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Enhancement of protein production by microalgae *Dunaliella salina* under mixotrophic condition using response surface methodology

S. Kadkhodaei[1], S. Abbasiliasi[1], T.J. Shun[2], H. R. Fard Masoumi[3], M. S. Mohamed[1], A. Movahedi[4], R. Rahim[5], A. B. Ariff[1]

[1]Department of Bioprocess Technology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia

[2]Institute of Bioscience, Universiti Putra Malaysia, Serdang, Selangor 43300 Malaysia

[3]Department of Chemistry, Faculty of Science, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia

[4]College of Forest Resources and, Environment of Nanjing Forestry University, Nanjing, Jiangsu, 210037, China.

[5]Department of Cell and Molecular Biology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia

Abstract

The present work was aimed at enhancing the protein production in *Dunaliella salina* by optimization of culture condition. The interactive effects of medium composition on protein production were optimized using response surface methodology (RSM). A mathematical model was proposed to describe the kinetics of *D. salina* growth. Results showed that the mixotrophic effects were in conjunction with that the mixture of NaCl and glucose. Based on the optimization study, protein production in *D. salina* could be markedly increased through glucose supplementation as low as 5 g/L. Optimal condition was achieved at 288, 288 and 312 h with a doubling time of 1.13, 3.03 and 5.17 days for D_1, D_2 and D_3 strains, respectively. The overall protein production was enhanced by 3.4, 3.9 and 14 times in D_1, D_2 and D_3, respectively. The microplate-based approach enabled us to screen large numbers of experimental cultures in a time and cost effectiveness manner. It also can be concluded that reducing the growth cycle through this cultivation system may expedite the analysis of transformants for production of recombinant proteins in microalgae.

Introduction

Dunaliella salina, unicellular halotolerant Chlorophyta microalgae, is capable of producing valuable compounds such as β-carotene and glycerine [1]. The Lack of cell wall makes it easily digestible in both humans and animals. In addition, this microalga is highly efficient in the conversion of light and nutrients into biomass. Hence, it could accumulate more dry weight and glycerol under suitable cultivation conditions [2] as well as high protein content (57% of dry matter) [3]. *D. salina* is one of the most widely used microalgal species for mass culture with an annual production of 1,200 tons [4]. The products derived from *D. salina* could be categorized into three groups: β-carotene extracts, *Dunaliella* powder for human use and dried *Dunaliella* for feed use [5].

One of the most recently used platforms, which is proposed for the purpose of molecular farming are microalgae as green "micro-bio-factories". These microorganisms are the best model to this end, since they have the combination advantages of both bacteria and yeast (in terms of growth rate and low cost growth requirements) and animal and plants (having the ability of posttranslational modifications). In view of the high growth rate, the scalability and biosafety considerations such as the lab-suited microorganisms in controlled environments, possibility of using various microalgal species have been tested as the production platform for recombinant proteins and engineering of metabolic pathways in the production of increased levels of desirable compounds [6,7]. One of the main issues pertaining to the above is the low production yield. Accumulation of foreign proteins could be influenced by both upstream (genomics, transcriptomics, proteomics and metabolomics levels) and downstream (cultivation condition and physiological aspects of the platform organism) processes. In the upstream process many features such as utilization of strong promoters and untranslated regions (UTRs), codon adaptation, RNA secondary structure and instability, protein production and purification strategies should be taken into consideration. In downstream processing the optimum cultivation conditions with high biomass concentration is required to get high recombinant protein yield. Factors influencing microalgae growth and development including light (both intensity and photoperiod), temperature, nutrient requirements (carbon and nitrogen source), pH, aeration and contamination, may indirectly play important roles in protein accumulation.

Factors affecting the production of *D. salina* have been widely studied and reviewed. The effective factors on the growth and composition of *D. salina* are mainly light (quality and quantity), salinity, temperature and pH. Amongst these factors, salinity affects the biomass and pigment content of *D. salina* more than any other factors. There is also a synergistic influence between salinity and illumination of light quality and quantity in *D. salina* for production of carotenes [1,8]. However, the influence of salinity is strain-dependent and only a few *Dunaliella* strains have the high carotenogenesis potential [9].

Two critical limiting factors, (1) labor costs and (2) low productivity, are involved in mass production of microalgal biomass. The use of efficient cultivation system such as the use of photobioreactor may be used to reduce the labor cost and to improve the production. High algal biomass (5 g/L) was obtained in cultivation using photobioreactor whereas very low algal biomass (0.5 g/L) was obtained in open pond system. Photobioreactors can effectively improve the labor costs and productivity (5 g/L algal biomass compared to 0.5 g/L in open ponds) [10]. In outdoor cultivation systems due to low light intensity, endogenous biomass consumption through respiration increases resulting lower productivity. The heterotrophic systems which rely only on the organic carbon source may be more costly than photoautotrophy. Furthermore, mixotrophic condition might reduce the cultivation costs compared to heterotrophic systems through shortening the growth cycle and thus increasing the biomass production [11].

Some microalgae are capable of transforming their growth habit from photoautotrophic to heterotrophic (utilizing organic materials as a source of energy for growth) or mixotrophic (combination of organic nutrition and light). This provides the feasibility of high growth rate and biomass production (up to 40 g/L) [12,13].

Specific requirements with reference to the production of protein from algae (*D. salina*) have been reported. Protein production in algae can be modified by altering the cultivation conditions. Certain combinations of influencing factors need to be optimized for improvement of the cultivation process. With regards to the complexity of the influencing factors an in-depth knowledge of these factors needs to be substantiated for subsequent application in the optimization process. Most studies carried out to date claimed validation by statistical analysis and the combination of variables with their values and limits were arbitrarily chosen based primarily on personal experiences [14,15].

Conventional methods in optimizing fermentation/cultivation process require treating each factor separately which are laborious, incomplete and time consuming. If several factors are to be considered simultaneously their interactions are not discernible even for the dominant ones. These conventional approaches did not yield reliable results either. In this respect response surface methodology (RSM) and the experimental factorial design have been successfully applied for optimization of various biomanufacturing processes, which could also be used to investigate the interacting factors [16,17].

RSM, a non-conventional approach, is a collection of statistical and mathematical methods that can be used to quantify the interaction between different factors. This approach provides statistically reliable results with fewer numbers of

experiments and is very useful for the development, improvement and optimization of the biomanufacturing processes [18]. The statistical designs of RSM provide alternative methodologies to optimize a particular process by considering the mutual interactions among the various factors. This alternative approach is advantageous as it simplifies the optimization process and reduces experimental costs. Besides, the most significant factors that influenced the maximum response could also be determined. The RSM is based on response analysis, which is influenced by specific factors with the objective to define the optimum condition of the response by these factors. It is difficult to demonstrate the methodology in the form of space dimension but could only be visualized in the mind. RSM is useful in the identification of the direction in the next experiment towards an optimum point. From the optimum or near optimum point on the response surface, the equation can be determined [19].

Microalgae as the lab-suited microorganisms in containment environments are among the best models for molecular farming. Most recently, these green "micro-bio-factories" are increasingly used as expression platforms. Most approaches for improvement of the recombinant protein production in molecular farming have been focused on upstream strategies. While the downstream strategies which also play critical roles in this regard, have less studied. In the present study, among the factors influencing productivity of the recombinant proteins in microalgae expression platforms we focused on downstream bioprocesses. The main objective of this study was to optimize the variables of microplate cultivation method of *D. salina* for enhancement of protein production using response surface methodology. The interactive effect of mixotrophy condition on growth rate, cell concentration and protein content were also analysed.

Experimental details

Microalgal Species and Cultivation Procedures

D. salina strains, D_1 and D_2 were obtained from the Department of Biology, University of Isfahan, Iran and D_3 (UTEX 1644) was purchased from UTEX Collection (Texas University of Austin, USA). A preliminary study to determine the preferred medium for growth of *D. salina* was carried out by comparing three different media including modified Johnson [10], 2x Erdschrider (UTEX, the culture collection of algae) and Ben Amotz [9]. The modified Johnson medium (M) demonstrated the ideal medium and was used for subsequent experiments. The strains were plate-streaked on solid medium containing ampicillin (100 µg/mL) and kanamycin (50 µg/mL). The stock culture preparation was carried out through inoculation of the liquid medium by a single colony obtained from the agar plate. This was performed for each strain separately from the plates that did not show any signs of contamination. The axenicity of stock cultures in terms of bacterial contamination was later confirmed through PCR amplification of 16S rDNA using primers as described by Weisburg *et al.* (1991) [20]. To start the cultivation, three different strains of *D. salina* (D1, D2 and D3) were cultured into four 96-well microplates (Nunc, Inc.). Two hundred microliters of aliquots were pipetted into each well. Four microplates were prepared with one as control (without microalgae) and two for mixotrophic cultures of 24 (M24) and 16 hours (M16) illumination, as well as one for heterotrophic condition in the absence of light (H). A light equipped incubator with controlled temperature at 25 °C and an irradiance of 40 $\mu mol/m^2.s^1$ supplied by a cool-white fluorescent light was used to cultivate microalgae for a period of two weeks. The cultures were agitated at 150 RPM using rotary shaker (Heidolph Titramax 1000) for 5 minutes, daily. Cultivation using microplates was conducted in triplicates for subsequent experiments.

Taxonomic Identification of the Strains

Three microalgae strains were identified by optical microscopy and 18S rDNA sequencing. Total genomic DNA was extracted according to Kadkhodaei *et al.* (2011) [21] and DNA quality and quantity were assessed through spectrophotometry and agarose gel electrophoresis. For molecular identification of the strains, four sets of primer pairs described by Gonzalez-Ballester *et al.* (2011) [22], Olmos *et al.* (2009) [23] and Wan *et al.* (2011) [11] were selected to amplify 18s ribosomal RNA. All PCR amplifications were performed in a final volume of 50 µL containing 50 ng of DNA template, 1x Buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.4 µM each primer, and 1 unit of Taq-polymerase (NEB, Ipswich, MA, USA). The temperature for primer annealing (T_a) was experimentally optimized using the gradient function of the thermocycler. The cycling program were performed with following conditions: 95 °C for 5 min, 35 cycles [94 °C for 30s, T_a for 30 s, 72 °C for 90 s], followed by a final extension step of 72°C for 10 min. The PCR products were visualize on 1.5% agarose (Amresco, Inc.) gel in TBE and staining with SYBR Gold™ (Invitrogen, Inc.) or GelRed™ (Biotium Inc., Hayward, CA). The fragments were then gel purified using Qiagen kit (Qiagen, Inc.). PCR products from each strain were sequenced with the primers used for amplification in an automatic DNA Genetic Analyzer (ABI Prism 3130 Genetic Analyzer) using the BigDye Terminator cycle sequencing kit v1.1 (Applied Biosystems). The resulting electropherograms were analyzed with CLC Main Workbench software v6.9.1 (CLCBio, Inc.) and subsequently aligned and phylogenetic dendrogram was created using Neighbor-Joining (NJ) algorithm. Bootstrap values based on the analysis of 1,000 bootstrap replicates were calculated to assess the confidence degree assigned to the nodes in the phylogenetic trees. The identity of PCR products was confirmed through BLAST (Basic Local Alignment Search Tool) from the database of the NCBI. Similarly, the strains sequences herein obtained and those present in NCBI database stored in a local database to perform a local alignment by the tool present in CLC software.

Analytical Procedures

Cell density was estimated by the mean number of cells which obtained from the direct cell counting. Three samples of each strain were used in cell counting using a hemocytometer and a light microscope (Nikon Eclipse TE200S). Maximum absorbance was inspected by scanning the culture sample from 400 to 700 nm with 10 $_{nm}$ increments (Supplementary Figure 2). The scanned data were normalized and plotted using Statistica software v.10 (StatSoft, Inc.). In order to determine the algal density, the highest absorbance value was used. The relationship between cell number and

spectrophotometer absorbance were expressed using a general power equation (Equation 1) [25] (Supplementary Table 2 and Supplementary Figure 3):

$$Y = a.(X)^b \quad \text{Equation (1)}$$

where;
Y: Absorbance
X: cell density (Cell/mL), and
a and b: calibration coefficients

The growth rates (day^{-1}), from the beginning to the end of the experiment as well as other growth parameters such as divisions per day and generation time were calculated according to Stein (1979) [26].

$$\text{Growth rate; } K' = Ln(N2 / N1) / (t2 - t1) \quad \text{Equation (2)}$$

where;
N_1 and N_2 are biomass at time1 (t_1) and time2 (t_2), respectively

$$\text{Division per day; } Div.day^{-1} = K' / Ln2 \quad \text{Equation (3)}$$

$$\text{Generation time; } Gen' t = 1 / Div.day^{-1} \quad \text{Equation (4)}$$

Glucose uptake was determined using the YSI bioanalyzer (Illinoise, USA) and expressed as the Equation 6.

$$= \Delta S_g / S_g \quad \text{Equation (6)}$$

where;
ΔS_g: is the total weight of glucose consumption from the beginning to the end of experiment,

S_g: is the weight of glucose supplemented to the medium at the beginning of cultivation

Biomass (X) measured as dry cell weight was estimated using filtration and oven drying according to the method suggested by Zhu and Lee (2002) [27]. Biomass yield was determined by the equation:

$$Y = (X_t - X_0)/(S_0 - S_t) \quad \text{Equation (7)}$$

where;
X_t: is the final biomass concentration (g/L);
X_0: is the initial biomass concentration (g/L); and
S_0 and S_t: are the initial and final concentrations of the carbon source (g/L), respectively.

Total nitrogen content was estimated by the micro-Kjeldahl method [28],[29] and then converted to protein using conversion factor of 5.95 which was suggested by López *et al.* (2010) [30] for microalgae and cyanobacteria undergoing rapid growth. To determine the protein content, three replicate samples of known volume and cell number were centrifuged in 3000 g and 4ºC for 15 min. The pellet was resuspended in 10 ml of sodium phosphate buffer containing 1% (w/v) sodium dodecyl sulphate (SDS) and the solution was sonicated on ice water (3 cycles) and then centrifuged to remove the pellet. The protein content was normalized to the cell numbers. Since the Kjeldahl nitrogen value has been shown to have better correlation with Lowry protein measurement for microalgae [30], this indirect protein content measurement was chosen in opposite to a direct protein measurement.

Experimental Design

Based on prior mixotrophic cultivations with modified Johnson medium, carbon source (glucose) and salinity (sodium chloride), which were the main factors affecting the growth performance of *D. salina* strains were selected as numeric experimental factors and photoperiod (periodic illumination times) and types of strains (D1, D2 and D3) were selected as categorical experimental factors. The initial ranges were selected according to preliminary studies and previous works. A central composite design (CCD) with a full-factorial design consisting of a two-factor-five-level pattern with 13 design points (nine combinations with five replicates on the center point) using response surface methodology (RSM) was applied in this study. The design matrix of CCDs used is presented in Table 2.
A polynomial model to predict the response and optimal levels were calculated using Equation 8.

$$Y = b_0 + b_1X_1 + b_2X_2 + b_{11}X_1^2 + b_{22}X_2^2 + b_{12}X_1X_2 \quad \text{Equation (8)}$$

where;
Y: is the response variable,
b_0: is interception coefficient,
b_1 and b_2: coefficients of the linear effects,
b_{11} and b_{22}: coefficients of quadratic effects,
b_{12}: coefficients of interaction effects for two variables and
X_1 and X_2: two different independent variables (X_1: glucose concentration, X_2: NaCl concentration)

The obtained experimental values were fitted by different polynomial equations which were provided in Design Expert software. Statistical analysis of data and plots were constructed using Design of Expert software version 7 (Stat Ease, Minneapolis, MN, USA), Sigmaplot 12.3 (Systat software, Inc.) and Statistica 10 (Statsoft, Inc). The analysis of variance (ANOVA) was employed to determine the significance of the model parameters in RSM. R^2 and adjusted R^2 values were calculated to evaluate the performance of the regression model.
The R^2 and adjusted R^2 were calculated using equation 9 and 10.

$$R^2 = \frac{\sum_{i=1-n}\left(X_i - y_{i,exp}\right)^2}{\sum_{i=1-n}\left(\bar{y}_i - y_{i,exp}\right)^2}$$

Equation (9)

where;
X: is predicted value of protein production,
$Y_{i,exp}$: is the observed value of protein production and
$\bar{y}_i$: is the average value of observed protein production.

The adjusted R^2 was calculated using equation 10,

$$Adjusted\ R^2 = 1 - [(1 - R^2) \times \frac{N-1}{N-K-1}]$$

Equation (10)

where;
N: is the total number of observations and
K: is the number of input variables.

Results and discussion

Molecular Identification

The axenicity of cultures was confirmed by PCR amplification of bacterial 16S rDNA (Fig. 1). On the other hand, the 18S rDNA sequences obtained for the strains demonstrated similar results, reflecting the high conservation of the molecular markers used as shown in Fig. 1. All PCR amplified products demonstrated a size between 1,600 and 1,750 bp. The phylogenetic tree obtained by Neighbor-Joining (NJ) determined the genetic distance and relationship among the studied strains and those already deposited in NCBI GenBank which all belonged to *Dunaliella* species. According to the genetic distances, strain D3 is far from the other two strains D1 and D2 which are more close to each other falling in the same subcluster (Fig. 2). Molecular identification of the microalgae strains revealed their relatedness to *Dunaliella salina.* The contaminations affect the growth parameters as well as interfere with protein content analysis of the microalgae cultures. Therefore, the axenity tests were performed as the prerequisite to validate the purity of the samples for further experiments. Phylogenetic analysis also confirmed the identification of species used in the study.

Growth Profile of D1, D2 and D3

All three strains exhibited classical sigmoidal growth patterns comprising an initial lag phase with slow growth, log phase with exponential growth, and final stationary phase (Fig. 3). The growth of all three strains increased and reached stationary phase on day 12. The growth rate of strain D_3 was demonstrated to be less than D_1 and D_2, respectively. In addition, the maximum cell density (N_{max}) of strains D_1 (2.27 x 10^6 cell/mL) and D_2 (1.67 x 10^6 cell/mL) was higher than D_3 (0.50 x 10^6 cell/mL) which showed a significantly lower growth rate at the end of day 14. The μ_{max} and t_d for D_2 is 2.1 and 1.3 fold higher than D_1 and D_3, respectively (Table 1, Supplementary Figure 1).

Optimization of Culture Conditions Using RSM

Table 3 provides ANOVA and statistical parameters for significance and intercorrelations among the factors for growth responses. In D_1, significant differences existed for both salt and glucose concentrations. In this case, A (glucose), B (salt) and B^2 are significant model terms. The model *F*-value of 37.23 implies that the model is significant with insignificant lack of fit. The predicted R^2 of 0.7889 is in reasonable agreement with the adjusted R^2 of 0.9379. The model also showed adequate precision by measurement of signal to noise ratio. The ratio greater than 4 is desirable. Thus, a ratio of 20.887 indicated an adequate signal [31].

RSM models sum of squares demonstrated that the quadratic type is the highest order polynomial regression that highly suitable to explain the relationship between the input variables and responses. The reduced polynomial equations for the biomass and protein production by strains D_1, D_2 and D_3 were constructed in terms of coded values and empirical equations after replacement of coded values with actual values are given in Table 2. Regression coefficients for the related equations were generated by nonlinear estimation and analysed in each culture as shown in equations 11 to 16.

Y_1: Biomass

D1: $Y_1 = 0.62-0.026(A)-0.11(B)-0.037(A)(B)-8.16E-03(A^2)-0.032(B^2)$ Equation (11)

D2: $Y_1 = 0.87-0.055(A)-0.25(B)-0.06(A)(B)-2.17E-03(A^2)+0.028(B^2)$ Equation (12)

D3: $Y_1 = 0.2-0.013(A)-6.58E-03(B)+2.92E-03(A)(B)+0.012(A^2)-0.011(B^2)$ Equation (13)

Y_2: Protein production

D1: $Y_2 = 0.89+0.15(A)+0.07(B)-0.2(A)(B)-0.092(A^2)-0.12(B^2)$ Equation (14)

D2: $Y_2 = 0.93+0.13(A)+0.12(B)-0.18(A)(B)+0.07(A^2)-0.1(B^2)$ Equation (15)

D3: $Y_2 = 0.58-0.17(A)+0.11(B)-0.017(A)(B)+0.074(A^2)-0.068(B^2)$ Equation (16)

where;
Y_1 and Y_2 are biomass and protein production, respectively. A and B are the coded values of the independent variables, glucose and NaCl, in the media, respectively. Statistical parameters of the models developed through RSM for *D.salina* strains have been provided in Supplementary Table 1.

In this study, CCD experiments revealed that high level of NaCl concentration (Run 8) has a negative effect on growth of *D. salina*, compared to the addition of a low NaCl concentration (Run 5) with glucose concentration in the middle point. As a comparison between runs 5 and 6, the response of specific growth rate was higher in run 5. The highest specific growth rate was observed in run 7 where the maximum specific growth rates (μ_{max}) were 0.49, 0.21 and 0.09 for strains D1, D2 and D3, respectively. The principal parameters such as glucose (X_1) and NaCl (X_2) enhanced biomass when their concentrations increased from low to high. It was also observed that variations in the NaCl concentrations caused a greater influence in biomass production as compared to glucose. The response surface curves (Figs. 4 and 5) showed the interactions of variables on microalgal growth during cultivation period of 14 days. Optimum results in biomass concentration were obtained when glucose was added to the central point with minimum NaCl concentration. In the optimization procedure, maximum predicted biomass was obtained when the concentrations of the factors were adjusted to 11.23, 2.76 and 0.01 g/L glucose and 0.55, 0.5 and 1.42 M NaCl, for D_1, D_2 and D_3, respectively (Table 4).

In strain D_3, maximum total protein content was observed in the media containing low–glucose concentration (5 g/L). While, in two other strains (D_1 and D_2) this was followed by two more peaks at 15 g/L in both 1 and 2 M NaCl treatments. The significant effect of glucose supplementation in different NaCl concentration on biomass and protein content is shown in Figures 4 and 5. Glucose supplementation increased the chlorophyll content, cell density and protein content in all strains. Comparison of maximum and minimum values in each strain revealed an increase of 2.61, 2.92 and 1.98 folds in optical density of strains D_1, D_2 and D_3, respectively. This increase in cell density was 9.3, 9.38 and 6.87 folds, respectively. For media without glucose addition, the protein content demonstrated 3.7, 3.94 and 1.01 folds increase in D_1, D_2 and D_3, respectively. When protein content was monitored among zero glucose treatments, a substantial variation was also observed at different NaCl concentrations. *D. salina* strains D_1, D_2 and D_3 showed 0.68, 1.68 and 1.84 folds increase ($P<0.01$), respectively, as NaCl concentration increased from 0.5 to 2.5 M. In the optimization procedure where the protein content is important in *D. salina* cultivation in media without glucose, NaCl concentration of 2.48, 2.22 and 2.02 M resulted in the best response for the strains D1, D2 and D3, respectively.

Composition of media for the cultivation of microalgae is a critical variable in defining cell density and growth rate. Organic carbon source supplementation supports rapid growth with high final cell concentration. Held (2011) [33] suggested that medium constituents play a more important role in providing energy rather than light. Carbon content showed to be a significant constituent of *D. salina* mixotrophic growth media. *D. salina* cultures grown in medium free from organic carbon source grew at considerably slower rates and much lower final densities. As a comparison, the cells E1 (D2-10-0.5), D12 (D2-15-1), E2 (D2-5-1), F7 (D2-0-2) and D5 (D2-0-1.5) can be visually distinguished among the others (Fig. 6).

This study produced results which corroborate the findings of a great deal of the previous work in this field. The findings of the current study are consistent with those of Wan *et al.* (2011) [11] who found that biomass and lipid production decreased at the highest glucose concentrations, but the content of protein and lipid were significantly augmented for mixotrophic conditions at least for some species. For the mixotrophic cultures in D1 and D2 at high glucose levels, much of the glucose was not consumed and remained in the medium. This is in agreement with the findings on the cultivation of microalgae *C. vulgaris* [34] and *C. sorokiniana* [11]. High glucose concentration in run 6 caused osmotic shock or osmotic stress. This might change the solute concentration around the microalgae cells, causing a rapid change in the movement of water across the cell membrane. In media containing high concentrations of any solutes (salts, substrates, etc.), water is drawn out of the cells through osmosis. In addition, such condition inhibits the transport of substrates and cofactors into the cell resulting in cell shocking. Cheirsilp and Torpee (2012) [35] reported that the chlorophyll content decreased with increasing initial glucose concentration for marine microalgae strains of *Nannochloropsis* and *Chlorella*. This is largely due to the increase of heterotrophic metabolism of glucose at higher concentrations of glucose.

On the economic side, carbon source supply exhibited to be one of the main input costs among all costs related to microalgae (*Spirulina*) cultivations [36], which holds true for *Dunaliella* cultures as well. Through optimization procedures in this study it was demonstrated that when there are limitations for the use of glucose (e.g. in economic side or contamination

considerations), the level of glucose as low as 11.55 g/L has equal influence to higher levels (17.74 g/L) in terms of desirability. Therefore, the growth of *D. salina* can be promoted with the lower level of glucose comparable with the higher concentrations resulting in a reasonable response for the further scale-up. In scaling up cultivations, production costs should be taken into account in economic side. However, the values of recombinant proteins will compensate these extra costs [8].

Protein is known as a critical factor in algal cell division and chlorophyll as the main pigment in photosynthetic system [2]. Quantitation of total protein has been used as the means to normalize cellular reactions for several decades; based on the premise that on average, each cell has the same amount of protein [32]. It has been demonstrated that the mixotrophic conditions (light and organic carbon supplementation) affect algal lipid, protein, carbohydrates and pigments biosynthesis differently. On the other hand, the level of protein content in microalgae (*C. vulgaris*) showed to be negatively correlated with carbohydrate and lipid content [34,37]. For example, increasing the initial glucose to 5 or 10 g/L improved the lipid yield in *C. sorokiniana* and *Chlorella*, respectively [11]. In the same study an increase of protein content in *D. salina* reported under mixotrophic condition with a maximum at 15 g/L of glucose, although it was minimal comparing the other studied microalgae species. This was confirmed in our study with a broader range of glucose combinations to various salt concentrations. In order to improve algal protein content through nutrient elements, nitrogen seems to be necessary for continuous protein biosynthesis which indirectly affects pigment formation as well. Our findings confirmed the former studies that supplementation of organic carbon source and energy (light and glucose) could convert the metabolic pathways in algal cells [34]. These results suggested that changes in the cellular biochemical composition were influenced by the trophic conditions and salt concentration in the medium.

In this study all experiments were done using microplate based system. Microplates not only offer the ability to measure many samples with very low volumes, but also the flexible array of wells density (6, 12, 24, 48, 96, 384, and 1536) which provide various options for the researcher to design the experiments based on the most appropriate sample number and reaction volume combination to fit their needs. In addition, automation can be easily performed for many repetitive and routine measurements since microplates have industry defined [32]**.** Therefore, utilizing the cost-effective microplate-based approach more samples can be analyzed and screened with less need to lab facilities and incubation room, saving costs particularly in case of application of expensive chemicals [38].

The standard protocols to estimate algal density include direct cell counting, measurement of chlorophyll content and absorbance correlations. In spectrophotometric methods, different reading wavelengths of 750_{nm} [25], 680_{nm} [39], 600_{nm} [32] and 540_{nm} [13] have been suggested to monitor algal growth. These values are correlated to the light absorbance of chlorophyll. A peaks could be observed (680_{nm}), representing the wavelength of maximum sensitivity to quantify *D. salina* samples. Therefore, all further analyzed samples were read in this wavelength. However, Held (2011) [33] used 600 nm as the wavelength of choice to monitor growth in order to avoid influence from absorbing material.

As the other important factors on growth of microalgae *D. salina* which should be taken care particularly for production of recombinant proteins include pH, temperature and light intensity. The optimum pH for growth of *D. salina* was reported to be 7.5-8.0 [10]. Microalgae are sensitive to temperature changes, thus maintaining constant temperature is important for stable long-term cultivation. Kim *et al.* (2012) [40] revealed that the optimum temperature conditions for growth of *Dunaliella* strains was 27°C. Light intensity is one of the most important factors in photoautotrophic conditions for photosynthesis in microalgae and it affects biomass productivity. The optimum light intensity for maximum productivity was 80 lEm^{-2} s^{-1} for *Dunaliella* strains. Light limitation will result in increasing pigment content of most species and shifts in fatty acid composition. In order to avoid photoinhibition, reduction in light intensity to some extent is preferable for many microalgae species. On the other hand, very low illumination might help green microalgae and cyanobacteria to survive in the vegetative state (not as cysts) for at least 6-12 months. However, the longer the stationary phase will result in longer lag phase when subculturing the cells in the fresh medium due to the shutdown of many biochemical pathways.

Conclusions

In summary, results from this study have demonstrated that *D. salina* can utilize glucose as the organic carbon substrate, and that the mixotrophic effects were in conjunction with that the mixture of NaCl and glucose for the production of biomass and protein. This alternative cultivation system could enhance the *D. salina* growth and biomass. It was observed that with an increase of glucose concentration from 0 to 15 g/L, biomass and protein content were increased. However, in the concentrations of glucose and NaCl higher than 15 g/L and 2.5 M, respectively, these responses were dropped dramatically. Based on the optimization procedure, protein production in *D. salina* was markedly enhanced by glucose supplementation as low as 5 g/L. The optimal response obtained through nonlinear estimation was for 288, 288 and 312 h, and a doubling time of 1.13, 3.03 and 5.17 days for D_1, D_2 and D_3 strains, respectively. The runs with unsupplemented glucose presented the lowest doubling times. In terms of using this technique for recombinant protein production, glucose could be utilized to enhance both the growth rate and protein content. On the other hand, reducing the growth cycle expedites the analysis of positive transformants. Therefore, since the production level of recombinant proteins in an expression host is related to the total protein content, the authors conceptualized it as an application of the study to enhance the recombinant protein in response to cultivation parameters in the genetically modified microalgae. To be cost-effective, many samples could be screened using microplate based system for various applications such as optimization of the cultivation condition and downstream bioprocesses as well as screening of the transformants in molecular farming and recombinant protein production in microalgae. Also, the use of this technique may help in enhancement of biomass and protein content of microalgae to be used in human and animal food and feed.

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Figures

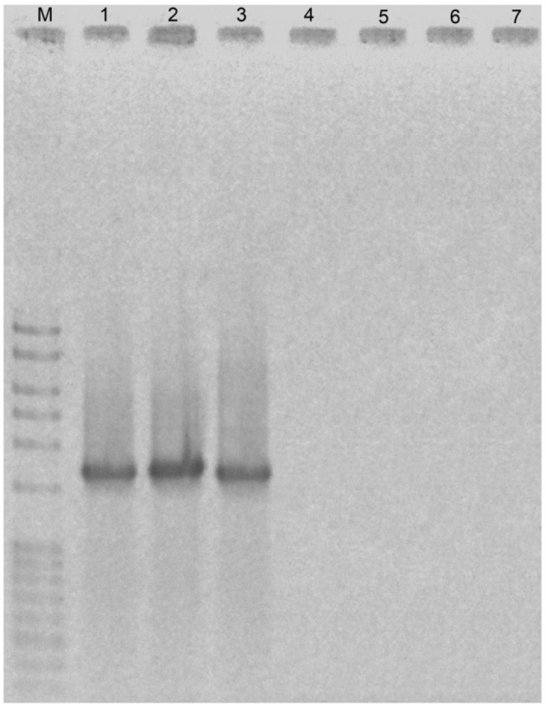

Figure 1. **Molecular identification (Lanes 1-4) and axenicity assessment (Lanes 5-7) of *Dunaliella* strains used in the study by 18S and 16S rDNA PCR amplifications, respectively.** Lane M: molecular size marker (100 bp DNA Ladder PLUS, 100–5000 bp; OZ Biosciences, France), Lane 1 and 5: D1, Lane 2 and 6: D2, Lane 3 and 7: D3, Lane 4: negative control (without DNA).

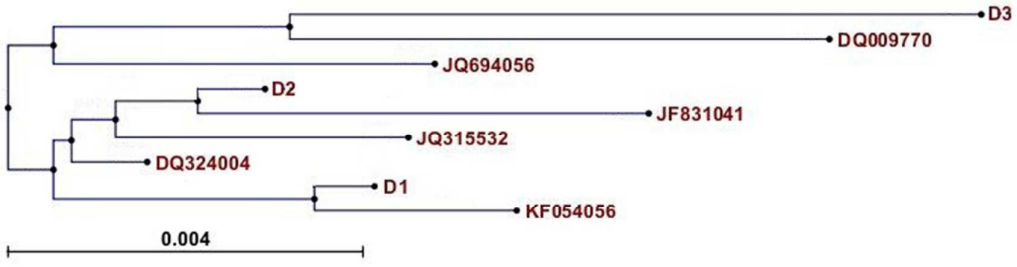

Figure 2. **Neighbor-Joining phylogenetic tree showing the relationships among the studied *Dunaliella* strains performed from the alignment of 18s amplicons and those reference sequences deposited to NCBI GenBank.** The phylogenetic tree was constructed using the neighbour-joining method (CLC Main Workbench 6). The scale bar represents the number of substitutions per nucleotide position, showing high similarity between the *Dunaliella* samples and GenBank sequences

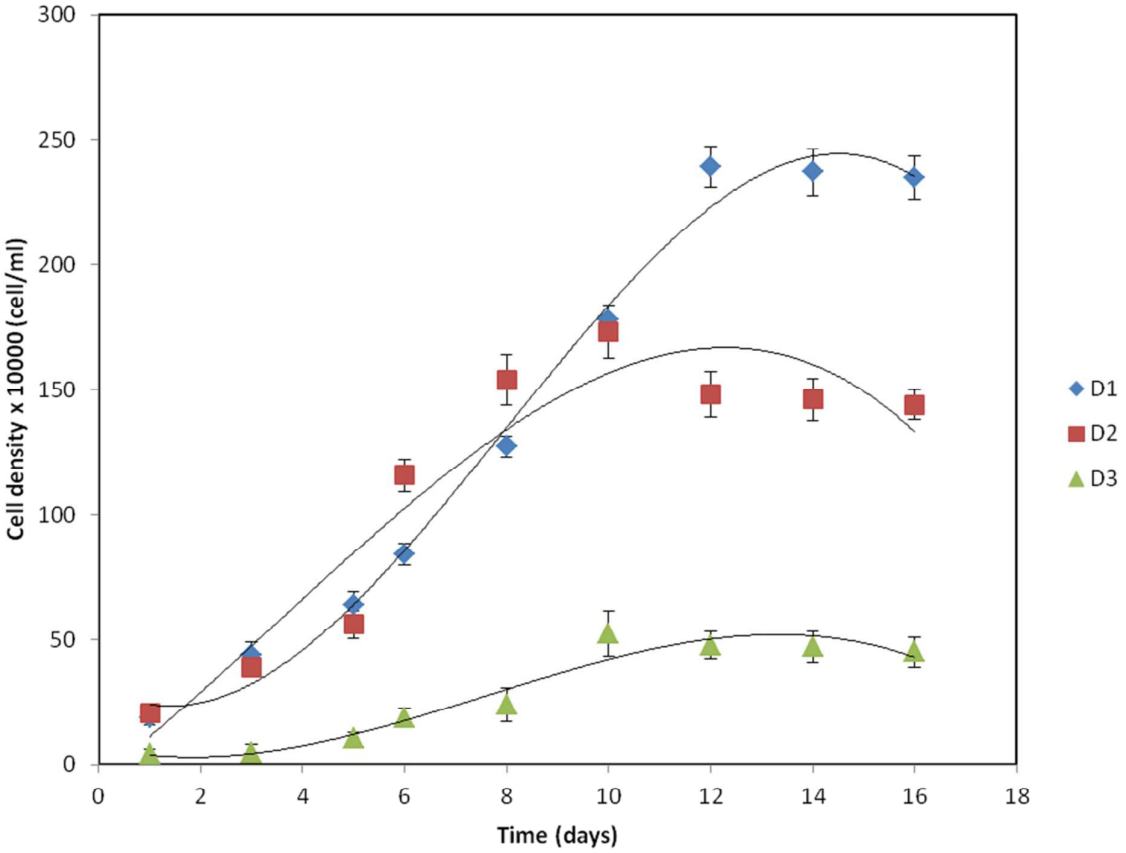

Figure 3. **Growth curves for three strains of *D. salina* based on cell density in M24.** The simulated and experimental data represented by solid lines and symbols, respectively

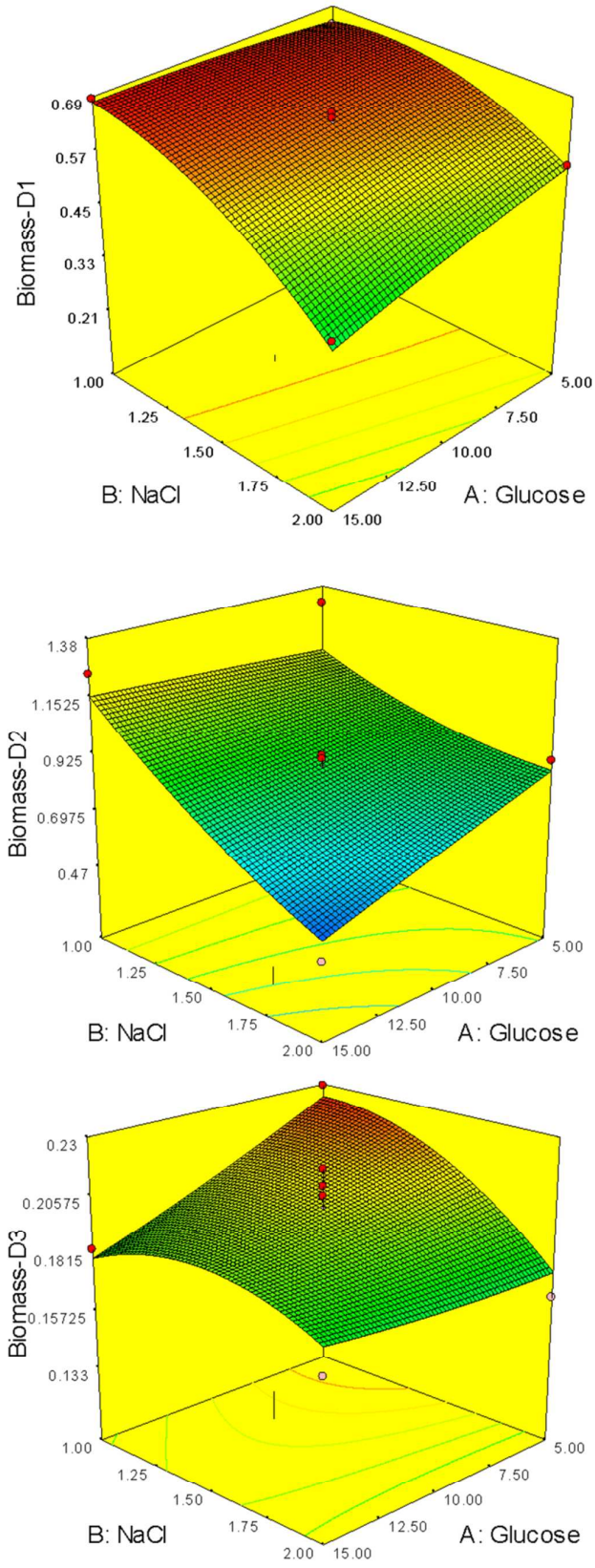

Figure 4. **Three dimension response surface and contour line plots for the impact of mixotrophic condition on *D. salina* cultures biomass (cell concentration).** NaCl: M, Glucose: g/L

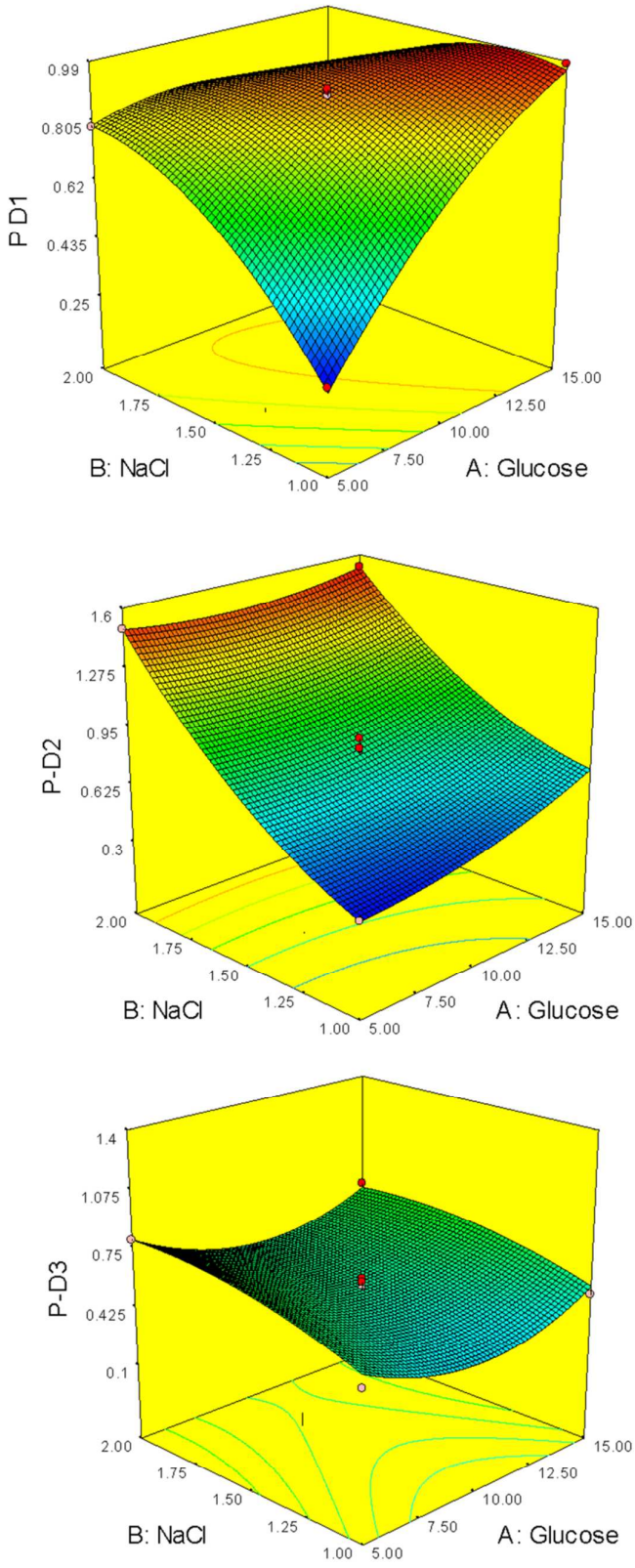

Figure 5. **The impact of glucose and NaCl on the maximum protein production by three *D. salina* strains showed as 3D response surface and contour line plots.** Protein (P): g/100g, NaCl: M, Glucose: g/L

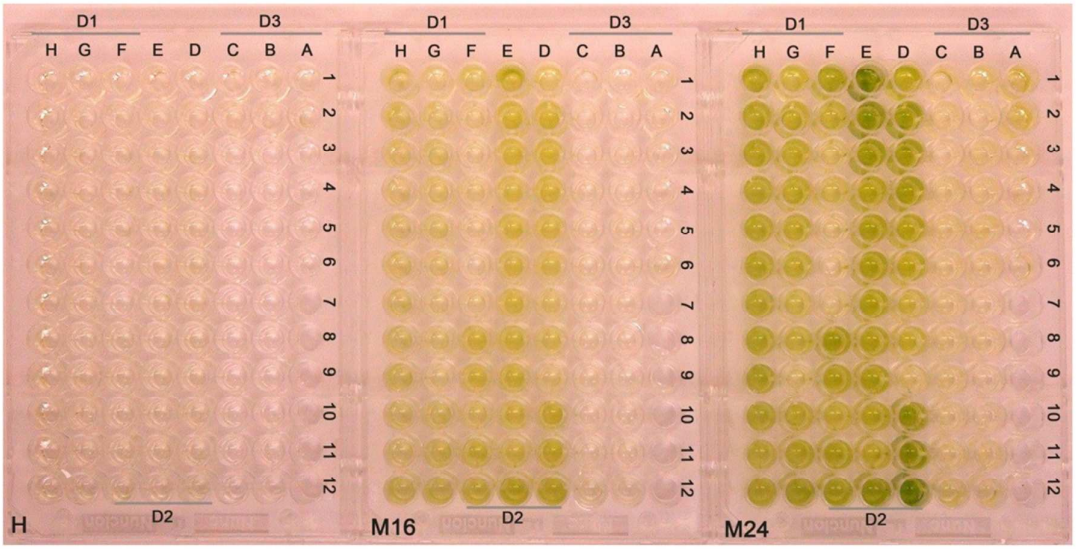

Figure 6. **A comparison between three mixotrophic (M24 and M16) and heterotrophic (H) conditions on the day eigth of cultivation for green algae *D. salina*.** The replicates were considered to be five for center points and three for the other points

Tables

Table 1. Growth performance for three strains of *D. salina* in M24.

Strain	μ_{max}	Div_{max} /day	Min t_d (h)	N_{max} (10^6 cell/mL)	D_{max}
D1	0.25	0.37	65.24	2.27	12
D2	0.52	0.75	32.11	1.67	12
D3	0.4	0.58	41.09	0.50	13

μ_{max}= Maximum growth rate, N_{max}= Maximum cell density, D_{max}= The day with maximum N_{max}, Min t_d=Minimum generation time. μ_{max}, N_{max}, D_{max} and Min t_d was calculated with the average values.

Table 2. Growth parameters calculated for each treatment in three strains of *D. salina* in M24.

Run	Glucose concentration (X_1)(g/L)	NaCl concentration (X_2)(M)	Growth rate		Protein production (g/100g)		Glucose consumption (g/L)
			Observed	Predicted	Observed	Predicted	
D1							
1	-1(5)	-1(1)	0.54 ± 0.04	0.54	0.27 ± 0.08	0.27	0.4 ± 0.07
2	1(15)	-1(1)	0.22 ± 0.07	0.23	ND*	ND*	2.4 ± 0.12
3	-1(5)	1(2)	0.64 ± 0.07	0.65	0.79 ± 0.08	0.79	1.52 ± 0.09
4	1(15)	1(2)	0.43 ± 0.09	0.41	0.71 ± 0.03	0.71	1.56 ± 0.08
5	-2(0)	0(1.5)	0.66 ± 0.05	0.68	ND*	ND*	0 ± 0.00
6	2(20)	0(1.5)	0.62 ± 0.07	0.61	0.83 ± 0.09	0.82	1.66 ± 0.08
7	0(10)	-2(0.5)	0.63 ± 0.07	0.66	0.27 ± 0.07	0.27	1.39 ± 0.15
8	0(10)	2(2.5)	0.60 ± 0.07	0.55	0.56 ± 0.05	0.56	1.89 ± 0.16
9	0(10)	0(1.5)	0.66 ± 0.08	0.63	0.89 ± 0.02	0.89	1.50 ± 0.10
10	0(10)	0(1.5)	0.54 ± 0.06	0.63	0.85 ± 0.09	0.89	1.55 ± 0.05
11	0(10)	0(1.5)	0.61 ± 0.03	0.63	0.89 ± 0.02	0.89	1.53 ± 0.04
12	0(10)	0(1.5)	0.65 ± 0.05	0.63	0.90 ± 0.07	0.89	1.52 ± 0.09
13	0(10)	0(1.5)	0.69 ± 0.03	0.63	0.91 ± 0.07	0.89	1.54 ± 0.08
D2							
1	-1(5)	-1(1)	1.31 ± 0.09	1.14	0.39 ± 0.01	0.39	1.00 ± 0.15
2	1(15)	-1(1)	1.24 ± 0.08	1.15	ND*	ND*	4.17 ± 0.29
3	-1(5)	1(2)	0.78 ± 0.04	0.76	1.49 ± 0.08	1.49	1.76 ± 0.09
4	1(15)	1(2)	0.47 ± 0.09	0.53	1.53 ± 0.08	1.53	1.97 ± 0.15
5	-2(0)	0(1.5)	0.91 ± 0.07	0.97	0.90 ± 0.09	0.9	0 ± 0.00
6	2(20)	0(1.5)	0.76 ± 0.06	0.75	1.26 ± 0.09	1.26	3.96 ± 0.40
7	0(10)	-2(0.5)	1.38 ± 0.09	1.48	0.43 ± 0.07	0.42	4.54 ± 0.32
8	0(10)	2(2.5)	0.53 ± 0.04	0.48	ND*	ND*	3.61 ± 0.21
9	0(10)	0(1.5)	0.92 ± 0.03	0.87	0.89 ± 0.07	0.83	2.67 ± 0.10
10	0(10)	0(1.5)	0.72 ± 0.04	0.87	0.82 ± 0.09	0.83	2.35 ± 0.09
11	0(10)	0(1.5)	0.86 ± 0.06	0.87	0.78 ± 0.03	0.83	2.54 ± 0.10
12	0(10)	0(1.5)	0.83 ± 0.08	0.87	0.83 ± 0.06	0.83	2.61 ± 0.08
13	0(10)	0(1.5)	0.91 ± 0.07	0.87	0.83 ± 0.03	0.83	3.00 ± 0.10
D3							
1	-1(5)	-1(1)	0.23 ± 0.06	0.22	0.49 ± 0.04	0.56	4.14 ± 0.55
2	1(15)	-1(1)	0.18 ± 0.07	0.19	0.50 ± 0.09	0.55	11.6 ± 0.87
3	-1(5)	1(2)	0.20 ± 0.01	0.2	0.80 ± 0.08	0.81	3.73 ± 0.27
4	1(15)	1(2)	0.17 ± 0.03	0.18	0.75 ± 0.06	0.73	10.92 ± 0.97
5	-2(0)	0(1.5)	0.27 ± 0.08	0.27	1.36 ± 0.08	1.33	0 ± 0.00
6	2(20)	0(1.5)	0.23 ± 0.03	0.22	0.10 ± 0.02	0.05	8.48 ± 0.73
7	0(10)	-2(0.5)	0.17 ± 0.06	0.17	0.46 ± 0.04	0.48	6.6 ± 0.71
8	0(10)	2(2.5)	0.15 ± 0.05	0.14	0.53 ± 0.02	0.56	6.62 ± 0.58
9	0(10)	0(1.5)	0.18 ± 0.08	0.2	0.51 ± 0.09	0.54	7.3 ± 0.63
10	0(10)	0(1.5)	0.22 ± 0.06	0.2	0.59 ± 0.06	0.54	6.33 ± 0.66
11	0(10)	0(1.5)	0.21 ± 0.03	0.2	0.57 ± 0.09	0.54	7.17 ± 0.70
12	0(10)	0(1.5)	0.20 ± 0.03	0.2	0.57 ± 0.04	0.54	5.67 ± 0.71
13	0(10)	0(1.5)	0.21 ± 0.04	0.2	0.49 ± 0.09	0.54	4.14 ± 0.69

ND*: Not detected

±: Standard deviation of triplicate data

Table 3. Analysis of variance (ANOVA) and coefficient estimate (CE) by regression model for the optimization of biomass by three strains of *D. salina* (D1, D2 and D3) after 12 days of cultivation.

Source	Coefficient	Standard	Sum of	Df	Mean	*F* value	Prob>*F*
D1	**Estimate**	**Error**	**Square**		**Square**		
Model			0.2	5	0.039	37.23	0.0001
Intercept	0.62	0.013					
A-Glucose	-0.026	9.35E-03	7.91E-03	1	7.91E-03	7.54	0.0286
B-NaCl	-0.11	9.35E-03	0.16	1	0.16	151.01	0.0001
AB	-0.037	0.016	5.53E-03	1	5.53E-03	5.27	0.0553
A^2	-8.16E-03	6.76E-03	1.52E-03	1	1.52E-03	1.45	0.267
B^2	-0.032	6.76E-03	0.023	1	0.023	22.32	0.0021
Residual			7.34E-03	7	1.05E-03		
Cor Total			0.2	12			
D2							
Model	0.87	0.046	0.82	5	0.16	13.23	0.0019
A-Glucose	-0.055	0.032	0.036	1	0.036	2.89	0.133
B-NaCl	-0.25	0.032	0.75	1	0.75	60.46	0.0001
AB	-0.06	0.056	0.015	1	0.015	1.18	0.3138
A^2	-2.17E-03	0.023	1.08E-04	1	1.08E-04	8.72E-03	0.9282
B^2	0.028	0.023	0.017	1	0.017	1.41	0.2732
Residual			0.087	7	0.012		
Cor Total			0.9	12			
D3							
Model	0.2	6.24E-03	0.01	5	2.07E-03	9.17	0.0056
A-Glucose	-0.013	4.33E-03	1.88E-03	1	1.88E-03	8.35	0.0233
B-NaCl	-6.58E-03	4.33E-03	5.20E-04	1	5.20E-04	2.31	0.1726
AB	2.92E-03	7.51E-03	3.40E-05	1	3.40E-05	0.15	0.7092
A^2	0.012	3.14E-03	3.13E-03	1	3.13E-03	13.89	0.0074
B^2	-0.011	3.14E-03	2.53E-03	1	2.53E-03	11.24	0.0122
Residual			1.58E-03	7	2.25E-04		
Cor Total			0.012	12			

Table 4. Predicted optimal levels for cell concentration and protein content by RSM predicted response on these levels.

Cell Concentration			
Strain	**Glucose (g/L)**	**NaCl (M)**	**Cell Conc. (x 10^6/mL)**
D_1	11.23	0.55	0.731
D_2	2.76	0.5	1.380
D_3	0.01	1.42	0.273
Protein Content			
Strain	**Glucose (g/L)**	**NaCl (M)**	**Protein (g/100g)**
D_1	14.93	1.33	0.982
D_2	20.00	0.96	1.607
D_3	0.00	2.02	1.291