J Am Oil Chem Soc (2011) 88:619–629
DOI 10.1007/s11746-010-1710-y

ORIGINAL PAPER

Optimization of Biomass and Arachidonic Acid Production by *Aureispira maritima* Using Response Surface Methodology

Sutanate Saelao · Akkharawit Kanjana-Opas · Songsri Kaewsuwan

Received: 24 March 2010 / Revised: 5 September 2010 / Accepted: 24 October 2010 / Published online: 19 November 2010

Abstract Statistically based experimental designs, based on the Plackett–Burman protocol, were applied to the optimization of biomass and arachidonic acid (ARA) production in *Aureispira maritima* shake-flask cultures. Tryptone and culture temperature were identified to have a significant effect on biomass production, whereas ARA production was only affected significantly by the pH and agitation rate. These four factors were subsequently optimized using response surface methodology. The validity of the optimum conditions was verified by separate experiments in which biomass and ARA yield were increased 4.02-fold (2.05 g l^{-1}) and 3.59-fold (21.50 mg g^{-1}), respectively, in 3-day fermentations. Under non-optimized culture conditions the corresponding values were 0.51 g l^{-1} and 5.99 mg g^{-1}, respectively. The results suggest that *A. maritima* might be a potential strain for further large scale investigations to determine whether this bacterium might be suitable for commercial production of ARA. To our knowledge, this is the first report of the statistically optimization of biomass and ARA production from the marine gliding bacterium *A. maritima*.

S. Saelao · A. Kanjana-Opas
Department of Industrial Biotechnology,
Faculty of Agro-Industry, Prince of Songkla University,
Hat Yai, Songkhla 90112, Thailand

S. Kaewsuwan (✉)
Marine Natural Products Research Unit,
Department of Pharmacognosy and Pharmaceutical Botany,
Faculty of Pharmaceutical Sciences,
Prince of Songkla University, Hat Yai,
Songkhla 90112, Thailand
e-mail: songsri.k@psu.ac.th; ksongsri@yahoo.com

Keywords *Aureispira maritima* · Arachidonic acid · Plackett–Burman design · Response surface methodology · Central composite design

Introduction

Arachidonic acid (5,8,11,14-*cis*-eicosatetraenoic acid, ARA) is an essential polyunsaturated fatty acid (PUFA) in human nutrition. Since it is a biogenetic precursor of the biologically active prostaglandins, thromboxanes, prostacyclins and leukotrienes, it possesses various physiological activities [1]. As a component of mature human milk, ARA is necessary for the neurological and neurophysiological development of both term [2] and preterm infants [3]. Many expert organizations, including the Food and Agriculture Organization/World Health Organization (FAO/WHO), recommend that ARA should be supplied as a supplement in infant feed formulas [4]. ARA has also found wide application in medicine, pharmacology, the cosmetic and food industries, and in agriculture [5].

Animal viscera, particularly porcine liver, are conventional sources of ARA. However, the ARA yield obtained is very low, and therefore difficult to industrialize. The production of ARA by microorganisms has therefore been gaining more interest. ARA production in bacteria, microalgae, and fungi has been studied [6–23], and it has been suggested that microbially produced ARA could be a convenient substitute for conventionally produced ARA. Production of ARA in microorganisms, such as microalgae (e.g. *Parietochloris incisa*, *Porphyridium cruentum*) and fungi (e.g. *Mortierella alpina*), appeared to be optimal under conditions of slow growth. However, slow growth rates are undesirable for a commercial productivity perspective [13, 24]. Bacteria cell cultures, on the other hand,

might offer an attractive alternative approach for the production of ARA.

Recently, a novel marine gliding bacterium, *Aureispira maritima* sp. nov., was isolated from marine barnacle debris collected from the southern coastline of Thailand. A phylogenetic analysis based on 16S rRNA gene sequences showed that this isolate formed a distinct lineage within the genus *Aureispira* in the family Saprospiraceae (phylum Bacteroidetes). This gliding bacterium contains a large amount of ARA (43.6% of total fatty acids). *A. maritima* can easily and rapidly be grown under axenic conditions in an easy to prepare medium at the optimum growth temperature of 30 °C and in the pH range of 6.0–8.0 [9]. Despite the increasing number of reports on isolation of novel marine gliding bacteria, little is known about their safety, hence great emphasis on safety assessment is further required. However, this strain shows optimal growth at 30 °C, whereas no growth occurs at 17 or 37 °C [9]. This might suggest that *A. maritima* reveals no pathogenic potential toward warm-blooded animals. It was therefore felt appropriate to conduct a systematic study to evaluate its potential as an alternative viable source for the production and commercialization of ARA.

The conventional approach for optimizing metabolite production normally involves investigation of one factor at a time, while keeping the others constant. This approach is tedious and lacks completeness for the prediction of responses under untested sets of variables. Furthermore, it does not demonstrate the interaction among all the factors affecting the responses. On the other hand, statistical methods, i.e., factorial experimental design, make possible the simultaneous study of numerous factors. These methods also allow the study of interactive effects of such factors together, and facilitate the prediction of the responses to values not yet tested in the experiment [25]. One of the statistical designs for the screening of the independent variables is the Plackett–Burman design. This protocol has frequently been used for screening the key factors affecting growth and productivity in cultures. It is a two factorial design, which offers the screening of a large number of independent factors in a small number of experiments [26–29]. Factors chosen for study could be either nutritional components or environmental conditions. It is only after the key factors have been screened that the optimization process can begin. Response surface methodology (RSM) is widely used for determination of the optimum values for product enhancement [30–32].

In this study, 15 factors affecting biomass and ARA production by the marine bacterium *A. maritima* TISTR 1715 were screened for their effects on biomass and ARA production using the Plackett–Burman design. A central composite design (CDD) was then applied to identify the optimum levels of the four most significant variables.

Experimental Procedures

Microorganism and Medium

The marine gliding bacterium, *A. maritima* TISTR 1715, was obtained from the Thailand Institute of Scientific and Technological Research (TISTR, Bangkok, Thailand), and was used throughout this study. The cells were cultured in 100-mm sterile petri dishes containing 20 ml of solid SAP 2 medium containing g l^{-1} of the following: yeast extract, 1; tryptone, 1; agar, 15; NaCl, 15; KCl, 0.35; $MgCl_2 \cdot 6H_2O$, 5.4; $MgSO_4 \cdot 7H_2O$, 2.7 and $CaCl_2 \cdot 2H_2O$, 0.5 at pH 8.0. The cells were kept at 25 °C and subcultured once every 7 days [8].

Inoculum Preparation

The cells grown on the solid medium were aseptically harvested and cultivated for 2 days in 250-ml sterile shake-flasks containing 100 ml of liquid SAP 2 medium at 25 °C in an orbital shaker (KMC-8480 SR-L, LMS, Japan) set at 200 rpm. After 2 days of growth, 10 ml of the homogenous cell suspension was transferred to 90 ml of fresh SAP 2 medium and further cultivated overnight until an optical density of 1.0 (660 nm) was achieved. These cultured cells were then used to inoculate the final production liquid medium at a 10% (v/v) level.

Dry Cell Weight Determination (Biomass)

For dry cell weight (DCW) determination, cell samples in the liquid media were harvested by centrifugation (WS-EPP-5403, Eppendorf, Hamburg, Germany) at 8,000 rpm for 15 min, and washed twice with distilled water. The fresh cells were then freeze dried overnight to a constant weight.

Fatty Acid Analysis

Total fatty acids from freeze dried cell samples (15 mg) were transmethylated with 2 ml of 2.5% sulfuric acid in methanol at 85 °C for 30 min. Fatty acid methyl esters (FAME) were then extracted into 1 ml of heptane, the organic layer evaporated to dryness with a stream of oxygen free nitrogen gas, and the residue dissolved in 500 µl of heptane before gas chromatography (GC) [33]. GC analysis of FAME was conducted using an HP 6890 Series gas chromatograph equipped with an HP-INNOWax capillary column (0.25 mm × 30 m × 0.25 µM), a flame ionization detector, using helium as the carrier gas. An aliquot (2 µl) of each sample extract was injected onto the GC column using the injector in the split mode. The initial column temperature was 185 °C (0.5 min) and this was

increased at a rate of 3.5 °C min^{-1} to 235 °C (14.3 min), and then maintained at 235 °C for 1.0 min. ARA methyl ester was identified by comparison with the retention time of the methyl ester of an authentic ARA standard (Nu-Check-Prep, Elysian, MN, USA). The amount of fatty acid was estimated from peak areas compared with calibration standards.

Experimental Design and Data Analyses

Plackett–Burman Design

Plackett–Burman design was employed for screening the important factors influencing production of biomass and ARA by cell cultures of *A. maritima* TISTR 1715. The variables evaluated are listed in Table 1. These include twelve nutritional (glucose, fructose, sucrose, KNO_3, NH_4NO_3, yeast extract, tryptone, NaCl, KCl, $MgCl_2{\cdot}6H_2O$, $MgSO_4{\cdot}7H_2O$ and $CaCl_2{\cdot}2H_2O$), three physical parameters (initial pH, temperature and agitation speed), and four dummy or unassigned variables. As shown in Table 2, the design matrix, developed according to the design of Wen and Chen [34], consists of 15 variables with 20 runs (N), with D_1 to D_4 being the dummy variables employed to evaluate the standard errors (SE) of the experiments. The inoculum prepared (see inoculum preparation section) was used to inoculate (10% v/v) in 250 ml sterile shake-flasks containing the total volume of 100 ml final production liquid medium. Incubation was for 3 days at 25 °C in an orbital shaker (Innova-4230, New Brunswick, USA), before determination of DCW and ARA production.

Table 1 Variables studied for biomass and ARA production by cell cultures of *A. maritima* TISTR 1715 using the Plackett–Burman statistical design technique

Code	Variable	High level (+)	Low level (−)
A	Glucose (g l^{-1})	10	1
B	Fructose (g l^{-1})	10	1
C	Sucrose (g l^{-1})	10	1
D	KNO_3 (g l^{-1})	5	0.5
E	NH_4NO_3 (g l^{-1})	5	0.5
F	Yeast extract (g l^{-1})	5	0.5
G	Tryptone (g l^{-1})	5	0.5
H	Initial pH (–)	8.0	6.0
I	Temperature (°C)	25	15
J	Agitation speed (rpm)	200	100
K	NaCl (g l^{-1})	15.0	7.5
L	KCl (g l^{-1})	0.35	0.18
M	$MgCl_2{\cdot}6H_2O$ (g l^{-1})	5.40	2.70
N	$MgSO_4{\cdot}7H_2O$ (g l^{-1})	2.70	1.40
O	$CaCl_2{\cdot}2H_2O$ (g l^{-1})	0.50	0.25
D_1	Dummy 1 (–)	–	–
D_2	Dummy 2 (–)	–	–
D_3	Dummy 3 (–)	–	–
D_4	Dummy 4 (–)	–	–

Statistical analyses were employed to identify the variables that had significant effects on the responses (i.e. biomass and ARA production). The effect of each variable ($E_{(X_i)}$) on each response was determined by subtracting the average response of the low level (R_i^-) from that of the high level (R_i^+) using the following standard equation:

$$E_{(X_i)} = \frac{2[\sum R_i^+ - \sum R_i^-]}{N} \quad (1)$$

where N is total number of experiments or runs ($N = 20$).

The effects of the dummy variables were used to calculate SE as follows:

$$\mathrm{SE} = \sqrt{\frac{\sum (E_\mathrm{d})^2}{n}} \quad (2)$$

where E_d is the effect of each dummy variable and n is the number of dummy variables ($n = 4$). The significance of each variable was determined using the Student's t test as follows:

$$t \text{ value} = \frac{E_{(X_i)}}{\mathrm{SE}} \quad (3)$$

The variables at or above the 95.0% confidence level ($p < 0.05$) were considered to have significant effects on the responses (biomass or ARA production).

Central Composite Design

Once the variables having the greatest influence on the responses were identified by the Plackett–Burman design, RSM was used to determine the optimum level of parameters for optimization of growth and ARA production. A 2^4 factorial central composite design (CCD) with 8 points, 16 quadrant points and 6 replicates at the center points leading to a table of 30 sets of experiments, was used to optimize the tryptone (X_1), initial pH (X_2), agitation speed (X_3) and temperature (X_4) for high biomass and ARA production. Coding of variables was carried out according to the following equation:

$$x_i = (X_i - X_\mathrm{cp})/\Delta X_i \quad i = 1, 2, 3, \ldots, k \quad (4)$$

where x_i is the dimensionless value of an independent variable, X_i is the real value of an independent variable, X_cp is the real value of an independent variable at the central point, and ΔX_i is the step change of variable i.

The relationship of the independent variables and the responses (biomass and ARA production) was calculated by the second-order polynomial equation:

Table 2 Plackett–Burman design matrix for evaluating variables influencing biomass and ARA production by cell cultures of *A. maritima* TISTR 1715

Run	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	D_1	D_2	D_3	D_4	Biomass production[a]	ARA production[b]
1	+	+	−	−	+	+	+	+	−	+	−	+	−	−	−	−	+	+	−	0.37	0.44
2	−	+	+	−	−	+	+	+	+	−	+	−	+	−	−	−	−	+	+	1.95	4.85
3	+	−	+	+	−	−	+	+	+	+	−	+	−	+	−	−	−	−	+	1.71	3.96
4	+	+	−	+	+	−	−	+	+	+	+	−	+	−	+	−	−	−	−	0.26	1.23
5	−	+	+	−	+	+	−	−	+	+	+	+	−	+	−	+	−	−	−	0.44	0.09
6	−	−	+	+	−	+	+	−	−	+	+	+	+	−	+	−	+	−	−	1.00	1.35
7	−	−	−	+	+	−	+	+	−	−	+	+	+	+	−	+	−	+	−	0.38	3.79
8	−	−	−	−	+	+	−	+	+	−	−	+	+	+	+	−	+	−	+	1.18	4.31
9	+	−	−	−	−	+	+	−	+	+	−	−	+	+	+	+	−	+	−	1.45	0.10
10	−	+	−	−	−	−	+	+	−	+	+	−	−	+	+	+	+		+	0.86	4.29
11	+	−	+	−	−	−	−	+	+	−	+	+	−	−	+	+	+	+	−	0.54	4.34
12	−	+	−	+	−	−	−	−	+	+	−	+	+	−	−	+	+	+	+	0.28	0.34
13	+	−	+	−	+	−	−	−	−	+	+	−	+	+	−	−	+	+	+	0.34	2.77
14	+	+	−	+	−	+	−	−	−	−	+	+	−	+	+	−	−	+	+	0.11	2.53
15	+	+	+	−	+	−	+	−	−	−	−	+	+	−	+	+	−	−	+	0.13	2.26
16	+	+	+	+	−	+	−	+	−	−	−	−	+	+	−	+	+	−	−	0.13	4.00
17	−	+	+	+	+	−	+	−	+	−	−	−	−	+	+	−	+	+	−	1.08	2.99
18	−	−	+	+	+	+	−	+	−	+	−	−	−	−	+	+	−	+	+	0.28	1.31
19	+	−	−	+	+	+	+	−	+	−	+	−	−	−	−	+	+	−	+	0.43	2.53
20	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	0.34	2.82

The four variables (D_1–D_4) are designed as "dummy variables". Biomass and ARA production under non-optimized conditions were found to be 0.51 ± 0.02 g l^{-1} and 5.99 ± 0.53 mg g^{-1}, respectively

+, high level; −, low level (see Table 1 for values); *ARA* arachidonic acid

[a] Represents mean of the responses for biomass production (g l^{-1}) based on three separate experiments

[b] Represents mean of the responses for ARA production (mg g^{-1}) based on three separate experiments

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{i=1}^{k} \beta_{ij} x_i x_j \quad (5)$$

where Y is the predicted response, β_0 is the model constant, β_i is the linear coefficient, β_{ii} is the quadratic coefficient, β_{ij} is the interaction coefficient, and k is number of factors. 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., Minneapolis, MN, USA). In addition, response surface and two-dimension contour plots were constructed for visual observation of the trend of maximum responses, and the interaction effects of the significant variables on the responses.

Experimental Validation of the Optimized Conditions

Two experiments were conducted in 250-ml shake-flasks containing the total volume of 100 ml final production liquid medium with 10% (v/v) inoculum as described above to verify the validity of the optimal conditions for maximum biomass (Experiment A) and ARA (Experiment B) production (Table 6). These experimentally validated optimal conditions were chosen for subsequent time course studies. Cell growth and ARA production curves of *A. maritima* TISTR 1715 were investigated over a period of 7 days using the optimal conditions. Biomass and ARA production were assayed every 12–24 h. Each of these experiments was carried out in triplicate, and the data calculated as means ± SE ($n = 3$).

Results and Discussion

Evaluation of Important Factors Affecting Biomass and ARA Production

Under non-optimized conditions, biomass and ARA production by *A. maritima* TISTR 1715 cultivated in liquid SAP 2 medium for 3 days were found to be 0.51 ± 0.02 g l^{-1} DCW and 5.99 ± 0.53 mg g^{-1} ARA. Since nutrients and physical parameters play an important role in the growth of microorganisms and their fatty acid

Table 3 Statistical analyses of Plackett–Burman design showing the calculated regression coefficient, *t* and *p* values, and confidence level for each variable for biomass and ARA production by cell cultures of *A. maritima* TISTR 1715

	Biomass production				ARA production			
	Coefficient	*t* value	*p* value	Confidence (%)	Coefficient	*t* value	*p* value	Confidence (%)
Glucose	−0.012	−1.229	0.286	71.4	−0.099	−0.372	0.728	27.2
Fructose	−0.010	−1.081	0.340	66.0	−0.213	−0.804	0.466	53.4
Sucrose	0.010	1.036	0.359	64.1	0.278	1.048	0.354	64.6
KNO_3	−0.010	−1.023	0.364	63.6	−0.111	−0.419	0.696	30.4
NH_4NO_3	−0.017	−1.850	0.138	86.2	−0.343	−1.293	0.266	73.4
Yeast extract	0.007	0.747	0.497	50.3	−0.364	−1.374	0.241	75.9
Tryptone	0.027	2.904	0.044	**95.6**	0.141	0.533	0.622	37.8
Initial pH	0.010	1.092	0.336	66.4	0.735	2.776	0.050	**95.0**
Temperature	0.027	2.852	0.046	**95.4**	−0.040	−0.151	0.887	11.3
Agitation speed	0.004	0.388	0.718	28.2	−0.927	−3.499	0.025	**97.5**
NaCl	−0.003	–0.339	0.752	24.8	0.261	0.987	0.380	62.0
KCl	−0.005	–0.526	0.627	37.3	−0.175	−0.660	0.545	44.5
$MgCl_2{\cdot}6H_2O$	0.005	0.504	0.641	33.9	−0.016	−0.060	0.955	0.05
$MgSO_4{\cdot}7H_2O$	0.011	1.126	0.323	67.7	0.367	1.387	0.238	76.2
$CaCl_2{\cdot}2H_2O$	0.003	0.269	0.801	19.9	−0.043	−0.163	0.879	12.1

$R^2 = 0.89$. The bold values indicate the significance at or above the 95.0% confidence level

ARA arachidonic acid

production, the Plackett–Burman design is a powerful technique for screening important variables. This technique has been used successfully by many investigators [34, 35]. Therefore, to enhance growth of *A. maritima* TISTR 1715, and increase ARA production, the Plackett–Burman design was firstly adopted to select the most significant factors. The data presented in Table 2 indicated that there was a wide variation in responses for biomass and ARA production, from 0.11 to 1.95 g l^{-1} and 0.09 to 4.85 mg g^{-1}, respectively, in the twenty trials. This variation reflected the significance of factors on the responses.

The statistical analysis of the data from the Plackett–Burman design experiments are shown in Table 3. Among fifteen variables studied, one nutritional factor (tryptone) and one physical factor (temperature) significantly affected the growth of cell cultures of *A. maritima* TISTR 1715, with confidence levels >95.0%. On the other hand, only physical parameters, including initial pH and agitation speed, were critically important for ARA production. The models of biomass and ARA production had coefficients of determination (R^2) of 0.89, which can explain 89% variability of data. Model terms having *p* values <0.05 are considered significant, and hence, tryptone, temperature, initial pH and agitation speed, having the lowest *p* values, were regarded as the most significant variables. This was subsequently tested experimentally using the RSM protocol. The exact optimal values of the individual factors are still unknown at this stage, but were determined by the subsequent CCD experiments.

Optimization of Screened Factors and an Analysis of Their Interactions

Based on the above screening tests by Plackett–Burman design, tryptone (X_1), initial pH (X_2), agitation speed (X_3) and temperature (X_4) exhibited high impact on biomass and ARA production by *A. maritima* TISTR 1715. A RSM by CCD was thereafter used to determine the optimum levels of these four significant parameters. The other components including, yeast extract, NaCl, KCl, $MgCl_2{\cdot}6H_2O$, $MgSO_4{\cdot}7H_2O$, $CaCl_2{\cdot}2H_2O$ were set to their level based on the SAP 2 medium [8]; meanwhile sucrose (10 g l^{-1}) was used as the carbon source. The observed responses (biomass and ARA production) are summarized in Table 4, and the results therein were analyzed by standard ANOVA as shown in Table 5.

A second-order polynomial mathematical model, incorporating the different interactions of low and high levels of different factors, is proposed with respect to growth (Eq. 6) and ARA production (Eq. 7).

$$\begin{aligned} Y_{\text{Biomass}\,(\text{g}\,\text{l}^{-1})} = {} & -19.4787 + 0.3570X_1 + 3.2544X_2 \\ & + 0.0285X_3 + 0.5132X_4 - 0.0148X_1X_2 \\ & + 0.0014X_1X_3 - 0.0024X_1X_4 \\ & + 0.0014X_2X_3 - 0.0020X_2X_4 \\ & - 0.0004X_3X_4 - 0.0259X_1^2 - 0.2147X_2^2 \\ & - 0.0001X_3^2 - 0.0097X_4^2 \end{aligned} \quad (6)$$

Table 4 Central composition design matrix for four variables in real and coded units (parentheses), along with the experimental values of the responses (biomass and ARA production) by cell cultures of *A. maritima* TISTR 1715

Run	Tryptone (g l^{-1}) (X_1)	Initial pH (X_2)	Agitation speed (rpm) (X_3)	Temperature (°C) (X_4)	Biomass production[a] (g l^{-1})	ARA production[b] (mg g^{-1})
1	2.5 (−1)	7.0 (−1)	70 (−1)	20 (−1)	0.91	14.99
2	7.5 (+1)	7.0 (−1)	70 (−1)	20 (−1)	0.83	8.48
3	2.5 (−1)	9.0 (+1)	70 (−1)	20 (−1)	1.00	12.50
4	7.5 (+1)	9.0 (+1)	70 (−1)	20 (−1)	0.69	12.26
5	2.5 (−1)	7.0 (−1)	170 (+1)	20 (−1)	1.03	14.53
6	7.5 (+1)	7.0 (−1)	170 (+1)	20 (−1)	1.99	14.65
7	2.5 (−1)	9.0 (+1)	170 (+1)	20 (−1)	0.99	11.51
8	7.5 (+1)	9.0 (+1)	170 (+1)	20 (−1)	2.04	15.81
9	2.5 (−1)	7.0 (−1)	70 (−1)	30 (+1)	0.49	8.07
10	7.5 (+1)	7.0 (−1)	70 (−1)	30 (+1)	0.97	2.75
11	2.5 (−1)	9.0 (+1)	70 (−1)	30 (+1)	0.48	10.64
12	7.5 (+1)	9.0 (+1)	70 (−1)	30 (+1)	0.33	5.37
13	2.5 (−1)	7.0 (−1)	170 (+1)	30 (+1)	0.56	7.79
14	7.5 (+1)	7.0 (−1)	170 (+1)	30 (+1)	0.84	9.86
15	2.5 (−1)	9.0 (+1)	170 (+1)	30 (+1)	0.65	4.88
16	7.5 (+1)	9.0 (+1)	170 (+1)	30 (+1)	1.15	8.56
17	0.8 (−1.68)	8.0 (0)	120 (0)	25 (0)	0.67	10.10
18	9.2 (+1.68)	8.0 (0)	120 (0)	25 (0)	1.91	15.67
19	5.0 (0)	6.3 (−1.68)	120 (0)	25 (0)	1.95	13.07
20	5.0 (0)	9.7 (+1.68)	120 (0)	25 (0)	0.34	1.83
21	5.0 (0)	8.0 (0)	36 (−1.68)	25 (0)	0.40	7.89
22	5.0 (0)	8.0 (0)	204 (+1.68)	25 (0)	1.25	13.64
23	5.0 (0)	8.0 (0)	120 (0)	16.6 (−1.68)	1.54	19.43
24	5.0 (0)	8.0 (0)	120 (0)	33.4 (+1.68)	0.59	5.79
25	5.0 (0)	8.0 (0)	120 (0)	25 (0)	1.96	14.24
26	5.0 (0)	8.0 (0)	120 (0)	25 (0)	1.80	11.47
27	5.0 (0)	8.0 (0)	120 (0)	25 (0)	1.89	12.63
28	5.0 (0)	8.0 (0)	120 (0)	25 (0)	1.90	14.51
29	5.0 (0)	8.0 (0)	120 (0)	25 (0)	1.83	12.71
30	5.0 (0)	8.0 (0)	120 (0)	25 (0)	1.93	13.87

ARA arachidonic acid

[a] Represents the mean of the responses for biomass production (g l^{-1}) based on three separate experiments

[b] Represents the mean of the responses for ARA production (mg g^{-1}) based on three separate experiments

$$\begin{aligned} Y_{\text{ARA (mg g}^{-1})} = & -99.1200 - 3.6031X_1 + 31.0034X_2 \\ & + 0.1813X_3 - 0.2744X_4 + 0.3027X_1X_2 \\ & + 0.0138X_1X_3 - 0.0125X_1X_4 \\ & - 0.0157X_2X_3 + 0.0194X_2X_4 \\ & - 0.0010X_3X_4 - 0.0116X_1^2 - 1.9984X_2^2 \\ & - 0.0003X_3^2 - 0.0068X_4^2 \end{aligned} \quad (7)$$

The ANOVA of the quadratic regression models for growth and ARA production indicated the "Prob>F" <0.0006, and 0.0020, respectively, which implied that both models confirm the significance of experimental data. Model coefficients estimated by regression analysis for each variable are also shown in Table 5. The significance of each coefficient was determined by p values. The smaller the p values, the higher the significance of the corresponding coefficient [36, 37]. According to the present model: the three linear (tryptone, X_1; agitation speed, X_3; temperature, X_4) and the three squared model (initial pH, X_2; agitation speed, X_3; temperature, X_4) terms were significant for growth, whereas only temperature (X_4, linear) and initial pH (X_2, squared model) were significant for ARA production. In addition, interactions between tryptone (X_1) and agitation speed (X_3) were significant for

Table 5 Analysis of variance (ANOVA) for response surface quadratic model of biomass and ARA production by cell cultures of *A. maritima* TISTR 1715

Variable[a]	Biomass production						ARA production					
	Coefficient estimate	SE	SS	*df*	*F* value	*p* value (Prob > *F*)	Coefficient estimate	SE	SS	*df*	*F* value	*p* value (Prob > *F*)
Model	−19.4787	0.130	8.970	14		**<0.0006**	−99.1200	0.97	409.600	14		**0.0020**
X_1	0.3570	0.070	1.060	1	10.10	**0.0062**	−3.6031	0.53	0.220	1	0.04	0.8536
X_2	3.2544	0.070	0.430	1	4.14	0.0600	31.0034	0.53	15.770	1	2.58	0.1289
X_3	0.0285	0.070	1.130	1	10.78	**0.0050**	0.1813	0.53	22.680	1	3.71	0.0731
X_4	0.5132	0.070	1.420	1	13.53	**0.0022**	−0.2744	0.53	224.53	1	36.75	**0.0001**
X_1X_2	−0.0148	0.081	0.022	1	0.21	0.6541	0.3027	0.62	9.160	1	1.50	0.2396
X_1X_3	0.0014	0.081	0.500	1	4.76	**0.0455**	0.0138	0.62	47.260	1	7.74	**0.0140**
X_1X_4	−0.0024	0.081	0.150	1	0.14	0.7139	−0.0125	0.62	0.390	1	0.06	0.8038
X_2X_3	0.0014	0.081	0.073	1	0.70	0.4158	−0.0157	0.62	9.840	1	1.61	0.2237
X_2X_4	−0.0020	0.081	0.002	1	0.015	0.9040	0.0194	0.62	0.150	1	0.03	0.8773
X_3X_4	−0.0004	0.081	0.170	1	1.65	0.2180	−0.0010	0.62	1.000	1	0.16	0.6916
X_1^2	−0.0259	0.080	0.430	1	4.08	0.0617	−0.0116	0.61	0.085	1	0.01	0.9074
X_2^2	−0.2147	0.080	0.750	1	7.17	**0.0172**	−1.9984	0.61	65.050	1	10.65	**0.0052**
X_3^2	−0.0001	0.080	1.750	1	16.69	**0.0010**	−0.0003	0.61	11.060	1	1.18	0.1984
X_4^2	−0.0097	0.080	0.960	1	9.20	**0.0084**	−0.0068	0.61	0.480	1	0.08	0.7836

The bold values indicate the significance at or above the 95.0% confidence level

SE standard error, *SS* sum of square, *df* degree of freedom, *ARA* arachidonic acid

[a] X_1, tryptone; X_2, initial pH; X_3, agitation speed; X_4, temperature

both biomass and ARA production. Other factors were found to be insignificant.

Response surface and contour plots were generated to allow the understanding of the main factors, as well as the interactions, of two factors, while maintaining other factors constant at the central point level.

The 2D contour plots are graphical representation of the regression equation generally used to visualize the relationship between the response and the experimental levels of each variable, and the type of interaction between the variables to deduce the optimum conditions [38].

Figure 1a and b shows the significant interaction occurring between tryptone (X_1) and agitation speed (X_3) for biomass and ARA production, respectively, while keeping the other two variables at their middle levels. The data obtained indicate that an increase in concentration of tryptone and agitation speed resulted in an increase in growth and ARA production. Tryptone is used as a complex nitrogen source in cell cultures. It has been reported that the growth rate and PUFA content of the diatom *Nitzschia laevis* increased when concentration of tryptone was doubled from 0.5 to 1.0 g l^{-1} [39]. However, utilization of an agitation speed higher than 170 rpm resulted in less biomass of *A. maritima*, since it is a non-sporulating, non-fruiting, gliding bacterium. The cells are 0.7–0.8 × 3–6 μm in size and usually form helical cells that are 1.5–2.0 μm wide and 20–90 μm long, with a twist occurring every 4–5 μm [9]. Therefore it was more sensitive to shear stress than other bacteria. In addition, agitation speed level is always an important factor in aerobic biological systems, since when the supply of oxygen is limited, both cell growth and product formation can be severely affected [40]. Higher agitation rates result in an increase in oxygen supply for growth, and can lead to an increase in the availability of intracellular molecular oxygen. This ensures optimum activities of the oxygen-dependent enzymes in PUFA biosynthesis [41–43]. However, the optimal dissolved oxygen concentration may vary with different spices. For example, the fungus *Thraustochytrium aureum* ATCC 34304 showed maximum biomass production with an agitation speed of 100 rpm, and the highest PUFA content in total lipids at 150 rpm, under optimal culture conditions. However, agitation speeds higher than 250 rpm physically disrupted the cells, so that the morphology was found to be severely changed and the PUFA content was also greatly reduced [44]. Higashiyama et al. [43] also found that a dissolved oxygen (DO) range of 10–15 ppm was optimum for maximum biomass and ARA yield in *M. alpina* cultures, but high levels of oxygen (average DO, 20–50 ppm) decreased ARA production due to cell adaptation by β-oxidation of the fatty acid.

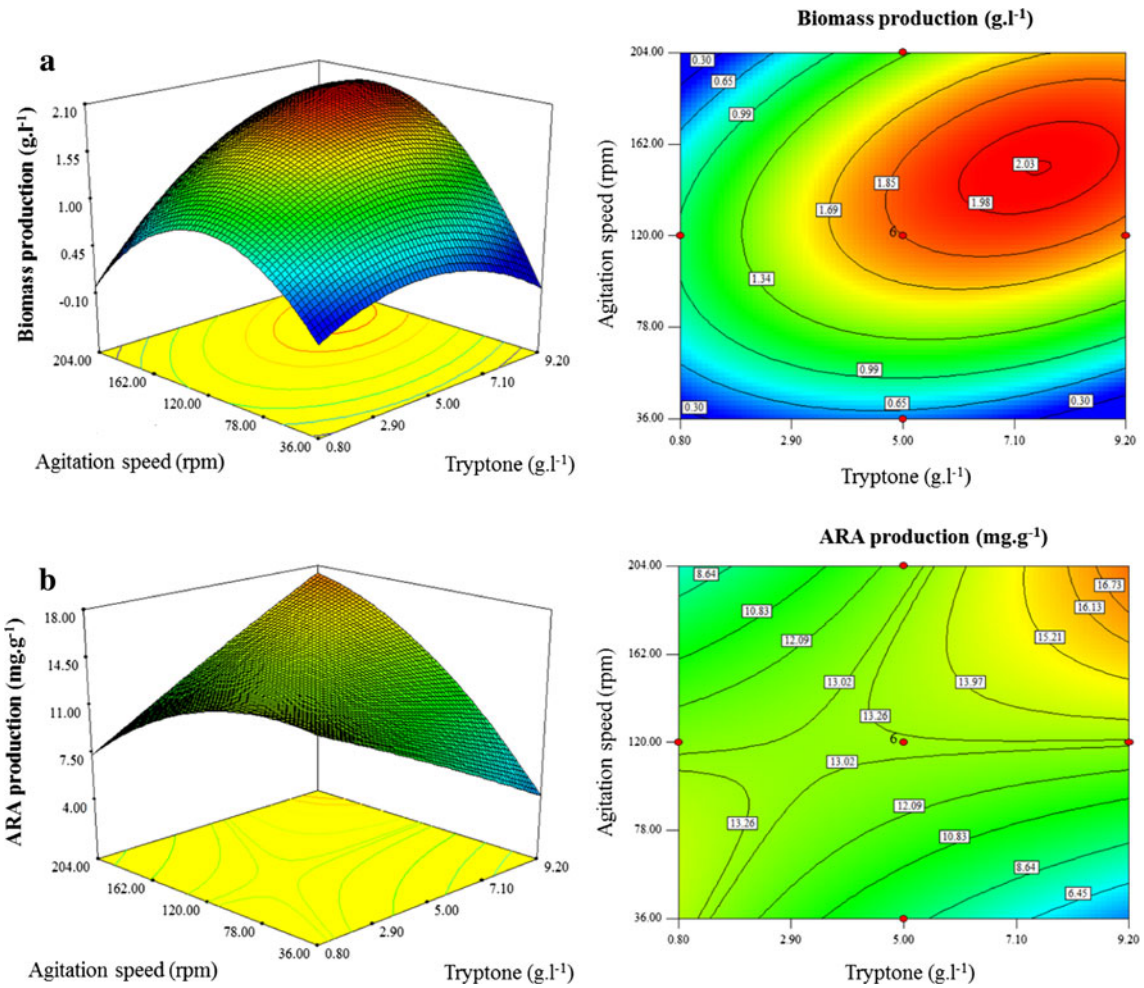

Fig. 1 Response surface and corresponding contour plots showing the effects of agitation speed and tryptone on biomass (**a**) and ARA (**b**) production. *ARA* arachidonic acid

Table 6 Predicted and actual biomass and ARA production by cell cultures of *A. maritima* TISTR 1715 using various culture conditions

Experiments[a]	Tryptone (g l^{-1}) (X_1)	Initial pH (X_2)	Agitation speed (rpm) (X_3)	Temperature (°C) (X_4)	Biomass production[b] (g l^{-1})		ARA production[b] (mg g^{-1})	
					Predicted	Actual	Predicted	Actual
A	7.7	7.5	154	21.6	2.18	1.80 ± 0.00	16.98	19.80 ± 2.78
B	9.0	7.9	170	17.8	2.07	2.05 ± 0.06	21.19	21.50 ± 0.25

The cells were cultured in 250-ml shake-flasks containing the total volume of 100 ml final production liquid medium with 10% (v/v) inoculum and incubated for 3 days

[a] A and B, experiments based on maximum growth and ARA production conditions, respectively

[b] Represents mean of the responses (biomass and ARA production) based on three separate experiments

Experimental Validation of the Optimized Culture Variables

The information from the equation models (Eqs. 6, 7) and the plots (Fig. 1), relating to the optimal levels of tryptone (X_1), initial pH (X_2), agitation speed (X_3) and temperature (X_4) for maximum biomass (Experiment A) and ARA (Experiment B) production are summarized in Table 6. The predicted and actual experimental responses (biomass and ARA production) for each set of variables are also

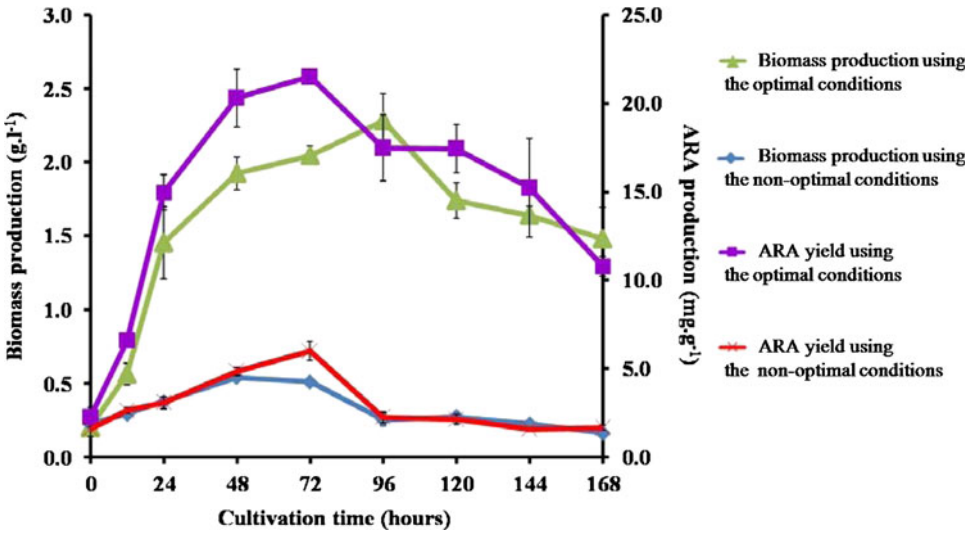

Fig. 2 Cell growth and ARA production curves for *A. maritima* TISTR 1715 grown under non-optimal and optimal conditions. *Vertical lines* indicate SE. *ARA* arachidonic acid

presented. Maximum production rates of 1.80 ± 0.00 g l^{-1} DCW and 19.80 ± 2.78 mg g^{-1} ARA, and 2.05 ± 0.06 g l^{-1} DCW and 21.50 ± 0.25 mg g^{-1} ARA, were obtained from experiments A and B, respectively. This represents 82–83 and 98–99% validity of the prediction models, respectively. Our study therefore suggests that the final optimal culture conditions would be tryptone 9.0 g l^{-1}, initial pH 7.9, agitation speed 170 rpm, temperature at 17.8 °C, sucrose 10 g l^{-1}, yeast extract 1 g l^{-1}, NaCl 15 g l^{-1}, KCl 0.35 g l^{-1}, $MgCl_2{\cdot}6H_2O$ 5.4 g l^{-1}, $MgSO_4{\cdot}7H_2O$ 2.7 g l^{-1}, and $CaCl_2{\cdot}2H_2O$ 0.5 g l^{-1}. These conditions would lead to maximum production of biomass of 2.05 ± 0.06 g l^{-1} and ARA of 21.50 ± 0.25 mg g^{-1}, which is 4.02-fold and 3.59-fold higher, respectively, than production rates in the preliminary non-optimization study (0.51 ± 0.02 g l^{-1} DCW, 5.99 ± 0.53 mg g^{-1} ARA).

Time course experiments for cell growth and ARA production were also conducted, and a comparison made between the non-optimized SAP 2 medium and the statistically optimized medium using Plackett–Burman and RSM protocols (Fig. 2). Cell cultures of *A. maritima* TISTR 1715 grow fastest under the statistically optimized conditions, the maximum biomass (DCW) achieved being 2.28 g l^{-1} on day 4 of culture. Maximum ARA production (21.50 mg g^{-1}) in the statistically optimized medium was achieved in the early stationary growth phase on day 3 of cultivation.

The production of ARA by fermentation with fungi and microalgae has been widely reported [12–24]. However, reports of bacterial ARA production are rare [7–11]. Our study is the first to report the optimization of culture conditions for biomass and ARA production by *A. maritima* TISTR 1715 even its productivity was lower than the fungi, especially *M. alpina* which is a current available source for industrial production. Nevertheless, ARA productivity of *A. maritima* TISTR 1715 was much higher than in the antarctic bacterium strain 651 [7], several algae [23], and some lower plants [33, 45] (Table 7). Moreover, the higher growth rate and easier handling of the bacterium make *A. maritima* a better choice for investigating it as a cheap versatile source of ARA. A further interesting feature is the simpler genetic information of prokaryotic in comparison to that of eukaryotic organisms which makes genetic manipulation easier, therefore, *A. maritima* TISTR 1715 is a potentially useful bacterial strain for developing rational strategies for enhancing the commercial production of ARA.

Conclusions

In the present investigation, successful attempts were made to improve biomass and ARA production in *A. maritima* TISTR 1715 using statistical approaches. The most significant factors were identified by the Plackett–Burman design. These data revealed that tryptone and culture temperature play a significant role in biomass production; the initial pH and agitation speed only affected ARA production. The optimum culture conditions for both cell growth and ARA production by *A. maritima* TISTR 1715 cultures were further derived using RSM. Cell growth and ARA production required relatively high levels of tryptone and agitation speeds. This is likely to be particularly important for scaling-up the cultivation process, in view of increased production costs. The information from our study is currently being used for the development of a larger scale cultivation process for *A. maritima* TISTR 1715 as an alternative source for commercial ARA.

Table 7 Biomass and ARA productivity by microorganisms

Microorganism	Culture time (days)	Biomass productivity[a] (g l^{-1} day^{-1})	ARA productivity[a] (mg l^{-1} day^{-1})	Reference
Bacteria				
Antarctic bacteria strain 651	10	n.s.	0.01	[7]
Aureispira marina gen. nov., sp. nov.	n.s.	n.s.	n.d.	[8]
Aureispira maritima sp. nov.	n.s.	n.s.	n.d.	[9]
Aureispira maritima TISTR 1715	3	0.68	14.69	Current study
Krokinobacter eikastus PMA-26T	3	n.s.	n.d.	[10]
K. diaphorus MSKK-32T	3	n.s.	n.d.	[10]
Plesiocystis pacifica SIR-1	3	n.s.	n.d.	[11]
Fungi				
Achlya sp. ma-2801	6	0.23	14.40	[12]
Mortierella alpina	7	4.46	267.14	[13]
M. alpina	8	4.63	967.50	[14]
M. alpina ATCC 32222	7	3.29	535.84	[15]
M. alpina I_{49}-N_{18}	6	4.60	758.33	[16]
M. alpina M_{18}	7	3.49	201.43	[17]
M. alpina ME-1	6.5	5.25	1,541.54	[18]
M. alpina 1S-4	8	6.33	1,362.50	[19]
M. alpina strain ZQ 9998	7	3.57	200.00	[20]
Pythium irregulare ATCC 10951	8	4.00	380.00	[21]
P. ultimum strain # 144	6	1.23	36.67	[22]
Algae				
Nannochloropsis oceanica	2.2	0.50	1.83	[23]
Oocystis sp.	2.9	0.23	0.12	[23]
Parietochloris incisa	38	0.55	70.18	[24]
Pavlova sp.	4.1	0.27	0.11	[23]
Phaeodactylum tricornutum	2.1	0.41	0.90	[23]
Rhodomonas baltica	4.1	0.09	0.02	[23]
Tetraselmis sp.	3.3	0.25	0.15	[23]
Lower plant				
Marchantia polymorpha	21	0.62	4.38	[45]
Physcomitrella patens	14	0.40	3.06	[33]

n.s. not stated, *n.d.* not determined by authors of cited references

[a] The biomass and ARA productivity data was calculated from information available in the cited references

Acknowledgments The authors wish to thank the Prince of Songkla University (PSU) for financial support. The Faculty of Agro-Industry, Faculty of Pharmaceutical Sciences and the Marine Natural Products Research Unit at PSU for providing laboratories facilities. Thanks are extended to the Thailand Institute of Scientific and Technological Research (TISTR) for providing the marine gliding bacterium, *A. maritima* TISTR 1715. The authors also thank Prof. L.A. Damani for helpful discussions, and for his scientific and editorial help with the manuscript.

References

1. Gill I, Valivety R (1997) Polyunsaturated fatty acids, part l: occurrence, biological activities and application. Trends Biotechnol 15:401–409
2. Brick EE, Garfield S, Hoffman DR, Uauy R, Birch DG (2000) A randomized controlled trial of early dietary supply of long chain polyunsaturated fatty acids and mental development in term infants. Dev Med Child Neurol 42:174–181
3. Bougle D, Denise P, Uimard F, Nouvelet A, Penniello MJ, Guillois B (1999) Early neurological and neurophysiological development of the preterm infant and polyunsaturated fatty acids supply. Clin Neuro Physiol 110:1363–1370
4. FAO/WHO (1995) Nutrition science policy. WHO and FAO joint consultation: fats and oils in human nutrition. Nutr Rev 53:202–205
5. Eroshin VK, Satroutdinov AD, Dedyukhina EG, Chistyakova TI (2000) Arachidonic acid production by *Mortierella alpina* with growth-coupled lipid synthesis. Process Biochem 35:1171–1175
6. Nichols DS, Nichols PD, McMeekin TA (1995) Ecology and physiology of psychrophilic bacteria from Antarctic saline lakes and sea ice. Sci Prog 78:311–347
7. Nichols DS, Brown JL, Nichols PD, McMeekin TA (1997) Production of eicosapentaenoic and arachidonic acids by an Antarctic bacterium: response to growth temperature. FEMS Microbiol Lett 152:349–354

8. Hosoya S, Arunpairojana V, Suwannachart C, Kanjana-Opas A, Yokota A (2006) *Aureispira marina* gen. nov., sp. nov., a gliding, arachidonic acid-containing bacterium isolated from the southern coastline of Thailand. Int J Syst Evol Microbiol 56:2931–2935
9. Hosoya S, Arunpairojana V, Suwannachart C, Kanjana-Opas A, Yokota A (2007) *Aureispira maritima* sp. nov., isolated from marine barnacle debris. Int J Syst Evol Microbiol 57:1948–1951
10. Khan ST, Nakagawa Y, Harayama S (2006) *Krokinobacter* gen. nov., with three novel species, in the family Flavobacteriaceae. Int J Syst Evol Microbiol 56:323–328
11. Iizuka T, Fudou R, Jojima Y, Hiraishi A, Ahn JW, Yamanaka S (2003) *Plesiocystis pacifica* gen. nov., sp. nov., a marine myxobacterium that contains dihydrogenated menaquinone, isolated from the Pacific coasts of Japan. Int J Syst Evol Microbiol 53:189–195
12. Aki T, Matumoto Y, Morinaga T, Kawamoto S, Shigeta S, Ono K, Suzuki O (1998) Lipid composition of a newly isolated polyunsaturated fatty acid-producing fungus, *Achlya* sp. ma-2801. J Ferment Bioeng 86:504–507
13. Zhu M, Yu LJ, Wu YX (2003) An inexpensive medium for production of arachidonic acid by *Mortierella alpina*. J Ind Microbiol Biotechnol 30:75–79
14. Zhu M, Yu LJ, Li W, Zhou PP, Li CY (2006) Optimization of arachidonic acid production by fed-batch culture of *Mortierella alpina* based on dynamic analysis. Enzyme Microb Technol 38:735–740
15. Jang HD, Lin YY, Yang SS (2005) Effect of culture media and conditions on polyunsaturated fatty acids production by *Mortierella alpina*. Bioresour Technol 96:1633–1644
16. Yuan C, Wang J, Shang Y, Guohong G, Yao J, Yu Z (2002) Production of arachidonic acid by Mortierella alpina I_{49}–N_{18}. Food Technol Biotechnol 40:311–315
17. Yu LJ, Qin WM, Lan WZ, Zhou PP, Zhu M (2003) Improved arachidonic acids production from the fungus *Mortierella alpina* by glutamate supplementation. Bioresour Technol 88:265–268
18. Jin MJ, Huang H, Xiao AH, Gao Z, Liu X, Peng C (2009) Enhancing arachidonic acid production by *Mortierella alpina* ME-1 using improved mycelium aging technology. Bioprocess Biosyst Eng 32:117–122
19. Higashiyama K, Yaguchi T, Akimoto K, Fukikawa S (1998) Effects of mineral addition on the growth morphology of and arachidonic acid production by Mortierella alpina 1S–4. J Am Oil Chem Soc 75:1815–1819
20. Lan WZ, Qin WM, Yu LJ (2002) Effect of glutamate on arachidonic acid production from *Mortierella alpina*. Lett Appl Microbiol 35:357–360
21. Cheng MH, Walker TH, Hulbert GJ, Rah Raman D (1999) Fungal production of eicosapentaenoic and arachidonic acids from industrial waste streams and crude soybean oils. Bioresour Technol 67:101–110
22. Gandhi SR, Weete JD (1991) Production of the polyunsaturated fatty acids arachidonic acid and eicosapenatenoic acid by the fungus *Pythium ultimum*. J Gen Microbiol 137:1825–1830
23. Patil V, Kallqvist T, Olsen E, Vogt G, Gislerød HR (2007) Fatty acid composition of 12 microalgae for possible use in aquaculture feed. Aquacult Int 15:1–9
24. Cheng-Wu Z, Cohen Z, Khozin-Goldberg I, Richmond A (2002) Characterization of growth and arachidonic acid production of *Parietochloris incisa* comb. nov (Trebouxiophyceae, Chlorophyta). J Appl Phycol 4:453–460
25. Box GEP, Hunter JS, Hunter WG (2005) Statistics for experimenters: design innovation and discovery, 2nd edn. Wiley, New York
26. Plackett RL, Burman JP (1946) The design of optimum multifactorial experiments. Biometrika 33:305–325
27. Vaidya R, Vyas P, Chhatpar HS (2003) Statistical optimization of medium components for the production of chitinase by *Alcaligenes xylosoxydans*. Enzyme Microb Technol 33:92–96
28. Djekrif-Dakhmouche S, Gheribi-Aoulmi Z, Meraihi Z, Bennamoun L (2006) Application of a statistical design to the optimization of culture medium for α-amylase production by *Aspergillus niger* ATCC 16404 grown on orange waste powder. J Food Eng 73:190–197
29. Chodok P, Kanjana-Opas A, Kaewsuwan S (2010) The Plackett–Burman design for evaluating the production of polyunsaturated fatty acids by *Physcomitrella patens*. J Am Oil Chem Soc 87:521–529
30. Francis F, Sabu A, Nampoothiri KM, Ramachandran S, Ghosh S, Szakacs G (2003) Use of response surface methodology for optimizing process parameters for the production of α-amylase by *Aspergillus oryzae*. Biochem Eng J 15:107–115
31. Liu C, Liu Y, Liao W, Wen Z, Chen S (2003) Application of statistically based experimental designs for the optimization of nisin production from whey. Biotechnol Lett 25:877–882
32. Boonsawang P, Wongsuvan T (2010) Nutrient optimization of polyhydroxyalkanoate production from palm oil fiber by *Ralstonia eutropha* TISTR 1095 using response surface methodology. Songklanakarin J Sci Technol 32:9–16
33. Kaewsuwan S, Bunyapraphatsara N, Cove DJ, Quatrano RS, Chodok P (2010) High level production of adrenic acid in *Physcomitrella patens* using the algae *Pavlova* sp. Δ^5-elongase gene. Bioresour Technol 101:4081–4088
34. Wen ZY, Chen F (2001) Application of statistically-based experimental designs for the optimization of eicosapentaenoic acid production by the diatom *Nitzschia laevis*. Biotechnol Bioeng 75:159–169
35. Song X, Zhang X, Kuang C, Zhu L, Guo N (2007) Optimization of fermentation parameters for the biomass and DHA production of *Schizochytrium limacinum* OUC88 using response surface methodology. Process Biochem 42:1391–1397
36. Karthikeyan RS, Rakhsit SK, Baradarajan A (1996) Optimization of batch fermentation conditions for dextran production. J Bioprocess Eng 15:247–251
37. Tanyildizi MS, Ozer D, Elibol M (2005) Optimization of α-amylose production by *Bacillus* sp. using response surface methodology. Process Biochem 40:2291–2296
38. Liu JZ, Weng LP, Zhang QL, Xu H, Ji LN (2003) Optimization of glucose oxidase production by *Aspergillus niger* in a bench top bioreactor using response surface methodology. World J Microbiol Biotechnol 19:317–323
39. Wen ZY, Chen F (2001) Optimization of nitrogen sources for heterotrophic production of eicosapentaenoic acid by diatom *Nitzschia laevis*. Enzyme Microbiol Technol 29:341–347
40. Wang SJ, Zhong JJ (2007) Bioreactor engineering. In: Yang ST (ed) Bioprocessing for value-added products from renewable resources. Elsevier, The Netherlands, pp 131–161
41. Gibbs A, Seviour R (1996) Does the agitation rate and/or oxygen saturation influence exopolysaccharide production by *Aureobasidium pullulans* in batch culture. Appl Microbiol Biotechnol 46:503–510
42. Singh A, Ward OP (1997) Microbial production of docosahexaenoic acid (DHA, C22:6). In: Neidleman SL, Laskin AI (eds) Applied microbiology. Academic Press, London, pp 271–321
43. Higashiyama K, Murakami K, Tsujimura H, Matsumoto N, Fujikawa S (1999) Effect of dissolved oxygen on the morphology of arachidonic acid production by Mortierella alpina 1S-4. Biotechnol Bioeng 63:442–448
44. Hur BK, Cho DW, Kim HJ, Park CI, Suh HJ (2002) Effect of culture conditions on growth and production of docosahexaenoic acid (DHA) using *Thraustochytrium aureum* ATCC 34304. Biotechnol Bioprocess Eng 7:10–15
45. Shinmen Y, Katoh K, Shimizu S, Jareonkitmongkol S, Yamada H (1991) Production of arachidonic acid and eicosapentaenoic acid by *Marchantia polymorpha* in cell culture. Phytochemistry 30:3255–3260