Statistical optimization of pigment extraction from *Acutodesmus reginae* sp.

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Abstract. Pigment is a natural colorant produced by an organism including microalgae. A locally isolated microalga, *Acutodesmus reginae* has shown potential in producing pigments. The intent of this study was to optimize the pigment yield from the unexplored *Acutodesmus reginae*. Various statistical software for experimental design as tools for optimization offers researcher to plan and able to determine cause and effect relationships in experiment widely applied nowadays. In this study, a 3-level factorial design in response surface method (RSM) used to analyse the effect of factors (type of solvent, extraction time) to determine the optimal parameters for chlorophyll a, chlorophyll b, carotene and phycocyanin pigment extraction yield. A total of 13 run were done using the combination of 95% ethanol, methanol and diethyl ether as the solvents at different extraction time, 15, 30 and 45 minutes. The used of RSM resulted in maximizing the yield of chlorophyll a, chlorophyll b, carotene and phycocyanin at 0.92 μg/mL, 0.64 μg/mL, 0.27 μg/mL and 1.70 μg/mL respectively using 95% ethanol within 45 minutes of homogenization with bead milling assisted. Pigments extraction was done under optimal conditions as predicted by RSM with desirability 0.730 for authentication of the pigment extraction observed. Analysis of the results indicates the experimental values acquired appropriate as the data estimated by the model indicating that the process optimization is reliable to predict the parameter for pigments extraction from *Acutodesmus reginae*.

1. Introduction

Microalgae are microscopic photosynthetic organisms, found in freshwater, marine and blossom a variety of habitats. Some microalgae have a good potential to produce valuable products such as carbohydrates, proteins, lipids as well as pigments due to their simple life cycle, simple growth requirements, fast growth rate and good adaptation [1]; [2]; [3]; [4]; [5]; [6]. Environmental and nutritional variables affect the productivity and cell composition of algal cultures, especially pigment composition. The climate in Malaysia is suitable for microalga cultivation as the sun shines around the year.

Producing natural pigments, environmental friendly and biodegradable pigments can replaces the toxic and hazardous pigment to human health. Pigments are extracted from microalgae to establish and improvise biorefinery approaches to the economy. However, the pigment composition is controlled by
rapid heating, photo-oxidative stress and bleaching effects [7]. Most microalgae are shade-adapted microorganisms, fast acclimatisation processes requiring of light harvesting machinery in response to light intensity and nutrient concentration [4]. Consequently, each microalgae strain, control-culture condition and type of extraction method requires processes designed to increase the production of enriched pigment composition.

Light is an essential condition for cell growth and multiplication for all photosynthetic microalgae and the consistency, intensity and length of light plays a critical role in the growth and physiology of microalgae. Photosynthetic cells can simultaneously generate pigment composition under the culture of stimulation during developing cell. Algae contain three major classes of photosynthetic pigments which are chlorophylls, carotenoid (carotenes and xanthophyll) and phycobilins. Chlorophyll a and b are the main pigments in microalgae as the chlorophyll absorbs blue and red wavelength and transmits green light. Production of coloring agents which are naturally non-toxic β-carotene from *D. salina*, *C. nivalis*, *Chlorococcum* spp., is used in to produce coloring agent for the food products such as chewing gum and cakes. The bioactive compounds from microalgae can be extracted using various solvents or buffers and analysed using particular techniques, such as spectrophotometric assays and chromatographic analysis, often significantly impacted pigment biomass [9]. In this study, a locally isolated microalgae, *Acutodesmus reginae* was selected for the development of valuable pigments. The selection is due to its resistance to bio-contaminants, rigid cell walls, rapid adaptation, reliability in various cultivation system and the efficacy of various biomass industrial processes [2]; [9]; [10];[11]. *A. obliquus* was isolated, characterised and cultivated under specifically enriched media growth to produce a uniform microalgae cell for the evaluation of pigment biomass using various techniques such as cell disruption and extraction methods. Development of knowledge in statistics and information technology, offers various statistical software for experimental design as tools for optimization in order to simplify the process and to view the effects of the variables and in relation with economics. Response surface methods (RSM) among the approach that offers the researcher to plan the approach and able to determine cause and effect relationships of parameter in the experiment [12]. This approach extensively applied in various areas of science because it reduces the number of experiment.

Hence, this study attempts to investigate pigment extraction from locally isolated *Acutodesmus* genus. In the present study, statistical analysis was used to study the extraction factors that effects the yield of pigments by implementing the response surface method.

2. Methods

2.1. Sample of study

*Acutodesmus reginae* was procured from culture collection in Institute of Bio-IT Selangor, Universiti Selangor, Malaysia.

2.2. Sample preparation

150 ml of microalgae was cultured in F/2 media for 2 weeks before used for pigment extraction experiments. Microalgae were centrifuged at 10000 rpm for 10 minutes at 4 °C. The supernatant was removed and the cell pallet was kept. Weight of microalgae used was 0.1 g in each run and broke with 5 g of glass beads (<107 µm) and the pigments were extracted using 50 ml solvents.

2.3. Optimization studies

Method of extraction was using homogenizer with bead milling assisted. The independent variable A (solvent selection) were 95% ethanol (1), methanol (2) and diethyl ether (3). The range of independent variable B (extraction time) were 15, 30 and 45 minutes in order to improve the pigment yield of Chlorophyll a, Chlorophyll b, Carotene and Phycocyanin. Variation of design level is shown in table 1.
Table 1. Variation of design level in actual and coded unit for three level factorial designs.

| 3 Levels | Unit | Parameter |
|----------|------|-----------|
| -1       | 0    | +1        |
| 1        | 2    | 3         | type Solvents (A) |
| 15       | 30   | 45        | minutes Time (B) |

The response surface method involving a central composite design was adopted in the investigation to define the relationship between the responses and the variables by a group of statistical estimation. A set of 13 experiments including were carried out using three level factorial design with two selected factors and the response in term of pigments concentration was observed. Thirteen runs of three level factorial designs were designed by response surface method (Design Expert 6.0.4) as shown in Table 2.

Table 2. Processing parameter variables for three level factorial designs.

| Run | Solvents (A) | Time (B) |
|-----|--------------|----------|
| 1   | 3            | 30       |
| 2   | 1            | 45       |
| 3   | 2            | 30       |
| 4   | 3            | 45       |
| 5   | 2            | 30       |
| 6   | 2            | 30       |
| 7   | 3            | 15       |
| 8   | 2            | 45       |
| 9   | 1            | 15       |
| 10  | 2            | 30       |
| 11  | 2            | 30       |
| 12  | 1            | 30       |
| 13  | 2            | 15       |

2.4 Spectrophotometric analysis
In spectrophotometric analysis, different pigments from microalgae absorbed different wavelengths of light between range of 400 nm and 700 nm. In this study 4 types of pigments chlorophyll a, chlorophyll b, carotenoid and phycocyanin were observed. Quantification of the pigment extracted was carried out by using composed equations [13].

2.5 Validation of experimental model
The statistical model was validated for all variables within the design to study the capability for pigment extraction.

3. Results

3.1. Optimization of extraction process for pigment extraction using response surface methodology approach
The effects of solvent selection and extraction time on pigment concentration were studied by respond surface methodology. The independent variables used were solvent (A) and extraction time (B) while the response variable of interest was pigment concentration (Y) with $Y_1$ for chlorophyll a, $Y_2$ for chlorophyll b, $Y_3$ for carotene, $Y_4$ for phycocyanin. The second order polynomial model was used in order to represent the response surface. The influence of two factors, time (15, 30 and 45 minutes) and type of solvents (95% ethanol, methanol, diethyl ether) on pigments (chlorophyll A, chlorophyll B, carotene and phycocyanin) extraction were evaluated using three level factorials designed. The yield of
pigments using *Acutodesmus reginae* at different conditions showed that the variation in pigment yield. This indicated that the selected factors had significant effects on pigment extraction process. The concentration of extracted pigments shown in Table 3.

Table 3. Effect of variables on yield of pigments.

| Run | Solvents (A) | Time (B) | Chlorophyll a | Chlorophyll b | Carotene | Phycocyanin |
|-----|--------------|----------|---------------|---------------|----------|-------------|
| 1   | 3            | 30       | 0.27          | 0.06          | 0.1      | 0           |
| 2   | 1            | 45       | 0.92          | 0.64          | 0.27     | 1.79        |
| 3   | 2            | 30       | 0.19          | 0.09          | 0.05     | 0.25        |
| 4   | 3            | 45       | 0.35          | 0.17          | 0.14     | 0           |
| 5   | 2            | 30       | 0.15          | 0.08          | 0.04     | 0.3         |
| 6   | 2            | 30       | 0.09          | 0.04          | 0.03     | 0.09        |
| 7   | 3            | 15       | 0.22          | 0.14          | 0.09     | 0.02        |
| 8   | 2            | 45       | 0.17          | 0.09          | 0.04     | 0.26        |
| 9   | 1            | 15       | 0.03          | 0.02          | 0.01     | 0.09        |
| 10  | 2            | 30       | 0.18          | 0.08          | 0.06     | 0.17        |
| 11  | 2            | 30       | 0.17          | 0.15          | 0.03     | 0.69        |
| 12  | 1            | 30       | 0.11          | 0.06          | 0.03     | 0.06        |
| 13  | 2            | 15       | 0.08          | 0.05          | 0.02     | 0.8         |

From this experiment, the highest yield for all type of pigments was observed in run 2, with extracted of 0.92 μg/mL Chlorophyll a, 0.64 μg/mL Chlorophyll b, together with Carotene at 0.27 μg/mL and 1.70 μg/mL of Phycocyanin. A significant improvement (p<0.05) can be seen resulting in 30, 32, 27 and 20-fold increase of each pigment respectively in comparison to trial 9 which recorded as the minimum yield of Chlorophyll a, Chlorophyll b, Carotene and Phycocyanin. Both run used similar solvent which was 95% ethanol but within 3 times longer durations. The organic solvent used in the extraction process as it is penetrated the cell membrane and dissolving the lipoproteins and lipids of chloroplast membranes [14]. The viability of chlorophyll extraction utilizing natural solvents accomplished through homogenisation, grinding, sonications or ultrasound. It has been found that cell disruption, achieved through grinding, homogenisation, ultrasound, or sonications, significantly improves the effectiveness of chlorophyll extraction using organic solvents. Besides, storage conditions of the filtered microalgae prior to the analysis and the duration of the extraction and the number of extraction steps employed in the analysis also affect the amount of pigments [15]. Simon and Helliwell [16] reported that, just a fourth of the potential chlorophyll a had the option to be extracted by an ideal technique without cell disruption and agree by the finding of this study.

Analysis of variance (ANOVA) was applied to determine the relationship between the process variables and the responses. The experimental results of the design were verified by looking at the correlation coefficient R² and statistical significance of model was verified by F-test. Combination of factors represents an interaction between the individual parameters.

An analysis of variance (ANOVA) was done in order to investigate the significance of the chosen model. ANOVA results are tabulated in Table 4. From the ANOVA model F-test, the model F value were 4.32, 4.67, 4.72, 5.96 respectively for Chlorophyll a, Chlorophyll b, Carotene and Phycocyanin which implies the model is significant for all studied pigments. There is only 3.8%, 3.12%, 3.32%, 1.60% chance that a model F value this large could occur due to noise. Value of Prob>F less than 0.05 indicate model terms are significant. In this case B, AB are significant model terms for three pigments except for carotene. Values greater than 0.100 indicate the model terms are not significant. Table 4 shows that the model selection for each responses is significant as Prob > F is lower that 0.05. model
for Chlorophyll a, Chlorophyll b, Carotene and Phycocyanin are 2FI model. Meanwhile model for carotene is quadratic model. The efficiency of the model was investigated by performing a lack-of-fit test. Results show that lack of fit is significant for both 2FI model that was chosen for Chlorophyll a, Chlorophyll b and Phycocyanin and the quadratic model that was chosen for the Carotene. The Quadratic Model (that was chosen for the Carotene) F-value of 25.32 indicates that the model is significant. For the 2FI model that was chosen for the Chlorophyll a and Chlorophyll b, the Model F-value of 30.63 and 14.48 respectively was implies that the model is significant. Values of Prob > F less than 0.05 indicate the quadratic model and the 2FI model terms are significant. R-square value for response Chlorophyll a, Chlorophyll b, Carotene and Phycocyanin are 0.5903, 0.6089, 0.7712 and 0.6651 respectively.

Table 4. Analysis of variance (ANOVA) for response surface model.

| Independent variables | Sum of squares | F value | Prob>F |
|-----------------------|----------------|---------|--------|
| **Chlorophyll a** | | | |
| Model | 0.36 | 4.32 | 0.038 | Significant |
| A | 0.008067 | 0.29 | 0.6018 |
| B | 0.21 | 7.44 | 0.0233 |
| AB | 0.14 | 5.23 | 0.0479 |
| Residual | 0.25 | | |
| Cor total | 0.61 | | |
| Lack of Fit | 0.24 | 30.63 | 0.0028 |
| **Chlorophyll b** | | | |
| Model | 0.19 | 4.67 | 0.0312 | Significant |
| A | 0.020 | 1.53 | 0.2472 |
| B | 0.079 | 5.95 | 0.0374 |
| AB | 0.087 | 6.53 | 0.0309 |
| Residual | 0.12 | | |
| Cor total | 0.31 | | |
| Lack of Fit | 0.11 | 14.48 | 0.0114 |
| **Carotene** | | | |
| Model | 0.046 | 4.72 | 0.0332 | Significant |
| A | 0.0006667 | 0.034 | 0.8582 |
| B | 0.018 | 9.35 | 0.0184 |
| AB | 0.011 | 5.63 | 0.0487 |
| Residual | 0.14 | | |
| Cor total | 0.059 | | |
| Lack of Fit | 0.013 | 25.32 | 0.0046 |
| **Phycocyanin** | | | |
| Model | 1.87 | 5.96 | 0.0160 | Significant |
| A | 0.61 | 5.87 | 0.0384 |
| B | 0.52 | 4.93 | 0.0534 |
| AB | 0.74 | 7.07 | 0.0261 |
| Residual | 0.94 | | |
| Cor total | 2.81 | | |
| Lack of Fit | 0.73 | 2.69 | 0.1792 |
Hence, the final equation in term of coded factor are as follow where \( A \) for solvents and \( B \) for extraction time

\[
\begin{align*}
Y_1 &= 0.23 - 0.037*A + 0.18*B - 0.19*A*B \\
Y_2 &= 0.13 - 0.058*A + 0.11*B - 0.15*A*B \\
Y_3 &= 0.032 + 0.0033*A + 0.055*B - 0.055*A^2 + 0.024*B^2 - 0.053*A*B \\
Y_4 &= 0.30 - 0.32*A + 0.29*B - 0.43*A*B
\end{align*}
\]

Therefore, the ANOVA analysis postulated that the model relevance for the pigment extraction by *Acutodesmus reginae* was within the limit of experimental factors.
Figure 1. Response surface plots of interaction terms showing significant impact on the yield of pigments extraction. Figure 1(a): Chlophyll a, Figure 1(b): Chlorophyll b, Figure 1(c): Carotene, Figure 1(d): Phycocyanin.

3.2. Response surface plot
The impact of interaction terms on the yield of pigments obtained by two factors illustrated by 3D response surface plots (Figure 1). It can be observed from plots that the optimal response was achieved by using at 45 minutes using 95% ethanol as the solvents. However, below these levels, a decline in the optimal response was observed. The similarities between the predicted and the actual value of the experiment which is the obtained response is depicted in Figure 1 and it can be clearly seen that the results are close to the predicted values by the design with 95% ethanol at extended time give higher yield.
3.3. Verification of the experiment

Once the optimum cultivation conditions were determined using RSM, verification experiments were conducted for further confirmation as suggested by the model in order to compare the experimental values for pigments extraction to the model predicted data. Pigments extraction was done under optimal conditions as predicted by RSM with desirability 0.730 for authentication of the pigment extraction observed. Analysis of the results shows the experimental values obtained was as good as the data predicted by the model indicating that the process optimization is reliable to predict the extraction of pigment.

Table 5. Validation of the predicted and experimental extraction variable.

| Pigment extraction variation | Predicted by statistical approach | Experimental result |
|-----------------------------|----------------------------------|---------------------|
|                            | Chlorophyll a | Chlorophyll b | Carotene | Phycocyanin | Chlorophyll a | Chlorophyll b | Carotene | Phycocyanin |
| 95% ethanol 45 minutes      | 0.637         | 0.449         | 0.219     | 1.343       | 0.442         | 0.289         | 0.1424    | 1.183       |

Conclusion

The statistical analysis proved to be beneficial and great tool in developing optimum extraction conditions. ANOVA of the model demonstrates that the models were significant. Statistical approach showed that the pigment extraction process was optimum using 95% ethanol within 45 minutes of homogenization with bead milling assisted. The software able to evaluate the effects and relation of factors in order to search optimum condition as well as predicting the response and checking the adequacy of the model without complex calculation to analyze data. Microalgae is a promising alternative source for pigments.

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Acknowledgments
The authors would like to acknowledge Universiti Selangor. This program was funded by Selangor State Government by ‘Geran Penyelidikan Negeri Selangor’ (GPNS).