Journal of Hazardous Materials 171 (2009) 619–625

Contents lists available at ScienceDirect

Journal of Hazardous Materials

journal homepage: www.elsevier.com/locate/jhazmat

Decolourization and detoxification of pulp and paper mill effluent by *Emericella nidulans* var. nidulans

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ARTICLE INFO

Article history:
Received 3 February 2009
Received in revised form 18 May 2009
Accepted 10 June 2009
Available online 18 June 2009

Keywords:
Comet assay
Decolourization
Detoxification
Optimization
Pulp and paper mill effluent
Taguchi approach

ABSTRACT

In this study geno-toxicity analysis along with effluent treatment was taken up to evaluate the efficiency of biological treatment process for safe disposal of treated effluent. Four fungi were isolated from sediments of pulp and paper mill in which PF4 reduced colour (30%) and lignin content (24%) of the effluent on 3rd day. The fungal strain was identified as *Emericella nidulans* var. nidulans (anamorph: *Aspergillus nidulans*) on the basis of rDNA ITS1 and rDNA ITS2 region sequences. The process of decolourization was optimized by Taguchi approach. The optimum conditions were temperature (30–35 °C), rpm (125), dextrose (0.25%), tryptone (0.1%), inoculum size (7.5%), pH (5) and duration (24 h). Decolourization of effluent improved by 31% with reduction in colour (66.66%) and lignin (37%) after treatment by fungi in shake flask. Variation in pH from 6 to 5 had most significant effect on decolourization (71%) while variation in temperature from 30 to 35 °C had no effect on the process. Treated effluent was further evaluated for geno-toxicity by alkaline single cell gel electrophoresis (SCGE) assay using *Saccharomyces cerevisiae* MTCC 36 as model organism, indicated 60% reduction.

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1. Introduction

Pulp and paper mills utilize huge amount of natural resources, inorganic and organic materials along with large volume of water in different stages of paper manufacturing. Pulping and bleaching are two main processes involved in the manufacturing of paper. In pulping, wood pieces are cooked at very high temperature and pressure in presence of chemicals to separate lignin and hemicelluloses from cellulose. Cellulose is used for making paper. In India kraft pulping is commonly practiced, using NaOH and sodium sulphide as cooking chemicals. The effluent generated at the pulping stage is dark brown in colour (black liquor) due to dissolved lignin, its degradation products, hemicelluloses, resin acids and phenols [1]. The pulp generated after pulping has residual lignin and hemicellulose. It is bleached using chemicals like chlorine, hydrogen peroxide, ozone, etc. The effluent generated at bleaching stage has absorbable organic halogens (AOX), peroxides and other such derivatives of lignin and hemicellulose [2]. It is estimated that 1 tonne of paper generates 150 m^3 of effluent which is extremely toxic in nature [1].

Various studies have reported detrimental effects of pulp and paper mill effluent on animals living in water bodies receiving the effluent. The effects are in form of respiratory stress, oxidative stress, liver damage and geno-toxicity [3–5]. A study in 1996 reported health impacts such as diarrhea, vomiting, headaches, nausea, and eye irritation on children and workers due to the pulp and paper mill wastewater discharged to the environment [6]. The effluent has high chemical diversity of organic chemicals present in it. Many of them are carcinogenic, mutagenic, clastogenic and endocrinic disrupters. A study on *B. subtilis* reported the mutagenic effect of the sediments contaminated by the effluent of kraft paper mill [7]. Another study reports the toxic and mutagenic effects of pulp and paper mill effluent contaminating lake Baikal [8]. Exposure to the effluent adversely affects diversity and abundance of phytoplankton, zooplankton and zoobenthos, disrupting benthic algal and invertebrate communities [9]. Therefore, it is obligatory to treat the effluent before disposal.

In general, the effluent is concentrated and then burned to generate electricity. While burning many toxic volatile organic compounds are formed. The alternative is adopting biological treatment methods that involve the use of fungi and bacteria. Among the two, use of fungi is common as they produce extracellular enzymes and can survive at higher effluent load than bacteria [10,11]. For treatment of effluent at the industrial scale it is important to optimize the various process parameters so that maximum colour and geno-toxicity reduction can be achieved by minimum input. In the present study pulp and paper mill effluent, generated at the pulping stage, was characterized together with isolation and identification of fungi from the contaminated site. For effective removal of colour and lignin from effluent various process parameters were

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doi:10.1016/j.jhazmat.2009.06.041

optimized. Further, comet assay was used to test the geno-toxicity of the treated effluent for safe disposal in the environment.

2. Materials and methods

2.1. Sites for sample collection and isolation of fungi

Sediments containing degraded wood and decomposed bagasse were collected in clean plastic containers from ANAND TISSUE PAPER MILL, Meerut, Uttar Pradesh, India for isolation of fungal strains. Sediments, decomposed wood and bagasse were mixed with sterilized water in the ratio 1:10 (w/v) and vortexed. It was kept standing at room temperature for 2 h. Then the supernatant was decanted, serially diluted with autoclaved double distilled water to 10^{-1}, 10^{-3} and 10^{-5} dilutions, spread on the potato dextrose agar plates and incubated at 30 °C for 4 days. The microbial colonies (fungal) appeared on the PDA plates were then isolated and purified [12].

2.2. Characterization of effluent

Effluent generated at the pulping stage was collected from ANAND TISSUE PAPER MILL in June, 2007 for treatment studies. They were stored at 4 °C until further use. The effluent was characterized for various physio-chemical parameters. pH was estimated using pH Meter (Cyberscan 51), colour by 2120 C Cobalt-platinate method [13], lignin by modified method of Pearl and Benson [14], COD by 5220 B open reflux method [13]. Anions, i.e., phosphate, nitrate and sulphate were estimated by ion chromatography using ICS-90 IC system DIONEX. The eluents used were 2.7 mM sodium carbonate ($NaCO_3$) and 0.3 mM sodium bicarbonate ($NaHCO_3$). Conductivity after chemical suppression was ca.14 μS/cm. The flow rate of 1 ml/min was maintained during the analysis. The anions were eluted out in order nitrate, phosphate and sulphate. Total time for each run was 22 min. Standards were prepared using Dionex anion standards. Sodium and potassium (cations) were estimated by Flame photometer and heavy metals including copper, zinc and nickel by Atomic adsorption spectrophotometer (AAS), 3030 G acid digestion method [13].

2.3. Screening and identification of fungal strains for effluent treatment

The four fungal strains (PF1, PF2, PF3 and PF4) isolated, as described above were screened for their decolourization and lignin reduction potential. MSM-effluent, i.e., MSM (in g/l: $Na_2HPO_4{\cdot}2H_2O$, 7.8; KH_2PO_4, 6.8; $MgSO_4$, 0.2; $Fe(CH_3COO)_3NH_4$, 0.01; $Ca(NO_3)_2{\cdot}4H_2O$, 0.05) having 10% pulp and paper mill effluent of pulping stage, inoculated with individual fungal isolates, were incubated at 30 °C, pH 7 in a rotary shaker, rpm 125 for 10 days [15]. The parameter colour and lignin were estimated at an interval of 0, 1, 2, 3, 5, 7 and 10 days. The experiments were conducted in triplicate.

On the basis of reduction in colour and lignin, the most potential strain was selected. The identity of this fungal strain was established by rDNA analysis. Genomic DNA was extracted and internal transcribed spacer (ITS) regions were amplified using primers ITS1* having sequence 5′-TCCGTAGGTGAACCTGCGG-3′ and ITS4* having sequence 5′-TCCTCCGCTTATTGATATGC-3′ [16,17]. The location of ITS1 and ITS2 regions on ribosomal DNA gene is shown in Fig. 1. ITS1* and ITS4* primers were used to amplify the rDNA sequence. It included partial 18S region, Complete ITS1 region, complete 5.8S region, complete ITS2 region and partial 28S region. The sequence of ITS1 and ITS2 regions reveals inter-specific variations [17]. The reaction mix (25 μl) consisted of buffer with $MgCl_2$ (2.5 mM), dNTP-2.0 μl (1 mM each), ITS1 (forward)-0.7 μl (10 mM), ITS4 (reverse)-0.7 μl (10 mM), Taq polymerase-0.5 μl (5 U/μl), DNA-20–50 ng and volume was made up by water. The programme for amplification was: initial denaturation step at 95 °C for 3 min, followed by 35 cycles of amplification at 95 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min and a final extension of 5 min at 72 °C. Amplified products were viewed after electrophoresis in 0.7% agarose gels, 1 μg/ml of EtBr and sequenced. The sequence was blasted using NCBI database and tools and phylogeny tree was constructed using Mega 4.0 software (www.megasoftware.net) [18].

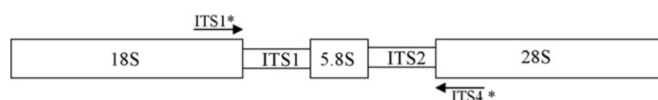

Fig. 1. The location of ITS1 and ITS2 regions in ribosomal DNA gene amplified by ITS1* and ITS4* primers for phylogenic placement of fungi.

2.4. Optimization of process parameters for decolourization of effluent

2.4.1. Screening for carbon and nitrogen sources

The most efficient fungal strain, having maximum potential to reduce colour and lignin content of the effluent, was optimized for maximum reduction in colour and lignin content of the effluent. The 10% effluent-MSM was used through out the experiments. Experiments were conducted to choose the best carbon and nitrogen source. Different carbon sources like sucrose, dextrose, sodium acetate and sodium citrate and different nitrogen sources like yeast extract, tryptone, peptone and sodium nitrate were tested. The experiment was conducted in triplicate.

2.4.2. Optimization using Taguchi approach

After selecting the most appropriate carbon and nitrogen sources Taguchi approach was used for optimization. Treatment of effluent in batch reactor is static design where the optimum levels for each parameters are determined to achieve the desired levels of output. The parameters that are not feasible to control and thus lead to variation in output are called noise. The purpose of optimization is to give robust design that is capable of giving desired output even in presence of noise. This is achieved by identifying the influence of individual parameters, establishing the relationship between variables and operational conditions and finally establishes the performance at the optimum levels obtained with a few well-defined experimental sets [19,20]. Taguchi approach involves two important features, use of orthogonal arrays for designing the experiments and analysis of variance (ANOVA) for generation and significance of results. The benefit of using this approach is drastic reduction in the number of experiments. In this study seven parameters at two levels were optimized. First parameter (factor) was temperature studied at 30 °C (level 1) and 35 °C (level 2), second rpm at 125 (level 1) and 150 (level 2), dextrose at 0.25% (level 1) and 0.50% (level 2), tryptone at 0.05% (level 1) and 0.10% (level 2), inoculum size at 5.0% (level 1) and 7.5% (level 2), pH at 5 (level 1) and 6 (level 2), and seventh parameter was duration studied at 12 h (level 1) and 24 h (level 2). Qualitek-4 software was used for designing of experiments and analysis [21]. Table 1 gives the details of eight experiments to be conducted according to L-8 orthogonal array. The number of experiments required to optimize seven parameters at two levels are 128 while they reduced to 8 by applying Taguchi approach.

Since the optimization was carried out for reduction in colour and lignin content, a combined index (overall evaluation criteria, OEC) was used. For calculation, overall evaluation criteria (OEC) assumed: X1 = numeric evaluation under criterion 1; X1ref = highest numerical value X1 can assume; Wt1 = relative weighting of criterion 1:

Table 1
Details of experimental runs according to L-8 orthogonal array.

Run	Temperature (°C)	rpm	Dextrose (%)	Tryptone (%)	Inoculum size (%)	pH	Duration (h)
1	1(30)	1(125)	1(0.25)	1(0.05)	1(5.0)	1(5)	1(12)
2	1(30)	1(125)	1(0.25)	2(0.10)	2(7.5)	2(6)	2(24)
3	1(30)	2(150)	2(0.50)	1(0.05)	1(5.0)	2(6)	2(24)
4	1(30)	2(150)	2(0.50)	2(0.10)	2(7.5)	1(5)	1(12)
5	2(35)	1(125)	2(0.50)	1(0.05)	2(7.5)	1(5)	2(24)
6	2(35)	1(125)	2(0.50)	2(0.10)	1(5.0)	2(6)	1(12)
7	2(35)	2(150)	1(0.25)	1(0.05)	2(7.5)	2(6)	1(12)
8	2(35)	2(150)	1(0.25)	2(0.10)	1(5.0)	1(5)	2(24)

1 and 2 denotes the two levels selected for optimization. Values in bracket are the actual values for that level.

Then OEC was calculated as:

$$OEC = \frac{X1}{X1ref} \times Wt1 + \frac{X2}{X2ref} \times Wt2 + \ldots \quad (1)$$

Colour is criterion 1 and lignin is criterion 2. The reduction in colour and lignin was measured in percentage thus highest numerical value for X1 ref and X2 ref was 100. Both the parameters are equally important thus relative weight (Wt1 and Wt2) for both the parameters was 50. X1 is the actual reduction in colour at each run and X2 is actual reduction in lignin at each run. All the values were feed in the formula and the single combined value (OEC) was calculated for each run. These values were used for analysis by Taguchi approach.

2.4.3. Analysis of data and confirmatory experiment

The optimum levels for each factor (parameter) was derived by analyzing the data of the above mentioned eight experiments using Qualitek-4 software (http://Nutek-us.com/wp-q4w.html) [21]. Apart from optimized conditions, the contribution of each individual parameter in colour and lignin reduction and the interactions among various parameters was also studied. The analysis was performed with "bigger is better" quality characteristics. This implies that the aim of optimization was to increase decolourization.

The effluent treatment was carried out at the optimum levels of process parameters and result was compared with the value predicted by the Taguchi model. This confirmatory experiment was repeated three times.

2.5. Analysis of geno-toxicity by comet assay (alkaline SCGE)

The comet assay (alkaline single cell gel electrophoresis, SCGE) is a fast, efficient and very sensitive method for detecting DNA damage in individual cells [22]. Alkaline comet assay was performed using *Saccharomyces cerevisiae* MTCC 36 [23]. In this study yeast was chosen as model organism for evaluation of geno-toxicity. Yeast cells were grown in MYPG media (0.3% malt extract, 0.3% yeast extract, 0.5% peptone, 1% glucose and pH 5.0). The cells in log phase of growth were treated (soaked) with the effluent for 6 h. The cells were mixed with low melting agarose and spread on slides. These slides were incubated with lyticase 20T (Sigma) for spheroplast formation at 30 °C for 1 h. After spheroplast formation the slides were incubated in lysis solution for 12 h at 4 °C. They were washed with electrophoresis buffer and further incubated in the same buffer for 1 h. Then electrophoresis was performed for 30 min at 25 V. After electrophoresis the gels were neutralized in neutralization buffer. Finally, the slides were stained with 4,6-diamidino-2-phenylindole, DAPI (2 μg/ml, 100 μl per slide) just before analysis.

The migration of (negatively charged) DNA fragments or DNA loops towards the positive pole takes place during electrophoresis. As a result, nuclei of cells having damaged DNA take the shape of falling comets having head and tail. Amount of DNA in tail, i.e., tail intensity increases with increasing DNA damage. The comets were analyzed using the fluorescence microscope with an excitation filter of 355 nm and a barrier filter of 450 nm. Images of randomly selected 50 comets were analyzed per sample. The fluorescence microscope was fitted with 100× oil immersion lens. The percentage of DNA in tail of nucleus of cell (comet) was calculated using CometScore™ Freeware software (www.tritekcorp.com). Percent DNA in tail of comets, i.e., tail intensity was calculated as a measure of geno-toxicity.

2.6. Statistical analysis

Data from the experiments was transferred to Microsoft Excel 2002 spreadsheets (Microsoft Corp., Redmond, WA) and analyzed using statistical functions of SPSS 10.0 and graphical functions of SigmaPlot 2001 (SPSS, Inc., Chicago, IL). Percent reduction in colour and lignin and tail intensity were used in one-way analysis of variance test (ANOVA) at $p \leq 0.05$, i.e., 95% confidence limit. Further Duncan test was applied to populations showing significant differences during screening of carbon and nitrogen sources and geno-toxicity analysis.

3. Results and discussion

3.1. Characterization of effluent

The effluent collected from ANAND TISSUE PAPER MILL, Meerut, Uttar Pradesh, India was highly alkaline in nature. This industry followed kraft pulping process using sugar cane bagasse: pH was 13, colour was 65,475 CU, lignin 163,741 ppm, COD 204,358 ppm, phosphate was below detection limit, nitrate 5852 ppm, sulphate 3746.3 ppm, sodium ions 11,650 ppm, potassium ions 7500 ppm, copper, zinc and nickel were below detection limit. Effluent was deep brown in colour due to the presence of lignin and its degradation products. The results indicated high values of COD, nitrates, sulphate, sodium and potassium ions because of various compounds present in the effluent. The source of sulphate ions in effluent was sodium sulphite used in the pulping process. The proteins present in wood or sugarcane bagasse contributes to the nitrates in the effluent. Some studies have reported the presence of metals in low amount in pulp and paper mill effluent. As such metals are not used in pulp and paper industry, however, the raw material used, i.e., wood may be contaminated by fungicides and biocides. Another source of contamination can be the use of wood from plants grown in metal containing soil and water [24]. The concentration of metals tested in this study were below detection limit as bagasse was used as raw material for preparation of pulp and metals were not used in any steps of the processes including preservation of raw materials.

3.2. Screening and identification of fungal strains for treatment of effluent

The fungi PF1, PF2, PF3 and PF4, isolated from the sediments of ANAND TISSUE PAPER MILL, Meerut, were tested for their ability to remove colour and lignin from the pulp and paper mill effluent.

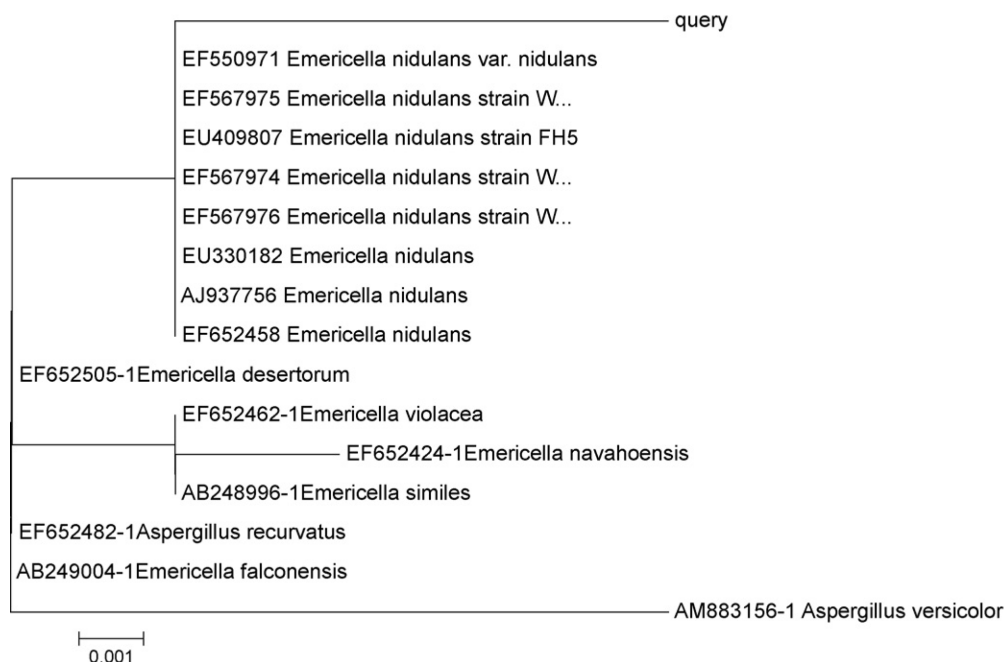

Fig. 2. Phylogenetic placement of PF4, *Emericella nidulans* var. nidulans (anamorph: *Aspergillus nidulans*), based on rDNA ITS1 and ITS2 regions analysis.

Initial colour was 6521 CU and lignin was 16,161 ppm. Maximum reduction in colour (21%) and lignin (19%) by PF1 was on 2nd and 3rd day, respectively. PF2 lead to 13% reduction in colour and 18% in lignin on day 1 and 3, respectively. The percent reduction in colour for PF3 was 17% on day 1 and 13% on day 3. PF4 lead to 30% (4565.6 CU) colour and 24% lignin (12,282 ppm) reduction on 3rd day. Among four fungi tested the most efficient strain was PF4 followed by PF1 > PF2 and PF3. Reduction in colour and lignin by all four fungal strains was significant at $p \leq 0.05$. After day 3 there was no significant reduction in colour or lignin by any of the fungi tested. This may be due to the effect of shaking and inhibition of growth of fungi in absence of proper carbon and nitrogen source [25]. The treatment of pulp and paper mill effluent has been studied using a number of fungi from quite some time. However, very few studies have optimized the process. A study used additional carbon and nitrogen for decolourization of effluent from bagasse based pulp and paper mills by white rot fungus *Schizophyllum commune* [26]. Another study reports effluent decolourization by using *Aspergillus fumigatus* by optimizing the process parameters using one parameter at a time approach [27]. Studies related to the significance and interaction of various parameters are lacking. Moreover, studies on the toxicity of the effluent report that chemical parameter cannot define the toxicity of the effluent accurately and additional toxicity tests are required [28].

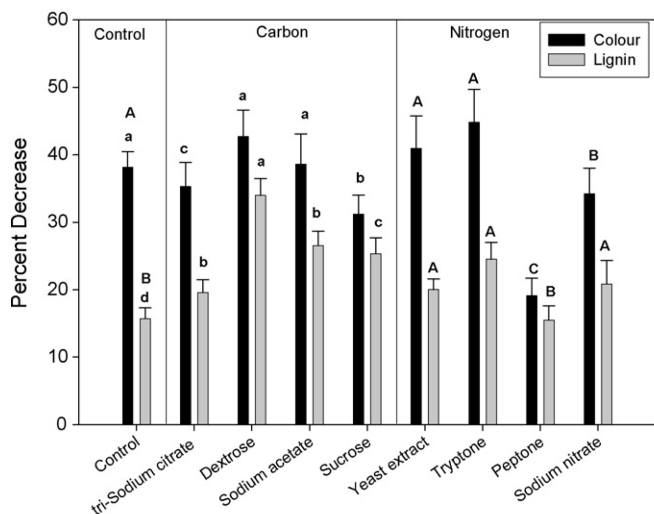

Fig. 3. Screening of carbon and nitrogen sources for *Emericella nidulans* var. nidulans for decolourization of pulp and paper mill effluent. Within each group (colour and lignin) values not followed by the same letter are significantly different at $p \leq 0.05$. Small letters are used for carbon sources and capital letters for nitrogen sources. Error bars are standard deviations.

The most efficient fungal strain PF4 was identified as *Emericella nidulans* var. nidulans (anamorph: *Aspergillus nidulans*) with 98% homology (Fig. 2). The sequence was submitted to the GenBank database under accession no. EU780786. *Emericella* is the sexual stage of *Aspergillus* and belongs to Ascomycota [29]. *Aspergillus* has been reported in association of bagasse [30]. This explains the presence of *Emericella nidulans* var. nidulans in the sediments, decaying wood and bagasse collected from ANAND TISSUE PAPER MILL which uses sugarcane bagasse as raw material.

3.3. Optimization of process parameters for decolourization of effluent

3.3.1. Screening for carbon and nitrogen sources

The 10% effluent-MSM was used through out the experiments having initial colour 6543 CU and 16,374 ppm lignin. For PF4, *Emericella nidulans* var. nidulans, the most suitable carbon source was dextrose with 42.7% reduction in colour (3747.2 CU) and 34% reduction in lignin (10810.7 ppm). The most suitable nitrogen source was tryptone where reduction in colour was 44.8% (3611.1 CU) and 24.5% in lignin (12960.3 ppm) (Fig. 3). The amount of carbon and nitrogen in available form are generally limiting in effluent. A study in 1988 reported the use of sucrose and ammonium chloride for treatment of paper mill effluent by *S. commune* [26]. In another study *Pleurotus ostreatoroseus* was used for the treatment of pulp and paper mill effluent and glucose was added [31]. Thus the use of co-substrates improved the decolourization potential of fungi [25].

Table 2
ANOVA for optimization of effluent treatment process by *Emericella nidulans* var. nidulans.

Col #/factor	DOF (f)	Sum of sqrs. (S)	Variance (V)	F-Ratio (F)	Pure sum (S')	Percent P (%)
1. Temperature	(1)	(5)		POOLED (CL = *NC*)		
2. rpm	1	120.125	120.125	240.25	119.625	16.626
3. Dextrose	1	15.125	15.125	30.25	14.625	2.032
4. Tryptone	1	21.125	21.125	42.25	20.625	2.866
5. Inoculum size	1	10.125	10.125	20.25	9.625	1.337
6. pH	1	512.000	512.000	1024.00	511.500	71.091
7. Duration	1	40.500	40.500	81.00	40.000	5.559
Other/error	1	5	5			0.489
Total	7	719.500				100.00

P (%) shows the contribution of each individual parameter in percentage.

3.3.2. Optimization using Taguchi approach

After selecting the most suitable carbon (dextrose) and nitrogen (tryptone) source, optimization experiments were designed and performed according to L-8 orthogonal array (Table 1). The results of colour and lignin reduction were converted to OEC values. OEC values for the eight experiments conducted were: run 1 had OEC value of 45.0, run 2 had 39.0, run 3 had 23.0, run 4 had 40.0, run 5 had 48.5, run 6 had 29.0, run 7 had 23.0 and run 8 had OEC value 44.5. These results were analyzed by Qualitek-4 software.

3.3.3. Analysis of data and confirmatory experiment

The effect of temperature variation from 30 °C (level 1) to 35 °C (level 2) on the process of effluent treatment was not significant. High rpm and high carbon content adversely affected the process of decolourization of the effluent as the OEC value decreased on moving from level 1 to level 2 (Fig. 4). The most suitable shaking condition was rpm 125, showed that fungus preferred slow agitation. One possible reason may be very rapid movement leads to the shear and tear of fungal mycelium [32,33]. Effluent seems to be deficient in nitrogen as increasing nitrogen content increased the colour and lignin reduction (Fig. 4). Decolourization was favoured by high inoculum size and longer duration but at lower pH. The process of decolourization was mediated by the fungus thus increasing the inoculum size from 5% to 7.5% increased decolourization of the effluent. Most of the studies report the pH range of 4–6 for treatment of pulp and paper mill effluent [26,34].

Studies that had been carried out previously restricted themselves to the optimization of parameters. There is no work about the relative influence of various parameters and their interactions. The results of ANOVA analysis for *Emericella nidulans* var. nidulans are presented in Table 2. Among all the parameters the most influential was pH (71%) followed by rpm (17%). Temperature had non-significant effect hence it was pooled and dropped from the analysis. Most significant interaction was in between dextrose and inoculum size (S.I. = 85%). And the least significant interaction was in between pH and duration (0.8%). This shows that most significant interaction took place in between two least significant parameters. If the significance level of the parameter is low, it does not imply that it is not important. It stresses that while varying the conditions from level 1 to level 2 (Fig. 4) for that parameter there is no significant effect on decolourization while the parameter in itself can be very important. After analysis the optimum conditions for effluent treatment by *E. nidulans* var. nidulans predicted by Taguchi model were temperature (30–35 °C), rpm (125), dextrose (0.25%), tryptone (0.1%), inoculum size (7.5%), pH (5) and duration (24 h) (Table 3). If experiments were performed at these optimum conditions then the expected performance predicted by the Taguchi model was 54.75 OEC value (Table 3). Before optimization it was 36.50.

The expected results predicted by Taguchi model (Table 3) were tested using MSM-effluent 10% having initial colour of 6533 CU and lignin 16319 ppm. The colour and lignin reduced

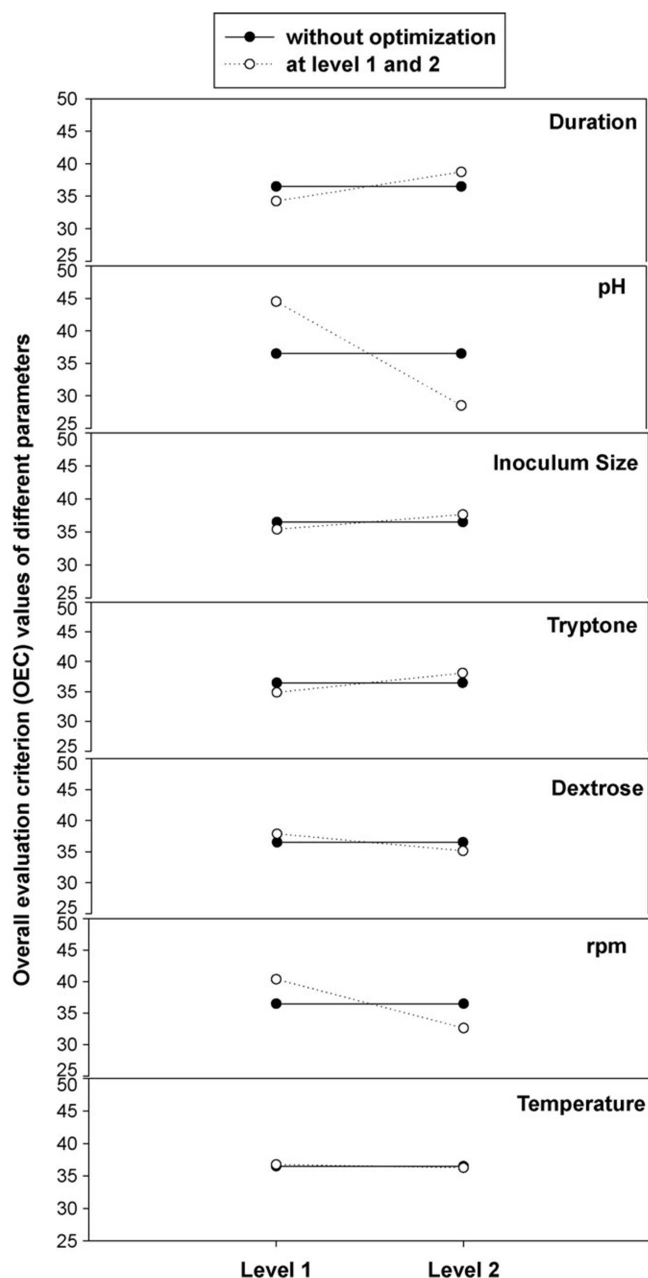

Fig. 4. Variations in OEC value of seven parameters (factors) at two levels, selected for optimization, for effluent treated by *Emericella nidulans* var. nidulans. OEC stands for overall evaluation criteria, an index used to combine the values of colour and lignin reduction.

Table 3
Optimized conditions and contribution of each individual parameter in decolourization by *Emericella nidulans* var. nidulans.

Factor	Value	Contribution (OEC)
rpm	125	3.875
Dextrose (%)	0.25	1.375
Tryptone (%)	0.10	1.625
Inoculum size (%)	7.5	1.125
pH	5	8.000
Duration (h)	24	2.250
Total contribution from all factors (*a*)		18.250
Performance without optimization (*b*)		36.500
Expected results at optimum conditions (*a* + *b*)		54.750

OEC stands for overall evaluation criteria, an index used to combine the values of colour and lignin reduction.

to 2177.66 ± 164.41 CU (66.66 ± 2.51%) and 10280.97 ± 326.38 ppm (37 ± 2%), respectively. OEC value was 51.8 ± 2.25. The treatment of effluent at the optimum conditions lead to an increase in decolourization potential by 31% for *E. nidulans* var. nidulans. A number of fungi have demonstrated decolourization potential. They include *Aspergillus fumigatus* and *Aspergillus flavus*, *Aspergillus foetidus*, *Aspergillus niger*, *Trametes versicolor*, etc. [27,35–37]. The decolourization potential of these fungi varies greatly ranging from values above 90% to below 30%. A study using *Trametes versicolor* showed 70% reduction in colour for effluent having 6600 CU after 9 days treatment [37]. Another study using the same fungi, i.e., *Trametes versicolor* strain B-7 lead to 93% decolourization, for effluent having 7000 CU, after 48 h at optimum pH, temperature and glucose concentration [38]. Sahoo and Gupta treated pulp and paper mill effluent using *Aspergillus fumigatus* isolate g7 at 30 °C, 200 rpm and glucose (20 g/l). Colour content reduced by 89% after 48 h [27]. Percent reduction in colour can vary with the initial colouring units of the effluent. *Aspergillus foetidus* showed 40% reduction in colour for effluent having 5888 CU (0.15% lignin) and 90% for 3874 CU (0.10% lignin content) [35]. Another study showed 56% reduction in colour after treatment for 48 h using *Aspergillus niger*. Initial colour was 1000 CU [36]. Some of the studies have optimized the process parameters; however, they have not evaluated the improvement in the process after optimization. Almost all the studies have concentrated on colour reduction while reduction in toxicity, though important, has not been evaluated.

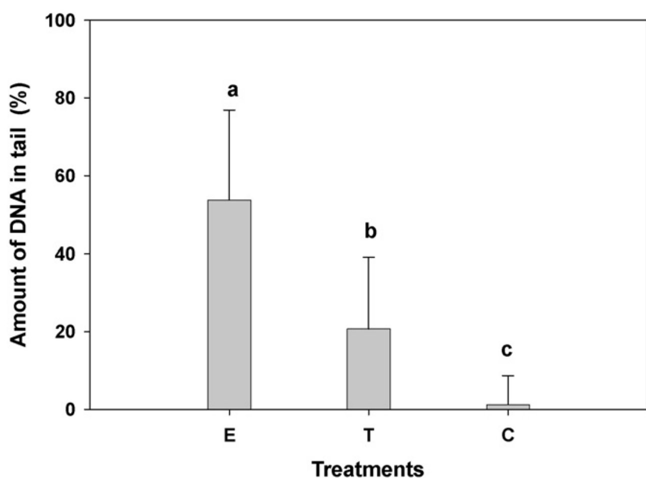

Fig. 5. Amount of DNA in tail of comets (nucleus of cell) for (E) untreated effluent, (T) treated effluent at optimized conditions and (C) autoclaved double distilled water. Values not followed by the same letter are significantly different at $p \leq 0.05$. Error bars are standard deviations.

3.4. Geno-toxicity analysis of effluent after treatment

Alkaline comet assay is sensitive to detect primary DNA lesions including single-strand breaks, double-strand breaks, incomplete excision repair sites and alkali labile sites that can be converted to DNA single-strand breaks during the test procedure [39]. Two controls were used, untreated effluent (E) and distilled water (C). Untreated effluent was used to compare the effectiveness of treatment in reducing geno-toxicity. Distilled water was used to see the effect of the process of comet assay on the nuclei. The effluent was genotoxic as comets were formed in cells treated with untreated effluent (E). No comets were observed in case of treatment (C). This implies that the process and the chemicals used during comet assay did not lead to DNA damage in cells. The average DNA in tail of nucleus, i.e., tail intensity was 54% in untreated effluent (E) which reduced to 21% in treated effluent (T). Treatment leads to significant ($p \leq 0.05$) reduction in geno-toxicity of the effluent (Fig. 5). The tail intensity was 60% less in treated effluent as compared to untreated effluent.

4. Conclusion

The results indicated:

1. *Emericella nidulans* var. nidulans (anamorph: *Aspergillus nidulans*) can efficiently decolourize and detoxify the effluent generated at the kraft pulping stage in pulp and paper mill.
2. Addition of dextrose and tryptone as co-substrates improved effluent treatment efficiency of *Emericella nidulans* var. nidulans.
3. After optimization of seven process parameters (temperature, rpm, dextrose, tryptone, inoculum size, pH and duration) using Taguchi approach, reduction in colour and lignin was 66.66% and 37%, respectively. Optimization improved colour and lignin reduction potential of *E. nidulans* var. nidulans by 31%.
4. pH was the most influential parameter. Fungus preferred slow agitation and temperature in the range 30–35 °C.
5. The effluent was genotoxic in nature and treatment by *Emericella nidulans* var. nidulans at optimum conditions reduced the geno-toxicity by 60%.

Acknowledgements

The authors thank University Grants Commission, New Delhi, India, for providing Senior Research Fellowship to AS and financial support. We are also grateful to Department of Biotechnology, Government of India, New Delhi, for financial support in the form of project, Mr. R.K. Roy for providing Qualitek-4 software, Mr. Kavinder for help during comet assay analysis, Mr Prashant and Mrs Garima for help during rDNA analysis. Authors thank editor and reviewers for their suggestions that helped immensely in giving the manuscript its present form.

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