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Optimization of glycerol production by the Moroccan strain of *Dunaliella salina* using response surface design methodology

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- ✓ *Dunaliella salina* (Morocco-10);
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Abstract

The Moroccan strain of the halophilic green algal, *Dunaliella salina*(Morocco-10) was used to assess optimal conditions for glycerol production according to the response surface methodology. The effects of four independents variables (salinity, incubation temperature, pH, and cultivation time on glycerol production (*Y*) were evaluated using the Box-Behnken Design method with 4-factors and 3-levels.Optimal conditions were estimated by statistical regression analysis and surface plots. The optimal production parameters for maximum glycerol production by *Dunaliella salina*(Morocco-10) were salinity 2.33 mol.L^{-1}, incubation temperature 36.36°C, pH 7.76 and a cultivation time of 120 h. The averaged experimental production of glycerol under these optimal conditions was found to be 270.03 ± 0.74 µg/ml, which agrees closely with a value of 269.94 µg/ml predicted by the model.

1. Introduction

Glycerol is a simple alcohol with two primary and one secondary alcohol functional groups [1]; it is also known as glycerin or propane-1. 2. 3-triol. It has a multitude of uses especially in the pharmaceuticalcosmetic, personal care and foods industries [2. 3]. It has also been considered as a feedstock precursor in several new chemical and biochemical processes [4]. Glycerol is produced by certain bacteria, yeasts and some microalgae [5]. Species of the halophytic microalga *Dunaliella* are the best known for autotrophic production of glycerol .

The halophylic algae are characterized by an elastic plasma membrane which enables cells to adjust their volume and shape rapidly in response to hypo or hyper-osmotic changes [6]. The *Dunaliella* genus shows a remarkable degree of adaptation to salt concentrations [7] and its glycerol production has been found to be proportional to the extracellular salt concentration [8]. *Dunaliella salina* is an important natural source of glycerol where it is produced by two different metabolic pathways. One is photosynthetic fixation of carbon dioxide. and the other is the starch conversion [9. 10]. Biosynthesis takes place in the chloroplast, but also in the cytosol [11]. This species can accumulate more than 50 % of the dry cell weight in glycerol [12] under optimal conditions [13]. The ability to grow at very high salt concentrations has made this microalga an attractive candidate for commercial production of glycerol [14. 15]. Several previous works have been interested in optimizatizing the cultivation of glycerol producing microalgal species [10. 16].

Nevertheless, to our knowledge.This study is the first to optimize glycerol production of the new Moroccan strain of *Dunaliella salina* (Morocco-10). In the present work. a response surface methodology (RSM) based upon the Box Behnken design (BBD) for optimization of the effects of physical parameters on glycerol

production in *Dunaliella salina* (Morocco-10) is used. Response surface methodology (RSM) is one of the most useful statistical methods since it is aimed specially for optimization the parameters for various processes in biotechnology [17]. This mathematical technique is also useful for designing experiments [18],developing models, exploring the relationships between design variables and responses, factors effects and searching for optimal conditions [19]. In this work, the effect of salinity, incubation temperature, pH and cultivation time on glycerol production by the Moroccan strain of *Dunaliella salina* (Morocco10) was studied and the optimal conditions for glycerol were determined.

2. Experimental details

2.1 Microorganism and Culture Conditions

2.1.1 Strain,media and maintenance of microalgal cells

Dunaliella salina (Morocco-10) was isolated from evaporating salt ponds at the Sidi Abed salt marsh (33°02N. 08°42'W) 35 km southwest of El Jadida. Morocco. This strain was identified with a traditional taxonomy key [20]. and confirmed with Basic Alignment Search Tool (Blast) of NCBI [21] and phylogenetic analysis of Internal Transcribed Spacer 2 (ITS2) sequences as described by [22]. *Dunaliella salina* cells were cultivated in the culture medium Semenenko-Abdullaev [23].The medium was sterilized at 121°C for 20 min before inoculation. To avoid precipitation, phosphate was autoclaved separately,solid KH_2PO_4 wasput in an oven over night at 120 °C and then mixed with sterilized water). During cell growth, 5mM carbon source ($NaHCO_3$) was added daily in order to avoid carbon limitation. For sterilization, solid $NaHCO_3$ was heated in an oven over night at 120°C and then mixed with sterilized water [24].

The Moroccan strain of *Dunaliella salina* (Morocco-10) was inoculated with 50 ml of Semenenko-Abdullaev culture medium in a 150 ml Erlenmeyer flask under aseptic conditions. The culture was stirred using an air pump kept at an average temperature of 25°C , and illuminated continuously in low light using under a white fluorescent lamp (40W) to promote cell multiplication. Periodically, part of the culture was diluted with fresh Semenenko-Abdullaev medium, to renew nutrient culture conditions.

2.1.2 Culture Conditions

A growth experiment for optimizing cultivation conditions was carried out during the growth phase. The experiment was performed in 1L Erlenmeyer flasks. The flasks were incubated at 25°C in an orbital incubator shaker (Ivymen System shaking incubator) and agitated at 150 rpm for 8 days under the natural light intensity and under 16:8-h light: dark cycle to obtain a final cell concentration of around 4×10^5 cell mL^{-1}. This culture was used as a standard inoculum for all cultivation experiments in shake flasks. In each Erlenmeyer, 100 ml of the stock suspension was added to 150 ml culture medium. In order to assess the effects of the physical parameters on glycerol production by *Dunaliella salina* (Morocco-10) in this study, salinities used were from 1.50 to 3 $mol.L^{-1}$., incubation temperatures were from 30 to 37°C , pH values were gradually adjusted from 7 to 8and the cultivation times were from 72 to 120 hours..

2.3 Determination of glycerol production

The amount of glycerol in the alga was determined by the chemical method of [9].This includes using the Malaprade and the Hantzsch reactions to measure glycerol concentration. In the Malaprade reaction, glycerol is converted with periodate into formaldehyde (Figure1) [25]. Then, in the Hantzsch reaction, formaldehyde is reacted with acetylacetone in presence of ammonia to form a dye yellow (Figure2). This dye, 3,5-diacetyl-1,4-dihydrolutidine (DDL), was then measured at 410 nm in a Helios Gamma UV/VIS Spectrophotometer [26].

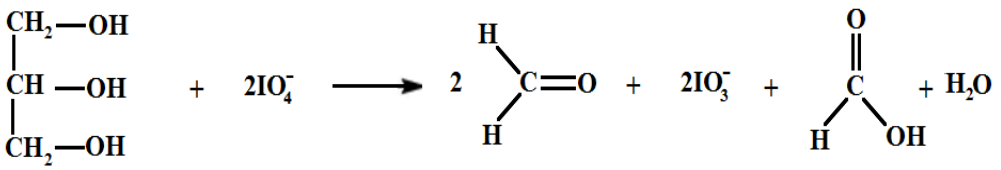

Figure1: Reaction scheme of the Malaprade reaction.

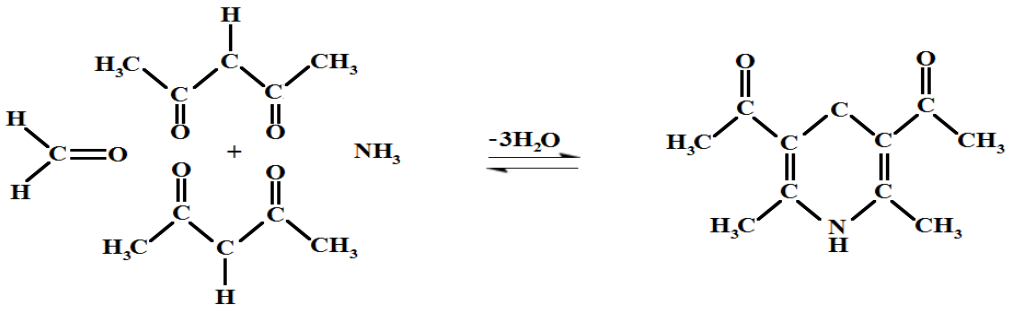

Figure2: Reaction scheme of the Hantzsch reaction

2.4. Experimental design and data analysis

The experimental optimization design was performed using Response Surface Methodology (RSM) with Box-Behnken Design (BBD) [27] .The experimental design uses four factors and three levels, including three replicates at the center point. Four factors namely, salinity (X_1, mol.L^{-1}), incubation temperature (X_2, °C),pH adjustment (X_3) and cultivation time(X_4,h)were coded according to three different ranges –1. 0and +1 (Table 1).

Table 1: Experimental ranges and values of the independent variables in the Box-Behnken design

Independent variables	**Units**	**Symbol**	**Coded levels** -1	0	+1
Salinity	mol L^{-1}	X_1	1.5	2.25	3
Incubation temperature	°C	X_2	30	33.5	37
pH	—	X_3	7	7.5	8
Cultivation time	h	X_4	72	96	120

The optimized response was glycerol production in µg ml-1 (Y). The complete optimization study comprised 27 combinations, which included three replicates for the center point (Table 2). The response function (Y) divided into the linear, quadratic and interactive terms as below:

$$Y= \beta_0+\beta_iX_i +\beta_jX_j +\beta_{ii}X_{2i} +\beta_{jj}X_{2j} +\beta_{ij}X_iX_j+ \varepsilon \qquad \text{(Eq. 1)}$$

Where Y is the dependent variable (glycerol production. µg mL-1);β_0 is constant.β_i,$\beta_{ii}$and $\beta_{ij}$are the regression coefficients for intercept, linear, quadratic and interaction terms, respectively; X_i and $X_j$are the independent variables and ε is the error term.

2.5. Statistical analyses

Analyses of variance (ANOVA) and statistics of the experimental data were carried out using Minitab 16.0 Statistical Software.

3. Results and Discussion

3.1.Devlopement of response surface model and data analysis

A Box-Behnken design (BBD) was used to elucidate the main effects and the interactions of the parameters involved: influence of salinity concentration (X_1), incubation temperature (X_2), pH of adjustment (X_3) and cultivation time (X_4) on glycerol production. All 27 experimental runs were evaluated using the Response Surface Regression (RSREG) procedure for RSM analysis and the second order model equation was obtained by using the statistic on the predicted model. The statistical significance of the quadratic polynomial model equations was evaluated by analysis of variance (ANOVA). Table 3 shows ANOVA of the model to explain the response of the dependent variable Y.

Table 2: Experimental design matrix and results of glycerol production from the Moroccan strain of *Dunaliella salina* (Morocco-10). See text for explanations of *X* values

Runs	X_1	X_2	X_3	X_4	Glycerol (µg ml^{-1})
1	1. 50	30. 0	7. 5	96	145.81
2	3. 00	30.0	7.5	96	162.85
3	1.50	37.0	7.5	96	213.50
4	3.00	37.0	7.5	96	234.32
5	2.25	33.5	7.0	72	169.95
6	2.25	33.5	8.0	72	200.95
7	2.25	33.5	7.0	120	242.72
8	2.25	33.5	8.0	120	292.51
9	1.50	33.5	7.5	72	169.36
10	3.00	33.5	7. 5	72	186.29
11	1. 50	33.5	7. 5	120	190.20
12	3.00	33.5	7.5	120	204.16
13	2.25	30.0	7.0	96	158.67
14	2.25	37.0	7.0	96	178.96
15	2.25	30.0	8.0	96	187.40
16	2.25	37.0	8.0	96	211.14
17	1.50	33.5	7.0	96	130.91
18	3.00	33.5	7.0	96	151.28
19	1.50	33.5	8.0	96	159.32
20	3.00	33.5	8.0	96	173.26
21	2.25	30.0	7.5	72	152.53
22	2.25	37.0	7.5	72	211.10
23	2.25	30.0	7.5	120	231.57
24	2.25	37.0	7.5	120	257.10
25	2.25	33.5	7.5	96	222.77
26	2.25	33.5	7.5	96	220.10
27	2.25	33.5	7.5	96	215.98

Table 3: Analysis of variance of the model results for glycerol production of the Moroccan strain of *Dunaliella salina* (Morocco-10).

Source	Degree of Freedom (DF)	Sum of squares (SS)	Mean of squares (MS)	*p*-value
Model	14	31125.4	2223.24	0.008
Square	4	11861.8	2964.44	0.008
Interaction	6	380.3	63.38	0.992
Residual Error	12	6259.3	521.61	—
Lack-fit	10	6235.3	623.59	0.019
Total	26	37384.7	—	—

Results show that the interaction term (X_1X_2; X_1X_3; X_1X_4; X_2X_3; X_2X_4; X_3X_4) did not contribute significantly to the model (P> 0.05), whereas the linear term (X_1; X_2; X_3; $X4$).The square term (X_1X_1; X_2X_2; X_3X_3; X_4X_4) and the total regression model (Y) were highly significant (P<0.05). Furthermore, the coefficient of determination (R^2) for Y was 0.832 implying that a second order model was suitable to represent the real relationships among the selected reaction parameters. Also, graphical analysis of the models (Figure3) was carried out by a normality test (the Anderson-Darling normality test) of the error terms using residuals of the dependent variable *Y*. The residuals portray a normal distribution because virtually all the points follow a straight line curve. Therefore, response surface model represented as a quadratic polynomial equation was statistically significant. The fitted model for glycerol production (Y) was given by following equation:

$$Y= -5810.13+331.33\ X_1 + 60.62\ X_2 + 1187.69X_3 - 2.22X_4 + 0.36X_1X_2 - 4.29X_1X_3 - 0.04X_1X_4 + 0.49X_2X_3 - 0.1X_2X_4 + 0.39X_3X_4 - 65.74\ X_1^2 - 0.74X_2^2 - 80.01X_3^2 + 0.02X_4^2 \quad \text{(Eq. 2)}$$

Where Y is the glycerol production (µg mL^{-1}).X_1 is the concentration of salinity (mol L^{-1}).X_2 is the incubation temperature (°C).$X_3$is the pH adjustment and X_4 is the cultivation time (h).

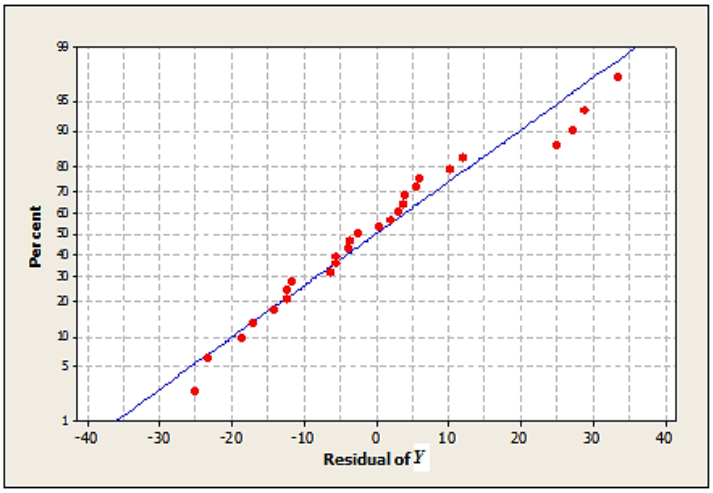

Figure3: Normal probability plots for error terms using residuals of the dependent variable according to the Anderson-Darling Normality test

3.2. Analysis of response surface plot

In order to study the effect of variation in factor levels on glycerol production and to define the optimal level for each variable, three-dimensional views of response surface and respective contour plots were used to demonstrate the effects and the interaction of the independent variables on the response (Figure 4). Temperature and salinity are both important environmental factors for glycerol production. Figure4A represents the response surface for interactive of salinity (X_1) and incubation temperature (X_2) on the response value (Y). An increased temperature with salinityup to the optimal point, increased glycerol production to the maximum level but a further increase in both decreased the glycerol production. The temperature optimum was around 36.36°C. This compares with an optimal growth temperature of 30°C determined by (Ben-Amotz and Avron. 1990) [7]. Garcia et al. (2009) [28] reported that 22°C as the optimum temperature for growth for Mexican species of *Dunaliella*and Ben –Amotz and Avron (1989) [29], 25-35°C for *Dunaliella bardawil*. This contrasting result can be explained by each strain having a different optimum temperature. Figure4B shows the effects of salinity (X_1) and pH adjustment (X_3) on the glycerol production. An increase in salinity and pH cause an increase in glycerol production to the optimum point.Further increases lead to a decrease in production decrease.

The effects of salinity (X_1) and cultivation time (X_4) on the glycerol production are shown in Figure4C. Maximum glycerol production for the Moroccan strain of *Dunaliella salina* (Morocco-10) occurred when salinity was at the optimum level (2.33 mol.L^{-1}) and the growth time was at the maximum. These results agree with previous studies showing that optimum growth on the Iranian strain of *Dunaliella salina* was obtained in 2 M NaCl [30]. Shariati reported that the optimum salinity for glycerol production of *D.salina*.*D.parva* and *D.pseudosalina* isolated from salt marshes of Iran was at 0.5 to 2 M NaCl [31].

Other studies reported that osmotic shock can affect glycerol production by *Dunaliella salina* when hyper-osmotic media was increased [32]. This is because glycerol is an osmotic stabilizer and probably this is the major function of glycerol in *Dunaliella salina* [33]. The effects of incubation temperature (X_2) and cultivation time (X_4) on the glycerol production are shown in Figure4E. Glycerol production of the studied microalgal strain was enhanced after increasing the temperature and the cultivation time. Fig.4.F shows the effects of pH (X_3) and cultivation time (X_4) on glycerol production. An increase in the cultivation time with pH up to the optimum point increased glycerol production to maximum level but a further increaseboth in the cultivation time and the pHdecreased the glycerol production.

In this studyoptimal pH for the Moroccan strain of *Dunaliella salina* (Morocco-10) is 7.76. These results agreed with those of Khalil et al. (2010) [34] where glycerol production by *Dunaliella bardawil* was significantly enhanced at pH 7.5.Other studies have indicated that the Namibian strains of *Dunaliella* showed glycerol production performance was better at initial pH levels (7.5 to 9) [35].

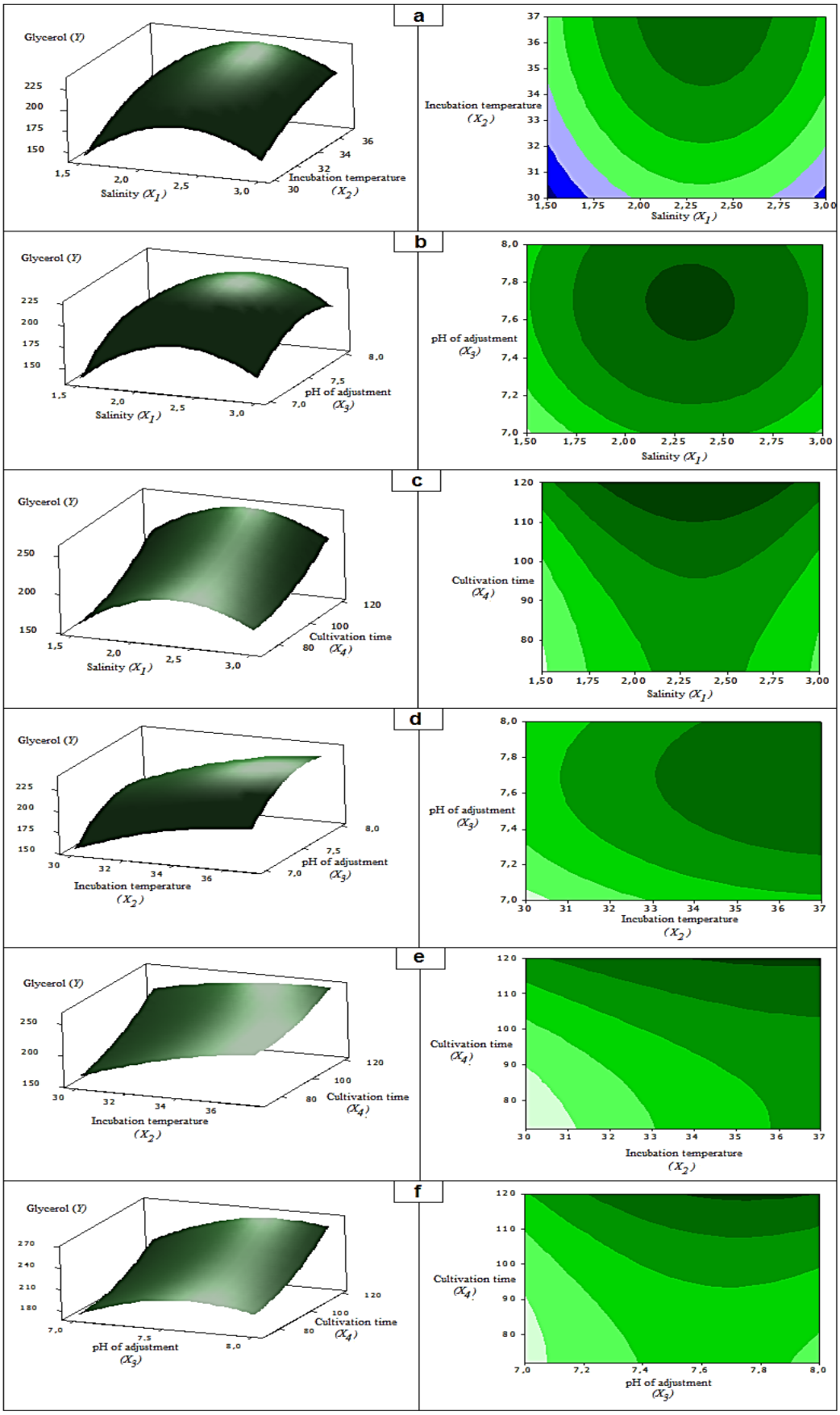

Figure4: Response surfaces plot and contour diagram of the independent variables effects on glycerol production

3.3. Optimization of glycerol production

In this studythe aim of optimization was to find the conditions which maximized glycerol production. Optimal values of the selected variables were estimated at a salinity of 2.33 mol.l-1 (X_1), temperature of 36.36 °C (X_2), pH of adjustment to 7.76 (X_3) and cultivation time of 120 h (X_4). From the model equation derived from optimal condition.The predicted value of glycerol production was 269.94 µg/ml of cultureafter 5days of culture.
In orderto verify this prediction, the experimental value was repeated three times and 270.03 ± 0.74 µg/ml was obtained. That result was in agreement with the predicted value. it also confirmed that the model used in this experiment is appropriate (Table 4). Production appeared to be a better function of time than in other study by Kaçka and Dönmez [36] whousing the Turkish strain T1showed glycerol production to be 452.57 µg / ml of culture after 60 days.

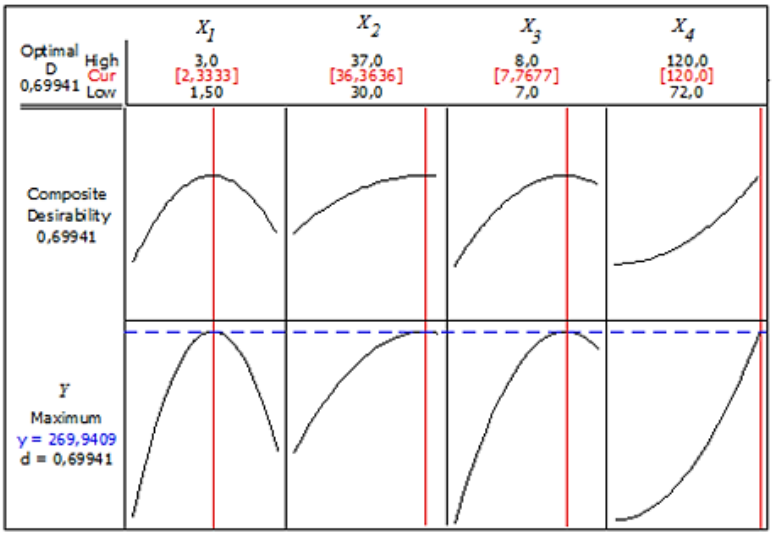

Figure5: The individual maximum responses of glycerol production

Conclusions

This study has shown that this Moroccan strain of *D. salina* (Morocco-10) is suitable for the producing large amounts of glycerol on a commercial scale. Morocco is favored by possessing many salt marshes with an equitableclimate for the growth of *D. salina*including strong sun light radiation to promote cell growth and glycerol production. Aqua-culture of this alga offers a potentially viable option for glycerol production in Morocco.

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