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Cost effective bioprocess for actual dye manufacturing industrial wastewater using statistical tool

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Dye contaminated wastewater is a cause of concern in textile and dyeing industry. By law, it is now mandatory to treat colour along with chemical oxygen demand (COD) from the effluent to required standards before discharging the same. In this context, bioremediation of textile effluent by bacterial consortium has gained considerable attention as it is relatively cost-effective and eco-friendly. Here, we propose a novel bacterial consortium consisting of *Bacillus* sp., *Proteus mirabilis*, *Alcaligenes faecalis* and *Bacillus cereus*, capable of removing 70% ADMI and 65% COD of effluent in mineral medium with 100% removal of heavy metals present in the effluent. Medium formulation by response surface methodology (RSM) method resulted in 89% COD reduction and 84% ADMI reduction from the SSDM effluent. The consortium was capable for degradation of SSDM effluent having as high as 21000 ADMI colour value. Analysis of untreated and treated SSDM effluent on UV-Vis spectroscopy and HPLC confirmed the mineralization of SSDM effluent. Induction of intracellular azo reductase (838%, 780%), NADH-DCIP reductase (288%, 216%) in addition to extracellular tyrosinase (102%, 309%) in F-MSMUJ and MSMYG medium, respectively indicates the vital role of oxido-reductive enzymes in the mineralization process. Single cell gel electrophoresis technique using *Allium cepa* showed decrease in tail length of comet for treated effluent compared to the untreated. Complete germination (100%) of plant seeds, *Vigna radiata* and *Trigonella foenum-graecum*, was achieved after treatment by the consortium in contrast to a meagre 20 and 30% germination of the respective plants in case of untreated effluent. Batch and fed batch treatment of SSDM effluent showed significant tolerance of consortium at high effluent load.

Keywords: *Alcaligenes faecalis*, *Bacillus cereus*, COD, Industrial dyes effluent, Jaggery, Phytotoxicity, Pollution, *Proteus mirabilis*, Response Surface Methodology (RSM), *Trigonella foenum-graecum*, Urea, *Vigna radiata*

Indiscriminate use of synthetic dyes in the textile industry has resulted in generating large volumes of dye contaminated wastewater[1]. Improper discharge of these effluents in water bodies affects the transparency to the extent of 10-200 mg/L dye concentration leading to aesthetic problems[2]. Earlier, as there was no colour discharge limits applied in wastewater treatment but only the permissible limits of chemical oxygen demand (COD), chemical coagulation followed by aerobic activated sludge treatment were used to treat dye containing effluent at common effluent treatment plant (CETP). However, of late, the government legislation became more stringent as in the developed countries with respect to the removal of colour along with COD from the industrial effluents[3].

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According to the Environment Protection Agency, it is mandatory to treat colour along with COD from the effluent to the required standards before discharging in sewage treatment plant and/or water bodies[1].

The effluents from textile and dyeing industries are relatively heavily coloured, with high concentrations of total dissolved solids, salts, pH and heavy metals, and also exhibit high BOD/COD values[4]. Jadhav *et al*.[5] has also reported presence of heavy metals like chromium, copper, lead, and nickel in textile dye effluent in Solapur, India. Further, direct and indirect toxic effects of the dyes and heavy metals can also lead to formation of tumours, cancers and allergies besides growth inhibition of bacteria, protozoan, algae, plants, animals and human beings[5,6].

Treating such a complex effluent containing heavy metals is a challenging. Biological treatments are relatively inexpensive and eco-friendly in removing dyes and metals from wastewater compared to the

available physicochemical treatment methods[1]. Therefore, using microbial consortia has received more attention, having considerable advantages, such as different strains may attack dye molecule at different positions or may use decomposition products produced by another strain for further decomposition[5]. Jadhav *et al*.[5] and Joshi *et al*.[7] have studied bioremediation of actual textile effluent by a bacterial consortium isolated from the local textile industries of Solapur and Ichalkarnji, India. But these reports have not taken into account the complex nature of the dye effluent containing varying concentrations of different dyes, salts and heavy metals. Though such data are essential for field application, the same have not been paid adequate attention.

At industrial level, one of the prime objectives is to reduce the cost of treatment process by optimizing the process parameters. The conventional classical optimization strategy (COS) is only time consuming but also fails to provide true optimum values. It involves changing one independent variable while keeping the other factors constant, especially the absence of interactions among factors[8,9]. However, response surface methodology (RSM) in combination with experimental design is an efficient strategy to determine the optimal conditions for a multivariable system. Recently, Asgher *et al*.[9] reported an optimization study of process parameters for textile effluent decolourization through RSM. However, work on an optimization of medium using crude substrates for efficient removal of dyes from the dye containing effluent through statistical methods is limited.

In this study, we evaluated the potential of available novel consortium to degrade and detoxify the actual industrial effluent, highly polluted in terms of colour and COD. Optimization of process variables was carried out including crude sources of nutrients through RSM to make the process economically feasible.

Materials and Methods

Sample collection

Soil samples were collected from dye contaminated areas of Shiv Shakti Dye Manufacturing (SSDM) Industry Pvt. Ltd. located at Vatva GIDC, Ahmedabad, India (N 22°; E 72°). Wastewater sample after the crystallization process of a dye manufacturing process called as dye mother liquor was collected in a sterile container from SSDM industry. The SSDM effluent was dark orange in colour and the exact composition was not disclosed due to privacy of industry. Physicochemical analysis of SSDM effluent *viz*. conductivity and pH were measured using a pH meter (Systronics, India, model 362). Chemical oxygen demand (COD) was determined by the standard potassium dichromate method[10]. The heavy metals were quantified using atomic absorption spectrophotometer (Elico, India, model SL-243). ADMI (American Dye Manufacturers Institute) colour value of SSDM effluent was measured using the ADMI 3WL tristimulus method[10]. The effluents used in the study were preserved in airtight plastic containers at room temperature until used.

Development of SSDM effluent decolourizing consortium

Enrichment for dye decolourizing microorganisms was initiated within 24 h of sampling. Soil sample was mixed thoroughly and 10 g well mixed sample was added in 90 mL sterile distilled water. The suspension was mixed thoroughly and allowed to settle for 15 min. Supernatants (10% v/v) from the resultant suspensions were used as an inocula in Erlenmeyer flasks of 250 mL filled with 100 mL sterile nutrient broth (Hi Media, India) medium containing 5% v/v SSDM effluent, the flasks were incubated under static condition at 30 ± 2°C for 48 h. Visual disappearance of colour of effluent was used as an indication of SSDM effluent decolourization. After decolourization was obtained, the enriched culture was re-inoculated (5% v/v) into fresh medium by adding SSDM effluent sterile 10% v/v for further enrichment purpose, and in this manner seven successive transfers were given.

For active inoculum development, acclimatized bacterial consortium was activated in 250 mL Erlenmeyer flask, containing 100 mL nutrient broth at 30 ± 2°C for 24 h under static condition. All decolourization experiments were carried out in triplicates in 250 mL Erlenmeyer flasks with 100 mL working volume of mineral salt medium (MSMYG) consisting (g/L): $MgSO_4$, 0.1; KH_2PO_4, 1.0; K_2HPO_4, 1.0; $(NH_4)_2SO_4$, 1.0; NaCl, 1.0 supplemented with 0.2% (w/v) each of yeast extract, glucose and 20% (v/v) SSDM effluent was inoculated with 10% (v/v) activated inoculum and incubated for different periods of time unless specified. Using the single variable approach, effect of different pH (4-11), temperature (20-50°C), salt concentration (2-12%), media constituents' concentration on decolorization of effluent by the consortium was checked.

Medium formulation, using crude substrates through RSM

Statistical methods for media optimization have proved to be a powerful and useful tool for biotechnology. Medium formulation using crude substrates by Response Surface Methodology (RSM) was carried out. The most popular RSM design is the Central Composite Design (CCD). Design Expert software 7.0.0 version (State-Ease, Inc., Minneapolis, USA) was applied to optimize and to ascertain individual and interactive effects of different variables (factors) through CCD[11]. The ranges of four important parameters were (A) jaggery (0.2-0.6% w/v); (B) urea (0.05-0.15% w/v); (C) NaCl (2.0-4.0% w/v); and (D) effluent (10-25% v/v). The percentage ADMI removal was the dependent response variable. The number of experiments (N) was estimated using Eq. (I)

$$N = 2n + 2n + nc \quad \ldots (I)$$

where, 'n' is the number of factors and 'nc' is the number of central points. Each of the independent variables was studied at five different levels as per CCD for four variables[12]. CCD consists of 16 factorial points, 8 axial points and 6 replicates resulted in 30 different combinations of experiments. Experimental runs number between 25 and 30 at the center point were used to determine the error of experiments and reproducibility of the data (Table 1). ADMI removal (response Y), was explained (Eq. II) as a second order response surface model with four independent variables

$$Y = \beta_o + \Sigma\beta_i X_i + \Sigma\beta_{ii} X_i^2 + \Sigma\beta_{ij} X_i X_j \quad \ldots (II)$$

where, β0, βi, βii, and βij represent, respectively, the constant process effect in total, the linear, quadratic effect of Xi and the interaction effect between Xi and Xj on ADMI removal of dye effluent. Analysis of Variance (ANOVA) was carried out to describe the coefficients of the quadratic equation. The 3D graphs were created to understand the interaction of different factors, and the graphs were used to evaluate the optimized components and conditions of the medium, which influences the responses. The point prediction is a unique feature of this software, which was used to confirm the obtained optimum value.

Table 1—Full factorial central composite design matrix for ADMI removal of SSDM effluent and results

Standard Order	Run Order	Jaggery A	Urea B	NaCl C	Effluent D	Experimental % ADMI removal	Predicted % ADMI removal	ADMI removal
1	20	0.20	0.05	2.00	10.00	74.94	74.83	3078
2	22	0.60	0.05	2.00	10.00	76.89	76.24	3158
3	27	0.20	0.15	2.00	10.00	83.46	82.40	3428
4	29	0.60	0.15	2.00	10.00	83.64	83.12	3435
5	2	0.20	0.05	6.00	10.00	66.15	65.60	2717
6	13	0.60	0.05	6.00	10.00	70.31	69.28	2888
7	21	0.20	0.15	6.00	10.00	66.24	65.63	2720
8	14	0.60	0.15	6.00	10.00	68.76	68.62	2824
9	19	0.20	0.05	2.00	25.00	50.36	48.43	3775
10	6	0.60	0.05	2.00	25.00	40.98	41.33	3072
11	1	0.20	0.15	2.00	25.00	59.30	60.06	4445
12	23	0.60	0.15	2.00	25.00	53.78	52.26	4031
13	3	0.20	0.05	6.00	25.00	48.68	48.94	3649
14	18	0.60	0.05	6.00	25.00	58.78	57.77	4406
15	16	0.20	0.15	6.00	25.00	54.45	53.02	4082
16	7	0.60	0.15	6.00	25.00	61.31	61.16	4596
17	26	0.00	0.10	4.00	17.50	36.78	37.95	2457
18	30	0.80	0.10	4.00	17.50	78.23	79.40	5226
19	11	0.40	0.00	4.00	17.50	56.67	57.84	3786
20	25	0.40	0.20	4.00	17.50	75.10	75.03	5017
21	24	0.40	0.10	0.00	17.50	77.34	77.48	5166
22	15	0.40	0.10	8.00	17.50	45.78	46.95	3058
23	12	0.40	0.10	4.00	2.50	91.00	91.33	1016
24	17	0.40	0.10	4.00	32.50	38.03	38.36	4102
25	28	0.40	0.10	4.00	17.50	64.23	64.85	4206
26	5	0.40	0.10	4.00	17.50	63.45	64.85	4155
27	9	0.40	0.10	4.00	17.50	66.52	64.85	4356
28	4	0.40	0.10	4.00	17.50	63.25	64.85	4142
29	8	0.40	0.10	4.00	17.50	65.08	64.85	4261
30	10	0.40	0.10	4.00	17.50	64.89	64.85	4249

Simultaneous time dependent removal of colour, COD and metals by consortium

Consortium was also examined for simultaneous removal of colour, heavy metals and COD of the system in the optimized F-MSMUJ medium at different time interval. At regular time intervals, aliquots were analyzed for ADMI, COD and heavy metals (quantitatively on AAS) along with growth detection in terms of dry biomass. In case of heavy metals, accumulation of them in bacterial cell biomass was qualitatively analyzed by X-ray diffraction (SEIFERT-FPM, model XRD7) method.

SSDM effluent degradation study

The SSDM degradation study was carried out using both, MSMYG and F-MSMUJ media for comparative analysis. For analysis of degradation of SSDM effluent spectrophotometrically, samples were collected at 0, 2, 4, 24, 48 h from the test flasks and supernatant after centrifugation was diluted as per the requirement and used to scan in the range of 200-800 nm by UV-Visible spectrophotometer. Absorbance of the samples was noted at λmax 500 nm. For confirmation of degradation of SSDM effluent, the metabolites produced in the broth during decolourization of the SSDM effluent were extracted with an equal volume of ethyl acetate. The extracts were dried by evaporation and the obtained residues were dissolved in 4.0 mL of HPLC grade methanol. These samples were further analyzed using HPLC (High performance liquid chromatography). HPLC analysis was carried out with a C18 column (250 mm × 4.6 mm, 5 mm) equipped with dual wavelength detector by isocratic method (Shimadzu SPD-20A, Japan). The mobile phase used was methanol: water (40:60) with a flow rate of 1.0 mL/min and 10 min run time. The 10 µL of the sample was manually injected into the injector port.

Enzyme analysis

In order to get additional insight of decolourization of SSDM effluent the analysis of activity of various oxidative and reductive enzymes was carried out. Activities of laccase, lignin peroxidase, azo reductase and NADH-DCIP reductase enzymes were analyzed spectrophotometrically as reported by Telke *et al.*[13]. Tyrosinase activity was determined in a reaction mixture (3.0 mL) containing 2.5 mL of sodium acetate buffer (20 mM, pH 4.0) and 100 µM of L-tyrosine. The reaction was started by adding 0.2 mL of enzyme solution and increase in absorbance was measured at 280 nm[14]. All enzyme activities were assayed in both cell free extract and the culture medium supernatant at room temperature. The reduction of DCIP was calculated using an extinction coefficient of 19 mM cm^{-1}. Extinction coefficient of oxidized ABTS (2,2-azinobis (3-ethyl benzothiazoline-6-sulfonic acid) was 3.6×104 $M^{-1}cm^{-1}$ at 420 nm and of methyl red 23 360 M^{-1} cm^{-1} at 430 nm.

Phytotoxicity and genotoxicity

As SSDM effluent contained dyes and heavy metals, toxicity posed by these compounds was necessary to analyze during degradation process. Phytotoxicity test was performed to assess the toxicity of the untreated and treated SSDM effluent with respect to two kinds of seeds commonly used in Indian agriculture; *V. radiata* and *T. foenum-graecum.* Ten seeds of each plant were watered everyday (5 mL) with untreated effluent (20% v/v), treated effluent and tap water (control). The experiment was carried out at room temperature. After seven days of treatment, % seed germination, length of shoot and root were recorded. Single cell gel electrophoresis (Comet assay) was also performed to assess the genotoxic effect of SSDM effluent and its metabolites as described by Achary *et al.*[15]. Small and uniform size of *Allium cepa* bulbs were exposed to water for root development. Developed roots were then exposed to SSDM effluent, metabolites and water (control). Stained slides were examined using the inverted microscope fitted with a camera. A computerized image analysis system (Comet version 1.5) was employed to measure % DNA damage (% T) and tail length (TL).

Batch and fed batch treatment for SSDM effluent

Effect of different SSDM dye effluent amounts on performance of decolourization by the consortium was studied in the range of 10 mL to 80 mL in system of total 150 mL volume containing 70 mL formulated MSM containing urea and jaggery (F-MSMUJ) medium. After addition of different volume of SSDM effluent into 250 mL Erlenmeyer flask all systems were made up to 150 mL using distilled water. All flasks were inoculated with 10% activated bacterial consortium and incubated under static conditions at 30ºC and from the supernatant percentage ADMI removal were calculated.

The decolourization capacity of the consortium was also studied by repetitive addition of SSDM effluent in the ongoing process flask without the addition of

further fresh MSMUJ medium. In each cycle, 10 mL SSDM effluent was added in test flask.

ADMI analysis

Percent decolourization was quantified using American Dye manufacturers Institute (ADMI) 3WL tristimulus method. In all the experiments of the SSDM effluent decolourization, the percent transmission of the sample was read at three different wavelengths *viz*. 590, 540 and 438 nm using UV-Visible spectrophotometer (Jasco V-530, Japan), the % ADMI removal values were measured and expressed as % ADMI removal as shown in Eq. III.

$$\text{ADAMI removal (\%)} = \frac{\text{Initial ADMI value - Final ADMI value}}{\text{Initial ADMI value}} \times 100 \qquad \text{(III)}$$

Data were statistically analyzed by one-way analysis of variance (ANOVA) with Tukey–Kramer multiple comparison test.

Results and Discussion

Development of consortium for SSDM decolourization - Physico-chemical characterization of the received SSDM effluent showed 42000 mg/L COD, 55080 mg/L total solids, 28571 ADMI colour value and the presence of heavy metals *viz*. 16.74, 20.37, 10.24, 3.48 mg/L Pb, Cu, Fe and Cr, respectively. The SSDM effluent also showed 45300, 9780, 55080 mg/L as $CaCO_3$, TDS, TSS and TS, respectively.

Efficient dye decolourizing consortium was enriched in just two transfers as samples were from dye contaminated soils. The consortium was consisting of *Bacillus* sp., *Proteus mirabilis*, *Alcaligenes faecalis*, and *Bacillus cereus*. Dye effluent contaminated sites of dye and textile industry is rich in biodegradative microbial flora which thrive there because of their metabolic adaptability[16]. Single variable optimization carried out (results not shown) and under all optimized conditions, the developed consortium was found to remove 72% ADMI and 65% COD of the SSDM effluent. Earlier, Joshi *et al*.[7] have reported 67% ADMI removal using nutrient rich medium. Here, the consortium has removed 72% ADMI removal using the minimal medium. It indicates that the consortium was capable of decolourizing the dye effluent efficiently even with minimum available nutrients and may be utilizing dye as major nutrient. Analysis of metals after treatment showed removal of all heavy metals from the initial SSDM effluent as bacteria are good accumulators of metals.

Medium formulation using crude substrates through RSM

Jaggery contains high amount of sucrose (60-85%) and was used as a carbon source which is economical (0.9 $/kg in place of glucose 12.38 $/kg). Jaggery is an agricultural product abundantly available in India and also easy to metabolize organic matter. Hence, it was selected as a carbon source for medium formulation study by statistical method. Similarly, locally available urea (0.25$/kg) which is 17 fold cheaper was selected as a nitrogen source in place of yeast extract (4.42$/kg). The substrate is the main cost component for industrial application of the biological process. Jaggery and urea are economically cheaper substrates for degradation of SSDM effluent in place of glucose and urea, respectively.

ANOVA analysis

The ANOVA for SSDM effluent decolourization revealed Model F-value of 131.44 indicating that the model was significant and only a 0.01% chance that this high Model F-value could occur due to noise. A '*P*' value less than 0.05 indicates that test parameter is significant at the 5% level of significance. The model's '*P*' value <0.0001 for experiment implies that the second-order polynomial models were highly remarkable and well fitted to the experimental results. The regression coefficients, 't' and '*P*' values for all the linear, quadratic and interaction effects of the variables are given in (Table. 2). The co-efficient for the linear effect of jaggery (P <0.0001), urea (P <0.0001), NaCl (P <0.0001) and dye effluent (P <0.0001) were highly significant. Pathak *et al*.[17] reported yeast extract (P=0.0005) as a significant variable for decolourization of synthetic dye mixture by isolate VS-MH2 through CCD method. While in this study, all four variables were highly significant (P <0.0001).

Apart from the linear effect of the variables in the decolourization process, the second order RSM also gives an insight into their quadratic and interaction effects. Among the higher order effects, the quadratic effect of jaggery (P=0.0002) was highly significant. Among the interaction terms NaCl and effluent (P <0.0001) and between jaggery and NaCl (P <0.0001) was statistically highly significant showing their strong interactive effects on ADMI removal. Interaction between urea and NaCl (P=0.0004), and between urea and dye effluent (P=0.0206) were also statistically significant. The fitted second order response surface model specified by Eq. II for % ADMI removal in coded process

variables is ADMI removal = (64.79) + (10.36×A) + (4.61×B) + (-7.89×C) + (-13.24×D) + (-0.17×A×B) + (2.28×A×C) + (-0.42×A×D)+(-1.89×B×C) + (1.01×B×D) + (4.14×C×D) + (-1.58×A2) + (0.51×B2) + (-0.57×C2) + (1.71×A×C×D) + (-1.87×A2×B) + (6.10×A2×C) + (3.07×A2×D) + (-9.68×A×B2) (IV)

Percent ADMI removal at specified combination of four variables can be predicted by substituting corresponding coded values in Eq. (IV). Using Eq. (IV), contour plots and three dimensional plots were prepared. The coefficient of determination, R2 for the above, predicting equation, was 0.99. Therefore, this equation can be used for predicting response at any combination of the four variables in the experimental range. In this study, the adjusted R2 and predicted R2 values were high (Adj R2, 0.98; Pred R2, 0.85) and close to the R2 value that ensured a satisfactory adjustment of the polynomial model to the experimental data.

Table 2—Analysis of variance (ANOVA) for a response surface quadratic model obtained from the experimental design

Source	Sum of squares	df	Mean square	*F* value	*P*-value (Prob>*F*)	Remarks
Model	5335.28	18	296.40	131.44	<0.0001	significant
A-jaggery	859.05	1	859.05	380.95	<0.0001	significant
B-urea	169.83	1	169.83	75.31	<0.0001	significant
C-NaCl	498.02	1	498.02	220.85	<0.0001	significant
D-effluent	1402.91	1	1402.91	622.12	<0.0001	significant
AB	0.49	1	0.49	0.22	0.6514	
AC	82.86	1	82.86	36.74	<0.0001	significant
AD	2.85	1	2.85	1.26	0.2850	
BC	56.89	1	56.89	25.23	0.0004	significant
BD	16.46	1	16.46	7.30	0.0206	significant
CD	274.48	1	274.48	121.72	<0.0001	significant
A^2	70.27	1	70.27	31.16	0.0002	significant
B^2	7.31	1	7.31	3.24	0.0993	
C^2	9.11	1	9.11	4.04	0.0696	
ACD	46.61	1	46.61	20.67	0.0008	significant
A^2B	18.59	1	18.59	8.24	0.0152	
A^2C	198.33	1	198.33	87.95	<0.0001	significant
A^2D	50.29	1	50.29	22.30	0.0006	significant
AB^2	500.07	1	500.07	221.76	<0.0001	significant
Residual	24.81	11	2.26			
Lack of Fit	17.53	6	2.92	2.01	0.2308	not significant
Pure Error	7.28	5	1.46			
Core Total	5360.09	29				

*BDL-Below Detectable Limit

Table 1 shows the percentage ADMI removal along with total ADMI removal of SSDM effluent by the consortium under the influence of individual four factors and their combinations. Range of percentage ADMI removal varied markedly from 36 to 91% in 30 experiments. The highest total ADMI removal was found in the standard run 18 (5226), followed by 21 (5166) and 20 (5017), in which jaggery concentration was 0.4-0.8%. The lowest percentage ADMI removal (36.78) was observed when jaggery was absent in the medium (standard run 17). These results proved the significance of carbohydrate source in medium for efficient decolourization of the dye effluent.

Even in the presence of 4.0-6.0% NaCl salt, the consortium was able to reduce more than 4000 total ADMI of of SSDM effluent (standard runs 11-12, 14-16, 18, 20, 21, and 24-30). Although, the presence of high salt (6%) and SSDM effluent (25%), did not considerably affect ADMI removal and found to remove more than 3400 total ADMI values of SSDM effluent, it proves significant tolerance of consortium towards the toxicity of salt as well as the effluent concentration. Even in case of more than 10000 initial ADMI values of SSDM effluent (32.5%) in the system (standard run 24), the consortium was capable of removing 4102 ADMI units within 24 h.

As per the results, maximum ADMI removal was obtained at jaggery (0.40%), urea (0.10%) with 17.5% effluent in the absence of NaCl salt. This formulated medium was further used in the remaining study (MSM containing 0.40% jaggery and 0.10% urea). Comparative experiment was carried out for effluent decolourization by the consortium between MSMYG and formulated MSM containing urea and jaggery (F-MSMJU) medium. Results are reported in (Table 3). Optimization of media constituents and use of crude substrates resulted in the enhancement of COD and ADMI removal by 24 and 13%, respectively. In other words, 3 fold increase in COD removal efficiency and 1.7 fold increase in ADMI removal efficiency of the

Table 3—Comparative ADMI, COD and heavy metals removal after optimization

Parameters	Initial value	MSMYG medium		F-MSMUJ medium	
	(0 h)	Residual value	Removal (%)	Residual value	Removal (%)
pH	8.00	7.4	-	7.4	-
ADMI	6680	1877	71	1068	84
COD (mg/L as $CaCO_3$)	7300	2550	65	803	89
Cu (mg/L)	3.47	BDL	100	BDL	100
Fe (mg/L)	1.80	BDL	100	BDL	100
Pb (mg/L)	2.86	BDL	100	BDL	100

consortium with F-MSMUJ compared to MSMYG medium. Application of RSM allowed consideration of interactive effects of media components that cannot be obtained without statistical model.

Interaction of variables

The 3D graphs of ADMI removal are shown in Fig. 1. Fig.1A depicts the influence of different jaggery and urea concentration on ADMI removal when other two variables NaCl and SSDM effluent concentration were held constant at mid-level (coded value 0). ADMI removal percentage values above 63.28 were obtained when jaggery and urea concentrations were at the actual value of 0.40 and 0.10% or slightly above it, respectively.

Fig. 1B reveals the behaviour of percentage ADMI removal with respect to changes in jaggery and NaCl salt concentration when other two variables were at mid-level. As jaggery concentration was increased, the percentage of ADMI removal also increased irrespective of change in salt concentration. Similarly, Fig. 1C shows the interaction of jaggery and effluent concentration on percentage ADMI removal of effluent. The effluent concentration also had significant influence as the toxicity of effluent to microorganisms increases at higher concentration. ADMI removal and jaggery concentration have shown positive correlation and the effluent concentration negative, i.e., the ADMI removal value increased with the increased jaggery concentration

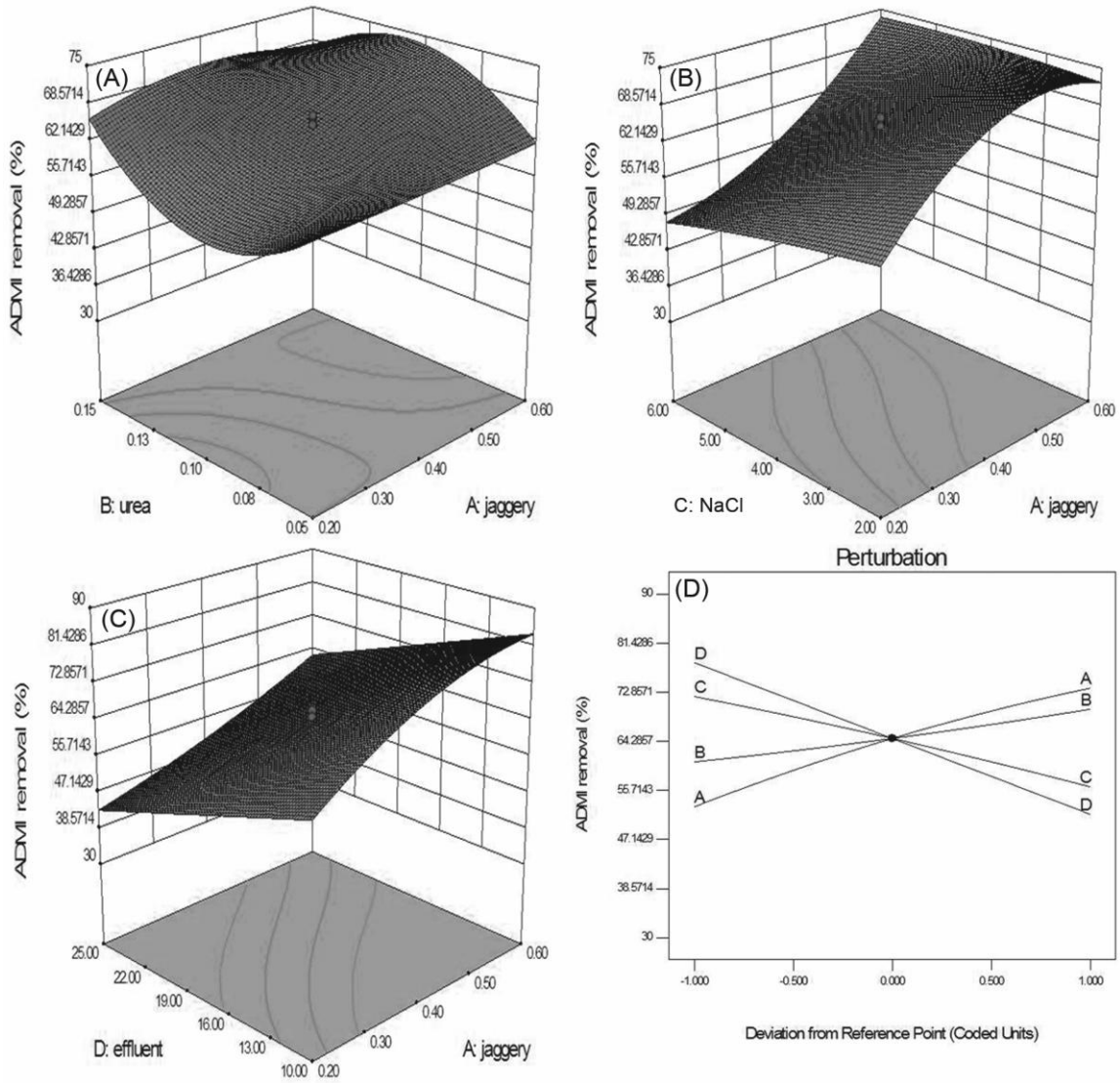

Fig. 1—Response surface graphs of (A) Jaggery and urea; (B) Jaggery and NaCl; and (C) Jaggery and effluent on ADMI removal. When the effect of two variables was plotted, keeping the other factors at their middle-level value (D) Perturbation plot

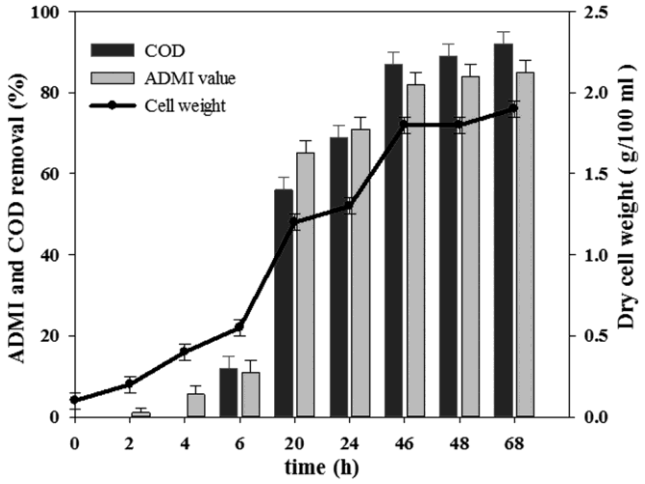

Fig. 2—ADMI and COD reduction of the SSDM effluent by consortium

and decreased effluent concentration. The perturbation plot compares the effect of all factors at a particular point within the design space. The response was plotted by changing one factor and holding other factors constant. Fig. 1D shows the comparative effect of all independent variables for the ADMI removal. The sharp curvature indicates that the dye removal efficiency was sensitive to all independent variables.

Simultaneous time dependent ADMI, COD, heavy metals removal

Maximum colour removal efficiency of the consortium was found to be 85% within 68 h under all optimized conditions in F-MSMUJ medium (Fig. 2). More than 70% ADMI was removed during the initial 24 h, and nearly 10% further removal was found in the next 24 h of incubation time. As shown, there is a minor difference in ADMI removal from 48 to 68 h incubation time. The COD analysis showed a 92% decrease in COD after 68 h indicating the mineralization of SSDM effluent due to microbial activity of the consortium. Treatment with bacterial consortium showed 100% removal of all the heavy metals present in the SSDM effluent. Accumulation of heavy metals in the bacterial cells of the consortium was analyzed using X-ray diffraction method. The X-ray spectra obtained with the cells exposed to SSDM effluent had several distinct peaks, indicating deposition or absorption of heavy metals (data not shown). Here, the graph of dry cell weight indicates that there is little change in growth or ceasing of growth due to nutrient depletion after 48 h. Hence, extended incubation after 48 h had had only negligible effect on the colour and COD removal by the consortium.

SSDM effluent degradation analysis

The time overlaid UV-Vis spectra of test samples collected during the decolourization experiment showed that the intensity of characteristic absorption peaks of dye effluent at 500 nm decreased drastically within 24 h and these peaks disappeared completely after 48 h (data not shown). Simultaneously, additional peaks appeared with higher intensity as compared to 0 h sample in the UV region (236 nm) at 24 h. It was found that decolourization was slow during the initial 4 h, then maximum decolourization took place within 24 h accounting for more than 60% decolourization of the dye effluent. Decolourization reached 70% in 48 h, indicating only 10% additional decolourization within the next 24 h. The UV-Vis spectra change represents the disappearance of dye and formation of metabolites during decolourization reaction.

HPLC analysis (Fig. 3A) of untreated SSDM effluent showed major peaks at 3.693, 3.846 and 4.027 min along with minor peaks at 2.728, 4.219 and 3.532 min. As the decolourization progressed the emergence of additional peaks were observed. Degradation of the SSDM effluent using F-MSMUJ medium (Fig. 3B) showed four major peaks at retention time 2.030, 2.580, 2.714 and 2.864 min and two minor peaks at retention time 3.594 and 4.352 min. In case of MSMYG medium (Fig. 3C), four major peaks at retention time 2.551, 2.728, 2.839 and 2.928 min along with comparative lesser intense peaks at 3.680, 3.862 and 4.035 min. Thus, HPLC analysis of treated effluent expresses the degradation of SSDM effluent into different compounds by bacterial consortium.

Enzyme analysis

Degradation of effluent using F-MSMUJ medium showed induction in the activity of intracellular azo reductase (838%) and DCIP reductase (288%), suggested their role in degradation of the SSDM effluent. The activity of lignin peroxidase and intracellular laccase was found to be affected adversely could be due to the toxicity of the dyes and metals present in the effluent. Extracellular laccase was also found to be induced up to 525% during the decolourization. There was moderate to slight induction in case of activity of intracellular (197%) and extracellular (102%) tyrosinase. While, degradation of effluent using MSMYG medium reported 780% azo reductase, 216% DCIP reductase, intracellular (225%) and extracellular (309%) tyrosinase along with 182% extracellular laccase. In

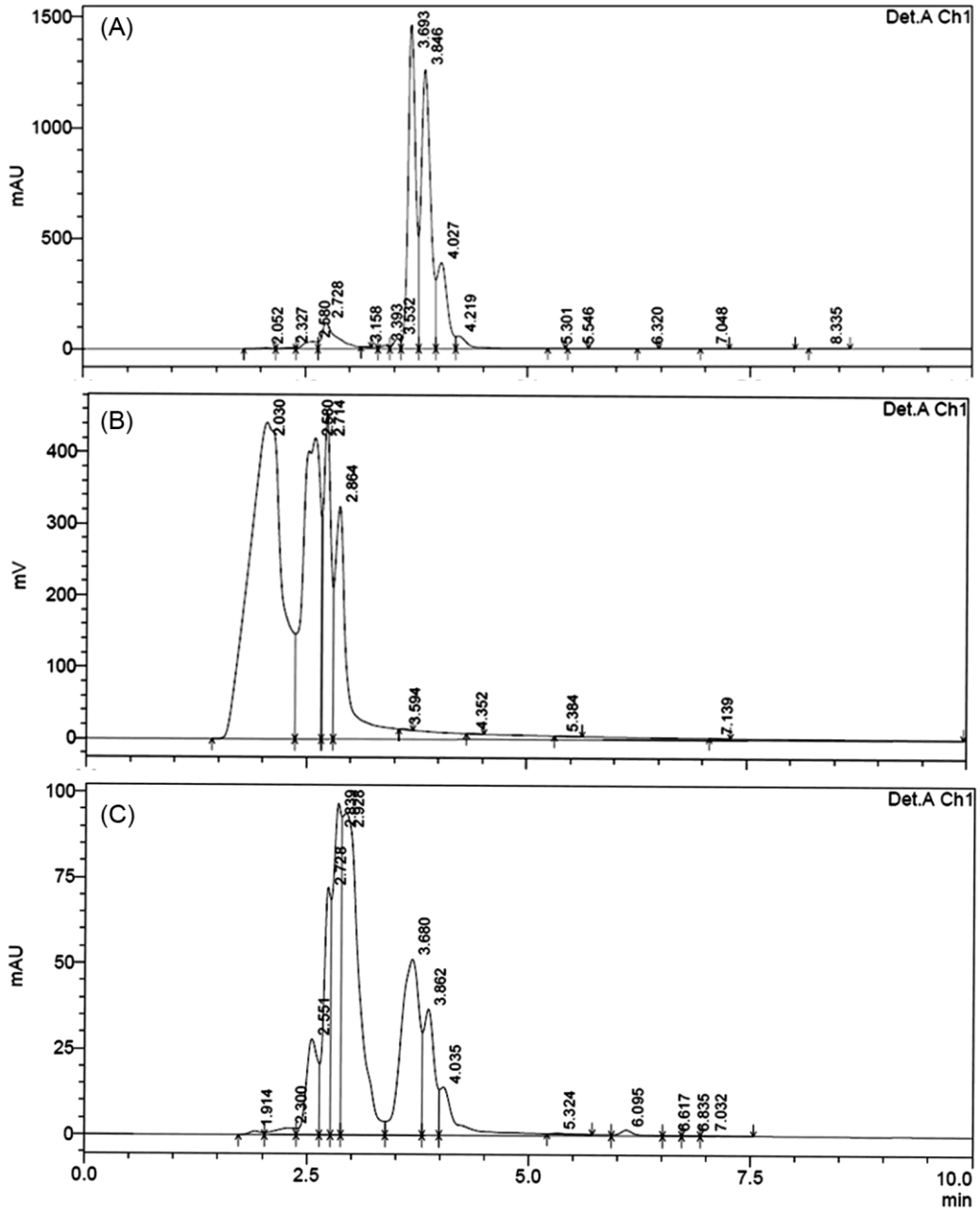

Fig. 3—HPLC chromatogram (A) untreated SSDM effluent; (B) treated SSDM effluent in F-MSMUJ; and (C) treated SSDM effluent in MSMYG

case of lignin peroxidase enzyme, slight induction of 115 and 153% were seen with F-MSMUJ and MSMYG medium, respectively. Results showed (Table 4) presence of all enzymes and significant induction of oxidoreductive enzymes might be due to the synergistic effect of bacteria present in the consortium.

Toxicity analysis of treated SSDM effluent

Use of untreated and treated dye effluent containing water for agriculture purpose has a direct impact on fertility of soil[18]. Hence, it is of concern to check the phytotoxicity and genotoxicity before and after treatment of SSDM effluent. Phytotoxicity study revealed toxicity of the SSDM effluent to *V. radiata* and *T. foenum-graecum*. Germination (%), shoot length and root length of both the plants are reported in (Table 5) and were found significantly higher for treated effluent as compared to the untreated SSDM effluent, indicated the decrease in toxicity after the treatment of the effluent. The germination of both

Table 4—Enzyme activities in control and after decolourization state

Enzyme assay	Enzyme location	Enzyme activity					
		F-MSMUJ medium			MSMYG medium		
		Control	Induced	Performance (%)	Control	Induced	Performance (%)
Lignin peroxidase[a]	Intracellular	3.18±0.5	3.67±0.6	115	2.05±0.3	3.14±0.2	153
	Extracellular	0.71±0.09	0.29±0.06	-	0.92±0.1	0.67±0.09	-
Laccase[b]	Intracellular	4.92±0.05	4.18±0.01	-	4.38±0.25	4.04±0.34	-
	Extracellular	2.37±0.03	12.45±2.78	525	3.49±0.4	6.38±2.00	182
Tyrosinase[a]	Intracellular	0.47±0.6	0.93±0.02	197	1.28±0.35	2.89±0.32	225
	Extracellular	0.46±0.05	0.47±0.00	102	1.38±0.05	4.26±0.27	309
Azo reductase[c]	Intracellular	0.87±0.08	14.29±0.9	838	1.16±0.35	9.05±0.2	780
NADH-DCIP reductase[d]	Intracellular	11.27±1.05	32.48±3.04	288	6.15±1.05	13.27±3.04	216

[[a] Activity in U/mL/min; [b] μM of ABTS oxidized/mL/min; [c]μM of methyl red reduced/mL/min; and [d] μM of DCIP reduced/mL/min. Values are mean of three experiments (±) SEM]

Table 5—Phytotoxicity and genotoxicity study of the SSDM effluent after degradation

Parameters	Control	F-MSMUJ		MSMYG	
		Untreated	Treated	Untreated	Treated
Phytotoxicity					
V. radiata					
Germination (%)	100	20	100	20	100
Plumule (cm)	12.7 ± 0.52	4.16 ± 0.67*	13.34 ± 0.53	3.46 ± 0.28*	11.18 ± 0.49
Radicle (cm)	8.15 ± 0.47	3.78 ± 1.28*	7.9 ± 0.72	3.36 ± 0.32*	5.4 ± 0.37
T. foenum-graecum					
Germination (%)	100	30	100	30	100
Plumule (cm)	4.42 ± 0.65	1.35 ± 0.17*	5.18 ± 2.25	1.18 ± 0.20*	4.66 ± 0.35
Radicle (cm)	3.12 ± 0.58	0.90± 0.09*	3.35 ± 1.07	1.28 ± 0.12*	2.86 ± 0.05
Genotoxicity					
DNA damage in cells of *Allium cepa*					
Mean TL (μm) ±SD	9.56 ± 2.38	39.05 ± 3.44*	9.28 ± 4.12	37.38 ± 3.78*	10.67 ± 2.19
Mean %T ± SD	27.02 ± 4.02	45.89 ± 4.24*	28.39 ± 3.58	47.28 ± 5.46*	29.47 ± 3.78

[*Values are the mean of three experiments SD (±), significantly different from the seeds germinated in control at, *P <0.001, by one-way analysis of variance (ANOVA) with Tukey Kramer Multiple Comparison Test]

plants' seeds: *V. radiata* (20%) and *T. foenum-graecum* (30%) inhibited with untreated effluent. In contrast, 100% germination of both plants' seeds was resulted for treated effluent. In addition, plumule length and radical length of above both plants were significantly higher for treated effluent as compared to the untreated effluent, indicated the detoxification after the treatment. Induction in shoot and root length confirms non-toxicity of treated SSDM effluent. Saratale *et al.*[18] and Telke *et al.*[13] have studied phytotoxicity for dye effluent but using *Sorghum bicolor* and *Phaseolus mungo*, which have also confirmed detoxification by consortium GR and *Pseudomonas* sp. SU-EBT.

In F-MSMUJ treated medium, the tail length (μM) and percentage of tail DNA (% of DNA in comet tail) for untreated effluent were 39.05 ± 3.44 and 45.89 ± 4.24, respectively, and in case of treated effluent, 9.28 ± 4.12 and 28.39 ± 3.58, respectively. While MSMYG treated medium, the tail length (μm) and percentage of tail DNA (% of DNA in comet tail) for untreated effluent were 37.38 ± 3.78 and 47.28 ± 5.48, respectively, and in case of treated effluent, 10.67 ± 2.19 and 29.47 ± 3.78, respectively. According to results showed (Table 5), DNA damage by treated effluent was lesser against the untreated SSDM effluent and also near to control. The consortium was found to be efficient for both degradation and detoxification of the SSDM effluent. Thus, treated dye effluent can be used for irrigation.

Batch and fed batch treatment for SSDM effluent

Consortium showed more than 60% ADMI removal of SSDM effluent in 48 h when the initial volume of SSDM effluent was lower than 30 mL (Fig. 4A). Above this initial concentration of SSDM effluent, significant reduction in the % decolourization by the consortium was observed, but

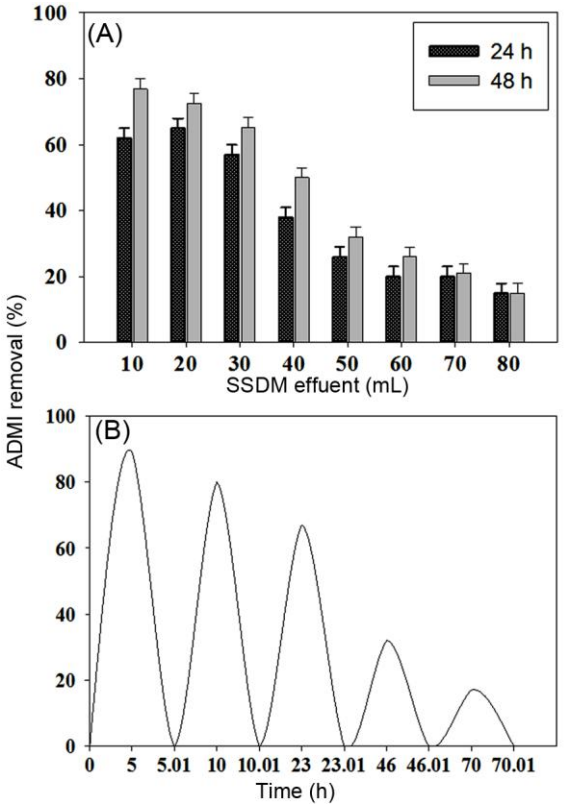

Fig. 4—Effect of (A) SSDM effluent concentration; (B) repetitive addition of SSDM effluent

no decrease in the amount of ADMI colour removal was recorded. As per the obtained results, extended incubation has minor effect on ADMI removal percentage at higher amount of effluent, as there is a minor difference in 24 h and 48 h result of 70 mL and 80 mL SSDM effluent. The decrease in the % decolourization at high concentration of SSDM effluent was due to increasing ADMI values due to the increased initial effluent amount in the system. Consortium decolourized 32% dye effluent, even when 80 mL SSDM present in the system. Nutrient rich medium or more incubation period may require for better effluent decolourization at higher concentration. This potentiality of the consortium suggests that it can be used for the treatment of dye containing wastewater of industries at commercial level.

The repeated use of the developed consortium is important in the commercial point of view. This study was carried out to examine the ability of the consortium to decolourize repeated additions of dye effluent at static conditions at 30 ± 2ºC. Developed consortium decolourized initial two additions of SSDM effluent within 10 h but the 3rd and 4th addition of SSDM effluent required more time and showed less than 70% ADMI removal. As illustrated in Fig. 4B, there was a drastic decrease in the ADMI removal in 5th cycle. Consortium showed decolourization up to 5 cycles within 70 h and further addition of dye effluent decreased percentage decolourization with the increase in the incubation time. Similar observations have been reported previously for decolourization of Acid Red 119 dye by *B. thuringiensis*, which showed that after 5 cycles gradually the time for decolourization increased[2].

Conclusion

Media optimization resulted in the formulation of cheaper medium which proved better for ADMI, COD and toxicity reduction. Media composition was found to be influencing selective induction of enzymes as well as phytotoxicity and genotoxicity. This protocol has potential of scaling up for lab scale reactor treatment of industrial effluent.

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