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Detection of persistent organic compounds from biomethanated distillery spent wash (BMDS) and their degradation by manganese peroxidase and laccase producing bacterial strains

Sangeeta Yadav and Ram Chandra*

Department of Environmental Microbiology, School for Environmental Sciences, Babasaheb Bhimrao Ambedkar University, Lucknow-226 025, India

**Corresponding Author email : rc_microitrc@yahoo.co.in*

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Abstract

Biomethanated distillery spent wash (BMDS) retains dark black colour with complex persistent organic pollutants even after anaerobic treatment. The specific ratio (4:3:1:1) of *Proteus mirabilis* (FJ581028), *Bacillus sp.* (FJ581030), *Raoultella planticola* (GU329705) and *Enterobacter sakazakii* (FJ581031) decolourised BMDS up to 76% within 192 hr along with degradation of persistent organic compounds in presence of glucose (1%) and peptone (0.1%). The colour removal ability was noted due to ligninolytic enzyme activity. Where the maximum manganese peroxidase was 1.93 U ml^{-1} and laccase activity equalled 0.84 U ml^{-1}. The gas chromatography–mass spectrophotometry (GC–MS) analysis confirmed the direct correlation between colourant and persistent organic pollutants due to simultaneous reduction of colour and pollutants present in BMDS. The seed germination test showed reduction of 75% toxicity after bacterial treatment process.

Key words

Degradation, Distillery effluent, GC-MS analysis, Metabolites, Phenolic

Introduction

Sugarcane molasses based distilleries are among the most polluting industries generating an average of 12–15l of spent wash for per liter of alcohol produced. There are about 319 distilleries in India, alone producing 3.25 x 10^9 l of alcohol and generating 40.40 x 10^{10} l of wastewater annually (Bharagava *et al.,* 2009). The biomethanated distillery spent wash (BMDS) become more intense, dark colour and viscous after conversion of degradable organic component into biogas and interaction of various pollutants (phenolics, sulphate, heavy metals, melanoidins etc) during anaerobic treatment. Though, about 2% melanoidin known as major colourant remains in the BMDS. Consequently, BMDS causes soil and water pollution due to high biological oxygen demand, chemical oxygen demand, total dissolved solids, phenolics, sulphate and phosphate (Bharagava *et al.,* 2008). In soil, it inhibits seed germination and reduces soil alkalinity as well as manganese availability (Rajasundari and Murugesan, 2011), whereas in aquatic system it reduces the penetration of sunlight which leads low oxygenation of water by reducing photosynthesis (Kumar and Chandra, 2006; Bharagava *et al.,* 2008). Ramakritinan *et al.* (2005) reported that respiratory processes in *C. carpio* due to distillery wastewater pollution induce towards anaerobiosis at organ level during the sublethal intoxication. Though, the treatment of distillery wastewater by physical or chemical methods such as ozonation, flocculation and activated carbon adsorption has been reported (Kim *et al.,* 1985; Migo *et al.,* 1997; Chandra and Singh, 1999; Chandra and Pandey, 2001), but it is not found feasible due to high cost and generation of secondary pollutants. It is also difficult to treat it by conventional biological treatment methods because melanoidins have antioxidant properties that render

them toxic to aquatic macro and microorganisms (Kumar *et al.,* 1997; Sirianuntapiboon *et al.,* 2004a; Chandra *et al.,* 2008). Moreover, most of the study has been focused only on the reduction of colour by measuring the absorbance at 475 nm, which is an absorption maxima of melanoidin. However, the information regarding the nature and fate of other pollutants on decolourisation of BMDS is unknown.

Biological decolourisation of melanoidins is mediated by non specific ligninolytic enzymes: manganese peroxidase and laccase. Though, the MnP and laccase have been predominantly reported in fungi (Kumar *et al.,* 1998; Raghukumar *et al.,* 2004; Pant and Adholeya, 2010), but at large scale application have limitations due to slow growth rate, unfavorable submerged aquatic environment and low pH range. Besides, some bacteria *Alkaligenes*, *Bacillus sp.*, *Lactobacillus hilgardii* W-NS, *Proteus mirabilis, Pseudomonas aeruginosa* PAO1, *Pseudomonas sp.* and *Acetogenic* have also been reported for melanoidins degradation (Kumar and Chandra, 2006; Mohana *et al.,* 2007). Recently few bacteria have been reported for the metabolization of melanoidins by MnP activity (Bharagava *et al.,* 2009). The objectives of this study were to identify the persistent organic compounds before and after decolourisation of BMDS by the bacterial consortium. Further, the role of MnP and laccase during the bacterial growth and BMDS decolourisation was also investigated.

Materials and Methods

The biomethanated distillery spent wash (BMDS) was collected from M/S Unnao Distillery and Brewery Ltd., Unnao (UP), India.

Quantitative evaluation of colour reduction from BMDS: The changes in pollution parameters [colour, biological oxygen demand (BOD), chemical oxygen demand (COD), total solids (TS), sulphate, nitrogen, phenol and phosphate] of BMDS before and after bacterial degradation were analyzed as per standard methods of APHA (2005). Nitrate, chloride ion and pH were determined using an ion meter (Orion autoanalyser model-960) by their respective ion electrode. The dissolved oxygen (DO) concentration in wastewater was monitored with a BOD probe and dissolved oxygen meter (Orion 835 A, Thermo Electron Corporation, USA). To measure the colour intensity of bacterial treated BMDS, bacterial biomass was separated from BMDS amended culture suspension after centrifugation at 5000 rpm for 10 min. The supernatant was taken and absorbance was measured at 475 nm. The percent decolourisation was expressed as the degree of decrease in absorbance at 475 nm against initial absorbance at the same wavelength. Major ions such as sulfate, phenol, phosphate were analysed by ion meter (Orion autoanalyser model-960) by using their respective selective electrode.

Optimization, degradation and decolourisation: To observe the optimum nutrient condition for decolourisation of BMDS by bacterial consortium, effect of different carbon (1%) and inorganic and organic nitrogen (0.1%) sources were observed along with basal salt media (KH_2PO_4, 0.1%; $MgSO_4 \cdot 7H_2O$, 0.05%) amended with BMDS (1200 ppm) by consortium having different inocula ratio of bacterial strains. The effect of variable glucose (1-1.5%) and peptone (0.1-0.3%) concentrations were also observed to explore the maximum decolourisation. In addition, the effect of the mixture of glucose, peptone along with other organic and inorganic nitrogen sources was also studied as shown in Table 1. Further, to investigate the maximum decolourisation of BMDS in presence of screened nutrient condition, effect of inocula size (2, 4, 6, 8 and 10%), shaking speed (100, 140, 180 and 220 rpm) and pH (4, 7, 10 and 13) was also studied. Similarly, effect of different concentrations of BMDS (600, 800, 1000, 1200 and 1400 ppm) on bacterial decolourisation was also evaluated.

After optimization of inoculum size, 8% of overnight grown culture of *Proteus mirabilis* (IITRM5; FJ581028), *Bacillus sp.* (IITRM7; FJ581030), *Raoultella planticola* (IITRM15; GU329705) and *Enterobacter sakazakii* (IITRM16; FJ581031) were inoculated in the 250 ml Erlenmeyer shaking flask with ratio of 4 : 3 : 1 : 1 at cell density of 3.50×10^4 cells ml^{-1}. These flasks containing glucose (1%), peptone (0.1%), yeast extract (0.1%), K_2HPO_4 (0.1%), $MgSO_4 \cdot 7H_2O$ (0.05%) and 1200 ppm of BMDS incubated at 35±2 °C in a rotary shaking incubator (Innova 4230, New Brunswick Scientific, UK) at 180 rpm for 8 days. Further, the growth and decolourisation was monitored spectrophotometrically (UV 2300 Spectrophotometer, Techcomp, Korea) by taking absorbance at 620 and 475 nm, respectively.

Ligninolytic enzymes activity during decolourisation of BMDS: To determine the production of MnP and laccase activity during the BMDS decolourisation at optimized condition, the culture supernatant was centrifuged at 5000 rpm for 10 min. The MnP and laccase activity was determined by the oxidation of phenol red (BDH Ltd.) and guaiacol, respectively (Miyata *et al.,* 2000; Bharagava *et al.,* 2009). For MnP activity, 5 ml of reaction mixture contained 1 ml sodium succinate buffer (50 mM, pH 4.5), 1 ml sodium lactate (50 mM, pH 5), 0.4 ml manganese sulphate (0.1 mM), 0.7 ml phenol red (0.1 mM), 0.4 ml H_2O_2 (50 mM), gelatin 1 mg ml^{-1} and 0.5 ml of enzyme extract. The reaction was initiated at 30°C by the addition of H_2O_2 and the rate of Mn^{3+}-malonate complex formation was monitored every minute by measuring the increase in absorbance at 610 nm. One milliliter of reaction mixture was taken from above solution and 40 ml of 5 N NaOH was added to stop the reaction. After every minute the same steps were repeated with 1 ml of the reaction mixture up to 4 min. One unit of enzyme activity is equivalent to an

absorbance increase of 0.1 unit min^{-1} ml^{-1} (Arora *et al.,* 2002). While, laccase reaction mixture containing 3.8 ml acetate buffer (10 mM, pH 5), 1 ml guaiacol (2 mM) and 0.2 ml of enzyme extract was incubated at 25ºC for 2 hr. The absorbance was read at 450 nm using spectrophotometer (Techcomp, UV-2300 spectrophotometer, Korea). Laccase activity was expressed as colorimetric unit ml^{-1} (CU ml^{-1}).

Analysis of BMDS degradation: To assess the degradability of BMDS, the TLC was done in the supernatant of control and bacterial treated BMDS obtained after centrifugation at 5000 rpm for 10 min. The sample was concentrated under vacuum condition and the dry residue obtained was dissolved in 10 ml acetone. Further, these samples were spotted on silica gel pre-coated chromatographic plate column (120 mesh; 75 x 3.2 cm; Gel 60/UV_{254}, S.D. fine chemical limited, India). The solvent system comprising acetic acid : chloroform : methanol : water (10 : 9 : 9 : 10 by volume) was used for separation of compounds. It was further observed under visible light through gel documentation system (Syngene, 230V-2A, UK). Further, the degradation of BMDS was confirmed through HPLC analysis. The bacterial decolourised and control samples of BMDS was analyzed by Waters, 515 HPLC system equipped with reverse phase column C-18 (250 mm x 4.6, particle size 5 µm) at 27°C and 2487 UV/VIS detector via millennium software. The control and degraded samples (20 µl) were injected into HPLC and monitored at wavelength 290 nm to assess the degradation of colourant (Bharagava *et al.,* 2009). Acetonitrile and water in the ratio of 70 : 30 (v/v) was used as mobile phase and flow rate was set at 1 ml min^{-1} in isocratic mode (Bharagava *et al.,* 2009; Chandra and Abhishek, 2011).

Analysis of persistent organic compounds : The persistent organic compounds present in control and degraded BMDS were extracted by ethyl acetate at acidic condition (pH< 2) as described previously (Bharagava and Chandra, 2009). For extraction of organic compounds, 25 ml of bacterial degraded and control (without bacterial inoculum) BMDS was acidified (pH 2.0) with 37% hydrochloric acid and then mixed with equal volume of ethyl acetate, the mixture was shaken continuously for 4 hr with intermittent rest for liquid-liquid extraction. The extraction was repeated successively three times to complete extraction of phenolics compounds as described earlier (Minuti *et al.,* 2006). The ethyl acetate was evaporated under vacuum and the dry residue obtained was dissolved in 10 ml acetone.

The above solution was derivatized with trimethylsilyl (TMS). In this method, 100 µl dioxane and 10 µl pyridine was added to samples followed by silylation with 50 µl trimethyl silyl [BSTFA (N, O-bis (trimethylsilyl) trifluoroacetamide) and TMCS (trimethyl chlorosilane)]. The mixture was heated at 60ºC for 15 min with periodic shaking to dissolve residues. An aliquot (2 µl) of silylated samples were injected in GC–MS (PerkinElmer, UK) equipped with a PE auto system XL gas chromatograph interfaced with a Turbomass mass spectrometric mass selective detector. The analytical column connected to system was a PE-5MS capillary column (length 20 m x 0.18 mm i.d, 0.18 µm film thickness). Helium gas was used as carrier gas with flow rate of 1 ml min^{-1}. The column temperature was programmed as 50 ºC (5 min); 50–300 ºC (10ºC min^{-1}, hold time: 5 min). The transfer line and ion source temperatures were maintained at 200 and 250ºC, respectively. A solvent delay of 3.0 min was selected. The chromatographic run time was maintained upto 35 min. In full-scan mode, the electron ionization (EI) mass spectra were recorded in range of 30–550 (m/z) at 70 eV. The organic pollutants and metabolic products were identified by comparing their mass spectra with that of National Institute of Standards and Technology (NIST) library available with instrument and by comparing the retention time with those of available authentic organic compounds (Chandra and Abhishek, 2011).

Toxicity evaluation of bacterial treatment and untreated BMDS : To evaluate the toxicity reduction of bacterial decolourised and control BMDS, the seed germination test with *Vicia faba* was conducted as per methods described by Nagda *et al.* (2006). The percent seed germination and α–amylase enzyme activity was evaluated in healthy seed of *V. faba* treated with different concentration of bacterial treated and untreated BMDS (0, 10, 25, 50, 75 and 100%). For the preparation of enzyme extract, twenty seeds from each treatment were homogenized with 0.1M sodium acetate buffer (pH 4.8), filtered through two layers of cheese cloth to remove large particles and the supernatant obtained was centrifuged at 15,000×g for 20 min. All the preparations were carried out at 4°C. The supernatant obtained was used as crude enzyme extract for α-amylase assay. For enzyme assay, the reaction medium (3 ml) contained 1ml of 0.1M acetate buffer, pH 4.8, 0.5 ml of enzyme extract diluted to 1ml using acetate buffer, and 1ml of 0.1% soluble starch solution. The enzyme extract was diluted to obtain an absorbance change of less than one during the enzyme assay. The reaction medium was incubated for 10 min at room temperature and then the reaction was stopped by adding 1ml of 0.1% iodine reagent and 3ml of 0.05N HCl. The absorbance was measured at 620nm and decrease in absorbance was expressed in terms of amylase activity (Bharagava and Chandra, 2010). The induction of α–amylase was also detected from purified enzyme by denaturing SDS-PAGE performed on 10% polyacrylamide gel.

Statistical analysis: To confirm the variability and validity of results, the data obtained was mean of triplicate which was subjected to statistical analysis using one-way analysis of variance (ANOVA) to make comparison between more than two means followed by Tukey's test using the Graph Pad software (Graph Pad Software, San Diego, Calif.) (Ott, 1984).

Results and Discussion

Decolourisation of BMDS: To explore the best carbon and nitrogen sources as nutrient for optimized bacterial consortium during the decolourisation of BMDS revealed that glucose (1%) supported maximum decolourisation as compared to other carbon sources (Table 1). The consortium could decolourise the BMDS up to 47% only in presence of 1% glucose. The order of different sugars for decolourisation were: glucose > sucrose > xylose > fructose = lactose > maltose > ribose (Table 1). Results revealed that bacterial consortium preferentially utilized the glucose for energy source. The increase of glucose concentration beyond 1% does not improve the decolourisation process. This might be due to formation of excess gluconic acid with increase of glucose concentration. Moreover, optimal glucose at 1% probably generate more redox mediators that acted as electron donors for the reduction of conjugated C=C and C=N bonds which impart the colour. In addition, the effects of different organic and inorganic nitrogen sources along with glucose showed that the organic nitrogen sources showed maximum decolourisation compared to inorganic at 0.1% concentration. This might be due to the direct utilization of organic nitrogen for biosynthesis of bacterial cell organelles which leads to the cell multiplication and caused the decolourisation of BMDS. In contrast, the inorganic nitrogen which mimicked the bacterial growth and decolourisation process of BMDS. This indicated that the

Table 1 : Effect of carbon, nitrogen and inoculums ratio on percent decolourisation of BMDS

Different nutrient	Inoculum ratio (IITRM5:IITRM7:IITRM15:IITRM16)					
	1:1:1:1	3:2:2:1	2:2:3:1	3:3:2:1	4:3:2:1	4:3:1:1*
Different carbon sources (1%)						
Glucose (G)	28±1.40	30±1.21	32±2.00	34±0.93	38±1.11	47±0.90
Sucrose (S)	33±1.05	31±1.76	33±1.53	33±0.35	36±1.00	39±0.82
Fructose (F)	18±0.82	17±0.99	19±0.95	21±0.76	20±0.53	29±0.40
Maltose (M)	21±0.76	20±0.75	23±2.31	19±0.12	26±0.34	27±0.50
Lactose (L)	18±0.45	19±0.86	20±0.84	21±0.19	24±0.46	29±0.43
Xylose (X)	20±0.33	22±1.65	27±1.20	30±0.26	31±0.52	32±0.33
Arabinose (A)	16±0.28	16±0.23	19±1.98	22±0.48	21±0.40	23±0.45
Ribose (R)	15±0.15	17±0.18	21±0.85	22±0.45	24±0.38	26±0.40
Different nitrogen sources (0.1%)						
G + Peptone (P)	31±1.23	33±1.18	36±1.56	37±1.65	38±0.78	50±0.58
G + Yeast Ext (Y)	28±0.54	29±2.87	32±1.78	36±1.45	35±1.45	46±0.83
G + Beef Ext (BE)	15±0.21	16±0.54	21±0.75	22±0.56	23±0.75	25±0.32
G + Urea	16±0.21	18±0.54	20±0.75	24±0.56	28±0.75	30±0.32
G + $NaNO_3$	11±0.01	13±0.78	15±0.82	18±0.34	19±0.54	21±0.40
G + NH_4Cl	14±0.21	15±0.74	16±0.11	18±0.30	20±0.43	23±0.48
G + $(NH_4)_2SO_4$	12±0.21	14±0.74	19±0.11	18±0.30	22±0.43	26±0.48
Effect of glucose concentration (%)						
G (0.1) + P (0.1)	08±0.40	09±0.21	10±0.14	11±0.93	10±0.11	11±0.40
G (0.5) + P (0.1)	14±0.34	15±0.41	17±0.46	18±0.32	20±0.31	24±0.90
G (1.5) + P (0.1)	15±0.21	17±0.32	19±0.28	20±0.52	22±0.37	26±0.44
Effect of peptone (%)						
G (1.0) + P (0.2)	31±1.23	34±1.21	37±1.10	38±0.66	39±1.21	51±1.34
G (1.0) + P (0.3)	32±1.00	33±1.43	38±1.84	39±1.11	41±1.44	52±0.82
G (1.0) + P (0.4)	32±1.76	34±1.35	39±1.45	40±1.93	42±2.01	53±1.28
Effect of glucose and nitrogen concentration in combination (%)						
G (1.0) + P (0.1) + Y (0.1)*	41±1.11	41±2.10	44±1.34	47±0.51	49±1.23	58±0.47
G (1.0) + P (0.1) + BE (0.1)	33±1.23	35±1.18	39±1.56	40±1.65	42±0.78	43±0.58
G(1.0) + P (0.1) + Urea (0.1)	30±0.34	28±0.54	30±0.22	32±1.00	35±0.99	40±0.47
G (1.0) + P (0.1) + $NaNO_3$ (0.1)	26±0.78	28±0.33	30±0.64	30±1.21	31±0.83	36±0.37
G(1.0) + P (0.1) + NH_4Cl (0.1)	27±0.84	29±0.73	31±0.47	33±1.35	34±1.83	37±1.74
G(1.0) + P (0.1) + $(NH_4)_2SO_4$ (0.1)	28±0.74	27±0.45	32±0.44	28±1.03	36±1.02	38±1.47

* : optimized conditions; Inoculum ratio = IITRM5:IITRM7:IITRM15:IITRM16 = *Proteus mirabilis* : *Bacillus sp.* : *Raoultella planticola* : *Enterobacter sakazakii*

Table 2 : Evaluation of the quantitative reduction of colour and other pollution parameters of BMDS before and after bacterial treatment

Parameters	Control	Bacterial treated		% Reduction
		4 days	8 days	
Colour appearance	Dark Black	Light brown	Light brown	
pH	8.00±0.32	6.50±0.33*	7.00±0.21*	12.51
Colour	115000±3450	38400±806*	27600±465*	76.00
BOD	12000±360	7284±124*	6452±167*	46.23
DO	5.00±0.23	2.00±0.06*	8.00±0.32*	-60.00
COD	40,000±387	19500±431*	12000±311*	70.00
TSS	28810±365	10475±345*	9870±167*	65.74
TDS	16542±264	10256±278*	8358±168*	49.74
TS	46516±673	18547±649*	16829±333*	63.82
TOC	7.16±0.35	5.54±0.86*	4.46±0.24*	37.40
Sulphate	2654±65.87	2045±68.45*	1254±36,85*	52.75
Phenol	437±14.85	213±8.52*	87±3.48*	80.00
Phosphate	1218±35.57	965±21.73*	745±35.76*	38.83
Potassium	410±18.78	60.97±2.11*	58.62±1.11*	85.70
Chloride	415±15.51	550±25.54*	701±21.35*	- 68.00
Sodium	54.12±1.34	30.56±0.61*	29.66±0.95*	45.19
Nitrate	21.07±0.63	17.58±0.60*	19.86±0.61ns	5.74
Heavy metals				
Copper	1.29±0.04	0.19±0.007*	0.11±0.006*	91.47
Cadmium	0.12±003	0.002±0.00*	0.001±0.00*	99.16
Zinc	11.24±0.56	0.90±0.06*	0.74±0.01*	93.41
Iron	50.64±2.53	29.45±0.94*	10.95±0.43*	78.38
Nickel	0.12±0.003	0.09±0.004*	0.04±0.003*	66.66
Manganese	2.94±0.14	1.92±0.23*	0.62±0.03*	78.91

All the values are means of three replicates ± SD. All values are in mg l^{-1} except for colour (Co–Pt) and pH. Negative sign shows, values increased after 8 days bacterial treatment. Statistical significance between the values of control to bacterial treated BMDS at different time in column was evaluated by ANOVA. Significance levels: *= p <0.001

bacteria require more energy and time in synthesizing amino acids for protein synthesis from inorganic sources (Kalil *et al.,* 2008). The 58% decolourisation of BMDS (1200 ppm) was noted optimum in media containing glucose (1%), peptone (0.1%), yeast extract (0.1%) by bacterial consortium having inoculum ratio IITRM5 (4) : IITRM7 (3) : IITRM15 (1) : IITRM16 (1) at 8% inoculum size (Table 1). This bacterial composition (4 : 3 : 1 : 1) might be effective for metabolisation of the BMDS. However, separate triplicate flasks were also inoculated with optimized inoculum ratio without addition of carbon and nitrogen sources. No significant bacterial growth and BMDS decolourisation was observed, this indicated that the carbon and nitrogen present in BMDS is not in bioavailable form therefore bacterial consortium could not utilize it. Hence, supplementary carbon (1% glucose) and nitrogen (0.1% peptone) was essential for bacterial growth. The presence of yeast extract (0.1%) with glucose and peptone enhanced the bacterial growth and decolourisation process (Table 1). The use of glucose and peptone as co-substrate for decolourisation of dye by bacteria has also been reported in previous study (Jirasripongpun *et al.,* 2007). The requirement of supplementary glucose, peptone and yeast extract as carbon and nitrogen sources has also been reported for decolourisation of anaerobically digested distillery effluent by other workers (Sirianuntapiboon *et al.,* 2004b; Mohana *et al.,* 2007). But, this is maximum concentration of BMDS and maximum level of decolourisation.

The maximum decolourisation with optimized inoculum ratio (4 : 3 : 1 : 1) was noted at lower concentration (600 ppm). However, there was a decrease in decolourisation with increase in BMDS concentration (800–1400 ppm) at 8% inoculum size (Fig. 1a). The significant decolourisation of BMDS was noted up to 1000 ppm with 8% inoculum size of bacterial consortium within 96 hr of incubation, further increased concentration mimicked the ability of consortium for BMDS decolourisation. This might be due to apparently because of the presence of high concentration of persistent organic compounds such as polyphenolics either alone or in the condensed forms with melanoidins having microbial growth inhibitory characteristics (Vahabzadeh *et al.,* 2004). In addition, BMDS decolourisation was further noted at high concentration with increase of inoculum size (2 to 10%). The optimum decolourisation (66%) of BMDS was noted at 1200 ppm by 8% bacterial consortium having strain ratio 4 : 3 : 1 : 1 within 168 hr incubation. But, inoculum size beyond 8% did not improve the BMDS decolourisation (Fig. 1b). This might be due to the requirement of more energy for bacterial cell multiplication at large inoculum size

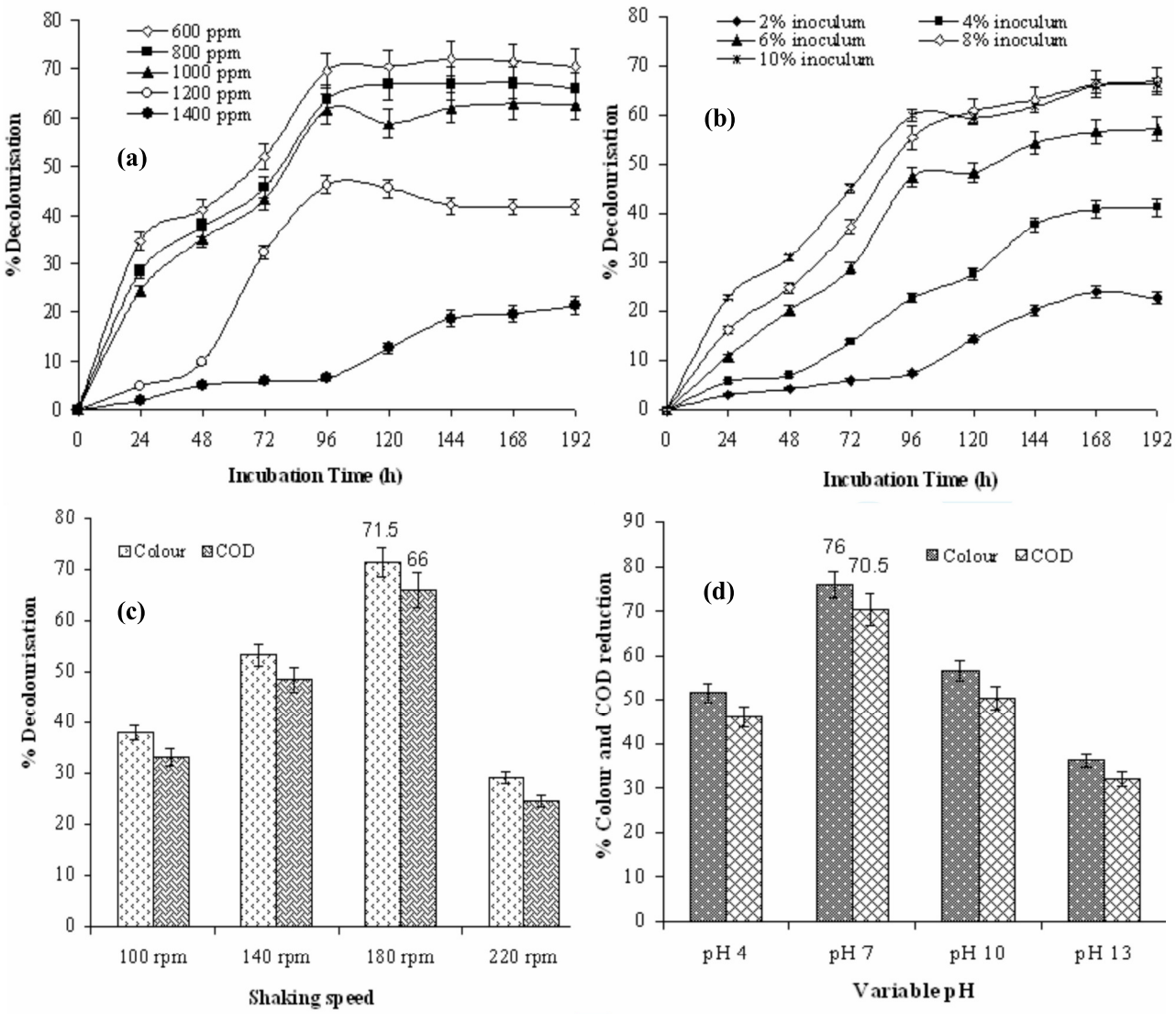

Fig. 1 : Effect of (a) BMDS concentrations, (b) inoculum size, (c) shaking speed and (d) pH on BMDS decolourisation by bacterial strains (4 :3:1:1)

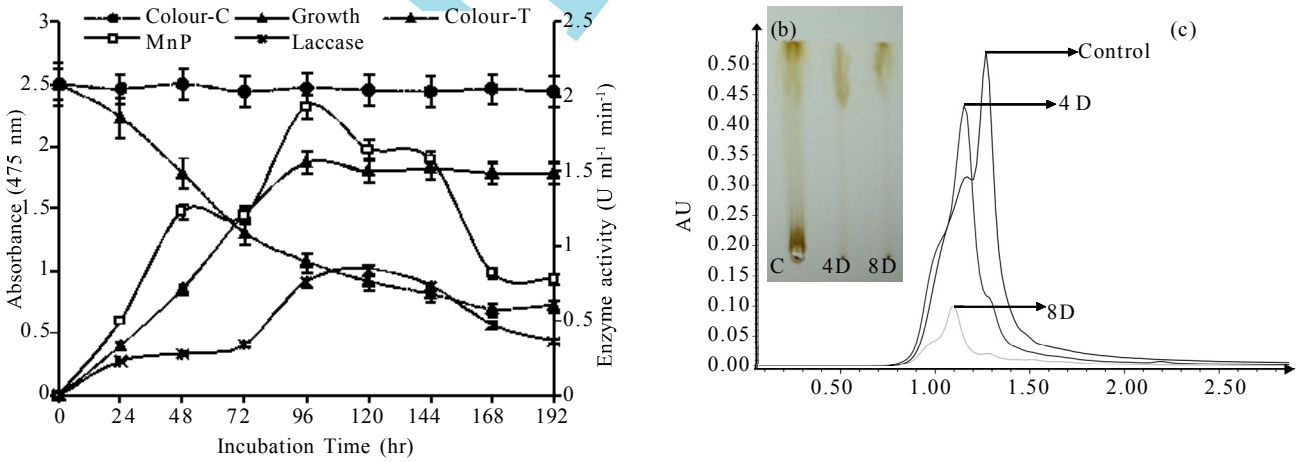

Fig. 2 (a-c) : Decolourisation was compared with manganese peroxidase (MnP) and (a) laccase activity, (b) TLC chromatogram and (c) HPLC chromatogram of BMDS before and after bacterial degradation at different time. C: untreated, T: bacterial treated, D: days

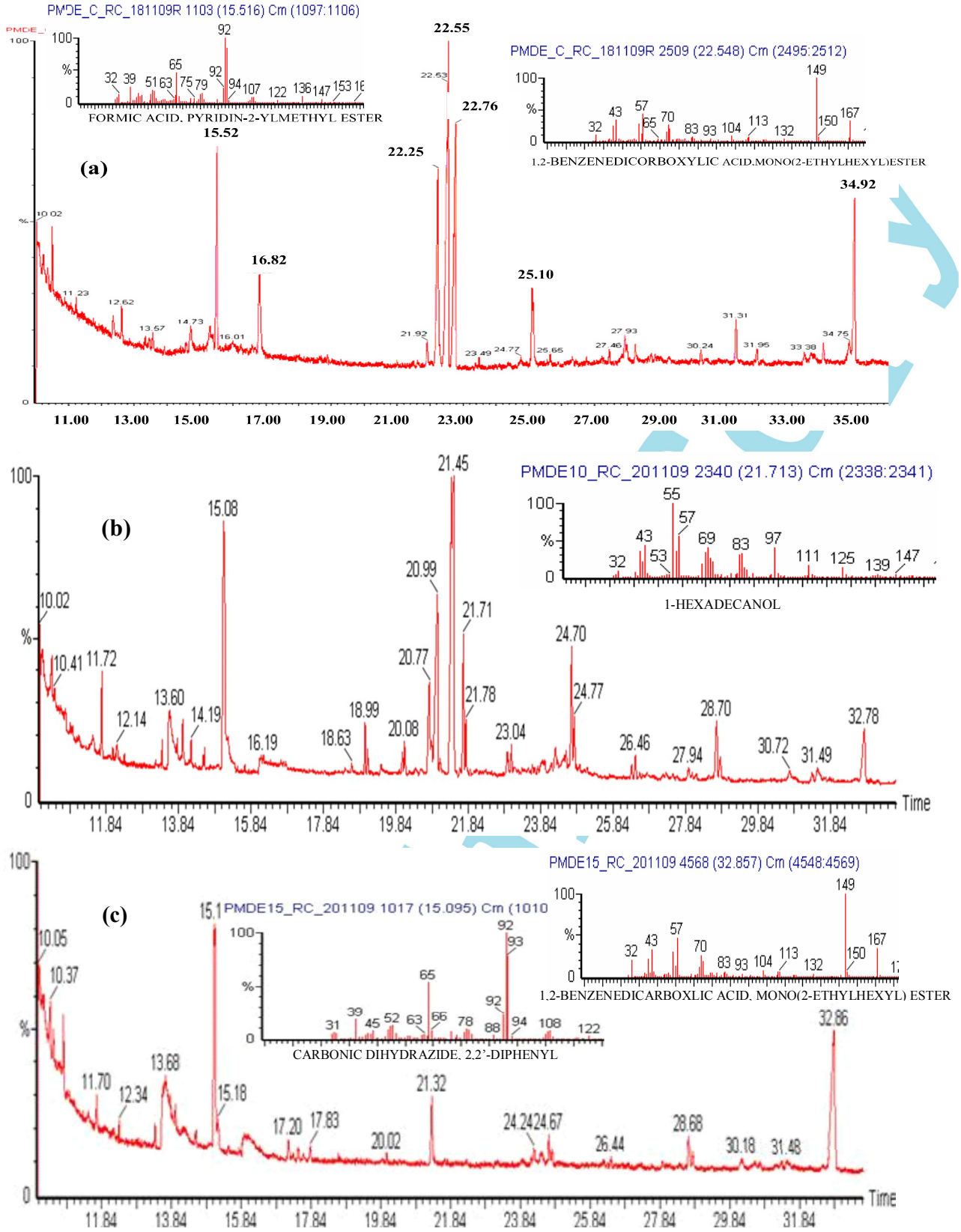

Fig. 3(a-c): GC–MS chromatogram of BMDS, (a) control, (b) after 4 days and (c) 8 days bacterial degradation

(Sirianuntapiboon *et al.,* 1988). Similarly, maximum COD and colour reduction were also noted at 180 rpm of shaking speed and 7.0 pH at optimized concentration (1200 ppm) with 8% inoculum having ratio 4 : 3 : 1 : 1 (Fig. 1c-d). This indicated that COD reduction is linked with colour. Similar trend of pollution reduction along with decolourisation has also been reported by Sangeeta and Chandra (2012). The increase of shaking speed beyond 180 rpm inhibited the percent COD and colour reduction. The reason for the less decolourisation at high shaking speed could be due to mechanical injury of bacterial cell (Sangeeta and Chandra, 2012).

Evaluation of the quantitative reduction of colour and other pollution parameters: The pollution parameters present in BMDS before and after bacterial treatment at different time interval are shown in Table 2. Apparent reduction in colour (76%) and COD (70%) was observed along with reduction of phenol and other pollutants at optimized conditions of BMDS degradation (Table 2). However, DO and chloride content were increased after bacterial treatment. The increased DO, chloride ion and reduction in COD might be due to the degradation of persistent organic compounds present in BMDS by bacterial consortium.

Ligninolytic enzyme for decolourisation of BMDS: Bacterial consortium showed much higher MnP activity than laccase during decolourisation of BMDS, this was noted maximum (1.93 U ml^{-1}) at 96 hr of its incubation period, further incubation of bacterial consortium subsequently suppressed the MnP activity. However, the maximum laccase activity (0.84 U ml^{-1}) was noted at 120 hr of bacterial incubation (Fig. 2a). The maximum enzyme activity coincided with maximum bacterial growth and decolourisation of BMDS. Several workers have reported ligninolytic enzyme in higher fungi showing the decolourisation of molasses melanoidin (Sahoo and Gupta, 2005; Shah *et al.,* 2005; Pant and Adholeya, 2007). Though, lignin peroxidase has been noted higher in fungus in various studies, while higher MnP activity in bacterial system is reported in recent observation (Bharagava *et al.,* 2008). The induction of MnP in culture supernatant was noted at 48 hr of bacterial growth and remained active up to 144 hr. While, the laccase induction was observed at 96 hr and its activity sustained up to 168 hr (Fig. 2a). This indicated the initial role of MnP in BMDS degradation. The report on MnP and laccase activity during decolourisation of BMDS in bacterial consortium is lacking. This is very important because its application at industrial scale is easy due to the fast bacterial growth in liquid media.

Studies on persistent organic compounds: The TLC analysis of bacterial degraded samples showed the disappearing of bands observed under visible light as compared to control (Fig. 2b). This indicated that the compounds present in BMDS were degraded by bacterial consortium. Therefore, the trailing of observed compounds in untreated BMDS (control) was disappeared after bacterial treatment indicated the degradation of BMDS (Fig. 2b). Further, the HPLC analysis of bacterial degraded BMDS showed the periodic reduction in peak area compared to control supported the decrease in colour intensity might be largely attributed to the bacterial degradation of colour containing compounds (Fig. 2c). Besides, the minor shifting of peaks from RT 1.20 to 1.05 min in HPLC chromatograph were noted in the bacterial degraded BMDS at 4th day, indicated the biodegradation of higher molecular weight compounds into lower molecular weight during bacterial growth. Subsequently, further reduction of absorption peak area at 8th day in chromatographic peak of bacterial degraded sample confirmed the biodegradation of colourant. Further, the characterization of persistent organic compounds through GC–MS analysis in ethyl acetate extracted sample

Table 3 : Compounds identified by GC-MS analysis in control and bacterial degraded BMDS

RT	Compounds	Samples		
		Control	Degraded	
			4 days	8 days
15.10	Carbonic dihydrazide, 2,2' diphenyl	-	-	+
15.52	Formic acid, pyridine-2-ylmethyl ester	+	-	-
16.82	Pyridine, 3-ethyl	+	-	-
20.99	DI-N-octyl phthalate	-	+	-
21.32	N-hexadecanoic acid	-	-	+
21.45	Dibutyl phthalate	-	+	-
21.71	1-Hexdecanol	-	+	-
22.25	DI-N-octyl phthalate	+	-	-
22.55	1,2-Benzenedicarboxylic acid, mono (2-ethylhexyl) ester	+	-	-
22.76	1,2-Benzenedicarboxylic acid, disooctyl ester	+	-	-
24.70	10-Methyl-9-nonadecene	-	+	-
25.10	N-hexadecanoic acid	+	-	-
32.86	1,2-Benzedicarboxylic acid, mono (2-ethylhexyl) ester	-	-	+
34.92	Formic acid, pyridine-2-ylmethyl ester	+	-	-

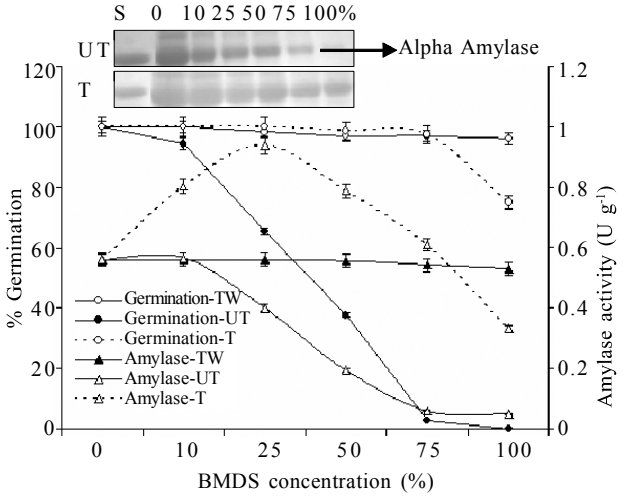

Fig. 4 : Percent germination and amylase activity shown by *Vicia faba*'s seeds treated with different concentration of bacterial treated and untreated BMDS. Inserted figure shows amylase on PAGE. S: Standard amylase, TW: Tap water, UT: Untreated BMDS, T: Treated BMDS

of control and bacterial degraded BMDS at 4 and 8 day are shown in Fig. 3a-c. The prominent compounds detected at RT 15.52, 16.82, 22.25, 22.55, 22.76, 25.10 and 34.92 min were formic acid, pyridine-2-ylmethyl ester, pyridine, 3-ethyl, DI-N-octyl phthalate, 1,2-benzenedicarboxylic acid, mono (2-ethylhexyl) ester, 1,2-benzenedicarboxylic acid, disooctyl ester, N-hexadecanoic acid and formic acid, pyridine-2-ylmethyl ester, respectively in control which disappeared during bacterial treatment (Fig. 3a-b and Table 3). Similar metabolic products have also been reported from fungal treated distillery wastewater from Cuba, Spain and Italy (Gonzalez *et al.,* 2000). Compounds like carbonic dihydrazide, 2,2' diphenyl (RT 15.10), N-hexadecanoic acid (RT 21.32 min) and 1,2-benzedicarboxylic acid, mono (2-ethylhexyl) ester (RT 32.86 min) were detected as persistent metabolic products after bacterial treatment (Fig. 3c and Table 3). While, the other detected persistent organic compounds were completely degraded after bacterial treatment. Thus, detected organic compound showed direct correlation with colourants present in BMDS sample.

Toxicity evaluation: The result of seed germination test revealed that untreated BMDS >10% (v/v) inhibited sprouting of seeds and amylase activity. However, 97% seed germination was noted in bacterial decolourised BMDS sample upto 75% concentration of BMDS, whereas seed germination was significantly inhibited at >75% of bacterial treated BMDS (Fig. 4). The amylase activity in *Vicia faba*'s seeds treated with BMDS decolourised sample was also noted higher than the seeds treated with untreated BMDS and tap water. However, enzyme activity (0.33 U grain^{-1}) was noted even in 100% treated BMDS. The higher percent seed germination and increase of enzyme activity in decolourised BMDS sample indicated that bacterial consortium could degrade and utilize various organic substances from the BMDS, which caused adverse effect on amylase activity responsible for seed germination. The inhibitory effects of BMDS on seed germination of different crop have also been previously noted (Ramana *et al.,* 2002; Sangeeta *et al.,* 2010).

The developed bacterial consortium could be used to scale up the decolourisation, degradation and detoxification process of BMDS for industrial application.

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References

APHA: American Public Health Association: American Water Works Association and Water Environment Federation, Standard Methods for the Examination of Water and Wastewater, 21st ed. APHA, Washigton (2005).

Arora, D.S., M. Chander and P.K. Gill: Involvement of lignin peroxidase, manganese peroxidase and laccase in degradation and selective ligninolysis of wheat straw. *Int. Biodete. Biodeg.*, **50**, 115–120 (2002).

Bharagava, R.N. and R. Chandra: Effect of bacteria treated and untreated post methanated distillery effluent (PMDE) on seed germination, seedling growth and amylase activity in *Phaseolus mungo* L. *J. Hazard. Mater.*, **180**, 730–734 (2010).

Bharagava, R.N. and R. Chandra: Isolation and characterisation of phenolic compounds by ^{1}HNMR and mass spectrometric analysis from sugarcane molasses post methanated distillery effluent. *Indian J. Environ. Protect.*, **29**, 873–88 (2009).

Bharagava, R.N., R. Chandra and V. Rai: Isolation and characterization of aerobic bacteria capable of the degradation of synthetic and natural melanoidins from distillery effluent. *World J. Microb. Biotech.*, **25**, 737–744 (2009).

Bharagava, R.N., R. Chandra and V. Rai: Phytoextraction of trace elements and physiological changes in Indian mustard plants (*Brassica nigra* L.) grown in post methanated distillery effluent (PMDE) irrigated soil. *Biores. Technol.*, **99**, 8316–8324 (2008).

Chandra, R. and A. Abhishek: Bacterial decolorization of black liquor in axenic and mixed condition and characterization of metabolites. *Biodegradation*, **22**, 603–611 (2011).

Chandra, R. and H. Singh: Chemical decolorization of anaerobically treated distillery wastewater. *Indian J. Environ. Protect.,* **19**, 833–837 (1999).

Chandra, R. and P.K. Pandey: Decolorization of anaerobically treated distillery wastewater by activated charcoal adsorption method. *Indian J. Environ. Protect.*, **2**, 132–134 (2001).

Chandra, R., R.N. Bharagava and V. Rai: Melanoidins as major colorant in sugarcane molasses based distillery wastewater and its degradation. *Biores. Technol.*, **99**, 4648–4660 (2008).

Gonzalez, T., M.C. Terron, S. Yague, E. Zapico, G.C. Galletti and A.E. Gonzalez: Pyrolysis/gas chromatography/mass spectrometry monitoring of fungal–biotreated distillery wastewater using *Trametes* sp. I-62 (CECT 20197). *Rapid Commun. Mass Spec.,* **14**, 1417–1424 (2000).

Jirasripongpun, K., R. Nasanit, J. Niruntasook and B. Chotikasatian: Decolourization and degradation of C.I. reactive red 195 by *Enterobacter sp. Thammasat Int. J. Sci. Technol.,* **12**, 6–11 (2007).

Kalil, M.S., H.S. Alshigab and W.M.W. Yusoff: Effect of nitrogen source and carbon to nitrogen ratio on hydrogen production using *C. acetobutylicum*. *American J. Biochem. Biotechnol.*, **4**, 393–340 (2008).

Kim, S.B., F. Hayase and H. Kato: Decolorization and degradation products of melanoidins on ozonolysis. *Agricul. Biol. Chem.,* **49**, 785–792 (1985).

Kumar, P. and R. Chandra: Decolorization and detoxification of synthetic molasses melanoidins by individual and mixed cultures of *Bacillus sp*. *Biores. Technol.,* **7**, 2096–2102 (2006).

Kumar, V., L. Wati, P. Nigam, I.M. Banat, B.S. Yadav, D. Singh and R. Marchant: Decolorization and biodegradation of anaerobically digested sugarcane molasses spent wash effluent from biomethanation plants by white-rot fungi. *Process Biochem.*, **33**, 83–88 (1998).

Kumar, V., L. Wati, P. Nigam, I.M. Banat, G. MacMullan, D. Singh and R. Marchant: Microbial decolorization and bioremediation of anaerobically digested molasses spent wash wastewater by aerobic bacterial culture. *Microbios,* **89**, 81–90 (1997).

Migo, V.P., E.J. Del Rosario and M. Matsumura: Flocculation of melanoidins induced by inorganic ions. *J. Ferment. Bioengin.*, **83**, 287–291 (1997).

Minuti, L., R.M. Pellegrino and I. Tesei: Simple extraction method and gas chromatography-mass spectrometry in the selective ion monitoring mode for the determination of phenols in wine. *J. Chromat.*, **1114**, 263–268 (2006).

Miyata, N., T. Mori, K. Iwahori and M. Fujita: Microbial decolorization of melanoidins containing wastewaters: Combined use of activated sludge and the fungus *Coriolus hirsutus*. *J. Biosci. Bioengin.,* **89**, 145–150 (2000).

Mohana, S., C. Desai and D. Madamwar: Biodegradation and decolourisation of anaerobically treated distillery spent wash by a novel bacterial consortium. *Biores. Technol.*, **98**, 333–339 (2007).

Nagda, G.K., A.M. Diwan and V.S. Ghole: Seed germination bioassay to assess toxicity of molasses fermentation based bulk drug industry effluent. *Electronic J. Environ. Agri. Food Chem.*, **5**, 1598–1603 (2006).

Ott, L.: An introduction to statistical methods and data analysis. 2nd Edn., PWS, Boston, Massachusetts (1984).

Pant, D. and A. Adholeya: Development of a novel fungal consortium for the treatment of molasses distillery wastewater. *Environmentalist*, **30**, 178–182 (2010).

Pant, D. and A. Adholeya: Enhanced production of ligninolytic enzymes and decolorization of molasses distillery wastewater by fungi under solid state fermentation. *Biodegradation*, **18**, 647–659 (2007).

Raghukumar, C., C. Mohandass, S. Kamat and M.S. Shailaja: Simultaneous detoxification and decolorization of molasses spent wash by the immobilized white-rot fungus *Flavadon flavus* isolated from the marine habitat. *Enzy. Microbial Technol.*, **35**, 197–202 (2004).

Rajasundari, K. and R. Murugesan: Decolourization of distillery waste water- role of microbes and their potential oxidative enzymes (Review). *J. Appl. Environ. Biol. Sci.,* **1,** 54–68 (2011).

Ramakritinan, C.M., A.K. Kumaraguru and M.P. Balasubramanian: Impact of distillery effluent on carbohydrate metabolism of freshwater fish *Cyprinus carpio*. *Ecotoxicology*, **14**, 693–707 (2005).

Ramana, S., A.K Biswas, S. Kundu, J.K. Saha and R.B.R Yadava: Effect of distillery effluent on seedgermination in some vegetable crops. *Biores. Technol.*, **82**, 273–275 (2002).

Sahoo, D.K. and R. Gupta: Evaluation of ligninolytic microorganisms for efficient decolorization of a small pulp and paper mill effluent. *Process Biochem.*, **40**, 1573–1578 (2005).

Sangeeta, Y. and R. Chandra: Biodegradation of organic compounds of molasses melanoidin (MM) from biomethanated distillery spent wash (BMDS) during the decolourisation by a potential bacterial Consortium. *Biodegradation*, **23,** 609–620 (2012).

Sangeeta, Y., R. Chandra and R. Vibhuti: Effect of biologically treated post methanated distillery effluent on seed germination and growth parameters of *Vicia faba*. *Indian J. Env. Prot.*, **30,** 253–365 (2010).

Shah, M.P., G.V. Reddy, R. Banerjee, P.R. Babu and I.L. Kothari: Microbial degradation of banana waste under solid state bioprocessing using two lignocellulolytic fungi (*Phylosticta spp*. MPS-001 and *Aspergillus spp.* MPS-002). *Process Biochem.,* **40**, 445–45 (2005).

Sirianuntapiboon, S., P. Phothilangka and S. Ohmomo: Decolorization of molasses wastewater by a strain No. BP103 of acetogenic bacteria. *Biores. Technol.*, **92**, 31–39 (2004a).

Sirianuntapiboon, S., P., Somchi and S. Ohmomo: Screening of filamentous fungi having the ability to decolorize molasses pigments. *Agric. Biol. Chem.*, **52**, 387–392 (1988).

Sirianuntapiboon, S., P., Zohsalam and S. Ohmomo: Decolorization of molasses wastewater by *Citeromyces sp.* WR–43–6. *Process Biochem.*, **39**, 917–924 (2004b).

Vahabzadeh, F., M. Mehranian and A.R. Saatari: Color removal ability of *Phanerochaete chrysosporium* in relation to lignin peroxidase and manganese peroxidase produced in molasses wastewater. *World J. Microbiol. Biotechnol.,* **20,** 859-864 (2004).