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OPTIMIZATION OF B-CAROTENE PRODUCTION IN *DUNALIELLA SALINA* TEOD. GROWN ON DIFFERENT NATURAL MEDIA

Kassem, A.M.; Khaleafa,A.f.; Shaalan,S.H. and Maghrabi,D.M.
Botany Department, Faculty of Science, Alexandria University,

Abstract

Synthesis of β-carotene and chlorophylls in *Dunaliella salina* cells grown on whey, fermentation liquor and molasses were studied. The obtained results indicated that : [1] Fermentation liquor at concentration of 0.4% in ¼ MH medium could replace the original MH medium. This state of culturing will save the chemicals used in the MH medium. [2] The amount of β-carotene synthesized in the presence of whey and fermentation liquor after 8 days of culturing were higher than the other culture treatments.[3] $CaCl_2.2H_2O$ was found to be a significant factor for the synthesis of β-carotene in *Dunaliella salina*.[4] NaCl was the most significant factor affecting β-carotene synthesis in *Dunaliella salina*. [5] β-carotene optimization obtained at a medium where all the factors were absent except NaCl.

Key words: Dunaliella salina, β-carotene, optimization

Introduction:

The halotolerant green alga *Dunaliella* is probably one of the most successful microalgae for the outdoor cultivation. Under appropriate growth conditions, *Dunaliella* accumulates massive amounts of highly precious products: β-carotene, glycerol, protein, carbohydrates, minerals, vitamins and others. Lacking a cell wall, dried cells are easily and fully digestible by humans and animals (Ben-Amotz and Avron, 1983a).

From all strains of *Dunaliella*, it was ascertained that *Dunaliella salina* Teod. and *Dunaliella bardawil* are the only species capable of producing relatively large amount of β-Carotene (Teodoresco, 1906; Massyuk, 1973; Ben-Amotz *et al.*, 1982). Natural β-carotene as found in *Dunaliella* and in most fruits and vegetables, contains a mixture of all-trans β-carotene and 9-cis β-carotene together with a few other stereoisomers (Bendich and Olson, 1989). Recent epidemiological and oncological studies suggest that normal to high levels of β-carotene in the body may protect it against cancer (Wald *et al.*, 1988).

The extent of β-carotene accumulation and the rate of synthesis, within the cells of *Dunaliella salina*, depend on certain physiological growth factors, namely light intensity, salt concentration, temperature and nutrient deficiency. Most parameters, which retard growth, induce β-carotene accumulation. However, the most studied one is the salt concentration (Ben-Amotz *et al.*, 1982; Loeblich , 1982; Milko,1963a ; Semenenko and abdullaev,1980; Avron and Ben-Amotz,1992).

The aim of this work is to study the principle growth features of the microscopic halotolerant green alga *Dunaliella salina* cultures with special reference to β-carotene production.

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Materials and methods:

Organism

Cells of *Dunaliella salina* were obtained from the algal culture collection of phycological laboratory, Botany Department, Faculty of Science, Alexandria University. The organism was originally collected from the brine water of salty lagoons with high irradiation zone at El-Mex district, Alexandria.

Culturing conditions

The axenic cultures were grown in MH medium (Loeblich, 1982), ¼ MH medium or ¼ MH medium with 0.4 % waste under controlled laboratory conditions (temperature at 25 ± 1°C, light at 4000 Lux, pH at 8.0 and a regime of 16 hr. light / 8 hr. dark cycles). Culture experiments were conducted in 250 ml. Erlenmeyer pyrex flasks, each contained 50-ml medium.

The wastes used in this work were:

(1)-Whey: obtained from the Department of Dairy Products, Faculty of Agriculture, Alexandria University.

(2)-Molasses and fermentation liquor: obtained from the Starch and Yeast Company at Alexandria.

β-carotene estimation

β-Carotene was estimated according to the method described by Jaspers, (1965).

Chlorophylls estimation

The concentrations of chlorophylls "a" and "b" were estimated according to the method descriped by Jeffry and Humphrey, (1975).

Statistical analysis:

The statistical methods applied in this work were those recommended by Greasham and Inamine, (1986) and Bloor and England, (1991).

Results and Discussion:

a- Biochemical analysis of *Dunaliella salina*:

Dunaliella salina cells cultured in MH medium, ¼ MH medium and ¼ MH medium with different wastes were subjected to β-carotene analysis at three periods of time (0, 8 & 18 day). In the case of ¼ MH medium and ¼ MH medium enriched with wastes, NaCl concentration remained unchanged as in case of the original medium. The results, which are present in table 1 revealed that the amounts of β-carotene synthesized in the presence of whey and fermentation liquor after 8 days of incubation were higher than the other culture treatments.

According to the results present in the Tables 1 & 2, the ratios of total chlorophylls to β-carotene after 8 days were 2.4, 2.1, 1.8, 5, 5.9 for MH medium, ¼ MH medium only, and 0.4 % whey, fermentation liquor and molasses in ¼ MH medium respectively. However, after 18 days, these ratios decreased for the MH medium, ¼ MH

medium and 0.4 % whey (2.1, 1.4, 1.6 respectively), while, for fermentation liquor and molasses the ratios remained nearly constant. These results were found to be in harmony with those obtained by Jeffrey (1961).

Table 1: The content of β-carotene (μg/ml) in *Dunaliella salina* grown on basal medium, 1/4 basal medium and 1/4 basal medium in addition to 0.4% concentration of whey, fermentation liquor and molasses wastes.

	Concentration of β-carotene (μg/ml)				
Time (day)	Basal medium	1/4 basal medium	Whey	Fermentation liquor	Molasses
0	0.03	0.03	0.03	0.03	0.03
8	0.655	0.377	0.869	0.778	0.075
18	0.356	0.280	0.491	0.323	0.039

Table 2: Total chlorophylls (μg/ ml) in *Dunaliella salina* grown on basal medium, 1/4 basal medium and 1/4 basal medium in addition to 0.4% concentration of whey, fermentation liquor and molasses wastes.

	Total chlorophylls (μg/ml)				
Time (day)	Basal medium	1/4 basal medium	Whey	Fermentation liquor	Molasses
0	0.200	0.200	0.200	0.200	0.200
8	1.564	0.790	1.586	3.900	0.439
18	0.756	0.381	0.810	1.785	0.237

b- Plackett and Burman experimental design:

The Plackett and Burman design (Greasham and Inamine, 1986) was used to determine the degree of significance of the different elements that constituted the MH medium on β-carotene synthesis. These elements were grouped into 7 variables; $MgCl_2.6H_2O$, $MgSO_4.7H_2O$, KCl, KNO_3, $NaHCO_3$, $CaCl_2.2H_2O$ & trace elements as a single factor. Tables (3&4) illustrate an array for n = 8 trials that will test n-1 independent variables. Each row represents one trial (MH medium) and each column represents a single variable. The + and – means that the element is present or absent within each trial respectively. Data recorded in table 4 showed that maximum β-carotene content (0.261μg /ml culture) obtained at trial m_4 where $CaCl_2.2H_2O$, $NaHCO_3$, KNO_3 & $MgSO_4.7H_2O$ were present. Also data showed that $CaCl_2.2H_2O$ had a highly significant effect while both, $NaHCO_3$ and trace elements had negatively significant effect on β-carotene synthesis in *Dunaliella salina* cells. These results helped us to use only 5 of these variables and organize a sophisticated protocol.

Table 3: The constituents of the different media (trials) used for Plackett and Burman matrix design.

	Variables (Factors)						
Trials (n=8)	$MgCl_2.6H_2O$	$MgSO_4.7H_2O$	KCl	Trace elements	KNO_3	$NaHCO_3$	$CaCl_2.2H_2O$
m_1	+	+	+	-	+	-	-
m_2	+	+	-	+	-	-	+
m_3	+	-	+	-	-	+	+
m_4	-	+	-	-	+	+	+
m_5	+	-	-	+	+	+	-
m_6	-	-	+	+	+	-	+
m_7	-	+	+	+	-	+	-
m_8	-	-	-	-	-	-	-

Table 4: Main effect and degree of significance of the different 7 factors based on Plackett and Burman design on the synthesis of β-carotene (μg/ ml) in *Dunaliella salina*.

	Variables (Factors)													
	$MgCl_2.6H_2O$		$MgSO_4.7H_2O$		KCl		Trace elements		KNO_3		$NaHCO_3$		$CaCl_2.2H_2O$	
Trials (m) (n=8)	+	-	+	-	+	-	+	-	+	-	+	-	+	-
m_1	0.198		0.198		0.198			0.198	0.198			0.198		0.198
m_2	0.201		0.201			0.201	0.201			0.201		0.201	0.201	
m_3	0.185			0.185	0.185			0.185		0.185	0.185		0.185	
m_4		0.261	0.261			0.261		0.261	0.261		0.261		0.261	
m_5	0.139			0.139		0.139	0.139		0.139		0.139			0.139
m_6		0.213		0.213	0.213		0.213		0.213			0.213	0.213	
m_7		0.118	0.118		0.118		0.118			0.118	0.118			0.118
m_8		0.109		0.109		0.109		0.109		0.109		0.109		0.109
Total	0.723	0.701	0.778	0.646	0.714	0.710	0.671	0.753	0.811	0.613	0.703	0.721	0.860	0.564
Main effect	0.006		0.033		0.002		-0.021		0.049		-0.005		0.074	
t-value	0.154		0.846		0.026		-0.513		1.563		-0.103		3.360	
degree of significance	P>0.10 n.s.		P>0.10 n.s		P>0.10 n.s.		P>0.10 n.s. (-)		P>0.10 n.s.		P>0.10 n.s. (-)		0.02>P>0.01 s.	

c- Factorial experiment:

Although Plackett and Burman design helped us to minimize the number of factors found in the MH medium, we still never test the significant effects of waste (fermentation liquor), NaCl and K_2HPO_4 that remained constant during this statistical technique. The main aim of this statistical design (Bloor and England, 1991) was to select elements rather than $CaCl_2.2H_2O$ which were the most significant for the synthesis of β-

carotene in *Dunaliella salina*. This factorial experiment (tabulated in Table 5) involved two levels for each of five media constituents (factors) a negative (-) level (half-fold of the factor concentration in MH medium) and a positive (+) level (one and half-fold of the factor concentration in MH medium). The number of trials = 2^n where n = the number of factors in the medium. Concerning β-carotene content in *Dunaliella salina*, the data recorded in Table 6 show 32 trials which conducted using 5 factors namely NaCl, fermentation liquor(w), K_2HPO_4, $CaCl_2.2H_2O$ and the remaining constituents of MH medium (M). For fermentation liquor (w) the positive (+) sign indicates 0.6% concentration, while the negative (-) sign indicates 0.2% concentration. Also, data in Table 7, clearly show that trials number 2 (only NaCl at (+) level) and number 25 (only K_2HPO_4 + medium (M) at (+) level) were the only significant trials at 1% and 5% probability of significance respectively. According to the above mentioned statistical results it could be concluded that NaCl was found to be the most significant factor affecting β-carotene content in *Dunaliella salina* because its significance was at 1% probability level while that of K_2HPO_4+ M was at 5% respectively. These results forced Sahar (1998) to modify Ben-Amotz medium (1987) in order to increase the synthesis of β-carotene in *Dunaliella salina* by increasing the concentration of NaCl from 1M to 3M and decreasing the concentration of nitrate and phosphate. The same trend was also followed by Markovits *et al.* (1993).

d- Steepest Ascent method:

In this method, a figure was generated for NaCl and K_2HPO_4+(M) which were found to be significant in the factorial experiment in order to optimize the yield of β-carotene giving 12 trials recorded in Table (8). The figures in the resultant column were divided by the number of trials performed, which was equal to 32. The figure thus multiplied by the unit of variation used in the factorial experiment (i.e. the extent of the + and - values used), which was 0.5 for all factors. The figure generated for each factor was then transformed relative to one of the factors, which was chosen arbitrarily. These final figures for each factor were then progressively added to (if they possessed a + sign) or subtracted from (if they possessed a - sign) the base level concentration of each factor until a reasonable series had been completed, or until one of the factors reached zero. From the data recorded in Table 9, it is clear that the organism cultured in trial number 3 synthesized the highest value of β-carotene. The concentration of β-carotene in this trial was 2.634 μg / ml i.e. 3.2 times compared to the blank. Medium of trial number 3 contained NaCl 1.7 times that of the blank, while, the other medium constituents were absent. The work of Loeblich (1969, 1974), Massyuk (1965), Milko (1963b), Semenenko & Abdullaev (1980), Ben-Amotz & Avron (1983b) concluded that *Dunaliella salina* accumulate β-carotene under environmental stress conditions such as high NaCl concentration, extreme temperature and pH values or nutrient deficiency. Ben-Amotz *et al.* (1982) found that increase in β-carotene to chlorophyll ratio is inverse relation to specific growth rate and this is the case also for our results. Lerche (1937) found that nitrogen and phosphorous deficiencies caused the cells of *Dunaliella salina* to redden, i.e. increase in β-carotene content. Also, Loeblich (1982) found that at high salinity the cells of *Dunaliella salina* contained more β-carotene and have a longer generation time than cells in low salinity. Finally, it is proposed that: in order to obtain maximum yield of β-carotene in *Dunaliella salina*, the organism must firstly cultured on medium suitable for optimization of growth, then the obtained crop is subjected to conditions of nutrient deficiency except NaCl salt.

Table 5: Arrangement of the five factors under study in a standard order at the factorial experiment.

Trial	Factor(s) under study	Factor level				
		NaCl	Fermentation liquor(W)	$CaCl_2.2H_2O$	K_2HPO_4	Medium (M)
1.	Base	-	-	-	-	-
2.	NaCl	+	-	-	-	-
3.	W	-	+	-	-	-
4.	NaCl,W	+	+	-	-	-
5.	$CaCl_2$	-	-	+	-	-
6.	NaCl,$CaCl_2$	+	-	+	-	-
7.	W,$CaCl_2$	-	+	+	-	-
8.	NaCl,W,$CaCl_2$	+	+	+	-	-
9.	K_2HPO_4	-	-	-	+	-
10.	NaCl,K_2HPO_4	+	-	-	+	-
11.	W,K_2HPO_4	-	+	-	+	-
12.	NaCl,W,K_2HPO_4	+	+	-	+	-
13.	$CaCl_2$,K_2HPO_4	-	-	+	+	-
14.	NaCl,$CaCl_2$,K_2HPO_4	+	-	+	+	-
15.	W,$CaCl_2$,K_2HPO_4	-	+	+	+	-
16.	NaCl,W,$CaCl_2$,K_2HPO_4	+	+	+	+	-
17.	M	-	-	-	-	+
18.	NaCl,M	+	-	-	-	+
19.	W,M	-	+	-	-	+
20.	NaCl,W, M	+	+	-	-	+
21.	$CaCl_2$, M	-	-	+	-	+
22.	NaCl,$CaCl_2$, M	+	-	+	-	+
23.	W,$CaCl_2$,M	-	+	+	-	+
24.	NaCl,W,$CaCl_2$,M	+	+	+	-	+
25.	K_2HPO_4,M	-	-	-	+	+
26.	NaCl,K_2HPO_4,M	+	-	-	+	+
27.	W,K_2HPO_4,M	-	+	-	+	+
28.	NaCl,W,K_2HPO_4,M	+	+	-	+	+
29.	$CaCl_2$,K_2HPO_4,M	-	-	+	+	+
30.	NaCl,$CaCl_2$,K_2HPO_4,M	+	-	+	+	+
31.	W,$CaCl_2$,K_2HPO_4,M	-	+	+	+	+
32.	NaCl,W,$CaCl_2$,K_2HPO_4,M	+	+	+	+	+

W: Fermentation liquor
M: MH medium constituents
+ : One and half-fold of the factor concentration in MH medium
- : Half-fold of the factor concentration in MH medium

Table 6: Responses (results) and statistical analysis of the factorial experiment for β-carotene synthesis in *Dunaliella salina*.

Trial no.	Factor(s) under study	Response β-carotene (μg/ml)	Effect total	Sum of squares
1	Base	0.599	104.215	total
2	NaCl	2.591	40.927	52.344
3	W	1.177	14.169	6.274
4	NaCl, W	2.410	- 1.403	0.062
5	$CaCl_2$	0.857	6.575	1.351
6	NaCl, $CaCl_2$	2.340	4.019	0.505
7	W, $CaCl_2$	1.937	1.215	0.046
8	NaCl, W, $CaCl_2$	4.787	- 0.751	0.018
9	K_2HPO_4	1.084	9.097	2.586
10	NaCl, K_2HPO_4	6.148	- 0.267	0.002
11	W, K_2HPO_4	3.077	- 5.337	0.890
12	NaCl, W, K_2HPO_4	6.277	- 6.753	1.425
13	$CaCl_2$, K_2HPO_4	1.967	- 11.951	4.463
14	NaCl, $CaCl_2$ K_2HPO_4	6.276	2.383	5.679
15	W, $CaCl_2$ K_2HPO_4	2.633	- 4.697	0.689
16	NaCl, W, $CaCl_2$ K_2HPO_4	4.882	2.507	0.196
17	M	1.585	6.131	1.175
18	NaCl, M	2.866	- 3.833	0.459
19	W, M	1.864	3.533	0.390
20	NaCl, W, M	6.056	5.229	0.854
21	$CaCl_2$, M	1.930	1.943	0.118
22	NaCl, $CaCl_2$, M	6.135	5.215	0.849
23	W, $CaCl_2$, M	3.532	0.655	0.013
24	NaCl, W, $CaCl_2$, M	6.893	- 4.611	0.664
25	K_2HPO_4, M	2.389	- 22.195	15.394
26	NaCl, K_2HPO_4, M	3.442	- 14.795	6.840
27	W, K_2HPO_4, M	3.408	- 0.277	0.002
28	NaCl, W, K_2HPO_4, M	3.847	2.311	0.167
29	$CaCl_2$ K_2HPO_4, M	1.518	- 4.007	0.502
30	NaCl, $CaCl_2$ K_2HPO_4, M	3.296	3.245	0.329
31	W, $CaCl_2$ K_2HPO_4, M	2.087	7.263	1.648
32	NaCl, W, $CaCl_2$ K_2HPO_4, M	4.325	7.151	1.598

W: Fermentation liquor
M: MH medium constituents

Table 7: The F-ratio and degree of significance of one and two-factors interactions for β-carotene content in *Dunaliella salina*.

Trial no.	Factor(s) under study	F-ratio	Degree of significance
2	NaCl	39.004	1 % level
3	W	4.675	n.s.
4	NaCl,W	0.046	n.s.
5	$CaCl_2$	1.007	n.s.
6	NaCl,$Cacl_2$	0.376	n.s.
7	W,$CaCl_2$	0.034	n.s.
9	K_2HPO_4	1.927	n.s.
10	NaCl,K_2HPO_4	0.001	n.s.
11	W,K_2HPO_4	0.663	n.s.
13	$CaCl_2$,K_2HPO_4	3.326	n.s.
17	M	0.876	n.s.
18	NaCl,M	0.342	n.s.
19	W,M	0.291	n.s.
21	$CaCl_2$,M	0.088	n.s.
25	K_2HPO_4,M	11.471	5 % level

Table 8: The figure generated for β-carotene optimization in Dunaliella salina using steepest ascent method.

	Trials (amounts in g / 100 ml culture)												
	Blank	1	2	3	4	5	6	7	8	9	10	11	12
NaCl	7.312	8.312	10.312	12.312	14.312	16.312	18.312	20.312	22.312	24.312	26.312	28.312	30.312
K_2HPO_4	0.0035	0.000											
$MgCl_2$	0.150	0.000											
$MgSO_4$	0.050	0.000											
KCl	0.020	0.000											
Trace elements*	0.100	0.000											
KNO_3	0.100	0.000											
$NaHCO_3$	0.0042	0.000											

* Amounts in ml / 100 ml culture.

Table 9: Content of β-carotene in *Dunaliella salina* obtained from each trial of the steepest ascent method after 8 days of culturing.

Trial number	β-carotene content (μg/ml)
Blank	0.829
1	1.519
2	2.522
3	2.634
4	2.408
5	1.993
6	1.143
7	1.084
8	1.040
9	1.035
10	0.819
11	0.643
12	0.335

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الملخص العربى

الإنتاج الأمثل للبيتا كاروتين فى طحلب دونالييلا سالينا النامى على منابت غير تقليدية

أمين قاسم ، عبد الفتاح خليفة، سامى شعلان و داليا مغربى

كلية العلوم – قسم النبات – جامعة الاسكندرية

تم فى هذا البحث دراسة كميات البيتا كاروتين و كذلك الكلوروفيللات فى خلايا طحلب دونالييلا سالينا المنماة على مخلفات طبيعية مثل شرش اللبن ومياه الفرز المتبقية بعد زراعة خلايا الخميرة و مولاس البنجر . وقد تم التوصل الى أن مخلف مياه الفرز بتركيز ٠,٤ % مضافا الى المستنبت الغذائى التقليدى بربع تركيزه (1\4 M H medium) يمكن أن يعطى نتائج مشابهة لنتائج المستنبت الأصلى مما يمكن استخدامه ليحل محل المستنبت الغذائى التقليدى ذو التركيز الكامل عند زراعة خلايا الدونالييلا سالينا مما يساعد فى توفير الكيماويات المستخدمة فى زراعة الطحلب. وكذلك تم التوصل الى أن شرش اللبن ومياه الفرز تحتل الصدارة فى انتاج البيتا كاروتين فى الطحلب عند زراعته لمدة ثمانية أيام. ومن أهم ما تم التوصل اليه أن عنصر كلوريد الصوديوم هو أهم العناصر على الإطلاق التى تؤثر فى انتاج البيتا كاروتين داخل خلايا الطحلب يليه فى ذلك كلوريد البوتاسيم ثنائي الماء