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Biodegradation of anaerobically treated distillery spent wash by *Aspergillus* species from a distillery effluent contaminated site

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ABSTRACT

This paper elucidated bioremediation process and its implementation to minimize the environmental load. Optimum chemical oxygen demand (COD) and decolorization have been achieved in the presence of glucose and peptone. Fungi are identified and isolated from a distillery effluent contaminated site. Identification has been carried out on the basis of morphology. Response surface methodology (RSM) based on central composite design (CCD) is employed to optimize operating parameters of the bioremediation process for the treatment of distillery spent wash. The effects of four independent parameters such as pH (X_1), carbon (fructose) concentration (X_2), nitrogen (peptone) concentration (X_3), and inoculums concentration (X_4) on the percentage decolorization and COD removal are investigated. A quadratic model is implemented to predict the decolorization and COD removal. Full factorial CCD of RSM is executed by using Minitab 18. The implication of independent variables and their relations are evaluated by analysis of variance. In order to achieve the maximum decolorization and COD removal, the optimum conditions are obtained by mathematical and statistical methods. The result illustrates that the maximum decolorization and COD removal efficiency could be achieved at optimum conditions (6 pH, fructose concentration 7 g L^{-1}, peptone 5 g L^{-1}, and 10% (w/v) inoculums concentration).

Keywords: Aspergillus; Chemical oxygen demand; Caramel; Biomethanated; Melanoidin; Native microbial consortium

1. Introduction

Due to globalization and rapid industrialization, the ecosystem is highly affected. Sugar molasses the by-product from sugar industry is used for the production of bioethanol. India is the fourth largest producer of ethanol in the world [1]. Biological treatment is the primary conventional treatment used to treat distillery spent wash (DSW). The most of organic load is removed except dark brown color prolonged and even increases due to repolymerization. Distilleries are the highest unpredictable industries all over the world, owing to different pigments existing during feedstock, tannic, humic acid, and melanoidin are due to Maillard reaction between sugar and amino acids. The presence of caramels are due to overheating of sugar molasses and furfurals which are due to acid hydrolysis causing dark brown color [2,3]. Distillery effluent is intensely dark brown in color that can block the sun radiation from streams and rivers, and also there is the deficiency of dissolved oxygen hence detrimental to aquatic life. This high load of environmental pollution results in eutrophication of groundwater [4]. Conventional activated sludge process is not effective to treat distillery effluent, as compounds present in sugar molasses are highly resistant to microbial attack [5]. Industries have implemented anaerobic digestion process (biomethanated) to minimize the

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pollution by generating the methane gas, and sludge, which reduces the chemical oxygen demand (COD) from 110,000 to 45,000 mg L^{-1}, and biochemical oxygen demand (BOD) from 55,000 to 28,000 mg L^{-1}. BOD:COD ratio indicates that the anaerobic process is the primary method to handle the effluent of distillery industry because by implementing biomethanated process effluents are not safe to dispose of as per the Central Pollution Control Board (CPCB) and Ministry of Environment and Forest (MoEF), India [6]. Naik et al. [3], Santal et al. [5], and Wagh and Nemade [7] reported that *Aspergillus* species such as *Aspergillus niger*, *Aspergillus niveus*, *Aspergillus fumigatus UB2 60*, *Aspergillus fumigatus G-2-6*, *Aspergillus oryzae Y-2-32*, *Aspergillus niger* UM_2, was having potential to remove the color and degradation of DSW [3,5,7]. Utilization of biological method is ecofriendly as well as economical to handle the cumbersome, toxic, and recalcitrant distillery effluent. Microbes like fungi are present in atmospheric conditions and even in soil at some amount. They release a fair amount of hydrolytic enzymes which are present in the form of saprophytes [8]. These groups of microbes have a strong influence on the ecosystem; hence they play a prominent role in assessing the biodiversity and functioning of extracellular enzymes affected by organic matter.

In the present investigation, microorganisms present in the distillery effluent contaminated site soil sample and raw spent wash are isolated and identified. These are then implemented to treat the anaerobically treated DSW. Response surface methodology (RSM) was implemented to recognize the result obtained during the experimental work.

2. Material and Methods

Different soil samples were collected from the contaminated site of Shri Dnyaneshwar distillery industry, Bhenda, Ahmednagar, Maharashtra, India, as several microorganisms were present in effluent disposal site. The soil sample was collected in a sterilized plastic bag and brought to microbiology laboratory of the department and immediately stored in a refrigerator at the temperature of 4°C, used for further analysis. Biomethanated sample and untreated spent wash were also collected to isolate the microorganisms present in the soil sample. Microorganisms present in the contaminated site are isolated. Identification of microorganisms has been carried out on the basis of morphotaxonomy from NFCCI, Agharkar Research Institute, Pune, Maharashtra, India.

2.1. Microbial enrichment

The sterilized nutrient agar (NA) media has been used for continuous enrichment of the microbial strains. In this investigation, for culturing the fungal strains pH of the media was adjusted to 6.0 ± 1.0. The composition of NA consists of 250 mL of distilled water, 1.25 g of peptone, 0.75 g of beef extract, 1.25 g of NaCl, and 3.75 g of agar. Hence, in order to avoid the contamination of the media used in the investigation, it was sterilized for 10–15 min.

2.2. Isolation of fungi from contaminated soil sample

The dilution series technique has been implemented to isolate the culture from the contaminated soil sample. Dilution series technique is the oldest and most efficient method to isolate fungi and bacteria colonies. In this technique, desired contaminated soil sample was diluted in a master test tube. The sample has been inoculated from the test tube. The spread method was implemented to inoculate the sample from diluted test tubes to NA media and then incubated the inoculated plate at 28°C for 48–72 h. After 3–4 d growth of culture was developed, then identification of culture was carried out on morphology basis.

2.3. Isolation of fungi from the treated and untreated spent wash sample

Spent wash (1.0 mL) was diluted up to 10^{-5} mL and shaken vigorously for proper mixing. After 2 h the supernatant was gradually poured and centrifuged at 1,000 rpm for 5 min. Sequential dilution of the supernatant in the order of 10^{-3}, 10^{-4}, 10^{-5} was done using 0.1 mL autoclaved double distilled water from each dilution was spread on different media and incubated at 30°C for 4 d. Spreading was done in triplicate.

2.4. Standard melanoidin preparation

The dark brown colored standard melanoidin was prepared in the laboratory by heating 1 M glucose with 0.5 M of glycine at 90°C and pH 5.5 for 6 h. The absorption maxima for regular melanoidin were designed at 450 nm by double beam spectrophotometer and a standard dose curve was prepared. For heating at 90°C hot air oven was used. The striking feature was that the absorption maxima for collected spent wash were measured at 450 nm. Hence, the further degradation studies were performed at 450 nm.

2.5. Bioremediation process

To study the degradation ability of native consortia of pure culture is isolated from a soil sample. Raw spent wash and biomethanated (anaerobically treated) spent wash sample were collected to find out fungi present in the sample. The sample has been inoculated on NA. Spent wash sample streaking on NA media was prepared on Petri plate. It was observed that very less colonies were found in a treated sample as compared with untreated spent wash. Colonies present on untreated spent wash is acidophilus bacteria, no fungus was found, the reason may be the acidic nature of spent wash and ingredient present in a raw spent wash. Colonies were not found on the treated sample because ampicillin were used to avoid the contamination, or used as an antibiotic for the good growth of fungus. The growth of fungus colonies on sludge and soil sample (moist form) was compared with other soil samples. This sample was hence selected for the experiment. Different colonies present on plates were separated and isolated and grown on different Petri plates. Fungi present in the indigenous soil sample, and raw spent wash were isolated, and cultural slant was prepared on potato agar.

3. Results and discussion

3.1. Bioremediation of color and COD by using Aspergillus culture

Aspergillus species have a great potential to decolorize and degrade the post-methanated distillery effluent,

and thus minimize the organic load of the environment. On the basis of morphotaxonomy, five different cultures have been identified such as *Aspergillus fumigatus* sp., *Aspergillus niger* sp., *Aspergillus niger* sp. mixed colonies of *Aspergillus oryzae* and *Alternaria* sp. *Aspergillus niger* sp. These cultures have been implemented to degrade the color and COD of DSW. Among these organisms tested *Aspergillus fumigatus* sp., degrade the color 56.5% and COD 42.7%. A similar report has been reported by Nagaraj et al. [3] and Omit Ohmomo et al. (1987). *Aspergillus niger* gr_1, reduces the color 81.5%, and COD 67.89%; *Aspergillus niger* gr_2, degrade the color 61.2%, and COD 48.3%; *Aspergillus niger* gr_3, diminish color 59.3%, and COD 47.2%. A similar result was reported in references [3,5,9,10]. Mixed colonies of *Aspergillus oryzae* and *Alternaria* sp., decolorize spent wash up to 41.2% and COD 29.7%. The related result has been reported by Yadav [9]. Fig. 1 illustrates the bioremediation of color and COD by cultures, among all culture, *Aspergillus niger* gr_1 shows optimum decolorization.

3.2. Effect of different concentration of carbon on color removal

During the early phase of microorganism growth, the carbon source is easily available which is added as medium and later on starts to degrade the DSW components for the carbon source. DSW contains a huge amount of sugar, and it is easily metabolisable and the carbon source is almost negligible. Therefore, the addition of readily available external carbon source like fructose is essential for its metabolism. To find out the influence of starch, fructose, glucose, and sucrose on color removal, the concentrations of carbon sources are varied from 1 to 7 g L^{-1}, while other parameters are kept constant such as temperature 35°C, pH 5.5, and peptone 5 g L^{-1}. For an initial period of 2 d, there was no significant effect in decolorization of spent wash. Optimum decolorization has been achieved 83.64% at 7 g L^{-1} fructose concentrations on the 12th day, no remarkable change has been observed for remaining days. Similar results were also reported by Aoshima et al. [11] and Hayase et al. [12]. The concentration of carbon sources, nitrogen sources, and at different pH, the concentration of glucose (2%) as carbon sources, peptone (0.5%) as nitrogen sources enhance the removal rate of color and COD of distillery effluent at pH 6 [13]. A similar result has been obtained by implementing the *Aspergillus niger* culture in the presence of carbon sources, magnesium sulfate, monopotassium phosphate, and ammonium nitrate. *Aspergillus fumigatus* identified and isolated from in situ soil sample has been found to be effective for decolorization of biomethanated post-treated distillery effluent [14].

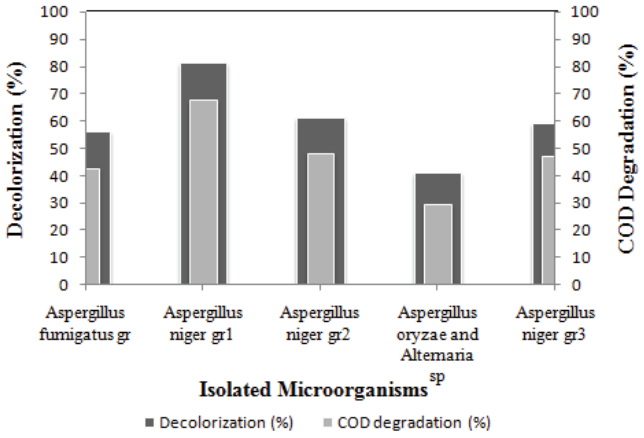

Fig. 1. Effect of isolated microorganisms on percentage decolorization and COD degradation.

3.3. Effect of different concentration of nitrogen on color removal

Different nitrogen sources such as peptone, ammonium nitrate, ammonium sulfate, and yeast extract are implemented on percentage decolorization of synthetic melanoidin pigment using different cultures. The low concentration of nitrogen source was reported as a great influence in decolorization of distillery effluent, especially peptone gave the highest intensification with decolorization for culture in the presence of carbon source [15,16]. Peptone (5 g mL^{-1}) gave optimum decolorization 83.64% on the 12th day. With increasing concentration there is lesser significance in decolorization as more nitrogen addition leads to initiate fungal growth. It was seen that lower concentration of peptone was used as nitrogen source for decolorization present in spent wash using *Phanerochaete chrysosporium* [17]. Laccase enzyme production was high on the 7th day and after that inhibited. In view to this, Viswanath et al. [18] reported that nitrogen source of peptone is the vital issue for laccase production. But in the investigation, it is seen that the production is high only at the low concentration of nitrogen source as compared with carbon source since surplus supplementation of nitrogen which in turn leads to inhibition of the basis to fungal growth.

3.4. Effect of temperature and pH on color removal

Temperature and pH are the key parameters which influence the decolorization rate. Decolorization has been studied by changing the temperature from 30°C to 45°C. Optimum decolorization has been recorded 83.64% at 35°C. At higher temperature, there was the reduction in color which manifest that organism could not survive at that temperature (as per the microbiology concern only thermophilic microorganism can survive at high temperature). Optimum growth of *Aspergillus niger* was found to be 0.86 g at the favorable condition for an incubation period of 7 d which assure the biodegradation of the spent wash. The most favorable pH for sorption was 5.5 and there was a reduction in the extent of bioadsorption potential with the increase in the pH of the solution [19]. Alkaline and neutral conditions are favorable for the growth of culture, indirectly decolorization rate decreases, the reason may be the enzymes formed by culture during the process were effective only in alkaline and neutral conditions. A similar result has been reported by Seyis et al. [20] for decolorization of molasses by implementing *Trichoderma* species. Similar result has been obtained by Ravikumar et al. [21] by implementing the fungus *Cladosporium cladosporioides* to treat the anaerobically treated DSW, he also discovered that acidic condition was more effective, maximum color removal was obtained 52.6% and COD reduction around 62.5% under the optimum condition of 5 g L^{-1} of fructose, 3 g L^{-1} of peptone, 5 pH, and 35°C temperature.

3.5. Effect of concentration of synthetic melanoidin pigment on percentage decolorization

The experiment was conducted in the presence of 7 g L^{-1} fructose and 5 g L^{-1} peptones with varying concentration of different color components present in DSW such as melanoidin, caramel, and ADP (alkaline degradation products). Percentage reductions in the concentration of synthetic colorants by using different species are mentioned in Fig. 2 [Initial concentration of different color pigments are: melanoidin 295 mg %, caramel 1,750 mg %, ADP 1,120 mg %, color O.D. 5.95]. Percentage degradation in the concentration of colorants in biomethanated distillery effluent by *Aspergillus niger* is mentioned in Table 1.

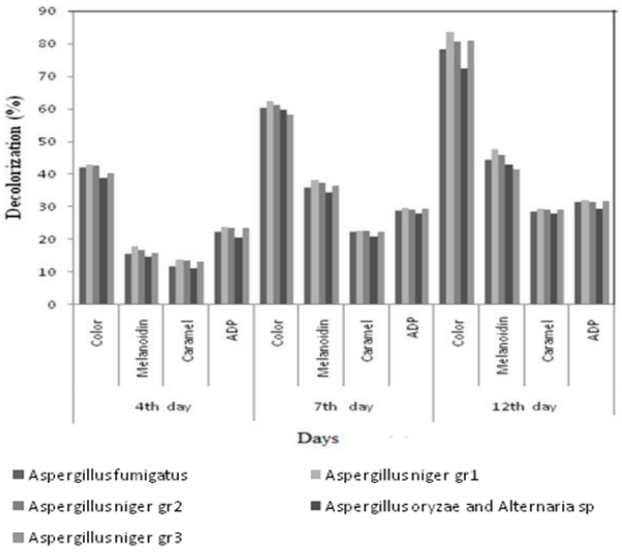

Fig. 2. Effect of concentration of synthetic melanoidin pigment on percentage decolorization.

3.6. Optimization of process parameters using design of experts (RSM)

In order to study the combined effect of different factors such as the pH (X_1), carbon (fructose) concentration (X_2), nitrogen (peptone) concentration (X_3), and inoculums concentration (X_4). Four-factor and four-level central composite design (CCD) were implemented to evaluate and optimize the effects of process variables on the responses such as decolorization and COD removal efficiency. Table 2 illustrates independent variables (pH, fructose, peptone, and inoculums concentration) and the concentration levels studied in the optimization design.

CCD matrix was generated using Minitab 18 (trial version) are shown in Table 3, and 30 trials were carried out as per the experimental plan. The regression model equations associated to elimination competence and procedure parameters were found to be:

$$\%\ \text{COD} = 77.7 - 16.05X_1 - 6.80X_2 - 4.77X_3 - 0.68X_4 + 0.240X_1 \times X_1 + 0.341X_2 \times X_2 - 0.927X_3 \times X_3 - 0.494X_4 \times X_4 + 0.858X_1 \times X_2 + 1.017X_1 \times X_3 + 1.314X_1 \times X_4 + 0.404X_2 \times X_3 + 0.223X_2 \times X_4 + 0.677X_3 \times X_4 \quad (1)$$

$$\%\ \text{Decoloization} = 66.0 - 17.69X_1 - 2.91X_2 + 2.68X_3 + 1.63X_4 + 0.546X_1 \times X_1 + 0.292X_2 \times X_2 - 1.245X_3 \times X_3 - 0.589X_4 \times X_4 + 0.358X_1 \times X_2 + 0.715X_1 \times X_3 + 1.547X_1 \times X_4 + 0.120X_2 \times X_3 + 0.150X_2 \times X_4 + 0.394X_3 \times X_4 \quad (2)$$

The mathematical model illustrates the correlation between the independent variables and dependent response (% COD and % decolorization). Information confirmed that RSM present in the run about linear, quadratic, and interaction effects of different factors by Fisher's F test and Student's t test. In general, lesser the value of p and larger the magnitude of t indicates that the corresponding terms are more significant. As revealed in Tables 4 and 5, it was discovered that the coefficient for the linear significance

Table 1
Percentage degradation in concentration of colorants in biomethanated distillery effluent by *Aspergillus niger*

Parameter	0th Day	4th Days	7th Days	12th Days	% Reduction in 12 d
Color (O.D. 475)	5.93	3.24	2.24	0.97	83.64
Melanoidin (mg %)	295	250	185	155	47.45
Caramel (mg %)	1,750	1,530	1,355	1,240	29.14
ADP (mg %)	1,120	855	790	760	32.14

Table 2
Independent variables (pH, fructose, peptone, and inoculums concentration) and the concentration levels studied in the optimization design

Variable	Factors	Studied range	Levels of variable				
			–2	–1	0	1	2
pH	X_1	3–6	3	3.5	4.5	5.0	6
Carbon (fructose; g L^{-1})	X_2	3–7	3	4	5	6	7
Nitrogen (peptone; g L^{-1})	X_3	1–5	1	2	3	4	5
Inoculums concentration (% w/v)	X_4	5–10	5	7.5	8.5	9.5	10

Table 3
The full factorial central composite design (CCD) matrix

Run	pH (X_1)	Carbon (fructose) X_2 (g L^{-1})	Nitrogen (peptone) X_3 (g L^{-1})	Inoculums concentration X_4 (% w/v)	% Decolorization	% COD removal
1	4.5	1	3.0	7.5	49.00	25.00
2	4.5	5	3	7.5	57.00	29.50
3	4.5	5	3	2.5	39.00	21.00
4	4.5	5	3	2.5	49.30	29.00
5	7.5	5	3.0	7.5	72.17	52.00
6	4.5	5	7	7.5	52.60	27.00
7	4.5	5	1	7.5	25.30	18.00
8	4.5	5	3	7.5	49.20	27.00
9	4.5	5	3	7.5	55.40	27.00
10	4.5	9	3.0	7.5	78.07	60.60
11	6.0	7	5.0	10.0	83.50	70.50
12	6.0	7	1	5.0	42.00	31.00
13	4.5	5	6.0	5.0	52.00	47.00
14	3.0	7	5.0	5.0	49.95	32.00
15	3.0	7	1	10	44.40	27.00
16	4.5	5	3.0	7.5	61.50	45.78
17	6.0	3	1.0	10.0	46.00	31.00
18	6.0	3	5.0	5.0	38.00	26.50
19	3.0	3	5.0	10.0	41.00	29.67
20	2.0	5	6.0	10.0	36.20	33.00
21	6.0	7	5.0	5.0	36.20	35.00
22	6.0	3	5.0	10.0	48.56	32.50
23	6.0	7	1	10.0	52.90	37.00
24	3.0	7	5.0	10.0	45.00	26.50
25	4.5	5	3.0	7.5	58.00	45.00
26	3.0	7	1	5.0	39.50	33.00
27	3.0	3	1	10.0	28.60	14.00
28	6.0	3	1	5.0	28.00	18.00
29	3.0	3	5	5.0	29.00	17.50
30	4.5	5	3	7.5	41.00	23.50

of pH was 0.004, carbon concentration (0.000) was highly significant for removal of COD and that of inoculums concentration 0.083 and 0.133 was least significant. The analysis of variance (ANOVA) of the regression model for these two quadratic equations was highly significant ($p < 0.05$). The results were obtained by analyzing the process by using Fisher's *F*-test. Increase in the value of *F* along with *p* value lesser than 0.05 shows high significance of regression model. Thus, it also indicates that the fructose (carbon concentration) and pH plays a very important role in degradation of COD as compared with inoculums and peptone. The analysis illustrated that these second-order polynomial equations might adequately predict the decolorization and COD removal by biodegradation process. The integrity of fit by the quadratic equation was verified by the determination of the regression coefficient. Values of R^2 for the quadratic equations were 90.03 and 86.43 for the decolorization and COD removal, respectively. Thus, it shows that total variation 9.97% for decolorization and 13.57% for COD removal. In the previous report, RSM was used to optimize the factors for decolorization of DSW through coagulation and fungal [22]. But as per the author's knowledge, there is no evidence of decolorization and COD biodegradation which simultaneously carried out by implementing biological treatment and its interpretation by mathematical and statistical modelling.

3.7. Model validation

In order to justify the RSM model results, experiment was conducted in set of three at the specified optimum process conditions (pH 6, fructose concentration of 7 g L^{-1}, peptone 5 g L^{-1}, and 10 (% w/v) inoculums concentration). Experimental result of decolorization and COD was found to be 81.5% and 67.89%, respectively. These obtained values were quite near to the RSM model values. This model proved to be useful in analyzing and predicting decolorization, COD, and optimum parameters for decolorization of DSW.

Table 4
ANOVA of the second-order polynomial equation for percentage COD removal (Analysis of variance for response surface quadratic model)

Source	DF	Sum of square	Mean square	*F* value	*P* value (Prob > *F*)	
Model	16	4,034.26	252.14	5.18	0.002	Highly significant
Blocks	2	427.16	213.58	4.38	0.035	Significant
Linear	4	1,981.37	495.34	10.17	0.001	Highly significant
X_1	1	588.36	588.36	12.08	0.004	Highly significant
X_2	1	1,080.44	1,080.44	22.18	0.000	Highly significant
X_3	1	171.57	171.57	3.52	0.083	
X_4	1	140.99	140.99	2.89	0.113	
Square	4	737.42	184.35	3.78	0.030	
$X_1 \times X_1$	1	8.00	8.00	0.16	0.692	
$X_2 \times X_2$	1	51.11	51.11	1.05	0.324	
$X_3 \times X_3$	1	377.51	377.51	7.75	0.016	
$X_4 \times X_4$	1	261.03	261.03	5.36	0.038	
Two-way interaction	6	888.31	148.05	3.04	0.044	
$X_1 \times X_2$	1	105.94	105.94	2.17	0.164	
$X_1 \times X_3$	1	149.02	149.02	3.06	0.104	
$X_1 \times X_4$	1	388.39	388.39	7.97	0.014	
$X_2 \times X_3$	1	41.70	41.70	0.86	0.372	
$X_2 \times X_4$	1	19.87	19.87	0.41	0.534	
$X_3 \times X_4$	1	183.40	183.40	3.76	0.074	
Error	13	633.32	48.72	–	–	
Lack-of-fit	10	398.33	39.83	0.51	0.817	
Pure error	3	234.99	78.33	–	–	
Total	29	4,667.58	–	–	–	

Table 5
ANOVA of the second-order polynomial equation for percentage decolorization (Analysis of variance for response surface quadratic model)

Source	DF	Adj SS	Adj MS	*F* value	*P* value (Prob > *F*)	
Model	16	4,947.19	309.200	5.40	0.002	Highly significant
Blocks	2	907.59	453.796	7.92	0.006	
Linear	4	2,141.29	535.321	9.34	0.001	Highly significant
X_1	1	412.10	412.096	7.19	0.019	Significant
X_2	1	925.41	925.414	16.15	0.001	Highly significant
X_3	1	376.12	376.121	6.56	0.024	Significant
X_4	1	427.65	427.655	7.46	0.017	Significant
Square	4	1,193.18	298.296	5.21	0.010	
$X_1 \times X_1$	1	41.42	41.419	0.72	0.411	
$X_2 \times X_2$	1	37.31	37.313	0.65	0.434	
$X_3 \times X_3$	1	680.21	680.211	11.87	0.004	
$X_4 \times X_4$	1	371.43	371.428	6.48	0.024	
Two-way interaction	6	705.13	117.522	2.05	0.131	
$X_1 \times X_2$	1	18.47	18.469	0.32	0.580	
$X_1 \times X_3$	1	73.57	73.574	1.28	0.278	
$X_1 \times X_4$	1	538.36	538.356	9.40	0.009	
$X_2 \times X_3$	1	3.70	3.696	0.06	0.803	

DF – Degrees of Freedom; MS – Mean Squares; and SS – Sum of Squares. (*Continued*)

Table 5 (Continued)

Source	DF	Adj SS	Adj MS	*F* value	*P* value (Prob > *F*)
$X_2 \times X_4$	1	8.99	8.985	0.16	0.699
$X_3 \times X_4$	1	62.06	62.055	1.08	0.317
Error	13	744.85	57.296	–	–
Lack-of-fit	10	431.68	43.168	0.41	0.873
Pure error	3	313.16	104.388	–	–
Total	29	5,692.04	–	–	–

4. Conclusions

Identified and isolated fungi from a distillery effluent contaminated site are suitable biosorbent for decolorization and degradation of DSW. Different parameters such as pH, the concentration of carbon sources, and nitrogen sources highly influence the color removal efficiency. This study presents a novel approach for enhancing decolorization and degradation of DSW by fungi present in the contaminated site under the optimum condition. Regression model revealed that carbon concentration and pH are the significant parameters. As per the experimental results, an empirical correlation between the response and independent variables was achieved and expressed by the second-order polynomial equation. The effect of the experimental parameters on the percentage COD removal and color removal of the distillery effluent was recognized by the RSM predicted by the model. The ANOVA demonstrate a high coefficient of determination value, thus ensuring an acceptable variation of the second-order regression model among the experimental data

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