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ORIGINAL PAPER

Biodegradation of kraft-lignin by *Bacillus* sp. isolated from sludge of pulp and paper mill

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Abstract Eight bacterial strains were isolated on kraft lignin (KL) containing mineral salt medium (L-MSM) agar with glucose and peptone from the sludge of pulp and paper mill. Out of these, ITRC-S8 was selected for KL degradation, because of its fast growth at highest tested KL concentration and use of various lignin-related low molecular weight aromatic compounds (LMWACs) as sole source of carbon and energy. The bacterium was identified by biochemical tests as Gram-positive, rod-shaped and non-motile. Subsequent 16S rRNA gene sequencing showed 95% base sequence homology and it was identified as *Bacillus* sp. In batch experiments, a decrease in pH was observed initially followed by an increase till it reached an alkaline pH, which did not alter the culture growth significantly. The bacterium reduced the colour and KL content of 500 mg l^{-1} KL in MSM, in the presence of glucose and peptone, at pH 7.6, temperature 30°C, agitation of 120 rpm and 6 days of incubation by 65 and 37% respectively. Significant reduction in colour and KL content in subsequent incubations is indicative of a co-metabolism mechanism, possibly due to initial utilization of added co-substrates for energy followed by utilization of KL as a co-metabolic. The degradation of KL by bacterium was confirmed by GC-MS analysis indicating formation of several LMWACs such as *t*-cinnamic acid, 3, 4, 5-trimethoxy benzaldehyde and ferulic acid as degradation products, which were not present in the control (uninoculated) sample. This favours the idea of biochemical modification of the KL polymer to a single monomer unit.

Keywords KL · L-MSM · Biodegradation · *Bacillus* sp. · GC-MS · Pulp paper sludge

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Introduction

The aromatic polymer lignin is a highly branched and heterogeneous three-dimensional structure made up of phenylpropanoid units which are interlinked through a great variety of different bonds (Brunow 2001). Lignin is well known for resistance to microbial degradation because of its

high molecular weight and presence of various biologically stable β-O-4 ether bonds, β-5 carbon-to-carbon and ether linkages (Argyropoulos and Menachem 1997). Microorganisms that degrade plant lignin via an oxidative process, are fungi (Tien and Kirk 1983), actinomycetes (Berrocal et al. 2000) and to a lesser extent, bacteria (Trojanowski et al. 1977). Study shows that the bacterial strains cleave intermonomeric linkages that are characteristic of lignin, but are probably unable to depolymerise the high molecular weight backbone of the lignin polymer (Kern and Kirk 1987) because, unlike fungi which secrete extracellular enzymes called ligninases (Hatakka 1994), the bacterial cells do not secrete lignin depolymerising enzymes (Vicuna 1993). However, the bacterial ability to use low molecular weight portion of lignin indicate that bacteria have many unique and specific enzymes with the ability to catalyse the production of various useful compounds (Masai et al. 1999). Due their productivity, bacterial enzyme systems are expected to serve as useful tools for the bioremediation of lignin from pulping effluent and its conversion into useful intermediate metabolites. Kraft lignin (KL), a waste polymer by-product from kraft pulping process of pulp and paper industry is disposed into the environment without considering the potential values of its derivatives causing adverse impact on natural flora, fauna as well as aquatic bodies due to dark colouration (Gaete et al. 2000). KL differs from natural lignin as it undergoes a variety of reactions including aryl–alkyl cleavages, strong modification of side chains, and various ill-defined condensation reactions causing the polymer to fragment into smaller water/alkali-soluble fragments (Chakar and Ragauskas 2004). Inspite of this, KL, though not identical to natural lignin, has been widely used as an experimental lignin for microbial degradation studies (Forney and Reddy 1979; Fiechter 1982; Perestelo et al.1989; Morii et al.1995). Several species of bacterial strains have been reported to degrade the lignin monomeric substructure model compounds (Vicuna 1987; Zimmermann 1990; Vicuna et al. 1993; Kumar et al. 2001), but only a few strains are able to degrade kraft-lignin from pulping industry. *Pseudomonas putida*, isolated from decomposing plant materials degrade polymeric kraft lignin by about 13% measured as klason lignin after 30 days of incubation (Perestelo et al.1989). Bacterial isolates from compost or soil, namely *Azotobacter*, *Bacillus megatarium* and *Serratia marcescens*, were capable of decolourising or solublizing industrial lignin (Perestelo et al.1989; Morii et al. 1995). However, the ligninolytic rates of reported bacterial species have been found to be much lower as compared to fungi (Ulmer et al. 1983). For this reason there is still a need for continued search of more efficient ligninolytic bacterial strains for bioremediation of lignin from pulp and paper mill wastewater. This paper has focused on this approach by isolation and identification of the bacterium and its possible use in the decolourisation and degradation of kraft-lignin. Further, this work has been extended to identify low molecular weight aromatic compounds to corroborate the bacterium KL degradation by GC-MS analysis.

Materials and methods

Chemicals

The reagents used in this work were of analytical grade. KL (molecular weight 28,000) and lignin-related low molecular weight aromatic compounds (LMWACs) such as vanillin (4-hydroxy-3methoxy benzaldehyde); *trans*-ferulic acid (Trans-4-hydroxy-3-methoxy cinnamic acid); *p*-hydroxybenzoic acid; gallic acid (3,4,5-trihydroxy benzoic acid); *trans*-cinnamic acid; guaiacol; syringic acid (4-hydroxy-3, 5-dimethoxybenzoic acid) were purchased from Sigma-Aldrich (Milwaukee, WI, USA) with 99% purity. The trimethyl silyl (TMS) a combination of BSTFA (N, O-bis (trimethylsilyl) trifluoroacetamide) and TMCS (trimethylchlorosilane) was procured from Supelco (Bellefonte, PA, USA). The KL powder was purified by treating it with sulfuric acid (1%) and washed extensively with de-ionised water to remove ash and other impurities. All solutions were prepared in Milli-Q water (Elix Millipore Purification System, France).

Collection of sludge sample

Activated sludge sample was collected from the effluent treatment plant of M/s Century Pulp and

Paper Mill Ltd, Lalkuan Nainital, Uttranchal (India) in sterile test tubes. The industry adopts kraft process for pulping of raw materials (mainly eucalyptus, bamboo and sugarcane bagasses). Subsequently this pulp is bleached through multistage chlorination. The effluent generated by this process contains high concentration of dissolved KL, chlorophenol and other soluble components of raw material, which subsequently undergo activated sludge process for the treatment after filtration of residual wood fibers. The sludge of treatment plant remains rich in lignin. Hence, the activated sludge samples were collected for the isolation of ligninolytic bacteria.

Isolation and screening of KL-degrading bacteria

One KL-degrading bacterial strain was isolated from the effluent sludge by enrichment culture technique (Morii et al. 1995) as described above. Briefly, one loopful of sludge was inoculated to 100 ml sterile mineral salt medium (MSM) containing KL 500 mg l^{-1} (designated hereafter L-MSM). MSM (pH 7.6) consisted of (g l^{-1} de-ionised water): Na_2HPO_4, 2.4; K_2HPO_4, 2.0; NH_4NO_3, 0.1; $MgSO_4$, 0.01; $CaCl_2$, 0.01 and trace element solution 1 ml l^{-1} (Pfenning and Lippert 1966). The bacterial strains could not grow using KL as single source carbon due to its high molecular weight. Therefore, through nutrient optimization process, 1.0% glucose and 0.5% peptone (w/v) were added in L-MSM-broth and—agar as growth supportive substrates. The flasks were incubated for 7 days on rotary shaker (120 rpm, Innova 4230 Refrigerated Incubator shaker, New Brunswick, Edison, New Jersey, USA) under aerobic conditions at 30°C. Samples from flasks exhibiting decolourisation were serially diluted and spread on L-MSM agar plates and incubated in dark at 30°C for 7 days. Eight phenotypically different bacterial colonies were picked and purified by repeated sub-culture in order to obtain pure isolates. The purity of stains was checked by microscope and these strains were named as ITRC-S1, ITRC-S2, ITRC-S3, ITRC-S4, ITRC-S5, ITRC-S6, ITRC-S7 and ITRC-S8. To select lignin-degrading bacterium, screenings were carried out on MSM-agar plate; (a) containing increasing concentrations of KL (100, 200, 400, 600 mg l^{-1}) supplemented with glucose and peptone and (b) containing various lignin-related LMWACs (50 mg l^{-1}) as sole source of carbon and energy. The plates were incubated at 30°C and growth was observed for 6 days.

Bacterium identification

16S rRNA gene sequencing

Though 16S rRNA gene is found conserved on evolutionary scale, it is still diverse enough to identify and classify the eubacteria (Amman et al. 1995). 16S rRNA gene sequencing involves amplification of target sequences using universal primers to yield a 1.5 Kb amplicon followed by sequencing and homology generation using ribosomal DNA database.

Total DNA was prepared from overnight grown cultures by a simple lysis protocol, as described earlier (Kapley et al. 2001). About 5 µl DNA was used to amplify the 16S rDNA gene using universal eubacterial primers (Narde et al. 2004). The PCR were performed under the following conditions: 35 cycles of denaturation at 94°C for 1 min, followed by annealing at 55°C for 1 min and extension at 72°C for 2 min. A 1,466 bp product was amplified using forward primer (27F) 5′-AGAGTTTGATCMTGGCTCAG-3′and reverse primer (1492R) 5′-TACGGYTAC CTTGTTACGACTT-3′. The reaction mixture contained 5 µl template, 1× PCR buffer, 200 µM each of dNTP's, 3.0 mM $MgCl_2$, 25 pmol of primer, and 2.5 units of Amplitaq DNA polymerase (Perkin Elmer) in a final volume of 50 µl. The amplification product was gel purified using QIA gel extraction kit, Qiagen, Germany, and sequenced using primer 27F. The sequence data was analysed by BLAST analysis and identified based on the closest identity of reported sequence data. The sequences were deposited in GenBank database (http://www.ncbi.nlm.nih.gov/) under accession No. AY952465. Phylogenetic Trees were constructed using the MegAlign software from LaserGene. Sequences or construction of the trees were downloaded from the NCBI GenBank

site. Standard ATCC strains were also used in tree construction.

Biochemical test for identification ITRC-S8

Strain ITRC-S8 was identified on the basis of colony morphology, Gram-reaction and biochemical tests as per the method described by Barrow and Feltham (1993).

Biodegradation of KL by strain ITRC-S8

Biodegradation experiment was carried out in 250-ml flask containing 100 ml sterile L-MSM (500 mg l^{-1} KL) at pH 7.6, which is greater than that found in pulp mill effluent (Raj and Chandra 2004). Culture suspension 1% (v/v) having an inoculum size (CFU)/ml 105×10^4 were inoculated into triplicate flasks. The inoculum was grown overnight in 100-ml flasks containing 50 ml MSM-broth. The flasks were incubated for 6 days on rotary shaker under aerobic conditions at 30°C and 120 rpm**.** Uninoculated medium was used as control in all cases. Samples were withdrawn periodically at 1-day intervals and analysed for bacterium growth, reduction of colour and residual KL content.

Analytical techniques

Cell growth was determined by measuring absorbance of inoculated sample at 620 nm (A_{620}) on spectrophotometer (UV-visible Cintra 40-GBC) using uninoculated medium as blank. Following removal of biomass (at 8,000×*g*, for 15 min), the colour intensity of culture supernatants was measured at 465 nm (A_{465}), after adjusting the pH of the supernatant to 7.6 using 12 M HCl. The amount of colour present was determined spectrophotometrically and was related to the absorbance of a PtCo standard solution at the same wavelength (Hernandez et al. 1994). For the estimation of residual lignin centrifuged supernatants from control and inoculated were acidified with 12 M HCl to pH 1–2 and then centrifuged at 12,000*g* for 10 min. Residual KL was obtained after each precipitate had been washed with de-ionised water and dried at 50–60°C for 48 h and weighed (Anthony et al. 1986). Kraft-lignin loss (%) in the supernatants decolourised by the strain was determined daily as dry weight (estimating 100% as the KL present in the same volume of uninoculated medium). All experiments were carried out in triplicates. The values are presented as mean ± standard deviation ($n = 3$).

GC-MS analysis for identification of low molecular weight aromatic compounds

Control and bacterial treated KL samples (50 ml) was centrifuged (8,000*g* for 15 min) to remove suspended solids. Supernatants were acidified to pH 1–2 with concentrated HCl and then thoroughly extracted with three volumes (50 ml) of ethyl acetate. The organic layer was collected, dewatered over anhydrous Na_2SO_4 and filtered through Whatman no. 54 filter paper. The residues were dried under a stream of nitrogen gas. The ethyl acetate extracts residues were analysed as trimethyl silyl (TMS) derivatives as described by Lundquist and Kirk (1971). Briefly, the method involved is as follows: To the sample, dioxane (100 µl) and pyridine (10 µl) were added followed by silylated reagent of 50 µl trimethyl silyl [BSTFA N, O-bis (trimethylsilyl) trifluoroacetamide) and TMCS (trimethylchlorosilane)]. The mixture was heated at 60°C for 15 min with periodic shaking to dissolve residues.

An aliquot of 1 µl of silylated compounds were injected in the injector port of GC-MS for analysis. The GC-MS equipped with a PE Auto system XL gas chromatograph interfaced with a Turbomass Mass spectrometric mass selective detector. The analytical column connected to the system was a PE-5MS capillary column (20m × 0.18 mm internal diameter, 0.18 µm film thickness). Helium was used as the carrier gas with flow rate of 1 ml min^{-1}. The column temperature program was 50°C (5 min); 50–300°C (10°C min^{-1}, hold time: 5 min). The transfer line and ion source temperatures were maintained at 200 and 250°C. A solvent delay of 3 min was selected. In the full-scan mode, electron ionization (EI) mass spectra in the range of 30–550 (*m*/*z*) were recorded at electron energy of 70 eV. All standard lignin-related aromatic compounds (1 mg) were derivatised and chromatographed

as above. The identification of low molecular weight lignin-related compounds as TMS derivatives derived from bacterial degradation was done by comparing their mass spectra with that of the mass spectra available in NIST library provided with the instrument and also by comparing the retention time with those of authentic compound available.

Results and discussion

KL purification

The purified KL was analysed by ICP as soluble solution at pH 9 and the results revealed the presence of Ca (2.0), Mg (2.5), Na (65), Fe (0.3), Al (0.2) and $SO4^{2-}$ (78 mg l^{-1}) respectively. We found the presence of sodium and sulphate ions while the other metal ions were in negligible quantity. Thus, lignin solution was sufficiently pure to avoid any adverse effect on bacterium enzymatic system.

Screening of KL-degrading bacteria

Eight different sizes of colonies of the bacteria isolated on L-MSM-agar plates were screened at increasing concentrations (100–600 mg l^{-1}) of KL and subsequently on various lignin-related LMWACs in order to obtain KL-degrader are summarised in Table 1. Though all the strains were able to grow on highest tested KL concentration, strain ITRC-S8 exhibited faster growth than others. Similarly, strain ITRC-S8 was the only strain, which grew on six of the seven LMWACs where these were the only source of carbon and energy while others grew only on few (Table 1). Since strain ITRC-S8 uses six LMWACs as sole source of carbon and energy for its growth, it was selected as lignin degrader because these LMWACs are the basic components of lignin moieties: *p*-coumaryl alcohol (H units) coniferyl alcohol (S units) and sinapyl alcohol (G units) that build the natural lignin polymer. Use of lignin-related LMWACs by bacteria as a sole carbon source has been taken as criteria for selection of ligninolytic bacterial strains (Gonzalez et al. 1986; Kato et al. 1998; Kumar et al. 2001).

Characterisation of bacterium

Partial sequence data of the isolates was analysed by BLAST using the program available online at Swiss Bioinformatics Institute (http://www.ch.embnet.org/software/aBLAST.html). BLAST analysis of lab isolate ITRCS-8 (accession no. AY952465) showed *Bacillus* sp. as the first twenty hits with more than 95% identical to 16S rRNA gene of *Bacillus*. Phylogenetic analysis was carried out using *Bacillus* strains reported in GenBank and the results are shown in Fig. 1. This isolate shows 95.8% identity with a *Bacillus thuringiensis* strain, accession no. DQ49995, and 95.2% similarity index with standard ATCC isolates *Bacillus thuringiensis* strain ATCC33679 and *Bacillus cereus* strain ATCC10987, respectively. However, to finally assign the strain at species level or for taxonomic characterization, a further study with DNA hybridization using standard strains is required. Nevertheless, looking at the objective of the studies, where the functional degradative properties could be exploited, identification has only been carried out at this level.

Results of biochemical tests for strain ITRC-S8 (Table 2) showed that the strain is Gram-positive, rod-shaped, non-motile; showing growth in 10% NaCl and is positive for oxidase, catalase and indole production, urease, citrate utilization and starch hydrolysis; but is unable to hydrolyse casein or produce acid from fructose, lactose and mannose. Based on these biochemical properties the strain shows close resemblance to *Bacillus* sp.

KL decolourisation/degradation

The biodegradation assay was carried out in 250-ml conical flask containing L-MSM, pH 7.6 in shake flasks at 30°C for 6 days. Figure 2a shows the growth of *Bacillus* sp. measured at 620 nm with respect to medium pH during biodegradation. A marked increase in culture absorbance was recorded since beginning, which continued till end of the experiment. The continuous monitoring of culture medium pH (Fig. 2a) revealed an initial significant reduction, the greatest fall being in the first day and when it reached upto pH

Table 1 Screening of tolerance patterns of bacterial strains on different concentrations of KL and on various lignin-related low molecular weight aromatic compounds

Bacterial Isolates	Isolates growth on MSM-agar plates containing KL at different concentrations (pH 7.6)				Isolates growth on MSM-agar plates containing lignin-related LMWACs at 50 mg l^{-1} (pH 7.6)						
	100 mg l^{-1} KL[a]	200 mg l^{-1} KL[a]	400 mg l^{-1} KL[a]	600 mg l^{-1} KL[a]	VA[b]	FA[b]	PA[b]	GA[b]	CA[b]	G[b]	SA[b]
ITRC-S1	++	++	+	+	–	–	+	–	–	+	–
ITRC-S2	+	+	+	+	–	+	+	–	–	+	+
ITRC-S3	+	+	+	+	–	+	–	–	–	+	+
ITRC-S4	++	++	++	+	–	+	+	+	–	–	–
ITRC-S5	++	++	++	++	–	++	++	–	–	–	+
ITRC-S6	++	++	++	++	–	–	+	–	–	+	–
ITRC-S7	++	++	++	++		–	+	–	–	–	–
ITRC-S8	+++	+++	+++	+++	+	++	+	–	++	++	++

[a] with 1.0% glucose and 0.5% peptone; [b] with 0.0% glucose and 0.0% peptone; [–] Indicating no growth; [+] Indicating slow growth; [++] Indicating good growth; [+++] Indicating fast growth. Abbreviation: VA (Vanillin acid), FA (Ferulic acid), PA (*p*-hydroxybenzoic acid), CA (*t*-Cinnamic acid), GA (Gallic acid), G (Guaiacol) SA (Syringic acid)

5.2. Thereafter, it increased to a level above its initial pH at the end of 6 days. However, the altered pH did not markedly affect the growth hence it may be related with bacterium metabolic activity in medium supplemented with glucose and peptone. Similar observations during the microbial degradation of alkali-lignin and pulp and paper mill effluent have been reported (Hernandez et al. 2001; Diez et al. 2002).

The reduction in colour and KL content by *Bacillus* sp. degraded sample, shown in Fig. 1b, reveals that in spite of fast bacterium growth, there was less reduction in colour and KL content present in the culture media during the initial 2 days of incubation. Following this, significant reduction in colour (65%) and KL content (37%) was observed at the end of 6 days of incubation indicating a co-metabolism process for KL degradation. This could be possibly due to the initial utilization of glucose and peptone as carbon and nitrogen sources by the bacterium for initializing their growth and subsequently utilization of KL as a co-substrate. Similar to this study, a co-metabolism mechanism in bacteria and fungi during lignin degradation of kraft pine lignin and synthetic [ring–^{14}C]-lignin (DHP) has been reported (Perestelo et al. 1989; Kirk et al. 1978). Further incubation did not cause marked reduction in colour and KL (data not shown). In this study the initial colour of KL was 1017 PtCo at 500 mg l^{-1}, but after *Bacillus* sp. treatment it reduced by 351 PtCO at the end of 6 days of incubation (data not shown).

No adsorption of KL on the surface of bacterial cells was observed which is a very common phenomenon during microbial degradation (Hernandez et al. 1994, 2001). This observation was confirmed by separating the bacterial biomass

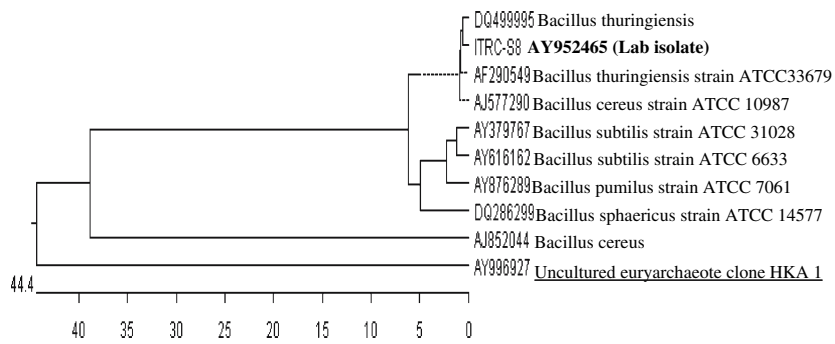

Fig. 1 Phylogenetic dendrogram based on the results of 16S rDNA sequence comparison

Table 2 Conventional tests for characterization of bacterium

Physiological and biochemical tests	Results
Shape	Rods
Gram reaction	Gram-positive
Motility	–
Growth in air	+
Anaerobic growth	–
Growth at 50°C	+
Growth in 10 % NaCl	+
Catalase	+ (Slow)
Oxidase	+
Oxidative/ Fermentative	Non sacchrolytic
Cellobiose	+ (Slow)
Fructose	+
Lactose	–
Mannose	–
Raffinose	+
Xylose	–
Casein hydrolysis	+
Starch hydrolysis	+
Citrate utilisation	+
H_2S production	+
Indole test	+
Urease test	+
Nitrate reduction	+ (Fast)

+, Positive; -, Negative

from the culture medium and washing it repeatedly with 1 M NaOH (Hernandez et al. 2001). Then suspension was filtered through Whatman No. 1 filter paper and colour content was estimated in the resultant filtrate at 465 nm, which showed no change in OD of filtrate indicating the reduction in colour was from biochemical modification of KL and not simply due to adsorption. No changes in colour or lignin content of KL were observed in uninoculated flasks (control).

GC-MS analysis of extract from control and bacterial treated sample

The degradation of KL by *Bacillus* sp. after 6 days of incubation was confirmed by GC-MS analysis. The Total Ion Chromatograph (TIC) corresponding to the compounds extracted with ethyl acetate from the acidified supernatants obtained from control and *Bacillus* sp.-inoculated sample are shown in Fig. 3 a and b and their peak identity is depicted in Table 3. Figure 3a, peak RT at 11.9 was identified as guaiacol, a single aromatic compound detected in ethyl acetate

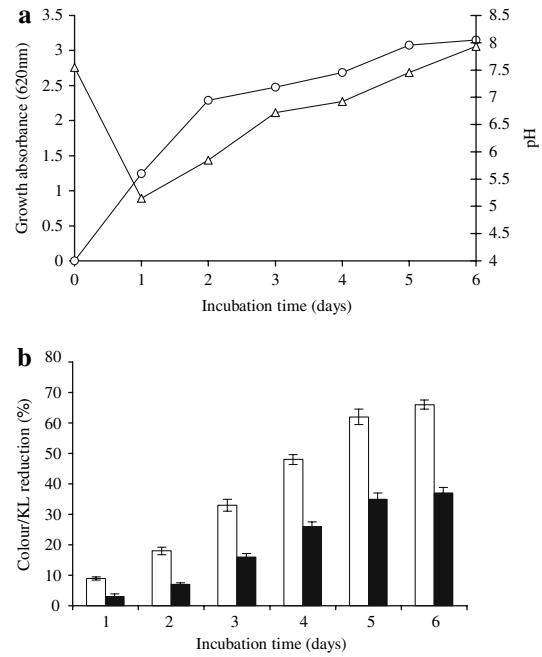

Fig. 2 Growth curve (o), variation in culture medium pH (Δ) (**a**) and reduction in colour (□) and KL (■) (**b**) of L-MSM sample inoculated by *Bacillus* sp during the course of time. The results represent the mean of three replicates for growth and pH while for colour and lignin loss represent standard deviation

extract of control, which may be attributed to chemical oxidation of lignin due to aeration and agitation.

TIC of sample inoculated by *Bacillus* sp. (Fig. 3b) showed a significant increase in number of peaks after 6 days of incubation as compared to control. Numbers of low molecular weight aromatic compounds (LMWACs) detected in inoculated sample were identified as a *t*-cinnamic acid (RT 16.5), 3, 4, 5-trimethoxy benzaldehyde (RT-18.6) and ferulic acid (RT-22.6). The presence of these LMWACs in the *Bacillus* sp.-inoculated sample is clear evidence of KL degradation, as these phenolic units are considered to be basic moieties that build natural lignin polymer. In addition to LMWACs, many acid-type compounds detected in ethyl acetate extract of control and inoculated sample (Table 3). It has been reported in literature that cupric oxide degradation of native lignin from different vegetal tissues showed that aldehyde-type compounds were always more abundant than ketone or

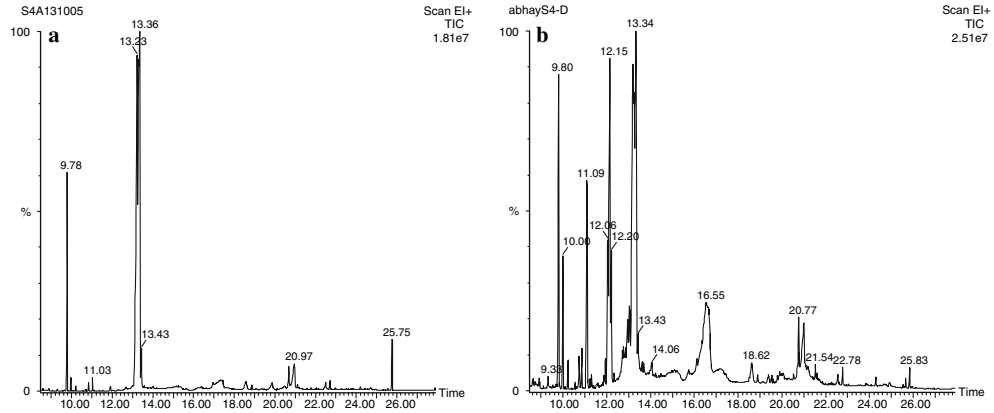

Fig. 3 Total ion chromatograph of ethyl acetate extracts from control (**a**) and from *Bacillus* sp.-inoculated (**b**) samples after 6 days. The MS-identified compounds with respect to their retention times are listed in Table 3

acid-type (Hedges and Ertel 1982). Further, the peak at RT 20.6, 20.8, 25.7 and 25.8 were identified as dibutyl phthalate, bis-(2-methoxy ethyl) phthalate, bis (2-ethylhexyl) phthalate and dio-octyl phthalate (Table 3) in control and bacterium inoculated extract. The phthalate derivatives such as butyl phthalate and bis (2-ethylhexyl) phthalate had been reported in literature (Shin and Lee 1999; Ksibi et al. 2003).

The degradation of KL by *Bacillus* sp. is higher than those of previous reports of Perestelo et al. (1994, 1996) by unicellular bacteria namely *Pseudomonas putida* which was only 1.4–2.1% and by *Serratia marcescens* up to 2%, although it solubilised 44% of lignin. The genus *Bacillus* has been shown to convert [^{14}C]-(side-chain)-lignin of spruce into $^{14}CO_2$ (Robinson and Crawford 1978) and to degrade acidolysis lignin (Odier and Monties 1977). Degradation of dealkalised lignin (molecular weight, 20,000) was 28% by termite guts bacteria, *Nasutitermes takasagoensis* (Kato et al. 1998) after 14 days incubation. Thus the *Bacillus* sp. has high capacity to decolourise/degrade KL and can be studied for treatment of pulp and paper mill effluent.

Conclusions

Results showed that the *Bacillus* sp., ITRC-S8, is a ligninolytic bacterium, significantly reducing the colour and KL content in L-MSM containing additional carbon and nitrogen source at pH 7.6, 30°C and agitation of 120 rpm. However, for the confirmation of *Bacillus* sp. ligninolytic machinery, a complete characterization for enzymes

Table 3 Compound identified as TMS derivatives in ethyl acetate extract from control and *Bacillus* sp. degraded KL samples as given in Fig. 3

Peak retention time (min)	Present in		Identified compounds
	Control	Inoculated	
9.3	–	+	Acetic acid
9.7	+	+	Propanoic acid
10.5	+	–	Ethanedioic acid
10.7	–	+	Furan carboxylic acid
10.8	–	+	Glyoxylic acid
11.0	+	+	Butanoic acid
11.3	–	+	3-Acetoxy butyric acid
11.9	+	–	Guaiacol[a]
12.0	–	+	Valeric acid
12.1	–	+	Hexanoic acid
13.1	–	+	Propanedioic acid
16.5	–	+	*t*-cinnamic acid[a]
18.6	–	+	3,4,5-trimethoxy benzaldehyde[a]
20.6	+	–	Dibutyl phthalate
20.7	+	+	Hexadecanoic acid
20.8	+	–	Bis- (2-methoxy ethyl) phthalate
22.6	–	+	Ferulic acid[a]
22.8	–	+	Octadecanoic acid
25.7	+	–	Bis- (2-ethylhexyl) phthalate
25.8	–	+	Di-octyl phthalate

[a] Confirmed by match of retention time (RT) with known standards

associated with lignin degradation is now necessary in order to establish their definitive role in the breakdown of the lignin. Nevertheless, the ability of this bacterial strain could be of interest for the treatment of pulp and paper mill effluent.

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