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Process optimization for biodegradation of black liquor by immobilized novel bacterial consortium

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ABSTRACT

Present investigation deals with the optimization of biodegradation process of black liquor (BL) performed by novel bacterial consortium which consist of two indigenous bacterial strains viz., *Bacillus megaterium* ETLB-1 and *Pseudomonas plecoglossicida* ETLB-3. During the process, dextrose as carbon; sodium nitrate as nitrogen; C:N ratio (2.5:1); temperature (35˚C); pH (8.0); and agitation rate (160 rpm) were observed as optimum conditions for bacterial consortium. Further, these conditions were assessed for the performance of immobilized consortium that exhibit conspicuous reduction in color (96.1%), lignin (91.5%), biological oxygen demand (96.7%), and chemical oxygen demand (86.4%) of black liquor. A maximum percent reduction of 90.7% in chlorophenols (up to 10.03 mg/L) with highest release of chloride ions i.e. 1,233 mg/L was recorded under optimum conditions. Biolignolytic activities with the presence of lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase (LAC) was observed as 6.94 U/ml, 9.35, and 8.96 U/ml at different time intervals viz. 120, 144, and 96 h, respectively, during the biodegradation study. Further, gas chromatography/mass spectroscopy revealed presence of certain organic acids, degradation of majority of the toxic compounds, and generation of certain high-value compounds.

Keywords: Biodegradation; Black liquor; Bacterial consortium; Parameters optimization; Cell immobilization

1. Introduction

Black liquor generated from pulp and paper industries is highly viscous and comprises complex chlorinated phenolic compounds. Pulp and paper industries involve two important processes viz., pulping (conversion of wood into pulp) and bleaching (decolorization of pulp) to generate pulp for paper manufacturing. During pulping, wood chips are generally converted into pulp by treating with solution of sodium hydroxide and sodium sulfate/sulfite at elevated temperature and pressure to separate the lignin and hemicelluloses fibers from cellulose. In this process, 90–95% of lignin is generated as effluent called as

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black liquor due to its dark brown color [1]. Another stage known as "bleaching", where, chlorine, hydrogen peroxide, ozone etc., are utilized for brightening the pulp, generates effluent consisting xenobiotic compounds (such as chlorinated lignin, resin acids, phenols, dioxins, furans, etc.), wood extractives, salts, and nutrients elements. These compounds are reported to affect aquatic life, increase soil salinity, deteriorates the soil structure, and cause nutrient imbalance in crops. Moreover, some of these pollutants are tend to persist in nature [2–4]. Although, black liquor can be used as a resource of high-valued chemicals which are of industrial importance due to the presence of lignin, some paper industries install recovery system, where the black liquor from pulping stage is removed to recover inorganic chemical and energy in evaporator and recovery boiler. However, most small paper industries do not use the recovery system and simply discard the black liquor directly or after primary treatment into the ecosystem [5]. Therefore, it is important to treat the black liquor prior to release into the environment. In recent trend, utilization of total chlorine-free (TCF) and Elemental chlorine-free (ECF) bleaching processes are emphasized to avoid generation of secondary pollutant. TCF involves the use of oxygen and peroxides, whereas ECF includes chlorine dioxide for bleaching. However, these two processes also have some negative impacts on environment such as; TCF involves chelation stage that may increase the metal load in effluents and ECF technique lead to the generation of chlorate which is a powerful herbicide [6].

Biological methods are quite preferable over the conventional bleaching and effluent treatments as they are economical and there is less possibility of secondary pollutants generation. Furthermore, microorganism can easily interact with pollutants, adapt physiologically and remain active in the stressed environment [7,8]. Biological degradation involves use of fungi, bacteria, and actinomycetes for the treatment of black liquor. Although, several studies have been done with fungi for lignin degradation due to their highly developed nonspecific ligninolytic system, but bacteria have also been proven to possess excellent degradation capability due to immense environmental adaptability and biochemical versatility [1,9–11]. Bioligninolysis is purely an enzymatic degradation process where three major biocatalysts viz., lignin peroxidase (LiP), manganese peroxidase (MnP), laccase (LAC) and some accessory enzymes are required for complete degradation of lignin [1]. However, presence of all essential enzymes in single micro-organism is difficult, but an active consortium of two or more microbes could overcome this problem [4].

Every technology requires optimization of process before its commercial application to establish best possible culture and environmental conditions by correlating all the parameters. For effective biodegradation therefore, it is essential to optimize the process as it makes the technology strong by improving the microbial activities [12]. Most of the studies have optimized the process of black liquor biodegradation with single bacterial or fungal strain, but very few reported to optimize the conditions for consortium [13–15]. Furthermore, use of immobilized cells for industrial application have been very useful as it provides long retention time (RT) for biomass, convenience in continuous operation, increase process stability and maintain the activities of enzymes [4,16]. Application of polymers, synthetic and inorganic materials for cell immobilization have several limitations such as immobilization cost, problems in diffusion of enzymes, mechanical stability, presence of toxicants (polyphenols), and generation of secondary pollutant [17]. However, use of natural structural material such as agro-industrial residue (AIR) for cell immobilization can provide an alternative to solve various problems associated with synthetic matrices. Corncobs are one such AIR generated from maize processing and largely investigated for cell immobilization in fermentation processes [18,19]. However, investigations on treatment of black liquor by immobilized bacterial consortium are still overlooking.

In our previous investigation, we have studied the degradation potential of novel bacterial consortium (*Bacillus megaterium* ETLB-1 (KC767548) + *Pseudomonas plecoglossicida* ETLB-3 (KC767547)) against the black liquor. Since the degradation potential of the micro-organisms could be influenced by several environmental and nutritional conditions, thus, to achieve maximum biodegradation of the pollutants/contaminants and for its successful application, it is necessary to optimize the process of biodegradation with the key environmental as well as nutritional parameters. Keeping this in view, present investigation was undertaken with the aim to identify the optimum environmental and culture conditions for the novel bacterial consortium, constructed from two indigenous bacterial isolates, for biodegradation of black liquor (BL). Furthermore, the effect of immobilization on degradation efficiency of bacterial consortium for BL along with ligninolytic activity was also investigated. Overall, the results of this study will add another arena to our previous findings which enables commercial application of indigenous bacterial consortium and their enzymatic system in industrial processes such as, effluents treatment, biobleaching of pulp, bioconversion of black liquor for

organic and inorganic chemicals, and high-value added products generation as well.

2. Materials and methods

2.1. Sample collection, construction of consortium, and media preparation

Black liquor samples were collected from the M/s Century Pulp Paper Mill Lalkuan, India and stored at 4°C until further utilization for analysis. Two indigenous bacterial strains *B. megaterium* ETLB-1 (KC767548) and *P. plecoglossicida* ETLB-3 (KC767547) previously isolated from soil contaminated with paper mill effluent were used for construction of consortium by mixing each in 1:1 ratio (v/v) [4]. For batch experiments, the minimal salts medium (MSM) was used which contains the following (g/L): $Na_2HPO_4{\cdot}2H_2O$, 7.8; KH_2PO_4, 6.8; $MgSO_4$, 0.2; $NaNO_3$, 0.085; $NH_4(CH_3COO)_3Fe$, 0.01; $Ca(NO_3)_2{\cdot}4H_2O$, 0.05, and trace element solution, 1 ml/L [14].

2.2. Physicochemical analysis

Black liquor samples were analyzed for physicochemical parameters such as BOD (5-d method), COD (open reflux method), total nitrogen (Macro Kjeldahl method), sulfate ($BaCl_2$ precipitation method), phosphate (spectrophotometrically after digestion), nitrate (Brucine method), total phenols (chloroform extraction method), sodium and potassium by flame photometer (Systronics 128, ASE Ltd), and TDS (total dissolved solids) as per standard methods for wastewater analysis [20]. pH was measured by digital pH meter of Elico, India. Ash content was determined gravimetrically by Tappi T 211 om-93 method after incineration of sample at 525–900°C. For lignin estimation, absorbance was taken at 430 nm, the values were compare with standard curve and transformed into lignin content (ppm) [14], Klason lignin (acid-insoluble lignin), and acid-soluble lignin was determined according to Zhu et al. [21]. Total carbohydrate in the sample was analyzed by high-performance anion-exchange chromatography with pulsed amperometric detector (HPAEC-PAD) [5]. Chloride content was estimated at 460 nm by UV–vis spectrophotometer (VARIAN 50Bio) using sodium chloride as standard solution [22]. Chlorophenols (pentachlorophenol—PCP) were analyzed by HPLC (Shimadzu Corporation, Kyoto, Japan) [23]. Heavy metals were detected using atomic absorption spectrophotometer (Shimadzu AA6701F). Color was estimated at 465 nm and absorbance value was transformed into color units (Co–Pt) [24].

2.3. Parameters optimization for black liquor biodegradation

The process of black liquor degradation by bacterial consortium was optimized with different nutritional and environmental parameters. For this, batch study was conducted in Erlenmeyer flasks (250 ml) containing autoclaved black liquor (100 ml) supplemented with MSM by changing one parameter and retaining others as constant. The flasks were inoculated with bacterial consortium and incubated for 168 h in rotary shaker. The bacterial consortium (1% v/v; OD_{600} = 2.0) comprising ETLB-1 (3.4×10^8 CFU ml^{-1}) and ETLB-3 (3.5×10^8 CFU ml^{-1}) was used for batch experiments of parameter optimization study. Various carbon (dextrose, malt extract, pulp, and wheat straw) and nitrogen (urea, ammonium nitrate, and sodium nitrate) sources at 0.2% w/v were screened first. After selection of appropriate carbon and nitrogen source other parameters such as C:N (0.5–5) ratio, temperature (20–45°C), pH (4.0–10.0), and agitation rates 100–200 rpm were optimized. pH was adjusted using different buffers: citrate (3.0–6.0), phosphate (6.5–8.0), and tris (8.5–9.0) to keep the pH of media constant. During optimization study, samples were extracted at different intervals up to 168 h of incubation and analyzed for changes in color, lignin, and cell growth.

2.4. Co-immobilization of bacterial cells

Immobilization of bacterial consortium was carried out by mixing 200 ml of pre-grown cultures (OD_{600} = 2.0) in 1:1 ratio (v/v) in Erlenmeyer flasks (500 ml) containing 100 g of processed corncob cubes [4]. Immobilized cells were removed from the flasks and used for degradation of black liquor.

2.5. Degradation study

The degradation batch experiments were carried out with two treatments (T1 and T2). T1 experiments were performed with co-immobilized cell in 500-ml Erlenmeyer flasks containing 250 ml of autoclaved black liquor supplemented with MSM and 10 g of co-immobilized material under optimized condition. A control batch experiments (T2) inoculated with biomaterial (10 g) without bacterial consortium was also run in parallel and monitored under the same conditions. All the experiments were performed in triplicate, with corresponding controls.

A small-scale bioreactor study was also conducted in a glass column (2.5 cm internal diameter and 30 cm height) with an air inlet at the bottom. The empty reactor was initially packed with of co-immobilized biomaterial (20 g) on a porous plate fixed at the

bottom. The degradation study was carried out under optimized conditions. Finally, autoclaved BL (1,000 ml) supplemented with MSM was fed into the column at a flow rate of 1 ml/min. Air was supplied from bottom of the reactor at a rate of 0.5 v/v per min. Samples were extracted from batch (T1 and T2) and the bioreactor experiments periodically up to 168 h (h) of incubation period, centrifuged at 10,000 rpm for 10 min and analyzed for physicochemical parameters.

2.6. Ligninolytic enzymes assay

Ligninolytic enzymes activity such as lignin peroxidase (LiP, EC1.11.1.14), manganese peroxidase (MnP, EC1.11.1.1), and laccase (LAC, EC 1.10.3.2) was monitored at 24 h time interval up to 168 h in the culture supernatant of T1, T2 (control) and bioreactor treatments. Lignin peroxidase activity was determined by the oxidation of dye Azure B in presence of H_2O_2 [25]. The reaction mixture contains 50 mM sodium tartrate buffer (pH 3.0), 32 μM Azure B, 500 μl of culture supernatant, 500 μl of H_2O_2 (2 μM), and reaction was monitored by measuring the changes in absorbance at 651 nm after 10 min. For manganese peroxidase activity measurement, reaction mixture consist the following: 500 μl culture supernatant, 500 μl of 1 mM phenol red, 100 μl of 25 mM sodium lactate, $MnS0_4$ (100 μM), and H_2O_2 (100 μM) in 1.0 ml of 20 mM sodium succinate buffer (pH 4.5). Reactions were carried out at 30˚C for 5 min and terminated with the addition of 2 N NaOH (40 μl) and absorbance was recorded at 610 nm [26]. Laccase activity was determined by the oxidation of 2,2´-azino-bis (3-ethylthiazoline-6-sulfonate) (ABTS) at 37˚C [27]. Reaction mixture (1 ml) contains the following: 600 μl culture supernatant, 300 μl sodium acetate buffer pH 5.0 (0.1 M), and 100 μl ABTS solution (1 mM). Oxidation reaction was measured at 420 nm wavelength for one minute.

2.7. Determination of cell growth

Growth of microbial cells in biomaterial and degradation medium as immobilized (mg/g) and free cells release (g/L), respectively, was determined at every 24 h of incubation period in T1 and T2 treatment according to Genisheva et al. [19]. For immobilized cell growth, approximately 1 g of biomaterial was separated from T1 and T2 treatments and placed in Erlenmeyer flask containing 20 ml of distilled water. The biomaterial was then separated, dried, and weighed until constant weight achieved. For estimation of free cells in the degradation medium, approximately 1 ml sample was extracted from T1 and T2 treatments, centrifuged at 10,000 rpm for 10 min and the recovered pallet was then suspended in 1 ml of distilled water and used for measuring the absorbance at 600 nm, which was correlated to a calibration curve (dry weight × absorbance).

2.8. Identification of metabolites

Culture samples (20 ml) were periodically withdrawn from control and treated black liquor and centrifuged at 10,000 rpm for 15 min to remove any microbial biomass. The obtained supernatants were then acidified to pH 2.0 by 1 N HCl and extracted with equal volume of ethyl acetate. For maximum extraction of aromatic compounds, the process was repeated three times. The collected organic layer was then dewatered over anhydrous sodium sulfate, filtered and vacuum dried. The obtained residues were derivatized with trimethylsilyl (TMS) using previously reported method [28]. The gas chromatography/mass spectroscopy (GC/MS) analysis was performed using the Advanced Instrumentation Research Facility (AIRF) of JNU, New Delhi. Identification of aromatic compounds and their degradation products was done by comparing the RT of obtained mass spectra with authentic compounds available at NIST-08 and Wiley-8 database libraries.

2.9. Statistical analysis

All experiments were conducted in triplicates and data were expressed as means ± standard error (S.E.) in tables and figures. Data was statistically analyzed by analysis of variance where the statistical significance of difference among the treatments was analyzed by SPSS statistical package (Statgraphics Plus V. 11) and expressed at 0.05 and 0.01 probability levels in appropriate places.

3. Results and discussion

3.1. Physicochemical characteristics of black liquor

The physicochemical characteristics of BL generated from century pulp and paper industry are presented in Table 1. Black liquor used in the present study showed following characteristics i.e. pH 10.2, color 9,732.80 Co–Pt, BOD: 3,285.61 (mg/L), COD: 8,928.57 (mg/L), TDS: 2,033.39 (mg/L), lignin: 2,584 (mg/L), Klason lignin: 2,130.6 (mg/L), acid soluble lignin: 129.4 (mg/L), ash content: 3.2%, carbohydrates: 0.41 (mg/g), chloride: 323 (mg/L),

Table 1
Physicochemical characteristics of black liquor used in the biodegradation study

Parameters	Untreated
pH	10.2 ± 0.37
Color (Co–Pt)	9,732.80 ± 72.52
BOD (mg/L)	3,285.61 ± 56.33
COD (mg/L)	8,928.57 ± 79.85
TDS (mg/L)	2,033.39 ± 40.21
Lignin (mg/L)	2,584 ± 69.36
Klason lignin (mg/L)	2,130.6 ± 82.49
Acid soluble lignin (mg/L)	129.4 ± 11.3
Ash content (%, w/w)	3.2 ± 0.36
Total carbohydrates (mg/g)	0.41 ± 0.67
Xylose (mg/g)	0.29 ± 0.03
Galactose (mg/g)	0.11 ± 0.01
Glucose (mg/g)	0.01 ± 0.01
Arabinose (mg/g)	ND
Mannose (mg/g)	ND
Chloride (mg/L)	323 ± 32.66
Chlorophenol (mg/L)	108 ± 15.40
Total phenol (mg/L)	499 ± 42.29
Total nitrogen (mg/L)	32 ± 6.70
Nitrate (mg/L)	57.8 ± 16.93
Sulfate (mg/L)	1,930 ± 27.16
Phosphate (mg/L)	29 ± 2.93
Sodium (mg/L)	160 ± 24.90
Potassium (mg/L)	25 ± 7.03
Cd (mg/L)	0.021 ± 0.005
Mn (mg/L)	0.1307 ± 0.037
Cu (mg/L)	0.1978 ± 0.061
Ni (mg/L)	0.1853 ± 0.043
Fe (mg/L)	1.3805 ± 0.096
Pb (mg/L)	0.0074 ± 0.012
Zn (mg/L)	0.2301 ± 0.053
Cr (mg/L)	0.0186 ± 0.033

Notes: Values are given as mean ± S.E.; ND = not detected.

chlorophenol: 108 (mg/L), total phenol: 499 (mg/L), total nitrogen: 32 (mg/L), nitrate: 57.8 (mg/L), sulfate: 1,930 (mg/L), phosphate: 29 (mg/L), sodium: 160 (mg/L) and potassium: 25 (mg/L) (Table 1). During the pulping process, high amount of sodium hydroxide and sodium sulfite are used that may contribute to the elevated levels of pH and sulfate in black liquor [29]. Various organic and inorganic compounds of lignin and its derivatives released during the pulp bleaching process contribute high COD in BL [30]. Heavy metals viz., Cd 0.021 (mg/L), Mn 0.1307 (mg/L), Cu 0.1978 (mg/L), Ni 0.1853 (mg/L), Fe 1.3805 (mg/L), Pb 0.0074 (mg/L), Zn 0.2301 (mg/L), and Cr 0.0186 (mg/L) in black liquor were also observed, but in trace amount.

3.2. Effect of environmental and nutritional conditions on biodegradation of black liquor

Various carbon (dextrose, malt extract, pulp, and wheat straw) and nitrogen (urea, ammonium nitrate, and sodium nitrate) sources were monitored for optimization of degradation process. Among all the tested carbon sources, dextrose was found to be the most desirable carbon source as compared to malt extract, wheat straw and pulp, which showed maximum reduction in color up to 3,351.68 Co–Pt (65.56%) and lignin up to 1,225.04 mg/L (52.59%) after 168 h of incubation period (Fig. 1(a)). Statistical analysis demonstrated that the process of decolorization and delignification were positively correlated with microbial growth and was significant at $p < 0.05$. Pulp was observed as relatively poorer carbon source allowing only 17.5% color and 13.9% lignin degradation with lower cell density (OD_{600}—1.18). Slow decolorization (4.6%) with least cell density (OD_{600}—1.05) was recorded in the absence of carbon source (control). The type of carbon requirement greatly varied with the organisms. Various studies have examined the effect of different carbon substrates on microbial decolorization of black liquor such as dextrose, sucrose, and glucose [11,14,15]. Among the various nitrogen sources, effective results were obtained from sodium nitrate with 68.27% (3,088.15 Co–Pt) reduction in color and 54.6% (1,174.17 mg/L) reduction in lignin (Fig. 1(a)). Lignin degradation is a nitrogen limiting process, but there have always been disputes for suitable nitrogen source for effective degradation. The enzymes responsible for lignin degradation are regulated by usable low nitrogen concentration in the surrounding, which stimulates the production of lignin degrading enzymes [31]. Earlier studies reported inorganic nitrogen sources to be more suitable for promoting ligninolytic enzymes production [32]. However, some studies suggested organic nitrogen forms to be more effective than inorganic nitrogen sources in lignin degradation [13,15]. In the present investigation, we used inorganic nitrogen source to make the process economically sustainable as organic source are costly and not easily available.

After selecting the most suitable carbon and nitrogen source, the effect of C:N ratio, temperature, pH, and agitation were optimized. It has been noticed that the C:N is better predictor of color and lignin degradation than the absolute amounts of carbon and nitrogen. The increase in nitrogen supply inhibits lignin degradation either by suppressing the ligninolytic system of micro-organisms or by generation of more recalcitrant compounds [33]. Therefore, supplements

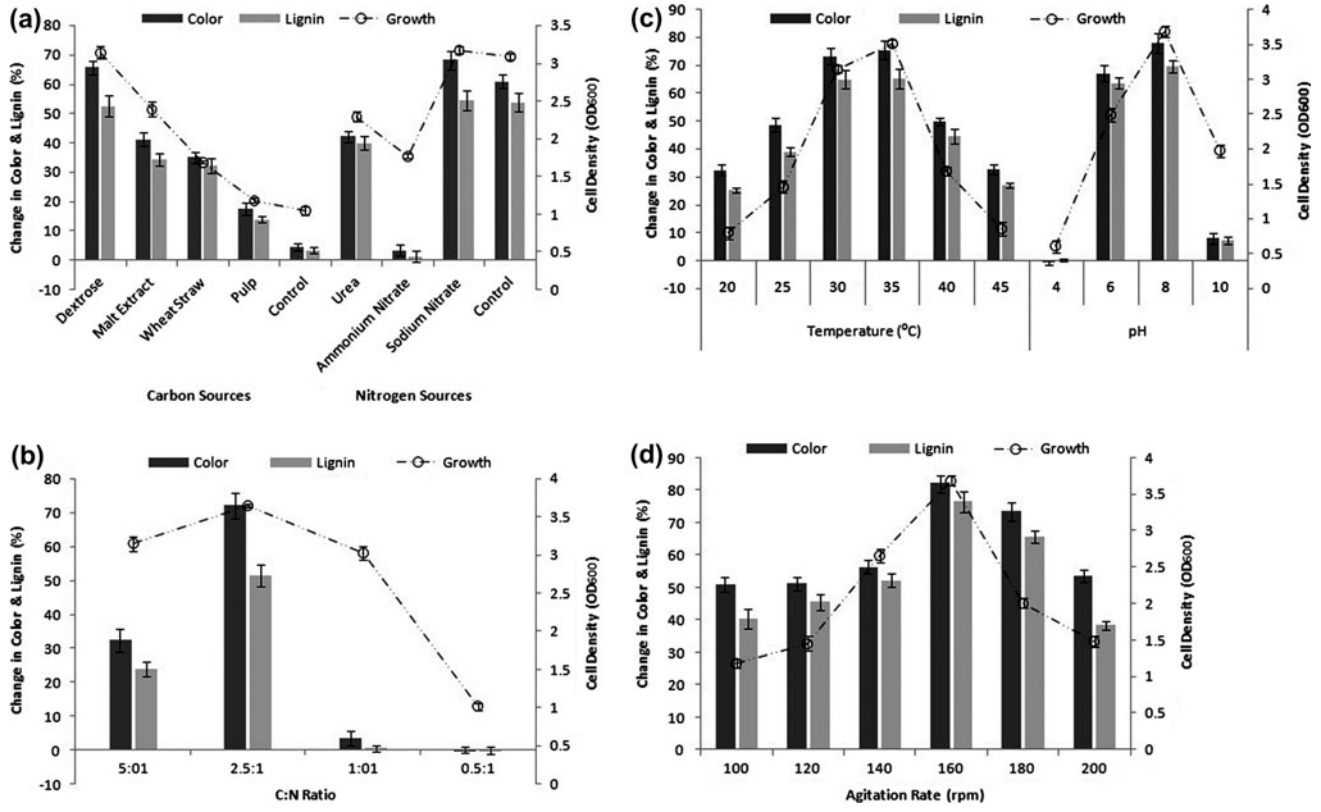

Fig. 1. Effect of different nutritional and environmental parameters on BL degradation process and growth of constructed consortium (a) nutritional parameters, (b) C:N ratio, (c) temperature and pH, and (d) agitation rate (values are means of triplicates, error bars shows ± S.E.).

of both carbon and nitrogen source is essential for efficient degradation. To optimize the carbon and nitrogen concentration (w/v), different ratio of C:N viz., 5:1, 2.5:1, 1:1, and 0.5:1 were undertaken. The ratio of 2.5:1 (i.e. 1%:0.4% of C:N) was observed to be most effective for reduction of color and lignin by bacterial consortium. Reduction in color and lignin of the effluent was recorded as 72% (2,714.54 Co–Pt) and 59.3% (1,051.5 mg/L), respectively, with cell density 3.643 at 600 nm after 168 h of incubation period (Fig. 1(b)). Incubation temperature is another important factor for regulation of microbial growth in degradation process. Maximum decolorization and delignification was recorded at 30 and 35˚C temperature with 2,389.53 Co–Pt (75.5%) and 900.72 mg/L (65.14%) at 35˚C. A sharp decline in decolorization and delignification was observed with increase in temperature beyond this temperature (Fig. 1(c)). The results show similar trends in earlier findings [14], who reported the optimum temperature of 35˚C for decolorization of pulp and paper mill effluent by *Bacillus* species.

pH 8.0 was found as optimum for decolorization and delignification of effluent (Fig. 1(c)). Optimum pH for bacterial decolorization and delignification of black liquor lies between 7.5 and 8.0 as presented in previous reports, such as 7.6 for *Aneurinibacillus aneurinilyticus* [28] and the corresponding values of pH for *Comamonas* sp. B-9 and *Cupriavidus basilensis* B-8 are 7.0 [9,10] and for *Bacillus* sp. is 7.6 [28]. Most suitable agitation rate was recorded as 160 rpm for decolorization and delignification by bacterial consortium. Maximum reduction was observed to be 82% in color and 76.3% in lignin (Fig. 1(d)).

3.3. Biodegradation of black liquor by immobilized bacterial consortium under optimized conditions

An increase in biodegradation was observed during the treatment of black liquor with co-immobilized cells under optimized condition. Dextrose as carbon; sodium nitrate as nitrogen; C:N ratio 2.5:1; temperature 35˚C; pH 8.0 and agitation rate 160 rpm were observed as optimum conditions that exhibit remarkable reduction in color, lignin, BOD, and COD up to 380 Co–Pt (96.1%), 219.64 mg/L (91.5%), 107.84 mg/L (96.7%), and 1,214.21 mg/L (86.4%), respectively, in contrast to control (T2 treatment), where the BL was

treated with biomaterial only (Fig. 2(a) and (b)). These changes can be attributed to the transformation of lignin by bacterial activities rather than biosorption on biomaterial. These observations are well in line with the previous findings of Malaviya and Rathore [34]. In the present investigation, reduction in pH value of degradation medium from 8.0 to near neutral was observed in T1 treatment (Fig. 2(a)). This can be attributed to the release of certain acidic compounds during bacterial metabolism. Aerobic degradation of chlorinated compounds like PCP results in release of hydrogen and chloride ions which indirectly contribute to lowering the pH value of degradation medium [35]. Bacterial metabolism also results in the release of hydrogen ions in degradation medium by active uptake of metals, which may result in reduction of pH levels [36]. Reduction in PCP concentration (from initial 108 mg/L to final 10.03 mg/L after 168 h) of BL was observed with the highest percent degradation (90.7%) of PCP (Fig. 3). Simultaneously, an increase in the release of chloride ions from initial 323 to 1,233 mg/L was recorded during biodegradation of

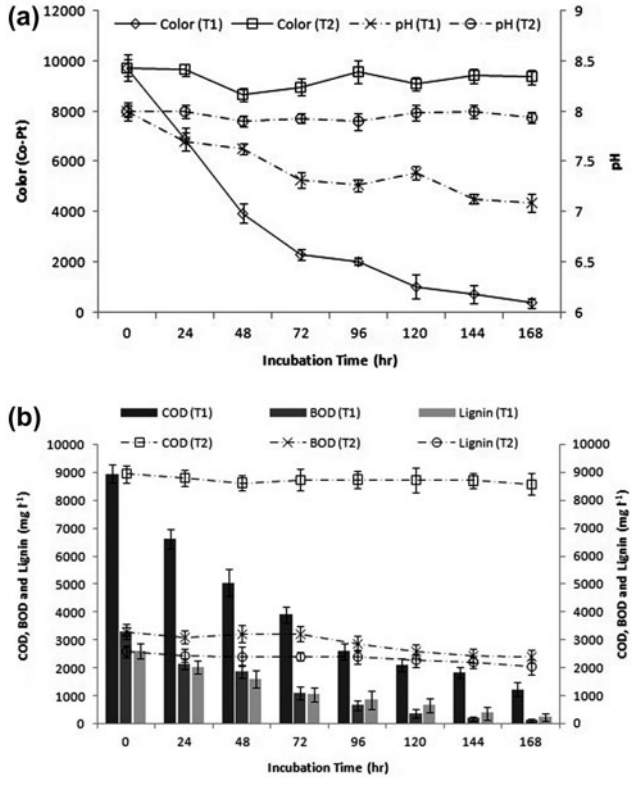

Fig. 2. Change in pollution parameters of black liquor during T1 (with immobilized bacterial consortium) and T2 (with biomaterial—control) treatment under optimum conditions (a) color and pH and (b) COD, BOD, and lignin (values are means of triplicates, error bars shows ± S.E.).

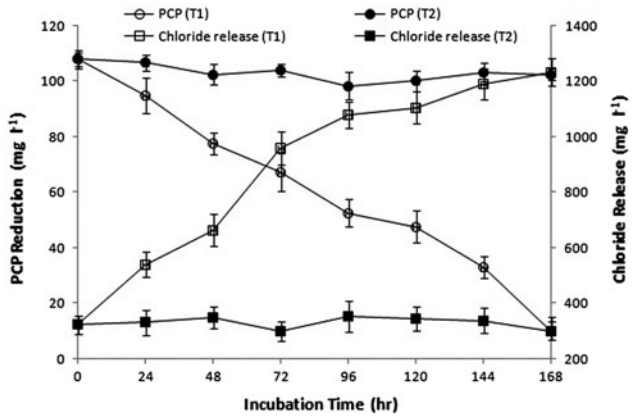

Fig. 3. Degradation of chlorophenol (PCP) and release of chloride during treatment of black liquor effluent by immobilized bacterial consortium (T1) and biomaterial (T2—control) (values are means of triplicates, error bars shows ± S.E.).

black liquor with co-immobilized bacterial consortium under optimum conditions (Fig. 3). Therefore, it can be stated that the two parameters were positively correlated ($R^2 = 0.95$) and significant at $p < 0.01$. Present findings can be easily corroborated with the previously reported work [4,37,38]. In order to compare the results of batch experiments, bioreactor study was carried out in packed bed column with all necessary arrangements. Significant reduction in color, lignin, BOD, COD, and PCP i.e. up to 214.1 Co–Pt (97.8%), 235.14 mg/L (90.9%), 167.5 mg/L (94.9%), 874.94 mg/L (90.23%), and 5.61 mg/L (94.8%) was observed during the treatment.

3.4. Ligninolytic enzymes activity and biomass formation

Ligninolytic enzymes activity was observed in culture supernatant during the degradation of BL, in T1, T2 (control), and bioreactor treatments at different incubation period. In the present study, a maximum of 6.94 U/mL, 9.35 U/mL, and 8.96 U/mL activities of LiP, MnP, and LAC at different time intervals viz., 120, 144, and 96 h, respectively, was recorded in T1 treatment (Table 2). Whereas, in T2 treatment (control) negative enzyme activity observed. Results of bioreactor study indicated that MnP (9.04 U/mL) was dominating enzyme followed by LAC (7.49 U/mL) and LiP (5.07 U/mL) at the end of treatment. Presence of ligninolytic activities in T1 and bioreactor treatments attributes the successful immobilization of bacterial consortium and the optimum conditions favors the increase in production of these enzymes as compared to our previously reported work [4]. The immobilized cell growth (biomass) and free cell release during the

Table 2
Ligninolytic enzyme activity during treatment of black liquor by co-immobilized bacterial consortium

	Estimation of enzymes (U/ml) during different treatment					
	LiP		MnP		LAC	
Incubation time (h)	T1	T2	T1	T2	T1	T2
24	2.31 ± 0.34	ND	3.41 ± 0.24	ND	5.12 ± 0.62	ND
48	3.08 ± 0.29	ND	4.72 ± 0.31	ND	5.85 ± 0.41	ND
72	4.92 ± 0.57	ND	6.09 ± 0.27	ND	7.99 ± 0.19	ND
96	6.16 ± 0.42	ND	7.92 ± 0.19	ND	8.96 ± 0.34	ND
120	6.94 ± 0.36	ND	8.53 ± 0.32	ND	8.93 ± 0.28	ND
144	6.72 ± 0.23	ND	9.35 ± 0.25	ND	8.96 ± 0.42	ND
168	6.45 ± 0.51	ND	8.91 ± 0.29	ND	8.84 ± 0.55	ND

Notes: Values are given as mean ± S.E. ND = not detected.

treatment process at different time intervals is shown in Fig. 4. A maximum of 13.83 mg/g of immobilized biomass was observed in T1 treatment after 168 h of incubation period. Whereas, an uneven change (release) of free cell concentration in degradation medium was recorded during the treatment hours. The two parameters significantly differs ($p < 0.05$) with each other. No biomass formation was observed in T2 treatment (control) (Fig. 4), where the degradation medium was inoculated with corncob cubes (biomaterial) without bacterial consortium. These results demonstrated that proper sterile conditions were successfully maintained and degradation of black liquor was done by bacterial activities. The voids present in biomaterial provide enough space for cell immobilization; therefore, immobilization process increases the biomass productions which indirectly improve the biodegradation of black liquor [4].

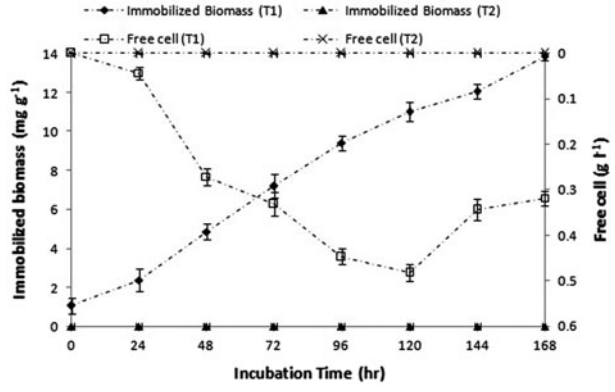

Fig. 4. Immobilized biomass formation and free cells released during the treatment of black liquor by co-immobilized cells (T1) and biomaterial (T2—control) (values are means of triplicates, error bars shows ± S.E.).

3.5. Identification of metabolites

The compounds present in untreated- and bacterial-treated BL samples were analyzed by GC/MS at different time intervals. The total ion chromatograph (TIC) of TMS derivatives of untreated and treated BL of different durations (0, 72, and 168 h) are shown in Fig. 5(a)–(c), respectively. GC/MS analysis indicated that major peaks are detected in the untreated (0 h) sample either disappeared or reduced after biodegradation. Some new but relatively small peaks in 72 and 168 h TIC of treated sample indicated production of metabolic products (Fig. 5(b) and (c)). The locations of the compounds observed in the TIC were identified by comparing with the available standards of the authentic compounds documented in NIST-08 and Wiley8 libraries (Table 3).

The major peaks detected in the BL samples at 0 h were identified as, erythro-pentonic acid (RT 10.352 min), arabino-hexonic acid (RT 10.699 min), 2-phenyl-3-((p-methoxyphenyl)amino)-3-phenylisoindolinone (RT 11.833 min), methylhydroquinon (RT 12.554 min), 3-vanillyl propanol (RT 13.345 min), 4,5,6,7-tetrahydroxy-1,8,8,9-tetramethyl-8,9-dihydrophenaleno[1,2-b]furan-3-one, (RT 14.358 min), dibutyl phthalate (RT 14.700 min), 1,4-dimethyl-1,2,3,4-tetrahydronaphthalene (RT 15.038 min), phthalic acid (RT 20.162 min), and 4H-1-benzopyran-4-one (RT 27.516 min) (Fig. 5(a)). Erythro-pentonic acid (isosaccharinic acid) and arabino-hexonic acid are sugar acid which may result by the degradation of carbohydrates in black liquor. 4H-1-benzopyran-4-one is an isomer of coumarin and a monomer unit of lignin [4]. Dibutyl phthalate is a phthalate derivative usually leach polyvinyl chloride goods and are suspected teratogens and endocrine disruptor, thus a major class of industrial contaminant [39], but phthalates may also be formed during microbial degradation of poly aromatic

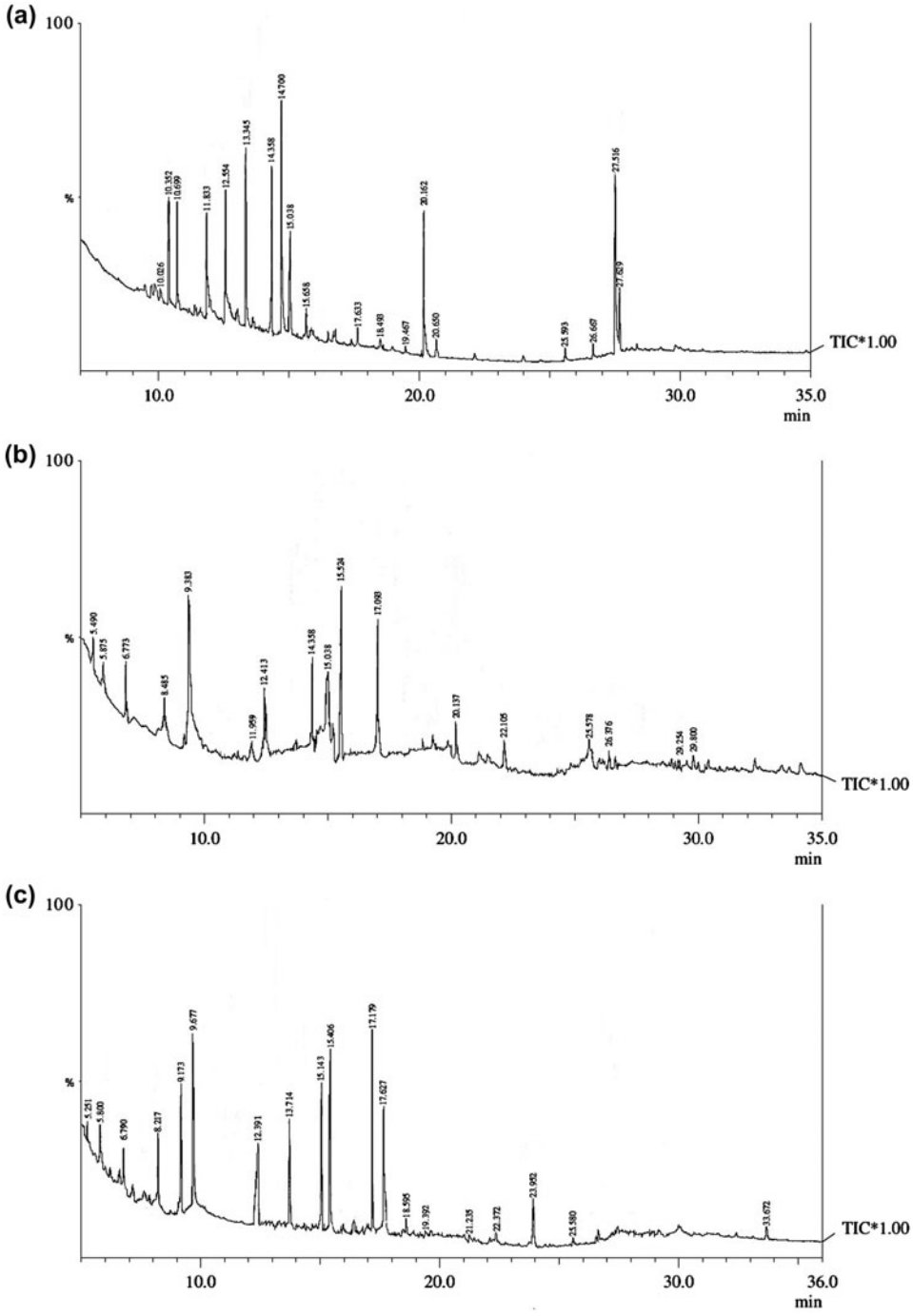

Fig. 5. Total ion chromatogram (TIC) of compounds extracted in ethyl acetate from black liquor at different time interval of treatment (a) 0 h, (b) 72 h, and (c) 168 h sample.

compounds [40]. Disappearance of dibutyl phthalate peak after 72 h of treatment confirms the detoxification of BL under optimum conditions (Fig. 5(b)). Presence of acidic compounds in the TIC of 72 and 168 h of bacterial-treated BL samples may be attributed to oxidative degradation of phenolic derivatives of lignin. During present investigation certain organic compounds (ferulic acid—RT 23.952 min and cinnamic acid—RT 17.627 min) of commercial importance were also observed in TIC of 168 h treatment of BL. Both ferulic and cinnamic acids are commercially important for their applications in food, flavor, and

Table 3
Compounds identified by GC/MS analysis of black liquor at different time interval of treatment

Identified compound	Retention time (min)	0 h	72 h	168 h
Benzeneacetic acid	5.490	−	+	+
Benzaldehyde	5.875	−	+	+
3-Butenoic acid	6.773	−	+	+
4-Hydroxy-benzoic acid	8.485	−	+	+
Hexadecanoic acid	9.383	−	+	+
3-Hydroxy-4-methoxybenzaldehyde	9.677	−	−	+
Erythro-pentonic acid	10.352	+	−	−
Arabino-hexonic acid	10.699	+	−	−
2-Phenyl-3-((p-methoxyphenyl)amino)-3-phenylisoindolinone	11.833	+	+	−
Propanedioic acid	12.413	−	+	+
Methylhydroquinone	12.554	+	−	−
3-Vanillyl propanol	13.345	+	−	−
1,2-Benzenedicarboxylic acid	13.714	−	−	+
4,5,6,7-Tetrahydroxy-1,8,8,9-tetramethyl-8,9-dihydrophenaleno[1,2-b]furan-3-one	14.358	+	+	-
Dibutyl phthalate	14.700	+	−	−
1,4-Dimethyl-1,2,3,4-tetrahydronaphthalene	15.038	+	+	−
1,6-Heptadiene	15.143	−	−	+
Octadecanoic acid	15.524	−	+	+
Nonadecanoic acid	17.093	−	+	+
Cinnamic acid	17.627	−	−	+
Phthalic acid	20.162	+	+	−
Ferulic acid	23.952	−	−	+
4H-1-benzopyran-4-one	27.516	+	−	−

Notes: +: Present; −: absent.

pharmaceutical industries. Formations of acidic compounds during bacterial biodegradation of lignin were also earlier reported by Chandra et al. [13] and Shi et al. [41]. GC/MS analysis therefore confirmed that immobilized bacterial consortium successfully biotransformed the BL samples under optimized conditions.

4. Conclusion

This investigation demonstrates that novel bacterial consortium possesses remarkable ability for degradation and detoxification of BL at optimum conditions. Optimization of nutritional and environmental parameters simply enhanced the decolorization efficiency of co-immobilized consortium by maintaining the immobilized cells and their ligninolytic activities. Results of bioreactor study provide a reasonable basis for the process evaluation of consortium based biological treatment on large scale. Degradation of toxic chemical compounds and generation of commercially important organic compounds manifest that the novel consortium possesses immense capacity for its applications in treatment of industrial effluent and also in production of high value products. The observations of present study could be further explored by studying the effect of continuous operation on cells and molecular changes occurred in the bacterial cells after immobilization.

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