

Isolation of fungi and optimization of process parameters for decolorization of distillery mill effluent

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Abstract Five different fungi isolated from distillery mill site in which two isolates (DF3 and DF4) had higher capabilities to remove color were identified as *Emericella nidulans* var. *lata* and *Neurospora intermedia*, respectively. Optimization of process parameter for decolorization was initially performed to select growth factors which were further substantiated by Taguchi approach in which seven factors, %carbon, %nitrogen, duration, pH, temperature, stirring and inoculum size, at two levels applying L-8 orthogonal array were taken for both fungi. Maximum color was removed at pH 3, temperature 30°C, stirring 125 rpm, dextrose (0.05%) and sodium nitrate (0.025%) by both fungi. After optimization, there was two-fold increase in color removal from 38 to 62% (DF3) and 31 to 64% (DF4) indicating significance of Taguchi approach in decolorization of distillery mill effluent. The mechanism for decolorization was determined by enzyme analysis, laccase and glucose oxidase, and indicated significant activity in DF3 as compared to DF4.

Keywords Decolorization · Distillery spent wash · *Emericella nidulans* var. *lata* · *Neurospora intermedia* · Taguchi approach

Introduction

Distilleries are one of the most polluting industries generating enormous amount of wastewater in which an average

of 10–15 l of effluent is generated with 1 l of alcohol production. The distillery wastewater known as spent wash is characterized by its dark brown color, high temperature, low pH, and high percentage of dissolved organic and inorganic matter. It also contains nearly 2% of the dark brown recalcitrant pigment called melanoidin (Kitts et al. 1993). Putriciable organics like skatole, indole and other sulfur compounds produces obnoxious smell in the effluent and when it comes in contact with high temperature, becomes more toxic to aquatic biota (Kitts et al. 1993). A typical distillery, which uses cane molasses as raw material for the production of ethanol, generates huge amount of spent wash daily.

Molasses has very high quantity of fermentable sugars (sucrose, glucose and fructose). These sugars react with amino acids, undergo Maillard reaction (amino carbonyl complex), and then polymerize to form melanoidin, which is a major color containing compound in the distillery effluent. Effluent so generated has very high chemical oxygen demand (COD) (80,000–100,000 mg/l) and biochemical oxygen demand (BOD) (40,000–50,000 mg/l), apart from low pH, strong odor and dark brown color (Raghukumar and Rivonkar 2001). Disposal of conventionally treated distillery spent wash is harmful as it contains mostly recalcitrant compounds, which are toxic to aquatic biota. Therefore, a comprehensive treatment strategy is required for decolorization of distillery spent wash to meet the discharge safety standards.

Decolorization of melanoidin in spent wash has been studied using activated carbon prepared from sugarcane bagasse (Bernardo et al. 1997), an inorganic flocculent (Migo et al. 1993), and using various microorganisms (NakajimaKambe et al. 1999). So far various studies have been carried out using fungi such as *Aspergillus fumigatus* (Ohmomo et al. 1987), *Phanerochaete chrysosporium*

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(Fahy et al. 1997), *Flavodon flavus* (Raghukumar and Rivonkar 2001). There are reports related to role of fungi in decolorization of melanoidin into mycelia (Ohmomo et al. 1988; Sirianuntapiboon et al. 1995; Pena et al. 1996). For effective decolorization it is essential to optimize the composition of culture media and culture conditions (Prakasham et al. 2005). In conventional methods numerous experiments have to be carried out to optimize all the parameters (factors) and to establish best possible culture condition by interrelating all the parameters. In these methods studying one variable at a time is long, cumbersome and not economical. Another approach is to use statistical tools and experimental designs (Stowe and Mayer 1999). Taguchi methods have been widely used to optimize the reaction variable by devising minimum number of experiments. This approach also facilitates to identify the impact of individual factor and find out the link between variables and operational conditions. Analysis of the experimental data using the ANOVA (analysis of variance) and factors effect gives the output that is statistically significant. This approach provides simple and efficient methodology for optimization with minimum number of experiments (Kackar 1985; Phadke and Dehnad 1988). Thus in the present study, fungi were isolated from distillery mill site and process parameters were optimized by a methodological application of Taguchi approach for optimizing the culture conditions to decolorize the distillery spent wash.

Materials and methods

Sampling site and microorganism

Fungal strains were collected from distillery effluent and sludge, Modi distilleries, Modinagar, Uttar Pradesh, India. For isolation of fungi capable of decolorizing distillery effluent, sludge and effluent were mixed and serially diluted in the order of 10^{-3} , 10^{-4} , 10^{-5} by using autoclaved double distilled water. Diluted samples (100 μ l) were spread on potato dextrose agar (PDA) plates, and kept at 30°C for 4 days. Fungal colonies that appeared on PDA plates were isolated and purified by repeated culturing (Thakur 2004). The fungal isolates were identified based on morphological structures as color, texture of the mycelia, spore formation and also by amplification of internal transcribed spacer (ITS) region of genomic DNA. For the preparation of pellets, isolates were grown in potato dextrose broth with streptopenicillin (100 ppm). The flasks were incubated at 30°C for 4 days in orbital shaker. Pellets of approximately 1.5–2 mm size were used for the treatment of distillery spent wash (Thakur 2004).

Identification of fungal strains

For identification of fungi, genomic DNA was isolated from pure fungal mycelia grown in PDB at 30°C for 7 days with modifications of procedure as described by Karakousis et al. (2006). In this method, 1 mg of freshly grown mycelium was frozen in liquid nitrogen and ground to fine powder using pre-frozen pestle and mortar (pre-sterilized by baking at 200°C, overnight). Extraction buffer (5 ml, 0.1 M Tris-Cl, 1.4 M NaCl, 0.02 M EDTA, 2% CTAB) preheated at 60°C was added along with β -mercaptoethanol (0.2%). It was mixed gently and incubated at 60°C for 1 h with periodic stirring. An equal volume of chloroform:isoamyl alcohol (24:1) was added to the crude extract and mixed at room temperature for few minutes. The content was centrifuged at 10,000 rpm for 10 min and supernatant was carefully decanted. Isopropanol (0.6th volume of ice cold) was added and solution was mixed and then kept at -20°C for 1 h. The mixture was centrifuged at 10,000 rpm for 10 min and the recovered pellet was washed with equal volume ethanol (70% chilled). Finally the pellet was air dried to remove all traces of ethanol and dissolved in TE (100 μ l). Internal transcribed spacer (ITS) region of the fungi were amplified using primers ITS1 having sequence 5' TCCGTAGGTGAACCTGCGG 3' and ITS4 having sequence 5' TCCTCCGCTTATTGATATGC 3' (Hortal et al. 2006). The reaction mix (25 μ l) consisted of buffer containing $MgCl_2$ -2.5 μ l (2.5 mM), dNTP-2.0 μ l (1 mM each), ITS1 (forward)-0.7 μ l (10 mM), ITS4 (reverse)-0.7 μ l (10 mM), Taq polymerase-0.5 μ l (5 U/ μ l), DNA-20–50 ng and volume was made up by water. The thermocycler program include an initial denaturation of 94°C for 5 min, followed by 35 cycles of amplification at 94°C for 1 min, 55°C for 1 min and 72°C for 1 min and a final extension of 7 min at 72°C. Amplified products were resolved on 0.7% agarose gels containing ethidium bromide (1 μ g/ml). The amplified products were sequenced. The resulting sequence was entered into the BLAST nucleotide search program of the National Center for Biotechnology Information to obtain closely related phylogenetic sequences. Pairwise alignments giving the closest match were chosen. The megablast algorithm was used and the phylogeny tree was constructed using Mega 3.1 software (Yang et al. 2007).

Culture condition and decolorization of distillery effluent

The fungal strains were screened for decolorization in minimal salt medium (MSM containing (g/l): $Na_2HPO_4 \cdot 2H_2O$, 7.8; KH_2PO_4 , 6.8; $MgSO_4$, 0.2; $Fe(CH_3COO)_3 NH_4$, 0.05; $Ca(NO_3)_2 \cdot 4H_2O$, 0.05 $NaNO_3$, 0.085; 10% distillery spent wash, at 3 pH and incubated at 30°C

(Thakur 2004). Decolorization of effluent was estimated on 3, 7, 10 and 15 days. Each experiment was performed in three replicates.

Decolorization assay

Color content was measured as described in the manual of Standard Methods for Examination of Water and Wastewater (APHA 2005). The sample was centrifuged at 10,000 rpm for 15 min to remove all the suspended matter. The supernatant was taken and absorbance was measured at 456 nm. The value of color was calculated in coloring unit (CU) by making standard curve.

Experimental design for optimization of process parameters

For the Taguchi design and analysis of results the Qualitek-4 version (7.6.0.3) software was used (Roy 2007). Traditional optimization methods involve variation of one parameter while keeping all other parameters constant and assessing the impact of that particular parameter on the process performance. In this study we have used Taguchi approach of orthogonal array and experimental designs that help to gain more information about the optimum conditions. It involves two important steps (1) the use of orthogonal arrays, (2) ANOVA for analysis. Orthogonal arrays are well-defined tables that are used to reduce the number of experiments to be conducted. Taguchi's L-8 orthogonal array table was used to carry out decolorization experiments by choosing seven parameters at two levels using (Table 1) (Roy 2007). In the orthogonal array of L-8 type, L and subscript 8 means Latin square and the number of experiments, respectively. Full factorial approach will

require 128 experiments to be conducted for optimizing a process while in fractional factorial using L-8 orthogonal array the number of experiments reduces to eight (Bakhtiari et al. 2006). After designing, the analysis of experimental data was done using ANOVA. Taguchi approach used ANOVA to evaluate which parameters were statistically significant in finding the optimum levels (Phadke and Dehnad 1988).

Screening experiments for decolorization of distillery spent wash

Screening experiments were performed to select most suitable carbon and nitrogen sources. Various carbon sources were used at an initial concentration 0.05% (w/v). MSM having distillery spent wash (5,000 C.U.) was taken as control. Batch study was conducted in an Erlenmeyer flasks containing 200 ml MSM along with 10% distillery spent wash, 5% (w/v) inoculum supplemented with different carbon sources (0.05%). Reduction in color was analyzed after 5 days. After selecting the most suitable carbon source, various nitrogen sources were screened at a concentration of 0.05% (w/v). Change in color was analyzed after 5 days. Same sets of experiments were carried out for both fungi.

Optimization of growth conditions by Taguchi approach

Once carbon and nitrogen sources were selected, the growth media was optimized for the optimum concentration of selected carbon and nitrogen sources, i.e., carbon (%) and nitrogen (%). Other process parameters such as pH, inoculum size, temperature, RPM and duration were

Table 1 Taguchi orthogonal array table of L-8 type and percentage decolorization in each run

No of experiment	Temperature	RPM	pH	Duration	Inoculum size	Carbon	Nitrogen	Decolorization (Percentage)	
								DF3 (<i>Emericella nidulans</i> var. <i>lata</i>)	DF4 (<i>Neurospora intermedia</i>)
1	1	1	1	1	1	1	1	62 ± 0.4	64 ± 0.3
2	1	1	1	2	2	2	2	30 ± 1.5	38 ± 0.6
3	1	2	2	1	1	2	2	18 ± 0.7	25 ± 0.4
4	1	2	2	2	2	1	1	54 ± 0.1.2	39 ± 1.5
5	2	1	2	1	2	1	2	22 ± 0.9	33 ± 0.3
6	2	1	2	2	1	2	1	30 ± 1.1	28 ± 0.5
7	2	2	1	1	2	2	1	20 ± 0.3	34 ± 0.6
8	2	2	1	2	1	1	2	36 ± 0.03	28 ± 1
Total contribution of all factors								33.75	27.87
Grand average of performance								32.75	36.12
Expected result at optimum condition								66.5	64

also optimized using their lower and higher levels which were coded as 1 and 2, respectively (Table 1). All the experiments were done in triplicate.

Analysis of the data

The data obtained after the eight experiments was processed in the Qualitek-4 software with quality characteristics for deriving the optimum level for each parameter and ultimately arriving at optimal culture conditions. Besides that, the contribution of each individual factor in total decolorization and the interactions among various parameters was also studied.

Validation

In order to validate the methodology used in optimization, the culture conditions obtained after optimization was tested and compared with the values predicted by the model. Experiment was repeated three times. Qualitek-4 software (Nutek Inc., MI) for automatic design of experiments using Taguchi approach was used in this study. This software is equipped to use L-4 to L-64 orthogonal arrays along with selection of 2–63 factors (parameters) with two, three or four levels. The automatic design option in the software allows selecting the array used and assigning factors to the appropriate columns. In this study L-8 orthogonal array was used with seven parameters at two levels (Dasu et al. 2003).

Estimation of enzymes for decolorization

To measure the activities of the enzyme the culture supernatant was obtained by centrifugation at $8,000\times g$ for 10 min. Four different enzymes, Lignin peroxidase (LiP), Manganese peroxidase (MnP), Laccase (Lac) and Glucose oxidase (GO) were estimated. LiP was measured through the oxidation of veratryl alcohol (VA) to veratryl aldehyde at 310 nm using Varian Carry 100 Bio Spectrophotometer (Tien and Kirk 1988). The reaction mixture consisted of 2 mM VA, 35 mM sodium tartrate buffer (pH 3.0), and enzyme. 1 U/ml of enzyme is defined as 1 μmol of veratryl alcohol oxidized to veratraldehyde per min. The reaction starts with the addition of 0.36 mM H_2O_2 . MnP activity was measured through the oxidation of Mn(II) to Mn(III) at 270 nm as described (Wariishi et al. 1992). The reaction mixture consists of 0.5 mM $\text{MnSO}_4\cdot\text{H}_2\text{O}$, 45 mM sodium malonate buffer (pH 4.5) and enzyme. The reaction started with the addition of 0.1 mM H_2O_2 . One U/ml of MnP was defined as 1 μmol of Mn(II) oxidized to Mn(III) per min. Laccase activity was measured by the oxidation of 2, 2'-azinodi-3-ethyl-benzothiazoline-6-sulfuric acid (ABTS) at 436 nm (Niku-Paavola et al. 1988). The reaction mixture

contains 10 mM ABTS, 85 mM sodium tartrate buffer (pH 3.0) and enzyme. One unit (U/ml) of enzyme activity was defined as the amount of enzyme required to oxidize 1 μmol substrates per min at 25°C. Glucose oxidase was measured by decrease in reducing sugars at 575 nm (Kona et al. 2001). In this case 0.2 ml of reducing sugar solution was incubated with 0.2 ml enzyme and citrate-phosphate buffer (1 ml, pH 5.6).

Nucleotide sequence accession number

Accession numbers for 18S rDNA partial sequences of strains DF3 and DF4 are EU741056 and EU741055, respectively.

Results and discussion

Isolation and screening of fungal strains for decolorization

Five different types of fungal strains appeared on the plates were isolated based on morphological differentiation of individual colonies. They were observed under a microscope, Olympus and Magnus MLX-TR, at 40 and 100 \times attached with a camera. Fungal mycelium, spores and the spore attachment were observed. They were further purified using PDA by repeated culturing and screened on the basis of their decolorization potential. The decolorization was measured after 1, 3, 7, 10 and 15 days (Fig. 1). Maximum reduction in color was observed on 10th day by DF3 (38%), followed by DF4 (31%), DF5 (23%), DF1 (9%) and minimum by DF2 (7%). The best performing fungal isolates DF3 and DF4 were acclimatized for 3 months at pH 3 with 10% distillery spent wash. After acclimatization the time required for decolorization reduced from 10 to 3 days.

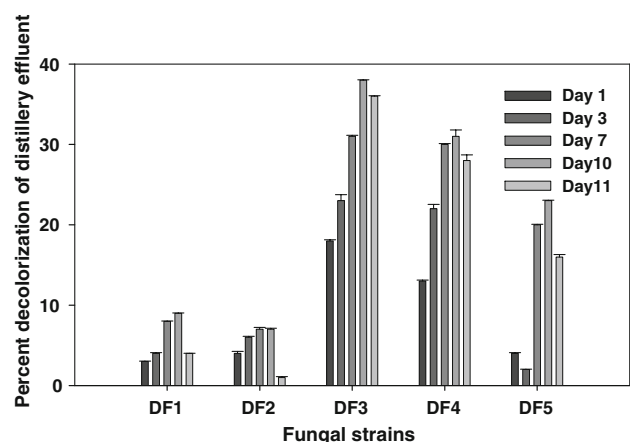


Fig. 1 Percent decolorization of distillery effluent by fungal isolates at different time interval in shake flask culture

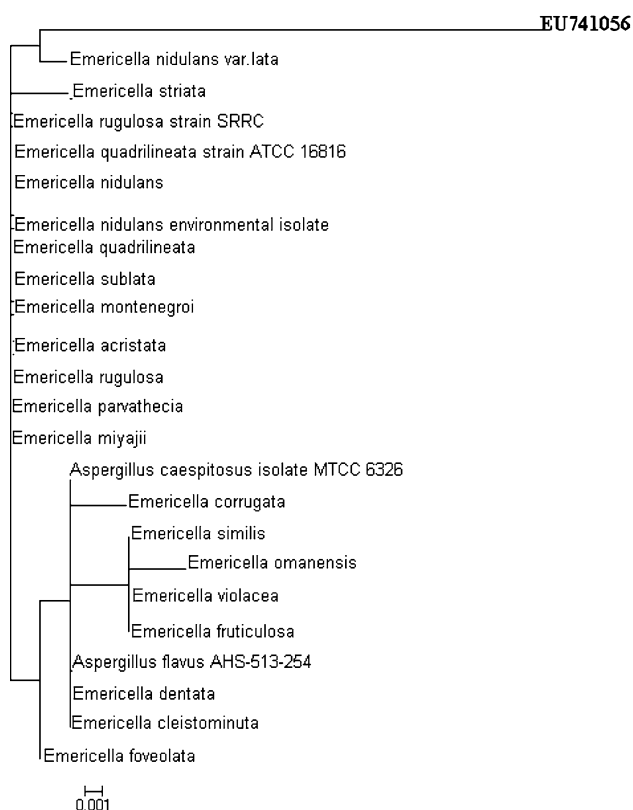


Fig. 2 Phylogeny tree of DF3 identified by ITS region of genomic DNA as *Emericella nidulans* var. lata obtained from distillery sludge and effluent. In figure scale 1 means substitution per 1,000 nucleotide

Identification and characterization of fungal strains

The two best performing strains (DF3 and DF4) were selected based on decolorizing potency of distillery mill effluent. The isolates were identified as *Emericella nidulans* var. lata (DF3) and *Neurospora intermedia* (DF4) after amplification and sequencing of ITS region of genomic DNA. The sequence of the amplified region, i.e., ITS1, 5.8S and ITS 4 of DF3 and DF4 was blasted using NCBI and the phylogeny tree was constructed using Mega 3.1 software. Figure 2 shows the phylogeny tree of DF3. The fungal strain was close to *Emericella nidulans* var. lata (DF3) having 98% homology. Similarly DF4 was identified as *Neurospora intermedia* (Fig. 3). However, this is the first report presenting the role of *Neurospora intermedia* (DF4) in decolorization studies.

Screening of growth factors for decolorization of distillery effluent

Among the various carbon sources (0.05%) maximum color was removed when effluent was treated by *Emericella nidulans* var. lata (DF3) in presence of dextrose

(41%) followed by sucrose (37%), sodium citrate (14%) and sodium acetate (8%), however, *Neurospora intermedia* (DF4) reduced color of the effluent in presence of dextrose (43%), sucrose (36%), sodium citrate (15%) and sodium acetate (8%) (Fig. 4). Result of the study indicated dextrose was the best carbon source for both fungi. In general, high carbon levels favor the process of decolorization, however, maximum decolorization of distillery spent wash in absence of additional carbon and nitrogen sources using soil as inoculum are also reported (Sirianuntapiboon et al. 2004; Adikane et al. 2006).

Effluent was further treated in presence of inorganic and organic nitrogen source (0.05%) like urea, yeast extract, sodium nitrate and ammonium nitrate, and the results indicated that maximum decolorization was achieved with sodium nitrate (33%), followed by ammonium nitrate (16%), urea (16%) and yeast extract (13%) by *Emericella nidulans* var. lata (DF3). Similarly in case of *Neurospora intermedia* (DF4) maximum reduction in color was achieved by sodium nitrate (30%) followed by ammonium nitrate (21%), urea (15%) and yeast extract (13%) (Fig. 4). Both strains preferably used inorganic nitrogen sources as compared to organic nitrogen sources for decolorization. Sirianuntapiboon et al. (2004) reported similar observations. Inhibitory effects of nitrogen on melanoidin decolorization by *Coriolus hirsutus* were observed (Miyata et al. 2000). The combined effect of the study says that decolorization was improved by the addition of carbon and nitrogen sources.

Determination of optimum conditions using Taguchi method

Taguchi method was used to identify the optimum conditions and select the parameter having the most significant step in decolorization of distillery spent wash. After selecting the most suitable carbon and nitrogen source, the effect of temperature, RPM, pH, inoculum size and duration and ratio of carbon and nitrogen sources were further optimized by using Taguchi approach for both strains (*Emericella nidulans* var. lata (DF3) and *Neurospora intermedia* (DF4)).

Experiments were conducted for optimization of increased decolorization according to the L-8 orthogonal array. Total eight experiments were conducted. Same sets of experiments (Table 1) were carried out with both fungi. Results showed that maximum decolorization achieved by *Emericella nidulans* var. lata (DF3) (62%) when experiment conditions were as follows: temperature 30°C, pH 3, agitation 125 rpm, duration 3 days, inoculum size 10% (w/v), carbon (dextrose) 0.05% (w/v), nitrogen (sodium nitrate) (0.025%) (w/v). For *Neurospora intermedia* (DF4) the optimum conditions for maximum decolorization (64%) were: temperature 30°C, pH 3, agitation 125 rpm, duration

Fig. 3 Phylogeny tree of DF4 identified by ITS region of genomic DNA as *Neurospora intermedia* obtained from distillery sludge and effluent. In figure scale 1 means substitution per 1,000 nucleotide

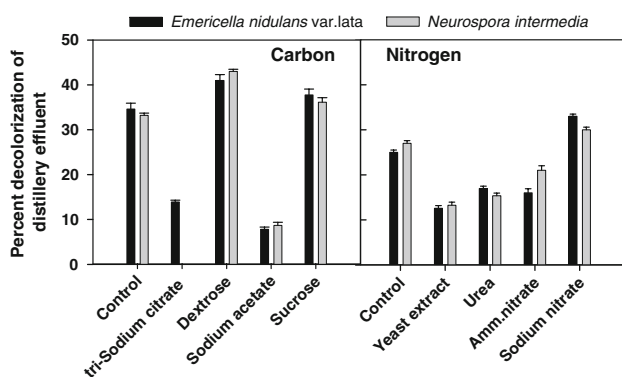
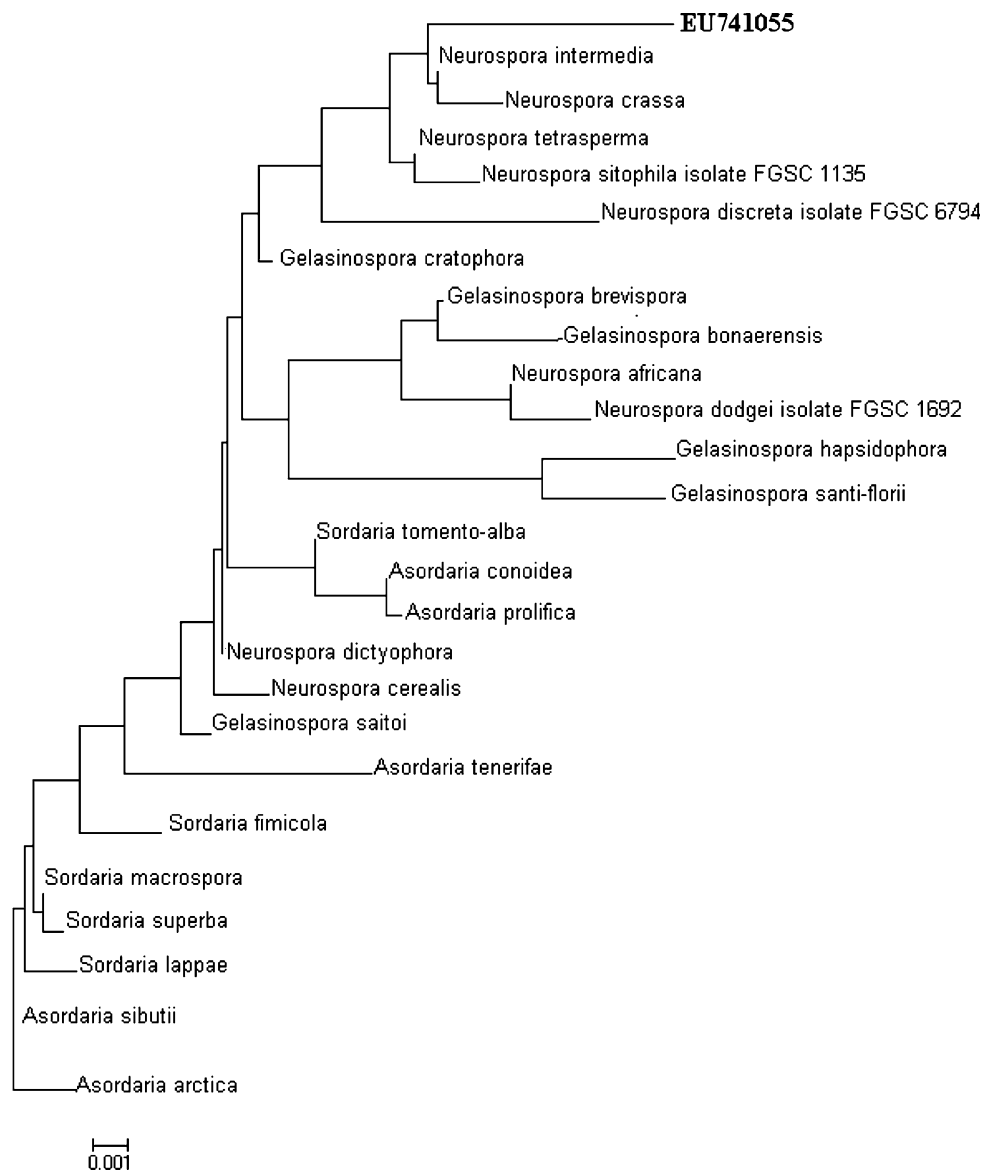
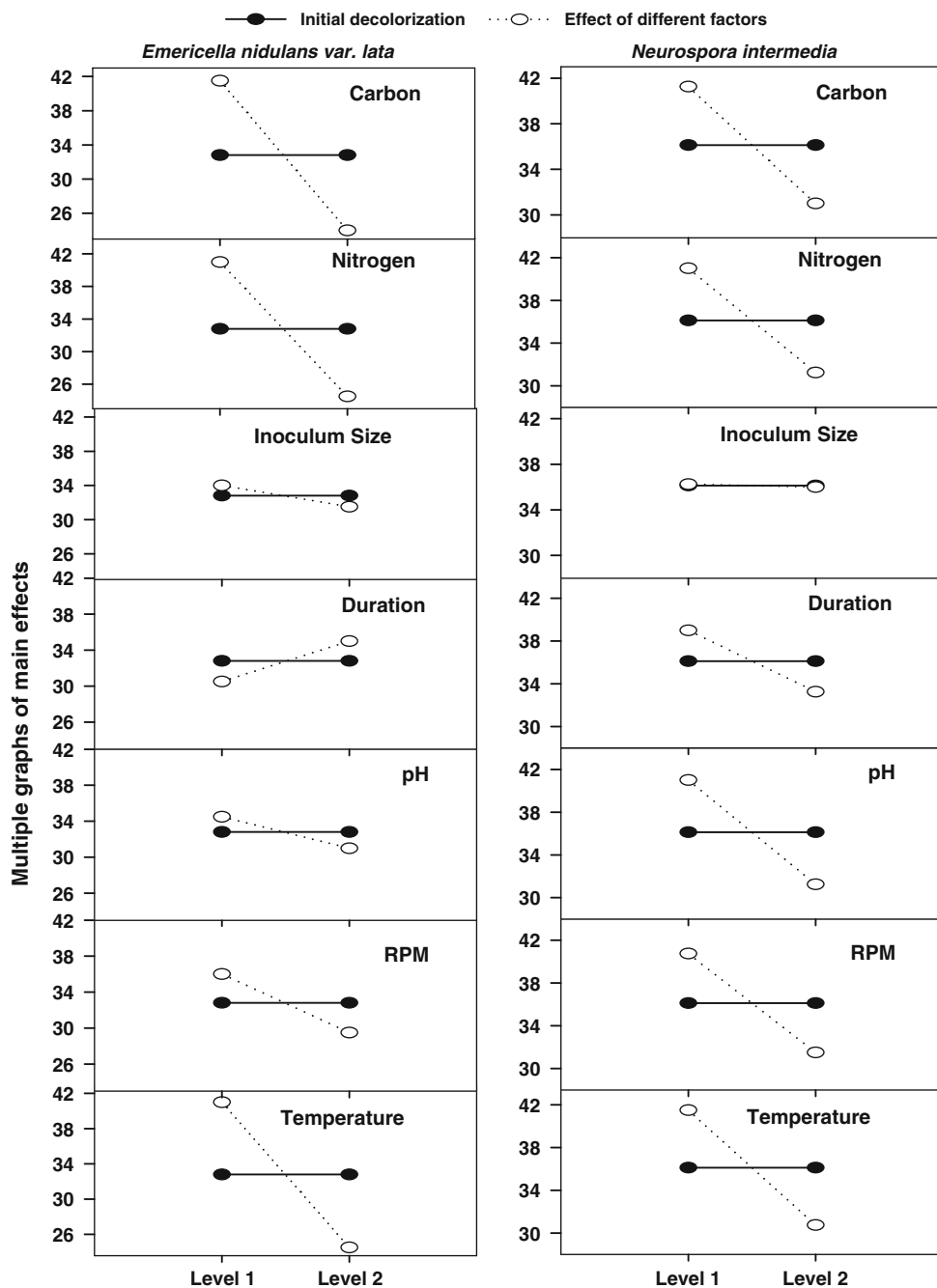


Fig. 4 Percent decolorization of distillery effluent in presence of carbon and nitrogen sources. In figure, is *Neurospora intermedia* and *Emericella nidulans* var. lata.

2 days, inoculum size 10% (w/v), carbon (dextrose) 0.05% (w/v), nitrogen (sodium nitrate) (0.025%) (w/v). In case of *Neurospora intermedia* (DF4) mycelium became dark, indicating that adsorption was the dominant phenomenon in the process of decolorization. However, in case of *Emericella nidulans* var. lata (DF3) the color of the mycelium remained unchanged, suggesting that the fungi might have metabolized various compounds present in the effluent in 3 days by secretion of enzymes responsible for decolorization.

Table 1 shows the results obtained after performing the experiments. The effective influence of a Taguchi L-8 orthogonal array designed experimental conditioned on maximum decolorization could be viewed and the data revealed variation in percentage decolorization of distillery

Fig. 5 Individual factor performance at different levels in decolorization of distillery mill effluent by fungi. In figure, —●— is initial decolorization and ···○··· effect of different factors



spent wash (Table 1). The observed variation certainly indicated the imperative role of optimization of all factors in achieving the best possible results. The results of decolorization of all eight experiments in both cases were fed in Qualitek-4 software for analysis. The optimization software provided an opportunity to study the multi interaction effect of the factors. Among all selected factors carbon and temperature had the highest positive impact on decolorization potency in case of *Emericella nidulans var. lata* (DF3) and *Neurospora intermedia* (DF4), respectively. The contribution of individual factor is the key to control

the biological processes. For each factor there is an optimum level. Figure 5 shows the increase or decrease in decolorization for each factor at different levels.

Carbon and nitrogen sources have extremely variable response in reference to decolorization. The optimum level of dextrose, most suitable carbon source, was 0.05% w/v i.e. level 1. Increasing the dextrose concentration 0.2% (level 2) reduced the decolorization drastically in both cases (Fig. 5). The percent of decolorization was increased in case of *Emericella nidulans var. lata* (DF3) when the factor duration was taken from level 1 (2 days) to level 2

Table 2 Analysis of variance of main effect of factors by *Emericella nidulans* var. *lata* (DF3) for efficient decolorization

Factor	Degree of freedom (<i>f</i>)	Sum of squares (<i>S</i>)	Variance (<i>V</i>)	<i>F</i> -ratio (<i>F</i>)	Pure sum	Percent of contribution (<i>P</i>)
1. Temperature	1	544.5	544.5	43.56	532	28.548
2. RPM	1	84.5	84.5	6.76	72	3.863
3. pH	1	24.5	24.5	1.96	12	0.643
4. Duration	1	40.5	40.5	3.24	28	1.502
5. Inoculum size	(1)	(12.5)	Pooled			
6. Nitrogen	1	544.5	544.5	43.56	532	28.548
7. Carbon	1	612.5	612.5	49	600	32.197
Other (error)	1	12.5	12.5			4.699
Total	7	1,863.5				100.00%

Table 3 Analysis of variance of main effect of factors by *Neurospora intermedia* (DF4) for efficient decolorization

Factor	Degree of freedom (<i>f</i>)	Sum of squares (<i>S</i>)	Variance (<i>V</i>)	<i>F</i> -ratio (<i>F</i>)	Pure sum (<i>S'</i>)	Percent of contribution (<i>P</i>)
Temperature	1	231.125	231.125	1,849	231	21.815
RPM	1	171.125	171.125	1,269	171	16.149
pH	1	190.125	190.125	1,521	190	17.943
Duration	1	66.125	66.125	529	66	6.233
Inoculum size	(1)	(0.125)	Pooled			
Nitrogen	1	190.125	190.125	1,521	190	17.943
Carbon	1	210.125	210.125	1,681	210	19.832
Other/error	1	0.125	0.125			0.085
Total	7	1,058.875				100.00%

(3 days). However, reverse effects were seen in case *Neurospora intermedia* (DF4). In case of pH change from lower level to higher level, a decrease in the decolorization efficiency was observed for both fungi which perhaps because of polymerization of colored compound (Shayegan et al. 2005).

An analysis of Variance (ANOVA) was conducted to determine the relative importance of each factor. The main objective of ANOVA was to conclude from the result that each factor causes how much variation relative to the total variation observed in the result. From the results of ANOVA (Table 2), it was concluded that % carbon had the largest variance; the temperature and %nitrogen indicated the second in case of *Emericella nidulans* var. *lata* (DF3). While in case of *Neurospora intermedia* (DF4) the highest variances were observed first for temperature, then carbon, pH and %nitrogen (Table 3). Inoculum size was pooled in both cases, as its relative variation seemed insignificant.

Validation experiments

Before optimization the average decolorization was 38 and 31% by *Emericella nidulans* var. *lata* (DF3) and *Neurospora intermedia* (DF4), respectively, and after optimization the expected decolorization was 66.5% (DF3) and 64%

(DF4). Actual decolorization was 62% by DF3 and 64% by DF4, which showed an almost two-fold increase. Thus the decolorization was significantly improved.

Mechanism for decolorization of distillery effluent by enzymes

The mechanism of decolorization of distillery effluent was determined by lignin peroxidase, manganese peroxidase, laccase and glucose oxidase enzyme analysis. The result of the study indicated no significant increase in peroxidase and oxidase enzyme in the culture supernatant of *Neurospora intermedia* (DF4), however, in the case of *Emericella nidulans* var. *lata* (DF3) the laccase and glucose oxidase activity in the culture supernatant was detected (Fig. 6). A marked increase in glucose oxidase and laccase activity was detected in the *Emericella nidulans* var. *lata* (DF3) treated effluent after 1 day, which reached to maxima after 48 h. The activity of enzyme decreased after 3 days.

Glucose oxidase activity has been reported in culture supernatant of *Flavodon flavus* during decolorization of molasses spent wash (Raghukumar et al. 2004). There are number of reports showing role of fungi in decolorization by adsorption of melanoidin to mycelia and role of lignolytic enzymes metabolized color containing chemical

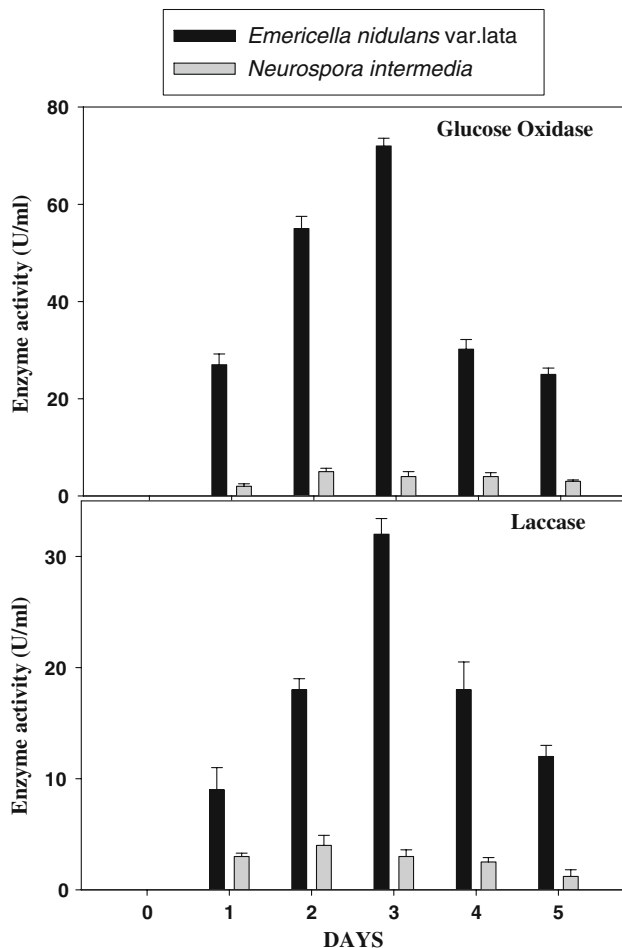


Fig. 6 Estimation of enzyme activity of Glucose oxidase and Laccase in *Emericella nidulans* var. lata (DF3) and *Neurospora intermedia* (DF4) in presence of 10% distillery effluent. In figure, is *Neurospora intermedia* and *Emericella nidulans* var. lata.

compounds in the effluent (Watanabe et al. 1982; Benito et al. 1997; Vahabzadeh et al. 2004). Our results indicated production of laccase and glucose oxidase in *Emericella nidulans* var. lata (DF3), which were not present in *Neurospora intermedia* (DF4). However, results of the study indicated reduction in color of distillery effluent by *Neurospora intermedia* (DF4), which might be due to biosorption of coloring material in the effluent in fungal biomass, increased by carbon and nitrogen sources. These growth factors do not enhance the decolorization directly but help by increasing the growth of the microorganism, hence contributing to decolorization.

Conclusion

Fungus isolated from distillery spent wash and sludge of distillery unit was identified by amplification and sequencing of ITS1 and ITS2 region of genomic DNA as

Emericella nidulans var. lata (DF3) and *Neurospora intermedia* (DF4), respectively. Both strains showed decolorization of distillery effluent after acclimatization and optimization. Most suitable nitrogen source was sodium nitrate and most appropriate carbon source was dextrose. Optimizing the growth factors and process parameters using Taguchi approach further enhanced the decolorization potential of strains. Before optimization the average decolorization of effluent by *Emericella nidulans* var. lata (DF3) was 38% and by *Neurospora intermedia* (DF4) was 31%. After optimization the actual decolorization increased up to 62 and 64%, respectively. Thus, the optimization technique used in this study could be widely used to enhance fermentation conditions. Enzymatic methods were employed to understand the mechanism of decolorization of distillery effluent in which the laccase and glucose oxidase activity was determined in DF3, however, no such activity was observed in the case of DF4.

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