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Optimization of Producing Liquid Fuel from Photosynthetic Algal Growth

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The green alga, *Dunaliella salina* under fed-batch cultivation produced 51.12 mg of hydrocarbon per liter with maintaining 0.313 (g dry wt/l). About 20% of hydrocarbon production yield based on dry biomass was obtained from both batch and fed-batch processes. Optimum culture conditions of light intensity, pH and salt concentration were obtained as 0.0080 (kJ/cm^2/h), 8.0 and 1.4 (g of NaCl/l), respectively by response surface analysis. The production of hydrocarbons in *D. salina* was closely correlated to cell growth. Fed-batch cultivation produced more hydrocarbons and maintained better cell growth than a batch process.

Much effort has been made to develop alternative energy sources to replace coal and petroleum for several decades (1, 9, 10). The production of ethanol from various sources has intensively been studied because it can partially substitute gasoline with relatively cheaper production costs than other alternative energy souces can. One of current interest in developing alternative energy is to utilize solar energy by solar cells and microorganisms (2), to directly use sun light or to produce other forms of energy such as hydrogen and hydrocarbons. It has been reported that only *Botryococcus braunii* can produce enough quantities of liquid hydrocarbons to meet economic feasibility (4, 5). However, this process has not been scaled-up yet to industrial application since biological conversion of light into chemical energy has not been completley understood and this system has less been analyzed to scale-up (11, 12). Therefore, we have screened new strains of photosynthetic organisms and then optimized for mass production of liquid fuel.

MATERIALS AND METHODS

Green alga and Culture Conditions

Dunaliella salina LB 200 (UTEX, USA) was grown in a 250 ml flask containing 100 ml of artificial seawater medium. This medium also contains 7.35% (w/v) of sodium chloride (pH 7.63). Detail composition of modified medium was described elsewhere (17). The flask was incuvated at 25℃ with 3.80×10^{-3} (kJ/cm^2/h) of light intensity in a rotary shaking incubator at 50 rpm for six to eight days before inoculating to a bioreactor for batch and fed-batch experiments. Light intensity was measured by Quantum sensor (Licor LS 190, USA) and controlled by changing the distance between the reactor and flourescent lamps (20 W white cool, Philips, USA). 10 ml of culture broth was filtered by 0.45 nm filter paper, and surface water in the paper was removed after washig the cells two or three times with distilled water. Then, cells on a filter paper were measured as fresh cell density. Dry cell density was measured by drying cells at 105℃ for one and half hours after filtering 15 ml of broth through a 0.45 μm filter paper (Milipore, USA).

Batch and Fed-batch Cultivations

Batch and fed-batch experiments have been carried out in a photobioreactor (working volume 12 l), which was made of acryl plates. Rectangular shape of the bioreactor (50×30×11 cm) was desingned to uniformly distribute the light intensity and 11 (cm) of light path was enough to make relatively uniform light intensity gradient inside the reactor by employing Lamber-Beer's law with 1.58 (l/g/cm) of extinction coefficient (13).

For batch and fed-batch experiments, 250 ml of liquid culture from a flask was inoculated into the bioreactor (10 l of initial working volume) where pH and temperature were automatically adjusted by switching on and

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off a hot plate stirrer and CO_2 gas flows. Once cell density reached to the maximum value, which was the ending point of batch cultivation, fresh media were added into the reactor by a peristaltic pump for fed-batch cultivation. Fresh media were added with 5 (ml/h) of constant flow rate until cell density was increased, then media flow rates were slowly increased up to 12 *l* of total working volume.

Purification and Analysis of Algal Hydrocarbons

Hydrocarbons secreted from cells were separated by adding two volumes of hexane into culture broth at room temperature, then evaporated. Cells were also disrupted by a sonicator (M-150, USA) for 5 min, then two volumes of hexane were added for one and half hours with stirring at room temperature. The hexane extracts were separated from cells by centrifugation at 100 rpm and then concentrated by evaporating remaining hexane.

Table 1. Values of independent variables and treatment conditions for randomized experimental design in batch cultivation of *D. salina*

Variables		Levels				
		−2	−1	0	1	2
C/N ratio	(X1)	0.0	0.75	1.50	2.25	3.00
NaCl conc.	(X2)	0.2	0.80	0.40	2.00	2.60
pH	(X3)	4.0	6.00	8.00	10.00	12.00

Treatment No.	X1	X2	X3
1	−1	−1	−1
2	1	−1	−1
3	−1	1	−1
4	−1	−1	1
5	1	1	−1
6	1	−1	1
7	−1	1	1
8	1	1	1
9	2	0	0
10	−2	0	2
11	0	2	0
12	0	−2	0
13	0	0	2
14	0	0	−2
15	0	0	0
16	0	0	0
17	0	0	0
18	0	0	0
19	0	0	0
20	0	0	0
21	0	0	0

Hydrocarbons from two portions were weighted to estimate the distribution ratio of them. Crude hexane extracts were separated by silica gel chromatographys. Silica gel (Merk 60GF 254) was activated for two hours at 110℃ and packed in a 2×20 cm column, then eluted with hexane (7). Purified hydrocarbons were identified by thin layer chromatography (TLC) and gas chromatography (MBM, West Germany) (14, 15) with eicosapentaenoic acid (EPA, $C_{20:5}$) as a standard. It has been reported that hydrocarbons from microalgae were similar to EPA and squalene (4, 8). Rhodamine 6G was used as an UV illuminating agent.

Optimazation of Culture Conditions

Response Surface Method (RSM) (6) was employed to optimize cell growth and hydrocarbon production in batch cultivation with three independent parameters of pH, NaCl concentration and C/N ratio in the media. Table 1 is an experimental design for RSM analysis with polynominal linear regression model. Experimental data were linearized by following equations:

$$X_1 \text{ code} = X_1 E^{-1.5}/0.75 \quad (1)$$

$$X_2 \text{ code} = X_2 E^{-1.4}/0.60 \quad (2)$$

$$X_3 \text{ code} = X_3 E^{-8.0}/2.00 \quad (3)$$

ANOVA method was used to correlate cell growth and product production with independent variables (6).

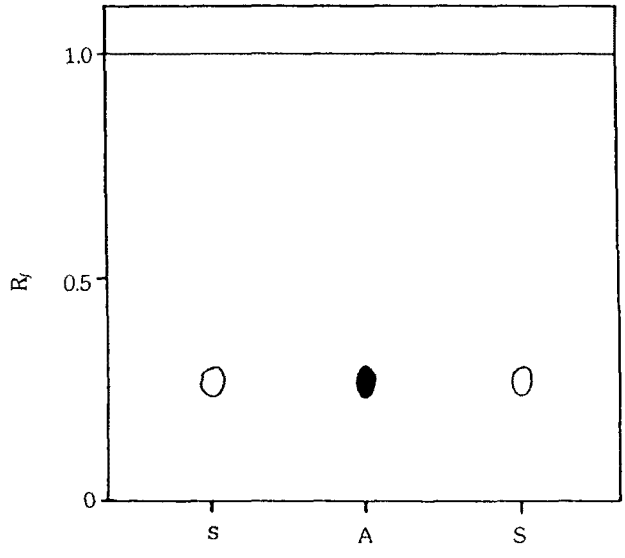

Fig. 1. Comparison of R_f values between the standard and samples from *D. salina* by thin layer chromatography. Solvent is the mixture of petroleum ether, diethyl ether and acetic acid (9:1:0.1, v/v).
S: standard (EPA); A: algal hydrocarbon.

RESULTS AND DISCUSSION

Fig. 1 is to compare hydrocarbons from *D. salina* to a standard by TLC, showing that hydrocarbon from algae has same R_f value as the standard. It possibly means that major portion of total hydrocarbons has twenty carbons in the chain. Then., Fig. 2 confirms the compositions of purified hydrocarbons by GC analysis, comparing to a standard EPA. Numbers in Fig. 2 are the identification numbers to compare peaks in samples and the standard. For example, number one in the A (standard) is the same hydrocarbon in number one of B (sample). It proves that $C_{20:5}$ is main hydrocarbon in hexane extracts from this algae because major peaks of the standard is $C_{20:5}$ as shown in Fig. 2.

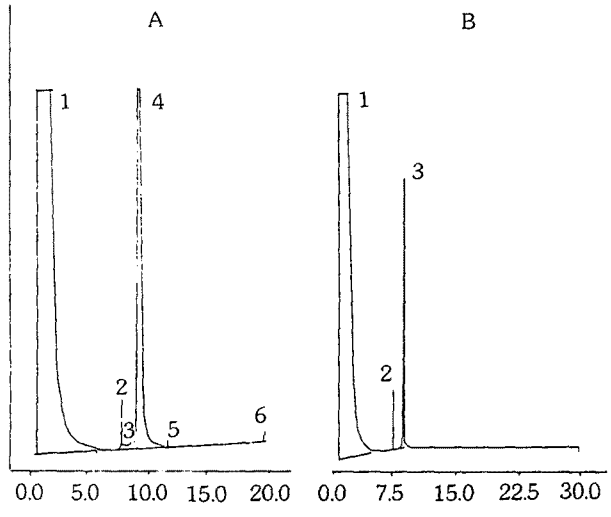

Fig. 2. Identification of hydrocarbons in the standard and samples by gas chromatography. Numbers in the figure are the identification digits between A and B.
A: standard (EPA); B: total hydrocarbon from *D. salina*.

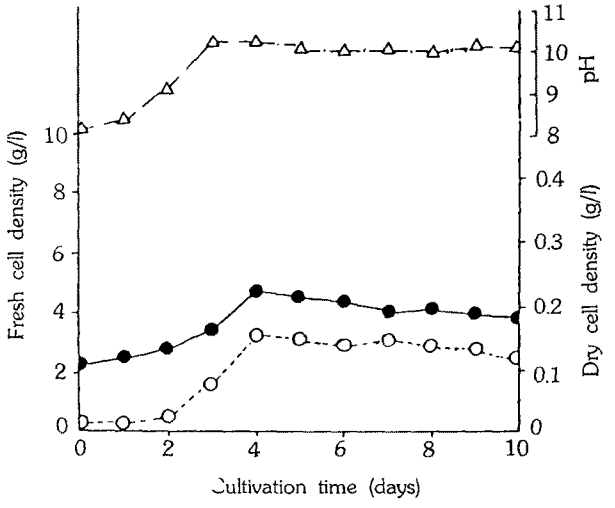

Fig. 3. Grwoth behaviour and changes of pH in batch cultivation of *Dunaliella salina*: Dark and open circles are fresh and dry cell densities, respectively. Triangles are the change of pH.

Fig. 3 illustrates growth behaviour and pH changes under batch cultivations of *D. salina*. Both dry and fresh cell densities were gradually decreased with slight increase of pH, up to 10. The differences between dry and fresh cell densities were relatively constant possibly due to constancy of cell components throughout the batch process, except for the lag period. Fig. 4 can be compared with the data from batch cultivation in Fig. 3. Higher maximum cell density and longer of cultivation time were maintained than those from the batch process: 8 (g of fresh cells/l) and 10 days vs 5 (g of fresh cells/l)

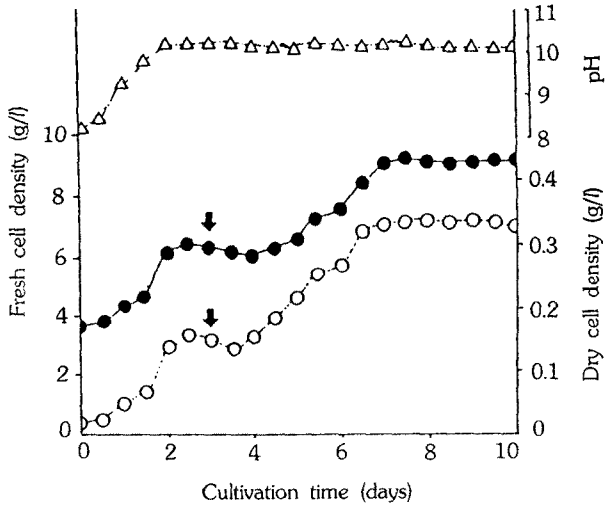

Fig. 4. Growth kinetics of cultivating *Dunaliella salina* under fed-batch condition and pH changes according to cultivation time: Dark and open circles are fresh and dry cell densities, respectively. Triangles are the change of pH and arrows are the points of feeding fresh media into the reactor.

Table 2. Results of cultivating *D. salina* under batch condition at various light intensities

Light intensity $I_o \times 10^3$ (kJ/cm^{2}h)	Cell density (g dry wt/l)	Total crude hydrocarbon (mg/l)
1.92	0.098	19.03
5.36	0.124	23.99
8.17	0.173	33.17
15.32	0.157	29.44
26.43	0.144	28.19

and 4 days, respectively. When cell growth reached to maximum cell density (at three days after starting batch cultivation), five ml of fresh media was constantly fed into the reactor every hour.

Table 2 is the result of cultivating *D. salina* under batch conditions as a function of light intensity for the production of hydrocarbons. As the light intensity was increased, both of the highest cell density and product production were obtained at 81.73×10^{-4} (kJ/cm^2/h) of light intensity. This optimum light intensity is similar to those for other algae (3). Media compositions were also optimized with three variables of NaCl, C/N ratio and pH in the media by RSM anlaysis as shown in Table 1. Fig. 5 is a NaCl vs pH contour plot to find optimal medium composition for the product production by utillizing data in Table 1 since pH and NaCl concentration played much important role in producing hydrocarbons than other combinations (C/N vs pH, etc.). Table 3 is the result of optimizing culture meida for both cell

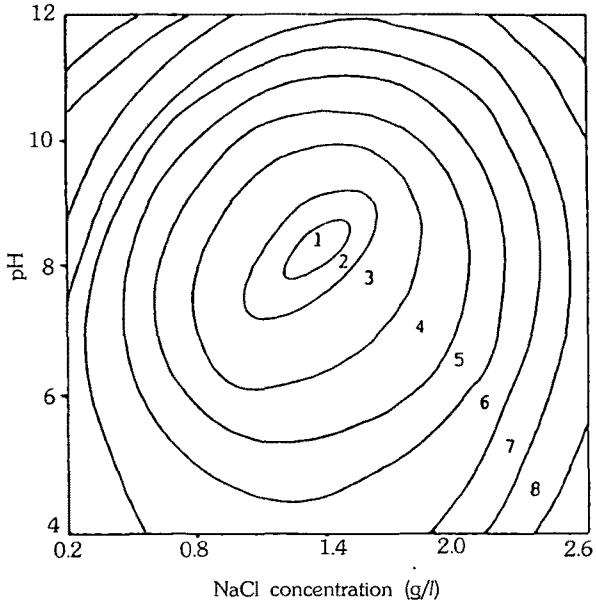

Fig. 5. Contour plot of NaCl versus pH at different hydrocarbon concentrations from cultures of *Dunaliella salina*. Estimated values of 28.05, 26.56, 25.24 and 23.84 (mg/l), respectively, from the center to the outside of circles in the plot.

Table 3. Optimized composition of culture medium for the growth and hydrocarbon production of *D. salina*+

Component	Concentration (g/l)
$MgSo_4\cdot7H_2O$	2.44
KCl	0.60
$NaNO_3$	1.16
$NaHCO_3$	1.37
$CaCl_2\cdot2H_2O$	0.30
KH_2PO_4	0.05
Tris buffer	0.10
Vitamin B_{12}	0.0013
A solution*	3 ml
B solution**	10 ml

+Final pH of this medium is 7.58 and 6.8% of NaCl concentration.

*Dissolved in 500 ml hot distilled water and 10 g Na_2-EDTA then added 500 ml of 0.1 N HCl dissolving 0.81 g of $FeCl_3$.

**H_3BO_3 3.426, $CoCl_2\cdot6H_2O$ 1.215, $MnCl_2\cdot H_2O$ 0.432, $ZnCl_2$ 31.5 and $(NH_4)_6Mo_7O_{24}\cdot4H_2O$ 31.2 (mg/l) and 1.0 (ml) of H_2SO_4.

Table 4. Comparison of hydrocarbon concentration between expermental data and estimated values by response surface anlaysis from Table 3

Treatment No.	Experimental data point	Estimated data point	Residuals
1	30	25.34	4.657
2	26	23.34	2.657
3	12	11.84	0.157
4	28	29.84	−1.843
5	24	15.84	8.157
6	30	23.84	6.157
7	18	14.34	3.657
8	16	14.34	1.657
9	0	6.16	−6.157
10	8	8.16	−0.157
11	0	3.66	−3.657
12	24	26.66	−2.657
13	32	33.66	−1.657
14	26	30.66	−4.657
15	24	24.90	−0.902
16	24	24.90	−0.902
17	24	24.90	−0.902
18	24	24.90	−0.902
19	24	24.90	−0.902
20	24	24.90	−0.902
21	24	24.90	−0.902

Table 5. Comparison of cell growth and hydrocarbon productivity between batch and fed-batch cultivations for the growth of *D. salina*

	Cell density (g dry wt/l)	Total crude hydrocarbon (mg/l)	Production yield (%)*
Batch	0.144	29.23	20.23
Fed-batch	0.313	51.12	16.33

*Estimated based on dry cell density.

growth and hydrocarbon production in *D. salina* from Fig. 5.

Table 4 is to compare the experimental data with estimated results from RSM analysis for hydrocarbon production. An estimated model well fits to actual results, having 2.58 of absolute average value of differences between two data points for all experimetns. Table 5 summarizes cell growth, hydrocarbon production and production yield from the culture of *D. salina* under batch and fed-batch conditions. Both cell growth and productivity were higher in fed-batch process than in batch culture. Total hydrocarbon production from fed-batch cultivation was two times higher than that from batch process. However, production yield based on dry cell was lower in fed-batch cultivation due to higher accumulation of cells. Therefore, specific product production of hydrocarbon for fed-batch system will be higher than for batch process. About 20% of hydrocarbons from dry biomass in fed-batch cultivation of *D. salina* are lower than 70% of production yield from *B. braunii* (16). However, *D. salina* produced most of C_{20} as shown in Fig. 1 and 2, compared to wide spectrums of saturated and unsaturated carbons from *B. braunii* (8). It must be an excellent merit in cracking algal hydrocarbons and maintaining high efficiency for fuels.

Acknowledgement

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