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Contributed Paper

Screening and Optimization of Squalene Production from Microalgae *Aurantiochytrium* sp.

Aeujkom Saengwong, Wichien Yongmanitchai and Duenrut Chonudomkul*
Department of Microbiology, Faculty of Science, Kasetsart University, Bangkok. 10900, Thailand.
* Author for correspondence; e-mail: fscidrc@ku.ac.th

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ABSTRACT

Microalgae are an alternative potential source of squalene because they grow rapidly, relatively easy to culture and accumulate large amounts of squalene. The objectives of this research are to isolate and screen squalene producing heterotrophic microalgae from mangrove forests of Thailand and to optimize culture conditions. Five hundred and eighty-five strains of the microalgae were isolated from the mangrove forests along the gulf of Thailand and southern, Thailand. After screening, the isolate S02-459 was selected for further studied due to its high squalene content. Sequencing of partial 18S rRNA gene revealed that it belong to the genus *Aurantiochytrium*. Evaluation of eight factors influencing growth and squalene accumulation, *i.e.,* glucose concentration, yeast extract, peptone, monosodium glutamate, pH, shaking speed, salinity and temperature were carried out by using Plackett-Burman design. Four factors, namely, glucose concentration, yeast extract concentration, peptone concentration and shaking speed were significantly ($p<0.05$) affected biomass and squalene production. Central Composite design was employed for optimized these four parameters for enhancement the squalene production. These factors were assessed using quadratic model. The coefficient of determination (R^2) of more than 0.9 and p-value less than 0.05 indicated that the model was acceptable. The optimized conditions from CCD showed that 30 g/L of glucose, 5.0 g/L of yeast extract, 20 g/L of peptone and shaking speed at 75 rpm were appropriate. Verifications of the predicted values were 41.19±1.86 mg/L of squalene production and 10.85±0.10 mg/g cell dry weight of squalene content. The deviation of experimental values and predicted values for squalene production and content were -1.81% and -6.78%, respectively.

Keywords: microalgae, *Aurantiochytrium*, squalene, optimization, central composite design

1. INTRODUCTION

Squalene (2, 6, 10, 15, 19, 23-hexamethyltetracosa-2, 6, 10, 14, 18, 22 hexaene) is a triterpene of hydrocarbon compounds with six double bonds. It was synthesized within cell of living organisms (plant, animal and human). It is an intermediate in the steroids and triterpene pathway [1]. Nowadays, squalene is used as an important ingredient in the cosmetic industry because of its function as an antioxidant. It is also

used in the pharmaceutical and medical applications due to its ability to inhibit tumor proliferation [2].

Conventional and commercial source of squalene is shark liver oils (400-800 g/Kg). However, sharks become endangered species because they were over fishing for their fins. At present, the population of sharks is declined at an accelerated rate. This situation will affect the marine ecosystem diversity. It is therefore becoming illegal to exploit shark liver oils for squalene. Searching for an alternative source of squalene from oil seeds and microorganism is become eminent. However, oil seeds such as olive, soy bean and pumpkin contain very low amounts of squalene [3]. Researchers considered microorganism is an alternative source of squalene because they can grow rapidly, have high squalene content and be able to produce in the industrial scale.

Today microalgae, especially the genus *Aurantiochytrium*, a heterotrophic marine microalga, gain more attentions because they accumulated large amounts of squalene in their cells. This microalga is a member of the Family Thraustochytriaceae in the Class Labyrinthulomycetes. Cell morphology is globose and produces biflagellate zoospore [4, 5].

Environmental factors have known to influence to metabolism of living microorganisms. The growth conditions such as temperature, pH, salinity and oxygen requirements, as well as nutrient composition, for example, carbon source and nitrogen source, were commonly considered to control growth and metabolic rates [6, 7]. Glucose plays an important role for lipid production of microorganisms [8]. It provides more energy when compared other carbon sources. Glucose provides 2.8 kJ/mol of energy while acetate produces only 0.8 kJ/mol [9]. Carbon source (glucose) and nitrogen source (yeast extract, peptone and monosodium glutamate) are essential nutrients to cell growth and squalene production. They have been reported as nutritional complement for heterotrophic cultivation [10-13].

In eukaryotic microorganism, sterols play an essential role in cell proliferation [14]. In sterol biosynthesis, the enzyme in this process required molecular oxygen for oxygenation of squalene to sterols. Squalene epoxidase (EC 1.14.99.7) and flavine-adenine dinucleotide (FAD) need molecular oxygen for oxygenation of squalene to 2, 3 oxidosqualene where 2, 3 oxidosqualene is cyclized to either lanosterol and cycloartenol [11, 14-17]. If this reaction does not receive oxygen, large quantities of squalene will be accumulated within the cells [6]. Therefore, the aeration rate in the culture medium is critical for optimization of cell growth and squalene production [6, 11]. The significance of culture temperature has been reported to influence biomass and squalene production form *Aurantiochytrium* sp. strain 18W-13a. Cultivation of the microalga at 15 °C enhanced growth enormously while high squalene production and squalene content were obtained at 25 °C [12]. Both salinity and pH were important components of algal culture. Thraustochytrids showed halophilic characteristic because they were commonly collected from mangrove ecosystem. As sea salt concentration increased, the biomass of *Aurantiochytrium* sp. KRS101 enhanced [18]. Salinity at 2.0 % was optimal for cell growth, while pH conditions in medium significantly affected the lipid production in Thraustochytrid [19].

Statistical experimental design, namely Plackett-Burman design (PBD) and Central Composite design (CCD) were commonly applies for studying optimal culture conditions. PBD was introduced for the first time in

1946 by Plackett and Burman [20]. This experimental design was used to evaluate the significant of each factor that influence growth and production of interesting compounds. In this study factors affecting growth and squalene production were determined from previously reports, After obtaining the critical factors, CCD and the response surface methodology were employed for optimization of culture condition [21]. There were numerous reports on using this statistical experimental design for optimization of growth and lipid production (squalene and docosahexaenoic acid (DHA)) by *Aurantiochytrium* and *Schizochytrium* [8, 13, 22]. The objectives of this study are to isolate and screen the squalene producing microalgae *Aurantiochytrium* spp. from mangrove forests of Thailand and to optimize the conditions for improvement of squalene production by using PBD design and CCD experiment.

2. MATERIALS AND METHODS

2.1 Isolation and Screening of Squalene-producing Microalgae

Mangrove leaves were randomly collected from mangrove forests along the gulf of Thailand and southern, Thailand. The microalgae were isolated by baiting method on GPY medium (0.2% glucose, 0.1% peptone and 0.5% yeast extract in 15 parts per thousand (ppt) sea water supplemented with 250 ppm sodium propionate and 200 ppm chloramphenicol). The typical creamy white colonies were picked up and streaked on GPY agar plate to obtain pure cultures. Under microscope cells are unicellular, thin cell wall and globose shape. The cultures were maintained in GPY agar slants and sub-cultured every two weeks. Screening for high squalene production was carried out in three steps. For primary screening, the stock cultures were activated on GPY agar for one day. The newly developed cultures were then transferred to GPY broth (3.0% glucose concentration, 1.0% peptone and 1.0% yeast extract in 15 ppt sea water) and inoculated at 25°C for 24 hours in a incubator shaker at 200 rpm. The high squalene-producing isolates were collected and subjected to secondary and tertiary screening. The highest squalene-producing isolate was selected for further study.

2.2 Inoculum Preparation

Inoculum was prepared by growing in 500 mL Erlenmeyer flasks containing 100 mL of the GPY and cultivated at 25°C for 24 hours in an incubator shaker (Bio-shaker BR-300LF, Taitec Corporation, Japan) at 100 rpm.

2.3 Cultivation Condition

The fully grown inoculum was transferred at pre-calculated volume to into 500 mL Erlenmeyer flasks containing 120 mL of the GPY broth to obtain the initial OD_{600} at 0.2 and incubated as above. The culture samples were collected at 6, 12, 15, 18, 21, 24, 27, 30, 33, 36, 39, 42, 45 and 48 hours for growth determination and squalene analysis.

2.4 Squalene Extraction

Squalene was extracted by the modified method of Bligh and Dyer [23]. Briefly, 10 mL of the culture sample was centrifuged at 3,300 rpm for 15 min. The cell pellet was made to suspension by adding 0.5 mL of distilled water and vortex for 15 sec. The cell suspension was extracted with 3.75 mL chloroform: methanol (1:2). After vortex for 1 min, 1.25 mL of chloroform was added and mixed for 1 min. Finally, 1.25 mL of distilled water was added and vortex for 15 sec. Phase separation was done by centrifugation at 3,300 rpm for 10 min. The bottom layer was collected for

further analysis by HPLC.

2.5 Squalene Analysis

The squalene extracts obtained were evaporated under vacuum and the remaining lipid fraction was dissolved by 0.5 mL of methanol: isopropanol (92: 8.0) and analyzed by HPLC. The system composed of pump (Waters model 510), UV/Vis detector Perkin Elmer, 758A) equipped with NovaPak C_{18} column (Waters Corporation, Maple Street/ Milford, MA 01757, USA) with methanol: isopropanol: acetic acid (91.95: 8.0: 0.05) as mobile phase at the flow rate of 1.0 mL/min. Detection was made at 205 nm. The squalene was identified by comparison with authentic standard (Sigma, St. Louis, USA).

2.6 Growth Determination

Cell growth was determined by measuring optical density (OD) at 600 nm using a UV/Vis spectrophotometer (PharmaSpec 1700, Shimadzu, Japan). The OD was converted to CDW by pre-determined cell factor of 0.506.

2.7 DNA Extraction and Analysis of 18S rRNA gene

The microalgae were cultivated in GPY broth and incubated at 25°C for 18-24 hours in an incubator shaker. Cells were collected and DNA was extracted following the method of Zhou et al. [24].

18S rRNA gene was amplified according to White et al. [25]. The partial sequences of 18S rRNA were compared through NCBI BLASTn (http:// www.ncbi.nlm.nih.gov). The phylogenetic tree was constructed by using MEGA version 6.0 (http:// www.megasoftware.net).

Table 1. The 12 run of Plackett-Burman design including the eight factors and three dummies.

Run	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	D_1	D_2	D_3	Biomass (g/L)	Squalene production (mg/L)	Squalene content (mg/g CDW)
1	+1	+1	-1	+1	+1	+1	-1	-1	-1	+1	-1	2.23	16.83	7.56
2	-1	+1	+1	-1	+1	+1	+1	-1	-1	-1	+1	2.11	14.22	6.74
3	+1	-1	+1	+1	-1	+1	+1	+1	-1	-1	-1	3.85	10.62	2.71
4	-1	-1	-1	+1	+1	-1	+1	+1	+1	-1	-1	5.28	2.68	0.51
5	-1	+1	+1	-1	+1	+1	-1	+1	+1	+1	-1	1.01	7.56	7.50
6	-1	-1	-1	+1	-1	+1	+1	-1	+1	+1	+1	0.55	0.11	0.20
7	+1	-1	-1	-1	+1	-1	+1	+1	-1	+1	+1	0.29	0.11	0.38
8	+1	+1	-1	-1	-1	+1	-1	+1	+1	-1	+1	1.86	10.91	5.86
9	+1	+1	+1	-1	-1	-1	+1	-1	+1	+1	-1	3.78	11.47	3.03
10	-1	+1	+1	+1	-1	-1	-1	+1	-1	+1	+1	4.86	4.75	0.98
11	+1	-1	+1	+1	+1	-1	-1	-1	+1	-1	+1	0.60	0.15	0.25
12	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	0.15	0.47	3.09

2.8 Evaluation of Factors Affecting Squalene Production by Plackett-Burman Design

In this study, eight factors including glucose concentration (g/L, X_1), yeast extract (g/L, X_2), peptone (g/L, X_3), monosodium glutamate (g/L, X_4), pH (X_5), shaking speed (rpm, X_6), salinity (%, X_7) and temperature (°C, X_8) were evaluated for their significance on growth and squalene production by PBD. Each factor was tested at two levels, low (-1) and high (+) as shown in Table 1. There were 12 runs and each run was repeated four times (Table 1). The algal-cultures were harvested at 30, 33 and 36 hours for measurement of growth and squalene production. All data were calculated by using the SPSS program version 11.5 (IBM corporation, USA.) to determine the confidence levels at greater than 95%.

2.9 Central Composite Design

Optimization of factors obtained from PBD were carried out by Central composite design (CCD). The total numbers of the CCD experiments were calculated according to the equation 1 [21].

$$(N = 2^k+2k+C_0) \quad (1)$$

Where N is the total number of experiments, k is the number of factors and C_0 is the number of central point. From 4 selected factors, 27 runs consist of 16 factorial portions, 8 axial points ($\alpha = 2$) and 3 replicates at central point (Table 2). Three replications were tested for each run. There were five levels ($-\alpha$, -1, 0, +1, $+\alpha$) of each in the experimental run and they were used for calculating the equation 2 [22].

$$xi = \frac{x_i - x_0}{\Delta x} \quad (2)$$

When xi is the dimensionless coded value of the factor X_i, X_0 is the value of the X_i at the central point and ΔX is step change. All data of the experiments were formulated using Design-Expert version 7.0.0 software (DX7) (Stat-Ease, Inc.; Minneapolis, MN, USA) for generation of quadratic model in surface response methodology.

3. Results and Discussion

3.1 Isolation and Screening of Squalene-producing Isolates

In Thailand, mangrove forests disperse along the coastline of Gulf of Thailand and Andaman Sea. Mangroves are the only forests situated at the confluence of land and sea in the world's subtropics and tropics [26]. Majority of plant in this specific ecosystem is mangrove (*Rhizophora* spp.). In this study, the sampling was collected from the mangrove forest of the gulf of Thailand coast and southern, Thailand. Including total 674 isolates (81 isolates from Nakhon Si Thammarat, 180 isolates from Surat Thani, 55 isolates from Chumphon, 22 isolates from Bangkok, 335 isolates from Prachuap Khiri Khan and 1 isolate from Phetchaburi). In the first screening, five hundred and eighty-five isolates of microalgae were tested. Thirty-nine of them produced squalene more than 50 mg/L. They were then subjected to secondary screening where seven strains still produced squalene up to 50 mg/L. In tertiary screening, two isolates, S02-459 and SS13-335 produce highest squalene and they were selected for further studies.

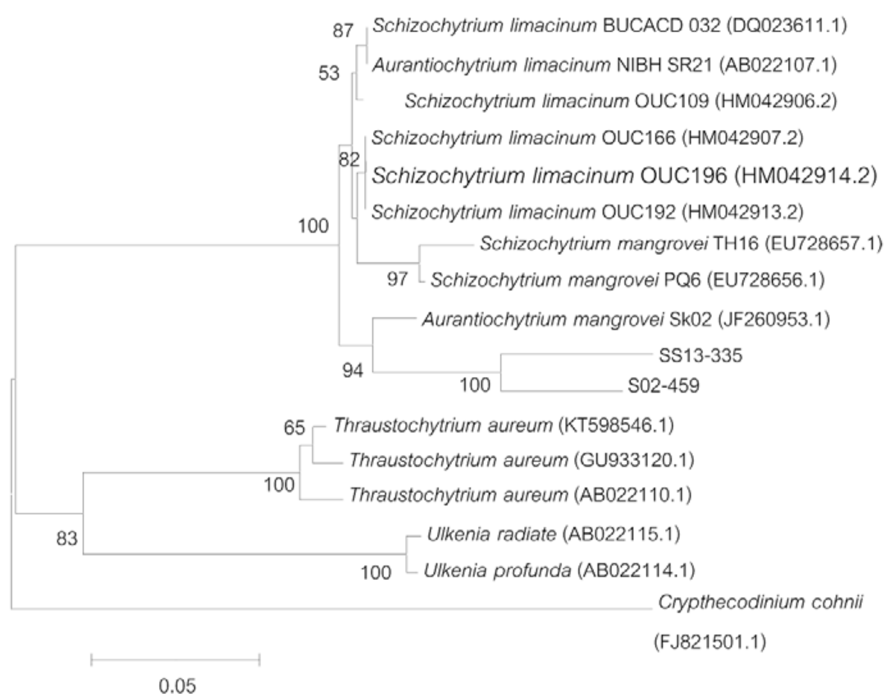

Figure 1. Phylogenetic tree of the 18S rRNA partial sequences of *Aurantiochytrium* sp. SS13-335 and *Aurantiochytrium* sp. S02-459.

3.2 Optimization of Cultivation Period

Since squalene is an intermediate in the synthetic pathway of sterols, delay in harvesting cells will seriously affect the squalene content. Both selected isolates were cultivated in GPY broth and harvested the cell suspension every 3 hour for the measurement of the optical density cell and squalene contents. The highest squalene production of 31.56 mg/L and squalene content of 12.18 mg/g CDW were attained in isolate S02-459 at 33 hours of cultivation period and the biomass was 2.59 g/L. For isolate SS13-335, the highest squalene production of 24.23 mg/L and squalene content of 13.31 mg/g CDW were examined at 27 hours of cultivation period with the biomass of 1.82 g/L. Therefore, the isolate S02-459 was chosen for the next experiment.

3.3 Identification of the Selected Microalgae by 18S rRNA Sequence Analysis

Alignment of 18S rRNA sequences of the isolate SS13-335 and S02-459 were conducted by using Clustal W program and compared with nucleotide sequences of thraustochytrids in the genera *Aurantiochytrium*, *Schizochytrium*, *Thraustochytrium, Ulkenia* and *Crypthecodinium cohnii* (FJ821501.1) as outgroup. The phylogenetic tree was constructed using neighbor-joining method in MEGA version 6.0. The isolate SS13-335 and S02-459 were closely with *Aurantiochytrium mangrovei* strain Sk02 (accession number: JF260953.1) (Figure 1). The maximum identity was 93% and 94%, respectively with high bootstrap values. The identity figures were actually quit low indicating that they might be a

novel species. However, isolate SS13-335 and S02-459 was renamed as *Aurantiochytrium* sp. SS13-335 and *Aurantiochytrium* sp. S02-459.

3.4 Plackett-Burman Design

The algal culture samples were collected at 30, 33 and 36 hours to determine the squalene production. The confidence levels of the most important factor at $p < 0.05$ indicated the statistical significance on the biomass and squalene production. The biomass, squalene production and squalene content were shown on Table 1. At 30, 33 and 36 hours of cultivation periods the R^2 values were 0.864, 0.881 and 0.913, respectively. The regression model consist of R^2 value > 0.75 (greater than 75%) indicated the good fit model. Therefore all of the experiments R^2 values were highly accepted [27]. At 30 hours of cultivation period, the biomass of all experiments were in the range of 0.15 to 5.28 g/L, squalene production 0.11 to 16.83 mg/L and squalene content of 0.20 to 7.56 mg/g CDW. While at 33 hours the biomass was 0.18 to 7.43 g/L, squalene production at 0.10 to 14.51 mg/L and squalene content 0.16 to 6.03 mg/g CDW. At 36 hours the biomass, squalene production and squalene content were slightly less ANOVA analysis of cultivation period revealed that 30 hours was appropriate for harvesting cells for squalene extraction. ANOVA analysis of the eight factors evaluated by Plackett-Burman design concluded that four factors consisted of glucose concentration, yeast extract, peptone and shaking speed influenced the squalene production ($p < 0.05$). Although, other factors, i.e., yeast extract, peptone, monosodium glutamate, pH, shaking speed, salinity and temperature, affected the biomass production ($p < 0.05$). Carbon source and nitrogen source play an essential nutrient for microalgal culture [7]. They play an important role for the cell growth and lipid production (such as squalene and DHA) in thraustochytrids [12, 28]. It has been reported that both yeast extract and tryptone were significantly increased the cell growth and squalene production in *Aurantiochytrium* sp. BR-MP4-A1. While monosodium glutamate influenced the cell growth [13]. In *Aurantiochytrium* sp. KRS101, Glucose concentration at 40 g/L and yeast extract 10 g/L were supplemented in medium, resulting in the enhancement of the highest lipid and DHA production [18]. The different glucose concentrations were found to affect the squalene content in *Aurantiochytrium mangrovei* FB3 [11]. The effect of oxygen concentration had shown to impact squalene epoxidase activity [6]. Squalene epoxidase is the key enzyme for catalyst of the first oxygenation step in sterol biosynthesis, which is associated to the accumulation of squalene within the cells [29].

3.5 Central Composite Design

The four most important factors obtained from Plackett-Burman design consisted of glucose concentration, yeast extract, peptone and shaking speed were optimized the enhancement of squalene production by 27 runs of the CCD. Data were analyzed by the software program [Design-expert® version 7.0.0. software (DX7)]. The experimental values were shown by quadratic model and surface plots.

Quadratic model for squalene production and squalene content:

$$Y_{squalene\ production} = 16.30 + 0.39x_1 + 3.49x_2 + 6.87x_3 - 5.68x_4 - 0.64x_1x_2 + 0.10x_1x_3 - 0.19x_1x_4 + 1.51x_2x_3 - 1.04x_2x_4 - 3.30x_3x_4 - 3.14x_1^2 - 1.21x_2^2 + 0.070x_3^2 - 0.63x_4^2 \quad (3)$$

$$Y_{squalene\ content} = 3.59 + 0.19x_1 + 0.74x_2 + 1.84x_3 - 1.95x_4 - 0.11x_1x_2 + 0.5x_1x_3 - 0.11x1x4 + 0.54x_2x_3 - 0.52x_2x_4 - 1.11x_3x_4 - 0.81x_1^2 + 0.4x_2^2 - 0.80x_3^2 + 0.53x_4^2 \quad (4)$$

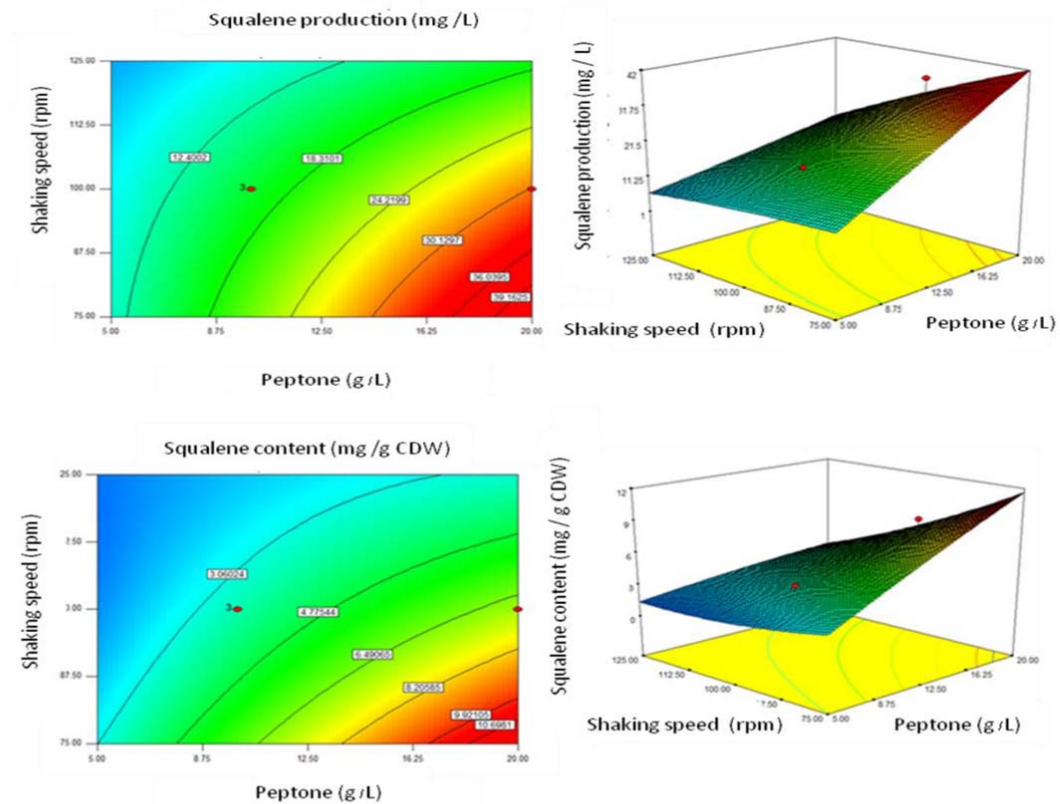

Figure 2. Two-dimensional response surface plots (left) and three-dimensional contour plots (right) of squalene production and squalene content between peptone and shaking speed.

In these experiments, the squalene production of S02-459 accumulated in the range of 1.00 to 34.41 mg/L. The biomass and squalene content varied between 0.97 to 6.02 g/L and 0.21 to 10.40 mg/g CDW, respectively. The highest squalene production was obtained in the run 22 consisted of 30 g/L glucose, 5.0 g/L yeast extract, 20 g/L peptone and shaking speed at 100 rpm. The squalene production of 34.41 mg/L was obtained at 33 hours of cultivation period. Table 2 showed code levels, the experimental values and predicted values at 33 hours. The predicted values of the biomass, squalene production and squalene content were very closed to the experimental values indicating the reliability of the quadratic model. Verification of the quadratic model (3-4) by prediction from surface plots of the optimized conditions for squalene production and squalene content were carried out. Analysis of variance for biomass, squalene production and squalene content reveal that *F*-value of 1.53 and *p*-value of 0.2341 of biomass indicated the selected four factors were not

significant (p<0.05). R^2 value (0.64) and R^2 adj. (0.22) of quadratic model were revealed that the model was not fit for biomass production. However, F-value of 29.99 and p-value of 0.0001 were highly significant for squalene production (p<0.05). The high R^2 value (0.97) and R^2 adj. (0.94) of quadratic model also indicated the good fit of the model. Consequently, F-value of 21.03 and p-value of 0.0001 were also significant for squalene content (p<0.05), as well as the high R^2 value (0.96) and R^2 adj. (0.92) of quadratic model.

Table 2. The experimental values and predicted values using CCD.

Run	X_1	X_2	X_3	X_4	Biomass (g/L)		Squalene production (mg/L)		Squalene content (mg/g CDW)	
					Predicted	Experimental	Predicted	Experimental	Predicted	Experimental
1	-1	-1	-1	-1	3.76	4.06	2.77	3.50	1.39	0.85
2	1	-1	-1	-1	3.42	3.80	5.02	5.67	1.90	1.49
3	-1	1	-1	-1	3.87	3.63	10.09	10.73	3.06	2.95
4	1	1	-1	-1	3.63	3.45	9.76	8.81	3.14	2.59
5	-1	-1	1	-1	3.73	3.67	19.87	17.62	5.89	4.77
6	1	-1	1	-1	3.51	3.55	22.53	23.28	7.02	6.65
7	-1	1	1	-1	3.75	3.47	33.22	33.26	9.72	9.94
8	1	1	1	-1	3.64	3.18	33.31	30.45	10.42	10.34
9	-1	-1	-1	1	3.77	4.55	0.47	1.00	0.99	0.21
10	1	-1	-1	1	3.56	4.60	1.94	1.42	1.05	0.31
11	-1	1	-1	1	3.98	5.70	3.63	2.40	0.57	0.42
12	1	1	-1	1	3.88	5.26	2.53	2.45	0.20	0.46
13	-1	-1	1	1	3.49	4.43	4.38	4.85	1.06	1.09
14	1	-1	1	1	3.41	3.96	6.26	3.29	1.73	0.98
15	-1	1	1	1	4.61	4.54	13.58	10.60	2.79	2.34
16	1	1	1	1	4.63	5.09	12.90	11.69	3.03	3.05
17	-2	0	0	0	5.92	5.41	2.97	3.59	-0.03	0.73
18	2	0	0	0	5.60	5.03	4.54	6.73	0.72	1.34
19	0	-2	0	0	2.41	0.97	4.48	4.38	2.68	4.33
20	0	2	0	0	3.74	4.11	18.43	21.35	5.65	5.38
21	0	0	-2	0	4.78	3.73	2.85	1.56	-0.40	0.42
22	0	0	2	0	4.50	4.48	30.31	34.41	6.94	7.50
23	0	0	0	-2	1.69	2.48	25.13	25.35	9.61	10.40
24	0	0	0	2	2.69	6.02	2.42	5.01	1.83	0.83
25	0	0	0	0	4.54	4.58	16.30	16.06	3.59	3.45
26	0	0	0	0	4.54	4.55	16.30	16.39	3.59	3.66
27	0	0	0	0	4.54	4.50	16.30	16.45	3.59	3.65

X_1: Glucose concentration, X_2: Yeast extract, X_3: Peptone and X_4: Shaking speed

Figure 2 showed three-dimensional response surface plots and two dimensional contour plots, which predicted the optimized conditions for squalene production and squalene content. The surface plots showed interaction between peptone and shaking speed when glucose concentration of 30 g/L and yeast extract of 5.0 g/L were fixed. Verification of the predicted values were performed with 30 g/L of glucose concentration, 5.0 g/L of yeast extract, 20 g/L of peptone and shaking speed at 75 rpm. The verification of predicted values of *Aurantiochytrium* sp. S02-459 achieved 41.19±1.86 mg/L of squalene production and 10.85±0.10 mg/g CDW of squalene content. The deviation of experimental values from predicted values for squalene production and squalene content were -1.81% and -6.78%, respectively. Under this condition the biomass at 3.74±0.17 g/L was obtained.

Several species of *Aurantiochytrium* spp. have been reported as a potential source of squalene. Optimization of culture conditions for the enhancement of squalene production is important in order to develop for commercialization. *Aurantiochytrium* sp. S02-459 achieved 41.19±1.86 mg/L of squalene production and 10.85±0.10 mg/g CDW of squalene content under the optimum culture conditions. In comparison, these values were much higher than the previous reported of Fan et al. [11]. An initial glucose concentration of 30 g/L squalene production and squalene content were higher but decreased at higher glucose concentration. In order to improve squalene production, terbinafine, an inhibitor of squalene epoxidase for enhancing sterol biosynthesis, was applied. Different terbinafine concentrations were supplemented in culture medium containing 30 g/L of glucose concentration. *Aurantiochytrium mangrovei* FB3 accumulated highest squalene at 2.90 mg/L and 0.53 mg/g CDW in culture supplement with 100 mg/L of terbinafine concentration, respectively. Chen et al. [13] revealed that *Aurantiochytrium* sp. BR-MP4-A1 produced 5.90 mg/L of squalene production and 0.72 mg/g CDW of squalene content under optimization of nitrogen sources. The resulted indicated that monosodium glutamate, yeast extract and tryptone were found to enhance the cell growth and squalene production. Nitrogen source is essential nutrient for microalgae culture. In *Schizochytrium* sp. S31, yeast extract was the most effective for biomass and DHA production [19]. Conversely, these values of this study were much less than the previously reported. It was been reported that *Aurantiochytrium* sp. 18W-13a achieved 0.9 g/L of squalene production and 171 mg/g CDW of squalene content in the optimal culture conditions containing 2.0% of glucose concentration, 50% sea water concentration at 25°C [12]. Similarly, *Aurantiochytrium* sp. strain Yonez5-1 achieved 1,073.66 mg/L of squalene production and 317.74 mg/g CDW of squalene content in GTY medium containing 2.0% of glucose concentration, 1.0% of tryptone, 0.5% of yeast extract and 50% artificial sea water with a reciprocal shaker at 100 strokes/min [30].

4. Conclusion

The thraustochytrid, *Aurantiochytrium* sp. S02-459 was isolated from mangrove forest of Thailand. Enhancement of squalene production of this strain by statistical experimental designs were successfully applied in this study. Plackett-Burman design suggested that four factors including glucose concentration, yeast extract, peptone and shaking speed were important for squalene production at significant level ($p<0.05$). The optimal culture conditions

under CCD experiment showed the influence to increasing of squalene production in *Aurantiochytrium* sp. S02-459 (41.19±1.86 mg/L) that it was much higher than original medium (31.56 mg/L of squalene production). The results of these experiments may provide the information for improvement of squalene production in other heterotrophic microalgae.

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