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Identification of low molecular weight aromatic compounds from the lignin degraded by *Bacillus* sp. using Gas Chromatography – Mass Spectrum

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Abstract

Sixteen bacterial strains were isolated from agricultural waste residues dumped soil sample. Out of the 16 isolates obtained through nutrient enrichment techniques one potential aerobic bacterial strain was capable of using all lignin preparations, which was identified as *Bacillus* sp. *Bacillus* sp decolourized lignin to a maximum level in 6 days. Initially with 48 h incubation the decolurization was slow with decreased pH. The release of low molecular weight aromatic compounds such as guaiacol, cinnamic acid, gallic acid and ferulic acid was detected using Gas chromatography - Mass Spectrum (GC-MS) and comparing the total Ion Chromotograph of ethyl acetate extract from lignin sample degraded by *Bacillus* sp. with uninoculated sample.

Keywords : *Bacillus* sp, GC-MS analysis, lignin

INTRODUCTION

Lignin is a highly branched aromatic polymer and heterogeneous three dimensional structure made up of polypropanoid units which are interlinked through a great variety of different bonds. Lignin is well known for resistance to microbial degradation because of its high molecular weight and presence of various biologically stable β-0-4 ether bonds, β-5 carbon to carbon and ether linkage. The plant lignin is degraded *via* an oxidative process with fungi, actinomycetes and to a lesser extent by bacteria (Trojanowski *et al*., 1977). Development of lignin degradation process has considerable potential for the production of a variety of chemicals. Fungal lignolytic enzymes have been proposed to release a number of low molecular aromatic compounds through oxidative changes and progressive depolymerization of lignin (Kirk and Farell, 1987). Cartwright and Holdum, (1973) isolated an *Arthrobacter* strain capable of utilizing enzymic lignin as a sole carbon source. Odier *et al.* (1981) reported the isolation of 11 Gram negative bacteria, identified as *Pseudomonas, Xanthomonas* and *Acinetobacter* sp. which were capable of assimilating poplar dioxane lignin without a co-substrate. Several *Nocardia* strains have been shown to decompose radiolabeled natural and synthetic lignin and to utilize as a carbon source. *Bacillus* strain and several *Streptomyces* strains have been shown to degrade radio labeled natural lignin.

Peanut hull contains more lignin (approximately 32 %) than most hardwoods and softwoods, and they are very resistant to biodegradation. Bacteria in particular, deserve to be studied for lignolytic potential because of their immense environmental adaptability and biochemical versatility. Due to their productivity, bacterial enzyme systems are expected to serve as useful tools for the conversion of lignin into various useful intermediate metabolites. Earlier studies have shown that some bacteria related species could metabolize lignin and lignin related model compounds to various low molecular weight compounds. However, the lignolytic rates of reported species of bacteria have been found to be much lower as compared to fungi. For this reason there is still a need for continued bioremediation of lignin from pulp and paper mill waste water. This paper is focused on this approach by isolation and identification of the bacterium and possible use in the decolourizaton and degradation of lignin. Further, an attempt has been made to identify low molecular weight aromatic compounds by Gass Chromatography - Mass Spectrum (GC-MS) analysis.

MATERIAL AND METHODS

Lignin degrading bacterial strains were isolated from solid waste dumped soil by enrichment culture technique (Morii *et al.,* 1995). One gram of soil sample was inoculated to 100 ml sterile mineral salt medium (MSM) containing Na_2HPO_4 (2.2 g), K_2HPO_4 (2.0 g), NH_4NO_3 (0.1 g), $MgSO_4$ (0.01 g), $CaCl_2$ (0.01 g) and trace element solution (1 ml) in 1000 ml of distilled water (pH 7.6) amended with 500 mg of lignin (separated from peanut hulls and named as dioxane lignin, klason lignin, HCl lignin and milled wood lignin) (Browning, 1967), and glucose (1 %) and peptone (0.5 %) were added into the medium as co-substrates. The flask was incubated for 7 days on rotary shaker under aerobic conditions at 30 ºC. Samples from the flask,

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exhibiting decolourization were serially diluted and spread on L-MSM agar plates separately and incubated at 37ºC for 7 days. Phenotypically different colonies were picked and purified by repeated subculture in order to obtain pure cultures.

Lignolytic activity was tested with simple plate test. Lignin Mineral Salt Medium (L-MSM) amended with 1 % phenol red (w/v) was inoculated with the test isolates and incubated at 37 º C for 7 days. The colour changes were observed. In addition to that these colonies were repeatedly streaked onto L-MSM agar plates and colonies obtained after four transfers were considered as positive colonies. Predominant isolates were identified on the basis of morphological and biochemical characteristics (Barrow and Feltham, 1993).

Biodegradation of lignin

Biodegradation was carried out in 250 ml conical flask containing L-MSM at pH 7.6 inoculated with test isolate having inoculum density of 105×10^4 CFU/ml and triplicates were maintained. The flasks were incubated at 30 º C for 7 days. Uninoculated medium was used as control in all cases. Samples were withdrawn periodically at 24 h intervals and were analyzed for bacterial growth, pH, reduction of colour and degradation. Cell growth was measured at 620 nm on spectrophotometer (UV visible Cintra 40 GBC) using uninoculated medium as blank. For the determination of colour reduction and degradation of lignin, samples were centrifuged at 5000 rpm for 30 min. The supernatant (1ml) was diluted by adding 3 ml phosphate buffer (pH 7.6) and the absorbance was measured at 465 nm (Morii *et al*., 1995) for colour reduction, and at 320 nm (Lara *et al*., 2003) for lignin degradation on UV-visible spectrophotometer. The pH of the medium was also measured by using a pH probe.

Gas chromatography - Mass Spectrum (GC - MS) analysis for identification of Low Molecular Weight Aromatic Compounds (LMWAC)

Control and bacteria treated samples (50 ml) were centrifuged (5000 rpm for 15 min) to remove the biomass. The supernatants were acidified to pH 1-2 with concentrated HCl and then thoroughly extracted with three volumes 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 extract residues were analyzed as TriMethyl Silyl (TMS) derivatives as described by Lundquist and Kirk (1971). In this method, 100 µl dioxane and 10 µl pyridine were added to the samples and silylated with 50 µl trimethyl silyl [BSTFA (N, O–bis (trimethylsilyl) trifluoroacetamide] and TMCS (trimethylchllorosilane). The mixture was heated at 60 º C for 15 min. with periodic shaking to dissolve the residues.

An aliquot of 1 µl of silylated compounds were injected in GC -MS equipped with a PE autosystem XL gas chromotograph interfaced with a Turbo mass spectrometric mass selective detector. The analytical column connected to the system was a PE -5MS capillary column (20 m x 0.18 mm internal diameter, 0.18 µm thickness). Helium was used as a carrier gas with flow rate of 1 ml min^{-1}. The column temperature programme was 50º C (5 min); 50-300º C (10º C min^{-1}, hold time: 5 min). The transfer time and ion source temperatures were maintained at 200 and 250ºC respectively. A solvent delay of 3.0 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 monomeric phenolic derivatives were determined and chromatographed as above. In order to identify the low molecular weight lignin related compounds as TMS derivatives derived from bacterial treatment was done by comparing their mass spectra with that of the NIST library available in the instrument and comparing the retention time with those of authentic compounds available.

RESULTS AND DISCUSSION

Among the 16 isolates grown on L-MSM plates with four different lignin preparation, only seven bacterial isolates grew predominantly and the remaining isolates did not grow upon transfer to fresh media. Although 7 isolates grew on four lignin preparation, only one bacterial isolate was capable of utilizing all the four prepared lignin as a carbon source (Table 1). Lignolytic activity of the isolates was visualized by change of indicator colour from phenol red to deep yellow orange at 144 h. Test isolate was Gram positive, rod shaped and nonmotile, showed growth in 10% NaCl, thermo tolerant (50 º C), capable of utilizing citrate as sole source of carbon and energy, hydrolysing the urea and reducing H_2O_2 and nitrate. Based on the biochemical characteristics it was identified as *Bacillus* sp.

The biodegradation assay was carried out with L-MSM broth in shake flask at 30 º C for 7 days. The growth response of *Bacillus* sp was measured in terms of absorbance at 620 nm and change of pH of the medium during biodegradation. A marked increase in absorbance was recorded and also there was variation in pH during biodegradation (Table 2). However, the altered pH did not markedly affect the growth and hence it could be related with bacterial metabolic activity in the medium supplemented with glucose and peptone. Similar observation during the microbial degradation of alkali lignin and paper and pulp mill effluents had also been reported earlier (Diez *et al*., 2002).

The reduction of colour and lignin content of the sample during degradation by *Bacillus* sp degraded sample was given in Table 2. It revealed that in spite of fast growth of bacteria, less reduction in colour and degradation of

Table 1. Growth of bacterial isolates on Mineral Salt Medium (MSM) with various lignin preparations

Bacterial isolates	Dioxane lignin	HCl lignin	Milled wood lignin	Klason lignin
Arthrobacter sp.	+++	+	+++	++
Bacillus sp.	+++	+++	+++	+++
Flavobacterium sp.	+	-	++	++
Micrococcus sp.	++	++	++	++
Pseudomonas sp.	+++	++	++	+++
Serratia sp.	++	-	+++	+++
Streptomyces sp.	++	+	++	++

+++ Fast and luxuriant growth
++ Slow growth
+ Very slow growth
_ No growth

Table 2. Biodegradation of lignin by *Bacillus* sp.

Day	Bacterial growth	pH	Colour reduction (%)		Lignin degradation (%)	
			Bacillus sp.	Control	*Bacillus* sp.	Control
1	7.7	7.1	38.8	0.0	11.9	0.00
2	7.5	5.8	38.1	0.01	20.0	0.009
3	7.5	5.4	38.9	0.02	24.8	0.009
4	8.0	6.7	41.1	0.02	30.1	0.009
5	7.0	7.8	52.4	0.03	48.4	0.009
6	7.2	7.9	55.2	0.04	63.9	0.009
7	6.0	7.9	55.8	0.04	67.8	0.009

Table 3. Compounds identified from the degraded lignin by *Bacillus* sp. using Gas Chromatography - Mass Spectrum

Peak Retention Time (min.)	Identified compounds	Degraded lignin by *Bacillus* sp.	Control
3.42	O Xylene	+	-
5.96	Propane 1,1 – diethoxy	-	+
8.49	Dodecane ,2,6,10 –triethoxy	+	-
11.9	Guaiacol[a]	+	-
13.7	Benzene ethanol	-	+
16.5	Cinnamic acid[a]	+	-
18.6	3,4,5 –trimethoxy benzaldehyde[a]	-	+
21.7	Gallic acid[a]	+	-
22.3	5 - Isopropylidine -3,3- dimethyl –dihydrofuron	-	+
22.6	Ferulic acid[a]	+	-
24.3	Benzyl butyl phthalate	+	-
24.6	Pyrrolo	-	+

[a] Confirmed by match Retention Time with known standards
+ denotes presence
- denotes absence

lignin was observed during the initial two days of incubation. Following this there was reduction in colour (55.8 %) and lignin content (67.8 %) on the seventh day of incubation. This could be possibly due to the initial utilization of glucose and peptone as carbon and nitrogen sources, respectively, by the bacterium during the initial phase of its growth and as a result these readily available nutrients could be depleted very fast. Hence, the organisms is forced to utilize the lignin as its energy source and hence the subsequent reduction in the lignin content which was indicated by changes in its intensity of colour. Similar observation was also reported during degradation of Kraft lignin by Perestelo *et al*. (1989).

Degradation of lignin by *Bacillus* sp after seven 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* inoculated sample is shown in Table 3 and their peak identities were also depicted in Figures 1 and 2. TIC of the sample inoculated with *Bacillus* sp. produced significant number of peaks after seven days of incubation as compared to control. Number of low molecular weight aromatic compounds were detected from the *Bacillus* degraded sample including guaiacol (RT 11.9), cinnamic acid (RT 16.5), gallic acid (RT 21.7) and ferulic acid (RT 22.6). Further, the peak at RT 3.42, 5.96, 8.49, 13.7, 18.6, 22.37, 24.3 and 24.6 were identified as xylene, propane, dodecane, benzene ethanol, trimethoxy benzaldehyde, 5-isopropylidine, benzyl butyl pthalate and pyrrolo, respectively, both in control and bacterium degraded samples. This could be attributed to the chemical oxidation of lignin due to aeration and agitation (Larrea *et al*., 1989). Phthalate derivatives had been detected and reported from fungal peroxidase degradation of lignosulfonate (Shin and Lee, 1999) and also from photo degradation of black liquor lignin. Apart from these aromatic compounds many acid type compounds were also detected due to degradation of graft lignin and pulp paper mill effluent (Gupta *et al*., 2005). The low molecular weight aromatic compounds identified in bacteria degraded sample favours the idea of biochemical modification of lignin to single aromatic unit by both strains and these aromatic compounds have been reported to be lignin degradation intermediates. Thus *Bacillus* sp has high capacity to decolourize and

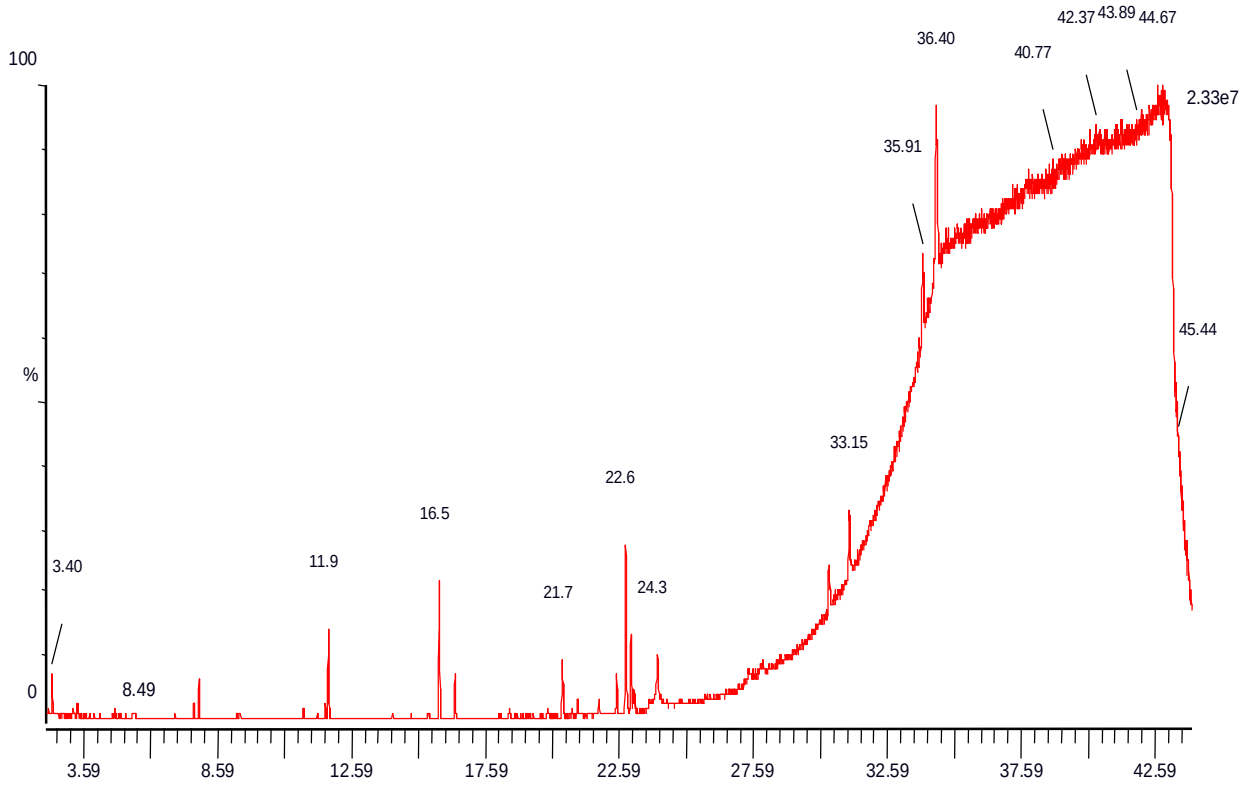

Figure 1. Gas Chromatography - Mass Spectrum analysis of *Bacillus* sp. degraded sample

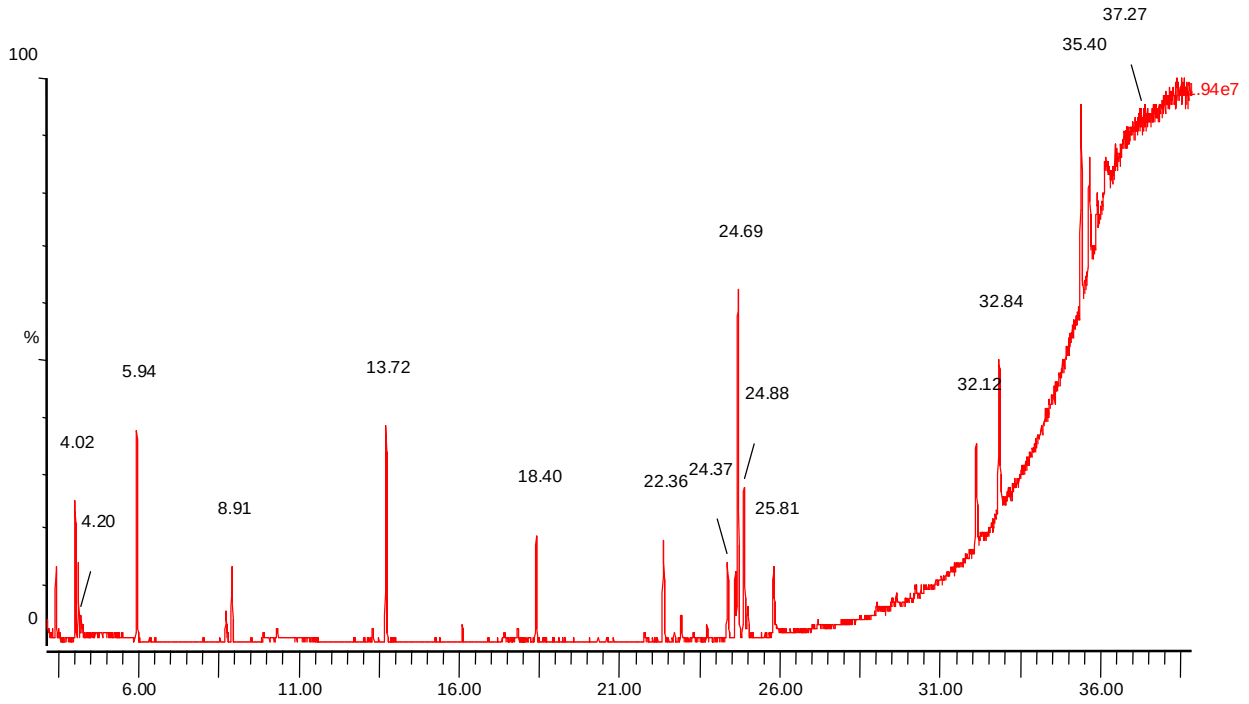

Figure 2. Gas Chromatography -Mass Spectrum analysis of control (uninoculated) sample

degrade lignin and hence it could be potentially used for the solid waste management.

REFERENCES

Barrow, G.I. and Feltham, R.K.A. 1993. *Cowan and Steel's Manual for the Identification of Medical bacteria*. 3rd ed, Cambridge University Press, Cambridge.

Browning, B.L. 1967. *Methods of Wood Chemistry*. Interscience Publishers, Inc., New York.

Catwright, N.J. and Holdom, K.S. 1973. Enzymatic lignin and its release and utilization by bacteria. *Microbios*, 8: 7-14

Deiz, M.C., Castillo, G., Aguilar, L., Vidal, G. and Mora, M.L. 2002. Operational factor and nutrient effect on activated sludge treatment of Pinus radiates kraft mill wastewater. *Bioresource Technol.*, 83: 131-138.

Gupta ,V.K., Ali, I., Saini,V.K., Gervan, T.V., Bruggen, B.V.D. and Vandecasteele, C. 2005. Removal of dyes from wastewater using bottom ash. *Ind. Eng. Chem. Res.,* 44: 365-364.

Kirk, T.K. and Farell, R.L. 1987. Enzymatic combustion the microbial degradation of Lignin. *Annu. Rev. Microbiol*, 41: 465-505.

Lara, M.A., Malaver-Rodriguez, A.J., Rojas, O.J., Holmquist, O., Gonzale, A.M., Bullon, J., Penaloza, N., Araujo, E. 2003. Black liquor lignin biodegradation by *Tramates elegans*. *Int. Biodeterior. Biodegrad*., 52: 167-175.

Larrea, L., Forster, C.F., Mele, D. 1989. Kraft lignin behaviour in diffused aeration of Kraft effluent. *Water Sci. Technol.,* 21: 241-253.

Ludquist, K. and Kirk, T.K. 1971. Acid degradation of lignin. *Acta Chem Scand.,* 25 : 889-894.

Morii, H., Nakamiya, K. and Kinoshita, S. 1995. Isolation of a lignin degrading bacterium *J. Ferment. Bioeng.*, 80: 296-299.

Odier, E., Jain, G. and Monties, B. 1981. Poplar lignin decomposition by gram negative aerobic Bacteria. *Appl. Environ. Microbiol.*, 41: 337-341.

Peresteol, F., Falcom, M.A. and OelaFuente, G. 1989. Biotrasformation of Kraft lignin Fractions by *Serratia marcesens*. *Lett. Appl. Microbiol.,* 10: 61-64.

Shin, K.S. and Lee, Y.J. 1999. Depolymerization of lignosulfonate by peroxidase of white rot basidiomycetes, *Pleurotus ostreatus. Biotechnol Lett.,* 212: 585-588.

Trojanowski J., Haider, K. and Sundaman, V. 1977. Decomposition of ^{14}C labelled lignin and phenols by *Nocardia* sp. *Arch. Microbiol.*, 114: 149-153.