Egyptian J. of Phycol. Vol. **4(1)**, 2003

NATURAL BYPRODUCTS AS NUTRIENT ADDITIVES FOR OPTIMIZATION OF PROTEIN CONTENT IN *DUNALIELLA SALINA* Toed CULTURES

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Abstract

The present paper aims to formulate a byproduct-supplemented medium suitable for the production of large amounts of protein rich *Dunaliella salina* biomass. Components of the MH basal growth medium were examined for partial replacement by cheese whey, beet molasses, and yeast fermentation liquor. Maximum growth rate was achieved after 8 days of incubation in the presence of 0.4% fermentation liquor that replaced ¾ of the weight of all medium components other than NaCl. Interestingly, the presence of fermentation liquor or cheese whey resulted in more than 1.5 and1.4-fold increases, respectively, in algal protein production. By application of three sequential multi-factorial experimental designs, medium composition was further optimized with respect to protein content in *Dunaliella* cultures. Among medium components, fermentation liquor was the most significant factor that affected the response. Compared to the basal condition, the optimized medium formula resulted in approximately a 1.7-fold increase in the protein content of *Dunaliella salina* cultures.

Key Words: *Dunaliella salina*, protein, optimization, byproducts.

Introduction

Animals and plants have always provided the main food sources. However, there have been many efforts to develop a source of protein independent of agricultural land use. The production of single cell protein as food is an active field of research in recent years (Mahasneh, 1997; Schwarz et al, 1995). Microorganisms, as protein producers, have some advantages over plants and animals. These non-conventional sources of food are of great nutritional value due to their high contents of proteins, vitamins and lipids and the general presence of a complete array of all essential amino acids (Thomas, 1984; Riviere, 1977; Boyd, 1973; Peppler, 1968; Dam et al., 1965). Also microorganisms have short generation times and can be easily modified genetically, cultivated in continuous cultures, and grown on raw materials.

The microscopic algae are the primary producers of the organic matter on which all other forms of aquatic life depend in their feeding. Serious attempts have

(ISSN: 1110-8649)

been initiated to use mass cultures for some special micro-algae that accumulate interesting products (Parkinson, 1987). Among those, *Dunaliella salina* seems to be promising for its utilization as a protein source for feeding. Due to lacking of cell walls, biomass of this halo-tolerant green alga is easily and fully digestible by humans and animals (Ben-Amotz and Avron, 1983). *Dunaliella* protein is similar, in composition, to other common plant proteins such as soybean meal, (Ben-Amotz and Avron, 1980). In addition to protein, *Dunaliella salina* is known to produce diverse metabolites such as glycerol (Thakur, 2000), carbohydrates (Shirai et al, 1998), β-carotene (Orset and Young, 2000), hydrocarbons (Park et al, 1998) and other metabolites.

The growth, metabolism and contents of *Dunaliella salina* cells are responsive to medium composition (Thakur et al, 2000; Yamaoka et al, 1994). The one-variable-at-a-time approach, used frequently to study the effect of culture environmental factors, consumes time and doesn't guarantee reaching the actual optima. Moreover, it ignores the interactive effects among different variables. On the other hand, multi-factorial experimental designs can effectively deal with technology optimization studies (Montgomery, 1991). These statistical techniques are suitable for designing experiments, building models, evaluating the effective factors, and most importantly, searching the optimum conditions of factors for a desirable response.

In this approach, the relative importance of environmental factors affecting the synthesis of protein in *Dunaliella salina* has been explored and their optimal levels were elucidated.

Materials and Methods

Organism

The experimental strain of *Dunaliella salina* was obtained from the algal culture collection of Phychological laboratory, Botany Department, Faculty of Science, Alexandria University. The organism was originally collected from the brine water of salty lagoons at El-Mex district, Alexandria, Egypt.

Culturing Conditions

The MH formula, as described by Loeblich (1982), was used as a basal growth medium. It contains (g/l): NaCl, 73.125; $MgCl_2.6H_2O$, 1.5; $MgSO_4.7H_2O$, 0.5; KCl, 0.2; KNO_3, $NaHCO_3$, 0.042; $CaCl_2.2H_2O$, 0.2 and K_2HPO_4, 0.035. The medium is supplemented by trace elements as described by Johnson et al. (1968). Axenic cultures were grown under aseptic conditions in the basal formula MH, ¼ MH (components, other than NaCl, were reduced to ¼ of their weights in the basal formula) or ¼ MH supplemented with indicated byproduct concentrations. The byproducts used in this work included cheese whey (obtained from the Department of Dairy Products, Faculty of Agriculture, Alexandria University), beet molasses and yeast fermentation liquor (obtained from the Starch and Yeast Company, Alexandria, Egypt). Experiments were conducted in 250 ml Erlenmeyer Pyrex flasks, each contained 50-ml medium. Initial pH was adjusted to 8.0. Cultures were

grown in an incubator at a temperature of 25 ± 1^0C and light of 4000 Lux with regime cycles of 16 h light and 8 h dark/24 h.

Growth Parameters

Growth of *Dunaliella salina* was estimated based on cell numbers, which were counted using a hemacytometer. Four replicas were taken to get the mean number of cells per ml culture. The growth rate, R (number of divisions per day) was calculated from the formula of Robert (1979):

$R = [3.322 / (t_2 - t_1)] [\log N_2/N_1]$

where t_1 = time at the beginning of the experiment, t_2 = time at the end of the experiment, N_1= number of cells/ml at t_1, N_2= number of cells/ml at t_2.

Protein Estimation

Total protein content in *Dunaliella salina* biomass was estimated calorimetrically as described by Hartree (1972).

Experimental designs

Plackett-Burman design: The most important environmental factors controlling protein synthesis in *Dunaliella salina*, were elucidated by applying the Plackett-Burman experimental design (Plackett and Burman 1946). It is a fraction of a two-level factorial design and allows the investigation of n-1 variables in n experiments. In the present work, seven variables were screened in eight combinations according to the design shown in Table 1 in the Results section. Examined independent variables included $MgCl_2.6H_2O$, $MgSO_4.7H_2O$, KCl, KNO_3, $NaHCO_3$, $CaCl_2.2H_2O$ and trace elements (treated as a single factor). The + and – are symbols used to indicate the presence or absence of variables, respectively. All trials were performed in duplicates and the average of observations was used as the response. The main effect of each variable was simply calculated as the difference between the average of measurements made at the presence (+) and the average of measurements observed at the absence (-) of that factor. For determination of variable significance, statistical *t*-values for equal unpaired samples were calculated with respect to the observation records.

Two-level factorial design: With respect to protein production by *Dunaliella salina, a* quick gross picture of the medium components, which are predicted to be essential, was generated by a two-level (2^n) factorial experimental design (Chatfield, 1975). Each of these variables was examined at a high setting (coded -) and a low setting (coded +), which are corresponding to the basal level ±50%. Consequently, the experiment included 2^5 or 32 combinations; each trial was performed only once. The arrangement of the experiment, as shown in Table 7, gave the chance for each medium constituent to be evaluated singly and in combination with any other component. The statistical analysis used was an analysis of variance. The three and four interaction sum of squares (SS) were combined and divided by their number to give the residual mean square. The *F*-ratio was calculated by dividing effect SS by the residual mean square.

Steepest ascent method. Medium components that affected the response significantly were simultaneously optimized by applying a single steepest ascent experiment (Bloor and England, 1991). In this method, relative concentration change units are chosen based on their corresponding effect total figures calculated from the 2^n experimental results.

Results and Discussion

Growth of *Dunaliella salina*

The growth of *Dunaliella salina* cells on the MH basal medium was monitored at different time intervals. The data graphed in Fig. 1 show that a lag phase of 5 days was required for a sharp exponential phase of biomass production that prolonged for 12 days. Further increase in cell number was observed until the 18th day of incubation and the maximum growth rate (0.671 divisions/day) was achieved after an incubation period of 8 days.

In another preliminary experiment, the nutritive values of some renewable economic byproducts were evaluated as partial medium supplements. *Dunaliella salina* cells were grown on ¼ MH enriched with 0.4% concentration of fermentation liquor, cheese whey, or molasses. Basal medium and ¼ MH cultures were used as controls.

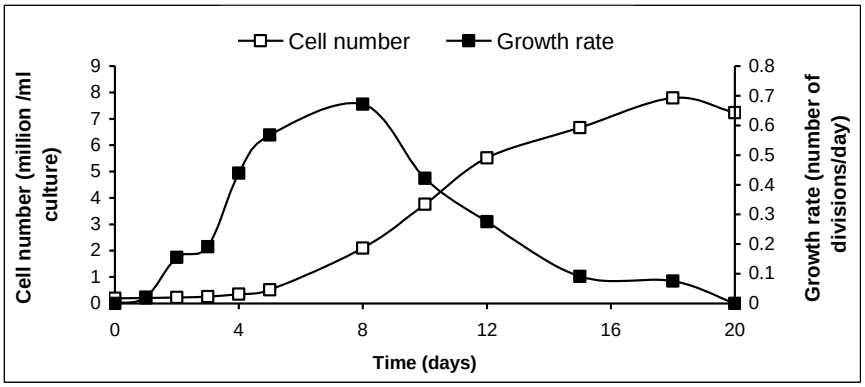

Figure 1: Growth of *Dunaliella salina* cells on MH basal medium.

The growth was monitored at different time intervals with respect to cell number. As shown in Fig. 2a, all examined nutrient additives dramatically affected algal growth. Highest biomass production results were recorded by the fermentation liquor culture, which clearly exceeded the basal condition, with an exponential growth phase of 18 days. On the other hand, cheese whey and molasses exerted growth inhibitory effects on *Dunaliella* cultures. As shown in the figure, the inhibitory effect of the later is clearly dramatic. Based on these results, calculations suggested that maximum growth rates in all cultures were achieved at the 8th day. These were 0.431, 0.657, and 0.547 divisions/day in the presence of cheese whey,

fermentation liquor, and molasses, respectively. Within these 8 days, proteins were maximally synthesized in the fermentation liquor culture with more than 10% and approximately 53% increases when compared to the complete basal medium and ¼ basal medium results, respectively (Fig. 2b). Proteins were also over-produced in the cheese whey culture with approximately 41% increase when compared to the ¼ MH control culture. These results coincide with those of other workers who reported that the source and concentration of organic nitrogen source in the culture media causes major changes in the growth and biochemical composition of marine algae, specially protein content (Myklestad and Haug, 1972; Parsons and Takahashi, 1973; Shifrin and Chishlom, 1981).

According to the biomass as well as protein content results of this experiment, fermentation liquor was chosen as an economic nutrient additive in the ¼ MH for *Dunaliella salina*.

Elucidation of medium components affecting protein expression by *Dunaliella salina*

The Plackett and Burman statistical design was applied to determine the degree of significance of medium components as regulators of protein synthesis in *Dunaliella salina*. Among medium constituents, 7 independent variables; including $MgCl_2.6H_2O$, $MgSO_4.7H_2O$, KCl, KNO_3, $NaHCO_3$, $CaCl_2.2H_2o$ and trace elements; were screened in this experiment. For simplicity, the complex component (trace elements) was treated as a single variable.

Dunaliella salina was cultured under the eight different conditions for 8 days, then cells were analyzed for protein content. The data recorded in Table 1 shows that maximum protein content (0.285 mg/ml) was achieved at trial 4 where $MgSO_4.7H_2O$, KNO_3, $NaHCO_3$ and $CaCl_2.2H_2O$ were present.

Based on these observations, the main effects and *t*-values were calculated for each independent variable. As shown in Table 2, the significance of examined nutrient elements can be arranged in the following order: $CaCl_2.2H_2O$ (significant), KNO_3, $MgSO_4.7H_2O$, KCl, trace elements, $NaHCO_3$, and $MgCl_2.6H_2O$. Furthermore, the main effect figures and the signs attributed to them suggest that the effects of trace elements, $NaHCO_3$, and $MgCl_2.6H_2O$ on the production of proteins by *Dunaliella salina* are slight and negative.

In the complete factorial experiment (2^n), the workers assumed that insignificant components might be still beneficial, possibly if they are introduced into the medium in other concentrations. Accordingly, factors appeared to be insignificant in the Plackett & Burman experiment were preferred to be further investigated as a single variable (M) rather than to be excluded from the medium. Examined variables included M, $CaCl_2.2H_2O$ (the significant variable), and the rest of medium components including fermentation liquor (W), NaCl, and K_2HPO_4. Each of these independent variables was examined at two different levels and the experiment was designed according to the 2^n standard order shown in Table 3.

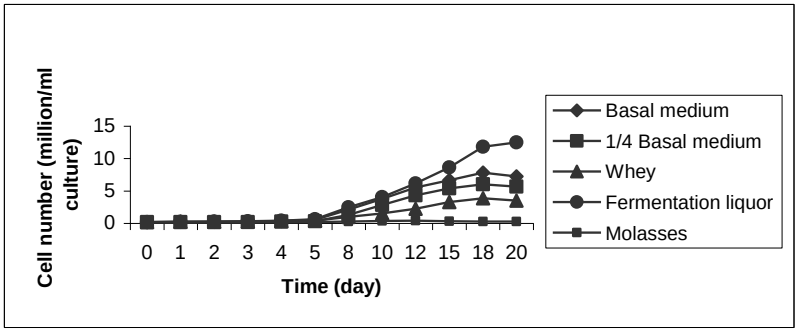

Figure 2a: Growth of *Dunaliella salina* cultured on basal medium (BM), 1/4 BM or 1/4 BM enriched with 0.4% whey, fermentation liquor, or molasses.

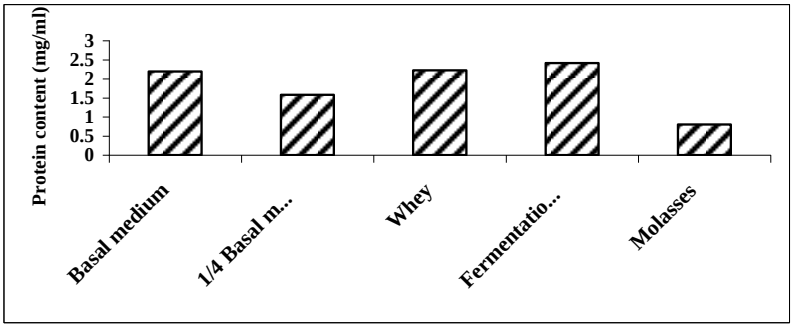

Figure 2b: Protein content of *Dunaliella salina* grown for 8 days on basal medium, 1/4 BM and 0.4% concentration of whey, fermentation liquor and molasses in 1/4 basal medium.

Table 1. Plackett-Burman design for 7 factors and experimental results with respect to protein production by *Dunaliella salina*

Trial	Independent variables							Response (Mg/ml)
	$MgCl_2$	$MgSO_4$	KCl	Trace element	KNO_3	$NaHCO_3$	$CaCl_2$	
1	+	+	+	-	+	-	-	**0.259**
2	+	+	-	+	-	-	+	**0.212**
3	+	-	+	-	-	+	+	**0.196**
4	-	+	-	-	+	+	+	**0.285**
5	+	-	-	+	+	+	-	**0.118**
6	-	-	+	+	+	-	+	**0.264**
7	-	+	+	+	-	+	-	**0.144**
8	-	-	-	-	-	-	-	**0.118**

Based on the observations of this experiment and calculations recorded in table 4, it is clear that fermentation liquor (W) was the most significant independent variable (99% level) that affected protein synthesis in *Dunaliella salina.* NaCl was also found to be significant, but to a less extent (95% level). The presented data indicate also that $CaCl_2.2H_2O$, K_2HPO_4 and M did not affect the response significantly, when each of them is evaluated singly. However, the two-factor interaction calculations suggest that $CaCl_2.2H_2O$ and M should be considered, as their combined action appeared to have a significant effect on the response. Consequently, W, $CaCl_2.2H_2O$ and M were designated as effective variables that require further optimization studies. Due to the positive signs attributed to significant effect total figures, it is also reasonable to predict that the optimum levels of these factors are most likely above their basal levels dictated by the MH original formula.

Optimization of protein production by *Dunaliella salina* cultures

The results obtained from the factorial experiment were used to optimize culture conditions for maximum protein production using the steepest ascent method (Bloor and England, 1991). This experiment contained four independent variables, W, NaCl, $CaCl_2.2H_2O$ and M. Due to its insignificant effect in the 2^n factorial experiment; K_2HPO_4 concentration was treated as a constant, which is introduced to all medium optimization trials at its basal level. Table 5 shows 15 statistically organized medium trials on which *Dunaliella salina* was cultured for 8 days then analyzed for protein content. The obtained results presented graphically in Fig. 3 proved that the protein content in the *Dunaliella* cultural trials was gradually increasing and reached maximum at trial number 10 where its record represented approximately 70% increase when compared to the basal condition. As shown in Table 5, levels of all independent variables in this experiment are increasing as the trial number is going up. This result coincided with the results obtained by Fabergas et al (1985, 1986) who found that great variations in protein content have been shown in cultures of different marine microalgae and batch cultures of *Dunaliella tertiolecta* at relatively high nutrient concentrations. However, a sharp decrease in the protein content was observed on other trials than that of the medium formula number 11. This could be a result of hyper-salinity stress or toxic effect of the heavy metal magnesium when present at high concentrations in the medium (Gerlach et al, 2000; Ford and Mitchell, 1992).

Table 2. Main effects and *t*-values calculated for each variable based on the observations of the Plackett-Burman experimental design.

Variable	$MgCl_2$	$MgSO_4$	KCl	Trace elements	KNO_3	$NaHCO_3$	$CaCl_2$
Main effect	**-0.007**	**0.051**	**0.033**	**-0.030**	**0.064**	**-0.028**	**0.079**
t-value	**-0.140**	**1.133**	**0.660**	**-0.600**	**1.422**	**-0.540**	**2.051**
Degree of significance	**p>0.1 n.s.**	**p>0.1 n.s.**	**p>0.1 n.s.**	**p>0.1 n.s.**	**p>0.1 n.s.**	**p>0.1 n.s.**	**0.1>p>0.05 s**

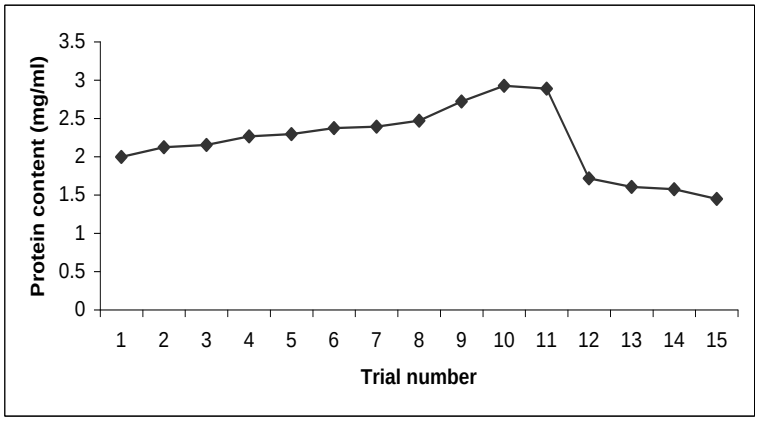

Figure 3: Protein contents in *Dunaliella salina* cultured (for 8 days) in the proposed trials of the steepest ascent method.

Table 3. Arrangement of the five factors under study in a standard orderat the factorial experiment.

Trial	Factor(s) under study	Factor level				
		NaCl	W	$Cacl_2$	K_2HPO_4	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=Factors appeared to be insignificant in the Plackett & Burman experiment

Table 4. Responses and statistical analysis of the 2^n factorial experiment with respect to protein production by *Dunaliella salina*

Trial	Factor(s) under study	Response (mg/ml)	Effect total	Sum of squares	*F*-Ratio
1	Base	**0.207**	**7.237**	**total**	
2	NaCl	**0.275**	**1.669**	**0.087**	**9.667***
3	W	**0.175**	**2.149**	**0.144**	**16.000****
4	NaCl, W	**0.296**	**0.507**	**0.008**	**0.889**
5	$CaCl_2$	**0.118**	**-0.017**	**$0.9x10^{-5}$**	**0.001**
6	NaCl, $CaCl_2$	**0.154**	**-0.477**	**0.007**	**0.778**
7	W, $CaCl_2$	**0.202**	**0.299**	**0.003**	**0.333**
8	NaCl, W, $CaCl_2$	**0.280**	**-0.339**	**0.004**	
9	K_2HPO_4	**0.113**	**0.025**	**$0.2x10^{-5}$**	**0.0002**
10	NaCl, K_2HPO_4	**0.233**	**0.173**	**0.0009**	**0.100**
11	W, K_2HPO_4	**0.343**	**0.277**	**0.002**	**0.222**
12	NaCl, W, K_2HPO_4	**0.688**	**0.287**	**0.003**	
13	$CaCl_2$, K_2HPO_4	**0.186**	**-0.781**	**0.019**	**2.111**
14	NaCl, $CaCl_2$ K_2HPO_4	**0.228**	**-0.421**	**0.006**	
15	W, $CaCl_2$ K_2HPO_4	**0.159**	**-0.989**	**0.031**	
16	NaCl, W, $CaCl_2$ K_2HPO_4	**0.296**	**0.153**	**0.0007**	
17	M	**0.107**	**-0.669**	**0.014**	**1.556**
18	NaCl, M	**0.144**	**-0.225**	**0.002**	**0.222**
19	W, M	**0.123**	**0.299**	**0.003**	**0.333**
20	NaCl, W, M	**0.285**	**-0.323**	**0.003**	
21	$CaCl_2$, M	**0.076**	**1.397**	**0.061**	**6.777***
22	NaCl, $CaCl_2$, M	**0.254**	**0.245**	**0.002**	
23	W, $CaCl_2$, M	**0.421**	**1.145**	**0.041**	
24	NaCl, W, $CaCl_2$, M	**0.489**	**-0.057**	**0.0001**	
25	K_2HPO_4, M	**0.086**	**-1.053**	**0.035**	**3.888**
26	NaCl, K_2HPO_4, M	**0.186**	**-0.509**	**0.008**	
27	W, K_2HPO_4, M	**0.123**	**-0.777**	**0.019**	
28	NaCl, W, K_2HPO_4, M	**0.243**	**-0.163**	**0.0008**	
29	$CaCl_2$ K_2HPO_4, M	**0.049**	**-0.163**	**0.0008**	
30	NaCl, $CaCl_2$ K_2HPO_4, M	**0.128**	**0.001**	**$0.3x10^{-7}$**	
31	W, $CaCl_2$ K_2HPO_4, M	**0.217**	**0.741**	**0.017**	
32	NaCl, W, $CaCl_2$ K_2HPO_4, M	**0.353**	**0.391**	**0.005**	

W=Fermentation liquor
M=Factors appeared to be insignificant in the Plackett & Burman experiment
F-ratios for interactions of more than two factors are neglected.
** Highly significant
* Significant

Table 5. The figures generated for protein optimization in *Dunaliella salina* cultures using the steepest ascent method

Factor	Concentrations[1] in trials 1-15														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
NaCl	8.100	9.676	11.252	12.828	14.404	15.980	17.556	19.132	20.708	22.284	23.860	25.436	27.012	28.588	30.164
W	1.400	3.400	5.400	7.400	9.400	11.400	13.400	15.400	17.400	19.400	21.400	23.400	25.400	27.400	29.400
$CaCl_2$	0.050	0.110	0.170	0.230	0.290	0.350	0.410	0.470	0.530	0.590	0.650	0.710	0.770	0.830	0.870
$MgCl_2$	0.375	0.825	1.275	1.725	2.175	2.625	3.075	3.525	3.975	4.425	4.875	5.325	5.775	6.450	6.900
$MgSO_4$	0.125	0.275	0.425	0.575	0.725	0.875	1.025	1.175	1.325	1.475	1.625	1.775	1.925	2.075	2.225
KCl	0.050	0.110	0.170	0.230	0.290	0.350	0.410	0.470	0.530	0.590	0.650	0.710	0.770	0.830	0.870
T.E.	0.250	0.550	0.850	1.150	1.450	1.750	2.050	2.350	2.650	2.950	3.250	3.550	3.850	4.150	4.450
KNO_3	0.250	0.550	0.850	1.150	1.450	1.750	2.050	2.350	2.650	2.950	3.250	3.550	3.850	4.150	4.450
$NaHCO_3$	0.010	0.023	0.036	0.048	0.061	0.074	0.086	0.099	0.111	0.124	0.137	0.149	0.162	0.174	0.187

[1] Concentrations are expressed in g/l except trace elements, which are presented as ml/l.

T. E. = Trace element

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استخدام المخلفات الطبيعية كمواد غذائية إضافية للحصول على أعلى محتوى للبروتين في مزرعة طحلب دوناليللا سالينا

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عنى هذا البحث بإيجاد مزرعة غير تقليدية من بعض المخلفات الملائمة لانتاج أعلى كمية من البروتين في طحلب دوناليللا سالينا. فقد تم إحلال جزئي للمزرعة الأساسية بمخلف شرش الجبن و المولاس و المخلف الناتج من تخمر الخميرة. و قد أثبتت النتائج أن نقص المكونات للمزرعة الأساسية إلى 4/3 وزن مكوناتها (ما عدا كلوريد الصوديوم) و في وجود 0,4 % من مخلف تخمر الخميرة أدى إلى حدوث أعلى سرعة لنمو الطحلب بعد فترة زراعة لثمانية أيام و في الوقت نفسه ازداد معدل بناء البروتين 1,5 إلى 1,4 مرة بالمقارنة بالمزرعة الأساسية بدون أي إضافات من المخلفات المستخدمة. و قد تم استخدام نظم إحصائيه خاصة للحصول على التركيز الأمثل للمخلف الذي يؤدى إلى بناء أعلى كمية للبروتين في طحلب دوناليللا سالينا. و قد أثبتت النتائج أن مخلف تخمر الخميرة هو الأفضل من بين المخلفات المستخدمة حيث أن المزرعة المقترحة عند التركيز الأمثل للمخلف أدى إلى زيادة بناء البروتين 1,7 مرة في مزرعة طحلب دوناليللا سالينا.