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Treatment of pulp and paper mill effluent by a novel bacterium *Bacillus* sp. IITRDVM-5 through a sequential batch process

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ABSTRACT

The various types of biorefractory organic pollutants present in pulp and paper mill effluent is obnoxious to the environment and severely affects the biotic and abiotic components. The present study investigates the isolation and characterization of a novel strain isolated from pulp and paper mill effluent. Among the isolates, the strain *Bacillus* sp. IITRDVM-5 showed maximum degradation and decolorization of 100% filter sterilized effluent, supplemented with only nitrogen and phosphorus in a sequential batch treatment. Under optimized conditions, the maximum reduction in TOC, COD, BOD and color was 82.22, 89.50, 93.33 and 73.01%, respectively after 72 h treatment. In addition, the reduction in total phenol, AOX and lignin was 88.5, 75.63 and 64.10%, respectively. The UV–Vis spectral scan identified the degradation pattern of contaminants during treatment. FTIR and GC-MS analysis indicated significant change in functional groups composition of organic pollutants and confirmed their transformation after treatment. Significant degradation of phenolic and chlorolignin derivatives was observed through GC-MS. Furthermore, the evaluation of toxicity with *V. radiata* and *T. foenum-graecum* seeds confirmed the toxic nature of effluent, of which detoxification was observed post bacterial treatment. The results highlight the degradation and decolorization potential of *Bacillus* sp. IITRDVM-5 and its future application in pulp and paper effluent treatment.

1. Introduction

The pulp and paper industry is one of the largest consumers of fresh water and generate a very high amount of effluent worldwide (CPCB, 2015; Kumar et al., 2014; Kumar et al., 2018). Due to the high volume of effluent and pollution load, pulp and paper mill industry has been classified under the category of 17 highly polluting industries in India (CPCB, 2015). The effluent in pulp and paper industry is generally produced during the process of pulping, bleaching and washing steps in pulp and paper mill. The effluent generated is loaded with high amount of organic compounds i.e., lignin fragments, hemi-cellulose, chlorophenolics, resins, fatty acids, and inorganic salts. Therefore, besides general parameters like pH, color, total dissolved solids (TDS), total suspended solids (TSS), total organic carbon (TOC), chemical oxygen demand (COD), biochemical oxygen demand (BOD), pulp and paper mill effluent is also characterized for absorbable organic halides (AOX), total phenol and extractable organic halides (EOX) (Abhishek et al., 2017; Hooda et al., 2018). The presence of toxic derivatives in the effluent is critical from the toxicological perspective owing to their recalcitrance, bioaccumulation and bio-magnification properties (ATSDR, 2015; González et al., 2010; Olaniran and Igbinosa, 2011).

Keeping the above in view, high cross media environmental impact in zero discharge schemes for water intensive industries have not been advocated by experts. Therefore, discharge of ecologically safe industrial effluents into the river system is being emphasized by water resource experts from the sustainability aspects of the river system. Although, the existing conventional treatment systems for pulp and paper mill effluent are able to remove most of the contaminants yet presence of choloro-organic derivatives in the treated effluent indicates the need for further upgradation. The chloro-organics are toxic to biotic components so their removal or discharge in ecologically safe limits is quintessential especially if the effluent is to be used for other purposes. There are several physical, chemical and biological treatment systems that have been established for the reduction of toxic recalcitrant organic derivatives from the paper mill effluent. Furthermore, physico-chemical processes are based on separation processes in which pollutants are not mineralized rather only change in state of pollutant or carrying medium takes place after the process. As a result, the process generates a low volume highly concentrated pollution stream, posing a major challenge for its further treatment before final disposal (Jamil

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et al., 2011; Chang et al., 2016; Rivera-Hoyos et al., 2018). Biological treatment displays the capabilities to mineralize the toxic organic compounds (Abhishek et al., 2017; Singh and Thakur, 2006; Yadav and Chandra, 2015). The efficiency of the existing conventional biological treatment can further be enhanced through bioaugmentation with potent chlorophenol and other toxic organic compounds degrading microbial strains so as to achieve ecologically safe discharge limits. There are several reports on isolation and characterization of toxic compounds degrading microbial strains but the major concerns associated with their field application are: use of additional carbon sources as a co-substrate (glucose, dextrose, and maltose), other media components (peptone, yeast extract, buffer) and long treatment time. Previous report on pulp and paper effluent treatment by *Citrobacter freundii* and *Serratia marcescens* used buffer, glucose and peptone and also treatment time was six days (Abhishek et al., 2017). Yadav and Chandra (2015) treated pulp and paper effluent using *Bacillus subtilis* and *Klebsiella pneumonia* supplemented with buffer, glucose and peptone for six days. Raj et al. (2014) used *Penibacillus* for effluent treatment augmented with buffer, glucose, peptone and yeast extract for six days. Similarly, Sharma et al. (2010) used *Bacillus subtilis* and *Achromobacter xyloxidans* for effluent treatment amended with glucose and yeast extract for seven days. There are reports where 100% effluent was used for treatment but were supplemented with carbon and nitrogen source (Abhishek et al., 2017; Haq et al., 2016). A study reported 100% effluent treatment using *Pseudochrobactrum glaciale, Providencia rettgeri* and *Pantoea sp.* Supplemented with dextrose and peptone for approximately 4 days (Chandra and Singh, 2012). Paliwal et al. (2015) used 100% effluent supplemented with dextrose and peptone for seven days. Tiku et al. (2010) successfully reduced the treatment time using the *Pseudomonas aeruginosa* and *B. megaterium* at the cost of glucose and sucrose addition. There are very limited reports available where only nitrogen and phosphorus were supplemented for effluent treatment (Hooda et al., 2015, 2018; Kumar et al., 2014). The biological treatment study comprising 100% effluent without any additional carbon source, less treatment time and strain enrichment will pave the way to develop a process that can be implemented in the industries.

The present study aims to investigate the above mentioned challenges associated with biological process of pulp and paper effluent treatment. In this study, an efficient bacterial strain was isolated and characterized for the treatment of 100% filter-sterilized effluents without any additional carbon source. The effluent was only supplemented with cost-effective nitrogen and phosphorus source. In order to reduce the treatment time and for strain enrichment, a sequential batch treatment process of effluent was performed with the isolate. The various physico-chemical parameters (TOC, COD, BOD, AOX, lignin, total phenol and color) were considered for the characterization. The degradation and decolorization of contaminants present in pulp and paper mill effluent were further optimized. The degradation of total phenol, lignin and chlorophenolics were also investigated. Furthermore, toxicological evaluation of effluent was performed with *V. radiata* and *T. foenum-graecum* seeds to illustrate the effectiveness of effluent treatment with the isolated strain.

2. Materials and methods

2.1. Sampling and characterization of pulp and paper mill effluent

The pulp and paper mill effluent and soil samples were collected from Star Paper Mills Limited (29.93° N, 77.56° E), Saharanpur, India. The effluent sample was collected from primary outlet (effluent from pulping and bleaching unit) of pulp and paper industry in sterilized plastic jerry-can. The fresh effluent sample was taken to the laboratory for the characterization of various physico-chemical parameters and then stored at 4 °C until complete analysis. The fresh effluent sample was characterized by various parameters i.e., pH, TOC, COD, BOD, total phenol, and color. The TOC was measured by High-Temperature Combustion method using TOC analyzer (Shimadzu, Swan Enviro-Analytical Pvt. Ltd., India). COD was analysed by open reflux method, color by cobalt-platinate method, total phenol by the direct photometric method and BOD by dilution methods respectively which was described in manuals of Standard Methods for the Examination of Water and Wastewater (APHA, 2012).

2.2. Sampling and isolation of microorganisms

The sediment sample was collected from the final discharged point of above mentioned pulp and paper mill for the isolation of potential microbe because the microbes inhabiting there are continuously exposed to the pollutants present in the effluent. The sediment was collected at a depth of 2 cm from the upper layer to isolate the active microbial strain. The sediment sample was suspended in autoclaved phosphate buffer saline (PBS) (1:10 w/v) by stirring and allowed to settle for 30 min. 0.1 mL of appropriate dilution (10^{-6}) was spread on the nutrient agar (NA) plates with composition (g L^{-1}) yeast extract, 1.5; beef extract, 1.5; peptone, 5.0; NaCl, 5.0 agar, 20, and pH 7.0. The plates were incubated at 37 °C for 24 h to isolate the distinct colony. An individual strain was selected on the basis of color, size and morphology and these strains were re-streaked on NA plates to further purify the isolate.

2.3. Screening and identification of potent bacterial isolate

For the screening of pulp and paper effluent contaminants degrading bacterial strain, the morphologically distinct and purified strains were further cultured on minimal salt medium (MSM) agar plates and broth having lignin as the sole carbon source. The modified composition of MSM (g L^{-1}) was $Na_2HPO_4{\cdot}2H_2O$, 7.8; KH_2PO_4, 6.8; $MgSO_4$, 0.2; $FeCl_3$, 0.05; $CaCl_2{\cdot}2H_2O$, 0.05; $MnSO_4$, 0.06; $CuSO_4$, 0.03; NaCl, 0.5 and NH_4Cl, 1.0, agar, 20 and 0.1% lignin (Kumar et al., 2015). The MSM culture was incubated at 37 °C for 72 h (150 rpm for broth) to screen the lignin degrading strains because lignin derivatives are the major contaminants present in effluent. The isolates showing growth on lignin were further used for the degradation of real effluent. For biodegradation experiments, the potential lignin degrading isolates were cultured in sequential batch experiment in a 2L conical flask with working volume of 500 mL filter-sterilized (pore size, 0.22 µm) effluent. Only nitrogen (N) and phosphorus (P) was supplemented to the effluent as urea and diammonium hydrogen phosphate (DAP) in the ratio (BOD) 100: (N) 5: (P) 1 (Kumar et al., 2014). The carbon content of urea and nitrogen content of DAP was taken into consideration during the experiments and result interpretation. The inoculum was prepared by growing the isolates separately in NB medium incubated at 150 rpm at 37 °C overnight. The cells were harvested at optical density (O.D.) 1, washed with sterile PBS and then used as inoculums in the ratio of 100 mL effluent: 2 mL inoculum (final O.D. 0.02). The flasks were incubated at 150 rpm for 48 h at 37 °C for acclimatization of isolates with the effluent. After the completion of the first cycle, the sample was allowed to settle for 1 h and then 90% of the effluent was removed and replenished with fresh filter sterilized effluent of same volume. The incubation time was reduced to 24 h and 5 cycles was performed with each isolates (Kumar et al., 2014). After completion of 5 cycles, the parameters TOC, COD, BOD and color were monitored for each isolate at 0 h, 24 h, 48 h and 72 h. The experiments were performed in triplicates.

On the basis of reduction in TOC, COD, BOD, and color, the most potent bacterial strain responsible for contaminant removal from effluents was selected and identified. The genomic DNA of the potential strain was extracted using genomic DNA extraction kit (Qiagen). The PCR was performed using forward primer (5′AGAGTTTGATCMTGGCT CAG3′) and reverse primer (5′TACGGYTACCTTGTTACGACTT3′) using genomic DNA as template for amplification. The condition of PCR was initial denaturation at 94 °C for 4 min followed by 35 cycles at 94 °C for

1min; annealing at 54 °C for 45 s, extension at 72 °C for 2 min and final extension for 10 min and 4 °C infinite. The amplified PCR product was purified by gel elution kit (Qiagen) and sent for sequencing at Eurofins genomics facility, Bangalore, India. The 16S rRNA gene sequence was blast searched against NCBI database for the identification of the strain. The phylogenetic tree was constructed using bootstrap, neighbor joining method of MEGA 6 software. The identified strain was maintained at 4 °C and as glycerol stock at −80 °C.

2.4. Optimization of pulp and paper mill effluent degradation

The degradation and decolorization of contaminants present in pulp and paper effluent was further optimized with the identified strain on the basis of three parameters viz., inoculum size, nitrogen and phosphorus concentration. The nitrogen and phosphorus were selected along with inoculums size for the optimization of organic pollutant degradation present in the paper mill effluent because these two are vital for the growth of microorganisms and synthesis of their enzymes. Furthermore, nitrogen deficiency has been linked with poor sludge settling due to filamentous and dispersed growth of microbial population (Kumar et al., 2014). The bacterial inoculum was varied between 2 to 8% v/v, nitrogen (urea) 0.07 gm to 0.28 gm and phosphorus (DAP) 0.0152 gm to 0.0608 gm during the sequential batch biological treatment. The TOC, BOD, COD, color, total phenol, lignin and AOX was estimated at 0 h, 24 h, 48 h and 72 h for the optimized culture.

2.5. Estimation of lignin and adsorbable organic halides (AOX)

Lignin content in the effluent was measured according to Pearl and Benson (1940). Briefly, 50 ml of the control and treated effluent was adjusted to pH 7.0 and mixed with 1 mL CH_3COOH (10%) and 1 mL $NaNO_2$ (10%) followed by incubation for 15 min. Further, 2 mL of NH_4OH was added and incubation for 5 min. Absorbance was measured at 430 nmagainst blank (control). The value of absorbance was expressed as lignin content (ppm) using the formula:

Lignin (ppm) = Absorbance/0.000247

AOX was estimated by Multi X 2500 halide analyzer (Jena, Germany). The control and treated sample was processed and the AOX content was calculated according to Nie et al. (2014).

2.6. UV–visible spectral scan and fourier transforms infrared spectroscopy (FTIR)

The UV–Vis spectral scan was carried out for the control and treated pulp and paper effluent in wavelength range 190–800 nm using Shimadzu 1800 spectrophotometer. The pH of control and treated samples were maintained at 7 before spectral scan. The wavelength scan was done using a quartz cuvette having 1 cm path length. All the spectra were collected at a scan rate of 0.1 nm and a scan speed of 1000 nm min^{-1}.

FTIR was done to identify the change in functional group composition of contaminants of before and after the effluent treatment. Equal amount of control and treated dried effluent powder sample was mixed with KBr in a ratio of 1/100 (w/w), milled, and homogenized into a very fine powder. Varian 7000 Fourier transform infrared spectroscopy (FTIR) spectrometer were used and analyzed according to Kumar et al. (2015).

2.7. Metabolites identification by GC-MS

The sample volume 50 ml of control and treated pulp and paper mill effluent was processed and analyzed with slight modification as described (Raj et al., 2014). Briefly, the effluents samples were centrifuged at 10000 rpm for 15 min to remove suspended solids and the pH of supernatant was adjusted 1–2. The supernatant was extracted with equal volume of ethyl acetate thrice. The extracted samples were derivatized using 100 μL BSTFA (N,O-bistrimethylsilyl trifluoroacetamide), 50 μL dioxane and 10 μL pyridine. Mixture was heated at 60 °C for 15 min with intermittent shaking. One-microliter of silylated extract was injected into GC-MS and analyzes for the identification of metabolites. The GC-MS was operated under following conditions initial temperature 60 °C for 3 min, temperature increased from 60 to 320 °C at a rate of 10 °C min^{-1}, and hold time 5 min. The analysis was done on Shimadzu GC-MS-QP 2010 Plus equipped with a capillary column Rtx-5MS (dimensions 0.25-μm film thickness, 0.25 mm ID, 30 m in length). Data were matched with the GC-MS inbuilt standard mass spectra library of NIST.

2.8. Toxicity test

The toxicity test of paper mill effluent was evaluated by seed germination method which gives the much authenticated results. It was performed with the seeds of *V. radiata*(mung bean) and *T. foenum-graecum* (methi) in the presence of tap water (TW) (consider as control), untreated effluent (UTE) and *Bacillus* sp. IITRDVM-5 treated effluent (TE), in petri plate containing sterilized filter paper (Chandra and Kumar, 2017; Santal et al., 2011). Prior to sowing the seed in petri plate, surface sterilization was done by the 0.1% $HgCl_2$ for 2 min, to prevent fungal contamination. The seeds were cultivated with equal amount of all liquid samples (TW, UTE, TE) and incubated at room temperature. The seed germination was observed with TW, UTE, 24 h, 48 h and 72 h treated effluent, from the day of sowing. The parameters covered such as Germination percentage (GP), Phytotoxicity percentage (PP), Tolerance index (TI), Relative toxicity (RT), Radical growth inhibition (RGI) and, Vigour Index (VI) for the toxicity assessment, each in triplicate, was performed as described by Chandra and Kumar (2017); David Noel and Rajan (2015) and Santal et al. (2011).

3. Results and discussion

3.1. Physico-chemical characteristics

The physico-chemical characterization of pulp and paper mill effluent is represented in Table S1. The nature of pulp and paper effluent was alkaline (pH 8.49) due to the kraft process used for pretreatment of biomass in the industry. The TOC, COD, BOD and color (PCU) of effluent was found to be 311.3 ± 5.56 mg L^{-1}, 648 ± 8.08 mg L^{-1}, 269 ± 10.81 mg L^{-1} and 1460 ± 7.93 (PCU) respectively (Table S1). The value of parameters vary due to heterogeneous characteristics of raw material used in the pulp and paper mill (Kumar et al., 2014). The toxicity is possibly due to the presence of lignin and its derivatives such as phenols, chlorophenols and other harmful lignocellulosic derivatives (Haq et al., 2016; Yadav and Chandra, 2015). So, the presence of lignin, total phenol and absorbable organic halides was also measured in the effluent and their concentration was observed to be 2425.1 ± 12.21 mg L^{-1}, 25.16 ± 0.33 mg L^{-1} and 11.9 ± 0.45 mg L^{-1} (Table S1). The phenolic and chlorophenolic compounds are generated during lignin depolymerization and chloride bleaching of pulp in paper industry (Bajpai, 2018).

3.2. Screening and identification of potent bacterial isolate

The bacterial strains were isolated from the sediment sample collected from the final discharged point of pulp and paper mill effluent. Indigenous microorganisms present in the polluted environment possess great potential to transform or degrade recalcitrant compound. Due to the continuous exposure to recalcitrant compounds these microorganisms develop resistance and capability to metabolize the contaminants present in the effluent. Morphologically distinct 16 autochthonous bacterial strains were isolated and cultured on lignin as sole

Table 1
Screening of strains on the basis of reduction in TOC, COD, BOD, and color of pulp and paper mill effluent.

S. No.	Strains	Reduction (%)			
		TOC	COD	BOD	Color
1	IITRDVM-1	67.38 ± 0.17	78.86 ± 0.66	58.00 ± 0.77	57.80 ± 0.74
2	IITRDVM-2	67.21 ± 0.60	76.76 ± 0.77	52.41 ± 0.92	62.00 ± 0.44
3	IITRDVM-3	66.42 ± 0.30	87.66 ± 0.06	86.06 ± 0.65	50.32 ± 0.58
4	IITRDVM-4	67.87 ± 0.05	87.90 ± 0.31	91.98 ± 0.74	58.93 ± 0.84
5	IITRDVM-5	68.07 ± 0.83	89.07 ± 0.98	93.71 ± 0.57	70.03 ± 0.62
6	IITRDVM-6	54.86 ± 0.82	80.30 ± 0.27	84.73 ± 0.57	47.41 ± 0.52
7	IITRDVM-7	57.65 ± 0.91	88.92 ± 0.06	90.00 ± 0.62	53.47 ± 0.47

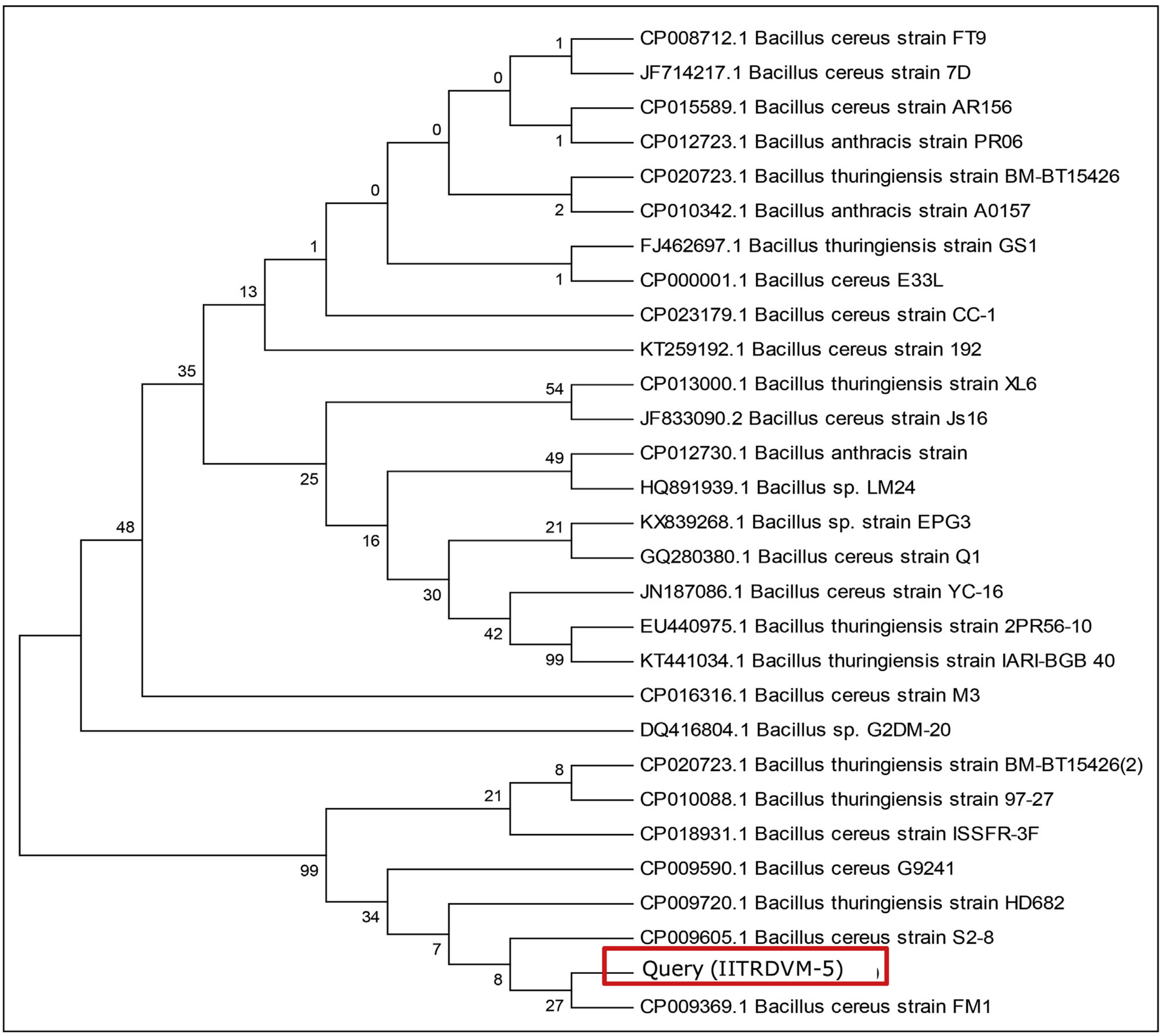

Fig. 1. Phylogenetic tree of *Bacillus* sp. IITRDVM-5 based on 16S rRNA gene sequence constructed by multiple sequence alignment with neighbor-joining method using MEGA 6.

carbon source. Out of the 16, only seven strains showed growth on MSM-lignin and were studied further (Table S2). These seven bacterial strains (IITRDVM-1, IITRDVM-2, IITRDVM-3, IITRDVM-4, IITRDVM-5, IITRDVM-6 and IITRDVM-7) were further screened on the basis of removal of TOC, COD, BOD and color using 100% filter-sterilized effluent without addition of buffer and additional carbon source. The screening parameters TOC, COD, BOD and color was monitored for three days because the maximum reduction was observed on day 3 and after that no significant reduction was observed (Table S3). On the basis of screening results, isolate IITRDVM-5 showed maximum removal of TOC, COD, BOD and color followed by IITRDVM-3, IITRDVM-4, IITRDVM-6 and IITRDVM-7. The reduction in TOC, COD, BOD and color observed for IITRDVM-5 was found to be 68.07 ± 0.83, 89.07 ± 0.98, 93.71 ± 0.57 and 70.03 ± 0.62% respectively (Table 1). A novel *Bacillus* sp. IITRDVM-5 was characterized as gram positive and also + ve for catalase, oxidase, citrate and nitrate, on the basis of morphology and biochemical test (Table S4 and Fig. S1). The optimum pH and temperature for *Bacillus* sp. IITRDVM-5 was found to be 7 and 37 °C respectively. The strain was further identified by 16S rRNA gene sequencing and the phylogenetic tree was constructed (Fig. 1). The blast result of best three hits showed 99% similarity to *Bacillus thuringiensis* strain 2PR56-10, *Bacillus thuringiensis* strain IARI-BGB 40 and *Bacillus cereus* strain YC-16. The 16S rRNA gene sequence of *Bacillus* sp. IITRDVM-5 has been submitted to GenBank with the assigned accession no. MK106115. This strain was further selected for detailed investigation for the degradation of pulp and paper mill effluent.

3.3. Degradation of pulp and paper mill effluent in optimized condition by Bacillus sp. IITRDVM-5

During the sequential batch biological treatment screening, *Bacillus* sp. IITRDVM-5 emerges as the potential strain for the treatment of pulp and paper effluent. This strain significantly reduces the TOC, BOD, COD and color. The strain *Bacillus* sp. IITRDVM-5 was further selected for the detailed characterization and optimization of pulp and paper mill effluent treatment. To reduce the pollution load, the pulp and paper mill effluent treatment was further optimized keeping in mind the feasibility of the process for industrial application. The important aspect considered while optimization are less resident time of effluent, no carbon source or buffer addition and low cost alternative of growth nutrient (nitrogen, phosphorus) for biodegradation. The parameters selected for optimization of pulp and paper effluent treatment are inoculums size, nitrogen and phosphorus concentration. Pulp and paper mill effluent is rich in carbon source but deficient in nitrogen and phosphorus. The reduction of TOC, COD, BOD and color of effluent was also studied without nitrogen and phosphorus supplementation. The TOC, COD, BOD and color reduction was found to be 67.27, 75.29, 75.55 and 61.5% respectively. For effective treatment of effluents, microorganisms require nitrogen and phosphorus for growth and metabolism of organic compounds. During optimization it was observed that inoculums size, nitrogen and phosphorusconcentration significantly influenced the effluent treatment. After optimization experiments, the maximum reduction in pollution load was observed at 8% v/v inoculums size, 0.28 gm L^{-1} nitrogen and 0.06 gm L^{-1} phosphorus content. The maximum reduction in TOC, COD, BOD and color was 82.22, 89.50, 93.33 and 73.01% respectively after 72 h with respect to control (Table S3 and Fig. 2). From the graph it was evident that 75, 85, 89 and 63% reduction in TOC, COD, BOD and color was observed after 24 h by *Bacillus* sp. IITRDVM-5 and acceptable limit of effluent COD discharge was achieved. The reduction in parameters after 24 h is due to the acclimatization of strain for five cycles prior to the experiment. The originality of the present work is 100% filter-sterilized pulp and paper effluent was used for treatment without addition of buffer and carbon source. A comparative analysis of bacterial mediated treatment of pulp and paper mill effluent has been shown in Table 2. The degradation of pulp and paper effluent contaminants was found to be significant compared to earlier studies. Abhishek et al. (2017) reported 61, 67 and 55% TOC, COD and color reduction of effluent supplemented with glucose (1%) and peptone (0.3%) after 6 days using bacterial consortium of *Citrobacter freundii* and *Serratia marcescens*. In a study, 78, 82 and 61% reduction in COD, BOD and color respectively, was reported from a sixth day culture of *Bacillus* sp. Supplemented with glucose and peptone (Raj et al., 2007). Compared to present study, a similar reduction in COD and BOD was observed using bacterial consortium augmented with dextrose (1%) and peptone (0.5%) after 84 h (Chandra and Singh, 2012). In a study, using only nitrogen ($NH_4(SO_4)_2$) and phosphorus (H_3PO_4) as supplement to *Brevibacillus agri* culture, and 69 and 47% reduction in COD and color respectively, was observed after 5 days of treatment (Hooda et al., 2015). Haq et al. (2016) reported a reduction of 85 and 72% in COD and color respectively after 144 h of treatment by *Serratia liquefaciens* supplemented with glucose (1%) and peptone (0.5%).

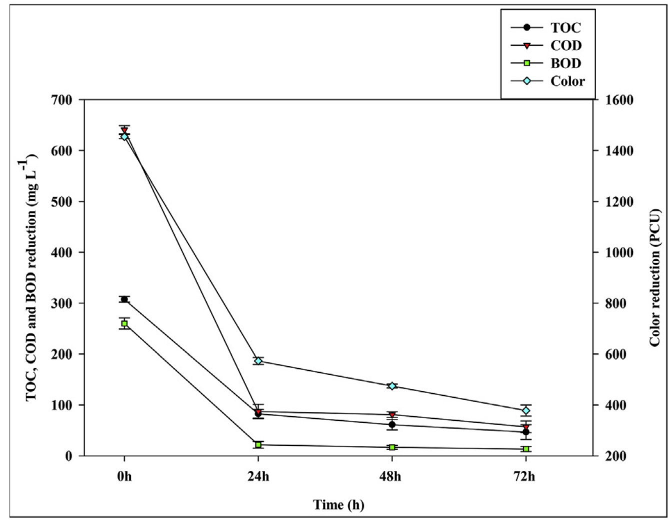

Fig. 2. Reduction of TOC, COD, BOD and color of pulp and paper mill effluent under optimized conditions by *Bacillus* sp. IITRDVM-5. The experiments were done in triplicates. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.4. Estimation of lignin, total phenol and adsorbable organic halides (AOX)

Bacillus sp. IITRDVM-5 was studied for the degradation or reduction of toxic compounds present in pulp and paper effluent. Lignin, phenolics and chlorolignin derivatives are the main components that are responsible for the toxicity of the effluent. Under the optimized culture condition, the reduction of lignin, total phenol and AOX by *Bacillus* sp. IITRDVM-5 was showed in Fig. 3. Lignin was reduced from 2425.1 ± 2.43 to 870.44 ± 19.43 mg L^{-1}, total phenol 25.1602 ± 0.90 to 2.88 ± 0.45 mg L^{-1} and AOX 11.9 ± 0.54 to 2.9 ± 0.25 mg L^{-1} after 72 h of growth. From the Fig. 3, it was evident that maximum reduction of these parameters occurred in first 24 h (more than 50%), thereafter slow reduction rate was observed. The strain was able to reduce 64.10 (lignin), 88.55 (total phenol) and 75.63% (AOX) from the pulp and paper mill effluent after 72 h treatment. The phenolic compounds, lignin derivatives, and AOX present in pulp and paper mill effluent is derived from depolymerization of lignin and bleaching of pulp with chlorine (Chandra et al., 2009; Haq et al., 2016; Yadav and Chandra, 2015). AOX are low molecular weight organic halides, generated during bleaching is toxic due to their ability to bio-accumulate in the fatty tissues of higher organisms (Kumar et al., 2018; Savant et al., 2006). Chlorinated dioxins are also present in very low concentration within the AOX aggregate. There are reports on reduction of lignin, AOX and phenolics from the pulp and paper effluent treated by individual isolate or consortia (Table 2). Lignin and its derivatives are the major contaminants present in the pulp and paper effluent. The percent removal of lignin obtained in the present study has been found to be higher than previously reported work on *Serratia liquefaciens* (58%), *Bacillus* sp. (53%), *Brevibacillus parabravis* (53.8%), *B. agri* (37%) and *Penibacillus* (54%) (Haq et al., 2016; Hooda et al., 2015, 2018; Raj et al.,2007, 2014). Furthermore, lignin removal by bacterial consortia was also found to be lower compared to present study (Abhishek et al., 2017; Chandra et al., 2007, 2009; Chandra and Singh, 2012). The reduction in total phenol achieved after only 3 days of treatment by *Bacillus* sp. IITRDVM-5 was found to be higher than that obtained by a *Penibacillus* sp. culture supplemented with glucose, peptone and yeast extract after six days of treatment (Raj et al., 2014). Moreover, a higher percent reduction in total phenol (95%) was

Table 2
Comparative studies of bacterial mediated treatment of pulp and paper mill effluent.

Bacterial Strain	Reduction (%)							Co-substrate	Time	References
	Color	COD	BOD	TOC	Lignin	AOX	TP			
Klebsiella sp., *Alcaligens* sp. and *Cronobacter* sp.	55	72.3	91.1	–[a]		–	–	urea and DAP	14–18 h	Kumar et al. (2014)
Serratia liquefaciens	72	85	–	–	58	–	95	1.0% glucose and 0.5% peptone	57 h	Haq et al. (2016)
Bacillus subtilis and *Klebsiella pneumonia*	80	73	62	–	–	–	–	glucose 1% and peptone 0.25%	144 h	Yadav and Chandra (2015)
Citrobacter freundii and *Serratia marcescens*	50–55	67	–	61	65	–	–	1% glucose and 0.3% peptone	144 h	Abhishek et al. (2017)
Brevibacillus parabrevis	59	62	–	–	53.8	–	–	Ammonium sulphate and phosphoric acid as N and P	120 h	Hooda et al. (2018)
Pseudochrobactrum Glaciale, *Providencia rettgeri* and *Pantoea* sp.	96.02	91	92.59	–	–	–	–	1% dextrose and 0.5% peptone	86 h	Chandra and Singh (2012)
B.megaterium ETLB-1 and *Pseudomonas plecoglossicida* ETLB-3	89.7	79.0	–	–	–	–	–	1% dextrose and 0.5% peptone	168 h	Paliwal et al. (2015)
Bacillus subtilis and *Achromobacter xyloxidans*	70	81	–	–	–	–	–	Yeast extract, 5.0 g L^{-1} and Glucose, 5.0 g L^{-1}	168 h	Sharma et al. (2010)
Paenibacillus sp.	68	78	83	–	54	–	86	1.0% glucose, 0.2% peptone and 0.1% yeast extract	144 h	Raj et al. (2014)
Bacillus sp.	61	78	82	–	53	–	–	D-glucose, 10.0(g L^{-1})and peptone, 5.0 (g L^{-1})	144 h	Raj et al. (2007)
Brevibacillus agri	47	69	–	–	37	39	–	Ammonium sulphate and phosphoric acid as N and P	120 h	Hooda et al. (2015)
Bacillus cereus and *Serratia marcescens*	62	90	70	–	54	–	–	1.0% glucose 0.5% peptone	68 h	Chandra et al. (2009)
Paenibacillus sp., *A.aneurinilyticus* and *Bacillus* sp.	69	–	–	–	40	–	–	D-glucose, 10 g L^{-1} and peptone, 5 g L^{-1}	144 h	Chandra et al. (2007)
Citrobacter freundii and *Citrobacter* sp.	79	82	–	–	60	79	–	1% glucose and 0.3% peptone	144 h	Chandra and Abhishek (2011)
Pseudomonas aeruginosa, *P. aeruginosa* and *B. megaterium*	76	70	76	–	–	–	–	20% glucose and 20%sucrose	24 h	Tiku et al. (2010)
Bacillus sp. and *S. marcescens*	80	99	98	–	–	–	–	1% glucose and 0.5% peptone	68 h	Singh et al. (2008)
Bacillus sp. IITRDVM-5	73	90	93	82	64	75	88	Urea and DAP	72 h	Present work

[a] Not available.

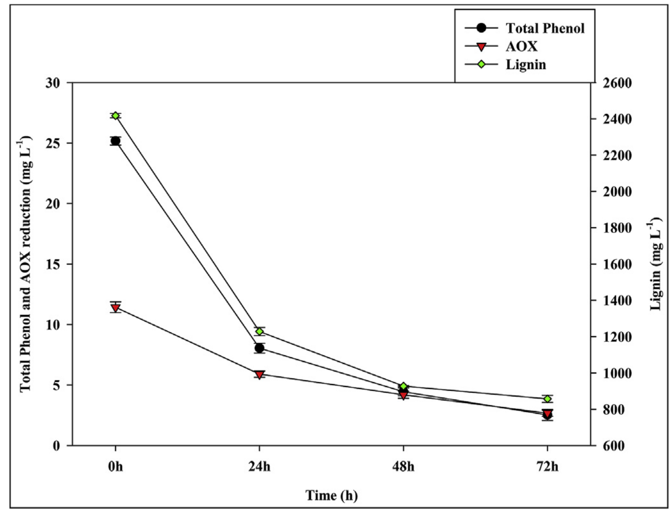

Fig. 3. Reduction of total phenol, lignin and AOX of pulp and paper mill effluent under optimized conditions by *Bacillus* sp. IITRDVM-5. The experiments were done in triplicates.

observed in a *Serratia liquefaciens* culture supplemented with glucose (1%) and peptone (0.5%) after six days of treatment (Haq et al., 2016). The AOX removal (75%) in the present study after 3 days of treatment was found to be much higher than that obtained by *Bravibacillus agri* (39%) after 5 days of treatment while comparable with consortia (79%) after 6 days of treatment (Hooda et al., 2015).

3.5. UV–vis spectroscopy analysis of pulp and paper effluent during treatment

The UV–Vis spectral scan of control and treated (24 h, 48 h and 72 h) pulp and paper mill effluent was done to identify the change in absorbance while degradation by *Bacillus* sp. IITRDVM-5. The UV–Vis scan of control and treated sample has been shown in Fig. 4. The two prominent absorption peaks can be observed around 225 and 280 nm. During the course of treatment, the absorbance of both the peaks decreased with time and the peaks almost disappeared after 72 h. The absorbance around this region has been observed for lignin and

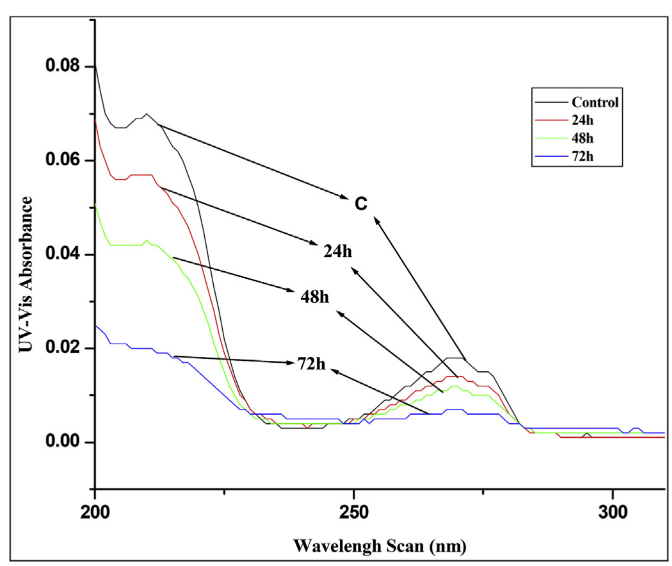

Fig. 4. UV–Vis spectroscopy analysis of pulp and paper mill effluent contamination degradation during the course of treatment under optimized condition by *Bacillus* sp. IITRDVM-5.

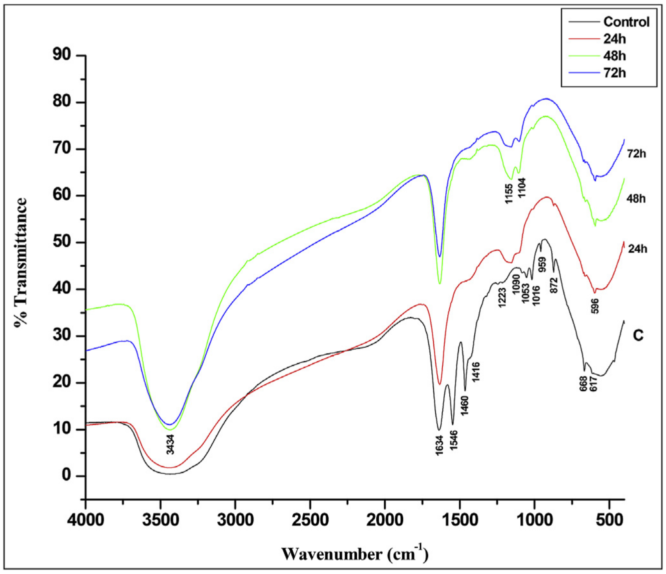

Fig. 5. FTIR spectra of pulp and paper mill effluent during the course of treatment under optimized conditions by *Bacillus* sp. IITRDVM-5.

chlorolignin derivatives (Chen et al., 2011; Kumar et al., 2015; Nie et al., 2014). Chen et al. (2011) developed an UV–Vis spectroscopy based method for the estimation of COD from the pulp and paper effluent and observed a similar absorption pattern. Compared to present study, a related UV–Vis spectrum was observed during the degradation of black liquor by white rot fungus *Trametes elegans* (Lara et al., 2003). The degradation of chlorophenols was studied spectrophotometrically in earlier study and the scan observed was in accordance to present study (Patel and Kumar, 2016). A similar UV–Vis spectra spectral pattern was observed during the kinetic study of a lignin model compounds and its chlorine dioxide derivative (Nie et al., 2014). The spectral analysis supported the degradation of the lignin and chlorolignin derivatives by *Bacillus* sp. IITRDVM-5.

3.6. FTIR analysis of pulp and paper mill effluent treatment

FTIR analysis of control and *Bacillus* sp. IITRDVM-5 treated effluent powder (24 h, 48 h and 72 h) was done qualitatively to identify the change in functional group composition during the course of degradation. FTIR spectra of control and treated sample powder have been shown in Fig. 5. There is loss of existing bands, reduction in intensity of bands and appearance of new bands were observed during the treatment. The band around 3500-3000 cm^{-1} was observed due to –OH stretching vibration of phenol, alcohols and organic acids present in lignin, cellulose and hemicelluloses (Zhang et al., 2018). The decrease

Table 3
GC-MS of ethyl acetate extract of control and *Bacillus* sp. IITRDVM-5 treated pulp and paper effluent.

S. No.	Retention time (RT)	Compound Name	Control (0 h)	Treated (72 h)
1.	7.66	3-chloro-3-trifluoromethyl-2thiabicyclo[2.2.1]hept-5-ene-2,2-dioxide	+	-
2.	7.84	1,2-di(2-pyrrolidinyl)-1,2-ethanedione	-	+
3.	7.95	3-hexadecene	-	+
4.	8.09	n-tridecane	-	+
5.	8.30	1,2,4,5-tetraethylcyclohexane	-	+
6.	9.76	sulfurous acid, 2-ethylhexyl isohexyl ester	-	+
7.	10.16	2,6-ditert-butyl-4-methylphenol	-	+
8.	10.92	(1,2-dimethylpropyl) cyclohexane	-	+
9.	11.35	eicosyl ethyl ether	-	+
10.	12.04	1-pentadecene	-	+
11.	12.07	phenol, 2-(aminomethyl)-	+	-
12.	12.50	(2-cyclohexyl-1-methylpropyl)cyclohexane	-	+
13.	13.05	methyl (3-oxo-2-[(2z)-2-pentenyl]cyclopentyl)acetate	-	+
14.	13.65	2-cyclohexyl-3-isopropyl-4-penten-2-ol	+	-
15.	15.67	2-hexyl-1-decanol	-	+
16.	16.09	4-chlorobenzoic acid, 4-hexadecyl ester	+	-
17.	16.13	2-ethyl-1-decene	-	+
18.	16.28	1-heptadecene	-	+
19.	17.21	hexanedioic acid, 3-methyl-, bis(trimethylsilyl) ester	-	+
20.	17.35	malonic acid, 2-butyl tetradecyl ester	-	+
21.	17.63	4-cyclohexyl-1-butanol	-	+
22.	17.76	n-nonylcyclohexane	-	+
23.	18.07	4-ethylbenzoic acid, 2-ethylcyclohexyl ester	+	-
24.	19.41	1,2-benzenedicarboxylic acid, dibutyl ester	+	+
25.	19.57	1-trimethylsiloxyhexadecane	-	+
26.	20.15	3,3,4-trimethyl-1-decene	-	+
27.	20.28	1-nonadecene	-	+
28.	21.44	4-propyl methoxybenzene	+	-
29.	22.88	succinic acid, dodec-2-en-1-yl 4-bromo-2-methoxyphenyl ester	+	-
30.	23.26	octadecoxy-trimethylsilane	-	+
31.	23.70	2-o-benzylpentofuranose	+	-
32.	23.98	1-nonadecene	-	+
33.	24.10	7-octen-2-ol, 2-methyl-6-methylene-	-	+
34.	25.84	chloroacetic acid, 4-hexadecyl ester	+	-
35.	25.92	*trans*-3,3,5-trimethylcyclohexyl chloroacetate	+	-
36.	27.40	docosyl alcohol	-	+
37.	27.95	2-(4′-chlorophenyl)-3-(4′-methylphenyl)butane	+	-
38.	28.06	3-benzyloxy-1,2-diacetyl-1,2-propanediol	+	-
39.	28.94	benzyl (phenyltelluro)formate	+	-
40.	29.52	1,2-benzenedicarboxylic acid	+	+
41.	29.71	2-(azidomethyl)-5-phenyltetrahydrofuran	+	-

(+) Present; **(−)** Absent.

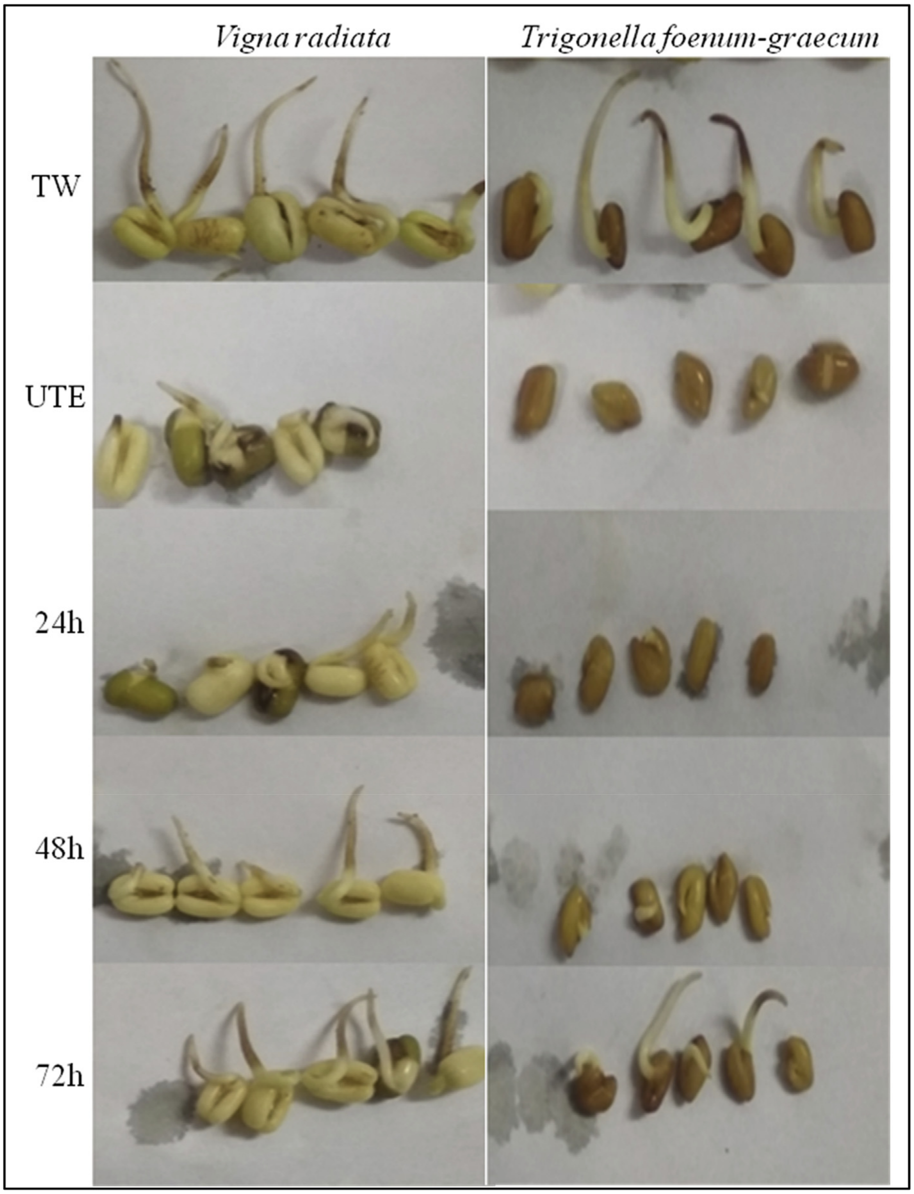

Fig. 6. Toxicity analysis of pulp and paper mill effluent through *V. radiata* and *T. foenum-graecum* seed germination.

in absorbance at 3500-3000 cm^{-1} during treatment indicates the degradation of phenols and other polymeric derivatives degradation by *Bacillus* sp. IITRDVM-5. There are various changes observed in the fingerprint region (1800-600 cm^{-1}) during the of degradation process. The band around 1800-1400 cm^{-1} i.e., 1634, 1546, 1460 and 1416 cm^{-1} denotes the aromatic skeletal vibration, aromatic C–O and C=O moieties, O–H conjugated C=O stretching, asymmetric bending in, methoxy in-plane bending vibration, –OCH_3 deformation, methyl and methylene group (Chattaraj et al., 2016; Kumar et al., 2015; Yadav and Chandra, 2018). During the treatment, reduction in 1634 cm^{-1}intensity was observed and removal of band at 1546, 1460 and 1416 cm^{-1} was observed after 24 h of treatment. The band 1223, 1090, 1053, 1016, 959 and 872 cm^{-1} signify the hemicellulose and cellulose moieties (C–O stretch O–H band and carboxylic acid) present in effluent. These bands were undetectable after 24 h of treatment. The band at 668 and 617 cm^{-1} showed the presence of halogenated compounds present in the effluent (Nie et al., 2015; Veluchamy and Kalamdhad, 2017). Complete removal of band at 617 cm^{-1} and reduction in intensity of band at 668 cm^{-1} was observed during the degradation process. The emergence of new bands at 1155, 1104 and 596 cm^{-1} were observed while degradation. These new bands indicate rearrangement and degradation hemicelluloses and lignin derivatives and alkyl halides throughout the degradation process by *Bacillus* sp. IITRDVM-5.

3.7. GC-MS analysis of metabolites present in pulp and paper effluent

The GC-MS analysis of ethyl acetate extracted control and treated sample was performed to identify the degradation or transformation of compounds present in the pulp and paper effluent by *Bacillus* sp. IITRDVM-5. The total ion chromatogram (TIC) of compounds present in control and treated sample has been shown in Figs. S2A and S2B and their identification is represented in Table 3. The loss or reduction in intensity of peaks from control sample and emergence of new peaks can be observed after treatment. The phenolic derivatives i.e., phenol, 2-(aminomethyl)-(RT 12.05), 4-ethylbenzoic acid, 2-ethylcyclohexyl ester (RT 18.07), 1,2-benzenedicarboxylic acid, dibutyl ester (RT 19.41), 4-propyl methoxybenzene (RT 21.44), 2-o-benzylpentofuranose (RT 23.70), 3-benzyloxy-1,2-diacetyl-1,2-propanediol (RT 28.06), benzyl (phenyltelluro)formate (RT 28.94) and 2-(azidomethyl)-5-

Table 4
Toxicity analysis of paper mill effluent by *V. radiata* and *T. foenum-graecum* seed germination.

Parameters	Radical Length (cm)	Germination Percentage (%)	Phytotoxicity Percentage (%)	Tolerance Index	Relative Toxicity (%)	Radical Growth Inhibition (cm)	Vigour Index
V. radiata							
TW	2.44 ± 0.24	100 ± 0.00	00	00	00	00	488.0 ± 48.6
UTE	1.013 ± 0.14	73.3 ± 11.5	58.5 ± 2.29	0.4 ± 0.02	26.6 ± 10.18	1.25 ± 0.17	250.38 ± 10.5
24 h	0.988 ± 0.06	93.333 ± 11.5	59.4 ± 1.6	0.4 ± 0.016	6.6 ± 11.5	1.45 ± 0.18	321.46 ± 61.2
48 h	1.393 ± 0.13	100 ± 0.00	42.8 ± 3.7	0.57 ± 0.037	00	1.04 ± 0.16	383.3 ± 35.5
72 h	2.026 ± 0.05	100 ± 0.00	16.3 ± 9.6	0.8 ± 0.09	00	0.4 ± 0.28	446.6 ± 20.2
T. foenum-graecum							
TW	1.4 ± 0.105	100 ± 0.00	00	00	00	00	280.0 ± 21.16
UTE	0.25 ± 0.15	26.6 ± 11.5	82.46 ± 9.5	0.17 ± 0.09	73.3 ± 11.5	1.15 ± 0.098	43.4 ± 17.36
24 h	0.24 ± 0.05	60.0 ± 20.0	82.4 ± 4.46	0.17 ± 0.04	40.0 ± 20.0	1.22 ± 0.08	99.06 ± 34.015
48 h	0.32 ± 0.055	73.3 ± 11.5	76.7 ± 4.9	0.23 ± 0.049	46.6 ± 30.5	1.07 ± 0.13	121.86 ± 14.45
72 h	0.99 ± 0.1	93.3 ± 11.5	28.7 ± 12.06	0.7 ± 0.1	6.6 ± 11.5	0.4 ± 0.2	222.86 ± 25.74

TW = Tap water, UTE = Untreated effluent.

phenyltetrahydrofuran (RT 29.71) was identified in the control sample. The detection of 2-o-benzylpentofuranose and 2-(azidomethyl)-5-phenyltetrahydrofuran in the control sample indicates cleavage of lignin-henicellulose linkage during biomass pretreatment. Furthermore, the presence of halogenated phenolic derivatives such as 4-chlorobenzoic acid, 4-hexadecyl ester (RT 16.09), succinic acid, dodec-2-en-1-yl 4-bromo-2-methoxyphenyl ester (RT 22.88) and 2-(4′-chlorophenyl)-3-(4′-methylphenyl)butane (RT 27.95) was detected in the control sample. The other chlorinated derivatives observed in the control sample are 3-chloro-3-trifluoromethyl-2thiabicyclo[2.2.1]hept-5-ene-2,2-dioxide (RT 7.66), chloroacetic acid, 4-hexadecyl ester (RT 25.84) and *trans*-3,3,5-trimethylcyclohexyl chloroacetate (RT 25.92) was also observed in control. The detection of phenolic, cholorophenolic and chlorolignin derivatives in control sample are generated from the lignin during pretreatment and bleaching of lignocellulosic biomass in pulp and paper mill (Yadav and Chandra, 2018). The detection of very few or transformed chloro derivatives in the treated sample indicate bacterial mediated dehalogenation of halophenols and halolignin derivatives (Chandra et al., 2009). After treatment all the phenolic and phenol halide derivatives were not detected after treatment. Several new peaks of small molecular weight compounds were detected in treated sample. The compounds such as 3-hexadecene, n-tridecane, 1,2,4,5-tetraethylcyclohexane, sulfurous acid, 2-ethylhexyl isohexyl ester, eicosyl ethyl ether, 1-pentadecene, (2-cyclohexyl-1-methylpropyl) cyclohexane and other cyclo and simple derivatives were observed. The low molecular weight simple compound produced after treatment were also reported earlier (Chandra and Singh, 2012; Raj et al., 2007; Yadav and Chandra, 2015). The presence of 1,2-benzenedicarboxylic acid (RT 29.52) was identified in control and treated sample and 2,6-ditert-butyl-4-methylphenol (RT 0.16) only in treated sample. During lignin degradation these two intermediates are generated and were detected in previous studies (Chandra et al., 2007; Kumar et al., 2015; Yadav and Chandra, 2015). The result indicates that *Bacillus* sp. IITRDVM-5 degraded or transformed both phenolic as well as phenol halide derivatives from the effluent.

3.8. Toxicity analysis of pulp and paper mill effluent

The toxicity of untreated effluent and *Bacillus* sp. IITRDVM-5 treated effluent was approved by the germination of *V. radiata* and *T. foenum-graecum* seeds in petri plate on day 3 of observation (Fig. 6). This test indicated the toxicity removal on the basis of seed germination or suppression and early seed growth. A comparative inspection demonstrated the growth of *V. radiata* seeds was more rapid as compared to *T. foenum-graecum* seeds. The various parameters for *V. radiata* and *T. foenum-graecum* were observed with raw and treated effluent. *V. radiata* showed GP (73.33 ± 11.54, 100 ± 0.00); PP (58.54 ± 2.29, 16.302 ± 9.66); TI (0.41 ± 0.02, 0.83 ± 0.09); RT (26.66 ± 10.18, 00); RGI (1.25 ± 0.17, 0.413 ± 0.28); VI (250.38 ± 10.57, 446.66 ± 20.23) whereas *T. foenum-graecum* GP (26.66 ± 11.54, 93.33 ± 11.54); PP (82.46 ± 9.55, 28.71 ± 12.06); TI (0.17 ± 0.09, 0.71 ± 0.12); RT (73.33 ± 11.54, 6.66 ± 11.54); RGI (1.15 ± 0.09, 0.41 ± 0.20) and VI (43.46 ± 17.36, 222.86 ± 25.74), after 72 h treatment. These parameters were also estimated for 24 h and 48 h treated effluent, and the results have been summarized in Table 4. The toxicity analysis of wastewater through seeds of both plants has been previously reported by the various researchers and the results were in tune with the previous studies (Bharagava and Chandra, 2010; Chandra and Kumar, 2017; Kumar and Chopra, 2012; Raj et al., 2014).

4. Conclusion

The novel isolate *Bacillus* sp. IITRDVM-5 was found to degrade variety of contaminants present in pulp and paper mill effluent. The sequential batch treatment of 100% filter-sterilized pulp and paper effluent by *Bacillus* sp. IITRDVM-5 confirmed the biodegradation and decolorization of effluent with. During the treatment, no addition carbon sources and buffer media was used. The bacterial mediated degradation of phenol, lignin and dehalogenation of contaminants was observed. UV–Vis spectral scan, FTIR and GC-MS analysis confirmed the degradation of pollutant present into the pulp and papermill effluent. Toxicity assessment using *V. radiata* and *T. foenum-graecum* seeds confirmed the detoxification of effluent after bacterial treatment. Therefore, *Bacillus* sp. IITRDVM-5 can be used as promising strain in treatment of biorefractory toxic compounds present in pulp and paper mill effluent.

Conflicts of interest

The authors declare that there is no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101232.

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