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# Response Surface Methodology of Astaxanthin Bioproduction from Agro-Industrial Wastes and Growth Kinetics of *Xanthophyllomyces dendrorhous* in Solid State Fermentation

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## Research Article

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**ABSTRACT**

In this study, solid state fermentation of three different agro-industrial wastes was performed for astaxanthin pigment biosynthesis using *Xanthophyllomyces dendrorhous* (ATCC 24202). Fermentation conditions were modeled and optimized by response surface methodology. 109.23 µg AX/gdw, 100.25 µg AX/gdw, and 66.03 µg AX/gdw were the maximum astaxanthin amounts obtained from wheat, lentil and rice wastes, respectively. Moisture content at a 90% level was indicated as the most effective parameter for all fermentation systems. Glucose consumption, cell and product formation as growth kinetics were implemented for the optimized waste, wheat, at the optimum conditions, and the experimental data were modelled. The yield coefficients of each kinetic parameter were calculated to represent productivity. The modelling, optimization and kinetic studies of solid state fermentation as well as the antioxidant capacity of the wastes fulfilled the above requirements.

## INTRODUCTION

The focus of astaxanthin pigment bioproduction which has unquestionable biological and commercial importance is to study and develop the process. Solid state fermentation (SSF), which is a commonly preferred method among researches due to its ability to provide natural character to targeted products, has been found nevertheless problematic in the production of astaxanthin using agro-industrial wastes. This is due largely to the fact that fermentation has serious effects on the production [1].

Turkey occupies an invaluable position internationally within the agricultural community as it produces and processes a large quantity of agricultural crops as well as produces cereal and legume wastes which are needed to be utilized as value-added products. The wastes of cereals and legumes are obtained following post-harvesting operations and industrial processing. Straw, husk, bran, grits and meals qualify as agricultural wastes (or crop residues) of cereal industry. The utilization of them in terms of providing efficiency and productivity in a fermentation system holds importance for the cost of the system and product yield [2,3].

A process which enables the large-scale production of microbial pigments must be elaborated clearly. There are crucial elements within the large-scale production of microbial pigments used in processed food products due to their great economic potential, namely; 1) Experimental design, 2) Medium composition, 3) Optimization of fermentation medium and conditions, 4) Evaluation of studies statistically [4-6]. Nevertheless, research is lacking regarding the production of astaxanthin from cereal and legume wastes via SSF technology in the framework of response surface methodology (RSM). Additionally, there exists no prior study which evaluates solid state fermentation kinetics, an entity that is difficult to determine due to medium solidity, homogeneity and separation problems.

The present study investigates the production of the astaxanthin pigment utilizing the wheat, lentil and rice wastes of selected yeast. The primary aim is to optimize fermentation parameters and determine kinetic parameters.

## MATERIALS AND METHODS

### Yeast Culture and Sample Preparation

*Xanthophyllomyces dendrorhous* (ATCC 24202, freeze-dried form) was purchased from American Type Culture Collection

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(Manassas, USA). It was maintained in YM (Yeast and Malt Extract) broth and YM agar at 20°C. The composition is: 3 g/L yeast extract (Merck, Germany), 3 g/L malt extract (Merck, Germany), 5 g/L peptone (Merck, Germany), 10 g/L dextrose (Sigma-Aldrich, Germany) and 20 g/L agar (Merck, Germany). The wastes of wheat, lentil and rice, supplied by Gaziantep, Turkey, were sieved to size 0.85 mm in order to obtain a uniform material for the fermentation system.

### Proximate Analyses

pH by pH meter (NEL pH890, Turkey); moisture content by drying oven (RT 500 W.C. Heraeus Hanau, Germany); ash by combustion oven (MF 120 Nüve, Turkey); protein by digestion unit (DK6 Velp Scientifica, Europe) and distillation unit (Kjeltec 2200 Foss, Sweden); and fat by Soxhlet apparatus (SER 148 Velp Scientifica, Europe) analyses were conducted for raw and fermented content as duplicate [7]. The phenol-sulfuric acid [8] method was applied to determine the sugar content spectrophotometrically by use of a double-beam UV/VIS Spectrophotometer (Lambda 25 UV/VIS Spectrophotometer, USA) of raw and fermented content as duplicate.

### Fermentation System and Pigment Analysis

250 mL Erlenmeyer flasks were used for the fermentation of each waste. 100 g flask content was sterilized by autoclave (HMC HV-85L, Germany) at 121°C for 15 minutes and afterwards was inoculated with 2% fresh culture. Incubation was carried out at the design temperatures for 12 days, as fermentation period which was determined by previous studies.

A 5 g fermented content and 20 mL pure methanol (Sigma-Aldrich, Germany) mixture set for 2 hours. After centrifugation (6000 rpm, 10 min), the supernatant was analyzed spectrophotometrically at 474 nm against the pure methanol blank [9]. The astaxanthin amount was calculated with regard to the standard curve and the results were explained as the mean of triplicate measurements.

### Modeling and Optimization

BBD (Design-Expert Version 7.1.5, Minneapolis, USA) was generated using three independent variables: moisture content (M.C.), temperature (T) and pH with three levels based on the yeast's optimum growth conditions (80% M.C., 20°C T, 4.5 pH). 17 runs with 5 center points **(Table 1)** were conducted. The data of the fermentation systems were evaluated by regression and ANOVA analyses within the scope of RSM in the Design-Expert program.

$$y=\beta_o+\beta_{1X1}+\beta_{2X2}+\beta_{3X3}+\beta_{12x1x2}+\beta_{13X1X3}+\beta_{23X2X3}+\beta_{11X11}+\beta_{22X22}+\beta_{33X33} \quad \text{Eq. (1)}$$

The quadratic model (Eq. 1) suggested by RSM is presented above where y is the response or dependent variable; βo is the model constant; $\beta_1$, $\beta_2$, $\beta_3$ are linear coefficients; $\beta$12, $\beta$13, $\beta$23 are cross-product coefficients (present the interactions between the variables); $\beta_{11}$, $\beta_{22}$, $\beta_{33}$ are quadratic coefficients [10]; and temperature $x_1$, moisture content $x_2$, and pH $x_3$ are independent variables.

**Table 1.** BBD design matrix and response results for three wastes; aAX: Astaxanthin, gdw: gram dry waste.

| Run | M.C.% | T (°C) | pH | Astaxanthin amount (µg AX/gdw[a]) | | |
|---|---|---|---|---|---|---|
| | | | | Wheat | Lentil | Rice |
| 1 | 80 | 15 | 3.5 | 95.00 ± 2.5 | 37.40 ± 1.0 | 66.03 ± 10.2 |
| 2 | 80 | 15 | 5.5 | 55.90 ± 1.2 | 31.50 ± 1.1 | 51.00 ± 1.9 |
| 3 | 70 | 15 | 4.5 | 34.98 ± 2.9 | 11.53 ± 1.0 | 21.71 ± 0.0 |
| 4 | 90 | 15 | 4.5 | 66.31 ± 1.8 | 38.02 ± 3.1 | 49.67 ± 0.0 |
| 5 | 70 | 20 | 3.5 | 28.96 ± 2.9 | 19.17 ± 0.0 | 17.04 ± 1.0 |
| 6 | 80 | 20 | 4.5 | 79.64 ± 5.2 | 49.44 ± 0.0 | 42.35 ± 2.5 |
| 7 | 80 | 25 | 5.5 | 70.76 ± 4.5 | 51.35 ± 5.6 | 34.68 ± 0.4 |
| 8 | 80 | 20 | 4.5 | 86.80 ± 8.7 | 59.59±3.1 | 55.16±1.6 |
| 9 | 80 | 20 | 4.5 | 60.85 ± 1.6 | 57.28 ± 8.6 | 46.13 ± 5.9 |
| 10 | 90 | 25 | 4.5 | 60.84 ± 2.2 | 90.51 ± 1.0 | 26.19 ± 2.1 |
| 11 | 80 | 25 | 3.5 | 72.84 ± 1.3 | 64.22 ± 2.6 | 43.57 ± 3.9 |
| 12 | 70 | 20 | 5.5 | 33.40 ± 5.3 | 70.20 ± 52.4 | 47.49 ± 1.6 |
| 13 | 90 | 20 | 5.5 | 109.23 ± 12.1 | 100.25 ± 0.0 | 32.73 ±1. 0 |
| 14 | 90 | 20 | 3.5 | 84.46 ± 0.0 | 75.15 ± 0.0 | 38.33 ± 1.7 |
| 15 | 80 | 20 | 4.5 | 88.99 ± 0.2 | 61.12 ± 2.1 | 50.47 ± 1.3 |
| 16 | 70 | 25 | 4.5 | 26.82 ± 2.8 | 16.70 ± 0.8 | 32.47 ± 8.8 |
| 17 | 80 | 20 | 4.5 | 87.70 ± 8.2 | 64.72 ± 5.4 | 48.90 ± 1.5 |

### Antioxidant Capacity Assay

A DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity was performed to determine the antioxidant activity (AC) of raw and fermented content [11]. A 5 g sample filled up to 50 mL with pure methanol (Sigma-Aldrich, Germany) was shaken (Innova

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40R New Brunswick Scientific, USA) at 250 rpm and 30°C for 2 h for execration. The filtrated and centrifugated extract was mixed with 60 µM DPPH radical (Sigma-Aldrich, Germany) in methanol. The reaction was implemented at dark and room temperatures as well as with the control sample (water). The measurements were carried out at 517 nm, and AC values were calculated by the following equation (Equation 2):

AC%=((ABS of control-ABS of extract))/(ABS of control ) × 100 Eq. (2)

**Kinetic Study**

Product formation (astaxanthin production), yeast growth (cell number) and sugar (glucose) consumption parameters were followed for the optimized waste during the fermentation period. The parameters were modeled by the SigmaPlot Version 11.0 (London, UK) program, which defines the logistic equation [12] for yeast growth and product formation (Eq. 3), by using a logistic-3 parameter equation for glucose consumption (Equation 4).

$$y=a/(1+((a/b)-1)\times\exp(-m\times x)) \quad \text{Eq. (3)}$$

Where y is response, a and b indicate cell number or astaxanthin amount, m is slope, x is time.

$$y=a/(1+(g/m)b) \quad \text{Eq. (4)}$$

Where y is response, g is glucose amount, a and b are coefficients.

Quantitative descriptions of bio-conversions were determined by yield coefficients −Yp/s for product/substrate, Yx/s for cell number/substrate, and Yp/x for product/cell number. These refer to the difference between the initial and final values of the kinetic parameters mathematically.

## RESULTS AND DISCUSSION

**Modeling and Optimization**

The astaxanthin amount produced by utilizing agro-industrial wastes was measured for each fermentation system **(Table 1)**. Optimized fermentation conditions are summarized for all wastes in **Table 2**. The data of wheat waste from previously published work was used to compare other wastes. The wheat waste was determined as the optimized waste by obtaining the maximum astaxanthin amount, 109.23 µg AX/gdw at the following conditions: 90% moisture content, 20°C temperature and 5.5 pH. Although the conditions were same with the wheat waste for the lentil waste, a greater astaxanthin amount was obtained from wheat waste fermentation. The reason for this could be related to nutrition content. When the values of protein, water, fat and ash are combined, the rest could be considered as total carbohydrates. The total carbohydrate amount of the wheat waste, 66.59 g/100 g was calculated as the maximum value. Accordingly, it can be stated that the greater the carbohydrate amount the greater the astaxanthin production. The total carbohydrate amount of the rice waste (51.71 g/100 g) was more than that of the lentil waste (49.35 g/100 g). However, the astaxanthin amount produced less than one-in-three by utilizing the rice waste. This might be explained by the higher protein content (36.96 g/100 g>13.42 g/100 g) of the lentil waste **(Figure 1)**.

**Table 2.** Response surface methodology results.

| Wastes | ANOVA parameters | | | | Optimized parameters | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Model | L. of fit[a] | $R^{2b}$ | Adj. $R^{2c}$ | | $x_1$ | $x_2$ | $x_3$ | $[AX]_d$ |
| Wheat | 0.0507 | 0.1701 | 0.8246 | 0.5991 | P | 21.5 | 86.9 | 5.5 | 95.4 |
| | | | | | A | 20 | 90 | 5.5 | 109.2 |
| Lentil | 0.0206 | 0.0183 | 0.8696 | 0.7019 | P | 23.4 | 90 | 3.5 | 97.8 |
| | | | | | A | 20 | 90 | 5.5 | 100.3 |
| Rice | 0.0688 | 0.0585 | 0.8053 | 0.5550 | P | 15 | 86.8 | 3.5 | 63.9 |
| | | | | | A | 15 | 80 | 3.5 | 66.0 |

P: Predicted, A: Actual, a: Lack of fit, b: R-squared, c: Adjusted R-squared, d: astaxanthin amount (µg AX/gdw)

Above 80% R2 value is a significant expression of the variability of response data for microbial processes. Although the highest coefficient of determination value belonged to the lentil waste (0.8696), a quadratic model fitted well (0.1701>0.1) only for the wheat fermentation system at ($p<0.1$) probability.

In previous studies, the maximum astaxanthin amount from ATCC 24202 was reached at 19.7°C and 6.0 pH using a synthetic medium in the scope of an experimental design generated by Ramírez et al. [13]. Ananda and Vadlani [14] studied wheat bran, full-fat rice bran (21% fat content) and defatted rice bran (2% fat content) to produce astaxanthin by ATCC 24202. At the end of 11 fermentation days, they managed to produce 66.75 µg AX/g substrate from wheat bran, 80.42 µg AX/g substrate from full-fat rice bran, and 16.94 µg AX/g substrate from defatted rice bran. Except for the full-fat rice bran, the other AX amounts are lower than the wastes employed in this study. The rice waste used has 16.31% fat content and 66.03 µg AX/gdw was produced. As it is understood, AX amount increases when the amount of fat increases in rice waste.

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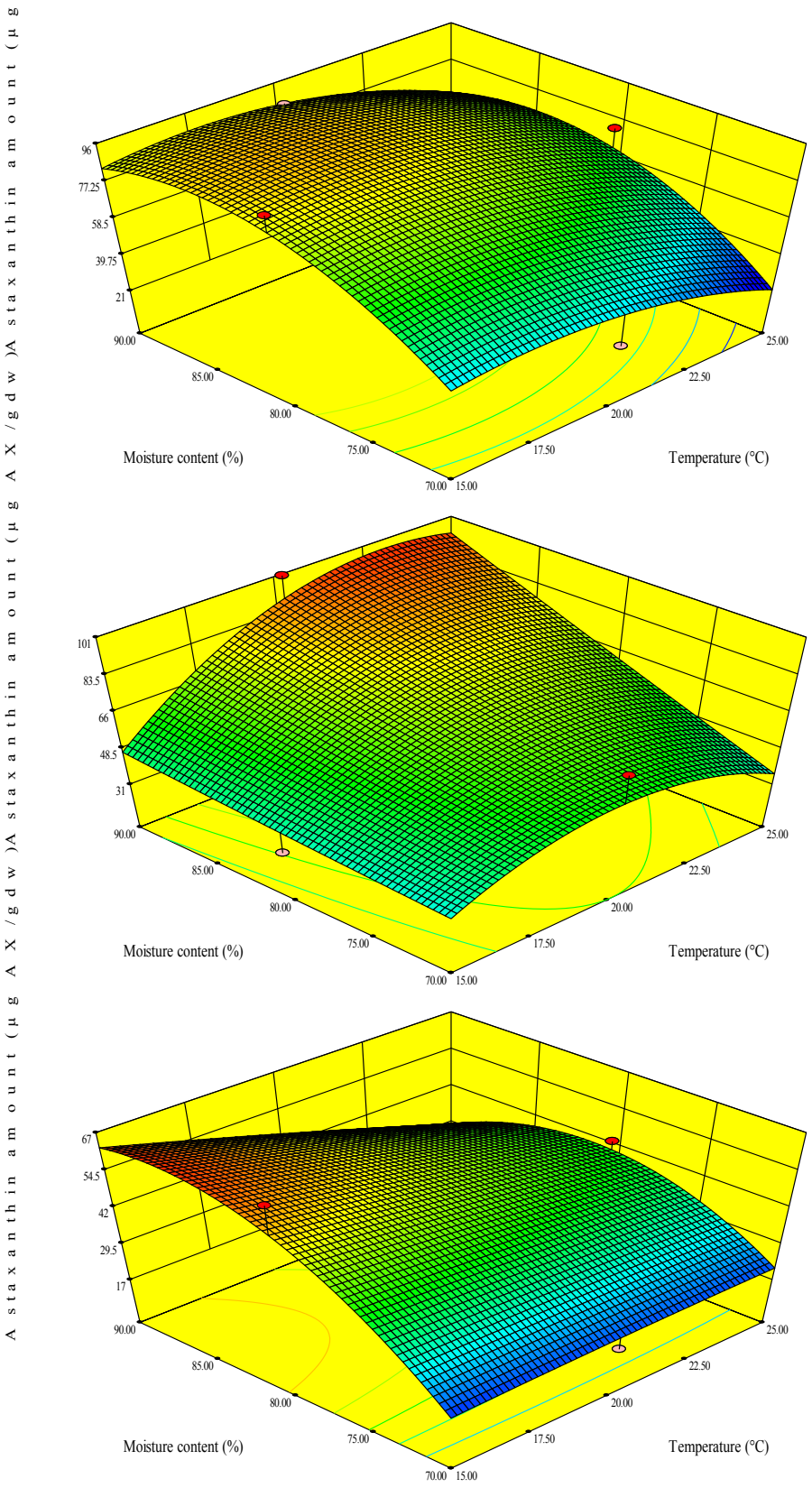


**Figure 1.** Optimized RSM plots demonstrate that the interactions of the most effected variables -moisture content and temperature- significantly for wheat (a), lentil (b), and rice (c) fermentation systems.

3D plots (A, B, C) of interactions of the most effective variables.

A second order polynomial equation with all coefficients and 3D-plots **(Figure 1)** is presented below to demonstrate the interactions of the most affected variables for the systems. Generally, for all fermentation systems, high moisture content and mild temperature values caused high AX yield. The interaction of low temperature and pH for the wheat and lentil fermentation systems

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increased the yield, whereas there was no effect for the rice. Moreover, the interaction with high moisture content engendered an increase in yield, despite whether the other parameter had a high or low value. It has been emphasized that moisture content is the most and critical parameter for SSF systems [12,15].

For wheat fermentation:

$$y=0.081-0.002617x_1+0.025x_2-0.001497x_3+0.0006725x1x_2+0.009256x1x_3+0.00508x2x_3-0.012x_{11}-0.022x_{22}+0.004801x_{33}$$

**Kinetic Study**

Three kinetic parameters for the optimized waste were followed during 12 days. Equation 3 was used to model the data of cell number and astaxanthin amount whereas Equation 4 was utilized to model the data of glucose amount. The results of the kinetic parameters and the evaluations of the models in terms of regression and statistical analysis are presented in **Tables 3 and 4**. Particularly high regression values were obtained for cell number and glucose amount models. A normality test, commonly employed to determine whether or not data are well-modeled, was applied in this study. All the parameters passed the test, which means that sample distributions were normal and the correlation between experimental and estimated data was normal. Plots in **Figure 2** show both experimental and estimated data for each parameter. So long as glucose was consumed, the yeast grew up and produced astaxanthin. The accordance of the shapes demonstrated this phenomenon. Yield coefficients in batch systems may refer productivity. Yp/s=0.1057 (µg AX/g glucose), Yx/s=0.25 × $10^8$ (number of cells/g glucose), Yp/x=3.68 × $10^{-9}$ (µg AX/number of cells) were calculated.

**Table 3.** Kinetic parameters and regression analysis results.

| Day | Astaxanthin amount (µg AX/gdw) | Glucose amount (mg G/gdw) | Cell number (cfu/gdw×$10^8$) |
|---|---|---|---|
| 1 | 66.25 ± 1.4 | 435.91 ± 6.7 | 0.09 |
| 2 | nd | nd | 0.01 |
| 3 | 58.35 ± 0.7 | 328.82 ± 1.7 | 0.03 |
| 4 | 70.07 ± 2.4 | 196.02 ± 3.3 | 0.97 |
| 5 | 65.74 ± 1.5 | 158.38 ± 2.9 | 0.51 |
| 6 | nd | 186.00 ± 2.6 | 3.91 |
| 7 | nd | 66.71 ± 0.0 | 18.55 |
| 8 | 72.36 ± 0.9 | 52.38 ± 1.4 | 14.00 |
| 9 | 66.50 ± 2.6 | 28.65 ± 2.9 | 25.45 |
| 10 | 90.20 ± 1.5 | 74.00 ± 1.0 | 113.64 |
| 11 | 103.19 ± 0.5 | nd | nd |
| 12 | 102.04 ± 0.4 | 97.16 ± 3.1 | 97.27 |

nd: Not Detectable

**Table 4.** Regression tools, statistical tests, equation coefficients.

| Sources | Product formation | Cell formation | Glucose consumption |
|---|---|---|---|
| $R^2$ | 0.84 | 0.93 | 0.92 |
| Adjusted $R^2$ | 0.78 | 0.92 | 0.90 |
| Normality test (Shapiro–Wilk) | 0.8935 (p) | 0.3056 (p) | 0.0978 (p) |
| a | 59.0 | 108.04 × $10^8$ | 462.33 |
| b | 61.6 | 2.27 | 2.23 |
| m | – 0.2 | 2.42 | 3.94 |

p: Passed

**Antioxidant Capacity of the Produced Astaxanthin**

The importance of the antioxidant power of astaxanthin pigment is obvious to researchers. This study investigated AC values before and after fermentation in order to determine astaxanthin presence in the SSF systems of wastes. The highest AC value (96.71%) related with astaxanthin amount was measured from the fermented wheat waste. The antioxidant capacities of the lentil waste (82.03%) and the rice waste (77.25%) were also determined, which the results were related with astaxanthin amount.

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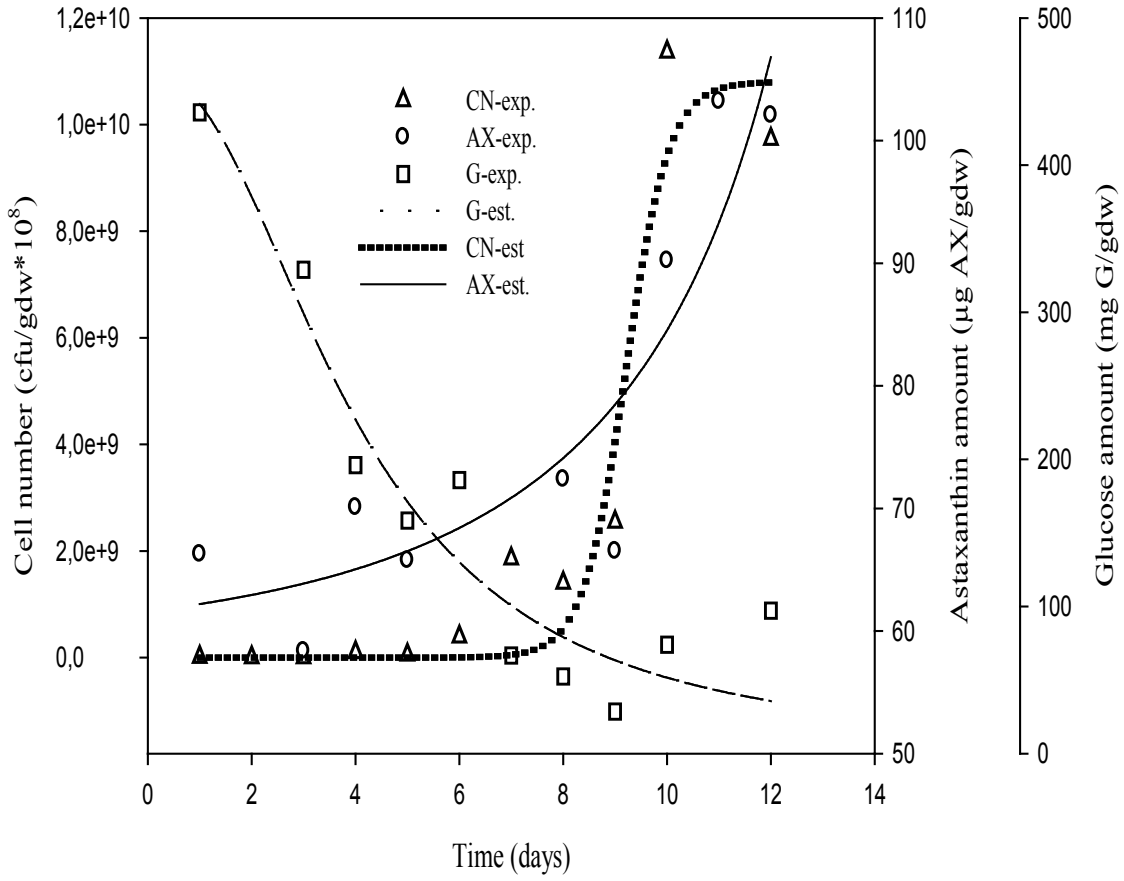


**Figure 2.** Experimental and estimated plots of kinetic parameters. The expressions of the terms used to reveal the kinetic parameters calculated depending on the equations, which have been explained in the text, are made as; CN-exp.: Cell Number experimental, AX-exp.: Astaxanthin experimental; G-exp.: Glucose experimental; CN-est.: Cell Number estimated; AX-est: Astaxanthin estimated; G-exp.: Glucose estimated.

## CONCLUSION

Key factors such as levels and effects of parameters, type of waste, fermentation period and significance of numerical analysis for the SSF of astaxanthin production were constituted by modeling and optimizing the fermentation conditions for three wastes within an experimental design. The study might be function as a basis for the performance of other related studies and developments. The optimized result deduced from RSM was that the most effective independent variable is moisture content. High moisture content might endanger the homogeneous distribution of mass and heat and, thus, easy accessibility of yeast to nutrients. As a result, growth and pigmentation were supported. The organic and inorganic content alterations of the wastes as well as the determination of the nutrition value of the products in terms of antioxidant capacity were also included in this study. The modeling of kinetic parameters and determination of the productivity for the process have been determined as difficult. Thus, further investigation is required in order to better understand and, hence, improve SSF systems.

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