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Lignin Decolorization and Degradation of Pulp and Paper Mill Effluent by Ligninolytic Bacteria

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A B S T R A C T

The aim of this research work is to isolate bacterial strains with high potential in the degradation and decolorization of lignocellulose compounds of paper mill effluent. Four bacterial strains were isolated from marine sediments and they were screened to their ability to degrade the lignin and decolorize the Century pulp and paper mill effluent. Among four bacterial strains, three bacterial strains *Bacillus subtilis*, *Bacillus endo-phyticus*, *Bacillus* sp. were capable of ligninolytic activity. Consortium made by these bacterial strains enhances the degradation of lignin as well as decolorization. Various nitrogen source, carbon source, pH, temperature and low molecular weight organic acids were used in the optimization process of decolorization and degradation of lignin in paper mill effluent. Maximum decolorization 68.29% was found at pH 7.92, temperature 33°C, in the presence of glucose (as carbon source) 0.99% and yeast extract (as nitrogen source) 0.36% when it was optimized through response surface methodology.

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INTRODUCTION

Among industries, pulp and paper mill industry has high rate of water consumption. In process of digestion of wood chips the liberated lignin generated dark brown color to the mill effluent. The color not only poses an aesthetic problem, but also inhibits the natural process of photosynthesis in the stream due to prevention for absorbance of sunlight. It's one of the oldest and major industries in India and effluent produced from the pulp and paper mill is dark brown in color and have high BOD, COD [1]. Lignin's toxicity causing serious problem in the receiving water body and land mass fertility in the presence of lignin with waste is obtained from the raw cellulosic materials and are not easily biodegradable. There are more than 60 large industrial units which manufacture paper and allied products. The paper making process requires large amount of water for the production processes, hence it is a water intensive process. This is because, without the physical properties of water, it would not be possible for a consistent structure to be achieved when the constituents of paper are processed in sludge. Consumption of water depends upon the raw material used in industrial processes. The natural raw material are used for the processes are wood, cellulose, which contain high amount of lignin [2].

The lignin is solubilized via degradation or derivatisation to free fibers for the manufacturing of paper and other products. Lignin is not easily degraded by physical, chemical and biological system; however the microorganism which contain enzymatic system can degrade lignin by oxidative process. Bacteria from several genera is reported to degrade the lignin but it is less in comparison to fungi they secrete the enzyme [3]. Bacterial species can degrade only one type of bond of the lignin so it is suitable to use mixed consortium to degrade the lignin in the effluent [4]. The lignin component is the more recalcitrant to microbial degradation and can act as a barrier to biodegradation of the more readily degradable polysaccharides. Thus, microbial degradation of the lignin component of

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lignocelluloses is generally the rate-limiting step in degradation of the associated polysaccharides [5].

The decomposition of lignin in nature has been considered for a long time to occur by the action of wood-rot fungi mostly of the *Basidiomycete* class. These microorganisms simultaneously decompose lignin and wood polysaccharides. Nevertheless, several reports brought strong evidence of the ability of certain bacteria to degrade lignin. Odier et al. [6] reported that the isolation of several bacterial strains able to degrade and assimilate isolated lignins. According to the results gained, there were eleven bacterial strains out of 122 soil isolates tested found to be able to grow by using poplar dioxane lignin as the sole carbon and energy source in mineral medium in aerobic conditions. In other words, these bacterial strains are able to produce ligninolytic enzyme to degrade lignin. The strains consisted of gram negative aerobic rods identified as *Pseudomonas*, *Xanthomonas* and *Acinetobacter*. Some other lignin degrading bacteria were identified, including *Corynebacterium*, *Agrobacterium*, *Aeromonas*, *Klebsiella* and 18 *Enterobacter*. These strains were also being able to assimilate different phenolic compounds considered as lignin related simple monomers. In salt marsh ecosystems, lignin degradation is an important biogeochemical process due to the high primary productivity in such ecosystem and the abundance of vascular plant derived lignocellulosic material [7]. Both bacteria and fungi can be involved in the lignin degradation. However, in aquatic environments, bacteria are probably responsible for the utilization of the most refractory components. In a salt marsh, bacteria mediate most of the degradation of lignin.

One of the ligninolytic potential marine bacteria was *Sagittula stellate*, as reported by Gonzalez et al. [7]. Laccase mediator system (LMS) could play an important role in the view of alternative bleaching processes in the pulp and paper industry. Bourbonnais and Paice [8] found that the substrate ranges of laccase can be extended to nonphenolic subunit of lignin by inclusion of a mediator, such as ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)). Reyes et al. [9] examined the immobilized *Coriolopsis gallica laccase* for test stability during repeated cycles of dye decolorization and the effect of Hydroxybenzotriazole (HBT), as a radical mediator, was used to extend the number of dyes decolorized. The dyes employed had different chemical structures, including anthraquinones, oxazines, phthalocyanines and azo compounds.

Response surface methodology (RSM) used here to determine optimum sets of operational variables of the decolorization process [10]. The statistical optimization of decolorization process will reduce the experimentation time; overall cost of the experiment. RSM is a collection of mathematical and statistical technique for empirical model building. By careful design of experiments, the objective is to optimize a response (output variable) which is influenced by several independent variables (input variables). An experiment is a series of tests, called runs, in which changes are made in the input variables in order to identify the reasons for changes in the output response. Originally, RSM was developed to model experimental responses [11] and then migrated into the modelling of numerical experiments and difference in the type of error generated by the response. In physical experiments, inaccuracy can be due, for example, to measurement errors while, in computer experiments, numerical noise is a result of incomplete convergence of iterative processes, round-off errors or the discrete representation of continuous physical phenomena [12]. In RSM, the errors are assumed to be random. The application of RSM to design optimization is aimed at reducing the cost of expensive analysis method (e.g. finite element method or CFD analysis) and their associated numerical noise. The objective of present research is to develop ligninolytic bacterial culture biodegradation of lignin by using statistical optimization of decolorization of paper mill effluent. For this study the effluent from Century pulp and paper mill was selected which is large scale paper mill and has advance treatment system located at Lalkuan, Nainital, Uttarakhand (India). The Century pulp and paper mill came in to existence in June 1984. This is a large scale pulp and paper mill which uses eucalyptus, bamboo and sugarcane bagasses as raw materials for production different grade writing and printing paper. Here Kraft process is used for pulping and bleaching is done using chlorine which produces chlorinated phenols and other toxic pollutants.

MATERIALS AND METHODS

Sampling location and collection:

For decolorization and lignin degradation analysis, effluent was collected in large jerry cane which was washed properly with detergent and rinse by hot water to remove detergent and then rinsed three times with laboratory distilled water. The sludge sample for bacterial isolation was collected in sterilized (at 121°C for 15 min) cotton plugged test tubes. The effluent sample was collected from the Century pulp and paper mill from the outside of the industry premises. The sludge for bacterial isolation was collected from the sea shore of Gujarat in sterilized test tubes.

Measurement of color:

The color unit (CU) of effluent was determined according to the Canadian Pulp and paper Association Standard Method [13]. All the samples of effluent were centrifuged at 8000×g for 15 min in centrifuge (Remi C-24) to separate suspended solids. From effluent, pH was

adjusted to 7.92 by addition of 2M NaOH in clear supernatant before measuring in UV-Visible Cintra 40-GBC spectrophotometer, against distilled water as a blank. Absorbance was transformed into standard color unit according to the following equation as follows:

$$Y = \frac{A_2 \times 500}{A_1} \quad (1)$$

where, A_1 is the absorbance of 500-CU of platinum cobalt (PtCo) standard (A_{465} = 0.1214) and A_2 is the absorbance of the effluent sample.

Estimation of lignin

During the time of incubation, lignin was degraded in marine salt medium was monitored by spectrophotometer according to Ulmer et al. [14]. In this method, 4.5 mL of 0.55% (w/v) NaOH was added to 0.5 mL sample. The sample was centrifuged at 8000×g for 30 min was diluted by adding 3.0 mL phosphate buffer and absorbance was measured at 280 nm on a UV-visible spectrophotometer. The degraded lignin percentage was calculated by using below equation:

$$\text{Lignin degradation (\%)} = \frac{\text{Initial OD}_{280} - \text{Final OD}_{280} \times 100}{\text{Initial OD}_{280}} \quad (2)$$

Inoculums preparation

Four different bacteria which are obtained from the screening process are now grown in the nutrient broth medium. Nutrient agar is a microbiological growth medium commonly used for the routine cultivation of non-fastidious bacteria. It is useful because it remains solid even at relatively high temperature. The growth of bacteria basically occurred on the surface nutrient agar grows on the surface and is clearly visible as small colonies. In nutrient broth, the bacteria grow in the liquid and are seen as a soupy substance, not as clearly distinguishable clumps.

These bacteria are inoculated in the Cotton-plugged Erlenmeyer conical flasks (250 mL) in nutrient broth medium and incubated for 24 hrs at 30°C in shaker at 110 rpm. This broth grown bacterial culture was used as inoculums for the lignin degradation and decolorization experiment.

Biodegradation of lignin by pure bacterial culture:

Marine Salt media each of 50 mL containing yeast extract 0.4%, glucose, 0.5%, lignin 0.5% tryptone 0.25%, sea salt composition was autoclaved in cotton-plugged Erlenmeyer conical flasks (250 mL) at 121°C for 15 min, glucose (0.5% w/v) and yeast extract (0.4% w/v) as a additional carbon and nitrogen source were added before sterilization. Before sterilization pH was adjusted to 7.6. Pure culture (1 mL) cells suspension of each bacterial strain per mL respectively were used to inoculate medium and incubated at 30°C on a rotary shaker (110 rpm) for six days. Samples from each flask were analyzed for pH, reduction in color, lignin at zero time and day to day up to days.

RESULTS AND DISCUSSION

Isolation, purification and screening lignin degrading bacterial strains

Based on different morphology of colony developed a total of 4 bacterial strains were isolated from marine sediments on marine salt agar plates containing glucose and yeast extract as a co-substrate. However by observing by microscope, it is found that all bacterial colonies are pure.

Lignin degradation/decolorization

Different bacterial strains (M1, M2, M3, M4) were grown in shake flask containing yeast extract 0.4%, glucose, 0.5%, lignin 0.5%, tryptone 0.25%, sea salt composition. The inoculated flasks were put in the shaker at 30°C at 110 rpm. Glucose is used as the carbon source and yeast extract is used as nitrogen source. After taking absorbance in spectrophotometer at 620 nm showed that growth reached maximum at 2 days for all bacterial strains and thereafter it decreases. Due to metabolic activity, pH of medium changes time to time. Since the color the lignin is ph dependent so the pH of supernatant after centrifuging the sample was adjusted at pH 7.6 by diluting the pH by phosphate buffer. After taking OD at 465 nm it is found that at 4th day M1 show negative result and M2 reduce color 34.21% and M3 reduce color 34.86% and M4 reduce color 32.22%.

The lignin degradation for M2 was 17.8% and for M3 it was 17.9% and for M4 it was 16.87%. After that consortium was made by combination of 3 bacteria (M2, M3, M4). Four combinations were made M2+M3+M4, M2+M3, M3+M4, M2+M4. In first consortium M2+M3+M4 decolorization % was maximum it was 45.80% at 6 day where as for M2+M3, M3+M4, M2+M4 it was 33.64, 40.19 and 31.77%, respectively. This lignin degradation % was 36.87% for M2+M3+M4, 21.32% for M2+M3 32.48% for M3+M4, 22.08% for M2+M4 (Figs 1-4).

Lignin degradation was also checked in the different nitrogen source such as peptone but its % decolorization % was less than that of nitrogen source of yeast extract (YE). The reduction of color resulting from lignin biodegradation has been assumed to be due to depolymerization of lignin polymers by bacterial ligninolytic systems [15](Perestelo et al., 1989). For further studies mixed consortium M2+M3+M4 was taken in consideration.

Lignin degradation/decolorization at different carbon and nitrogen source

In order to grow in nature or in the laboratory, a bacterium must have an energy source, source of carbon and nitrogen. The carbon requirements of organisms must be met by organic carbon (a chemical compound with a carbon-hydrogen binding by CO_2. For decolorization experiment different carbon source was selected to achieve maximum decolorization different carbon sources such as glucose, saccharose, maltose, xylose fructose [16](Donderski et al., 1998) and nitrogen source (yeast extract and peptone) was observed with these carbon sources.

Different combination of carbon and nitrogen sources was tested in which 0.1% lignin was taken and carbon and nitrogen % was 1% both. the combination of carbon source and nitrogen source such as Glucose + YE, Saccharose +YE ,Maltose + YE, Xylose +YE ,Fructose + YE was tested for three days of degradation/decolorization studies. It was found that on the 3rd day max decolorization was in Glucose and yeast extract which was 33.02% where as in the Saccharose +YE and Maltose + YE the decolorization % was 17.81% and 22.40%, and in Xylose +YE & Fructose + YE it shows 10.23% and 12.35%. Yet growth was maximum in Saccharose +YE as in Donderski et al. [16] studies.

When the same carbohydrate was used with peptone it was found that on 3rd day the decolorization % was maximized in Saccharose + Peptone but it was less than that of glucose + yeast extract.

Decolorization of lignin at different pH and temperature

Hydrogen ion concentration and temperature plays an important role for bacterial enzymatic activity to degradation of lignin and decolorization. Each bacterium has specific temperature and pH requirement at which induction of particular enzymes are occurred. Besides, at low pH the solubility of high molecular weight fragments derived from lignin is reduced and affect the availability of compounds to bacteria in liquid medium. Therefore in present research work it has attempted to observe the effect of pH (acidic to alkaline) and temperature on decolorization of lignin by bacterial strains consortium (M2+M3+M4). pH range was from 6 to 9 and temperature was from 15°C to 40° C. Different flask was put on the incubation for six days and in shaker at 110 rpm. It was found that growth was occurring at temperature 15 to 40°C and decolorization was taking place at pH 6 to 9. It was found that at temperature 40°C the decolorization was not significant. Maximum decolorization was occurring at pH 7 and 35°C which was 57.93% and lignin degradation was 45.75%. It was found at pH 7 and 30°C the decolorization percentage was 57.24%, where as decolorization at pH 8, 30°C and pH 8, 35°C and pH 9, 30°C and pH 9, 35°C were 54.84, 55.84, 54.07 and 51.85%, respectively (Figs. 7 and 8).

Statistical optimization for decolorization of pulp and paper mill effluent using Response surface methodology (Box Behnken Design)

As an important subject in the statistical design of experiments, the Response Surface Methodology (RSM) is a collection of mathematical and statistical techniques useful for the modeling and analysis of problems in which a response of interest is influenced by several variables and the objective is to optimize this response [17]. The first goal for Response Surface Method is to find the optimum response. When there is more than one response then it is important to find the compromise optimum that does not optimize only one response [18]. The graphical representation of their functions is referred to as response surfaces and this approach is used to describe the individual and interactive effects of the process variables and their subsequent effects on the response. The main objective of RSM is to determine the optimum set of operational variables of the process [19]. The statistical experimental designing of decolorization process can reduce the process variability, experimentation time, overall cost with improved process output. BBD, a spherical, revolving design, consisting of a central point and the middle points of the edges of the circle circumscribed on the sphere, is suitable for constructing second-order polynomial models [20]. BBD requires relatively few combinations of variables for determining the complex response function and it does not contain those combinations for which all variables are at their highest or lowest levels simultaneously. The number of experiments (N) required for the development of BBD can be determined as $N = 2k(k-1) + C_0$, where k is the number of variables and C_0 is the number of central points. For a four-factor BBD, three to six center points are recommended [21].

In our experiment four variables were taken pH, temperature, concentration of glucose and concentration of lignin. The number of experiments (N) required for the decolorization process was $N=2\times4(4-1)+3 = 27$, where no of centre runs was taken as 3. After defining the range of each of the process variables through initial experiments, they were coded to lie at ±1 for the factorial points and 0 for the center points. The actual values of the variables were transformed into their respected coded values as [22] as given below:

$$Z_i = \frac{X_i - X_0}{\Delta X_i} \quad (3)$$

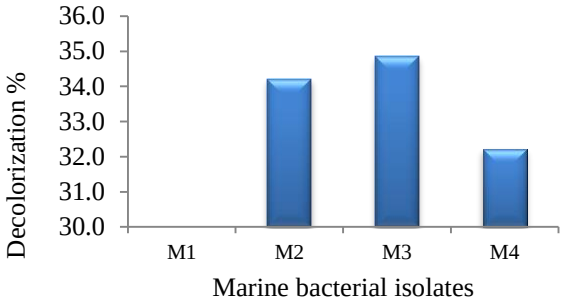

Figure 1. Decolorization of lignin by different Marine bacterial isolates at 4th Day

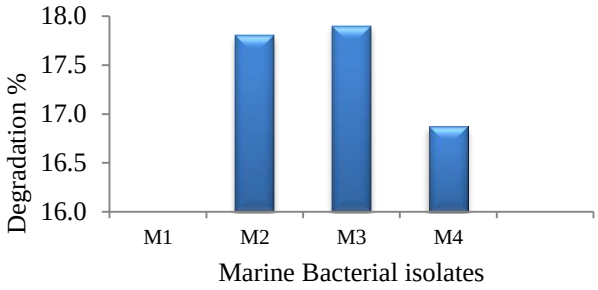

Figure 2. Degradation of lignin by different Marine bacterial isolates at 4th Day

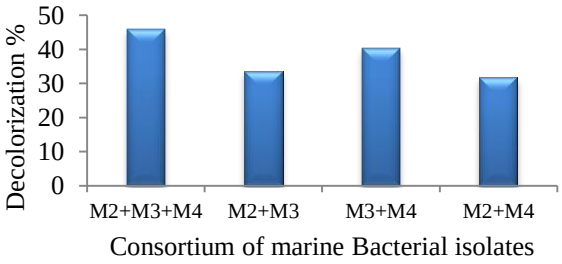

Figure 3. Decolorization of lignin by Marine Consortium

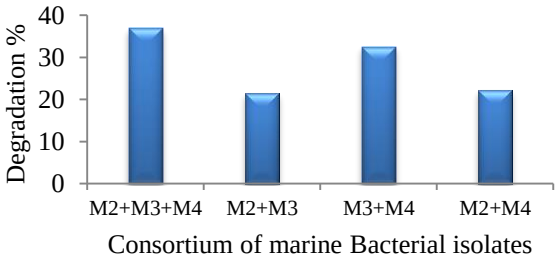

Figure 4. Degradation of lignin by Marine Consortium

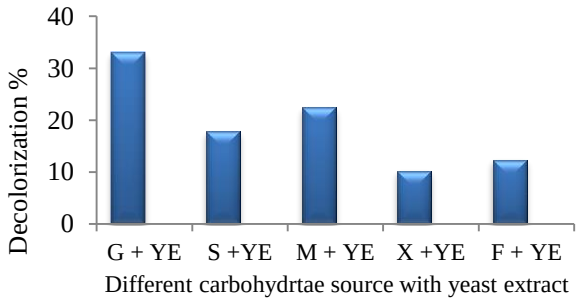

Figure 5. Decolorization by consortium with different carbon and Nitrogen source at day 2 where G= glucose, S= Sachrrose, M= Maltose, X = Xylose , F= fructose & YE= yeast extract

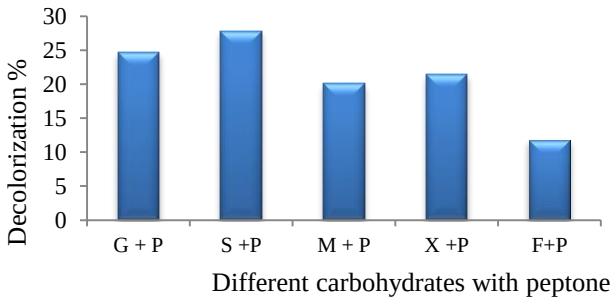

Figure 6. degradation of lignin by consortium with different carbon and Nitrogen source at day 3 where G= Glucose, S= Sachrrose, M= Maltose, X = Xylose, F= fructose & YE= yeast extract

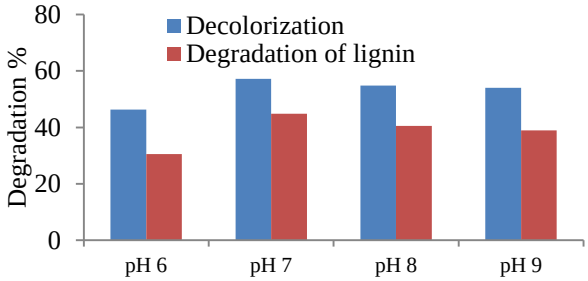

Figure 7. Decolorization of paper mill effluent at different pH at temperature 30°C

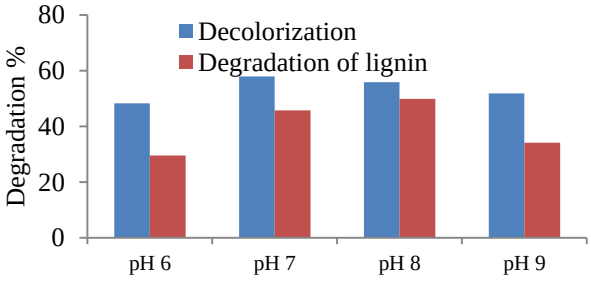

Figure 8. Decolorization of paper mill effluent at different pH at temperature 35°C

TABLE 1. BBD matrix with four independent variables (natural and coded values) and corresponding decolorization percentage

Exp no.	Factor 1 A: pH		Factor 2 B: Temp (°C)		Factor 3 C: Glucose %		Factor 4 D: Yeast %		Response 1 Decolorization %
	A	C	A	C	A	C	A	C	
1	7.50	0	25.00	0	0.625	0	0.625	0	57.12
2	7.50	0	25.00	0	1.000	+1	0.250	-1	59.89
3	7.50	0	25.00	0	0.250	-1	0.250	-1	55.78
4	7.50	0	25.00	0	0.250	-1	1.000	+1	54.45
5	9.00	+1	25.00	0	0.250	-1	0.625	0	50.94
6	6.00	-1	35.00	+1	0.625	0	0.625	0	56.36
7	7.50	0	25.00	0	0.625	0	0.625	0	57.12
8	7.50	0	15.00	-1	0.250	-1	0.625	0	30.45
9	6.00	-1	25.00	0	0.625	0	0.250	-1	47.79
10	9.00	+1	25.00	0	0.625	0	0.250	-1	51.67
11	6.00	-1	25.00	0	0.250	-1	0.625	0	44.94
12	7.50	0	15.00	-1	1.000	+1	0.625	0	33.25
13	9.00	+1	15.00	-1	0.625	0	0.625	0	22.45
14	7.50	0	25.00	0	1.000	+1	1.000	+1	52.75
15	9.00	+1	35.00	+1	0.625	0	0.625	0	58.78
16	9.00	+1	25.00	0	0.625	0	1.000	+1	53.94
17	7.50	0	35.00	+1	0.625	0	0.250	-1	63.79
18	7.50	0	35.00	+1	0.625	0	1.000	+1	56.54
19	7.50	0	35.00	+1	1.000	+1	0.625	0	67.49
20	7.50	0	15.00	-1	0.625	0	0.250	-1	32.47
21	7.50	0	35.00	+1	0.250	-1	0.625	0	54.78
22	6.00	_1	25.00	0	1.000	+1	0.625	0	51.58
23	9.00	+1	25.00	0	1.000	+1	0.625	0	54.78
24	7.50	0	25.00	0	0.625	0	0.625	0	57.12
25	6.00	-1	15.00	-1	0.625	0	0.625	0	25.15
26	7.50	0	15.00	-1	0.625	0	1.000	+	20.18
27	6.00	-1	25.00	0	0.625	0	1.000	+1	43.63

where, Z_i is the dimensionless coded value of the i^{th} independent variable, X_i is the uncoded value of the i^{th} independent variable, X_0 is the uncoded value of the i^{th} independent variable at the center point, ΔX_i is the step change value.The four variables which were selected according to the experiment such as pH, temperature, glucose % and yeast extract % and their coded value and step changes. are given below. +1 was given to the highest values and -1 for the lowest value and mid values of both were coded as 0. Step change was the difference between -1 to 0 or 0 to +1. All the experiment was conducted according to the BBD, A set of 27 experiments was conducted in duplicate according to the variables selected and according to the range: pH 6-9, temperature 15°C to 35°C, glucose and yeast extract 0.25% to 1.0%. The media used for growth of marine bacteria was same as previous experiment as marine salt NaCl 23.375 g/L, $MgSO_4.7H_2O$ 4.925 g/L, $CaCl_2.2H_2O$ 1.11 g/L, KBr 0.2025 g/L, KCl0.745 g/L, $MgCl_2.6H_2O$ 40.625 g/L and transferulase 1 mM . All the experiment was carried out for 6 days as maximum decolorization was found after 6 days and agitated at 110 rpm (Table 1).

Modeling:

The response variable Y (Decolorization %) of paper mill effluent can be expressed as

$Y=f (X_{pH} , X_T , X_G , X_Y)$

where; X_{pH}, X_T, X_G, X_Y are the coded values for the four process variables. The selected relationship being a second degree response surface expressed as below

$Y= \beta_0+ \beta_1 X_{pH} + \beta_2 X^2_{pH} + \beta_3 X_T + \beta_4 X^2_T + \beta_5 X_G + \beta_6 X^2G + \beta_7 X_Y + \beta_8 X^2_Y + \beta_9 X_{pH}X^T + \beta_{10} X_{pH}X_G + \beta_{11} X_{pH}X_Y + \beta_{12} X_TX_G + \beta_{13} X_TX_Y + \beta_{14} X_GX_Y$

The model coefficients (βi) are estimated and used for predicting the response values for different combinations of the coded values of the variables.

Model validation

The validation experiment was performed by several sets of different combination of variables (pH, temperature, Glucose % and yeast extract %) each within their experimental ranges. The model predicated value and experimental value were used to determine the validity of model (Table 2).

TABLE 2. The actual (A) and coded (C) values of the independent variables set and corresponding values of the response variables

Experiment	pH		Temperature		Glucose %		Yeast extract %		Decolorization%	
No.	A	C	A	C	A	C	A	C	Expt	Pred
1	6.5	-0.67	20	-0.50	0.30	-0.86	0.30	-0.86	41.69	42.48
2	7.0	-0.33	25.0	0.00	0.50	-0.33	0.50	-0.33	56.92	55.58
3	8.0	0.33	30.0	+0.50	0.70	0.20	0.70	0.20	60.92	62.91
4	8.5	0.67	35	1.00	0.90	0.73	0.90	0.73	61.84	63.01

Expt = Experimetal and Pred = Predicted

Experimental design and regression model

The individual and interactive effects of the selected variables on decolorization of paper mill effluent were investigated using the BBD approach. The minimum and maximum decolorization was observed to be 20.18 and 67.49%, respectively. Polynomial regression modeling was performed between the response variable and the corresponding coded values (X_{pH}, X_T, X_G, X_Y) of the four different process variables, and finally, the best-fitted model equation was obtained as

Final Equation in Terms of Coded Factors:

Decolorization =

$+57.12+1.93\ X_{pH} +16.15\ X_T+2.37\ \ X_G-2.49\ X_Y + 1.28\ X_{pH}\ X_T - 0.70\ X_{pH}X_G + 1.61\ X_{pH}\ \ X_Y + 2.48\ \ X_T\ X_G + 1.26\ X_T\ X_Y - 1.45\ X_G\ \ X_Y - 5.97\ X^2_{pH}\ -11.01\ X^2_T +0.17\ X^2_G -2.11\ X^2_Y$

Final Equation in Terms of Actual Factors:

Decolorization =

$-180.98394+37.93111\ pH+5.85617*\ temp\ +4.11296*\ glucose\ -11.27037*\ yeast+0.085333*\ pH\ *\ temp-1.24444\ \ *\ pH\ *\ glucose\ +2.85778*\ pH\ *\ yeast\ +0.66067*\ temp\ *\ glucose\ +0.33600*\ temp\ *\ yeast\ -10.32889*\ glucose\ *\ yeast-^2.65259*\ pH^2-0.11008\ *\ temp^2\ +1.17630\ *\ glucose^2-15.00148\ *\ yeast^2$

The quadratic model was used to evaluate the influence of the process variables on the decolorization of paper mill effluent using bacterial consortium. The ANOVA results suggest that the model was highly significant.

Model Optimization:

On the basis of best fitted equation according to the model optimization of variables were done to get the maximum decolorization % result. There was 55 optimized results were found (Table 3) according to the variables it was found that the result no 9 was the maximum optimized condition for our model which shows 68.29 % decolorization. From the model optimization it was found that the best combination for decolorization process was pH 7.92, temperature 33.03°C, glucose 0.99% and yeast extract 0.36% (Table 4).

The ANOVA results suggest that the model was significant, as evident from the Fisher's F test (F model=32.76) with a very low probability value (p value <0.0001). The goodness of fit of the model was checked by the coefficient of (multiple) determination (R^2) between the experimental and model predicted values of the response variable. R^2 value provides a measure of how much variability in the observed response values can be explained by the experimental factors and their interactions. A fairly high value of R^2 (0.975) indicated that most of the data variation was explained by the regression model (Fig. 9). Moreover, a closely high value of the adjusted coefficient of determination (R^2 adj=0.945) also showed a high significance of the model. Values of "p" less than 0.0500 indicate model terms are significant. In this case A, B, C, D, A^2, B^2 are significant model terms. Values greater than 0.1000 indicate the model terms are not significant.

In terms of the regression coefficients the pH (X_{pH}), Temperature (X_T), Glucose% (X_G), quadratic term of glucose% (X^2_G) and yeast extract % (X^2_Y) , and the interactive terms ($X_{pH}X_T$, $X_{pH}\ X_Y$, $X_T\ X_G$, $X_T\ X_Y$) exhibited positive relationship with the decolorization process, whereas yeast extract % (X_Y), and quardatic term of temperature (X^2_T) and pH (X^2_{pH}) and interactive terms ($X_{pH}\ X_G$,$X_G\ X_Y$) exhibted negative relationship with the decolorization process. The percent contribution (PC) of each of the individual term in final model was computed (Table 6) using the SS (Sum of squares) values of the corresponding term. The PC of a term is obtained as the ratio of SS of an individual term to that of sum of SS for all the terms [23] (Yetilmezsoy et al., 2009), as

$$PC = \frac{SS \times 100}{\sum SS} \quad (4)$$

From the table 6 it was showed that the temperature is most significant term in decolorization process its contribution is 73.91% which is far greater than other variables used in decolorization process.

Three-dimensional response surface and contour plots

In order to gain better understanding of the effects of the independent variables and their interactions on the dependent variable, 3D response surface and contour plots for the measured responses were constructed based on the quadratic model. A circular contour of response surfaces indicates that the interaction between the corresponding variables is negligible. In contrast, an

TABLE 3. Different optimization result on the basis of variables in the experimental range

Number	pH	Temp	Glucose %	Yeast %	Decolorization %
1	7.82	31.34	0.98	0.52	67.6225
2	7.59	34.41	0.96	0.39	67.7793
3	7.70	31.97	0.97	0.33	67.9353
4	7.85	33.06	0.99	0.58	67.7903
5	7.49	32.97	0.97	0.36	68.0176
6	7.44	33.64	0.96	0.48	67.6455
7	7.50	32.70	0.99	0.46	68.1609
8	7.21	33.70	0.99	0.29	67.7441
9	7.92	33.03	0.99	0.36	68.2933
10	7.68	32.58	0.95	0.52	67.5879
11	7.71	34.20	0.95	0.48	67.6233
12	7.77	33.18	0.98	0.59	67.7266
13	7.77	31.35	0.97	0.25	67.5375
14	7.68	31.98	0.96	0.42	67.8224
15	7.77	34.20	0.96	0.54	67.6213
16	7.71	33.66	0.96	0.32	67.9025
17	7.80	32.67	0.94	0.47	67.6539
18	7.92	33.80	0.95	0.49	67.5261
19	7.30	33.03	1.00	0.30	68.1980
20	7.54	30.68	0.99	0.29	67.7022
21	8.09	34.06	0.98	0.44	67.7776
22	7.79	32.05	0.97	0.50	67.7332
23	7.62	34.20	0.96	0.37	67.7976
24	7.04	33.61	1.00	0.27	67.4920
25	8.06	34.40	0.97	0.43	67.5996
26	7.99	33.74	0.98	0.58	67.5638
27	7.83	32.62	0.93	0.35	67.4929
28	7.60	31.29	0.98	0.54	67.5052
29	7.22	34.86	0.99	0.39	67.4968
30	7.74	34.95	0.96	0.48	67.5505
31	7.72	33.27	0.94	0.45	67.7354
32	7.56	33.58	0.94	0.48	67.5419
33	8.01	33.69	1.00	0.31	68.0409
34	7.61	31.10	0.97	0.38	67.7409
35	8.23	32.14	1.00	0.36	67.4978
36	8.06	31.39	1.00	0.44	67.7000
37	7.95	31.68	0.99	0.54	67.5881
38	7.66	31.47	0.96	0.28	67.5780
39	7.39	31.89	0.99	0.54	67.5067
40	7.76	33.25	0.98	0.61	67.5689
41	7.95	34.76	0.99	0.54	67.8586
42	7.87	32.54	0.96	0.45	67.7726
43	8.19	33.87	1.00	0.50	67.7414
44	7.93	32.97	0.95	0.50	67.6044
45	7.81	31.96	0.99	0.28	68.0833
46	7.34	32.72	0.97	0.47	67.6412
47	8.02	32.87	0.96	0.43	67.6846
48	7.43	33.86	0.96	0.32	67.7368
49	7.80	34.79	0.98	0.40	67.9592
50	7.38	31.66	0.97	0.40	67.6947
51	7.31	31.92	0.98	0.32	67.8456
52	8.16	34.35	1.00	0.62	67.3623
53	8.28	34.68	1.00	0.62	67.0489
54	6.72	31.82	1.00	0.25	66.3605
55	6.86	29.49	1.00	0.25	65.8982

TABLE 4. ANOVA of Response Surface Quadratic Model for decolorization of paper mill effluent

Source	Sum of Squares	df	Mean Square	F Value	p-value	Remarks
Model	4186.88	14	299.06	32.76	< 0.0001	significant
XpH-pH	44.51	1	44.51	4.87	0.0475	significant
XT-temp	3129.55	1	3129.55	342.79	< 0.0001	significant
XG-glucose	67.21	1	67.21	7.36	0.0188	significant
XY-yeast	74.5	1	74.5	8.16	0.0144	significant
XpHXT	6.55	1	6.55	0.72	0.4134	
XpH XG	1.96	1	1.96	0.21	0.6514	
XpH XY	10.34	1	10.34	1.13	0.3083	
XT XG	24.55	1	24.55	2.69	0.127	
XT XY	6.35	1	6.35	0.7	0.4206	
XG XY	8.44	1	8.44	0.92	0.3553	
X2pH	189.98	1	189.98	20.81	0.0007	significant
X2T	646.31	1	646.31	70.79	< 0.0001	significant
X2G	0.15	1	0.15	0.016	0.9015	
X2Y	23.74	1	23.74	2.6	0.1329	
Residual	109.56	12	9.13			
Lack of Fit	109.56	10	10.96			
Pure Error	0	2	0			
Cor Total	4296.44	26				

TABLE 5. Multiple regression results and significance of the components for the quardatic model

Factor (coded)	Parameter	Coefficient	Effect	Sum of squares	PC
Intercept	β0	57.12	-	-	-
XpH-pH	β1	1.93	3.86	44.51	1.05
XT-temp	β2	16.15	32.3	3129.55	73.91
XG-glucose	β3	2.37	4.74	67.21	1.59
XY-yeast	β4	-2.49	-4.98	74.5	1.76
XpHXT	β5	1.28	2.56	6.55	0.15
XpH XG	β6	-0.7	-1.4	1.96	0.05
XpH XY	β7	1.61	3.22	10.34	0.24
XT XG	β8	2.48	4.96	24.55	0.58
XT XY	β9	1.26	2.52	6.35	0.15
XG XY	β10	-1.45	-2.9	8.44	0.2
X2pH	β11	-5.97	-11.94	189.98	4.49
X2T	β12	-11.01	-22.02	646.31	15.26
X2G	β13	0.17	0.34	0.15	0
X2Y	β14	2.11	4.22	23.74	0.56

elliptical or saddle nature of the contour plots indicates that the interaction between the corresponding variables is significant [24] (Liu and Chiou, 2005). Almost parallel contour lines suggest that there is no significant interaction. In contour plots, the upper horizontal axis represents the dependent variable.

Fig. 10 showed 3D response surface and contour plots for decolorization by Marine consortium as a function of temperature and effluent pH at constant initial inoculums size (1 mL of inoculate of each M2, M3, M4=3 mL).

The decolorization % increased with increasing temperature and with the pH of the paper mill effluent within their respective experimental ranges. However, the influence of temperature on decolorization was very significant and influence of pH is not significant as such temperature. It also appears from experimental results. It may be because of different growth pattern of bacteria in different temperature. The elliptical view of the lines in 3D contour plot suggested for significant interactive influence of these two variables on the decolorization process.

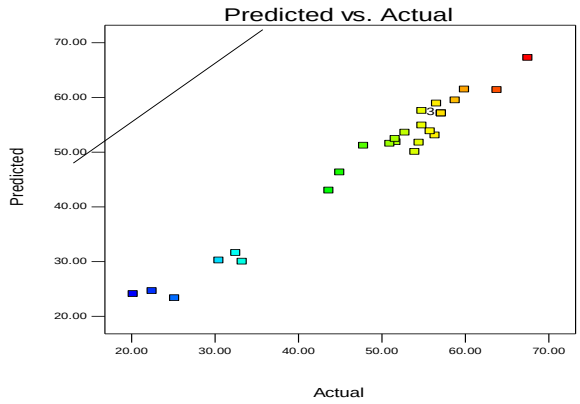

Figure. **9**. Plot of the measured and model predicted values of the response variable

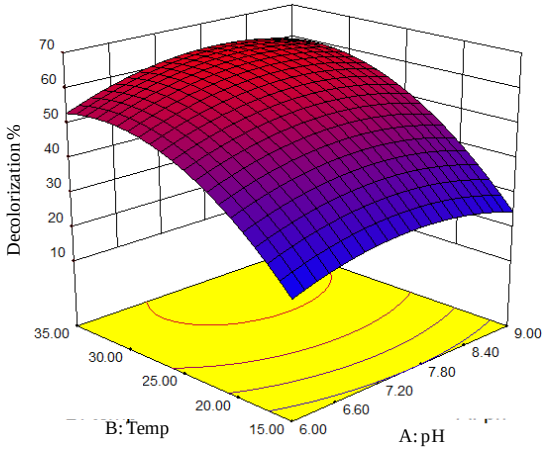

Figure 10. The 3D a response surface and b contour plots showing effect of Glucose and pH on the decolorization process

Fig. 11 showed the 3D response surface and contour plots for decolorization by Marine consortium as a function of Glucose % and effluent pH at constant initial inoculums size (1 mL of inoculate of each M2, M3, M4=3 mL). In this decolorization % increased with increasing effluent pH but there is no significant effect of glucose on the decolorization process. Almost parallel lines in 3D contour plot Fig. 11 suggested almost no interactive influence of these two variables on the decolorization process.

Fig. 12 showed the 3D response surface and contour plots for decolorization by Marine consortium as a function of Yeast extract % and effluent pH at constant initial inoculums size (1 ml of inoculate of each M2, M3, M4=3 mL). Figure showed that with increasing yeast extract % decolorization % decreases and with increasing pH the decolorization % increases.

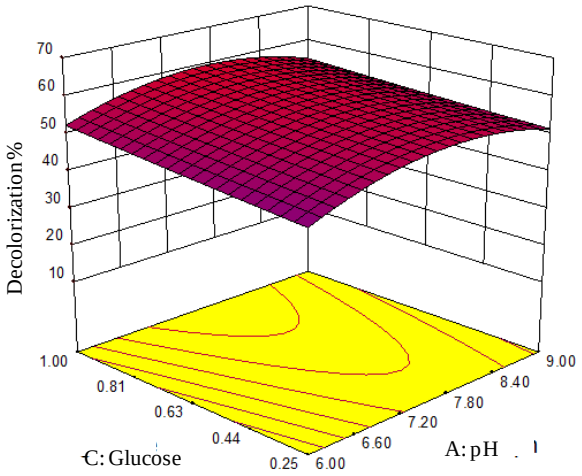

Figure 11. The 3D a response surface and b contour plots showing effect of Glucose and pH on the decolorization process

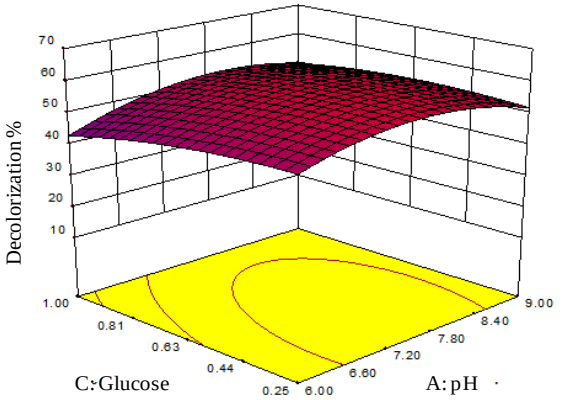

Figure 12. The 3D (a) RSM and (b) contour plots showing effect of yeast extract % and pH on the decolorization process

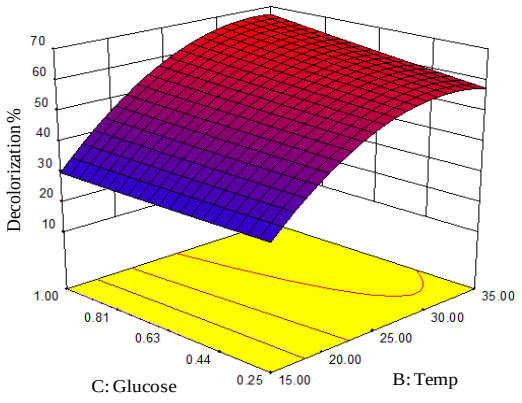

Figure 13. The 3D a response surface and b contour plots showing effect of Glucose% and Temperature on the decolorization process

Fig. 13 showed the 3D response surface and contour plots for decolorization by Marine consortium as a function of Glucose % and Temperature at constant initial inoculums size (1 mL of inoculate of each M2, M3, M4=3 mL). Figure showed that with increasing temperature the decolorization % increases whereas with increasing in glucose percentage, the decolorization percentage was decreased but it was a minor change.

Fig. 14 showed the 3D response surface and contour plots for decolorization by Marine consortium as a function of yeast extract % and Temperature at constant initial inoculums size (1 mL of inoculate of each M2, M3, M4=3 mL). Fig. 14 showed that with increasing yeast extract % the decolorization % decreases. Almost parallel line suggested almost no interactive influence of these two variables on the decolorization process.

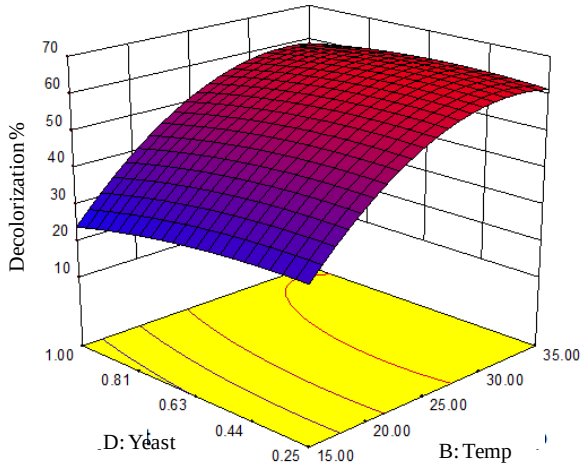

Figure 14. The 3D (a) RSM and (b) contour plots showing effect of yeast extract % and temperature on the decolorization process

Fig. 15 showed the 3D response surface and contour plots for decolorization by Marine consortium as a function of yeast extract % and Glucose % at constant initial inoculums size (1 mL of inoculate of each M2, M3, M4=3 mL). Figure showed that with increasing yeast extract % decolorization % decreased it may be because of melanoid pigments present in the yeast which may impart their color also, whereas with increasing glucose % the decolorization % increased.

CONCLUSIONS

Present study of color removal was based on physico-chemical and biotechnological methods. The problems underlying the physico-chemical treatment are those associated with cost and reliability. Biotechnological method has the potential to eliminate/reduce the problems associated with physico-chemical methods. Among four bacterial strains, three bacterial strains Bacillus subtilis, Bacillus endophyticus, Bacillus sp. were capable of ligninolytic activity. Consortium made by these bacterial strains enhances the degradation of lignin as well as decolorization. Various nitrogen source, carbon source, pH, temperature and low molecular weight organic acids were used in the optimization process of decolorization and degradation of lignin in paper mill effluent. Maximum decolorization 68.29% was found at pH 7.92, temperature 33.03°C, in the presence of glucose (carbon source) 0.99% and yeast extract (nitrogen source) 0.36% when it was optimized through response surface methodology.

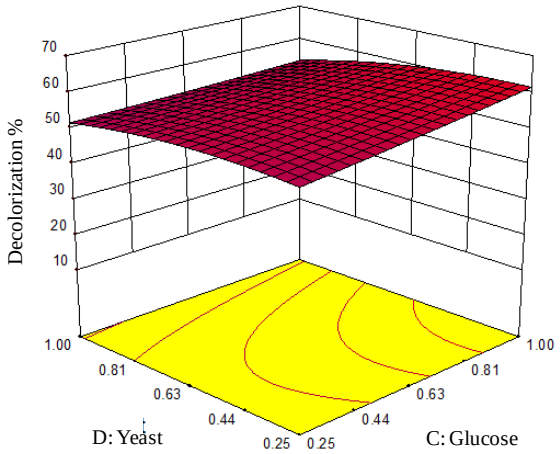

Figure 15. The 3D RSM (a) and (b) contour plots showing effect of yeast extract % and Glucose % on the decolorization process

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Persian Abstract

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چکیده

هدف این کار تحقیقاتی جداسازی گونه های باکتریایی با توانایی بالا در تجزیه و رنگ زدایی ترکیبات لیگنوسلولزی خروجی کارخانه کاغذ می باشد. ۴ گونه باکتریایی از رسوبات دریایی جداسازی شدند و آنها از نظر توانایی تجزیه کردن لیگنین و رنگ زدایی خروجی کارخانه کاغذ و خمیر غربال گری شدند. در میان ۴گونه باکتریایی ، ۳گونه باکتریایی *Bacillus Subtilis, Bacillus endo-phyticus, Bacillus Sp*. قادر به فعالیت تجزیه لیگنین شدند. کنسرسیوم (consortium) ساخته شده به وسیله ی این گونه های باکتریایی تجزیه لیگنین را به خوبی رنگ زدایی افزایش داد. منبع نیتروژنی متنوع ، منبع کربنی ،pH ، دما و اسیدهای آلی با وزن مولکولی پایین در بهینه سازی فرایند رنگ زدایی و تجزیه لیگنین در خروجی کارخانه کاغذ استفاده شدند. ماکسیم رنگ زدایی ۶۸,۲۹٪ در pH ۷/۹۲، دمای ۳۳درجه سلسیوس ، در حضور گلوکز (به عنوان منبع کربنی) ۰,۹۹٪ و عصاره مخمر (به عنوان منبع نیتروژنی) ۰,۳۶٪ بدست آمده با استفاده از (RSM) بهینه شد.