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MASSIVE PRODUCTION OF SOME ECONOMICALLY IMPORTANT METABOLIC COMPOUNDS IN *DUNALIELLA SALINA*. PART III: GROWTH AND CARBOHYDRATE OPTIMIZATION.

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Abstract

The nutrient factors controlling growth and carbohydrate production by *Dunaliella salina* were statistically analysed. The obtained results indicated that: (i) *Dunaliella salina* grows better at media having lower sodium chloride concentrations rather than higher ones. (ii) Sodium chloride at high concentrations had a negatively significant effect on carbohydrate production by *Dunaliella salina*. (iii) Reduction of sodium chloride concentration in the basal medium from 1.25M to 0.75M led to maximum carbohydrate production by *Dunaliella salina*.

Key words: *Dunaliella salina*, carbohydrate, optimization.

Introduction

Photosynthesis is the most abundant energy-storing and life-supporting process on earth. It is not surprising, therefore, that utilization of the photosynthetic machinery for the production of energy, chemicals and food by mass culturing of microalgae has a particular appeal. Serious attempts to utilize mass culture systems for marine microalgae were initiated in the last few years, prompted by requirements in the mari-culture food chain biotechnology, the search for lipid-producing microalgae as a means for large scale biological energy storing system (S.E.R.I., 1985) and the investigation of specific algae which accumulate industrially interesting products (Parkinson, 1987).

The halotolerant green alga *Dunaliella salina* has several features, which made it favorite for mass-cultivation (Ben-Amotz and Avron, 1983). Many workers revealed that different species of *Dunaliella* can tolerate a very wide range of salinity variations up to saturation of sodium chloride (Marre *et al*., 1958; Trezzi *et al*., 1965 & Borowitzka and Brown, 1974). On the other hand, the reduction in algal growth as a response to increase in salinity has been recorded in both fresh water and marine algal species (Selter and Green Way, 1979 &Mohy El-Din, 1992).

Carbohydrates, the direct photosynthetic products, were found to be quantitatively and qualitatively affected by salinity, Ahmed *et al*.,(1985) and

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Shafea, (1987). Higher plants were recorded to accumulate soluble sugars and polyols at the expense of other sugar fractions in response to salinity (Karadge and Chavan, 1983; Imamul-Hug and Larther, 1984 & Weimberg *et al*., 1984). The monosaccharides, glucose and fructose might be important in osmoregulation at high salinity level (Weimberg *et al*., 1984). Similarly, accumulation of carbohydrates, was also recorded in fresh water algae as well as in marine species in response to salinization treatments (Weincke and Lauchli, 1981; Edmann,1983 & Warr *et al*., 1985). The accumulation of these soluble compounds could be derived from current photosynthesis or from stored compounds such as polysaccharides and glycoproteins. In this respect, Wegmann (1971) found that in *Dunaliella*, the stored starch was consumed for glycerol synthesis. The capacity of *Dunaliella* for osomoregulation has been attributed to the accumulation of glycerol in the cells (Ben-Amotz and Avron, 1973). Sucrose which originated partially from photosynthesis was also recorded to be accumulated in some salinized organisms (Wegmann, 1968). In *Dunaliella*, sucrose accumulation was originated from hydrolysis of stored carbohydrates (Wegmann, 1969). Therefore, the aim is to determine which nutrient factor (s) controls the optimization of growth and the synthesis of these metabolites. To confirm this, thirty-two trials have been experimentally studied for each case. The basal medium (MH medium, Loeblich 1982) was mainly used to test the state of algal growth and carbohydrate content under controlled conditions.

Materials and Methods

Organism

The organism used in this work was the unicellular green alga, *Dunaliella salina*. It was obtained from the algal culture collection of phycological laboratory, Botany Department, Faculty of Science, Alexandria University. The organism itself was originally collected and isolated from the brine water of salty lagoons with high irradiation zone at El-Mex district, Alexandria.

Growth Medium

The axenic cultures of *Dunaliella salina* was grown in MH medium (Loeblich 1982) composed of the following constituents:

Salts	Amount / litre
NaCl	73.050g
$MgCl_2.6H_2O$	1.500g
$MgSO_2.7H_2O$	0.500g
KCl	0.200g
$CaCl_2.2H_2O$	0.200g
KNO_3	1.000g
$NaHCO_3$	0.043g
* KH_2PO_4	0.035g
EDTA	1.890mg

$FeCl_3.6H_2O$	2.440mg
$ZnCl_2$	0.041mg
H_3PO_3	0.610mg
$CoCl_2.2H_2O$	0.015mg
$CuCl_2.2H_2O$	0.041mg
$MnCl_2.4H_2O$	0.410mg
$(NH_4)_6\ Mo_7O_{24}\ .4H_2O$	0.300mg

PH was adjusted at 7.5

* Potassium phosphate solution was autoclaved separately and added aseptically to the sterilized medium to avoid the precipitation of phosphate.

Culture conditions

The axenic cultures was grown in MH medium (Loeblich 1982) under controlled laboratory conditions (temperature at 25 ± 3 °C and light at 4000 lux). Culture experiments were conducted under a regime of 16 hours light / 8 hours dark in 250 ml Erlenmeyer flasks, each contained 100 ml medium.

Growth Determination

Growth of *Dunaliella salina* was determined by cell counting every two days. However, the following estimates were also calculated as criteria for growth.

The relative growth rate (K`) (Fogg 1975):

$$K` = \frac{\log N - \log No}{t}$$

Where:

Log N = log number of cells after time (t) days.

Log N_0 = log number of cells at the initial time.

The generation time:

$$G.T. = \frac{0.301}{K`}$$

Where:

0.301 = Growth constant.

K` = Relative growth rate.

Growth rate R (division / day) (Stein, 1973):

$$R = \left[\frac{3.322}{T_2 - T_1}\right] - \log\frac{N_2}{N_1}$$

Where:

3.322 = Growth constant.

T_1 = time at the beginning of the experiment.

T_2 = time at the end of the experiment.

N_1 = number of cells at the beginning of the experiment.

N_2 = number of cells at the end of the experiment.

Measurement of total carbohydrate content

Total carbohydrate contents were estimated according to the method described by Dubois *et al*. (1959).

Statistical analysis

The statistical methods used were those recommended by Davis (1979) & Bloor and England (1991).

Results and Discussion

In the present work the massive production of carbohydrate content in *Dunaliella salina* was experimentally discussed.

Growth Optimization

Preliminary results showed that maximum growth rate was observed between the eight and tenth days. Regarding the effect of different minerals used in the basal medium on growth optimization and carbohydrate production, a sophisticated statistical treatment (consisting of 1^{ry} and 2^{ry} factorial experiments) was done.

Primary factorial experiment.

The media constituents were grouped into five factors, a-(NaCl), b-($MgCl_2.6H_2O$, $CaCl_2.2H_2O$ & KCl), c-($MgSO_4.7H_2O$, KNO_3 & $NaHCO_3$) d-(KH_2PO_4) & e-(trace elements). Each factor had two levels, a negative level (-) of half concentration present in the basal medium and a positive level (+) of one and half concentration present in the basal medium. A combination of these factors was arranged in table (1) representing 32 trials (no. of trials = 2^n where n= number of factors) (Bloor and England 1991).

The results of growth optimization tabulated in table (1) indicate that trial number 31 is the optimum for growth. The generation time in this trial was the lowest (1.98 days) while the relative growth rate was the highest (0.135). However, at the basal medium the generation time was 3.1 days. In this trial (number 31) NaCl (factor a) was the only factor in the negative level.This indicates that *Dunaliella* grows better at media having lower sodium chloride concentrations rather than higher ones. Many authors recorded the reduction in marine and fresh algal growth as a response to increase in salinity (Setter and Greenway 1979; Allam 1989&1994 and Mohy Eldin 1992). In the trial number 32 (in which all the factors in the positive level) the generation time was 2.23 days. However, the generation time of the other trials ranged from 2.37 to 3.27 days.

Table (1): Growth of *Dunaliella salina* cultured on different trials having standarly arranged five factors for the primary factorial experiment. a-(NaCl), b-($MgCl_2.6H_2O$, KCl & $CaCl_2.2H_2O$), c-($MgSO_4.7H_2O$, KNO_3 & $NaHCO_3$), d-(KH_2PO_4), e-(trace elements).

Trials	Factor Level					Generation Time (Days)	Relative Growth Rate (K`)
	a	b	c	d	e		
1	-	-	-	-	-	2.87	0.105
2	+	-	-	-		2.55	0.113
3	-	+	-	-	-	2.89	0.102
4	+	+	-	-	-	3.20	0.093
5	-	-	+	-	-	2.76	0.093
6	+	-	+	-	-	3.17	0.093
7	-	+	+	-	-	2.39	0.116
8	+	+	+	-	-	3.14	0.093
9	-	-	-	+	-	3.27	0.090
10	+	-	-	+	-	3.07	0.100
11	-	+	-	+	-	2.49	0.123
12	+	+	-	+	-	2.45	0.129
13	-	-	+	+	-	2.87	0.097
14	+	-	+	+	-	2.76	0.100
15	-	+	+	+	-	2.66	0.108
16	+	+	+	+	-	3.07	0.094
17	-	-	-	-	+	3.01	0.096
18	+	-	-	-	+	2.49	0.118
19	-	+	-	-	+	2.59	0.113
20	+	+	-	-	+	2.53	0.111
21	-	-	+	-	+	3.27	0.091
22	+	-	+	-	+	2.79	0.095
23	-	+	+	-	+	3.07	0.092
24	+	+	+	-	+	2.87	0.097
25	-	-	-	+	+	2.55	0.114
26	+	-	-	+	+	3.01	0.091
27	-	+	-	+	+	2.41	0.120
28	+	+	-	+	+	3.04	0.099
29	-	-	+	+	+	2.79	0.101
30	+	-	+	+	+	2.37	0.120
31	-	+	+	+	+	1.98	0.135
32	+	+	+	+	+	2.23	0.114
Basal medium						3.10	0.096

Carbohydrate Optimization.

A-*Primary factorial experiment*

Data in table (2) represents the primary factorial experiment for carbohydrate content under the two levels of five factors (a,b,c,d,&e) (Bloor and England 1991). These data showed that the highest carbohydrates content (90.5 μg/ml) was recorded from trial number 27 which was 2.3 folds that of the basal medium. It is clear that all the trials with factor "a" at the positive level showed low carbohydrate content, while the reverse was observed for factor "a" at the negative level. This confirms the results obtained by Wegman (1971); Ahmed *et al*. (1985) and Shafea (1987), who reported that rise of salinity level caused reduction in carbohydrate content in *Dunaliella* sp. Recently, Cowan et al. (1992) assertened that hyposalinity results in the conversion of glycerol to starch by *Dunaliella salina*, while Rhodes (1987) reported that under hypersaline conditions, glycerol increases and carbohydrates decrease.

From the results obtained for the significance values (table 3), it appeared that factor "a" (NaCl) and factor "b" ($MgCl_2.6H_2O$, $CaCl_2.2H_2O$ and KCl) significantly affected the carbohydrate production by *Dunaliella salina* at 1% and 5% probability level respectively. Since factor "b" is a collection of chloride salts, another experiment (secondary factorial experiment) was carried out to elucidate the effect of each salt on carbohydrate production by *Dunaliella salina*.

Table(2): Statistical analysis concerning the effect total and sum of squares of the primary factorial experiment for carbohydrate production by *Dunaliella salina*.

Trials	Factor Level					Carbohydrate (μg/ml)	*Effect Total	Sum Of Squares
	a	b	c	d	e			
1	-	-	-	-	-	65.0	1631	Total
2	+	-	-	-	-	21.5	-578	10440.1
3	-	+	-	-	-	59.5	186	1081.1
4	+	+	-	-	-	36.0	-3	0.3
5	-	-	+	-	-	60.0	88	242.0
6	+	-	+	-	-	25.5	67	140.3
7	-	+	+	-	-	82.5	51	81.3
8	+	+	+	-	-	50.0	-14	6.1
9	-	-	-	+	-	45.5	-30	28.1
10	+	-	-	+	-	18.5	-67	140.3
11	-	+	-	+	-	62.5	-43	57.8
12	+	+	-	+	-	32.0	-48	72.0
13	-	-	+	+	-	67.5	5	0.8
14	+	-	+	+	-	40.0	68	144.5
15	-	+	+	+	-	81.0	-76	180.5
16	+	+	+	+	-	45.5	-45	63.3
17	-	-	-	-	+	60.0	46	66.1
18	+	-	-	-	+	38.0	-69	148.8
19	-	+	-	-	+	76.5	-25	19.5

Table (2) continue

20	+	+	-	-	+	38.0	-24	18.0
21	-	-	+	-	+	63.5	-35	569.5
22	+	-	+	-	+	24.5	78	190.1
23	-	+	+	-	+	76.0	-2	0.1
24	+	+	+	-	+	54.0	31	30.0
25	-	-	-	+	+	87.5	-15	7.0
26	+	-	-	+	+	16.0	-94	276.1
27	-	+	-	+	+	90.5	-30	28.1
28	+	+	-	+	+	24.5	19	11.3
29	-	-	+	+	+	56.0	-74	171.1
30	+	-	+	+	+	33.5	79	195.0
31	-	+	+	+	+	71.0	23	16.5
32	+	+	+	+	+	29.0	-72	162.0
Basal medium						39.5	-	-

- Effect total =results of calculations using the method of Yates, 1937.

Table (3): F-ratio and significance values of trials at one factor effect and two factors interaction of the primary factorial experiment for determination of carbohydrate production by *Dunaliella salina*.

Trials	F-Ratio	Significance
2 (a^+,b^-,c^-,d^-,e^-)	106.79	1%
3 (a^-,b^+,c^-,d^-,e^-)	11.06	5%
5 (a^-,b^-,c^+,d^-,e^-)	2.48	NS
9 (a^-,b^-,c^-,d^+,e^-)	0.29	NS
17 (a^-,b^-,c^-,d^-,e^-)	0.68	NS
4 (a^+,b^+,c^-,d^-,e^-)	0.00	NS
6 (a^+,b^-,c^+,d^-,e^-)	1.43	NS
10 (a^+,b^-,c^-,d^+,e^-)	1.43	NS
18 (a^+,b^-,c^-,d^-,e^+)	1.52	NS
7 (a^-,b^+,c^+,d^-,e^-)	0.83	NS
11 (a^-,b^+,c^-,d^+,e^-)	0.59	NS
19 (a^-,b^-,c^-,d^-,e^+)	0.20	NS
13(a^-,b^-,c^+,d^+,e^-)	0.00	NS
21(a^-,b^-,c^+,d^-,e^+)	5.83	NS
25 (a^-,b^-,c^-,d^+,e^+)	0.07	NS

NS = results of trial not significant. 1% = results of trial significant at 1% level.
5% = results of trial significant at 5% level.
Significance points for F-ratio were calculated using 1, 16 degrees of freedom.

B-*Secondary factorial experiment*

During this experimental design, four factors were studied; a-(NaCl), b_1-($MgCl_2.2H_2O$), b_2-($CaCl_2.2H_2O$) and b_3-(KCl) (Bloor and England 1991). The

concentration of each of the above mentioned nutrient factors was conducted under both negative and positive levels, while the other media constituents were kept at their negative levels. Under these conditions, sixteen trials were carried out. Statistical analysis of results of secondary factorial experiment was tabulated in tables (4 & 5). From these results, it is noticeable that the highest carbohydrate production is observed with trial number 13 where factors a&b_1 were at the negative level. It is also noticeable that factor "a" had the same effect as in the primary factorial experiment. This in a good agreement with the results obtained by many workers; Ahmed *et al*. (1985); Rhodes (1987); Shafea (1987) and Cowan *et al*.(1992). Also, carbohydrate content at trial number "1" (where all studied factors are at the negative level) was 1.3 fold that of trial number "16" (where all studied factors are at the positive level). From the statistical data it is clear that trial number "2" in which factor "a" is at the positive level (while all other factors are at negative level) showed a highly significant effect (negatively effect) on carbohydrate production. On the other hand, any one of the other studied factors never had a significant effect on carbohydrate production separately.

Table (4): Statistical analysis concerning the effect total and sum of squares of the secondary factorial experiment for carbohydrate production by *Dunaliella salina*.

Trials	Factor Level				Carbohydrate (µg /ml)	Effect Total	Sum Of Squares
	a	b_1	b_2	b_3			
1	-	-	-	-	172	2795.9	Total
2	+	-	-	-	155	-667.1	27813.9
3	-	+	-	-	185.8	-224.5	3150.0
4	+	+	-	-	125	-133.1	1107.2
5	-	-	+	-	233.3	52.1	169.7
6	+	-	+	-	142	-258.5	176.4
7	-	+	+	-	211.8	82.9	429.5
8	+	+	+	-	94.5	154.7	1495.8
9	-	-	-	+	224.5	157.1	1542.5
10	+	-	-	+	211.3	-94.3	555.8
11	-	+	-	+	205.8	-54.1	182.9
12	+	+	-	+	92.5	6.5	2.6
13	-	-	+	+	258.8	-35.5	78.8
14	+	-	+	+	113.3	3.1	0.6
15	-	+	+	+	239.5	188.5	2220.8
16	+	+	+	+	130.8	119.1	886.6

Table (5):F-ratio and significance values of the trials at one factor effect and two factors interaction of the secondary factorial experimentfor carbohydrate production by *Dunaliella salina*.

Trials	F-Ratio	Significance
2 (a^+,b_1^-,b_2^-,b_3^-)	30.19	1%
3 (a^-,b_1^+,b_2^-,b_3^-)	3.42	NS
5 (a^-,b_1^-,b_2^+,b_3^-)	0.18	NS
9 (a^-,b_1^-,b_2^-,b_3^+)	1.67	NS
4 (a^+,b_1^+,b_2^-,b_3^-)	1.20	NS
6 (a^+,b_1^-,b_2^+,b_3^-)	4.53	NS
10 (a^+,b_1^-,b_2^-,b_3^+)	0.60	NS
7 (a^-,b_1^+,b_2^+,b_3^-)	0.47	NS
11 (a^-,b_1^+,b_2^-,b_3^+)	0.20	NS
13 (a^-,b_1^-,b_2^+,b_3^+)	0.09	NS

Significance points for F-ratio were calculated using 1, 5 degrees of freedom.

C- *Media optimization for maximum carbohydrate production.*

From the results obtained from secondary factorial experiment it was found that sodium chloride at the positive level had a negatively significant effect on carbohydrate production by *Dunaliella salina*. Therefore, sodium chloride was changed in the basal medium at the following concentrations 1.5, 1.25, 1, 0.75, 0.50 and 0.25 M (basal medium has 1.25 M sodium chloride). The growth and carbohydrate content of *Dunaliella salina* were estimated every 48 hours for 10 days. Results of carbohydrate production were graphed in Figure (1). From these results, it is noticeable that maximum carbohydrate production was achieved at medium containing 0.75 M sodium chloride. Also, results of generation time (table 6) revealed that medium containing 0.75 M sodium chloride produced lowest generation time. Therefore, this concentration of sodium chloride could be considered as the optimum for higher growth and maximum carbohydrate production. Our results obtained for the primary and secondary factorial experiments agreed with those of Kalpan *et al*. (1980) and Cowan *et al*. (1992). This gives us a license to modify the basal medium for optimization of carbohydrate production by *Dunaliella salina*.

Table (6): Generation time of *Dunaliella salina* cultured in different experiments for carbohydrate optimization.

Experiment	Generation Time (Days)
(1) 1.50 M NaCl	2.47
(2) 1.25 M NaCl	2.41
(3) 1.00 M NaCl	2.49
(4) 0.75 M NaCl	2.39
(5) 0.50 M NaCl	2.62
(6) 0.25 M NaCl	3.27

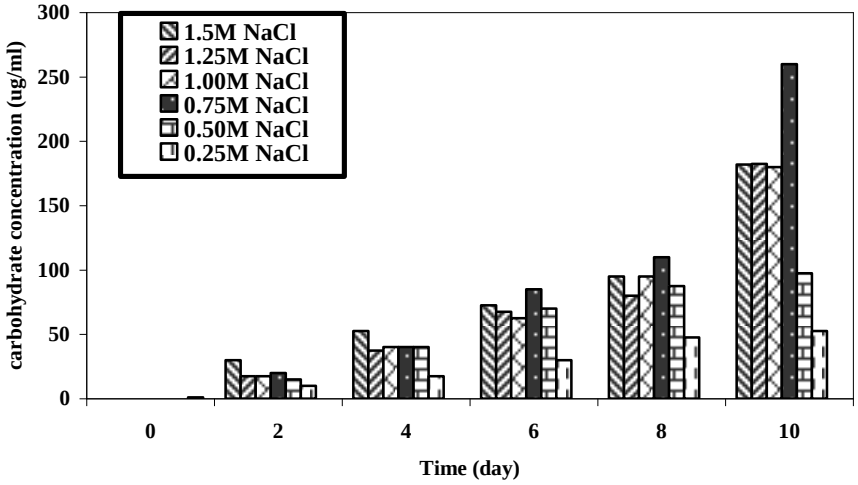

Figure (1). Carbohydrate production

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الإنتاج الكمي لبعض المركبات الأيضية ذات القيمة الإقتصادية الهامة في طحلب دونالييللا سالينا. الجزء الثالث: النمو والإنتاج الكمي للكربوهيدرات

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عني هذ البحث بدراسة العوامل المغذية التي تتحكم في تنظيم النمو والإنتاج الكمي للكربوهيدرات في طحلب دونالييللا سالينا باستخدام تجارب ودراسات احصائية. وقد اوضحت نتائج الدراسات الإحصائية التي تم الحصول عليها على الآتي: (1) قيم دلالات النمو لطحلب دونالييللا سالينا كانت عند اعلاها في المزرعة المحتوية على التركيزات المنخفضة عنه عند التركيزات العالية لكلوريد الصوديوم. (2) تركيز كلوريد الصوديوم العالي له تاثير سلبي على الإنتاج الكمي للكربوهيدرات في الطحلب. (3) الإنخفاض في تركيز كلوريد الصوديوم في المزرعة الأساسية من 1.25 مول الى 0,75 مول ادى إلى الحصول على التركيز الأمثل لانتاج اعلى كمية من الكربوهيدرات في طحلب دونالييللا سالينا.