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Use of response surface methodology to optimise carotenogenesis in the microalga, *Haematococcus pluvialis*

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Abstract

The factors controlling biomass production and the synthesis of astaxanthin esters in the microalga *Haematococcus pluvialis* (CCAP 34/7) have been investigated using a statistical approach employing response surface methodology (RSM). The culture conditions required for optimal growth and carotenogenesis in this alga are very different. Of particular importance is the photon flux density: for growth the optimum is 50–60 μmol m^{-2} s^{-1}, whereas the optimum for astaxanthin synthesis is much higher at $\sim$ 1600 μmol m^{-2} s^{-1}. The addition of low levels of NaCl to the medium also stimulates to a small extent synthesis of astaxanthin, but photon flux density remains the overriding factor. The optimal temperature for this strain is quite low at 14–15 °C. RSM has been shown to be a rapid and effective technique leading to the optimisation of algal culture conditions. This statistical approach can be applied readily to the majority of microalgae and their products.

Introduction

A number of microalgae have the ability to accumulate large amounts of commercially valuable biochemicals (Borowitzka & Borowitzka, 1988; Borowitzka, 1995). Perhaps the best known example is the production of β-carotene by the halophilic alga *Dunaliella salina*. Other algae (e.g. *Chlamydomonas nivalis*, *Haematococcus* spp.) have been reported to accumulate the carotenoid astaxanthin, when cultured under specific, generally growth-limiting, conditions (Goodwin, 1980). Whilst the commercial production of *Dunaliella* is now well established in many countries (Borowitzka & Borowitzka, 1988), the large scale synthesis of astaxanthin by microalgae is hampered by problems associated with process design and scale-up. As carotenoid synthesis in algae such as *Haematococcus* is reported to be greatly stimulated under growth-limiting conditions (Borowitzka *et al.*, 1991; Boussiba & Vonshak, 1991; Droop, 1955; Goodwin & Jamikorn, 1954; Kobayashi *et al.*, 1992; Spencer, 1989), any commercial process for their production must address the, not insubstantial, problem of combining biomass production and product formation.

Major improvements in the productivity of many microbial fermentation processes are generally ascribed to the development of superior strains via genetic manipulation. As yet such techniques have been limited to only a few cases in the development of large scale algal fermentation processes. There are, however, other parameters such as the nutritional and physical environment to which an organism is exposed and which are known to alter growth and product yield significantly. However, media commonly used to screen for industrially important microbial metabolites frequently do not become part of the process definition. Undesirable characteristics of these media include economically unattractive nutrients, support of sub-optimal productivity and support of the synthesis of closely related product components. Optimum values of temperature and pH are also important factors in the optimisation of the fermentation process. However, consideration should be given to interactions between the parameters and to the effects of increased scale on so-called optimum conditions (Winkler, 1987). The

resulting medium should support not only good algal growth but also maximise product yield, reduce the synthesis of compounds closely related to the product, and enhance product recovery.

Once a suitable strain for a particular microbial product has been selected, a medium for optimal productivity can be devised. The process may then be categorised, for example, as growth-linked or growth-dissociated (Gaden, 1959), as subject to repression by a specific nutrient assimilation rate or as a secondary metabolite (Bushell, 1989). Preliminary formulation work using an empirical approach can be enhanced considerably by the use of factorial and other numerical experimental designs (Greasham & Inamine, 1986). The first step in the use of statistical designs is to identify the most important nutritional and physical parameters independently, and determine the effect of these on growth and/or product yield. The problem of being confronted with many variables which may be of possible importance, and the pressure to select and optimise the most important variables as efficiently as possible, led to the widespread adoption of the procedure developed by Plackett and Burman (1946).

The next step in the optimisation procedure is the determination of the optimum level of each of the previously identified independent variables. Response surface methodology (RSM) is one such technique that can be applied. RSM is a heuristic approach and does not guarantee the achievement of the unique optimum. Optimization needs to take account of non-linear responses, and for this purpose a full quadratic model is required. Each variable is tested at a minimum of three values, the upper and lower values being spaced equally from a centre point value. Once again the experimental design is a fraction only of the full factorial, but the Plackett-Burman design cannot be used because it does not evaluate non-linear responses where they occur. Instead, designs such as the Box-Wilson (1951) which accommodates two-way and quadratic effects are used. Trials are made in random sequence and the coefficients calculated. The output from these computations is depicted as contours of responses plotted against two independent variables. Several contour plots are required to accommodate three or more variables. Whilst these plots (the response surfaces) may directly reveal the optimum condition, or suggest the region where the optimum is to be found, it is also possible that they do not indicate the unique optimum but rather a saddle point or ridge. Finally, confirmation experiments are carried out once the value of each variable for optimum response has been estimated. Whilst RSM has been successfully applied to microbial systems, there are few, if any, reports of its use for microalgal culture.

This paper reports on the use of RSM techniques to optimise the production of astaxanthin in *H. pluvialis*. RSM techniques were applied to astaxanthin production with the identification of three key independent variables with the aim of optimising the nutritional and physical parameters to support maximum biomass production in *H. pluvialis*.

Materials and methods

Optimum nitrogen source for the growth of H. pluvialis

H. pluvialis 34/7 was obtained from the Culture Collection of Algae and Protozoa (U.K.) and was cultured in 250-mL Erlenmeyer flasks under constant illumination (supplied by a combination of fluorescent and water-cooled tungsten-halogen lights) at 19 °C in BBM (Nichols & Bold, 1964) modified to pH 7.0. A range of nitrogen sources were also tested in the medium, namely sodium nitrate, sodium nitrite, ammonium chloride, urea and potassium nitrate at a concentration of 1.2 mM. The dry cell weight of the cultures was determined after 3 weeks cultivation by removal of 5-ml aliquots, washing in distilled water and drying at 100 °C to constant weight (24 h).

Optimisation of biomass production

Two sets of biomass optimisation experiments were carried out to determine the optimum conditions. In the first biomass optimisation experiment green motile cells of *H. pluvialis* were cultured in 250-mL Erlenmeyer flasks in modified BBM under continuous light. The three key independent variables identified for growth were (i) nitrogen concentration, (ii) photon flux density (PFD) and (iii) temperature. Algal cultures were grown at three different levels of each parameter and in all combinations: nitrogen (0.15, 1.47 and 2.79 mM; PFDs (10, 40 and 70 μmol $m^{-2}s^{-1}$); temperature (15, 22 and 29 °C). The response curves obtained from these experiments were then used to modify these values for a second (confirmation) set of optimisation experiments: nitrogen (0.37, 1.47 and 2.57 mM); PFDs (10, 50 and 90 μmol m^{-2} s^{-1}); temperature (11, 15 and 19 °C).

Optimisation of astaxanthin production

The three key independent variables studied for astaxanthin production were: (i) salt concentration; (ii) temperature; and (iii) PFD. Low media nitrogen levels are known to be very important in stimulating carotenoid synthesis in many algae and the majority of studies are in agreement that the optimum nitrogen concentrations for astaxanthin synthesis are close to zero (Borowitzka *et al.*, 1991; Goodwin 1980). A statistical approach such as RSM is however not suited to the identification of an optimum which may be at zero. The algal cultures were initially exposed to three different levels of each parameter and in all combinations. However, because of the observed high optimum for the PFD used in these experiments it was not possible to maintain temperatures of the culture lower than 24 °C for an extended period. The salt concentrations used were initially 100, 170 and 240 mM NaCl but later optimised to 10, 50 and 90 mM NaCl and the PFDs investigated were 30, 630 and 1230 μmol m^{-2} s^{-1}.

The astaxanthin content of a fixed volume of the cultures was determined after 3 weeks cultivation by reversed-phase HPLC analysis (Tsavalos *et al.*, 1992; Harker & Young, 1995) using an absorption coefficient of $A^{1\%}_{1cm} = 2100$ (in hexane). Carotenoid standards were kind gifts of *F. Hoffmann* La Roche (Basel) Ltd.

Statistical design and analysis

Statistical design and analysis was achieved using three-level factorial experiments which constituted the basis of the statistical designs used. Computation was carried out using a Ness-286 computer using the University of Nebraska Response Surface Methodology program for regression analysis. The program used for response surface plotting was Systat 5.1 on a Macintosh Centris 650.

Results

Optimum nitrogen source for growth of H. pluvialis

The dry cell weights of the algal cultures cultivated in the various nitrogen sources for 3 weeks are given in Table 1. This indicates that urea and sodium nitrate were the best nitrogen sources for supporting maximum algal growth (in subsequent optimisation experiments, urea was used). The growth of the alga in ammonium chloride was significantly lower in comparison to the other nitrogen sources and was probably the result of large pH fluctuations observed in these cultures during cultivation (data not shown).

Table 1. Yield of cells of *H. pluvialis* cultured in a range of nitrogen sources (n=3; standard error <3.9%)

Nitrogen source	Mean dry cell weight ($g\,l^{-1}$)
Ammonium chloride	0.54
Potassium nitrate	1.10
Sodium nitrate	1.12
Sodium nitrite	1.02
Urea	1.22

Table 2. Optimum levels of key independent variables for (A) growth and (B) astaxanthin synthesis in *H. pluvialis* as determined by RSM.

Key independent variable	Optimum level of key variable as determined by RSM
A. Growth/Biomass production	
Temperature	14–15 °C
Photon flux density	50–60 μmol $m^{-2}s^{-1}$
Urea concentration	2.5–3.0 mM
B. Astaxanthin synthesis	
Temperature	14–15 °C
Photon flux density	1600–1700 μmol $m^{-2}s^{-1}$
NaCl concentration	25–30 mM

Optimisation of biomass production

The dry cell weights of the algal cultures cultivated under the range of physical and nutritional conditions after three weeks were used to calculate the RSM regression equations (equations 1 and 2) and coefficients (data not shown). Using the values obtained from the regression equations and coefficients, contour maps showing the response curves were then plotted (Figs 1, 2). These can be used to determine the optimum conditions when the values formulated by the RSM regression equations and coefficients result in a contour map in which the lines orbit a central point.

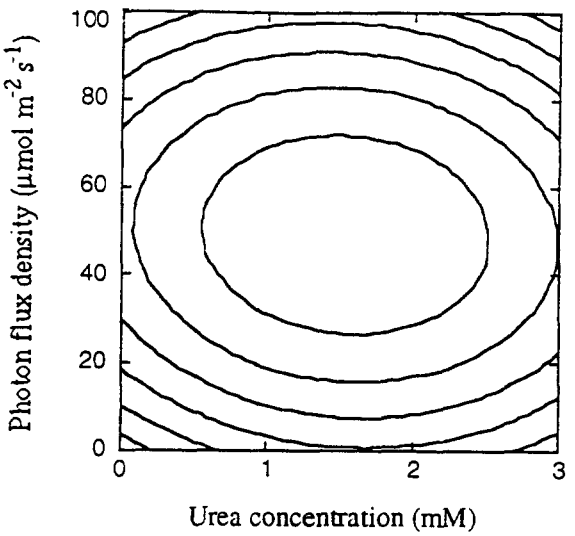

Fig. 1. Contour map showing response curves used to determine the optimum nitrogen (urea) concentration and photon flux density for algal biomass production.

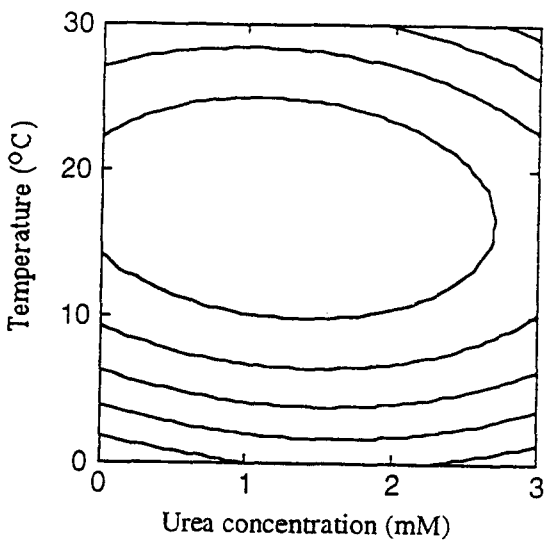

Fig. 2. Contour map showing response curves used to determine the optimum nitrogen (urea) concentration and temperature for algal biomass production.

The two co-ordinates of this central point define the optimum conditions of the dependent variable for that contour map. If no central point is observable on the contour map then the identification of the optimum conditions is not possible.

The contour maps shown in Figs 1 and 2 indicate that the urea concentration corresponding to maximum algal biomass production was approximately 1.5 mM. The PFD corresponding to the maximum level of algal biomass production was approximately 50 μmol m^{-2} s^{-1} and the optimum temperature required for the maximum production of algal biomass was 14–15 °C. Since the optimum temperature for the maximum production of algal biomass was not clearly defined in these initial experiments, a second series of experiments was performed, using more carefully selected parameters for each variable (the results of the first experiments were used to determine the levels of parameters to be tested). The dry weights of the algal cultures following three weeks growth were again used to calculate the RSM regression equations (equations 3 and 4) and coefficients (data not shown) and the contour maps were plotted (Figs 3, 4). These indicate that the PFD corresponding to the maximum rate of algal biomass production was close to that determined in Fig. 1 at 60 μmol m^{-2} s^{-1}. The optimum temperature to achieve maximum algal growth was clearly identified at 14 °C, whilst, in this case, the optimum urea concentration for algal growth was determined to be higher at 3.0 mM.

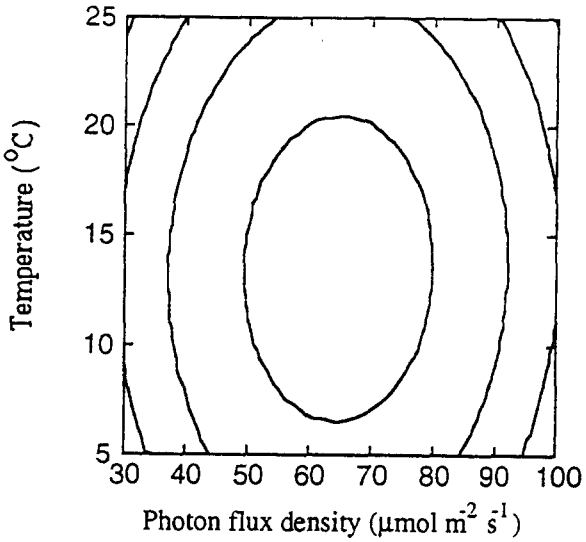

Fig. 3. Contour map showing response curves used to determine the optimum photon flux density and temperature for algal biomass production.

Optimisation of astaxanthin production

The astaxanthin content of algal cultures cultivated under the various salt concentrations and PFDs after seven days was determined and used plot the contour maps shown in Figs 5 and 6 (from RSM regression equations 5 and 6 and coefficients – data not shown). Figure 6 indicates that the optimum PFD for algal growth lies between 1600–1700 μmol m^{-2} s^{-1} and

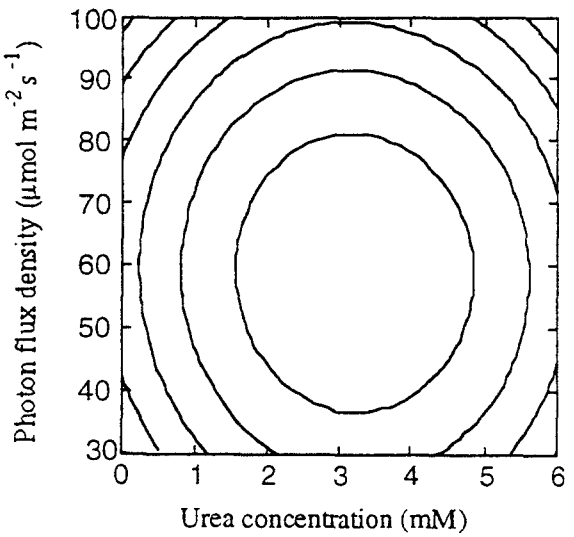

Fig. 4. Contour map showing response curves used to determine the optimum concentration of nitrogen (urea) and the photon flux density for algal biomass production.

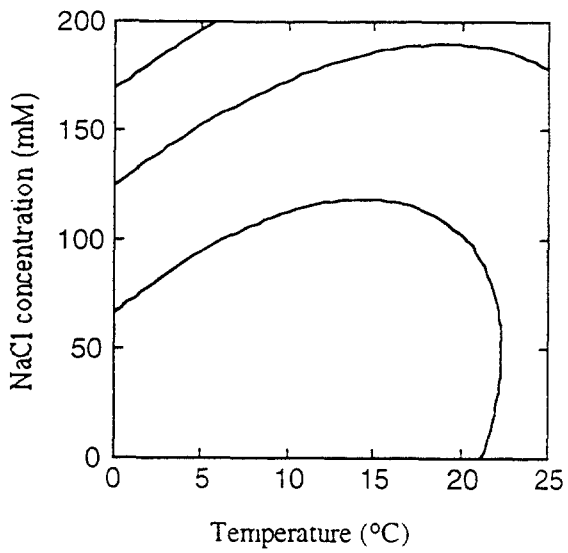

Fig. 5. Contour map showing response curves used to determine the optimum temperature and NaCl concentration for astaxanthin production from *H. pluvialis*.

the optimum salt concentration for algal astaxanthin production is in the range 25–30 mM NaCl.

Discussion

There have been few studies of the nutrient and culture condition requirements of *H. pluvialis*, with particular emphasis on those requirements which result in the optimal production of astaxanthin. The few

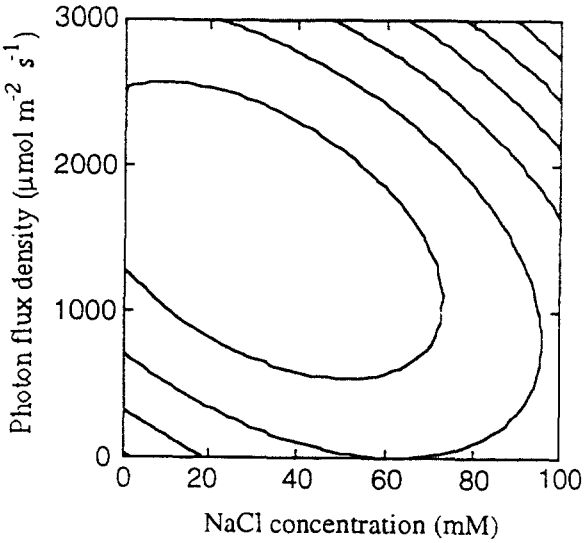

Fig. 6. Contour map showing response curves used to determine the optimum photon flux density and NaCl concentration for astaxanthin production from *H. pluvialis*.

previous investigations into the growth requirements for *H. pluvialis* have yielded complex relationships between nutrient levels, growth rate, cell yield, cell type and product (astaxanthin) formation.

In the present study the best nitrogen source for biomass production was identified as urea, agreeing with a previous report (Borowitzka *et al.*, 1991) for *Haematococcus* spp. The use of an ammonium salt as a nitrogen source in the present study resulted in particularly poor levels of biomass. Previous investigations with *H. pluvialis* have also shown that nitrate nitrogen is preferred to ammonium nitrogen (Proctor, 1957), although Stross (1963) noted that exponentially growing cells at acid pH preferred ammonium nitrogen. *H. pluvialis* has been reported to differ from most other microalgae in preferring nitrate nitrogen sources to ammonium nitrogen, at least under laboratory culture conditions (Syrett, 1962). Cultivation of the alga in fast-growing, high cell density cultures in ammonium nitrogen may lead to high cell mortality rates because of the rapid acidification of the medium due to ammonium uptake and metabolism (Borowitzka & Borowitzka, 1988).

The key independent variables tested in the biomass and astaxanthin production optimisation experiments, all proved to be important factors in determining the overall levels of cell growth/product yield. The data obtained in the initial experiments to optimise growth conditions indicated the range of parameter values where the optimum could be found. Just as important,

the initial optimisation experiments also indicated the range of parameter values where the optimum could not be identified. A second series of optimisation studies allowed a narrower range to be used and a more accurate determination of conditions to be made. This trial and error approach is a significant component in the optimisation procedure.

The biomass optimisation experiments identified the optimum levels of all the key independent variables tested. Table 2A shows the optimum levels identified to achieve maximum biomass production in *H. pluvialis*. The temperature required for optimum biomass production was between 14–15 °C, in agreement with the findings of Borowitzka & Borowitzka, (1988). The optimum concentration of urea required for algal growth was 2.5–3.0 mM and the optimum PFD for biomass production was in the range 50–60 μmol m^{-2} s^{-1}.

The astaxanthin production optimisation experiments identified the optimum levels of two of the three key independent variables investigated (Table 2B). The optimum NaCl concentration required for astaxanthin production was low at 25–30 mM. The optimum PFD identified for astaxanthin production was 1600–1700 μmol m^{-2} s^{-1}. This is much greater than any value in the literature for the induction of astaxanthin formation in *H. pluvialis*. Although this value lies outside the tested range, it would be difficult to optimise this further due to the great difficulties encountered when working with such high PFDs. Unfortunately, the optimum temperature for astaxanthin production could not be identified due to practical considerations as the light source used radiated large amounts of heat. The amount of heat produced made it impossible to cultivate the cultures below 24 °C. Even had 24 °C been used as a minimum point to investigate the optimum temperature, this value would have been much greater than the optimal temperature required for astaxanthin production. Therefore, the optimum temperature could not be identified under these conditions. Because of the temperature problems of using high light sources, the best light source for commercial astaxanthin production is natural sunlight.

Overall the use of RSM to optimise biomass production and ultimately astaxanthin production in *H. pluvialis* was very effective. It should, however, be noted that this data is pertinent to only this particular strain of the alga and temperature and other optima may be somewhat different for other strains. The optimisation experiments were designed around a two stage production process using *H. pluvialis* to produce astaxanthin: the first stage involved the bulking up of algal biomass, and the second involved the induction of astaxanthin synthesis.

The use of RSM involved carrying out a relatively low number of trials. If an empirical approach had been employed to optimise the key independent variables in the optimisation experiments, it would have been necessary to vary each nutrient and physical parameter independently and determine the effect of this on algal growth/product yield (evaluating nutrients at four concentrations, for example, would require 256 separate trials). RSM avoids the need to carry out this number of trials, but is nonetheless a potent technique in optimising algal fermentation processes.

RSM allows the visual representation of response surfaces as a direct means of locating maximum points, and with the availability of suitable computer programs, the mathematical manipulations are reduced to routine procedures. This leaves the way open for RSM to be applied to any algal fermentation process to optimise key parameters and achieve maximum algal growth/product yield. The next stage would be to continue the optimisation process during the scale-up of the fermentation process. This can be achieved using the simplex optimisation method. The simplex method (Spendley *et al.*, 1962) for two-parameter optimisation would involve three initial trials which may be represented by the vertices of a triangle in a plot with concentrations of the parameters as axes. Having determined the algal growth/product yield, the trial with the lowest growth/yield is identified and a further triangle, a reflection of the first, is constructed opposite the lowest growth/yield trial. The process is continued until the optimum point is located at which point the 'simplex' circles around it. This enables a fully optimised large scale algal fermentation process to be developed.

Acknowledgments

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Regression equations:

Regression equation 1 (see Fig. 1):

[**Predicted Biomass** = 0.144164 + 0.2940778 * urea concentration + 1.698319 $\times 10^{-2}$ * PFD + -5.050581 $\times 10^{-4}$ * urea concentration * PFD + -8.800137 $\times 10^{-2}$ * urea concentration^2 + -1.64815 $\times 10^{-4}$ * PFD^2]

Regression equation 2 (see Fig. 2):

[**Predicted Biomass** = 0.1000167 + 0.1400093 * urea concentration + 5.810618 $\times 10^{-2}$ * temperature + -1.955085 $\times 10^{-3}$ * urea concentration * temperature + -4.290296 $\times 10^{-4}$ * urea concentration^2 + -1.601872 $\times 10^{-3}$ * temperature^2]

Regression equation 3 (see Fig. 3):

[**Predicted Biomass** = 9.143954 $\times 10^{-2}$ + 2.460869 $\times 10^{-2}$ * PFD + 2.365008 $\times 10^{-2}$ * temperature + 2.226146 $\times 10^{-5}$ * PFD * temperature + -1.927704 $\times 10^{-4}$ * PFD^2 + -9.306559 $\times 10^{-4}$ * temperature^2]

Regression equation 4 (see Fig. 4)

[**Predicted Biomass** = 0.1194797 + 0.2160638 * urea concentration + 2.122043 $\times 10^{-2}$ * PFD + -1.04673 $\times 10^{-4}$ * urea concentration* PFD + -3.287115 $\times 10^{-2}$ * urea concentration^2 + -1.772189 $\times 10^{-4}$ * PFD^2]

Regression equation 5 (see Fig. 5):

[**Predicted Astaxanthin Content** = 184.6898 + 2.839204 * temperature + -0.3448339 * salt concentration + 2.761079 10^{-2} * temperature * salt concentration + -0.2118014 * temperature^2 + -2.68544 10^{-3} * salt concentration^2]

Regression equation 6 (see Fig. 6):

[**Predicted Astaxanthin Content** = 9.259102 + 0.6659855 * salt concentration + 3.630979 10^{-2} * PFD + -2.214323 10^{-4} * salt concentration * PFD + -5.347514 10^{-3} * salt concentration^2 + -9.530493 10-6 * PFD^2]

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