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The use of *Chlorella vulgaris* in reducing the organic load of poultry slaughterhouse wastewater: Modeling and optimization of influential factors in the process

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Abstract

Background: Wastewater from poultry slaughterhouses is a serious environmental threat if they are incompletely treated. Recently, the utilization of microalgal species has gained significant attention for treating such wastewater. *Chlorella vulgaris* is one of the most efficient microalgae for treating poultry slaughterhouse wastewater due to its exceptional capacity for N, P, and chemical oxygen demand (COD) removal. This study aimed to investigate the impact of initial total nitrogen concentration, total phosphorus, photoperiod, and cultivation time on reducing the organic load and enhancing the production of biomass in slaughterhouse wastewater.

Methods: Samples were collected from the effluent of a poultry slaughterhouse and underwent qualitative analysis. *C. vulgaris* was cultivated in the BBM culture medium. Experiments were designed using the response surface method. The designed experiments were then carried out and the obtained data were subjected to analysis of variance (ANOVA), resulting in fitted models for the data on organic load removal and biomass production. Numerical optimization was performed to optimize COD removal and increase algal biomass. Finally, the models were validated.

Results: The results demonstrated that the quadratic model has a good fit for COD removal and biomass increase data. In the optimal conditions, including TN = 600 mg/L, TP = 34 mg/L, culture duration = 15 d, and photoperiod = 12.6 hr, the COD removal efficiency and algal biomass production were 93.88% and 92.37%, respectively.

Conclusion: *Chlorella vulgaris* exhibits significant potential for the removal of the organic load from poultry slaughterhouse effluent. Also, substantial algal biomass is generated, which can be used in various areas such as livestock feed and sanitary uses.

Keywords: Poultry, Wastewater treatment, Chemical oxygen demand, *Chlorella vulgaris*, Analysis of variance

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Introduction

The poultry industry has experienced remarkable growth since 2018, driven by the increasing demand for white meat, with a 3% increase compared to the average annual production of 2018 (1). In this industry, freshwater is employed for various processes, including bird washing, cleaning, cooling, waste transport, and slaughtering (2). Poultry slaughterhouses are among the establishments that consume considerable amounts of water, consequently, generating a substantial volume of wastewater. Poultry slaughterhouse wastewater, due to its high organic load, is composed of a mixture of various fats, suspended solids, proteins, blood, and nutrients such as nitrogen and phosphorus, all originating from slaughter and cleaning activities (2). It is considered one of the most polluted types of wastewater. The high nitrogen and phosphorus content is primarily responsible for the eutrophication phenomenon in open waters, which has been recognized as a significant environmental issue over the past few decades (3). Additionally, poultry slaughterhouse wastewater contains a significant amount of organic matter, primarily derived from protein substances and blood. This high organic content poses several issues, including foul odors, decay, and the creation of a favorable environment for the proliferation and accumulation of insects and carriers (2,4). Therefore, if this wastewater is discharged into the environment without proper treatment, it can pose a severe threat (1,2,5).

Various methods, including chemical coagulation, electrocoagulation, dissolved air flotation, advanced oxidation processes, and aerobic-anaerobic digestion (6), have been traditionally employed for treating wastewater from poultry slaughterhouses (5).

The drawbacks of physical and chemical methods include the requirement for a substantial amount of space, high energy consumption and the necessity for chemical usage, necessitates intricate and costly equipment, the need for complex equipment and sensitivity to variations in operational conditions, and the generation of toxic byproducts (7,8). Some disadvantages of biological treatment include sensitivity to environmental conditions and large system dimensions (9).

Therefore, these conventional methods have been deemed inefficient and costly. Consequently, there has been growing interest in exploring the potential of biological treatment utilizing microalgae species as a promising alternative for the future (5,10,11).

Algae-based biological treatment methods generally exhibit lower energy consumption compared to traditional methods. Consequently, this can lead to reduced energy costs and operational expenses, as well as a decreased need for chemical additives. Ultimately, these advantages can result in savings in chemical costs during the wastewater treatment process (12).

Utilizing microalgae in wastewater treatment is considered more environmentally friendly and suitable than bacteria-based treatments, as it effectively reduces pollutants and disease-causing agents (13). Additionally, this method offers the advantage of potential utilization of the sludge generated during the treatment process for fertilizer production and the development of other bio-products, an area currently being investigated and studied (14). Several studies have been conducted using different species of algae for wastewater treatment. Among these species, *Chlorella vulgaris* stands out as one of the most effective microalgae for treating this particular type of wastewater. This is primarily attributed to its remarkable ability to remove nutrients (N and P) and reduce chemical oxygen demand (COD) levels (1).

Chlorella vulgaris is a single-celled, freshwater green alga that can thrive in both heterotrophic and autotrophic conditions and different environments. It can effectively remove nutrients and toxins from various agricultural and industrial waste and by-products (15,16), making it a valuable tool for waste management (15,17). Studies have shown that *C. vulgaris* has an 86% removing nitrogen and a 70% removing phosphorus, surpassing other reservoirs in terms of growth rate. Additionally, *C. vulgaris* biomass is rich in proteins (18,19) and other nutrients, making it suitable for use as a nutritious feed or as a source of biochemicals (20). However, it is important to note that the water used for cultivating *C. vulgaris* must be purified and free from hazardous substances such as heavy metals, toxic compounds, and pathogens. Under controlled and clean conditions, *C. vulgaris* can also serve, as a food source for humans. From a nutritional perspective, *C. vulgaris* contains not only proteins but also chlorophylls rich in magnesium, free radicals, and antioxidants (such as phenols, carotenoids, and polyunsaturated fatty acids) (3,8).

Several studies have assessed the biological removal of organic nitrogenous and phosphorous compounds from wastewater using microalgae. Katiyar et al investigated the removal of nitrogen, phosphorus, and total organic carbon (TOC) from municipal wastewater using two distinct Chlorella species. Both Chlorella species demonstrated notable abilities in removing a significant proportion of the total nitrogen and phosphorus from wastewater. Furthermore, TOC removal efficiency was reported to be 95% and 98% for *Chlorella minutissima* and *Chlorella sorokiniana*, respectively (21). In another study, Soto et al investigated the removal of biochemical oxygen demand (BOD) and COD from sugarcane distillery wastewater using *C. vulgaris*. The findings suggested reductions in COD and BOD by up to 49% and 70%, respectively, accompanied by the peak algal biomass production of 10.50 ± 0.92 g/L. Moreover, the wastewater's toxicity was decreased by over 90% (17,22). Akhavan et al. conducted a study on urban wastewater treatment deploying microalgae. They discovered that the microalgae reduced the COD, total nitrogen, and phosphates by about 50%, 25%, and 50%, respectively (17).

The response surface methodology (RSM) is a set of mathematical and statistical techniques used to analyze the relationship between responses and multiple independent variables. It involves constructing empirical models and designing experiments to estimate the effects of variables, including main effects, interaction effects, and second-order effects. RSM aims to establish a regression model connecting responses to the investigated factors. The methodology includes screening experiments, analysis of variance, regression to develop a response-fitting function, and optimization to determine optimal levels of independent variables. RSM uses statistical principles to minimize the number of experiments and identify efficient conditions for desired outcomes (23-25).

This study aimed to evaluate the performance of *C. vulgaris* microalgae in removing COD from poultry slaughterhouse wastewater. Furthermore, the study aimed to optimize the treatment process using RSM to determine the most effective conditions for achieving high efficiency in COD removal.

Materials and Methods

Wastewater sampling

The required wastewater samples were collected from the treatment plant of a poultry slaughterhouse in Qazvin province. These samples were preserved and transported to the laboratory in compliance with the procedures delineated in "Standard Methods for the Examination of Water and Wastewater" (26). As a preliminary step, a qualitative analysis of the wastewater was undertaken. The details regarding the quality characteristics of the wastewater being studied can be found in Table 1 (5,20). Then, based on the results of previous studies on the treatment of poultry slaughterhouse wastewater using *C. vulgaris* algae and conducting several preliminary laboratory experiments, the range for each independent variable being studied was established.

Experimental design

In this study, four independent variables including initial concentrations of total nitrogen and total phosphorus, photoperiod, and cultivation time, were considered. The dependent variables were the efficiency of COD removal and the concentration of dry biomass. Following the establishment of the range for each independent variable, the experiment design was implemented using RSM.

The polynomial quadratic model illustrated in Equation 1 was employed for result prediction.

$$Y = \beta_0 + \sum_{i=1}^{k} \square i.xi + \sum_{i=1}^{k} ii.xi^2 + \sum_{ii\le j}^{k} \sum_{j}^{k} ij.xi.xj + \ldots + e \quad (1)$$

In this equation, Y represents the response variable, i and j are linear and quadratic coefficients, respectively, β denotes the regression coefficients, k is the number of factors studied and optimized in the experiments, and e is the random error.

The factors under study and the level of each in actual and coded values are presented in Table 2.

Table 1. Characteristics of chicken slaughterhouse wastewater treatment plant effluent

Parameters	Mean ± SD
COD (mg/L)	300 ± 50
BOD (mg/L)	120 ± 35
TOC (mg/L)	225 ± 12
TSS (mg/L)	465 ± 35
TN (mg/L)	600 ± 15
TP (mg/L)	60 ± 4
Turbidity (NTU)	500 ± 50
pH	8.0 ± 0.2

Abbreviations: BOD, biochemical oxygen demand; COD, chemical oxygen demand; TSS, total suspended solids; TP, total phosphorus; TN, total nitrogen; TOC, total organic carbon; SD, standard deviation.

Subsequently, the design of experiments was conducted using the Box-Behnken design, a subset of the RSM. The number of required experiments was determined according to equation 2.

$$N = 2^k + 2(k) + cp \quad (2)$$

In this equation, N is the number of experiments, k is the number of factors, and cp is the number of experiments at the center point. The experiments in this study included 16 factorial experiments (2^k), 8 axial or star-shaped experiments (2k), and 5 repeated experiments at the center point. In total, 29 experiments were conducted. The experiment design matrix, along with the sequence of their execution, the actual outcomes obtained in the lab, and the results projected by the fitted model based on the experimental data, are all presented in Table 3.

Experiments were conducted in the lab according to the experimental design matrix, and the results were recorded. The collected data were then subjected to analysis of variance (ANOVA) and regression analysis to derive the model fitted to the data. The experimental design and statistical analyses were carried out using the Design Expert version 11 software. Following the preliminary ANOVA, any statistically insignificant factors within the equations were removed, leading to the adjustment of the resultant models. ANOVA results of COD removal and dry biomass increasing are shown in Table 4. The plots of the comparison of actual and predicted values, and distribution of residuals relative to the order of experiments for COD removal and algal biomass increasing are presented in Figure 1.

The surface responses of the quadratic model, with two variables maintained at a midrange value and the other two varying within the experimental ranges, are shown in Figures 2 and 3.

The optimization for removing COD and the augmentation of *C. vulgaris* biomass was performed using numerical methods in the software. To validate the findings derived from the final modified models, two supplementary experiments were executed under the conditions predicted by each model. The laboratory results were compared with the model predictions to assess their accuracy, and the validation outcomes are presented in Table 5. The validation results are presented in Table 5. The importance of each factor was confirmed

Table 2. Levels of independent parameters and their coded values for the Box-Behnken design

Parameters	Unit	Symbol	Range and levels		
			-1	0	+1
Total nitrogen	mg/L	A	250	425	600
Total phosphorus	mg/L	B	33	49	65
Culture duration	day	C	5	10	15
Photoperiod	h	D	8	16	24

Table 3. Design of experiments matrix for COD removal and dry biomass increasing their actual and predicted results

Standard order	Run	Factors				COD Removal Efficiency (%)		Biomass Increasing (%)	
		TN Initial Concentration (A) (mg/L)	TP initial Concentration (B) (mg/L)	Photoperiod (d)	Cultural Duration (D) (h)	Experimental	Predicted	Experimental	Predicted
18	1	600.00	49.00	5.00	16.00	65.34	63.35	73.37	73.06
5	2	425.00	49.00	5.00	8.00	62.8	65.30	55.35	54.31
10	3	600.00	49.00	10.00	8.00	81.44	81.70	73.15	74.64
23	4	425.00	33.00	10.00	24.00	79.47	79.97	77.81	76.61
2	5	600.00	33.00	10.00	16.00	74.01	76.57	86.71	85.74
11	6	250.00	49.00	10.00	24.00	80.96	82.18	74.44	73.42
13	7	425.00	33.00	5.00	16.00	64.08	64.68	60.96	61.83
21	8	425.00	33.00	10.00	8.00	81.47	77.55	69.33	69.29
8	9	425.00	49.00	15.00	24.00	85.21	83.99	87.53	88.21
9	10	250.00	49.00	10.00	8.00	60.72	63.14	73.06	71.55
20	11	600.00	49.00	15.00	16.00	85.93	85.68	93.91	95.03
14	12	425.00	65.00	5.00	16.00	75.52	68.91	67.53	69.61
27	13	425.00	49.00	10.00	16.00	80.18	76.54	86.44	84.33
3	14	250.00	65.00	10.00	16.00	77.16	77.65	82.68	83.29
1	15	250.00	33.00	10.00	16.00	58.64	59.56	68.46	71.42
4	16	600.00	65.00	10.00	16.00	76.18	76.54	88.04	84.73
19	17	250.00	49.00	15.00	16.00	64.65	63.88	92.23	92.42
24	18	425.00	65.00	10.00	24.00	93.86	95.02	81.45	81.38
22	19	425.00	65.00	10.00	8.00	82.06	78.80	74.28	75.37
16	20	425.00	65.00	15.00	16.00	81.32	82.20	94.89	94.49
29	21	425.00	49.00	10.00	16.00	73.6	76.54	85.16	84.33
15	22	425.00	33.00	15.00	16.00	79.52	78.87	93.04	91.43
28	23	425.00	49.00	10.00	16.00	75.52	76.54	83.9	84.33
25	24	425.00	49.00	10.00	16.00	72.38	76.54	82.98	84.33
12	25	600.00	49.00	10.00	24.00	82.23	81.30	84.12	86.10
26	26	425.00	49.00	10.00	16.00	81.04	76.54	83.19	84.33
17	27	250.00	49.00	5.00	16.00	69.98	67.47	61.15	59.91
6	28	425.00	49.00	15.00	8.00	84.95	86.96	87.96	87.97
7	29	425.00	49.00	5.00	24.00	87.65	86.92	67.76	67.39

with a Pareto chart, which is shown in Figure 4. In this figure, the percentage contribution (P_i) is defined as the ratio of the sum of squares for each factor (SSA) to the total sum of squares (SST), as stated in equation 3.

$$Pi(\%) = \frac{SS_A}{SS_T} \quad (3)$$

Chlorella vulgaris Cultivation

Chlorella vulgaris algae were purchased from the Academic Center for Education, Culture, and Research of Iran and transferred to the laboratory following the provided guidelines. Before commencing the cultivation process, the cultivation room was sterilized using UV lamps for half an hour. To avoid contamination of the algal growth environment, all laboratory instruments were sterilized in an oven at 180 °C for an hour. In addition, Erlenmeyer flasks containing the growth medium were thoroughly sterilized in an autoclave. Initially, 500 mL of the purchased algae stock was cultivated in each of the 2-L Erlenmeyer flasks containing 1.5 L of sterile BBM (Bold Basal Medium) cultivation medium. Algae cultivation was carried out at room temperature (25 ± 2 °C). Artificial light produced by fluorescent lamps continuously illuminated the algal growth medium. The amount of light irradiated on the surface of the flasks containing the algae was 65 micromoles per square meter per second (3510 lux). The cultivation medium was continuously aerated and mixed with sterilized air, which was filtered (carbon dioxide content ≈ 0.039% volume/volume). The experiments were conducted following the experimental design matrix (Table 2). Each experiment was conducted in a 500 mL flask, with each flask containing 250 mL of pre-cultivated algae from the initial phase and 250 mL of

Table 4. ANOVA results of COD removal and dry biomass increasing using *Chlorella vulgaris*

Source	Sum of Squares		d.f	Mean Square		F Value		*P*-value Prob > F	
	COD	Dry Biomass		COD	Dry Biomass	COD	Dry biomass	COD	Dry biomass
Model	1986.53	3095.18	14	141.89	221.08	14.73	57.29	< 0.0001	< 0.0001
A-TN	234.26	186.28	1	234.26	186.28	24.32	48.27	0.0002	< 0.0001
B-TP	199.35	88.35	1	199.35	88.35	20.69	22.89	0.0005	0.0003
C-Day	263.30	2226.05	1	263.30	2226.05	27.33	576.87	0.0001	< 0.0001
D-Photo	260.77	133.20	1	260.77	133.20	27.07	34.52	0.0001	< 0.0001
AB	66.83	41.54	1	66.83	41.54	6.94	10.76	0.0196	0.0055
AC	167.96	27.77	1	167.96	27.77	17.44	7.20	0.0009	0.0178
AD	94.58	22.99	1	94.58	22.99	9.82	5.96	0.0073	0.0285
BC	23.23	5.57	1	23.23	5.57	2.41	1.44	0.1427	0.2495
BD	47.61	0.43	1	47.61	0.43	4.94	0.11	0.0432	0.7437
CD	151.17	41.22	1	151.17	41.22	15.69	10.68	0.0014	0.0056
A^2	167.50	8.37	1	167.50	8.37	17.39	2.17	0.0009	0.1630
B^2	2.95	23.50	1	2.95	23.50	0.31	6.09	0.5886	0.0271
C^2	12.14	61.96	1	12.14	61.96	1.26	16.06	0.2806	0.0013
D^2	204.57	297.36	1	204.57	297.36	21.24	77.06	0.0004	< 0.0001
Residual	134.87	54.02	14	9.63	3.86				
Lack of fit	74.38	45.58	10	7.44	4.56	0.49	2.16	0.8346	0.2386
Pure error	60.49	8.45	4	15.12	2.11				
Cor total	2121.40	3149.20	28						< 0.0001

Other Statistical Parameters

COD	Mean = 76.48	S.D = 3.10	R^2 = 0.94	Adj.R^2 = 0.87	Pred.R^2 = 0.75	C.V = 4.06	A.p = 15.89
Dry biomass	Mean = 79.00	S.D = 1.96	R^2 = 0.98	Adj.R^2 = 0.97	Pred.R^2 = 0.92	C.V = 2.49	A.p = 28.824

wastewater sample. The total nitrogen and phosphorus levels in the wastewater sample were adjusted according to the specifications of each experiment in Table 2. Throughout all the experiments, similar to the initial algal cultivation phase, the illuminance at the surface of the cultivation flasks was 65 µmol per square meter per second, and the ambient temperature of the cultivation was maintained at the laboratory temperature (25 ± 2 °C). After the cultivation period, the contents of each flask were evaluated to measure the concentration of the dry cell mass and to determine the COD concentration. The diagram of the study method is shown in Figure S1.

COD concentration determination

The COD concentration was determined by filtering a specific volume of the algal culture medium through pre-weighed cellulose acetate filters with a nominal pore size of 0.45 µm. The COD level in the filtered liquid was then measured using method 5220D, as outlined in the "Standard Methods for the Examination of Water and Wastewater". The analysis was conducted using a reactor and a spectrophotometer (model DR 6000, HACH) (26).

Determination of dry biomass concentration

To determine dry biomass concentration, a specified volume of the algal culture medium was filtered using pre-weighed cellulose acetate filters with a nominal pore size of 0.45 µm (vacuum pump model DV-42N-250, J/B Industries Inc). The residual biomass on the filter surface was then rinsed with deionized water, twice the initial volume of the sample, and dried in a desiccator. Finally, the dry weight of the remaining biomass was recorded (27).

Results

Table 1 illustrates the quality characteristics of the slaughterhouse wastewater studied. Notably, the wastewater has a considerably high organic load. Thus, its direct application for activities such as irrigating agricultural lands, replenishing water sources, or industrial uses is not feasible. Hence, this study investigated the possibility of diminishing the organic load to acceptable levels for environmental discharge.

Table 2 illustrates the independent variables under study and their range of variations, both in their real and coded forms. As shown in Table 2, each variable was examined at three concentrations and levels.

The design matrix of experiments and actual results along with the predicted results by the model are presented in Table 3.

Figure 1 (A and B) illustrates the comparison between

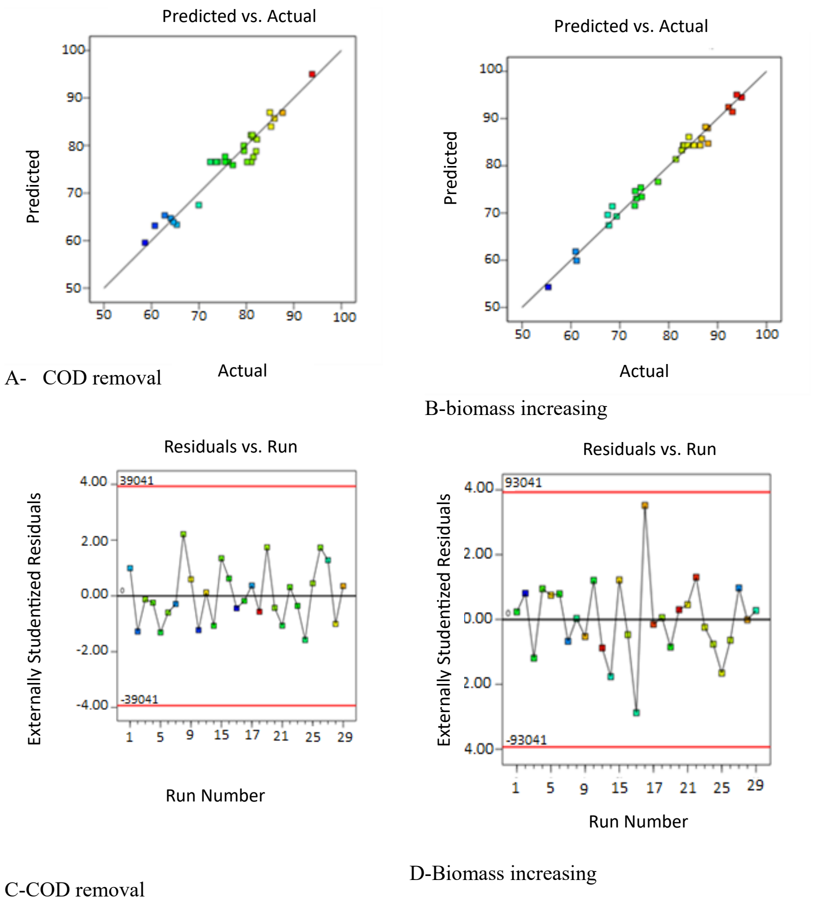

Figure 1. (A and B) distribution of predicted values versus actual values and (C and D) distribution of residuals relative to the order of experiments

the predicted values and the laboratory-measured values. It can be observed that there is a consistent relationship between the data obtained from the laboratory and the predicted data for all the responses. The data points are roughly aligned along a straight line, indicating a linear relationship and adherence to a first-degree equation (y = x). This implies that the distribution of the data follows a normal pattern. Figure 1 (C and D) illustrates the distribution of residuals concerning the sequence of performed experiments utilized to inspect the independence of residuals. The data's independence is deemed acceptable without a discernible trend, such as a sinusoidal pattern in these charts. No such pattern that could refute the independence of data is observed in this graph. Therefore, the assumptions of data independence and normal distribution of data are confirmed.

Table 4 presents the results of the analysis of variance for the collected data. At a confidence level of 95%, it is apparent that the primary impacts of the independent variables including total nitrogen (A), total phosphorus (B), contact time of wastewater and Algae (C), and duration of the light cycle (D), were significant in terms of both COD reduction and augmentation of biomass. In the context of COD reduction, the interactions of AB, AC, AD, BD, and CD were statistically significant. Conversely, concerning the enhancement of algal biomass, the interactions of AB, AC, AD, and CD were deemed significant. Moreover, considering the quadratic effects of independent variables, terms A^2 and D^2 were found significant for COD reduction, while terms B^2, C^2, and D^2 were observed significant for the increase in algal biomass.

The "lack of fit test" quantifies the deviations of the data around a fitted model. A significant result in this test implies that the model is not well-fitted to the data (19). In this study, the lack of fit values for the second-order models fitted for COD and algal biomass were 0.8346 and 0.2386, respectively. Hence, their insignificance indicates an acceptable fit of the derived models to the data. The coefficient of determination (R^2) indicates the proportion of the total variation of the predicted response explained by the model and shows the ratio of the total sum of squares from the regression (SSR) to the total sum of squares (SST). A larger R^2 value, close to 1, is desirable, and a required agreement with the adjusted R^2 (Adj.R^2) is necessary (19). Moreover, in this study, a high predicted R^2 (Pred.R^2) denotes the power of the derived models in predicting the results. According to the results presented in Table 4, for both responses, the R^2, Adj.R^2, and Pred. R^2 values are approximately at a high level, validating the good fit of the extracted models to the data.

The coefficient of variation (CV) provides an assessment of the consistency or reproducibility of the

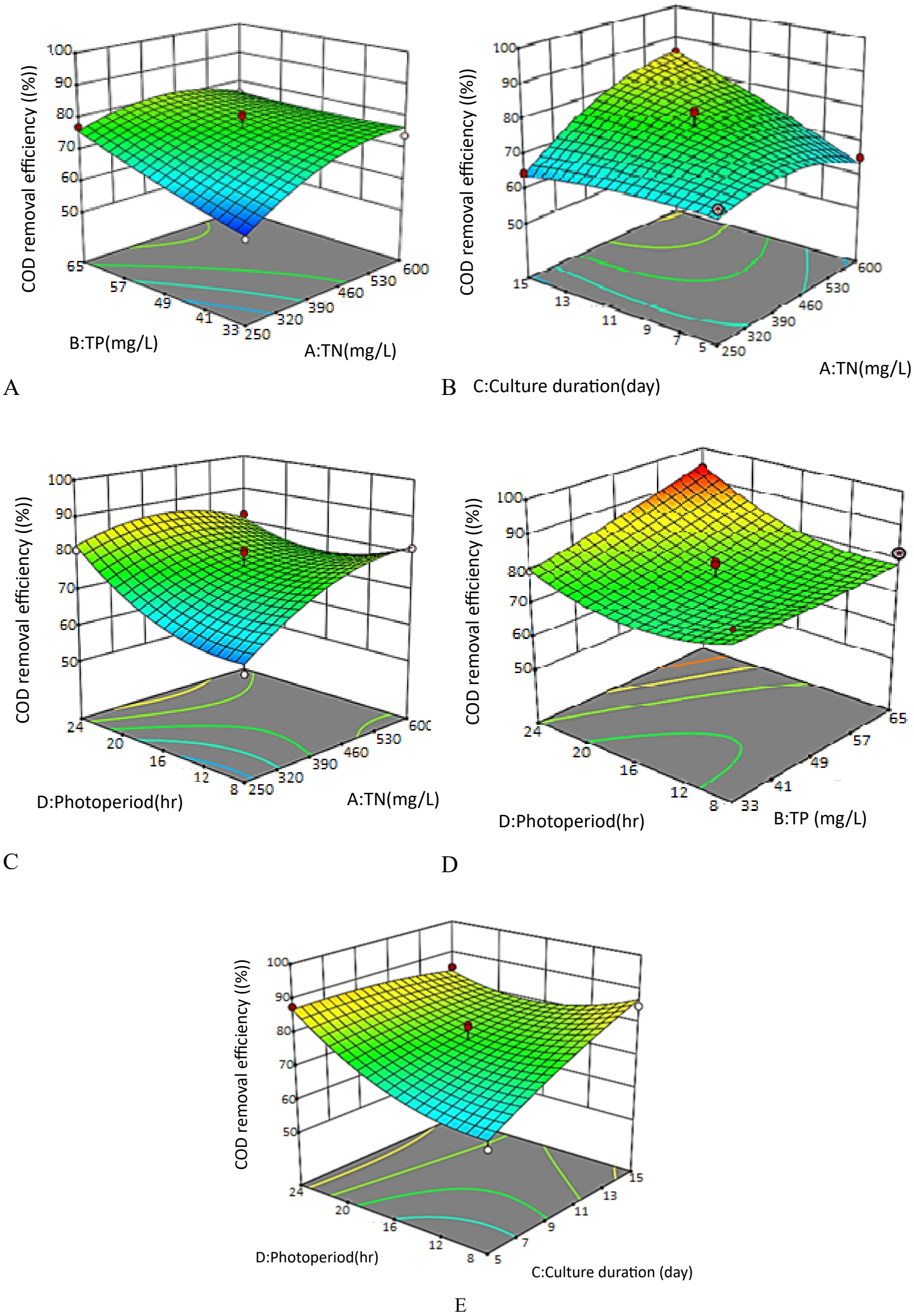

Figure 2. 3D plots of mutual effects of variables in reducing COD

model outcomes. A model is deemed to have satisfactory reproducibility if its CV value is below 10% (19). In this research, the CV values for COD and algal biomass were respectively 4.06% and 2.49%, underscoring the high level of reproducibility for the derived models. Furthermore, the adequate precision (AP) values demonstrate the ability of the model to predict the efficiency of COD removal and increase in algal biomass. Essentially, this index measures the "signal-to-noise ratio," with ratios greater than 4, indicating that the signal is strong enough to guide the design of space (20).

Equations 4 and 5 illustrate the final adjusted models for the removal of COD by *C. vulgaris* algae and the consequent increase in algal biomass, respectively. These models were derived after discarding terms that were not statistically significant.

$$\text{COD removal efficiency} = 76.54 + 4.42A + 4.08B + 4.68C + 4.66D - 4.09AB + 6.48AC - 4.86AD + 3.45BD - 6.15CD - 5.08A^2 + 0.67B^2 - 1.37C^2 + 5.62D^2 \quad (4)$$

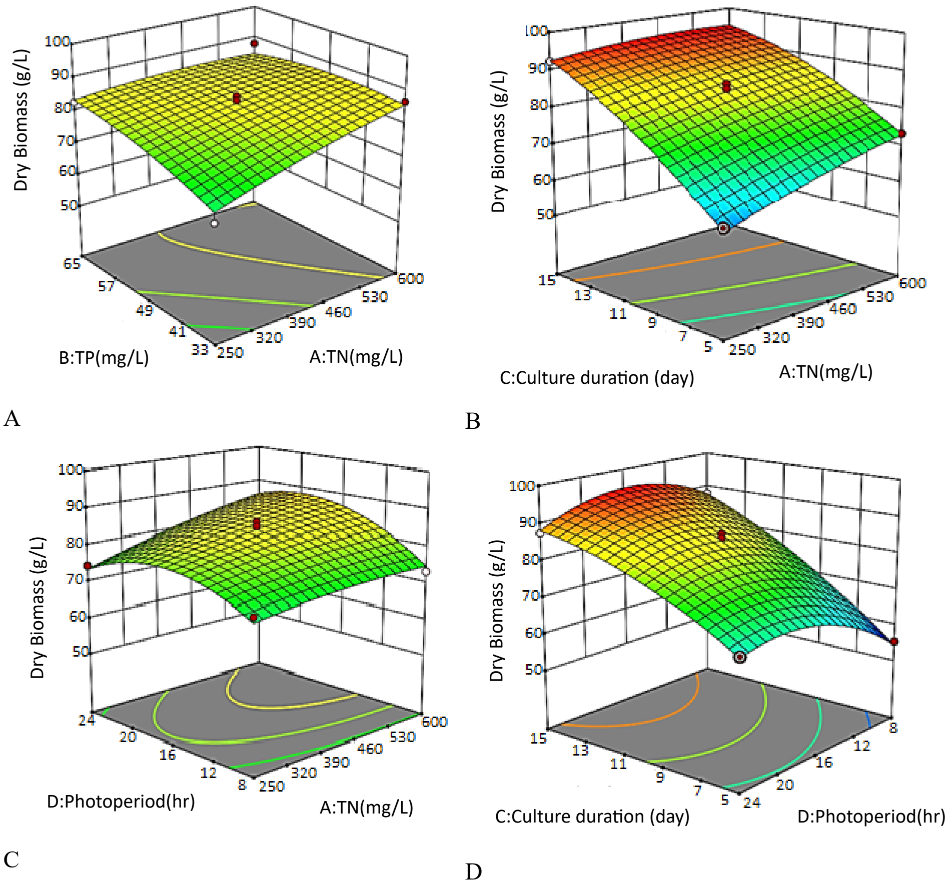

Figure 3. 3D plots of mutual effects of variables in increasing algal biomass

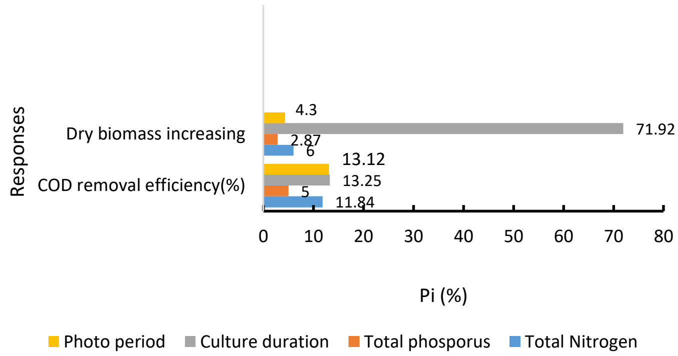

Figure 4. Pareto effects analysis of the effects of factors on target responses for treatment of poultry wastewater

Table 5. Verification of developed models for removal of COD and increasing Biomass at optimum conditions

Optimum Conditions	COD removal efficiency (%)		Biomass increasing (%)	
	Actual	Predicted	Actual	Predicted
Total nitrogen = 600 mg/L Total phosphorus = 34 mg/L Culture duration = 15 day Photoperiod = 12.5 h	87.50	93.88	84.60	92.37
Error	6.38		7.77	
SD	±2.49		±3.13	
Desirability			0.97	

$$\text{Dry biomass increasing} = 84.33 + 3.94A + 2.71B + 13.62C + 3.33D - 3.22AB - 2.64AC + 2.40AD - 3.21CD - 1.90B^2 - 3.09C^2 - 6.77D^2 \quad (5)$$

Subsequently, while other independent variables were kept constant at their mid-range values, three-dimensional plots were constructed to illustrate the pairwise interaction effects of the remaining variables. These are depicted in Figures 2 and 3.

Process optimization

After investigating the interaction effects of variables in the COD removal and algal biomass increase, numerical optimization was carried out in the utilized software. The factors were selected in the "In range" condition, and the responses were set to "Maximize". The optimization results, along with the validation of the model's predictions against experimental results, are presented in Table 5.

As observed in Table 5, the data predicted by the model aligns well with the experimental data.

Figure 4 presents the impact of each independent parameter on the reduction of organic load and the increase in dry biomass. The chart demonstrates that the culture duration exerted the most significant influence on biomass increase, accounting for 71.92% of the observed variation, as well as organic load reduction, accounting for 13.25%. Regarding COD removal, the duration of light exposure contributed 13.12% to the observed variation, while the influence of total nitrogen was 11.84%. On the other hand, the impact of total phosphorus on both biomass production and organic load reduction was relatively minimal, accounting for only 5%. Variables such as total nitrogen (6%), light cycle (4.3%), and total phosphorus (2.87%) did not exhibit a significant effect on algal biomass production.

Discussion

According to Table 3, the highest efficiency in removing organic matter was 93.86%, observed in test 18. This result was achieved under conditions of a total nitrogen concentration of 425 mg/L, a total phosphorus concentration of 65 mg/L, a cultivation period of 10 days, and a light cycle duration of 24 hr. On the other hand, the maximum percentage increase in the dry mass of biomass, which amounted to 94.89%, occurred under conditions where the total nitrogen and phosphorus concentrations were 425 and 65 mg/L, respectively, the cultivation period was 15 days, and the light cycle duration was 16 hr. These results suggest that with an increase in the concentration of nitrogen and phosphorus, algal growth increases while the organic load decreases.

COD represents the total organic load (dissolved and suspended) of wastewater. In this study, the efficiency of organic loading removal in the studied mixotrophic culture medium was approximately 93.86%. These results are higher than the average COD removal (78%) of *C. pyrehadidosa* in municipal wastewater (7) and by *C. vulgaris* from Swine wastewater (76%) (8).

Figure 2A assesses the interactive effect of total nitrogen and phosphorus on the reduction of organic load while keeping the two variables photoperiod and cultivation time, constant at mid-range. As depicted in the figure, the efficiency of organic load removal increases with the elevation of nitrogen and phosphorus concentrations, continuing up to around 80%. Under these conditions, the concentrations of nitrogen and phosphorus are approximately 450 and 65 mg/L, respectively. Beyond this point, as nitrogen levels continue to increase, the efficiency of organic load removal shows a downward trend. *C. vulgaris* cells assimilate inorganic phosphorus in the form of orthophosphate to produce phospholipids, ATP, and nucleic acids (7). It is important to note that phosphorus removal by microalgae is generally slower and less efficient compared to nitrogen removal (28).

The interactive effects of total nitrogen and cultivation time on organic load removal are shown in Figure 2B. According to the figure, the efficiency of organic load removal increases with the elevation of total nitrogen and cultivation time to the extent that the highest efficiency of organic load removal reaches approximately 85%.

According to Figure 2C, initially, the efficiency of removal increases with the rise in both the photoperiod and total nitrogen concentration. The maximum removal efficiency is obtained with a 24-hour photoperiod and a total nitrogen concentration of approximately 425 mg/L. Beyond this point, an increase in nitrogen concentration does not result in a noticeable change in COD removal efficiency.

As depicted in Figure 2D, with the increase in total phosphorus and photoperiod, the efficiency of organic load removal exhibits an increasing trend. The maximum organic load reduction occurs at a 24-hour photoperiod and a total phosphorus concentration of 65 mg/L, reaching approximately 90%.

Figure 2E shows that with an increase in cultivation time and photoperiod, the efficiency of COD removal rises. The maximum efficiency of organic load removal, around 85%, was achieved at a cultivation time of 10 days and a photoperiod of approximately 24 hr. Beyond this point, the removal efficiency remained fairly constant with an increase in cultivation time.

Photoperiod is a highly effective factor in reducing organic load. As depicted in Figure 2 (C-D-E), the highest efficiency of organic load reduction corresponds to a photoperiod of 24 hours. The results of the study by Terán Hilares et al showed that in the removal of organic load from poultry slaughterhouse wastewater pre-treated with the acidic precipitation method, the use of *C. vulgaris* algal culture demonstrated that within 48 hr of exposure to wastewater and algae under continuous illumination with a white LED lamp (440 µmol m-2s-1 of photon flux density), the removal of the organic load reached 83%, which is consistent with the findings of the present study. The slight difference observed in the removal efficiency of the organic load could be attributed to the difference in wastewater culture duration to algae and the initial characteristics of the studied wastewater (1). Also, under these conditions, the removal rates of total nitrogen and total phosphorus have been reported to be 58% and 60%, respectively.

Azam et al utilized the *Chlorella pyrenoidosa* algae for treating wastewater from cattle, sheep, and buffalo slaughterhouses. Their findings revealed that with an initial COD of 1714 mg/L, the efficiency of organic load removal reached 50% without any dilution of the wastewater (29). Soto and colleagues' research on treating wastewater containing sugarcane vinasse with *C. vulgaris* algae demonstrated a COD and BOD removal efficiency of about 49% and 70%, respectively, achieved over a 5-day cultural duration without light. This was coupled with the peak algal biomass production of 10.50 ± 0.92 g/L (22), which is inconsistent with the results of the present study. Considering that Soto and colleagues' experiment was conducted without light, the photosynthesis light cycle likely did not occur, suggesting that only the bioabsorption of organic materials was operational. As illustrated in Figure 2B, increasing the cultivation duration and nutrient quantities, such as nitrogen, enhanced the removal efficiency of COD.

Alazaiza et al demonstrated that using *C. vulgaris* for municipal wastewater treatment resulted in an 84% COD reduction over a 5-day algae culture duration to the wastewater, along with a maximum biomass yield of approximately 350 mg/L after 12 days of cultivation (30). Similarly, Salgueiro et al reported a 71% COD removal efficiency and a 91% increase in algal biomass over a cultivation period of nine days, utilizing *C. vulgaris* for the treatment of synthetic wastewater with an initial COD of 960 mg/L (31). These studies are consistent with the present research. It can be suggested that *C. vulgaris* not only absorbs nitrogen and phosphorus from its growth environment but also assimilates the organic materials present, leading to an increase in algal biomass and a reduction in the environmental organic load.

As shown in Figure 3A, the efficiency of algal biomass increases, indicating an upward trend with the increase in phosphorus and nitrogen concentrations. This trend continues until the phosphorus and nitrogen concentrations reach approximately 65 and 550 mg/L, respectively. Beyond this point, further increases in total nitrogen and decreases in phosphorus yield only a slight increase in algal biomass. According to this figure, the effect of nitrogen on increasing algal biomass is greater than that of phosphorus, which is supported by the results presented in Table 4. These results confirm a more significant impact of total nitrogen (F-value = 48.27) compared to the effect of total phosphorus (F-value = 22.89). The results of the study conducted by Azizi et al indicated that during a one-day exposure of the output wastewater from a paper factory, which underwent a treatment process involving an active sludge system along with membrane filtration, to *C. vulgaris* algae, significant algal growth was achieved. This was accomplished along with a decrease of 57% in nitrates and 43% in phosphates over a 24-hour photoperiod (32).

Figure 3B shows the interaction effects of total nitrogen and culture time on biomass growth efficiency. As depicted, biomass production demonstrates an increasing trend with extended culture duration. In contrast, an increase in total nitrogen presents a diminishing trend. According to this figure, the effect of culture time on increasing biomass is significantly higher than the effect of total nitrogen, which is confirmed by the higher impact of culture time (F-value = 576.87) compared to total nitrogen (F-value = 48.27), as indicated in Table 4.

As the concentration of total nitrogen increases and the duration of light exposure extends, there is a corresponding increase in algal biomass (Figure 3C). Peak growth efficiency, which is around 85%, is achieved at an approximate total nitrogen concentration of 550 mg/L and a photoperiod between 16 and 20 hours.

Figure 3D illustrates the impacts of both the cultivation duration and photoperiod on the enhancement of algal biomass. However, the influence of the cultivation period (F value = 576.87) is considerably more substantial than that of the photoperiod (F value = 34.52).

In a study conducted by Khoyi and Seifabadi, it was reported that the biomass concentration of *C. vulgaris* increased when the light phase was extended in light/dark cycles. Specifically, changing the light/dark (L/D) cycle from 8/16 hours to 16/8 hours increased biomass concentration (33). These findings align with the results of the present study, as depicted in Figure 3D, where it is evident that biomass increases with photoperiods and cultivation duration. Additionally, Hee- Choi reported that the growth of *C. vulgaris* in brewery wastewater exhibited an upward trend over a 15-day cultivation period with a 16-hour light cycle (34).

Microalgae typically obtain energy and carbon in a photoautotrophic manner (in the presence of light and inorganic carbon such as CO_2). However, certain algal species are capable of growing in the presence of both light and organic carbon (16,35). In the present study, as detailed in Table 3, the highest percentage increase in dry biomass was observed in experiment 20, with lab results and model predictions showing increases of 94.89% and 94.43%, respectively. This outcome was achieved with nitrogen and phosphorus concentrations of 425, and 65 mg/L, respectively, a light cycle of 16 hours, and a cell cultivation period of 15 days. Additionally, Figure 3A highlights the impact of nutrients in boosting algal biomass growth.

Chlorella vulgaris is capable of reducing COD through the mixotrophic pathway in the presence of light and organic carbon (9). However, if the organic carbon in the culture medium has a low biodegradability, it can lead to a decrease in the rate of organic matter consumption and algae growth.

The efficiency of COD removal by mixotrophic growth depends on the initial COD concentration. Therefore, the higher the initial COD concentration, the higher the COD removal efficiency (8).

Generally, microorganisms can survive by utilizing light and CO_2 (or organic carbon) as sources of energy and carbon through photoautotrophic metabolism. Many algal species, such as *Chlorella*, can assimilate organic compounds in the presence of sunlight as a preferred carbon source (36,37).

Abeliovich and Weisman have also reported that both groups of algae and bacteria consume organic carbon through mixotrophic or heterotrophic metabolism as a carbon source (38).

Vazirzadeh et al reported that the optimal algal growth was obtained at comparatively low nitrogen concentrations coupled with high phosphorus levels. Their findings indicated that the maximum biomass concentration could be achieved with an approximately 17-hour light cycle and a cultivation duration of close to 11 days, which is consistent with the present research results. Abolhasani, in their research, demonstrated that *C. vulgaris* is capable of thriving in wastewater with high nitrogen and phosphorus content. With a light cycle of 16 hours, the algae could grow efficiently and reduce the pollutants in the wastewater, which is consistent with the findings of the present study (27).

Chlorella vulgaris, due to its high tolerance in environments rich in organic load and nutrients, has been employed in the treatment of various wastewater and biomass production. The study conducted by Mujtaba et al demonstrated the algae's potential to reduce the organic load from synthetic wastewater. They used simultaneous cultivation of suspended activated sludge and immobilized *C. vulgaris* in a reactor. Within 2 days, the COD removal efficiency reached 94%-96%, and biomass production attained 2.2 g/L. They concluded that *C. vulgaris* is highly effective in diminishing the organic load and promoting biomass production, which confirms the findings of our study (35). Also, in another study, Yue Wang reported that by cultivating *C. vulgaris* in pig farm wastewater, about 60%-70% of COD could be removed within 2 days while also enhancing algal biomass (18).

Wang et al also reported that in the treatment of pre-treated chicken manure wastewater using *C. vulgaris* that the maximum accumulation of algal biomass (0.53 g/L) was from cultivation in wastewater pre-treated with electrolysis for 2 hours (20).

Conclusion

This study demonstrated that *C. vulgaris* exhibits substantial effectiveness in mitigating the residual organic matter present in the wastewater from poultry slaughterhouses. Notably, the algae displayed considerable growth in these conditions, suggesting its potential for future applications. Given the high cost associated with the preparation of the BBM culture medium, it is feasible to consider the use of this wastewater as an alternative growth medium for cultivating algae for non-human-oriented purposes.

The results of this study indicate that the second-degree polynomial model provided a satisfactory fit with the collected data. The lack of fit for the second-order models constructed for the responses of this study, namely COD removal and algal biomass production, was 0.8346 and 0.2386, respectively, confirming the good fit of the models. Also, the high R^2, consistency with Adj.R^2, and a high Pred. R^2 indicate the good predictive capacity of the models.

The findings revealed that under optimized conditions, both the efficiency of COD removal and the increase in algal biomass surpassed 90%. This signifies the significant potential of *C. vulgaris* in diminishing the organic load and boosting algal biomass. The desirability of the model for prediction outcomes was approximately 97%. Among the independent variables affecting COD removal from wastewater by algae and algal biomass growth, the duration of algae and wastewater exposure exhibited the most substantial influence.

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Competing interests

None.

Ethical issues

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Supplementary File

Supplementary file 1 contain Figure S1.

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