Int. J. Adv. Eng. Pure Sci. 2023, 35(2): 177-182
DOI: 10.7240/jeps.1174562

RESEARCH ARTICLE / ARAŞTIRMA MAKALESİ

Evaluation of Potential Environmental Risks of Graphene-Based Materials by Examining the Effect of rGO on the Microbial Activity of *P. Chrysosporium.*

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Abstract
Graphene has been used in various applications in many fields. In recent years, its annual output has reached one hundred tons. Graphene has shown great potential in analytics, medicine, electronics, energy, agriculture, and environmental remediation. With increasing applications and production, the environmental risks and hazards of graphene have increased public concern. It was a key issue in environmental risk assessments of graphene materials. Microbial degradation of graphene and graphene oxide and its degradation by fungi in the environment have been previously studied. However, reduced graphene oxide (rGO) was difficult to degrade by fungi, and there were limited studies on this subject. In this study, the white rot fungus *Phanerochaete chrysosporium* was incubated with the culture system rGO for one week. The independent variables of microorganism concentration, pH, and rGO concentration were analyzed with the Box Behnken statistical method using response surface methodology. The potential environmental risks of graphene-based materials were assessed by examining the effect of rGO on the microbial activity of *P. chrysosporium*. The results revealed that rGO inhibited microbial activity during incubation and acted as an inhibitor in the medium. In addition, pH was found to be effective in inhibiting the environment, while microbial activity decreased at low pH. Moreover, *P.chrysosporium* was thought to degrade the oxygen groups on the rGO surface due to its decomposition ability. To test the environmental impact of graphene-based materials in general, it was aimed at unraveling the structure-activity relationships of the fungus *P. chrysosporium.*
Keywords: Reduced graphene oxide, White rot fungus, Box Behnken Design, Response surface methodology

I. INTRODUCTION

In recent years, rapid population growth, depletion of water resources, and drought have especially threatened clean and drinking water resources (Cordell et al., 2011). Owing to human influences, industrial activity, and natural activities, there was a lot of wastewater output. Due to the increasing pollution and scarcity of water, Nature magazine emphasized that "more than one trillion people in the world do not have access to clean water, and life is getting worse and worse" (Edokpayi et al., 2020). In particular, developing industries and wastewater with increasing production capacity polluted existing clean water resources. Therefore, it was important to treat wastewater before discharge. Existing physical (Fu and Wang, 2011), chemical (GracePavithra et al., 2019), and biological (Dodbiba et al., 2015) technologies were applied to remove contaminants from wastewater. However, the ineffectiveness of traditional treatment methods in the treatment of new generation pollutants led to the search for new treatment methods. Recent studies reported that the application of carbon-based nanomaterials is effective in wastewater treatment (Selvaraj et al., 2020). Nanomaterials could be defined as materials with a minimum external size ranging from 1 to 100 nm, while nanoparticles were defined as objects consisting of three external dimensions at a particular nanoscale (Rebello et al., 2021). Nanomaterials such as graphene, graphene oxide (GO), reduced graphene oxide (rGO), and carbon nanotubes were used in wastewater treatment. Graphene-based materials were applied in an integrated manner in physico-chemical (membrane, Fenton process, electro-Fenton process, photocatalysis, photoelectrocatalysis) methods (Madenli et al., 2021).

Carbon-structured materials such as graphene have demonstrated successful performance in the removal of pollutants from wastewater. However, it has been reported that it may pose a significant threat to the environment and human health if released into nature. (Yang et al., 2019). When released into the environment, they could interact with living organisms and enter living cells through penetration and endocytosis. Placing graphene inside the cell not only damaged the cell membrane of living things, but also

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Submitted: 13.09.2022, **Revised:** 02.05.2023, **Accepted:** 09.05.2023

negatively affected the DNA helix structure (Zhao et al., 2014). When the ecotoxicity of graphene in fungi, algae, bacteria, and plant species was examined, it caused stress in nutrient environments and a decrease in oxidative chains. (Efremova et al., 2015; Hu et al., 2015). The viability potential of *E. coli* bacteria when exposed to GO and rGO was reported to be 69.3% and 47.4%, respectively. Liu et al., (2011). Begum et al., (2011) investigating the effect of graphene on plants, reported that they found damage to leaf cell lesions and cell membranes as a result of phytotoxicity symptoms when they examined cabbage, tomato, and spinach. Considering that this condition destroys cell structures and hinders the environment, graphene and its derivatives were predicted to be potentially toxic materials for the environment (Arvidsson et al., 2013).

White rot fungi (WRF) had an important role in the CO_2 balance in the world's carbon cycle. WRFs were unique microorganisms with their ability to degrade lignin and similar structures (Gao et al., 2010). WRF with this capability was preferred in the evaluation of the environmental impact of graphene since it was used in the separation of aromatic hydrocarbons in previous studies (Yang et al., 2019).

The aim of our study was to reveal the effect of rGO on potential microbial activity. *P. chrysosporium* was exposed to rGO at different concentrations to monitor media inhibition and microbial activity. The total protein amount was measured during *P. chrysosporium* incubation. How rGO affects microbial growth against microbial concentration and pH factors was investigated using the Box-Behnken design (BBD) under RSM with Design Expert 13.0. The model fit and significance of variables were evaluated by analysis of variance (ANOVA). The results clearly showed the concentration-dependent toxicity of rGO to *P. chrysosporium*. It has been observed that low doses of rGO reduce the effect of microbial growth. It was determined that the morphological structure was deteriorating. Attention was drawn to the issue of ensuring the safety of graphene for living life.

II. MATERIALVE METHOD

2.1. Preparation of GO/rGO

The Modified Hummers Method (Hummers and Offeman 1958; Liu et al. 2011) was used to synthesize graphene oxide. This process recognized as being a quicker and more effective way to produce GO. The GO was first synthesized by oxidizing graphite flakes, and the next process involves reducing the GO to obtain rGO. The GO synthesis involved the use of graphite powder, sulfuric acid (H_2SO_4, 98%), potassium permanganate ($KMnO_4$), and hydrogen peroxide (H_2O_2, 30%). GO was converted into rGO using hydrazine hydrate, a reductant chemical (Stankovich et al., 2007).

2.2. *P. chrysosporium* Medium Composition

The *P. chrysosporium* strain used in this investigation were taken from the Nigde Omer Halisdemir University Environmental Microbiology Laboratory's culture collection. *P. chrysosporium* growing medium, Potato Dextrose Agar (PDA), was obtained from Merck and prepared in accordance with the directions on the PDA package. *P. chrysosporium* was cultured in a PDA plate for five days at 30 °C, and then it was used to inoculate 250 mL erlenmeyer flasks with 50 mL of stock basal medium, which was made by combining glucose (2 g/L) with other nutrients in Milli-Q water. The stock basal medium also included the following additional ingredients:
2 g/L peptone, 2 g/L KH_2PO_4, 0.1 g/L $CaCl_2$, 0.5 g/L $MgSO_4.7H_2O$, 0.001 g/L thiamine, and 1 mL/L trace elements. In 1 L of ultrapure water, 0.08 g of $CuSO_4.5H_2O$, 0.05 g of $NaMoO_4.2H_2O$, 0.07 g of $MnSO_4.4H_2O$, 0.043 g of $ZnSO_4$, and 0.05 g of $Fe(SO_4)_3$ were dissolved. After being placed into test tubes with cotton plugs, the solution was autoclaved at 121 °C (1.2 atm pressure) for 15 minutes.

2.3. Total Protein Analysis

The amount of protein formed in the incubation *of P. chrysosporium* with added rGO was calculated by the modified Lowry method. Solution A (2% (w/v) potassium sodium tartrate and 10% (w/v) Na_2CO_3) was added to the diluted sample. After the tubes were kept in a water bath set at 50 °C for 10 minutes, after cooling to room temperature, 0.1 mL of B (2% (w/V) potassium tartrate + 1% (w/v) $CuSO_4.5H_2O$ + 4% (w/v) NaOH) solution was added. The solutions were kept at room temperature for 10 minutes. Then, 3 mL of C solution (Folin Ciocalteu diluted 1:15) was added, mixed and kept in a 50°C water bath for 10 minutes. After the tubes were cooled to room temperature, they were read against the Bovine serum albumin standard at 650 nm in UV-VIS spectroscopy.

2.4 RSM Experimental Design

The Box-Behnken design, which makes use of face points to highlight possible parameter interactions, minimizes the number of tests while maximizing total protein. That proves more useful than other approaches due to these properties. In this study, three independent variables were determined to the parameters that rGO has an effect on PC microbial activity. Independent variables were determined as pH, microorganism concentration and rGO. These three factors had been also studied by Box-Behnken Design (BBD). BBD contains 17 separate experimental sets. The experimental design was given in Table 1. The results of the experimental designs were analyzed and interpreted using the Design Expert statistical software (Design Expert version 13).

Table 1. Experimental design of Statistical model for incubation of *P. chrysosporium* and rGO

Standard	Order	rGO (mg/L)	Microorganism (%)	pH
1	4	0.05	2	5
2	17	0.15	2	5
3	6	0.05	10	5
4	10	0.15	10	5
5	2	0.05	6	4.5
6	11	0.15	6	4.5
7	14	0.05	6	5.5
8	13	0.15	6	5.5
9	9	0.1	2	4.5
10	1	0.1	10	4.5
11	15	0.1	2	5.5
12	16	0.1	10	5.5
13	8	0.1	6	5
14	7	0.1	6	5
15	3	0.1	6	5
16	5	0.1	6	5
17	12	0.1	6	5

III. RESULTS AND DISCUSSION

3.1. Box-Behnken Statistical Analysis via RSM

The design matrix was used by determining the minimum and maximum levels of the three independent variables of *P. chrysosporium* concentration (%), rGO concentration (mg/L), and pH. Total protein ratios in *P. chrysosporium* microbial activity ranged between 0.1 mg/L and 4.15 mg/L. The regression equation, which was an empirical relationship in coded form between the test variables for total protein values obtained from the three-level BBD matrix, is given below.

Total protein= +0,3948-0,0552A-0,03681B+0,6442C+0,6595AB-0,3620AC-0,2914BC+0,1431A2+1,45B2-0,0103C2+0,0000ABC+1,37A2B-0,6503A2C+0,5613AB2+0,0000AC2+0,0000B2C+0,0000BC2 +0,0000A3+0,0000B2 +0,0000C2

The model equation and accuracy were performed using ANOVA analysis of variance (Table 2). It was an analysis that breaks down elements found in sources of variation to determine the accuracy of hypotheses about the total variation in a data set and the variables of the statistical model. The statistical significance of the above equation was determined by the F value. In addition, the Model F value of 160.31 showed that the model was significant. In addition, the interactions of the independent variables with each other were interpreted with the p value. As seen in Table 2., the p value for the total protein value after incubation of *P. chrysosporium* with rGO was 0.0001. This value was important for the model. Because of P values less than 0.0500 showed that the model terms were important. In this case, A, B, C, AB, AC, BC, A^2, B^2, A^2B, A^2C, AB^2 were important model terms. Values greater than 0.1000 showed that the model terms were not significant (Körbahti and Tanyolaç, 2008). In other words, it has been obvious that could be an important connection between the parameters and that the analyzed factors have been an impact on the total protein output. In determining the effect of rGO on *P. chrysosporium* microbial activity, the cubic model coefficient R^2 was obtained as 0.9979. These R^2 values were quite high. As in Fig 1, there was a good correlation between the actual and predicted values. Additionally, in Fig 1, the data points were straight lines, indicating the accuracy of the predicted and actual values. As a result, when the model and term coefficients were examined, the total protein result values showed high significance and conformity with the statistical model.

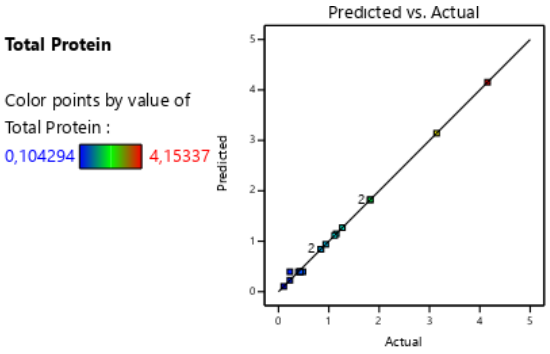

Figure 1. Correlation of actual versus predicted response for rGO incubation with *P. Chrysosporium*

Table 2. ANOVA variant analysis

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	18,94	12	1,58	160,31	< 0.0001	Significant
A.rGO	0,0122	1	0,0122	1,24	0,3281	
B.*P.chrysosporium*	0,5420	1	0,5420	55,06	0,0018	
C.pH	1,66	1	1,66	168,63	0,0002	
AB	1,74	1	1,74	176,75	0,0002	
AC	0,5241	1	0,5241	53,24	0,0019	
BC	0,3397	1	0,3397	34,51	0,0042	
A^2	0,0862	1	0,0862	8,76	0,0416	
B^2	8,89	1	8,89	902,95	< 0.0001	
C^2	0,0004	1	0,0004	0,0454	0,8416	
ABC	0,0000	0				
A^2B	3,73	1	3,73	378,60	< 0.0001	
A^2C	0,8458	1	0,8458	85,93	0,0008	
AB^2	0,6302	1	0,6302	64,03	0,0013	
Pure Error	0,0394	4	0,0098			
Cor Total	18,97	16				
				R^2	0,9979	
				Adjusted R^2	0,9917	

3.2. Effect of Independent Variables

Three independent variables (microorganism concentration, pH and rGO concentration) were determined in the incubation of *P. chrysosporium* white rot fungus with rGO. Total protein analyzes were performed to test how independent variables affected microbial activity in incubation with rGO. The effects of independent variables on total protein results are given in 3D graphics in Fig 2.

pH, rGO concentration and *P. chrysosporium* spore concentrations were prepared according to BBD and taken into rGO and *P. chrysosporium* incubation medium. The effects of the parameters on the incubation environment were seen in the graphs given in Fig. 2. In Fig. 2a, the rGO concentration was 0.09 mg/L and the P. chrysosporium concentration was 2%, while the total protein amount was maximum 3.14 mg/L. Total protein production stopped when the rGO concentration reached the maximum (0.15 mg/L) and the *P. chrysosporium* concentration was 6%. In other words, as rGO increased, it inhibited the P. chrysosporium culture system. In addition, when the *P. chrysosporium* concentration reached 10%, the total protein value was 1.82 mg/L. This showed that the high amount of spores stressed the environment and stimulated growth. Xie et al., (2016) reported that 4 mg/mL graphene oxide stimulated growth and mass loss in culture in a 14-day incubation of *P. chrysosporium.*

In Figure 2b, the effect of rGO and pH on 2% *P. chrysosporium* concentration was determined. At pH 5.5 and concentration of 0.1 mg/L, the total amount of protein was 3.14 mg/L. As the pH increased, it was revealed that while the rGO concentration was at the optimum level, it partially affected the incubation environment. As the pH decreased and the rGO concentration increased, the medium became inhibited and total protein production ceased. Total protein analysis was also a parameter that allowed to have an idea about the enzymes of the microorganism. *P. chrysosporium* was known to contain a number of extracellular oxidative enzymes, oxidases and peroxidases. All experimental results indicated that rGO did not support the growth medium of the fungus by disrupting the enzyme structure of *P. chrysosporium*. Yang et al., (2019) observed an oxidation process by breaking down the oxygen groups on the surface of the *P. chrysosporium* fungus on the surface of rGO.

Fig. 2c showed the effects of pH and *P. chrysosporium* concentrations on protein production at 0.15 mg/L rGO concentration. When P. chrysosporium was at 10% spore concentration and pH 5, the total protein amount reached 4.15 mg/L. Maximum total protein value was observed at these parameter values. In microbial activities, pH was a factor determining the vital activity of the microorganism. Total protein production ceased as the pH decreased in the incubation media and the PC concentration decreased. Ming et al., (2018) examined the toxicity of carbon nanotubes to *P. chrysosporium* in their study, they reported that *P. chrysosporium* did not affect the growth environment when the pH increased from 4.5 to about 4.8. Yang et al., (2018) reported that when the effect of *P. chrysosporium* on rGO was examined, the incubation medium took an acidic state. They reported that this situation may be caused by microbial metabolism of *P. chrysosporium* and may lead to mass loss. The environment may become acidic due to the enzymes produced during the metabolomics activity of the fungus. While pH was found to be effective in the inhibition of the medium in our study, Ma et al., (2020) reported that pH was not effective in the toxicity of *P. chrysosporium* nanodiamond. Although different nanomaterials had inhibitory effect, the effect of parameters changed.

Nogueira et al., (2015) reported that when they examined the effect of GO in the green algae *Raphidocelis subcapitata*, algae growth was slowed and the toxic effect of GO was observed on algae density and autofluorescence. Akhavan and Ghaderi, (2010) examined the toxicity of graphene and the graphene oxide incubation with gram-positive bacteria *Staphylococcus aureus*, and reported that bacteria grew on the surface of graphene after 1 hour in the incubation medium and 5-13% of the bacteria survived. (Ma et al., 2020) investigated the effect of *P. chrysosporium* on another nanomaterial, nanodiamonds. In the results they obtained, they emphasized that the fibrous structure of *P. chrysosporium* micelles was disrupted, the cell wall and its damage were broken, and it also prevented the environment. In general, it can be said that nanomaterials inhibit microbial activity in vivo and act as inhibitors.

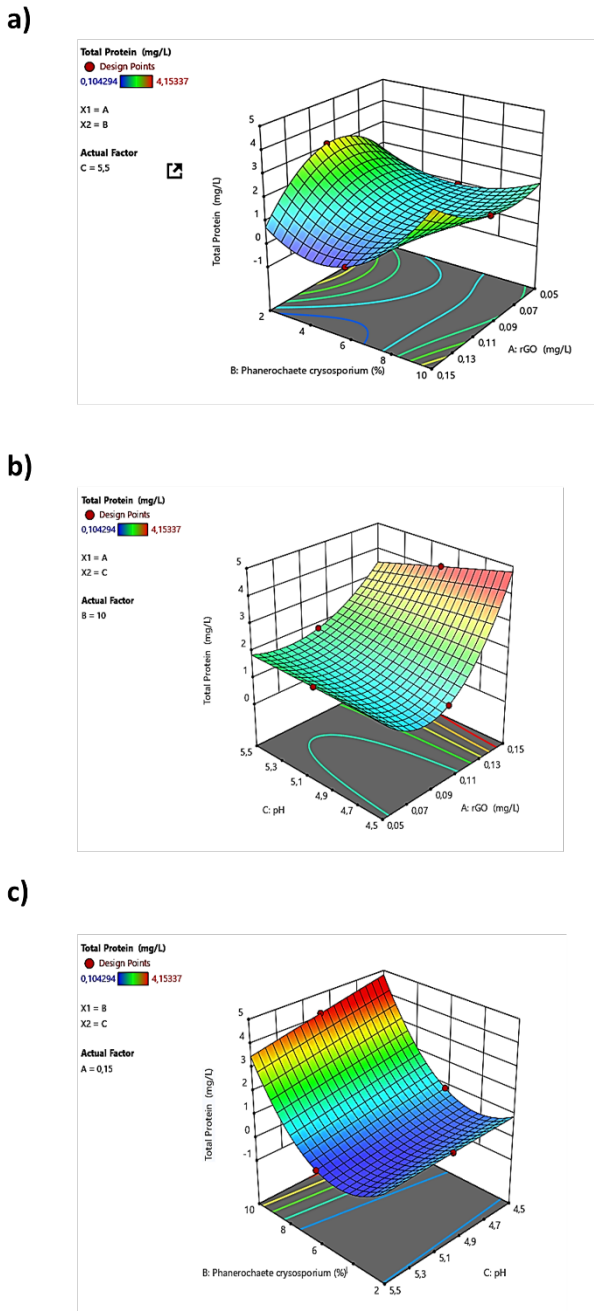

Figure 2. 3D response surface plots showing the effects of various parameters on *P. chrysosporium* microbial activity in rGO; a) *P. chrysosporium* and rGO concentration, b) pH and rGO concentration and c) *P. chrysosporium* concentration and pH

IV. CONCLUSION

As a result, rGO dose dependent toxicity of *P. chrysosporium* was evaluated. The maximum protein value was determined as 4.15 mg/L even at low rGO concentration. It was determined that the medium was inhibited and microbial growth was stimulated as the rGO concentration increased. Hyphae development was observed when the intermediate protein content increased during microbial growth, and this was thought to affect the morphological structure. Total protein analyzes were also seen as a tool to have an idea about the enzyme production of the microorganism. *P. chrysosporium* was known to contain a number of extracellular oxidative enzymes, oxidases and peroxidases. It was thought that this situation might cause graphene to inhibit oxidative enzymes when present in aqueous environments and affect microbial activities in ecological systems. We hoped that our results would spark further interest in the ecotoxicology of graphene-based materials.

ACKNOWLEDGMENT

This work was funded by The Scientific and Technological Research Council of Turkey (TUBITAK) with the project numbered 120Y087.

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