Biodegradation and Decolorization of Distillery Spent Wash with Product Release by a Novel Strain *Cladosporium cladosporioides*: Optimization and Biokinetics

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Owing to rapid developments in reducing investment costs for the treatment of industrial effluent and gain through release of product, this investigation focuses on simultaneous treatment and product release. Tests were conducted to reveal the potential of *Cladosporium cladosporioides* for biodegradation and decolorization of distillery spent wash. A 2^4 full factorial central composite experimental design of Response Surface Methodology was conducted obtaining a maximum decolorization of 62.5 % and Chemical Oxygen Demand (COD) reduction of 73.6 % at optimized conditions (fructose concentration of 7 g L^{-1}, peptone 2 g L^{-1}, 6 pH and 10 % (w/v) inoculum concentration). Results of the kinetic study revealed that the decolorization process follows first order kinetics with half life saturation period of 5.02 days. The products released during the degradation were separated, analyzed using Gas Chromatography-Mass Spectrometry (GC-MS), and identified through the National Institute of Standards and Technology (NIST) library.

Key words:

Cladosporium, decolorization, response surface methodology, central composite design, distillery spent wash

Introduction

Pollution control is one of the prime concerns of society today. Untreated or partially treated wastewaters and industrial effluent discharges into natural ecosystems pose a serious environmental problem.[1] The alcohol industry is one of the major agro-based industries, which utilizes molasses as raw material for the production of rectified spirit and ethanol. The fermentation process using molasses yields large volumes of dark brown and highly toxic wastewater containing considerable amounts of organic compounds. Although most of the organic compounds are removed by means of conventional biodegradation treatment, the dark color due to the presence of melanoidin-type compound remains the problem of these process.[2] Microbial degradation and decolorization of industrial waste is an environment-friendly and cost competitive alternative to chemical decomposition.[3,4] Earlier literature reported that several fungi *Phanerochaete chrysosporium* JAG-40, *C.versicolor, Aspergillus* species, *P.pinophilum* and *A.gaisen, Emericella nidulans* were used for the treatment of distillery spent wash.[5–10] However, it is important to improve performance of the systems and increase product yield without increasing the cost. The method used for this purpose is called optimization. Conventional and classical methods of studying a process by maintaining other factors involved at an unspecified constant level does not depict the combined effect of all the factors involved. This method is time-consuming and requires a number of experiments to determine optimum levels, which are unreliable. These limitations of classical methods can be eliminated by optimizing all the affecting parameters collectively by statistical experimental design such as Response Surface Methodology (RSM). RSM was used as an optimization tool to optimize the process parameters required to treat effluents through effective electrooxidation, photo-fenton oxidation, biodegradation, adsorption.[11–14] The main objective of RSM was to determine the optimum operational conditions for the system as well as determine the region that satisfies the operating specifications.[15] Best operating conditions for the target value of color removal from distillery spent wash with *Moringa oleifera* seeds as coagulant was achieved using RSM.[16] Since biodecolorization af-

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ter biomethanation is a complicated system, it is important to be familiar with the combined interactions among the factors involved in the process. To the best of our knowledge, an effective treatment involving the effect of interaction among various parameters using central composite design during biological decolorization process and also biokinetic study in the presence of *Cladosporium sp*. has not been reported. So, the objective of this investigation was to optimize the parameters involved in the decolorization of anaerobically treated distillery spent wash, and reveal its interaction through RSM. Central composite design was used to derive a reduced polynomial model and the results were validated. Further investigation was carried out in biokinetics and a suitable model was proposed for the growth of the organism.

Materials and methods

Materials

Dioxane, pyridine, trimethyl chlorosilane, lactophenol blue, hydrochloric acid, sodium hydroxide, mercuric sulfate crystals, sulfuric acid – silver sulfate reagent, potassium dichromate, ferroin indicator, ferrous ammonium sulfate were the chemicals used for the analysis of the spent wash and characterization of the organism. All the chemicals used were purchased from Hi-Media Laboratories Pvt. Ltd, Mumbai, Maharashtra, India and were of analytical grade. The anaerobically treated distillery spent wash (ADSW) after biomethanation from anaerobic digester was collected aseptically from the distillery division of Bannari Amman Sugars Limited, Periyapuliyur, Erode District, Tamilnadu, India.

Microorganism and inoculum preparation

The spent wash collected was centrifuged at 4200Xg for 15 minutes before use in order to remove the suspended solids, and stored at 4 °C. The stored ADSW was filtered through (Whatman No: 42) filter paper and diluted with deionized water (Millipore Direct – Q.3 UV). ADSW contaminated soil sample was collected from the disposal site near the same distillery unit and was serial diluted. Several organisms were isolated from the soil and screened for decolorization ability of ADSW using giant colony and shake flask method. *C. cladosporioides* showed higher efficiency and was used for this investigation.[17] Fungal inoculum was prepared by growing the culture from the slant in a 50 mL Potato dextrose broth at 37 °C for 7 days. The culture was maintained in a 250 mL conical flask, incubated with orbitary shaker at 250 rpm until the phase of spores formation was observed. Further reseeding was carried out every 25 days to maintain the active population. All the biochemicals used for this work were obtained from M/s Himedia Ltd., Bangalore, India.

Sequencing of 18S rRNA gene and genetic analysis

The genomic DNA was extracted using a fungi DNA Kit . The sequencing PCR was set up with ABI-BigDye® Terminatorv3.1 Cycle Sequencing Kit. The 18S rRNA gene sequence data was aligned with publicly available sequences obtained from GenBank of NCBI release and analyzed to reach identity. PCR amplification of about 529 base with 969 bits was done using universal primer. The gene sequence had been deposited in NCBI gene bank with an accession number JN592511.

Optimization of process parameters using Response Surface Methodology

Experiments with four independent variables Fructose (X_1), Peptone (X_2), pH (X_3), and Inoculum concentration (X_4) at four coded levels (ά, -1, 1,-ά) as shown in table 1, and percentage decolorization as dependent response variables, were conducted using the experimental design obtained by the full factorial Central Composite design (CCD) using RSM. Initially, the parameters were optimized using single factorial experimental design.[18] The optimum parameter values obtained from single factorial experiment were then converted to uncoded units using equation 2 by assigning those values as center points. A 2^4 full factorial experimental design with 30 experiments was employed, which included sixteen trails for factorial design, eight trails for axial points, and six trails for replication of the central points based on the pattern generated through software. The experiment was conducted as per the design matrix with 100 mL of spent wash medium in a 250 mL conical flask. Each flask was incubated at 37 °C in a 250 rpm orbitary shaker for 10 days. During the process, an equal volume of sample was collected at regular intervals and analyzed for optical density at 475 nm. The percentage of decolorization was obtained in each experiment as response in each trial, which is the average of duplicates. By using the response values, the Analysis of variance (ANOVA) table and regression information were generated with the help of the Design of experiments software Design Expert 8.0 program to find out the interaction between the variables and the response. Further, based on the 'P' and 'T' value, the significant factors were determined. For statistical calculation, independent variables were coded as:

$$x_i = \frac{\left(X_i - X_o\right)}{\delta X_i} \tag{1}$$

Where X_i is the experimental value of variable, X_o is the midpoint of X_i, δX_i is the step change in X_i and x_i is the coded value for X_i ; i = 1,2,3,4. Experimental design with coded levels of variables using CCD is shown in Table 2.

The behavior of the system was explained by the following second-order polynomial equation:

$$y = \beta_o + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ij} x_i^2 + \sum\sum_{i<j} \beta_{ij} x_i x_j + \in \quad (2)$$

Where y is the percentage of color removal, β_0 is offset term, β_i is the coefficient of individual effect β_{ii} is the coefficient of squared effect and β_{ij} is the coefficient of interaction effect. Further, the optimal values were calculated using equation (2).[19] The goodness-of-fit of the regression model and the significance of parameter estimates were determined through appropriate statistical method. Further ANOVA and regression information for reduced model was generated and the optimized solution of the experimental conditions was obtained by analyzing the response surface and contour plots. Three-dimensional response surface and contour graphs were plotted on the basis of the reduced model equation to reveal the interaction among the variables and to determine the optimum value of each factor for maximum percentage decolorization. The experiment was carried out with the optimum process parameters to verify the percentage decolorization and also the reduction in Chemical Oxygen demand (COD) of the spent wash medium.

Growth kinetics

The batch data obtained during experimental run with the optimized process parameters was used to interpret the kinetic information. Reports emphasize that biokinetics used in biodegradation plays a major role in designing an economic and efficient reactor for the treatment of effluent plants. Hence, knowledge on cell growth kinetics is important to know the strength and ability of the microorganism.[20] Initially, the rate of COD reduction was assumed to be following first-order kinetics and was tested with

$$-r_A = -\frac{dC_A}{dt} = KC_A^n \quad (3)$$

Equation (1) is rewritten as

$$-r_A = -In\left(\frac{C_A}{C_{Ao}}\right) = Kt + A \quad (4)$$

Where C, concentration of the spent wash in terms of COD (mg L^{-1}); K, first-order specific reaction rate constant, h^{-1}; t, time in days; and A, constant. Half-life saturation period of the first-order biodegradation was obtained using

$$t_{1/2} = \frac{\ln(2)}{K} \quad (5)$$

The rate of disappearance of the COD demand, which is considered as substrate concentration for the organism, was observed for regular interval of time. Using equation (5), the experimental data was fitted to evaluate the specific degradation reaction kinetic constant and the order of the reaction. Simultaneously, Biomass concentrations and CO_2 release were noted. Automatic on-line analysis of CO_2 in the exit gas from the flask allows the real time kinetic information of the culture. The response of CO_2 release was correlated with other factors such as biomass, percentage decolorization and COD reduction. The percentage release of CO_2 was measured by using CO_2/O_2 gas analyzer (New Brunswick Scientific, USA, EX-2000) which contains infrared zirconium oxide type sensor with biocommand multiloop controller. The microbial growth kinetics has been well documented by an empirical model proposed by Monod (1942). The model introduced the concept of growth-limiting substrate.

$$\mu = \frac{1}{X}\left(\frac{dX}{dt}\right) = \frac{\mu_{\max} S}{K_S + S} \quad (6)$$

Rearranging the above equation leads to

$$\frac{1}{\mu} = \left(\frac{K_S}{\mu_{\max}}\right)\frac{1}{[S]} + \frac{1}{\mu_{\max}} \quad (7)$$

Where μ is the specific growth rate, μ_{max} is Maximum specific growth rate, S = Substrate concentration, and K_s = Substrate saturation constant. Specific growth rate for various substrate concentration were determined using semi log plot of biomass versus time. Maximum specific growth rate and kinetic constant was calculated from the slope and intercept of the plot $1/\mu$ vs 1/[S] of the equation (7).

Biokinetic model parameter estimation

The classical method of obtaining kinetic constants is to linearize kinetic models. Non-linear least squares computer fitting of data model equation has been used. The non-linear least square fitting routine of Matlab 6.5 software package was used to fit the kinetic models to different batch experimental data. The parameters of Monod, Haldane and Yano were fitted to the experimental calculated phenol consumption and degradation.[21] In this investigation, three models Monod, Haldane (Andrew, 1968), and Yano and Koga (1969) were used to fit the ki-

netic models to different batch experimental data. Biodegradation kinetics constants were estimated using MATLAB – 7.06 software package. The following models were used:

$$Haldane(Andrews,1968): r_s = \frac{r_{s,\max} S}{K_s + S + \frac{S^2}{K_i}} \quad (8)$$

$$Monod(1949) := \frac{r_{s,\max} S}{K_s + S} \quad (9)$$

$$YanoandKonga(1969): r_s = = r_{s,\max} \frac{C_S}{K_s + C_S + \frac{C_S{}^2}{K_i} + \frac{C_S^3}{K_2^2}} \quad (10)$$

Analytical method

During biodegradation of the distillery spent wash, samples were analyzed for produced metabolite compounds using GC-MS analysis. Before analysis, the samples were processed following the protocol.[22] The treated samples collected were centrifuged to remove suspended solids and microbial solid broth. Supernatant samples from the centrifuge were acidified to pH 2 using hydrochloric acid and extracted with ethyl acetate for first stage and methanol for the second stage using soxhlet apparatus. The organic layer was collected, dewatered over anhydrous Na-SO_4 and filtered. The residues were dried under a stream of nitrogen gas. The extract was analyzed as trimethylsilyl derivatives. In this method, 100 μl dioxane and 30 μl pyridine were added to the extract sample followed by silylation with 50 μl trimethylsilyl and trimethyl chlorosilane. The mixture was heated at 60 °C for 15 minutes and placed in the orbital shaker to dissolve the residue. The analysis was carried out using gas chromatography-mass spectroscopy (Thermo GC-TRACE ULTRA VER:5.0, Thermo MS DSQ-II) equipped with a capillary column (DB-35–Nonpolar column 0.25 μm film thickness × 0.25 mm id × 30 m). One microliter of each extract was injected to analyze at preset conditions of 40–270 °C. Carrier gas used was helium with a flow rate of 1.0 mL min^{-1}. The data obtained was compared with NIST library inbuilt standard chemical library system of GC-MS.

Results and discussion

Strain identification

The isolated strain was taxonomically identified based on the method of 18S rRNA genes and physiological characteristics. Sequence alignment in NCBI revealed a 99 % similarity to the sequence of *Cladosporium cladosporioides* strain which is shown in Table 1. 18SrDNA partial sequence of strain *C.cladosporioides* submitted to NCBI had been assigned the nucleotide database accession number JN592511 from Gene Bank, Bethesda, Maryland, USA. The identified gene sequence was as follows:

ATCATTACAAGTGACCCCGGTTTACCACCGG
GATGTTCATAACCCTTTGTTGTCCGACTCTGT
TGCCTCCGGGGCGACCCTGCCTTCGGGCGG
GGGCTCCGGGTGGACACTTCAAACTCTTGC
GTAACTTTGCAGTCTGAGTAAACTTAATTAA
TAAATTAAAACTTTTAACAACGGATCTCTTG
GTTCTGGCATCGATGAAGAACGCAGCGAAA
TGCGATAAGTAATGTGAATTGCAGAATTCAG
TGAATCATCGAATCTTTGAACGCACATTGCG
CCCCCTGGTATTCCGGGGGGCATGCCTGTTC
GAGCGTCATTTCACCACTCAAGCCTCGCTTG
GTATTGGGCAACGCGGTCCGCCGCGTGCCTC
AAATCGACCGGCTGGGTCTTCTGTCCCCTAA
GCGTTGTGGAAACTATTCGCTAAAGGGTGCT
CGGGAGGCTACGCCGTAAAACAACCCCATT
TCTAAGGTTGACCTCGGATCAGGTAGGGATA
CCCGCTGAACTTAAGCATATCAATTAAGCGG
AGGA

Table 1 – *Nucleotide sequence percentage homology table indicating the identity with neighbors*

Sequences producing significant alignments:1287			
accession	description	query coverage	max ident
EU497957.1	Cladosporium cladosporioides strain F28b	99 %	99 %
HM535372.1	Cladosporium sp. E-19	100 %	99 %
EF405864.1	Cladosporium cladosporioides strain Hu01	99 %	99 %
JF449742.1	Uncultured Cladosporium clone OS_2w_G08	100 %	99 %
EF029809.1	Cladosporium sp. HKB6	99 %	99 %
GU909522.1	Uncultured Capnodiales clone 9SB1cb01	99 %	99 %
HQ211815.1	Uncultured Cladosporium clone 6_j08	99 %	99 %
GQ153060.1	Dothideomycetes sp. 11048	100 %	99 %
EU670719.1	Cladosporium cladosporioides strain F29	100 %	99 %

Optimization of process parameters using RSM

The optimum value obtained through the single factorial experiment to achieve maximum percentage decolorization was used as center point for deciding experimental range and levels of independent variables, as shown in Table 2.

Table 2 – *Experimental range and level of independent variables*

Independent variables	Low level	High level
Carbon (Fructose)(g L^{-1})	3	7
Nitrogen (Peptone)(g L^{-1})	2	5
pH	3	6
Inoculum Concentration (%w/v)	5	10

Center composite design matrix was generated using RSM as shown in Table 3, and 30 experiments were carried out as per the experimental design. Using the experimental results, the regression model equation relating to removal efficiency and process parameters were found to be:

$$\eta = 36.2 + 5.095x_1 + 0.705x_2 + 3.2675x_3 + \\ + 0.9825x_4 + 0.5275x_1^2 + 3.7387x_2^2 - \\ - 0.12625\,x_3^2 + 1.21125x_4^2 - 0.27875x_1x_2 + \\ + 0.8075x_1x_3 + 0.23x_1x_4 + 0.255x_2x_3 + \\ + 1.995x_2x_4 - 3.0575x_3x_4. \quad (8)$$

Where x_1 is the concentration of fructose, x_2 is the concentration of peptone, x_3 is the effect of pH, and x_4 is the effect of inoculum concentration. This mathematical model explains the relationship between the independent variables and the dependent response (% decolorization). As shown in Table 3, it was observed that the coefficient for the linear effect of carbon concentration 0.0003, pH 0.0083 was highly significant, and that of peptone 0.5218, inoculum concentration 0.3752 was least significant. Whereas the coefficient of quadratic effect of CC 0.0659 and DD 0.0083 was highly significant. It was also observed that the interaction effect AC 0.0124 was highly significant, while AB 0.9249 was found to be least significant. Hence, the linear effect and interaction between carbon concentration and pH was high compared to other parameters for the response. The analyses were done by means of Fisher's 'F'-test. Generally, the high 'F' value with a low probability 'P' value lower than 0.05 indicates high significance of the regression model.[23] So, it was identified that fructose concentration and pH plays a major role compared to the concentration of inoculum and peptone.

Table 3 – *Full factorial central composite design matrix (CCD)*

Run	Carbon (Fructose) X_1(g L^{-1})	Nitrogen (Peptone) X_2 (g L^{-1})	pH X_3	Inoculum concentration X_4 (w/v)	% Decolourization
1	1	3.5	4.5	7.5	21
2	7	2	6	10	57.2
3	7	2	6	5	48.5
4	5	6.5	4.5	7.5	35
5	3	5	6	5	32.5
6	3	2	6	10	32
7	7	5	6	5	38.2
8	5	3.5	4.5	7.5	36.2
9	7	2	4	5	24.3
10	5	3.5	2.5	7.5	23
11	3	5	4	10	31.5
12	3	2	4	10	31
13	3	5	4	5	22.47
14	3	5	6	10	29.95
15	5	3.5	4.5	2.5	27.5
16	5	3.5	4.5	12.5	18
17	5	3.5	7.5	7.5	31
18	5	3.5	4.5	7.5	36.2
19	5	6.5	4.5	7.5	37
20	5	3.5	4.5	7.5	36.2
21	5	3.5	4.5	7.5	36.2
22	7	5	4	10	28.28
23	3	2	4	5	27.68
24	5	3.5	4.5	7.5	36.2
25	7	2	6	10	62.5
26	7	5	4	5	42.5
27	7	2	4	10	30
28	9	3.5	4.5	7.5	52.8
29	3	2	6	5	19.5
30	5	3.5	4.5	7.5	36.2

From the ANOVA summary table 4, the model was found to be statistically significant ($P < 0.01$) at 99 % confidence level. Further, the fitting of the experimental data to the regression model was checked and exactly explained by the value of determination coefficient ($R^2 = 0.8551$). It indicates that approximately 14.49 % of the total variations in the percentage decolorization were inadequately explained by the model (Eq.8). In order to confirm the ade-

quacy of the model fits, F-value should be greater than the tabulated value of the F-distribution for a certain number of degrees of freedom.[24] Here the F-ratio obtained, 6.32, is clearly greater than the tabulated F (1.94 at 95 % significance) which confirms the fit of the model. Hence, the estimated model fits the experimental data adequately. Based on the results, reduced model was calculated and the regression model was found to be:

$$\eta = 36.2 + 5.095x_1 + 3.2675x_3 - \\ - 0.12625\ x_3^2 + 1.21125x_4^2 + 0.8075x_1x_3 \quad (9)$$

Table 4 – *ANOVA results for the equation of Design Expert 8.0*

Source	Sum of squares	DF	Mean Square	F Value	P Value Prob > F
Model	2472.50	14	176.60	18.97	0.0060
A-carbon	623.02	1	623.02	22.47	0.0003
B-Nitrogen	11.93	1	11.93	0.43	0.5218
C-pH	256.24	1	256.24	9.24	0.0083
D-Inoculum concentration	23.17	1	23.17	0.84	0.3752
AB	4.45	1	4.45	0.16	0.6943
AC	223.65	1	223.65	1.07	0.0124
AD	0.26	1	0.26	0.01	0.9249
BC	23.47	1	23.47	0.85	0.3721
BD	1.24	1	1.24	0.04	0.8352
CD	10.43	1	10.43	0.38	0.5488
A^2	1.45	1	1.45	0.05	0.8222
B^2	1.78	1	1.78	0.06	0.8032
C^2	109.17	1	109.17	1.94	0.0659
D^2	256.41	1	256.41	1.25	0.0083
Residual	418.82	15	27.93	–	–
Lack of Fit	415.96	10	41.60	–	–
Pure Error	2.85	5	0	–	–
Corrected Total	2891.32	29	–	–	–

An earlier report revealed that RSM had been utilized to optimize the factors for the decolorization of distillery spent wash through coagulation.[20] But to our knowledge, there have been no reports on RSM study of fungal decolorization of distillery spent wash. Response surface and contour plots to estimate the percentage decolorization over independent variables are shown in the figures. Fig. 1 presents the combined interaction effects of the parameters AC, AB, AD,BC,BD, and CD on percentage decolorization as response. It was observed that the interaction effect of AC is higher than the other effects. This is because the organisms acquire sufficient carbon source from the spent wash in an optimum ph level, leading to an increase in decolorization. The percentage decolorization increased to a maximum of 62.5 % with carbon concentration of 7 g L^{-1} and pH 6. Generally, circular contour plots indicate that the interactions between parameters are negligible. On the contrary, elliptical ones indicate evidence of interactions.[25] The convex response surface suggested well-defined optimum variables (Fructose and pH) and that the decolorization increased with increase in fructose and pH up to 7 g L^{-1} and 6 respectively, then declined with further increase of these two parameters. The coordinates of the central point within the highest contour levels will represent the optimum values of the respective constituents. It was observed that maximum of 62.5 % decolorization and COD removal of 73.6 % (Initial 34,800 mg L^{-1}, final 9,170 mg L^{-1}) was obtained with fructose concentration of 7 g L^{-1}, Peptone 2 g L^{-1}, inoculum concentration 10 % (w/v), at pH 6, which was found to be same as that of experimental value.

Model valediction

In order to confirm the validity of the RSM model result, a confirmation experiment with triplicate set was conducted at the specified optimum process conditions (fructose concentration of 7 g L^{-1}, peptone 2 g L^{-1}, 6 pH and 10 % (w/v) inoculum). The percentage decolorization was found to be 60.5 %, which was close to the RSM result. Thus, the model was useful to predict the percentage decolorization and also the optimum process parameters for decolorization of distillery spent wash. However, interesting information was found after carrying out the same experiment in triplicate in the absence of nitrogen source and found that peptone was not required for the decolorization of the spent wash.

GC-MS analysis report

Microorganisms serve as an important tool for the simultaneous biodegradation and release of valuable compounds during the degradation of distillery spent wash. GC-MS Chromatogram of compounds extracted after the biodegradation of the distillery spent wash by *C.cladosporioides* are shown in Fig. 2. Identified metabolites and degradation products formed during the treatment of distillery spent wash are shown in Table 5. It was observed from NIST data base that some valuable compounds have been produced during the metabolic reaction of the organic compounds present in the spent wash, which can be purified further and added a market value.

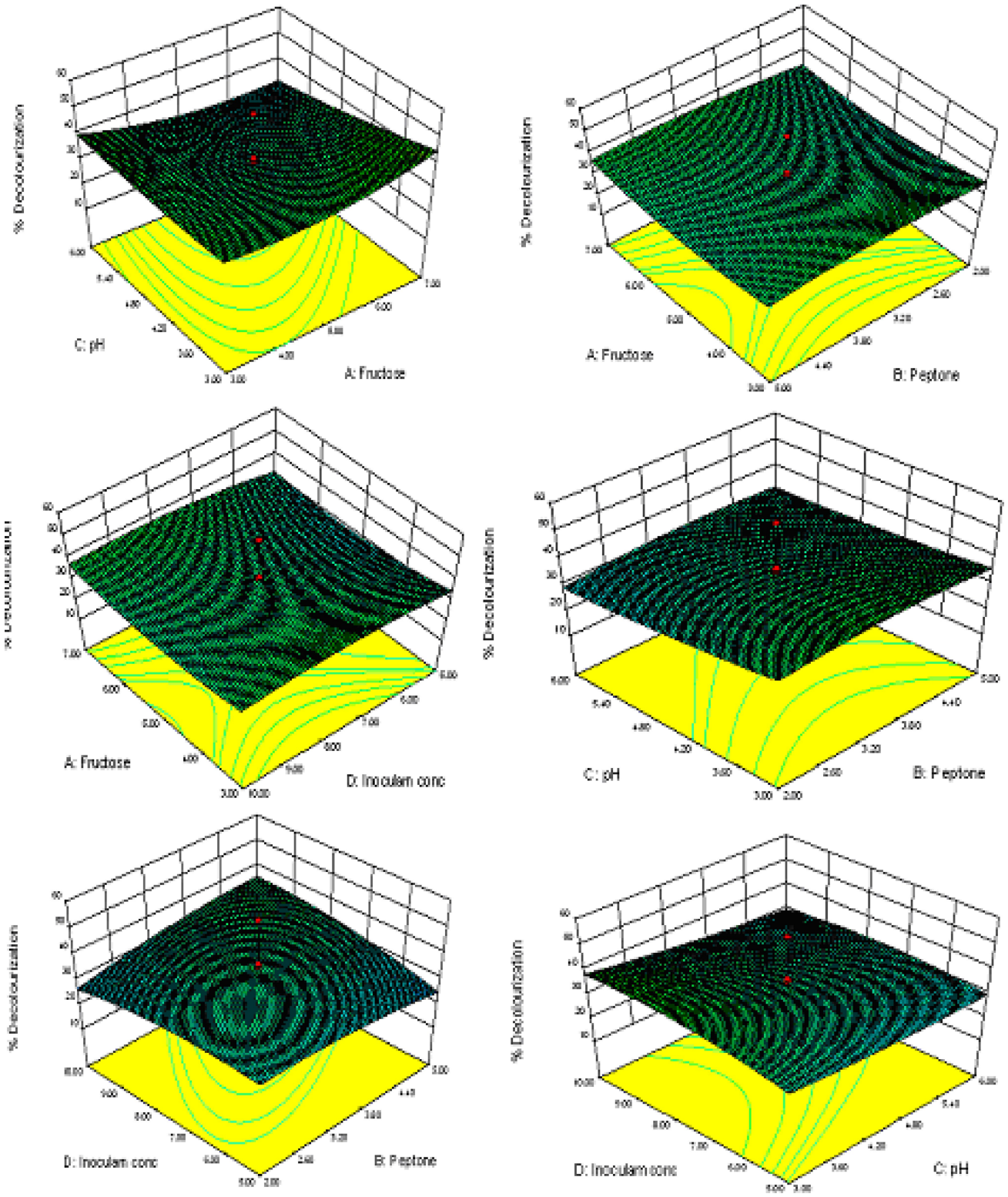

Fig. 1 – *Surface and contour plot showing the combined effect of the parameters AC, AB, AD, BC, BD, and CD on percentage decolorization*

Biokinetic study

Experimental data were fitted with equation (5) and the data analysis of the kinetic information from Fig. 3a revealed that the biodegradation of the distillery spent wash follows first-order rate equation with kinetic coefficient as $K = 0.138$ day^{-1} with constant $A = 0.020$. The half-life saturation period of the reaction was found to be 5.02 days. The R^2 value of 0.9670 with error of 0.009 reveals the better fit of the first-order equation. Specific growth rate increases with initial concentration of spent

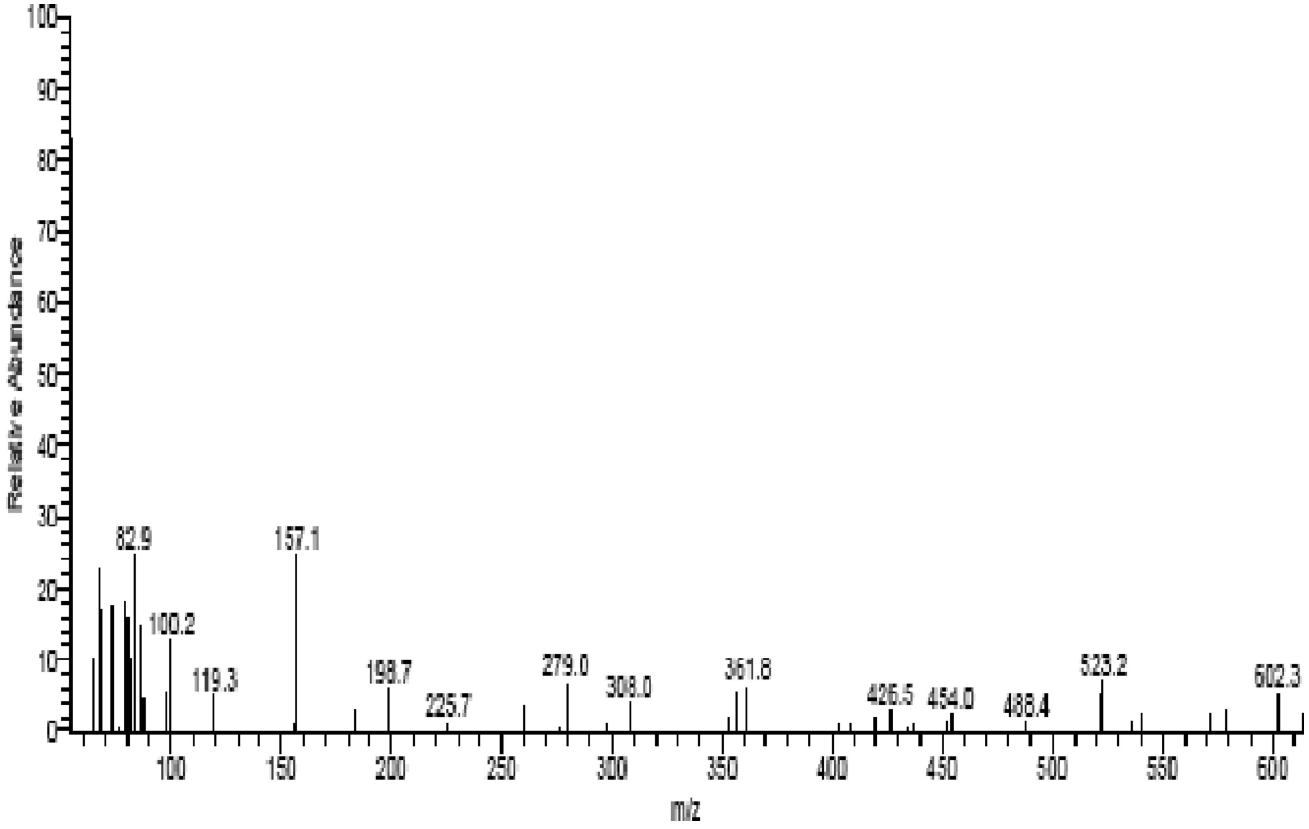

Fig. 2 – *GC-MS Chromatogram of compounds extracted from Spent wash treated using C.cladosporioides*

Table 5 – *Identified product released during the degradation of spent wash in using C.cladosporioides*

SI	RSI	Compound Name	Molecular Formula	Molecular Weight	Area %
416	833	4-Methylenebicyclo[5.3.0]-2-oxadecan-3-one	C10H14O2	166	3.08
411	832	8-NONEN-1-OL	C9H18O	142	3.08
400	906	2-OCTENYL ACETATE	C10H18O2	170	3.08
378	841	1,6-Heptadiene (CAS)	C7H12	96	3.08
370	849	(Cyanomethyl)cyclohexane	C8H13N	123	3.08
346	807	N-ethyl methylketene imine	C5H9N	83	3.08
344	884	2,7-Octadieniol acetate	C10H16O2	168	3.08
298	857	But-4-enyl but-3-ene-1-sulfonate	C8H14O3S	190	3.08
297	842	But-3-enyl Prop-2-enesulfonate	C7H12O3S	176	3.08
289	852	6-Hydroxy-hexanenitrile	C6H11NO	113	3.08
284	877	à-4,4-Bis(2,3-epoxypropyl)pent-1-ene	C11H18O2	182	3.08
274	814	3,5-trans-3-(Methylsulfonyloxy)-5-[(E)-1-pentenyl]-4,5-dihydro-2(3H)-furanone	C10H16O5S	248	3.08
260	824	4-Methylhex-5-en-1-al	C7H12O	112	3.08
225	818	5-Bromo-1-hexene	C6H11Br	162	3.08
125	839	1-(Hydroxymethyl)-3-methylene-1-cyclobutanol	C6H10O2	114	3.08
116	853	Cyclohexyl(2-methylenecyclopropyl)carbinol	C11H18O	166	3.08
114	839	cis-1-Bromo-2,2,3-trimethylcyclopropane	C6H11Br	162	3.08
112	807	2,5-Divinyl-tetrahydrothiophen-1,1-dioxide	C8H12O2S	172	3.08
107	851	à-4,4-Bis(2,3-epoxypropyl)pent-1-ene	C11H18O2	182	3.08
96	985	(1R,2R,3S,4Z)-1-(4'-Methyl-2'-trichloromethyl-2'-oxazolin-4'-yl)-1-hydroxy-2,3-O-isopropylidene-11-(2''-hexyl-1',3''-dioxolane-2-yl)-4-ene-2,3-undecanediol	C28H46Cl3NO6	597	3.08

wash until a maximum growth rate 0.0457 h^{-1} was reached, beyond which the rate was found to decrease. Earlier, it was reported that for *Aspergillus fumigatus* maximum specific growth rate was 0.0685 h^{-1} with R^2 value of 0.98.[26] In most of the work, fitness of the model is valued with the regression coefficient value which is to be close to value of 1. But still for some closer values the model is not validated. Therefore, R^2 value along with residual plot will give a best fit for the given model The residual plot is based on the difference between the calculated and measured values. If the residual values are randomly distributed and do not show a clear trend, then the model represents data correctly. Fig. 3b represents the random nature of the residual plot and confirms the fitness of the data to the model. Similar trend and nature of randomness in residual plot was found in the specific growth model of *Pseudomonas putida*.[27]

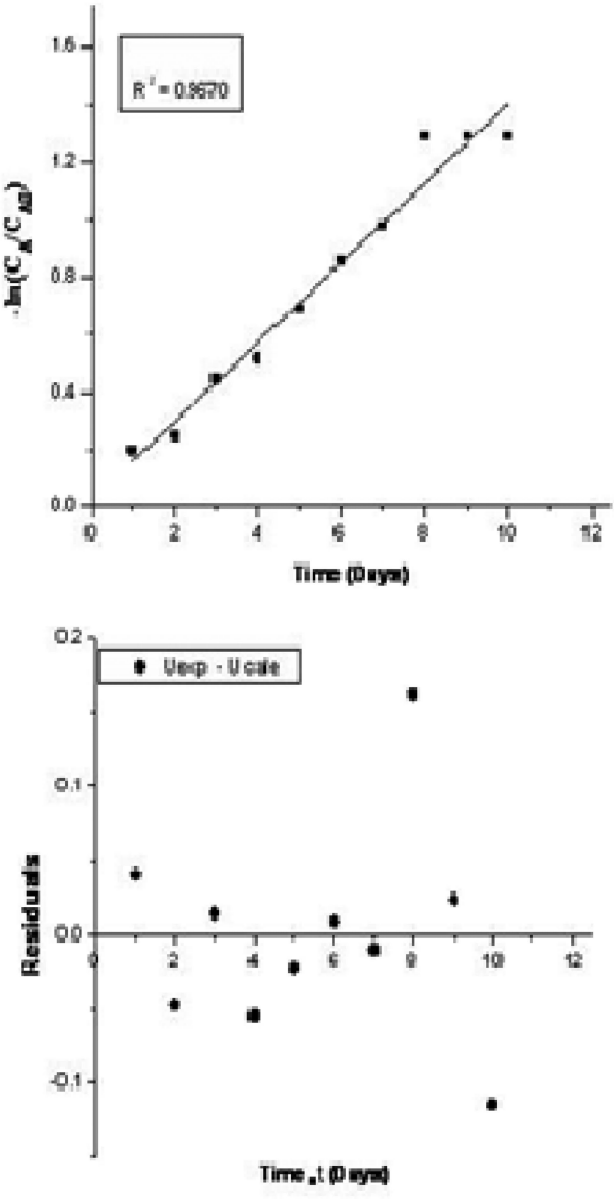

Fig. 3 – *a) Fitting of the experimental data with the first-order kinetic equation. b) Residual plots for the fitting of the first order kinetic equation*

Growth model and kinetic constants

From Fig. 4 it was observed that the rate of CO_2 released during the metabolism reached a maximum of 1.44 % (v/v) and then decreased. The growth of the organism attained a maximum of 8.2 g L^{-1} on the 7th day with giving a maximum decolorization of 62.5 % and COD removal of 73.6 %. This is mainly due to the presence of laccase enzyme secreted by the organism**.** Laccase and MnP enzymes extractacted from Fusarium verticillioides TERIDB16 were used for the decolorization of undiluted distillery effluent and obtained a maximum decolorization (37 %) using the enzyme extract of Pleurotus florida EM1303.[28] The treatment of undiluted distillery effluent for 40 days using fungal consortium on a bioreactor resulted in 61.5 % decolorization and 65.4 % COD reduction .[29] During the exponential phase of 2–5 days biomass growth and COD reduction the profile expressed a steep rise in the values with the utilization of spent wash and fructose as major source. Further, the biomass growth reached a stationary phase between days 6–8 followed by a decline phase. The maximum release of CO_2 release suggests that metabolic activity of the organism is high during the stationary phase. The rate of CO_2, biomass production are directly associated with melanoidin and organic maters con-

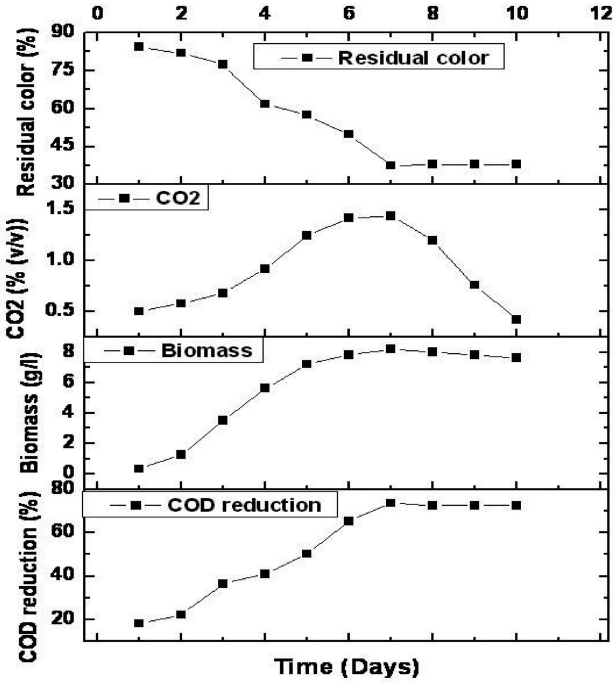

Fig. 4 – *Comparative plots of the experimental values as a function of time*

sumed during the decolorization. The production of CO_2 is mainly due to the sugar content present in the spent wash and the respiratory metabolism of the organism. The fit of the experimental data to Monod growth model with $\mu_{mas} = 0.282$ h^{-1} and constant $K_S = 12.94$ with R^2 value of 0.9655 indicates the suitability of the model for the growth of organism. This clearly indicates that the percentage decolorization of spent wash follows growth-associated kinetics. The experimental results of specific substrate (spent wash in terms of COD) consumption rate variation with time was fitted to three inhibition models of Haldane (Andrew, 1968), Yano and Koga (1969) and Teissier (Edwards, 1970) and is shown in Fig. 5. The kinetic parameters of the models (rsmax, Ks and Ki) were estimated using the non-linear regression routine of MATLAB 7.0. The kinetic parameters of the model were found to be K_S (mg L^{-1}) 79.78, R_{max} (h^{-1}) 0.678,K_I 7.25 and 34.56. Results revealed that among the three models used, the experimental data was best fitted with Andrews ($R^2 = 0.959$) while for the regression coefficient values were found to be Yano and Koga (0.9308) and Teissier ($R^2 = 0.941$).

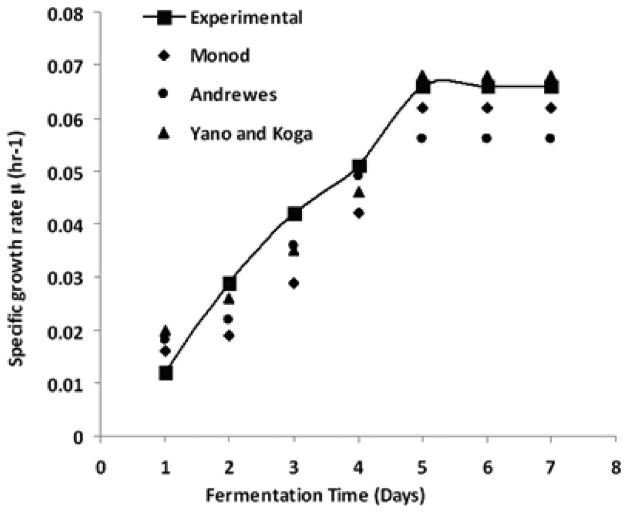

Fig. 5 – *Comparison of experimental data with models*

Conclusion

In summary, we have reported a novel fungi *Cladosporium cladosporioides* F28b with accession number JN592511 and proved that it has the ability to biodegrade and decolorize anaerobically treated distillery spent wash, with the optimized parameters obtained using RSM. From this investigation, it was observed that the percentage color removal efficiency is influenced by fructose concentration, pH, and inoculum concentration. The results of individual and combined interaction effects among the parameters revealed that peptone is very least significant and is not required for the growth of microbial decolorization, which was favorable for fixing a low processing cost. High regression coefficient values and random distribution nature of residuals with no trend confirm the biokinetic information. These results will facilitate the streamlining of our approach to bioreactor with the conduct of minimum experiments and low cost treatment.

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List of symbols

Symbols – Units
g L^{-1} – Grams per liter
w/v – Weigh per volume
°C – Degree centigrade
mg L^{-1} – Milligrams per liter
b_0 – Coefficient of interception
b_i – Coefficients of linear effects
b_{ii} – Coefficients of quadratic effects
b_{ij} – Coefficients of interactive effects (i < j)
X_i – Process variables
R^2 – Coefficient of determination
y – Percentage of color removal
β_0 – Offset term,
β_i – Coefficient of individual effect
β_{ii} – Coefficient of squared effect
β_{ij} – Coefficient of interaction effect
μ_{max} – Maximum specific growth rate
K_s – Substrate saturation constant
μ – Specific growth rate
K_i – Substrate Inhibition constant

List of abbreviations

COD – Chemical Oxygen demand
rRNA – Ribosomal ribonucleic acid
PCR – *Polymerase Chain Reaction*

NIST– National Institute of Standards and Technology
RSM – Response surface methodology
CCD – Central composite design
ANOVA – Analysis of variance
NCBI – National Center for Biotechnological Information
GC-MS – Gas Chromatography Mass Spectrometry
ADSW – Anaerobically treated distillery spent wash

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