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Optimization of Process of Parameters for Bacterial Decolorization of Paper Mill Effluent and Its Metabolite

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Abstract

Decolorization of pulp effluent was optimized by bacterial consortium using response surface methodology. The degree of significance of the proposed design was evaluate using *p*-value, *F*-value, R^2 and adjusted R^2. According to design, the predicted results were close resembled to the expected experimental values under optimum conditions (Carbon source 2%, agitation speed 200 rpm and pH 8.0), which indicated good quality evaluation of experimental data. The analysis of effluent after bacterial treatment compared to its control indicated that the consortium utilized the constituents of pulp paper effluent rather than biotransformation. In the knowledge of author's, this study reveals a great use of statistical optimization for effective bacterial treatment of pulp paper effluent (in terms of saving time, manual efforts) for environmental safety point of view.

Keywords: Statistical optimization, bacteria, RSM, pulp paper effluent, GC-MS

Contents

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1. Introduction

Pulp and paper mill are major industrial sectors using a huge amount of lignocellulosic raw materials and water during manufacturing processes, and releasing lignin, chlorinated lignosulphonic acid, chlorinated resin, chlorinated phenol and chlorinated hydrocarbon in the effluent. Discharge of inadequate treated effluent from the pulp and paper industry causes slime growth, thermal impacts, scum formation, color problems, and loss of aesthetic beauty in the environment (D Pokhrel et al., 2004).During the last decade various physico-chemical treatment methods developed for the removal of color and toxicity of pulp and paper mill effluents. However, these methods are less desirable than biological treatment because of the cost effectiveness (MT Das et al., 2012). The biological methods tried so far, most of the literature is confined to a few genera of white rot fungi. But, bacteria seem to be more effective than fungi for the bioremediation of environmental pollutants due to their immense environmental adaptability and biochemical versatility (N Jain et al., 1997; S Singh et al., 2008; JJ Ko et al., 2009). Although, decolorization of pulp paper effluent is well reported by pure culture (H Morii et al., 1995; N Jain et al., 1997; MT Das et al., 2012), the results of such studies are not necessarily relevant to the field because microorganisms in nature grow mostly in mixed condition.

The conventional orthogonal approach (one-factor at a time) for optimization of the process parameters requires a very large number of experimentation, which would be very expensive and time consuming. To overcome these drawbacks, one of the statistical design tools, so called Response surface methodology (RSM), can be used for the process optimization, and prediction of interaction between several process variables. RSM is a collection of statistical techniques for designing experiments, building models, evaluating the effects of factors and searching for the optimum conditions (KP Singh et al., 2012).

The most important part before applying the RSM methodology is the selection of appropriate design of experiment. It has a large influence on the building of response surface and thus, on the precision of its prediction. In the present research work, three bacterial strains (*Paenibacillus* sp., *Aneurinibacillus aneurinilyticus, and Bacillus* sp.) have been isolated on the basis of their color and lignin content reduction efficiency in mineral salt medium at pH 7.6 containing 1000 mg/l kraft lignin (data not shown). A central composite design (CCD) was employed for the optimization of bacterial decolorization of pulp paper mill effluent. Further, by taking advantage of gas chromatography-mass spectrometry analysis, decolorization of pulp paper mill effluent was done which will be useful for the management of pulp paper mill effluent.

2. Materials and Method

Sampling site and sample collection

Effluent was collected from wash machine section of *M/s.* Century Pulp Paper Mill, Lalkuan, Nainital, Uttrakhand, India located (79 °10’E longitude and 29 °3’N latitude) at foot hills of Himalayas. The samples were taken into pre-sterilized container (20 liter capacity), test tubes, and immediately preserved at 4 °C in June 2012. Further, the physico-chemical parameters were accomplished as described in standard methods for the examination of water and wastewaters (APHA, 2005). Metals and different salts (chloride, sodium and nitrate) were analyzed by atomic absorption spectrophotometer and ion meter (Orion Model 960) using selective ion electrode.

Micro-organism and culture conditions

Isolated bacterial strains *Paenibacillus* sp., *Aneurinibacillus aneurinilyticus, and Bacillus* sp.were used for the treatment of pulp paper mill effluent (data not shown). The cultures maintained at -20 °C were used to inoculate L-MSM agar plates of following composition (mg/ml): K_2HPO_4, 85; KH_2PO_4, 17; $MgSO_4$, 30; $FeSO_4 \cdot 7H_2O$, 30; CaSO4, 30; $MnSO_4 \cdot H_2O$, 30; $(NH_4)_2\ SO_4$, 17; trace element solution (1 ml/l). After 6th day of growth on solid medium at 30 °C these strains were developing as a consortium in the chemostate by continuous enrichment mineral salt media (MSM) along with kraft lignin (1000 mg/l) and pentachlorophenol (500 mg/l).

The stability of consortium (mixture of *Paenibacillus* sp., *Aneurinibacillu saneurinilyticus, and Bacillus* sp.) was maintained in same media. For decolorization study, pulp paper mill effluent having COD 23,700± 707; BOD 8,250±102 and initial color 6700 CU was used throughout the experiment. Two percent (v/v) overnight grown suspension of constructed consortium (innoculum size 31 x 10^4, 28x10^4 and 33x 10^4 for *Paenibacillus* sp., *Aneurinibacillus aneurinilyticus, and Bacillus* sp.,respectively) was transferred aseptically to 250 ml flask containing 98 ml pulp paper mill effluent. During the decolorization period, samples were taken at before and after treatment and analyzed for reduction of pollution parameters (color, COD and BOD) and toxicity evaluation.

Reduction of pollution parameters

Reduction in color in control and treated samples were accomplished as described in standard methods for the examination of water and wastewaters (APHA, 2005). Absorbance value (for color) were transformed into color unit (CU) according to the equation CU = 500 A_2/A_1, where A_1 is the absorbance of 500-CU platinum-cobalt standard solution (A_{465} = 0.129) and A_2 is the absorbance of effluent sample.

Experimental Set up

Experiments were conducted to choose the best carbon source, Agitation speed; pH; temperature and incubation time. After selecting the three most influencing factor, RSM inbuilt with CCD was employed. The three parameters (factors) i.e. pH (X_{pH}), Agitation (X_{Agi}) and Carbon source (X_c) were investigated at five levels (-√2,-1, 0, +1, +√2) each to optimize the dependent response variables i.e. % decolorization (Y1). The study included 15 experiments with 5 runs at central level as replicates. A regression model was proposed, and results were analyzed using Design Expert software version 7.0 (Minitab Institute, USA). The responses (Y_1 and Y_2) were related to chosen variables by full second-order polynomial (quadratic) model. The second-order model in terms of coded variables can be expressed as follows:

$$Y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_4 x_1 x_2 + \beta_5 x_1 x_3 + \beta_6 x_2 x_3 + \beta_7 x^2_1 + \beta_8 x^2_2 + \beta_8 x^2_3 \qquad (1)$$

Where 'Y' is the predicted response; β_0 is intercept term, β_1, β_2 and β_3 are first order linear coefficients; β_4, β_5 and β_6 are the interaction coefficients; while β_7, β_8 and β_9 are the second order quadratic coefficients. All 15 experiments were conducted in triplicates to take the mean value.

Metabolite characterization by GC-MS analysis

The samples (untreated and treated) were centrifuged and extracted thrice with equal volume of ethyl acetate. The ethyl acetate extract was vacuum dried and residue was dissolved in HPLC grade acetonitrile and used for metabolites characterization (Chandra and Abhishek, 2011). For GC-MS analysis, the dry residues of ethyl acetate extracts were derivatised with trimethylsilyl (BSTFA (N, O-bis (trimethylsilyl) trifluoroacetamide) TMCS).An aliquot of 1 µl of silylated compounds was injected into the GC-MS equipped with a PE Auto system XL gas chromatograph interfaced with a Turbo mass Mass spectrometric mass selective detector. The analytical column connected to the system was a PE-5MS capillary column (20 m x 0.18 mm i.d., 0.18 µm film thickness). Helium gas with flow rate of 1 ml min^{-1} was used as carrier gas. The column temperature was programmed as 50 °C (5 min); 50–300 °C (10 °C min^{-1}, hold time: 5 min).

Table 1. Physico-chemical parameters of pulp paper mill effluent. All the values are in mg/l except color and pH; BDL: below detection limit; *Permissible limit of trace elements in wastewaters (USEPA, 2000).

S. No.	Parameters	Pulp paper mill effluent	Permissible Limit (EPA 2000)*
1.	pH	8.0±1.0	5-9
2.	TDS	1023±9.2	-
3.	COD	23,700±707.2	120
4.	BOD	8250±102.0	40.0
5.	BOD/COD ratio	3.4±0.3	-
6.	Color (CU)	6700±56.3	Colorless.
7.	Lignin	1500±18.4	-
8.	Total Nitrogen	116±32.8	25.0
9.	Sulphate	993±6.3	-
10.	Phosphate	8.3±0.3	-
11.	Nitrate	173.30±6.2	10.0
12.	Heavy metals		
	Cd	BDL	0.01
	Cr	BDL	-
	Cu	0.09±0.14	0.20
	Fe	10.22±9.02	5.00
	Ni	5.03±1.02	0.20
	Zn	9.83±1.13	2.00
	Pb	BDL	0.05
	Mn	0.04±0.04	0.20

Table 2. Experimental design layout as per CCD along with the values of response variables. [x]the responses were the mean values of at least three experiment with ±1.1-4.21% standard deviation.

S. No.	pH	Agitation speed (rpm)	Carbon source (%)	% [x] Decolorization
1.	8	200	0.59	70
2.	7	130	1	73
3.	8	200	2	87
4.	8	200	3.41	80
5.	8	200	2	88
6.	8	200	2	86
7.	9	270	1	71
8.	8	300	2	84
9.	9.41	200	2	72
10.	7	270	3	73
11.	8	200	2	83
12.	8	200	2	84
13.	9	130	3	78
14.	6.59	200	2	72
15.	8	110	2	78

Table 3. Compound identified as trimethylsilyl (TMS) derivatives from control and treated samples

S.No	Compounds	RT	Control	Treated by Consortium
1.	Acetic acid	11.4	+	+
2.	2-Hydroxymethyl cyclopropane carboxylic acid	12.97	+	-
3.	2-Methoxyphenol	14.68	+	-
4.	Phthalic anhydride	15.7	+	-
5.	2,6-Dimethoxy phenol	15.96	+	-
6.	2-Methoxy-4-ethyl-phenol	16.38	+	-
7.	Unidentified compound	16.6	-	+
8.	Benzene acitic acid	17.58	+	-
9.	3-Allyl-6-methoxyphenol	19.04	+	-
10.	Ethanone, 1-(-4-hydroxy-3,5-dimethoxyphenyl)	19.97	+	-
11.	Benzoic acid	20.17	+	-
12.	1,2-Benzene dicarboxylic acid	20.95	+	-
13.	Hexadecanoic acid	21.68	+	+
14.	2-Methoxy-4-(1-propenyl) phenol	22.26	+	-
15.	Clionasterol acetate	22.29	+	-
16.	Octadecane	23.07	-	+
17.	Octadecanoic acid	23.29	+	-
18.	Methoxycinnamic acid	24.50	+	-
19.	Trimethyl-salyl	26.1	+	+

3. Results and Discussion

Chemical analysis of pulp paper mill effluent & bacterial consortium

The collected effluent was found slightly alkaline (pH-8) in nature and dark brown in color with high COD (23,700±707 mg/l), BOD (8,250±102 mg/l), color 6700 CU, nitrates (173±6.2 mg/l) and sulphate (993±6.3 mg/l) (Table 1). The concentration of metals in pulp paper mill effluent was 10.22, 5.03 and 9.83 mg/l for Fe, Ni and Zn, respectively which are very high than the permissible limit as reported in USEPA 2000. In the present work, three strains (*Paenibacillus* sp., *Aneurinibacillusaneurinilyticus, and Bacillus* sp.) were combined to construct a simple consortium for checking its generalized acceptance of its degradability and good controllability for treatment of industrial effluent.

Optimization of pulp paper mill effluent by RSM

Except glucose, all other tested carbon sources were less effective for pulp paper mill effluent treatment. RSM was employed to characterize the individual and interactive effects of process parameters on % decolorization for the treatment of pulp paper mill effluent. The results showed that a Carbon source 2% with agitation speed 200 rpm and pH 8.0 formed the best combination to increase decolorization up to 88 % (Table 2). The second-order model was hypothesized and statistically evaluated by analysis of variance (ANOVA) which gave the following regression equation for the % decolorization (Y_1).The regression model explains perfectly the experimental range studied. The observed % decolorization varied between 70-88% and the model prediction match these observational results satisfactorily. Thereafter, 3D plots for the measured responses (% decolorization) were constructed based on quadratic model. The influence of three different process parameters on decolorization are visualized in the 3D response surface plots (Fig. 1).

Metabolite characterization after decolonization

The GC–MS analysis of control sample has shown major peaks between 10 and 28 retention time (RT-min) (Fig. 2), detail of compounds generated from lignin and other phenolic compounds due to the decolorization process is listed in Table 3. The phthalate derivative had been also detected from photodecolorization of black liquor lignin as well as fungal peroxidase decolorization of lignosulfonate.

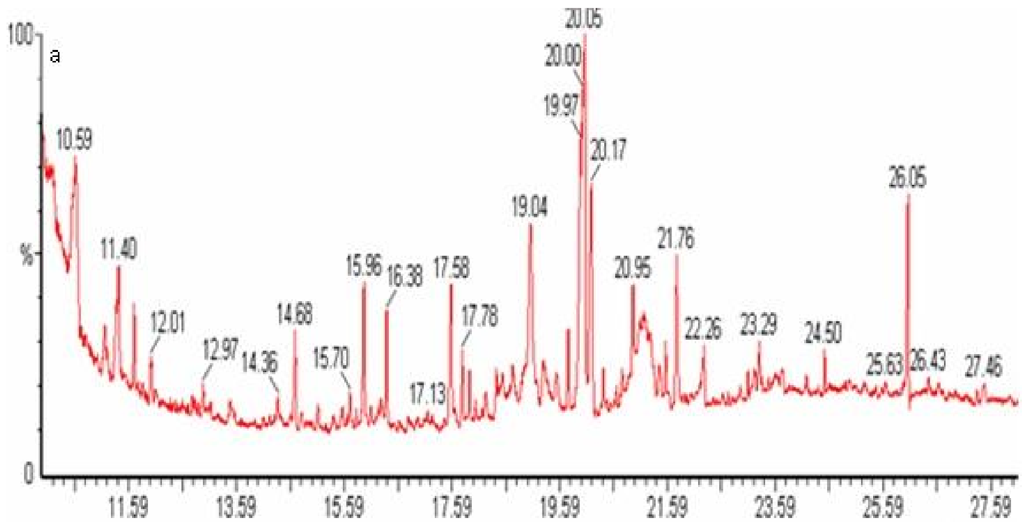

Figure 1. The 3D response surface plot showing effect of pH and Carbon Source; pH and Agitation (rpm),Agitation (rpm) and Carbon Source on % decolorization (A, B, C) of pulp paper mill effluent

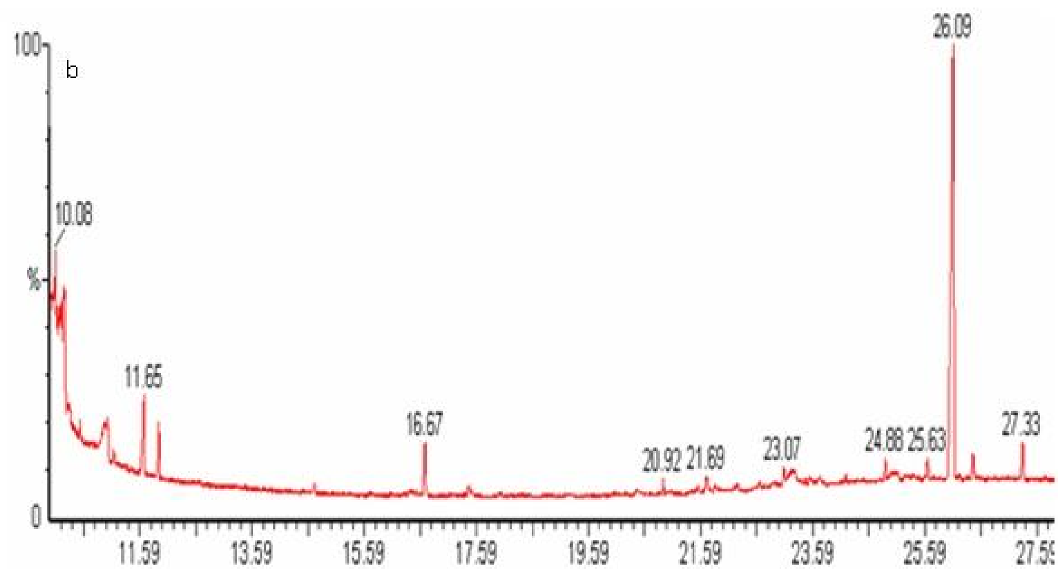

Figure 2. GC MS chromatogram of control (A); and bacterial consortium treated sample (B) of pulp paper mill effluent.

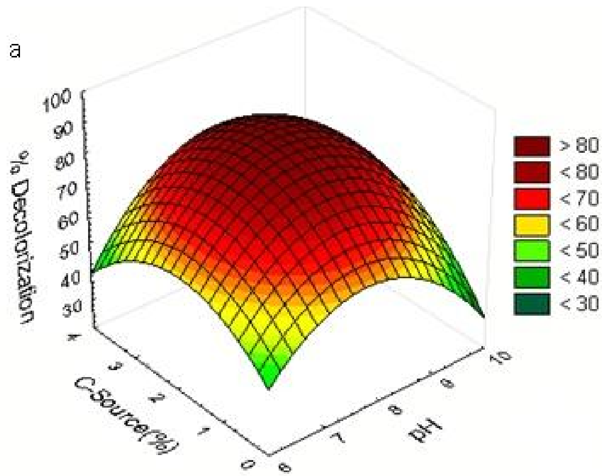

Figure.3

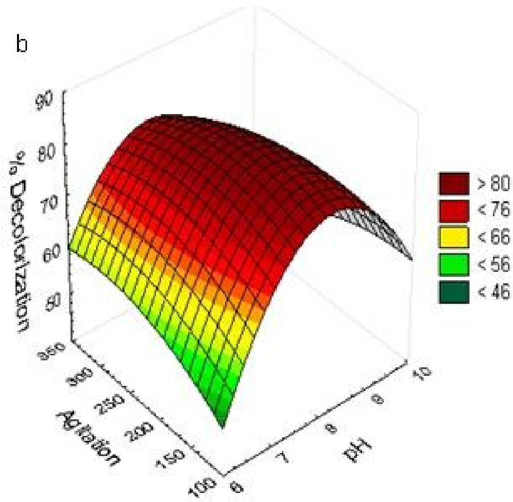

Figure.4

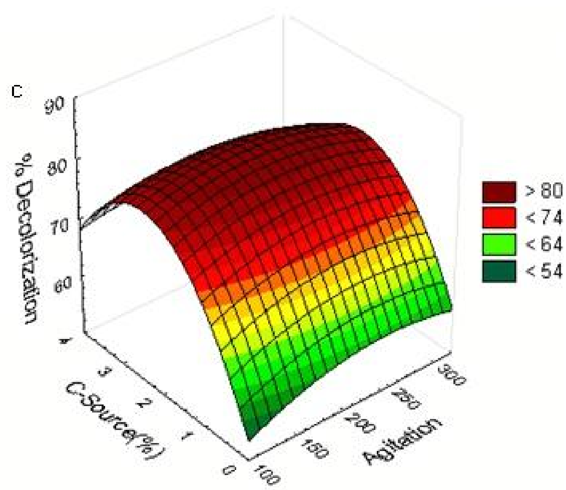

Figure.5

Discussion

Pulp-paper effluent was found slightly alkaline in nature and dark brown in color with high pollution parameters. The source of sulphate ions in effluent might be sodium sulphite, which is used in pulping process and the nitrates detected in effluent indicated the presence of nitrogen in lignin (A Singhal et al., 2009). These metals might be added in pulp paper effluent because of corrosion of digestion vessels and possibly due to bioaccumulation of these metals by plants which are used as raw material.

Due to the continuous presence of microorganism in their harsh environment with organic pollutants, genetic potency develops to degrade these compounds. Such type of strains can be enriched in the presence of metabolites to toxic compounds and significant strains will be evolved with the process of adaptation. To our knowledge, almost all microorganisms used for pulp paper effluent treatments were isolated from contaminated water or soil (A Raj et al., 2007; A Singhal et al., 2009; MT Das et al., 2012; Chandra et al.2011), and they could only handle, diluted or naturalized effluent. In the present work, three strains (*Paenibacillus* sp., *Aneurinibacillus aneurinilyticus, and Bacillus* sp.) were combined to construct a simple consortium for checking its generalized acceptance of its degradability and good controllability for treatment of industrial effluent. The bacterial consortium could adapt to extreme environments much better than axenic condition (data not shown), and make extreme environmental bioremediation (especially in the higher pH environment) easier and more practical. The effect of different process parameters i.e. carbon sources (glucose, galactose, dextrose and fructose); Agitation speed (100-300 rpm); pH (6–9); temperature (30-40 °C) and incubation time (12-72h) was optimized by the conventional (one-variable-at-a-time) method inbuilt with Their main first-order effects, interactions and second-order effects were determined through CCD by employing RSM. The results showed that a Carbon source 2% with agitation speed 200 rpm and pH 8.0 formed the best combination to increase decolorization up to 88 %. The second-order model was hypothesized and statistically evaluated by analysis of variance (ANOVA) which gave the following regression equation for the % decolorization (Y_1).The regression model explains perfectly the experimental range studied. The observed % decolorization varied between 70-88% and the model prediction match these observational results satisfactorily. The model adequacy was further checked using ANOVA.

The degree of significance and accuracy of the proposed regression model was evaluate using *p*-value, *F*-value, R^2 and adjusted R^2. The model *F*-values is 10.43 (*p*-value: 0.0094) imply that the model is significant (YJ Lee et al., 2012). The mathematical models' signal to noise ratio was well in control, as assessed by the values of adequate precision is 7.701, which were quite higher than the standard value of 4 (S Singh et al., 2008). For a model to fit the experimental data, the R^2 value should be close to 1. In present study, the R^2 value 0.95 also implies that 95 % of variations were explained by the independent variables within the range studied, and only 5 % of the total variations cannot be explained by the model. Moreover, result also showed that the values of R^2 for both the responses were in reasonable agreement with the adjusted R^2. This ensured a satisfactory adjustment of a quadratic model to the experimental data. In overall, results of the ANOVA indicate that the proposed model is statistically significant, and can be used to navigate the design space.

Pulp paper effluent is a complex aqueous system and it is difficult to comprehensively investigate the changes during the decolorization process. A lot of studies were focused only on optimization of the processes itself while few reports emphasized on the compositional changes during the bio-treatment process. The total ion chromatogram (TIC) of treated sample showed the massive consumption of compounds as compared to control indicated that the bacterial consortium has strong ability to utilize its constituents as carbon, nitrogen and energy source. Many aromatic compounds were detected in control sample, such as 2-methoxy phenol (Guaiacol) (RT 14.5, min), Benzene acetic acid (RT 17.5, min), Benzoic acid (RT 20.1, min) as low-molecular-weight phenolic units of lignin (A Raj et al., 2007; C Yang et al., 2008; JJ Ko et al., 2009). The phthalate derivative had been also detected from photo decolorization of black liquor lignin as well as fungal peroxidase decolorization of lignosulfonate (YJ Lee et al., 2012). Apart from this finding, other workers have also reported more acid-type compounds than aldehyde and ketone-type due to decolorization of lignin (R Chandra et al., 2012). Unfortunately, compounds like RT-16.6 min in bacterial treated sample that could not be identified by mass spectrometry. On the other hand, compounds such as trimethylsilyl (RT 26.1, min) remain unchanged because it is a derivatising agent which is used during the derivatization process (R Chandraet al., 2011; A joshi et al., 2013).

4. Conclusion

In this study we analyzed the analysis of effluent after bacterial treatment compared to its control indicated that the consortium utilized the constituents of pulp paper effluent rather than biotransformation. In conclusion, this study reveals a great use of statistical optimization for effective bacterial treatment of pulp paper effluent (in terms of saving time, manual efforts) for environmental safety point of view.

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