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Acutodesmus obliquus CN01 as new potential source of eicosatetraenoic acid: Enhancing omega-3 fatty acids production by Acutodesmus obliquus CN01 by medium optimization using Taguchi method

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Abstract: *Acutodesmus obliquus* is widely known as oleaginous microalgae that is able to produce α-linolenic acid (ALA). In this study, this microalgae was detected to produce eicosatetraenoic acid (ETA) which is a very rare omega-3 fatty acid. Recently, there has been renewed interest in ETA which has shown to have a pivotal role in anti-inflammatory activities. ETA is of significance due to their diverse functions that are little investigated. The aim of the study was to enhance the yield of ALA and ETA produced by *Acutodesmus obliquus* CN01 by optimising various culture conditions using Taguchi method that are much simpler (minimum number of experiments) with time and cost-saving. Four factors studied were carbon, nitrogen, salinity, and phosphorus. Taguchi method was performed to evaluate the optimisation of omega-3 fatty acids production under different culture conditions. The results demonstrated that the optimal culture conditions can be obtained by following the combination of 0.5 gL^{-1} of sodium acetate, 0.015 gL^{-1} of sodium nitrate, 0.58 gL^{-1} of sodium chloride and 0.0002 gL^{-1} of dipotassium hydrogen phosphate concentration. Carbon is observed as the main factor contributing to omega-3 fatty acids production. All sets of experiments produced both ALA and ETA. The result suggested that *Acutodesmus obliquus CN01* has shown to be potential omega-3 fatty acids producer including the rare ETA which is of beneficial to the pharmaceutical industry and also food industry.

Keywords: ***Acutodesmus obliquus*, omega-3 fatty acids, α-linolenic acid, eicosatetraenoic acid, Taguchi Method**

1. Introduction

Recently, there has been renewed interest in microalgae as a novel "green technology" [1]. Previous studies have reported that microalgae have advantages of biomass with high economic potential [1]. Existing research recognizes the crucial role played by microalgae, which include anti-inflammatory, antidiabetic, antioxidant, anticoagulant, immunomodulatory, antimicrobial effects, UV protective and tyrosinase inhibitory [2].

Acutodesmus obliquus is widely known as fresh-water oleaginous unicellular green microalgae that is able to produce ALA. *Acutodesmus obliquus* are oxygenic photosynthetic organisms that are of interest due to its fast growth and easy to cultivate [3]. It is mesophilic microalgae that can grow between 15 to 40 °C [1] and has a very short (less than 24 hours) cell cycles [4].

Fatty acids are valuable biocomponents that are rapidly accumulated in microalgae. Omega-3 fatty acids has significant roles in cell signalling, ameliorate cardiovascular diseases, membrane structure and function [5]. ALA (C18:3ω3) acts as a precursor of omega-3 metabolic pathway [5].

Eicosatetraenoic acid (ETA, C20:4ω3) is an omega-3 fatty acids that involved in anti-inflammatory effects [5]. To date, ETA has received limited attention in the research literature. There is little published data on biochemical function of ETA due to rarity of ETA in natural oils and many studies only focused on the health benefit of ALA [5]. However, pure ETA is extremely expensive and difficult to obtain, which also limits the investigation on the role of ETA [5]. Exploring new microalgal strains as a potential source of ETA has raised a great interest. This is due to continuing concern of increasing demand from the growing world population for high purity of omega-3 fatty acids especially ETA [5].

Orthogonal arrays (OA) is the key for the development of Taguchi method [6]. L_9 of Taguchi's orthogonal array, and signal-to-noise (S/N) ratio were employed to identify the optimal media parameters and effectiveness of this approach.

To enhance ALA and ETA production, Taguchi method was conducted to evaluate the optimization of ALA and ETA production under different culture conditions. To date, this was the first study to demonstrate the application of Taguchi's method to enhance ALA and ETA production. In this study, the main focus was on ALA and ETA production by optimizing the key media component. In order to obtain optimal culture medium, choosing the best nutrient concentration was one of the major obstacles in enhancing omega-3 fatty acids production. Results obtained from Taguchi method were compared with analysis of variance (ANOVA). Optimization of microalgae cultivation has emerged as powerful platforms for production of biocomponents with high quality that can enhance the production efficiency of microalgae and reduce its cost [7].

In this study, *Acutodesmus obliquus* CN01 was found to accumulate a considerable amount of ALA and ETA. The main objective of this study is to improve ALA and ETA production in *Acutodesmus obliquus* CN01 by optimising culture conditions using the Taguchi method.

2. Materials and Method

2.1. Microalgal species and culture conditions

The freshwater species, *Acutodesmus obliquus* (CN01, MJIIT-UTM, Malaysia) was obtained by researchers from Universiti Teknologi Malaysia. *Acutodesmus obliquus* CN01 were cultured in autoclaved AF6 medium as described in Othman et al. [8]. Cultures were grown for 12 h photoperiod at 25°C under light intensity of approximately 80μmol photons/m^2/s with continuous aeration [8]. The cultures were grown until it reached stationary phase. The biomass was then freeze-dried and stored in the -80 °C freezer until further use.

2.2 Orthogonal array design and analytic strategy

In this study, four variable factors (each has three levels), i.e. carbon, nitrogen, salinity, and phosphorus, were selected for this optimization. As shown in Table 1, the parameters that were optimised are sodium acetate concentration (0.5000, 1.0000, and 2.0000 gL^{-1} [9]) as carbon source, sodium chloride concentration (0.58, 1.45, and 5.80 gL^{-1}[10]) as salinity source, dipotassium hydrogen phosphate concentration (0.0002, 0.0005 [11] , and 0.0015 gL^{-1} [12]) as phosphorus source and sodium nitrate concentrations (0.0150 [13], 0.0200, and 0.1000 gL^{-1} [13]) as nitrogen source.

Table 1 : Factors and levels in the Taguchi method for optimisation of omega-3 fatty acids production

Serial number	Factor	Level 1	Level 2	Level 3
1	Carbon	0.5000	1.0000	2.0000
2	Nitrogen	0.0150	0.0200	0.1000
3	Salinity	0.5800	1.4500	5.800
4	Phosphorus	0.0002	0.0005	0.0015

The combination of factors level for optimal response of omega-3 fatty acids production were identified by using Taguchi method. Table 1 represents the factors and levels in the Taguchi method for optimisation of omega-3 fatty acids production that was obtained via literature studies.

Table 2 represents the orthogonal array (OA) of L_9. Orthogonal array of L_9 was chosen as the experimental design to study the four factors with three different levels each. Columns in Table 2 indicate the levels of each factor and each row indicates the set of experiments that need to be run. L_9 means there are 9 trials in the experiment. Hence, using Minitab 17, the optimal conditions are determined by the highest S/N ratio obtained from combination of level of each factors.

Table 2 : L_9 orthogonal array of Taguchi method

	C1	C2	C3	C4
	Carbon	**Nitrogen**	**Salinity**	**Phosphorus**
1	0.5	0.015	0.58	0.0002
2	0.5	0.020	0.94	0.0005
3	0.5	0.100	1.45	0.0015
4	1.0	0.015	0.94	0.0015
5	1.0	0.020	1.45	0.0002
6	1.0	0.100	0.71	0.0005
7	2.0	0.015	1.45	0.0005
8	2.0	0.020	0.71	0.0015
9	2.0	0.100	0.94	0.0002

The omega-3 fatty acids molecule results obtained from OA were measured and S/N ratio was calculated to optimise the experimental data. For S/N ratio, the larger the better function (larger response is better) was selected.

2.3 Fatty acids analysis

ALA and ETA in *Acutodesmus obliquus* CN01 is detected after microalgae reached the stationary phase. The fatty acids are prepared as described in Othman et al [8]. Shimadzu GCMS-QP2010 (Shimadzu, Kyoto, Japan) which was equipped with a CP-cil 5 CB column (50 m x 0.32 mm x 0.12 mm film thickness) was used for gas chromatography/ mass spectrometry (GC/MS) analysis. Helium was used as the carrier gas with 1 mL/min of column flow rate. The injector temperature was kept at 320 °C, with an oven temperature programmed from 60 °C to 130 °C at a rate of 20 °C/min after 1.5 min hold at 60 °C , from 130 °C to 320 °C at a rate of 4 °C/min, and then finally maintained at 320 °C for 18 min. Percent relative to the peak area was used for quantification of fatty acids. The FAMEs content was calculated as mentioned by Corro et al [14].

2.4 Analysis of variance (ANOVA)

Response of each factor in orthogonal experiment was evaluated by using ANOVA. The effect of experiment parameters (sodium acetate, sodium nitrate, sodium chloride and dipotassium hydrogen phosphate) were investigated using ANOVA. Factors that has P- value that is less than 0.05 ($p<0.05$) was considered as the most significant factor in this study.

3. Results and Discussion

3.1 OA analysis and S/N ratio

For optimisation purposes, 9 sets of experiments were conducted according to suggestions by the Taguchi method. This optimisation was conducted to escalate the omega-3 fatty acids production in *Acutodesmus obliquus*.

Experiment No.	C1	C2	C3	C4	Omega-3 fatty acids molecule detected in percentage (%)		S/N ratio
	Carbon	Nitrogen	Salinity	Phosphorus	ALA	ETA	
1	0.5	0.015	0.58	0.0002	8.6	7.3	18.6697
2	0.5	0.020	0.94	0.0005	3.8	4.1	11.5728
3	0.5	0.100	1.45	0.0015	6.8	5.0	16.6502
4	1.0	0.015	0.94	0.0015	2.4	2.7	7.6042
5	1.0	0.020	1.45	0.0002	1.9	3.8	5.8451
6	1.0	0.100	0.71	0.0005	1.8	1.5	5.1055
7	2.0	0.015	1.45	0.0005	2.7	2.2	8.5302
8	2.0	0.020	0.71	0.0015	2.4	2.4	7.6042
9	2.0	0.100	0.94	0.0002	3.3	0.6	10.3703

Table 3 : Omega-3 fatty acids molecule detected after optimisation

From Table 3, only ALA was detected while neither EPA nor DHA was identified. From the previous study, *Acutodesmus obliquus* usually has the ability to produce ALA but it is very rare to produce ETA. ETA was an intermediate molecule between stearidonic acid and EPA. The most striking results to emerge from the data is that this optimisation was successful in producing ETA that was very rare to this species. This is an interesting and remarkable finding. According to previous study, this is the first study to reveal the presence of ETA in *Acutodesmus obliquus* [4, 15]. This result is somewhat counterintuitive.

From this set of experiments, the first set of experiments seems to be the optimal condition because it had the highest content of ALA and ETA which are 8.6 and 7.3% respectively. Therefore, this condition was considered as an optimum culture condition for *Acutodesmus obliquus* CN01. After S/N ratio was analysed, S/N ratio data and its responses were converted to Figure 1 for selecting the best and optimal factor level. From Figure 1, combined factor with the highest S/N ratio was identified as optimal setting.

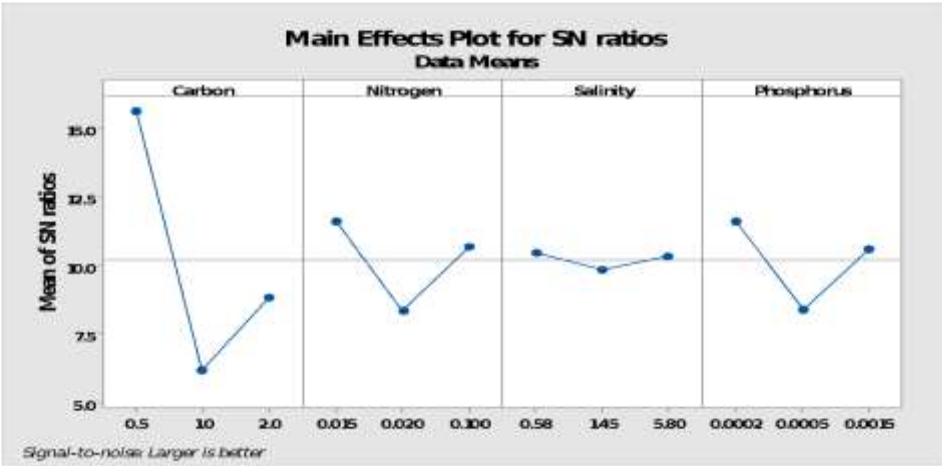

Figure 1 Control factor effects on omega-3 fatty acid production

From Figure 1, 0.5 gL^{-1} of carbon, 0.015 gL^{-1} of nitrogen and 0.0002 gL^{-1} of phosphate has a larger impact in optimising the medium composition for omega-3 fatty acids production. 0.58 gL^{-1} and 5.80 gL^{-1} of salinity yield nearly equal S/N ratio, but 0.58 gL^{-1} salinity was selected due to the maximum response of S/N ratios obtained in Figure 1.

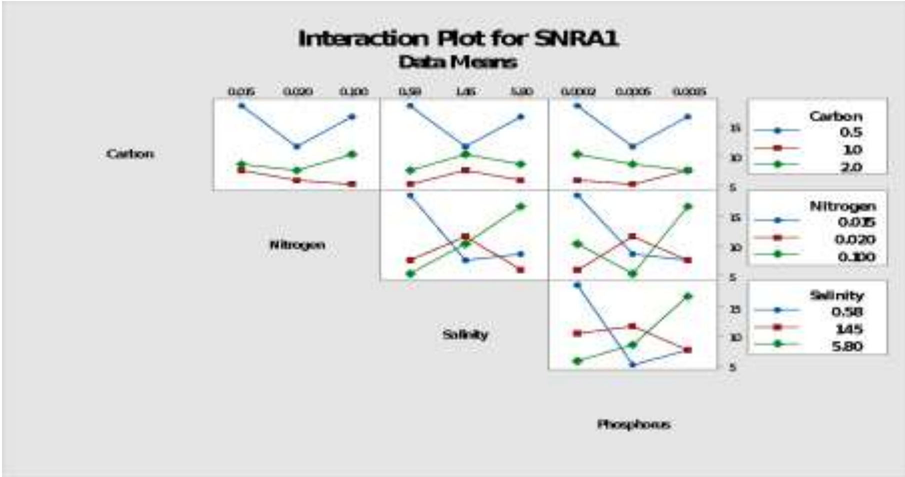

Figure 2 Interaction effects plot for S/N ratios

Figure 2 represents the interaction plot for S/N ratios for combination of each factors. The optimal conditions could be determined from these graphs.

Overall, from Table 4, these results indicate that carbon sources are the major factors that affect ALA and ETA production followed by nitrogen, phosphorus, and salinity respectively.

Table 4 : Response Table for Signal to Noise Ratios (Larger is better)

Level	Carbon	Nitrogen	Salinity	Phosphorus
1	15.631	11.601	10.460	11.628
2	6.185	8.341	9.849	8.403
3	8.835	10.709	10.342	10.620
Delta	9.446	3.261	0.611	3.226
Rank	1	2	4	3

From Table 5, 0.5 gL^{-1} of sodium acetate (level 1), 0.015 gL^{-1} of sodium nitrate (level 1), 0.58 gL^{-1} of sodium chloride (level 1), and 0.0002 gL^{-1} of dipotassium phosphorus (level 1) respectively, were considered as the optimum conditions for ALA and ETA production for this microalga in this study.

Table 5 : Optimum values of each factors and their respective levels

Parameter	Optimum value
Carbon	0.5
Nitrogen	0.015
Salinity	0.58
Phosphorus	0.0002

Parameters	Degree of Freedoms	Sum of Squares	Mean Square	F-value	P-value
Carbon	2	142.43	71.217	12.57	0.007
Nitrogen	2	17.04	8.518	0.32	0.737
Salinity	2	0.630	0.3148	0.01	0.989
Phosphorus	2	16.34	8.168	0.31	0.747
Total	8	176.44			

3.2 ANOVA analysis

From Table 6, only carbon has a significant effect towards this experiment ($p<0.05$). Meanwhile, other parameters such as nitrogen, salinity and phosphorus are not significant to this experiment. What is interesting in this data is that ANOVA revealed similar conclusions as S/N ratio results. These results suggest that carbon is the most significant factor for medium optimization in this study.

Table 6: ANOVA results for signal-to-noise ratio for parameters involve in omega-3 fatty acids production

4.0 Conclusion

This is the first study that employed Taguchi method in optimising culture medium for omega-3 fatty acids production in *Acutodesmus obliquus* CN01 in different factors (carbon, nitrogen, salinity and phosphorus). Optimal level for each factor was determined by adopting L_9 Taguchi's OA. The optimal factors for medium optimisation in this study are: 0.5 g/L of sodium acetate (level 1), 0.015 g/L of sodium nitrate (level 1), 0.58 g/L of sodium chloride (level 1), and 0.0002 g/L of dipotassium phosphorus (level 1). Interestingly, S/N ratio and ANOVA results revealed similar conclusions which demonstrated that carbon is the most significant factor among other parameters studied. Further research could usefully explore *Acutodesmus obliquus* CN01 as a new source of ETA to ameliorate inflammatory**.**

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions:

All authors mentioned contributed significantly, directly and intellectually to the work, and authorized it for publication.

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