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Degradation of kraft lignin and simultaneous decolorization of pulp and paper mill wastewater by bacillus spp

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Abstract
The characteristics of effluent revealed that it was dark brown in color with 1692 PtCo CU having high chemical oxygen demand values, biological oxygen demand, slightly alkaline pH and contained high load of inorganic and organic constituents, well above the prescribed standards. This study gives out the degradation and decolorization of black liquor by potential bacterium isolated from timber soil sample. The isolate was identified as *Bacillus velezensis* based on 16s rRNA gene sequencing and biochemical analysis. This bacterium was able to reduce color (28.83%), liginin (27.15%), BOD (96.21%), COD (80.71%) and total phenol (15.55%) from pulp and paper mill wastewater after 168h of treatment at 40 ºC, pH 7.6. During the course of degradation significant change in functional group composition was found through FTIR analysis. The degradation and decolorization analysis indicated potential application of the isolate *Bacillus velezensis* in treatment of lignin containing pollutants and Kraft lignin (KL) valorization.

Keywords: *Bacillus velezensis*, decolorization and degradation, pulp and paper mill wastewater

Introduction

The presence of organic compounds (liquid and solid waste) in the paper industry has become a global trepidation due to their enormous toxicological impact on biotic and abiotic constituents. Pulp and Paper mill industry effluent has high amount of halides, lignin derivatives chlorophenols that are generated in the bleaching section. The aquatic living community has been affected due to their 50 physicochemical properties (bioaccumulating higher persistence and carcinogenic substances) present in the food web (Kumar *et al*., 2018; Singh *et al*., 2008) [7, 10]. For the treatment of these contaminants, various physical and chemical conventional methods were adapted but they require high energy or generation of free radicals (Kumar *et al*., 2018; Toczyłowska-Mamińska, 2017). Aerobic process was adopted for Industrial effluent treatment, which was economical over the other management strategies. The complex characteristics of deleterious waste present in the effluent depend upon the chemical compositions and cross-linking due to covalent and non-covalent forces (Sanchez, 2009) [4, 9]. So, there is a strong need to establish a process that could solve these problems and the sequence batch microbial process using bacterial strain is one of them. By using microorganisms or their metabolic products in the biodegradation of organic compounds (especially environmental pollutants) has been advanced significantly during the past three decades because of advancement in biotechnology. It has been found that huge numbers of microbes cohabit and undergo different forms of interactions in almost all natural soil environment. Many biodegradable synthetic and natural organic chemicals are readily biodegradable in the environment by organisms that require enzymes and metabolic pathways. Such organisms are usually isolated from different habitats by researchers. Biodegradation of materials involves secretion of extracellular enzymes to degrade the substrate or uptake via transport system, initial proximity, allowing absorption or physical access to the substrate, followed by intracellular metabolism. studied Bioremediation of wastewater by ligninolytic *Serratia liquifaciens* was studied and found to reduce COD (85%), color (72%), lignin (58%) and phenol up to (95%) of real effluent (Haq *et al*., 2016) [5]. axenic and co culture conditions *Klebsiella pneumoniae* and *Bacillus subtilis* were applied for the treatment of effluent (discharged from rayon grade pulp industry). Maximum decolorization (80%) and reduction of COD (73%) and BOD (62%) were observed by consartium than in axenic condition (Yadav and Chandra, 2015) [12]. Kumar *et al*. (2020) studied treatment of pulp and paper mill effluent by bacteria - *Pseudomonas putida*. The strain was efficient for utilization of phenol & cresol, co- metabolism of chlorinated compound and decolonization. After 24 hr of incubation, the removal efficiency of these three strains and their combinations for color, biological oxygen demand and chemical oxygen demand varied from 40% to 45%, 35% to 42% and 30% to 40%, respectively. Use of paper mill sludge and sewage sludge powder as phosphorus and nitrogen sources with bacterial consortium (*Bacillus* sp. *Pseudomonas sp.* and *Pseudomonas stutzeri* were studied for the treatment of paper industry wastewater. The maximum reductions in total organic carbon (TOC), chemical oxygen demand (COD), biochemical oxygen demand (BOD 5day) and color at PMSP 3.16 g/500 mL were 79.7, 78.3, 83.2, 33 and 73.9%, whereas at SWSP 1.392 g/500 mL were 72.6, 71.4, 76.1 and 69.3%, respectively compared to their respective controls (Sonkar *et al*., 2020). Microbial enzymes have shown a promising future for bioremediation of effluents related to the paper mill effluent. Pentachlorophenol therefore it needs to be removed from the environment because it is extremely

hazardous to living cells. Microorganisms (fungi and bacteria) have the potential to degrade phenolic compounds e.g. Bacillus stearothermiphilus, Pseudomonas putida, Coricus versicolor, Sphingomonas chlorophenol, Fusarium sp, Bacillus subtilis and P. aeroginosa (Adamu, 2020). Hence, the aim of the present study is to investigate a latent bacterial culture capable of decolorizing and detoxifying toxicological load for safe disposal.

Material and Methods

1. Sampling of effluent

In sterile plastic containers the effluent sample was collected from the discharge point of pulp and paper mill (Madhya Bharat pulp and paper mill, Champa, Raipur, Chhattisgarh, India). This was transported with the help of ice to the laboratory and stored at 4 °C for further analysis (Garg *et al*., 2012).

2. Bacterial culture and maintenance

A previously isolated, screened and identified bacterial culture *B velezensis* was employed in this study. The bacterium was screened to detect enzyme which degrade lignin (lignin Peroxidase (LiP), Peroxidase (MnP), Manganese and laccase). The strain was subcultured and preserved on NAM slants and stored at 4 °C.

3. Preparation of inoculum

In 100 ml sterilized nutrient agar medium the bacterium inoculum was prepared by transferring a loopful of culture and incubated at 30±1 °Cfor 24 h in an incubator shaker. The inoculum size used for decolorization and degradation studies was 5 (v/v) of *B. velezensis* containing 100 X 10^5 CFU/ml.

4 screening for KL decolorization and degradation

The bacterial strain was qualitatively and quantitatively screened for the presence of ligninolytic enzymes i.e. MnP, LiP and laccase. Decolorization of lignin mimic dyes was assessed in agar plates (Tian *et al*., 2016).

5 Culture condition for wastewater treatment by RSM (Predesigned CCD modeling)

The decolorization and degradation behavior of KL using the most potential strain was determined by inculation of effluent (10%) in the optimized MS medium. 5% (v/v) overnight grown bacterial suspension (inoculums size 100 X 10^5 cells) were inoculated aseptically In axenic condition (Chandra and Abhishek, 2011) [2]. The uninoculated and inoculated samples were incubated at 40◦C for 7 days.

6 Analytical determinations

The uninoculated and inoculated samples were submitted to IITR (Lucknow) for physicochemical analysis. Samples was withdrawn for the analysis of color, lignin, bacterial growth, pH, BOD, COD, TOC and total phenol. Physicocemical parameters parameters were determined as per standard methods of APHA (1999).

6.1 Biological oxygen demand (BOD)

BOD of the effluent was determined by 5 days test as per standard method of water and waste water analysis (APHA, 1999). In this method, airtight BOD bottles were filled with sample to overflowing and incubating at 20± 1ºC for 5 days. The incubation period in tropical and subtropical belts, where the rate of metabolic activities and temperature are higher, should be at 27˚ C for three days (BOD, 27˚C). To avoid oxygen stress in the contaminated water samples, it is necessary to oxygenate/aerate/as dilute to water sample with BOD free water. Titrimetric azide modification method was used to measure BOD of sample. Before and after of incubation Dissolved oxygen concentration was measured and the BOD was calculated from the difference between initial and final DO.

6.2 Chemical oxygen demand (COD)

The COD is used as a measure of oxygen equivalent to the quantity of oxidant consumed in a contaminated sample under specific condition of oxidizing agent, temperature and time. The COD of wastewater sample was determined by open reflux method as described in APHA (1999). Small portion of wastewater was taken and diluted to 50.0 ml with distilled water. 1g of $HgSO_4$ was added and dissolved by adding 5 to 10 ml sulfuric acid reagent. The reaction mixture was cooled with subsequent addition of $K_2Cr_2O_7$ (25 ml). About 70 to 75 ml of sulfuric solution was added in the sample and refluxed for 2 h. After refluxing the sample mixture was diluted to about twice its volume with distilled water. The excess $K_2Cr_2O_7$ was titrated with 0.25 N standard ferrous ammonium sulfate (FAS) solution using 2-3 drops of ferroin indicator. The first sharp color change from blue green to reddish brown was considered as end point. For blank sample, 50.0 ml of distilled water was titrated in the same way (APHA, 1992):

6.3 Total Phenol

A colored antipyrine dye in the presence of potassium ferricyanide was formed when phenols (Steam distillable) react with 4-aminoantipyrine at pH 7.9±0.1. About 250.0 ml of waste water (pH 4.0) was taken for distillation. After distillation of 225.0 ml of sample, 25.0 ml of warm water (distilled) was added, and the distillation was continued till the final distilled volume reached to 250.0 ml. A series of 100.0 ml standards containing 0.1, 0.2, 0.3, 0.4 and 0.5 mg phenol was prepared from stock solution. For blank, 100.0 ml distilled water was taken. The sample (100. 0 ml distillate), standards and blank were treated by addition of 2.5 ml NH_4OH (0.5 N) solution and pH (7.9±0.1) was adjusted with phosphate buffer. After that 4-aminoantipyrine solution (1.0 ml), potassium ferricyanide solution (1.0 ml) were added and the sample mixture was left for fifteen minutes. The absorbance of standards and sample against the blank at 500 nm was recorded for plotting calibration curve (APHA, 1999).

6.4 Total organic carbon (TOC)

Control and degraded samples of effluent were sent to Water Testing Laboratory (Centre for Ground Water Recharge), Raipur, Chhattisgarh for analysing the sample.

6.5 Color measurement

To remove the biomass inoculated and uninoculated (control) were centrifuged (at 8000 rpm for 30 min). Supernatant (1 mL) was diluted (by adding phosphate buffer (3 mL) pH 7.6) and at 465 nm absorbance was measured using UV–Vis Spectrophotometer (Systronics, India). The color unit was calculated by:

$CU\ (PtCo) = 500 \times (A_2/A_1)$

Where,
A_1 = absorbance of 500-CU platinum cobalt standard solution ($A4_{65}$ = 0.132)
A_2 = absorbance of the sample.
Color removal % = (A–B)/A X 100
Where
A = color unit (uninoculated sample)
B = color unit (inoculated samples)

6.6 Lignin estimation
Pearl and Benson method was used to estimate lignin content. The inoculated (Bacterial degraded samples) and control (uninoculated) were centrifuged (at 8000 rpm for 30 min) to remove the biomass. CH_3COOH (10 %) one ml and $NaNO_2$ one ml were added to 50 mL of sample and kept it for 15 min. NH_4OH (2 ml) was added and then reaction mixture was left for 5 min. CH_3COOH (10 %) one ml and NH_4OH two ml were added to 50 mL distilled water for the blank. $NaNO_2$ (10 %) one ml was added After 15 min. Absorbance was measured at 430 nm. Lignin content was determined by following formula
Lignin (ppm) = Absorbance/0.00024

7. Analytical methods
FTIR analysis
To assess the decolorization and degradation of wastewater through axenic culture, FTIR analysis was done. Experiments were conducted in cotton plugged conical flask (250 ml) MS-effluent media. In axenic condition, 5% (v/v) overnight grown bacterial suspension was inoculated aseptically to flask. In mixed culture condition, 1.7% (v/v) of inoculums from each culture was added to obtain inoculums size 5% (v/v). The control and inoculated flasks were incubated at 40˚C for 7 days. Supernatant was analyzed by Varian 7000 Fourier transform infrared spectroscopy (FTIR) spectrometer at attenuated total reflectance mode in the spectra range 4000 to 500 cm^{-1} and the spectra was recorded using 64 scans per sample (Madan *et al*., 2015). Analysis was facilitated by National Institute of Technology, Raipur, Chhattisgarh, India.

Results and discussion
1 Effluent treatment by axenic culture using predesigned CCD modelling of RSM
The physicochemical analysis of pulp and paper mill effluent used in this study are presented in the Table 1. The color and lignin content in the effluent recorded as 1583 mg/Land 1692 CU.the presence of carbohydrate and organic acids were found to indication of high BOD. Lignin present in effluent (high molecular weight) was found to cause high COD and color in pulping effluent. Lignin content do not cause high BOD. The pH of effluent was recoded as 8.74 and it was adjusted to pH 7.6 for experiments.
At optimized condition KL (600 mg/L in MSM) reduced to 56.16% and color reduced to 40.39% at pH 7.6. Therefore, this bacterial strain was used for the treating the effluent for lignin degradation and to examine their potential to decolorize the final effluent which are normally discharged into the environment. The decolorization and degradation experiments were conducted in 100 ml, MS-effluent amended with appropriate amount of carbon and nitrogen source by inoculating single and mixed bacterial strains and incubated at 40˚C for 7 days. The bacterial growth, lignin content and reduction of color during the sequence of water treatment treatment revealing that primarily bacterium attained good growth and remained lively for up to 5 days. After that they started slightly to drop, however the reduction of color and lignin was started after 2 days of incubation. It directs that bacterium primarily utilized growth supportive substrates and subsequently chromophoric componds, thereby reduces lignin content and color in the effluent. The ANOVA result showed that there was significant color reduction (28,83 $F= 10.54$ $p<0.05$) and lignin (27.95% $F=88.40$ $p<0.01$) within 7 days of incubation. The results revealed that the reduction of color from the effluent depends on the reduction of lignin content. The initial color unit and lignin content represented as 1692 cu and 1583 mg/L, but it was reduced to 1285 cu and 1051 mg/L during the degradation of effluent by *bacillus velezensis.* Drop in pH was noticed, the preliminary pH value 8.74 got decreased to 8.31 on the 7 day. The cause behind such fluctuation in pH can be directly associated with bacterial enzyme activity during the conversion of complex organic compound chlorinated organic and lignin compounds present in the effluent.
The concentration of chlorinated organic and lignin compounds present in the effluent is responsible for COD, BOD and toxicity. The reduction in this strain reduced BOD (96.21 %), COD (80.71%) and total phenol (15.55 %). The highest reduction in the level of BOD was observed. The decline of COD and total phenols during these experiments can be considered as the result of degradation of lignin and chlorinated organic compound present the effluent.
This effluent causes severe aquatic and soil contamination because of its dark color and high pollution load. They reduce the DO level in aquatic system which unpleasantly affects the flora and fauna of the water body (Garg and tripathi, 2011) [3]. Anaerobic oxidation results putrefying odour of the receiving water body. The BOD is an important indicator of organic matter which shows the presence of easily biodegradable compound such as organic acids and carbohydrates. Along with the BOD, TOC and COD are particularly important for characterization of industrial effluent and treatment. Thus, the untreated effluent used in the study is dangerous to discharge into water bodies and onto land. In the present study, experiments were designed in laboratory scale to address the above issue. The treatment of pulp and paper mill effluent in the laboratory scale was attempted by axenic and mixed bacterial cultures. Treatment in axenic condition was performed by *B. velezensis* and in mixed condition by *B. velezensis*, *B. aryabhatti* and *B. subtilis*. The optimum conditions of our previous study such as pH, carbon, temperature, nitrogen at 168 h designed by CCD modeling of RSM were used for the treatment of pulp and paper mill effluent media to analyze its color, lignin,

COD, BOD, TOC and Phenol. Singh *et al*. (2007) [11] studied sequential anaerobic and aerobic treatment in two step bioreactors for removal of color in the pulp and paper mill effluent. Reduction of color and lignin was 59%, 71% by *Microbrevis luteum*. The potential bacterial strains *Klebsiella pneumoniae* and *Bacillus subtilis* were isolated screened and applied to analyse degradability of kraft lignin in axenic and mixed culture conditions. Mixed culture efficiently decolorizes KL (80%) (Yadav and Chandra, 2015) [12]. Another bacterial strain isolated from pulp and paper mill effluent and identified as *Serratia liquefaciean* for detoxification of pulp and paper mill effluent. This strain significantly reduced color by 72% and lignin by 58% (Haq, 2016) [5]. During the treatment process of pulp and paper mill effluent, variation in pH have also been found by all these bacterial strains.

Table 1: Physicochemical parameters of effluent discharged from pulp and paper mill

Sample no	Parameters	Wash machine			Result
		Inland surface water	Public Sewer	Land for irrigation	
1	pH	5.5-9.0	5.5-9.0	5.5-9.0	8.74
2	BOD (mg/L)	30	350	100	14284.0
3	COD (mg/L)	250	NS	NS	34680.0
4	Total Solids (mg/L)	NS	NS	NS	25974.0
5	Dissolved Solids (mg/L)	NS	NS	NS	23492.0
6	Suspended Solids (mg/L)	100	600	200	2482.0
7	Fixed Solids (mg/L)	NS	NS	NS	19162.0
8	Volatile Solids (mg/L)	NS	NS	NS	3812.0
9	Chloride (mg/L)	NS	NS	NS	15.78
10	Phosphate as P (mg/L)	5.0	NS	NS	8.96
11	Sulphate as So4-2 (mg/L)	NS	NS	NS	962.6
12	Fluoride (mg/L)	2.0	1.5	NS	1.74
13	Oil & Grease (mg/L)	10	20	10	1246.0
14	Nitrates as NO3 (mg/L)	10	NS	NS	13.8
15	Total Nitrogen (mg/L)	NS	NS	NS	35.88
16	Cyanide (mg/L)	0.2	2.0	0.2	.089
17	Sulphide (mg/L)	2.0	NS	NS	3.62

Table 2: Physicochemical parameters of effluent before and after treatment by axenic and consortium

Physicochemical parameters	Physicochemical value of untreated effluent	Physicochemical value of treated effluent
		Axenic (B. *velezensis*)
Ph	8.74	8.31
Colour	1692 CU	1285 CU
Lignin	1583 mg/L	1051 mg/L
BOD	14284.0 mg/L	540 mg/L
COD	34680.0 mg/L	6688 mg/L
TOC	1639 mg/L	1384.4 mg/L
Phenol	31.76 mg/L	14.64 mg/L

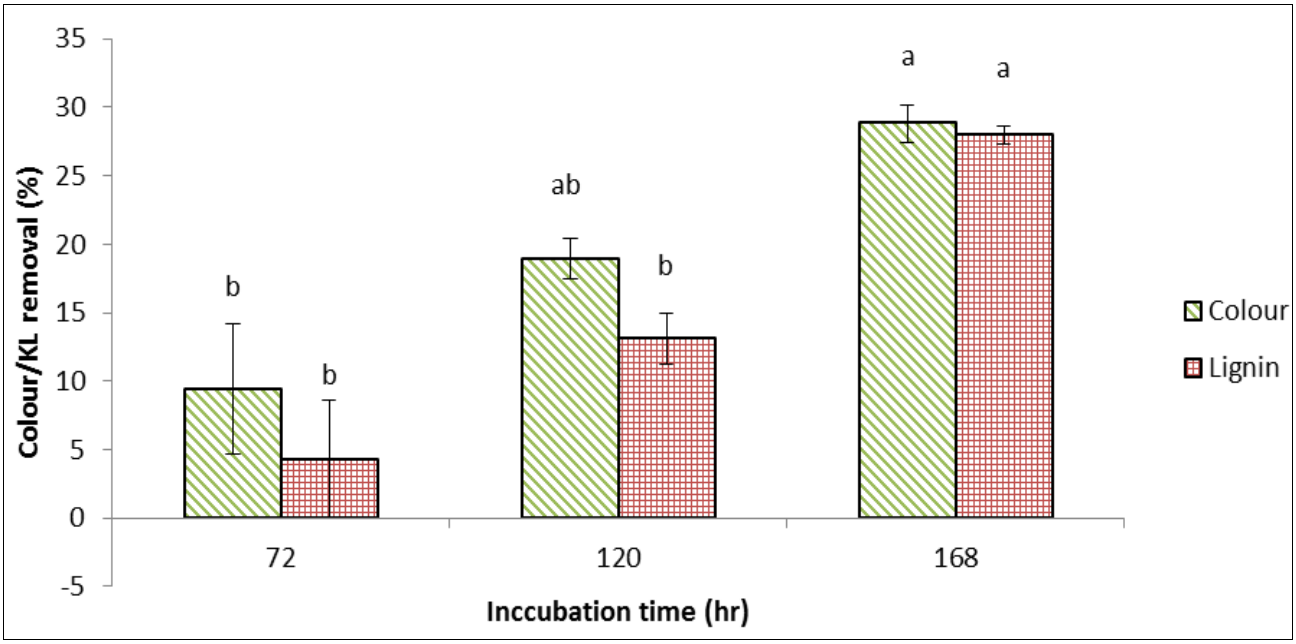

Fig 1: Lignin degradation of decolorization of pulp and paper mill effluent by *Bacillus velezensis*

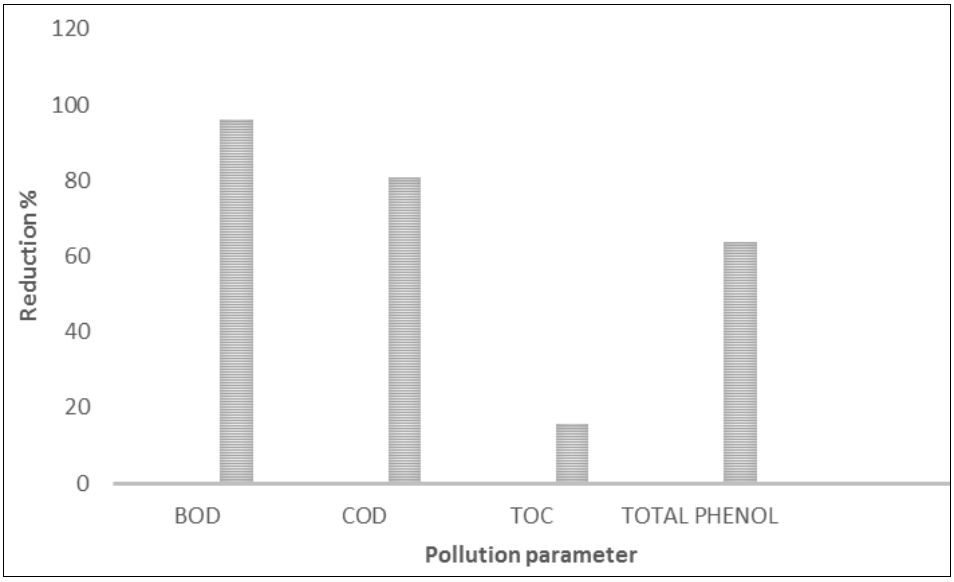

Fig 2: Reduction in pollution parameter of waste water by *Bacillus velezensis*

2 FTIR analysis

To investigate the qualitative changes (changes in functional group composition) that occurred after bacterial treatment of KL, the uninoculatd (control) and bacterial treated effluent samples were taken and FTIR analysis was carried out. FTIR spectra are shown in Fig. 3 and the assignment of functional group was done according to previous studied (Jahan *et al*., 2007: Hage *et al*., 2009: Liu *et al*., 2014) [4, 6]. In control sample the absorption band at around 3500-3000 and 2073 cm^{-1} was present. In control band at 2073 was disappeared during the course of treatment by axenic culture. In control band at 1639 cm^{-1} was assigned to aromatic skeletal vibrations and 1640 $cm^{-1}$was found after axenic treatment.

Table 3: Assignment of characteristics bands of the infrared spectra of the lignin samples studied in control, B. velezensis and consortium treated effluents samples

Functional group assignment	Control (cm−1)	7-day treated By *B. velezensis* (cm^{-1})
O-H stretching	3450	3456
C–H stretch in aromatic –OMe and side chain –CH_2 and CH_3	2073	-
C–H stretch in methyl		
C=O stretching of aliphatic acetate		
C=O stretch in unconjugated ketone, carbonyl, and ester groups		
O–H and conjugated carbonyl stretching	1636	1640

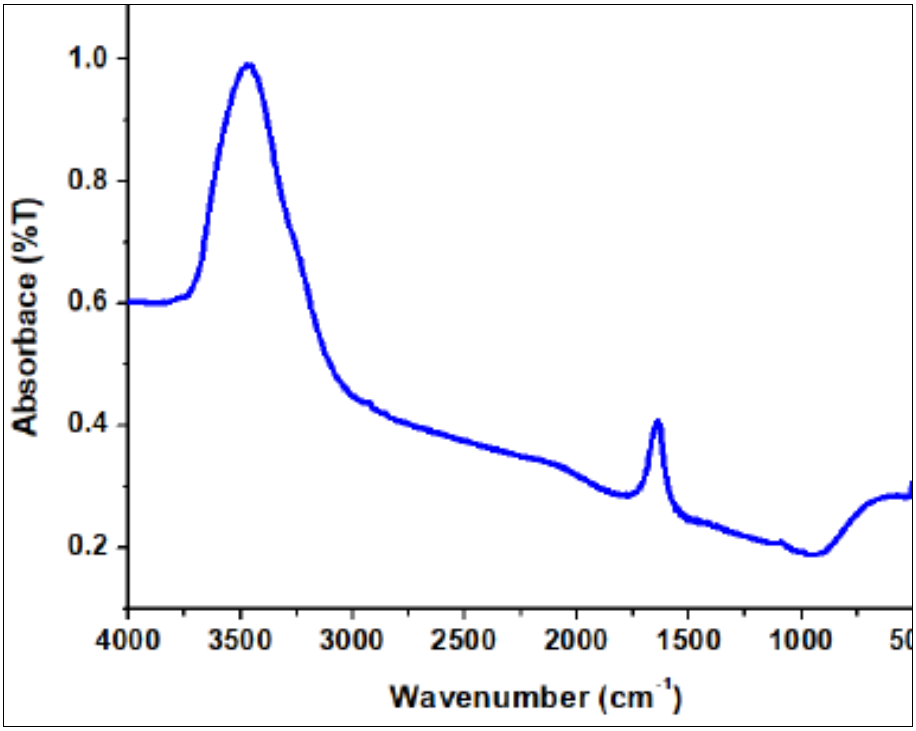

Fig 3: FTIR spectra of *B. velezensis* treated effluent after 7 days of incubation.

Conclusions

Bacillus velezensis, a potential ligninolytic strain was isolated from soil contaminated site. This strain was applied for the remediation of paper and pulp wastewater. It efficiently reduced the pollution load of pulp and paper mill effluent was decline when treated with *Bacillus* velezensis. Toxicity of effluent was also reduced significantly. The bacterium was capable of producing ligninolytic enzymes i.e. LiP, MnP and Laccase while growing in L-MSM. The decolorization and reduction of other pollution loas was observed as the bacteria increases. It can be concluded that effluent decolorization and lignin degradation using Bacillus velezensis is an efficient and economic process capable of removing color (28.83%), liginin (27.15%), BOD (96.21%), COD (80.71%) and total phenol (15.55%) from pulp and paper mill waste water after 168h of treatment. The bacterial cultures were able to co-metabolically remove lignin, using primary source of nutrients such as glucose and peptone. Based on the pollution reduction activities and detoxification, it can be used as a potential organism for further treatment studies.

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