell cultures of *P. patens* containing a high quantity of ADA could be considered as a potential alternative source of this important fatty acid.

Nutritionally enhanced food oils derived from agriculture biotechnology offer great promise in providing a sustainable and plentiful supply of high value fatty acids.

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Outputs

1. Pichit Chodok, Akkharawit Kanjana-Opas, **Songsri Kaewsuwan**. Plackett-Burman design for evaluating the production of polyunsaturated fatty acids by *Physcomitrella patens*. Journal of the American Oil Chemists' Society. (Accepted: 13 December 2009). (Impact Factor 2008=1.504)

2. **Songsri Kaewsuwan** , Nantavan Bunyapraphatsara, David J. Cove, Ralph S. Quatrano, Pichit Chodok. High level production of adrenic acid in *Physcomitrella patens* using the algae *Pavlova* sp. Δ^5-elongase gene. Bioresource Technology. (Accepted: 30 December 2009). (Impact Factor 2008=4.453)

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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* L_{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:

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

For GLA production:

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

For ALA production:

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

For EDA production:

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

For DHGLA production:

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

For ARA production:

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

For EPA production:

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

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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