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ORIGINAL PAPER

Isolation and characterization of alkalotolerant bacteria and optimization of process parameters for decolorization and detoxification of pulp and paper mill effluent by Taguchi approach

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Abstract Four different bacterial strains were isolated from pulp and paper mill sludge in which one alkalotolerant isolate (LP1) having higher capability to remove color and lignin, was identified as *Bacillus* sp. by 16S RNA sequencing. Optimization of process parameters for decolorization was initially performed to select growth factors which were further substantiated by Taguchi approach in which seven factors, % carbon, % black liquor, duration, pH, temperature, stirring and inoculum size, at two levels, applying L-8 orthogonal array were taken. Maximum color was removed at pH 8, temperature 35°C, stirring 200 rpm, sucrose (2.5%), 48 h, 5% (w/v) inoculum size and 10% black liquor. After optimization 2-fold increase in color and lignin removal from 25–69% and 28–53%, respectively, indicated significance of Taguchi approach in decolorization and delignification of lignin in pulp and paper mill effluent. Enzymes involved in the process of decolorization of effluent were found to be xylanase (54 U/ml) and manganese peroxidase (28 U/ml). Treated effluent was also evaluated for toxicity by Comet assay using *Saccharomyces cerevisiae* MTCC 36 as model organism, which indicated 58% reduction after treatment by bacterium.

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Introduction

The pulp industry is one of the most polluting industries in the world. One of the reasons for the pollution is that the industry uses woody and non-woody lignocellulosic compounds as raw material. During the preparation of pulp, huge amount of inorganic compounds are used which react with lignin like compounds in lignocellulose and forms toxic organic compounds in pulp and paper mill effluents. Various chemicals are used during the cooking process to remove lignin. Delignification of wood pulp is a necessary step to improve the quality of paper because lignin causes paper to turn yellow. Due to this process, effluent emerging from pulp and paper mill has large amount of lignin and its derivatives. Pulp and paper mill effluent (black liquor) is characterized by its dark brown color, high temperature, strong odor, highly alkaline pH, very high chemical oxygen demand (COD; 200,000 mg/l) and biochemical oxygen demand (BOD; 40,000–50,000 mg/l). It is estimated that one tonne of paper generates 150 m^3 of effluent which is huge in volume and extremely toxic in nature (Pokhrel and Viraraghavan 2004). Disposal of conventionally treated black liquor is harmful as it contains a large number

of organic compounds, some of which such as dioxins, furans, and polycyclic aromatic hydrocarbons (PAHs) etc. may be carcinogenic and mutagenic to aquatic biota. Color of black liquor is mainly due to lignin and its derivatives and polymerized tannins, which are recalcitrant in nature due to the presence of carbon-to-carbon biphenyl linkages (Ali and Sreekrishnan 2001; Crooks and Sikes 1990; Pokhrel and Viraraghavan 2004). Various studies have reported the detrimental effects of pulp and paper mill effluent such as respiratory effect, oxidative stress, liver damage and genotoxicity (Schnell et al. 2000; Vass et al. 1996). Therefore, a comprehensive treatment strategy is required for decolorization of effluent to meet the discharge safety standards.

Various physiochemical methods like application of acids to precipitate lignin and then burning this lignin are in use, however, they are inefficient, costly and produce large amount of sludge which are difficult to handle. As sludge is huge in amount, landfilling is generally not feasible. On burning, volatile organic toxic compounds like dioxins are formed. Some pulp and paper mill use recovery boilers to recover and burn much of the black liquor they produce, generating steam and recovering the cooking chemicals (sodium hydroxide and sodium sulfide which are used to separate lignin from the cellulose fibres needed for papermaking). The dry solids content of separated black liquor is increased by removing water from the black liquor in the evaporation process. Sodium and sulfur are recovered from the black liquor by burning the black liquor in recovery boiler. This chemical reaction and burning of organic materials releases a considerable amount of heat energy. The heat is recovered by transferring it through water-filled tubes in walls of the recovery boiler. The water vaporizes into steam and electricity is produced from steam with a turbine. Another possible solution for black liquor treatment is biological methods, i.e., the use of fungi and bacteria. A variety of white-rot, soft-rot, and brown-rot fungi have been proved to be lignin degraders. Moreover, it is now generally agreed that lignin degradation is not an ability limited to the "white-rot" fungi. Along with these fungi, numerous bacteria such as *Pseudomonas* sp., *Flavobacteria*, *Xanthomonas* sp., *Bacillus* sp., *Aeromonas* sp., *Cellulomonas* sp., etc. have been reported to decompose lignin and its derivatives (El-Bestawy et al. 2008; Kirk et al. 1977). The contributions of bacteria have been reported for utilization of low-molecular weight lignin oligomers as the sole source of carbon and energy that produce enzymes and cleave intermonomeric linkages of lignin (Vicuna et al. 1993). Bacteria play a pivotal role in depolymerizing lignin in aquatic ecosystem because wood degrading bacteria have a wider tolerance of temperature, pH and oxygen limitations than fungi (Vicuna 1988).

For effective decolorization it is essential to optimize the composition and conditions of culture media (Prakasham et al. 2005). In conventional methods numerous experiments have to be carried out to optimize all the parameters and establish best possible culture conditions by correlating all the parameters. In these methods studying one variable at a time is cumbersome and uneconomical. Another approach is to use statistical tools and experimental designs (Stowe and Mayer 1999). Taguchi methods have been widely used to optimize the culture conditions by devising minimum number of experiments and to identify the impact of individual factor. Analysis of the experimental data using the ANOVA (analysis of variance) gives statistically significant output (Kackar 1985; Phadke and Dehnad 1988). Therefore, in this study, bacteria was isolated and identified from the contaminated sites to optimize process parameters for effective removal of color and lignin in the wastewater. It is believed that effluent and sludge are contaminated by highly toxic and recalcitrant compounds; therefore methods like comet assay were developed to test the toxicity of the treated effluent for safe disposal in the environment.

Materials and methods

Sampling site and isolation of bacteria

In this study, bacterial strains were obtained from pulp and paper mill effluent and sludge, which were collected from the Century pulp and paper mill, Lalkuan, Uttarakhand, India (29° 24′N, 79° 28′E). Sediments were dissolved in autoclaved water (1:10 w/v) by mild stirring. The supernatant containing bacteria was poured in minimal salt medium (MSM) in a chemostat. Chemostat culture was set with culture condition as stirring, 150 rpm; temperature, 30°C. The composition of MSM (g/l) was: $Na_2HPO_4{\cdot}2H_2O$, 7.8; KH_2PO_4, 6.8; $MgSO_4$, 0.2; $NaNO_3$,

0.085; $NH_4(CH_3COO)_3Fe$, 0.01; $Ca(NO_3)_2{\cdot}4H_2O$, 0.05, trace element solution, 1 ml/l, containing lignin (1%) as the carbon (C) source (Thakur et al. 2001). The pH of the chemostat was kept constant at 7 by 1 M NaOH. Culture medium for inoculation was collected from chemostat when bacterial growth became stable. The growth of the bacterial cells was determined by turbidity after measuring optical density (OD) at 595 nm. Serial dilution of the supernatant of the order of 10^{-3}, 10^{-4} and 10^{-5} was done and spread on agar plate. The bacterial colonies appearing on agar plates were morphologically characterized and purified by repeated culturing. They were observed under a microscope, Olympus and Magnus MLX-TR, at 40× and 100× attached with a camera. The morphologically distinct bacterial strains were screened for decolorization and delignification in MSM with 10% black liquor, at pH 7 and incubated at 30°C (Thakur 2004). Decolorization and delignification of effluent was estimated after 48 h.

Decolorization assay and measurement of lignin

The sample was centrifuged at 8,000 rpm for 15 min to remove all the suspended matter. Color content was measured as described in the manual of Standard Methods for Examination of Water and Wastewater (APHA 2005). Absorbance of supernatant was measured at 456 nm.

For measuring lignin, the pH of the supernatant was adjusted to 7.0 with 2 M NaOH. The sample (50 ml) was mixed with 1 ml CH_3COOH (10%) and 1 ml $NaNO_2$ (10%). After 15 min, 2 ml of NH_4OH was added. It was then left for 5 min and absorbance was measured at 430 nm. For Blank, 1 ml CH_3COOH (10%) was added in 50 ml distilled water and 2 ml NH_4OH. After 15 min, 1 ml of $NaNO_2$ (10%) was added (Pearl and Benson 1940). The absorbance value was transformed into lignin content (ppm) using the following formula:

$$\text{Lignin (ppm)} = \text{Absorbance}/0.000247$$

16S rDNA sequence analysis of the most potent bacterial strain

Genomic DNA of bacteria was isolated using QIAamp DNA Mini Kit. 16S rRNA gene was amplified using primers Po 5′ (GAGAGTTTGATCCTGGCTCAG) 3′ and P_6 5′ (CTACGGCTACCTTGTTACGA) 3′ with a thermo cycler (Applied Biosystem, USA) (Scortichini et al. 2002). The amplified DNA was purified using Qiaquick PCR Purification Kit (Qiagen), adjusted to 200 ng μl^{-1} and sent for sequencing. The amplified 16S rRNA sequences were compared with the nucleotide sequences present in the GenBank using the standard BLASTN site at NCBI server (http://www.ncbi.nlm.nih.gov/BLAST) (Altschul et al. 1990). The alignment of the sequences was done using CLUSTAL W program (version 1.8.3). From the aligned sequences neighbour-joining dendrogram was constructed with Mega 3.1 software (Kumar et al. 2004).

Experimental design for optimization of process parameters

For Taguchi design and analysis of results the Qualitek-4 version (7.6.0.3) software was used (Roy 2001; Roy 2007). In this study, we used Taguchi approach of orthogonal array and experimental designs that help to gain more information about the optimum conditions. Apart from optimized conditions, the contribution of each individual factor in color and lignin reduction and the interactions among various factors was also studied. Both orthogonal arrays and ANOVA were used for this purpose. 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 (Table 2; Roy 2001). 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, experimental data was analyzed using ANOVA. Taguchi approach used ANOVA to statistically significant parameters in finding the optimum levels (Phadke and Dehnad 1988).

Screening for decolorization and removal of lignin of pulp and paper mill effluent

Screening experiments were performed to select most suitable carbon (C) and nitrogen (N) sources. Various C sources were used at an initial concentration of

1.5% (w/v). MSM having black liquor (5,000 C.U.) was taken as control. Batch study was conducted in Erlenmeyer flasks containing MSM along with 10% black liquor, 2.5% (w/v) inoculum supplemented with different C sources (1.5%). After selecting the most suitable C source, various N sources were screened at a concentration of 0.05% (w/v). Change in color and lignin was analyzed for 2 days.

Optimization of growth conditions by Taguchi approach

Once C and N sources were selected, the growth media was optimized for the optimum concentration of selected C and N sources. Other process parameters such as pH, inoculum size, temperature, rpm and duration were also optimized. Table 1 shows the parameters and levels used in this experiment.

Analysis of the data using overall evaluation criteria

The data obtained from eight experiments was processed in the Qualitek-4 software with quality characteristics for determination of optimal culture conditions. Along with this, the contribution of each individual factor in total decolorization and lignin reduction was studied by estimating performance at optimum conditions. Since the optimization was carried out for both reduction in color and lignin a combined index (overall evaluation criteria–OEC) was used. For calculation OEC assume:

X1 = Numeric evaluation under criterion 1
X1ref = Highest numerical value X1 can assume
Wt1 = Relative weighting of criterion 1

Table 1 Parameters and levels used for optimization of the experiment

Parameters	Level 1	Level 2
Temperature	30°C	35°C
RPM	200	250
pH	7	8
Duration	24 h	48 h
Inoculum size	4% (w/v)	5% (w/v)
Carbon	1.5% (w/v)	2.5% (w/v)
Black liquor	7.5% (v/v)	10% (v/v)

Then OEC was calculated as:

$$OEC = \frac{X1}{X1ref} \times Wt1 + \frac{X2}{X2ref} \times Wt2 + \cdots$$

In this experiment color is criterion 1 and lignin is criterion 2. The reduction in color and lignin was measured in percentage thus highest numerical value for X1ref and X2ref 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 color 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.

Software

Qualitek-4 software (Nutek Inc., USA) for automatic design of experiments using Taguchi approach was used in this study (Roy 2007). 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. This is user-friendly window based software that 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).

Analysis of the data and validation

The data obtained after the 8 experiments were processed in the Qualitek-4 software with quality characteristics for deriving the optimum level for each parameter and ultimately arriving at optimal culture conditions. The contribution of each individual factor in total decolorization and delignification was studied. The analysis was performed with "bigger is better" quality characteristics. In order to validate the methodology used in optimization, the culture conditions obtained after optimization were tested and compared with the values predicted by the model.

Estimation of enzymes for decolorization

To measure the activities of the enzyme the culture supernatant was obtained by centrifugation at $8000 \times g$ for 10 min. Four different enzymes, Lignin peroxidase (LiP), Manganese peroxidase (MnP), Laccase (Lac)

and Xylanase 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 activity is defined as amount of enzyme required to oxidize 1 mol of VA 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 (Wariishi et al. 1992). The reaction mixture consists of 0.5 mM $MnSO_4 \cdot H_2O$, 45 mM sodium malonate buffer (pH 4.5) and enzyme. The reaction started with the addition of 0.1 mM H_2O_2. 1 U/ml of MnP was defined as enzyme required to oxidize 1 µmol of Mn(II) to Mn(III) per min. Lac 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. The xylanase activity was measured by determining the amount of reducing sugar released from oat spelt xylan. The reaction mixture consisted of 1% xylan in 100 mM Tris–HCl buffer (pH 8.0) and enzyme (Ratanakhanokchai et al. 1999). After incubation for 15 min at 50°C, the increase in the amount of reducing sugar was determined by the Somogyi–Nelson method (Nelson 1944). One unit (IU) of xylanase activity was defined as the enzyme necessary to catalyze the production of 1 µmol of xylose equivalent per min.

Analysis of toxicity by comet assay (Alkaline SCGE)

Alkaline comet assay was performed using *Saccharomyces cerevisiae* MTCC 36 as model organism for evaluation of toxicity (Miloshev et al. 2002). Yeast cells were grown in MYPG media (0.3% malt extract, 0.3% yeast extract, 0.5% peptone, 1% glucose, pH 5.0). The cells in log phase of growth were 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. Comets were analyzed using the fluorescence microscope with an excitation filter of 355 nm and a barrier filter of 450 nm. Fifty comets were analyzed per sample. The fluorescence microscope was fitted with 100× oil immersion lens. The percentage of DNA in tail was calculated using CometScore^{TM} Freeware software (www.tritekcorp.com). The comets were divided into five classes on the basis of amount of DNA in tail (Miyamae et al. 1998). Class I less than 1% DNA in tail (intact nucleus), Class II had 1–20% DNA in tail, Class III had 20–50% DNA in tail, Class IV it was 50–75% DNA in tail and Class V having comets with more than 75% DNA in tail. The incidence of comets in these classes in each sample was studied.

Results and Discussion

Isolation and screening of bacterial strains for decolorization

Four different types of bacterial strains appeared on the plates were isolated based on morphological differentiation of individual colonies. The decolorization was measured after 6, 12, 24 and 48 h. Maximum reduction in color and lignin was observed at 48 h by LP1 (25%, 28%), followed by LP2 (17%, 24%), LP3 (17%, 21%) and minimum by LP4 (17%, 17%), respectively (Fig. 1).

Molecular characterization and identification of bacteria

On the basis of decolorization potential, one bacterium was selected among all the four strains for further studies and analyzed by sequencing the amplified 16S rRNA gene. Pair wise alignments giving a closest match of 99% with sequences analyzed were chosen. LP1, showing 99% homology with *Bacillus* sp.

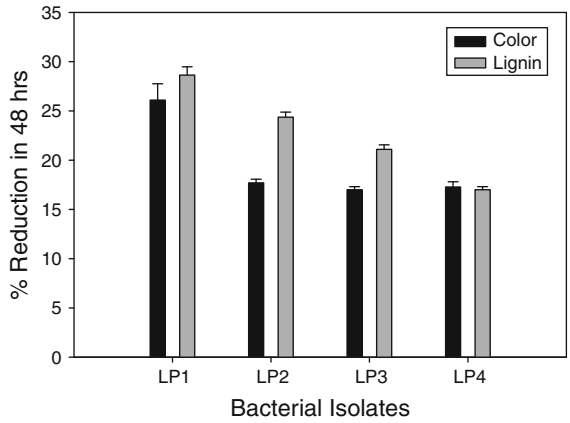

Fig. 1 Percent decolorization and delignification of black liquor of pulp and paper mill by different bacterial isolates at 48 h in shake flask culture (*Error bars* are standard deviations)

sequence of the 16S rRNA gene was submitted in genbank with accession no. (EU741057; Fig. 2). Phylogeny tree was drawn using MEGA 3.1 software (Kumar et al. 2004). Molecular techniques based on 16S rDNA genes have provided new insights to elucidate microbial community. Sequencing of 16S rRNA genes has been very useful for describing the compositions of bacteria (Giovannoni et al. 1990).Various other bacteria have been also reported for decolorization and delignification (El-Bestawy et al. 2008; Kirk et al. 1977).

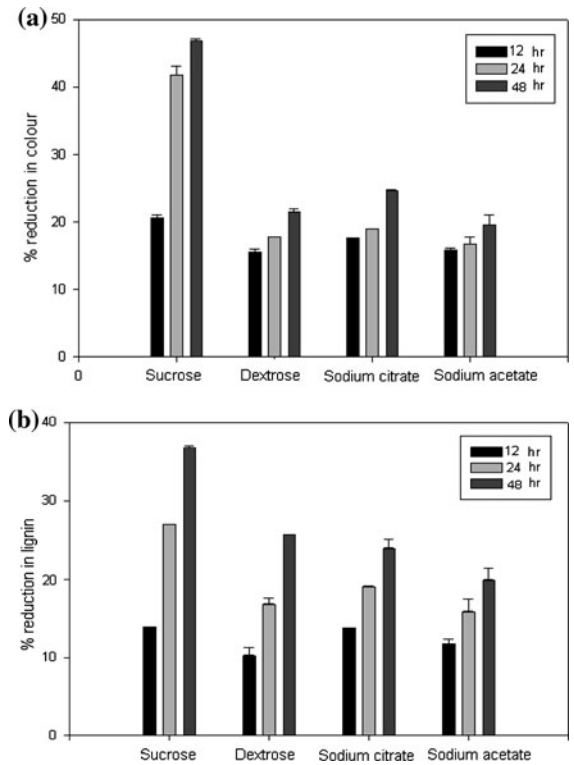

Fig. 3 Percent decolorization (**a**) and delignification (**b**) of black liquor in presence of different carbon sources (1.5% each) before optimization. Maximum decolorization (46.8%) and delignification (37.1%) was obtained by sucrose at 48 h (*Error bars* are standard deviations)

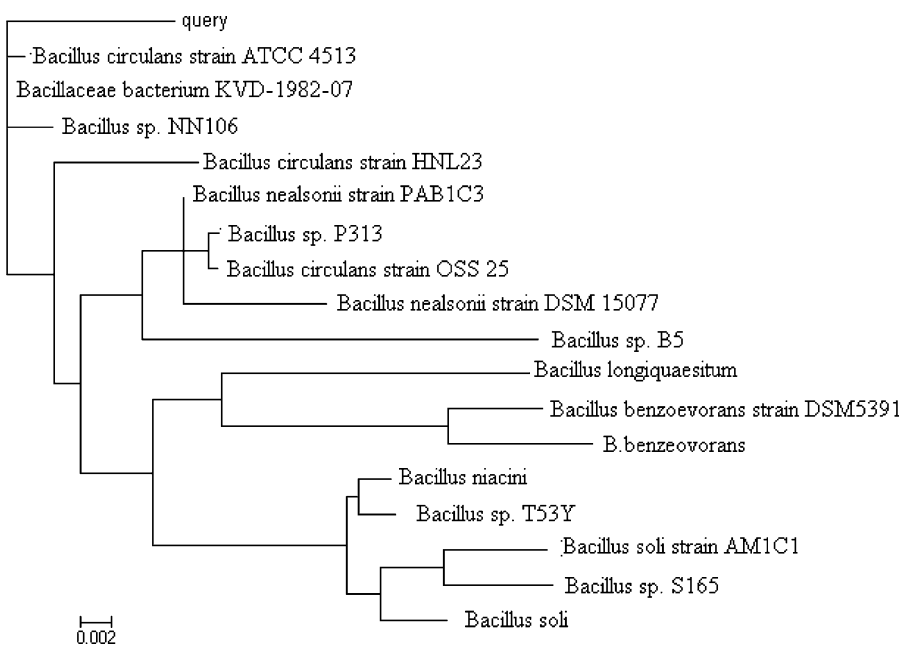

Fig. 2 Phylogenetic tree of lignin-degrading bacterial strain, *Bacillus* sp., based on 16S rDNA gene sequence. Boot strap consensus tree was drawn by multiple sequence alignment with neighbour-joining method using software MEGA 3.1

Screening of growth factors for decolorization of pulp and paper mill effluent

Bacillus sp. strain LP1 was applied for the evaluation of decolorization and delignification in presence of various parameters. Initially various C sources (1.5%) were tested in which LP1 removed color and lignin in presence of sucrose (46.8%, 37.1%) followed by dextrose (23%, 25.8%), sodium citrate (23.7%, 25.9%) and sodium acetate (21.2%, 18.6%), respectively (Fig. 3a, b). Effluent was further treated in presence of inorganic and organic N source (0.05%) like urea, yeast extract, sodium nitrate and ammonium nitrate, and the results indicated that N has negative impact on growth and decolorization. Screening experiments were performed in advance, in presence of C and N sources to optimize the remaining process parameters by Taguchi approach. The decolorization activity depends upon cell metabolism, which is regulated by media composition such as C and N sources in addition to other process parameters like pH, incubation, temperature and aeration. In general, high C levels favor the process of decolorization (Jadhav et al. 2008; Nagarathnamma and Bajpai 1999), however, maximum decolorization of effluent in absence of additional C and N sources using soil as inoculum are also reported (Adikane et al. 2006; Sirianuntapiboon et al. 2004). N nutrients are the most important factors to be considered in the culture conditions. In *Trametes versicolor* cultures, decolorization of Kraft bleach effluent has been shown to occur under N deficient conditions (Bajpai et al. 1993). Addition of high amount of N sources decreased the decolorization efficiency of the microbes. On the contrary, decolorization by microbial consortium of *Galactomyces geotrichum* and *Bacillus* sp., has been investigated using N rich media (Jadhav et al. 2008).

Table 2 Taguchi orthogonal array table of L-8 type and percentage decolorization in each run

S.No	Temperature	RPM	pH	Duration	Inoculum size	Carbon	Black liquor	Decolorization	Delignification
1	1	1	1	1	1	1	1	23 ± 0.4	15 ± 0.3
2	1	1	1	2	2	2	2	50 ± 1.5	50 ± 0.6
3	1	2	2	1	1	2	2	31 ± 0.7	24 ± 0.4
4	1	2	2	2	2	1	1	28 ± 0.1	35 ± 1.5
5	2	1	2	1	2	1	2	34 ± 0.1	43 ± 0.5
6	2	1	2	2	1	2	1	64 ± 0.6	51 ± 0.3
7	2	2	1	1	2	2	1	48 ± 0.3	30 ± 0.6
8	2	2	1	2	1	1	2	33 ± 0.03	30 ± 1
Total contribution of all factors								33.75	27.87
Expected result at optimum condition								70	55

Table 3 Optimum conditions and performance

S. No	Parameter	Level description	Level	Contribution
1	Temperature	35	2	4.728
2	RPM	200	1	4.656
3	PH	8	2	1.666
4	Duration	48	2	4.708
5	Inoculum size	5.0	2	1.093
6	Sucrose (%C)	2.5	2	7.791
7	Black liquor	10	2	1.906
Total contribution from all factors				26.547
Current grand average of performance				36.738
Expected result at optimum condition (OEC value)				63.286

Determination of optimum conditions using Taguchi method and validation experiment

Taguchi method was used to identify the optimum conditions and select the most significant parameter in decolorization of effluent. After selecting the most suitable C and N source, temperature, rpm, pH, inoculum size and duration were further optimized by using Taguchi approach. Eight experiments were conducted for optimization of increased decolorization and delignification according to the L-8 orthogonal array. The results of experiments in this section showed that maximum decolorization and delignification achieved by *Bacillus* sp. (63 and 51% respectively) when experiments condition were as follows: temperature 35°C, pH 8, agitation 200 rpm, duration 48 h, inoculum size 4% (w/v), C (sucrose) 2.5% (w/v). Table 3 is showing optimum conditions to have maximum decolorization and delignification (70 and 56%, respectively). Table 2 shows the results obtained after performing the experiments. The effective influence of a Taguchi L-8 orthogonal array based experimental conditions on maximum decolorization could be viewed and the variation in percentage decolorization of black liquor is obvious (Table 2). The observed variation certainly indicated the crucial role of optimization of all factors in achieving the best possible results. The results of decolorization of all eight experiments in both the cases were fed in Qualitek-4 software for analysis. The optimization software provided an opportunity to study the multi interaction effect of the factors. Figure 4 represents contribution of each individual factor on effective decolorization. Among all selected factors C had the highest positive impact on decolorization potency (Fig. 4). The contribution of individual factor is the key to control the biological processes. For each factor there is an optimum level.

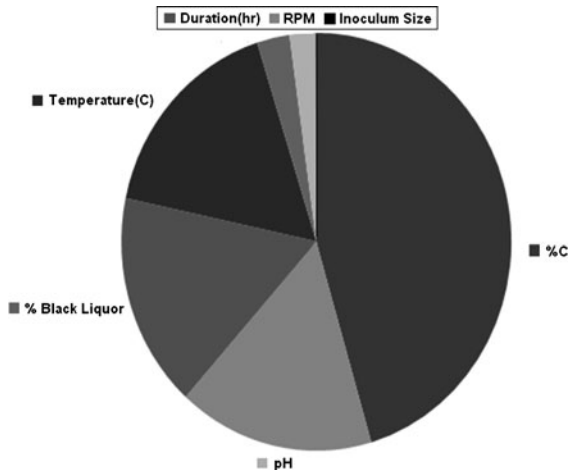

Fig. 4 Percent contribution of individual factor and interaction influences for *Bacillus* sp. % carbon was the most important factor among all factors

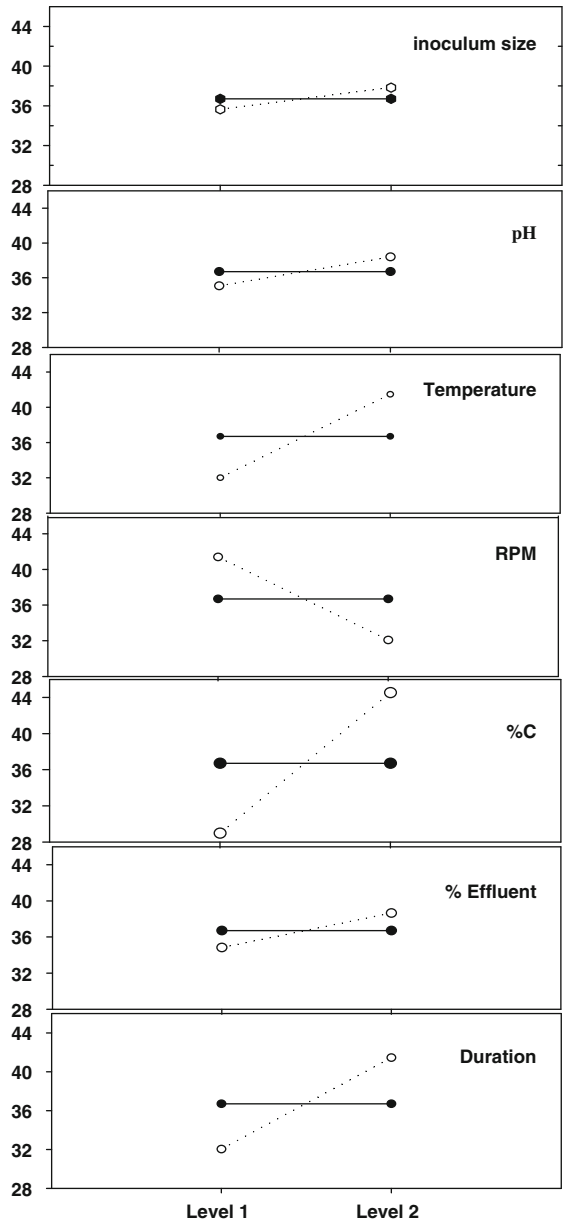

Fig. 5 Individual factor performance at different levels in decolorization and delignification of pulp and paper mill effluent by *Bacillus* sp. (*filled circle*: before optimization; *solid line*: after optimization)

Table 4 Analysis of variance of main effect of factors by *Bacillus* sp. for efficient decolorization

Factor	Degree of freedom (f)	Sum of squares (S)	Variance (V)	F-Ratio (F)	Pure Sum (S')	Percent of contribution (P)
Temperature	1	178.888	178.888	–	178.888	16.622
RPM	1	173.445	173.445	–	173.445	16.116
pH	1	22.211	22.211	–	22.211	2.063
Duration	1	177.378	177.378	–	177.378	16.482
Inoculum size	1	9.57	9.57	–	9.57	0.889
Carbon	1	485.627	485.627	–	485.627	45.124
Black liquor	1	29.07	29.07	–	29.07	2.071
Total	7	1076.192	0.125	–		100%

Figure 5 shows the increase or decrease in decolorization for each factor at different levels. C and N sources have extremely variable response in reference to decolorization. The optimum level of sucrose, most suitable C source, was 2.5% w/v, i.e., level 2. N has negative impact on decolorization. Bourbonnais and Paice (1987) tested *Bacillus cereus* and two strains of *Pseudomonas aeruginosa* for the decolorization of bleach Kraft effluent. In previous studies also decolorization (92%) of effluent in presence of glucose in 24 h. is reported (Nagarathnamma and Bajpai 1999). Most significant interaction is in between pH and % effluent (S.I. = 80%). This shows that most significant interaction took place in between two least significant factors (Table 5). In order to conduct the analysis of relative importance of each factor, an ANOVA was conducted. From the results of ANOVA (Table 4), it was concluded that % C had the largest variance; the duration, temperature and rpm indicated almost same variance. Bacteria showed more decolorization at alkaline pH, i.e., pH 8 which is adequate for treatment of pulp and paper mill effluent because lignin molecules have a tendency to undergo self-condensation, particularly in acid media, explaining its resistance to degradation to simple molecular species (Ali and Sreekrishnan 2001).

Table 5 Interactions in between different factors along with significance index (SI) for *Bacillus* sp

S.No	Interacting factor pairs	Columns	SI (%)	Col.	Opt.
1	pH × % Black liquor	3 × 6	80.34	5	(1,2)
2	pH × Inoculum size	3 × 4	73.86	7	(2,1)
3	Inoculum size × % Black liquor	4 × 6	70.95	2	(2,2)
4	Rpm × Duration (h)	2 × 7	62.33	5	(1,2)
5	Rpm × pH	2 × 3	50.38	1	(1,2)
6	Temperature × pH	1 × 3	49.61	2	(2,2)
7	Inoculum size × % C	4 × 5	37.76	1	(1,2)
8	Rpm × inoculum size	2 × 4	29.04	6	(1,2)
9	pH × % C	3 × 5	19.65	6	(1,2)
10	pH × Duration	3 × 7	17.15	4	(2,2)
11	Temperature × % C	1 × 5	8.73	4	(2,2)

Before optimization the average decolorization and delignification was 25 and 28%, respectively, by bacteria and after optimization the expected decolorization and delignification was 70 and 55%, respectively, with optimal conditions. A validation experiment was performed using software derived optimum conditions. Actual decolorization and delignification was 69 and 53%, respectively, which validates the software results.

Enzymes involved in decolorization and delignification process

The mechanism of decolorization and delignification of black liquor was determined by LiP, MnP, Lac and xylanase enzyme analysis. The result of the study indicated no significant increase in LiP and Lac enzyme but a marked increase in xylanase and MnP activity was detected in the treated effluent after 1 day which reached to maxima after 48 h (54 and 28 U/ml, respectively) in optimum culture conditions in presence of black liquor (10%). The data of the study indicated that after third day the activity of enzymes was decreased (Fig. 6).

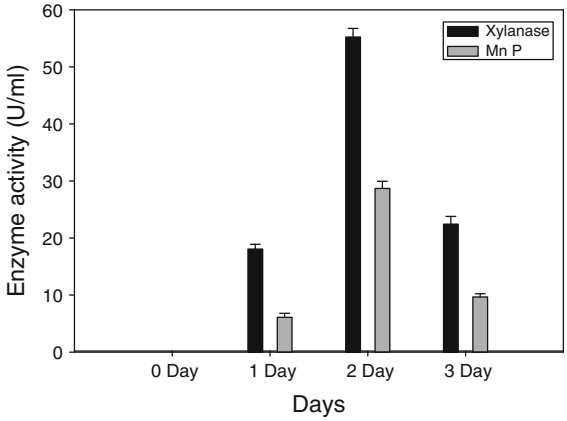

Fig. 6 Estimation of enzyme activity of Xylanase and Manganese Peroxidase (MnP) in *Bacillus* sp. in presence of 10% black liquor

The pulp and paper mill effluent has phenolics, lignin, hetero-polysaccharides (xylan) as its constituents which can induce production of xylanase and lignolytic enzymes. In our experiment, culture media has black liquor (10%) which acted as inducer for xylanase and MnP production. Decolorization of pulp and paper mill effluent due to Xylanase in *B. pumilus* CBMAI 0008 and MnP in *Paenibacillus* sp. CBMAI 868 is reported in previous studies (Moriya et al. 2005; Oliveira et al. 2009).

Toxicity analysis of effluent after treatment

Pulp and paper mill effluent is extremely toxic in nature due to the various chemicals present in it. An important objective of effluent treatment is reduction in toxicity (Pokhrel and Viraraghavan 2004). Thus, comet assay was performed to check the toxicity of the effluent after treatment with *Bacillus* sp. Untreated effluent (E) and distilled water (W), both were taken as positive and negative control. The formation of comets shows that the effluent is genotoxic by nature (Fig. 7). From the graph it is clear that treatments are efficient in reducing toxicity as there is a clear shift of comets to lower classes. In untreated effluent (E) class IV is dominant. The bulk of the comets have shifted to class II and class III after treatment (T; Fig. 9). The average DNA in tail was 58% in untreated effluent (E) which reduced to 24% in treated effluent (T) and the treatment was significant at $P \leq 0.05$ (Fig. 8). There was 58% reduction in percentage tail DNA in treated effluent as compared to untreated effluent. A study on *B. subtilis* reports the mutagenic effect of the sediments contaminated by the effluent of Kraft paper mill (Kinae et al. 1981). Another study reports the toxic and mutagenic effects of pulp and paper mill effluent contaminating Lake Baikal (Lindstrom-Seppa et al. 1998).

Conclusion

In this report a bacterium isolated from pulp and paper mill sludge was identified as *Bacillus* sp. by 16S rDNA analysis. Decolorization and delignification of pulp and paper mill effluent were evaluated by *Bacillus* sp. using Taguchi approach. Bacterium was able to remove color (69%) and lignin (53%) in alkaline condition mainly due to production of xylanase and MnP enzyme. It is worth noting the ability of *Bacillus* sp. to decolourize the direct wastewater that is characterized by a high pH value. Comet assay was used to determine the reduction in toxicity. There was

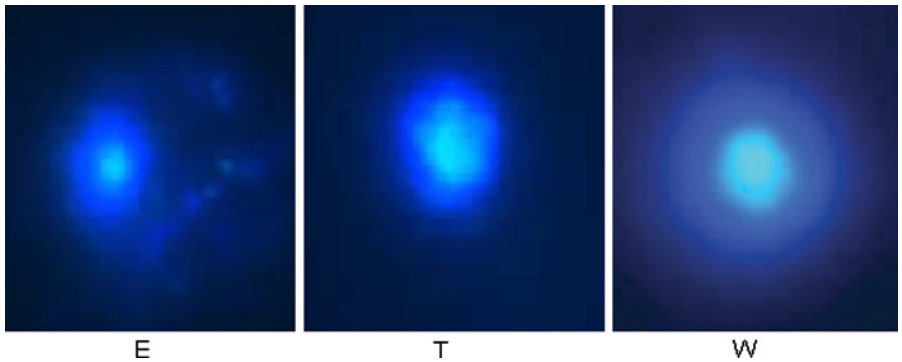

Fig. 7 Formation of comets in effluent (E), treated effluent (T) and control (W)

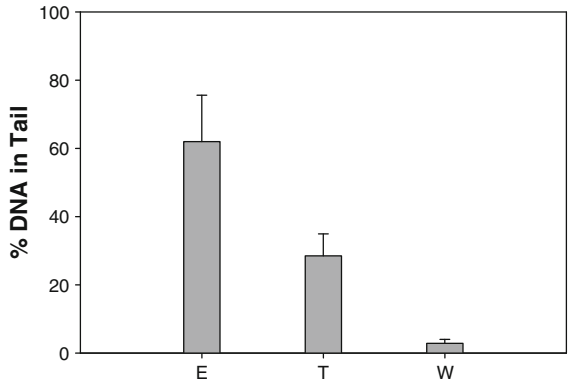

Fig. 8 Percentage changes in DNA present in tail of comets. Within each group, values not followed by the same letter are significantly different at $P \leq 0.05$ (*Error bars* are standard deviations)

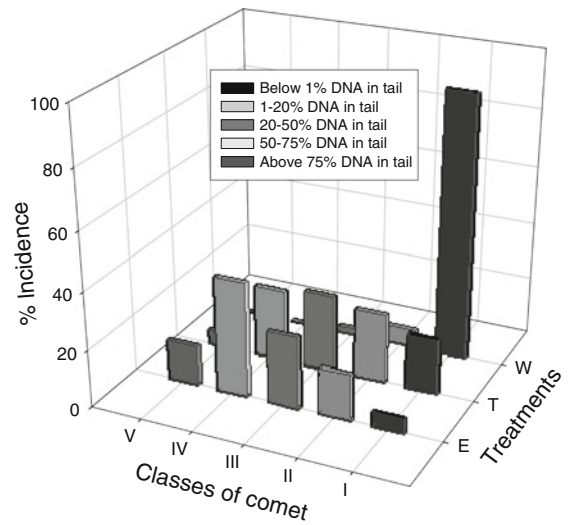

Fig. 9 Distribution of comets in class I–V, depending on percent DNA in tail, for (E) untreated effluent (T) treated effluent at optimized conditions and (W) distilled water

58% reduction in toxicity which indicates treatment of effluent and safe disposal of it into the environment.

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