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ORIGINAL PAPER

Biodegradation of organic compounds of molasses melanoidin (MM) from biomethanated distillery spent wash (BMDS) during the decolourisation by a potential bacterial consortium

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Abstract Molasses melanoidin (MM) is a major pollutant in biomethanated distillery spent wash (BMDS) due to its recalcitrant properties. The 75% colour and 71% COD of MM (1,000 ppm) were reduced with developed bacterial consortium comprising *Proteus mirabilis* (IITRM5; FJ581028), *Bacillus* sp. (IITRM7; FJ581030), *Raoultella planticola* (IITRM15; GU329705) and *Enterobacter sakazakii* (IITRM16, FJ581031) in the ratio of 4:3:2:1 within 10 days at optimized nutrient. Bacterial consortium showed manganese peroxidase and laccase activity during MM decolourisation. The dominant growth of *R. planticola* and *E. sakazakii* was noted in consortium during MM decolourisation. The comparative GC–MS analysis of extracted compounds of control and degraded samples showed that most of the compounds present in control were completely utilized by bacterial consortium along with production of some metabolites. The developed bacterial consortium could be a tool for the decolourisation and degradation of melanoidin containing BMDS.

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Introduction

Molasses melanoidin (MM) is dark brown to black coloured natural condensation polymer of sugars and amino acids; they are produced by the non-enzymatic browning reactions known as Maillard reactions (Plavsic et al. 2006). MM is widely discharged in huge amount as environmental pollutant by various agro-based industries especially from distilleries and fermentation industries (Kumar and Chandra 2006). Melanoidin remains about 2% in molasses based distillery effluent as major colouring recalcitrant pollutant together with metals and phenolics (Manisankar et al. 2004; Chandra et al. 2008). It is hardly degraded by microbes and behaves as anionic hydrophilic polymers, which has high binding tendency with metal cations result into more complex nature of effluent (Migo et al. 1997; Plavsic et al. 2006). Moreover, phenolics present in effluent makes more complex compounds with heavy metals (Chandra et al. 2008).

Melanoidin containing sugarcane molasses based distillery wastewater is the major source of soil and water pollution even after anaerobic digestion due to high biological oxygen demand (BOD, 23000), chemical oxygen demand (COD, 47400), total dissolved

solid (TDS, 10480), phenolics (510), sulfate (3786) and phosphate (739 mg l^{-1}) (Bharagava et al. 2008). Its discharge in the soil inhibits seed germination, decrease soil alkalinity as well as manganese availability whereas in aquatic system, it blocks the sunlight penetration and photosynthesis (Kumar and Chandra 2006; Bharagava et al. 2008). Hence, its degradation prior to discharge is essential for the environmental safety.

Though, the various effective physical and chemical methods such as ozonation, flocculation and activated carbon adsorption etc. have been attempted for the removal of melanoidin and biomethanated distillery spent wash (BMDS) decolourisation (Kim et al. 1985; Migo et al. 1997; Chandra and Pandey 2001). But, these methods were not found feasible due to high cost and generation of secondary pollutants. In order to search a sustainable technology for the decolourisation and detoxification of BMDS, various fungal strains such as *Penicillium decumbens*, *Aspergillus* sp., *Aspergillus niger*, *Flavadon flavus, Phanerochaete* sp., *Phanerochaete chrysosporium*, *Trametes versicolor, Coriolus* sp., *Pleurotus florida*, *Aspergillus flavus*, *Alternaria gaisen* and *Fusarium monoliforme* (Gonzalez et al. 2000; Chopra et al. 2004; Raghukumar et al. 2004; Pant and Adholeya 2009) as well as bacterial strains such as *Pseudomonas*, *Bacillus, Alkaligenes* and *Lactobacillus* has been discussed by several authors (Ohmomo et al. 1985; Kumar et al. 1997; Kumar and Chandra 2006; Mohana et al. 2007). The manganese peroxidase (MnP) has been reported in bacteria as extracellular enzyme for decolourisation of melanoidin (Bharagava et al. 2009). The involvement of MnP and laccase in white rot fungus for degradation of various biopolymers (lignin and tannin) has also been reported (Arora et al. 2002; Rubia et al. 2002). But, the detail role of MnP and laccase in bacteria for decolourisation of melanoidin present in molasses yet to be investigated.

Recently, it has been reported that phenolics and other organic compound contribute significant role in colouration of anaerobically digested distillery effluent (Morales et al. 2005; Bharagava et al. 2008). However, the complete knowledge regarding the degradability of organic compounds along with MM present in BMDS by bacteria is lacking. In addition, the relation between the melanoidin and other co-pollutants (phenolics) present in BMDS during biological decolourisation is completely unknown. Hence, this study has been focused on degradation of MM and phenolics contents, extracted from BMDS and characterization of metabolic products after bacterial degradation. In addition, MnP and laccase activity was also investigated in bacteria during this study.

Materials and methods

Collection of BMDS and extraction of MM

BMDS was collected from extended aeration lagoon at M/S Unnao distillery and brewery Ltd. Unnao (UP), India located 26.48°N, 80.43°E. This distillery uses sugarcane molasses as raw material with installed capacity of alcohol production $9{,}000 \times 10^3$ l annually leads to discharges approx 370×10^3 l wastewater per day. The MM content in BMDS was extracted, using equal volume of BMDS and isopropanol (v/v) in separating funnel and mixed vigorously. Subsequently, precipitate at bottom of the separating funnel was separated and dried in a hot air oven at 50°C (Kalavathi et al. 2001; Bharagava et al. 2009). This dried powder was designated as MM and it was used throughout this study.

Physico-chemical analysis of MM solution used in the study

The changes in physico-chemical parameters (colour, BOD, COD, total solid (TS), sulphate, nitrogen and phosphate) in MM solution before and after bacterial degradation were analyzed as per standard methods (APHA 2005). Nitrate, chloride ion and pH were determined using an ion meter (Orion autoanalyser model-960) by their respective ion electrode. To measure the colour intensity of bacterial treated MM media, media was centrifuged at 10,000 rpm for 15 min to remove bacterial biomass. 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.

Construction of bacterial consortium inoculum ratio and size for optimal decolourisation

For construction of active bacterial consortium a loop full of pure pre-isolated bacterium (*Proteus mirabilis*,

Bacillus sp., *Raoultella planticola* and *Enterobacter sakazakii*) was inoculated in sterilized 50 ml mineral salt (MS) medium containing K_2HPO_4 (0.1%) and $MgSO_4 \cdot 7H_2O$ (0.05%) amended with MM (1,000 ppm) and incubated at 35 ± 1°C and 120 rpm. After 48 h the equal volume (1 ml) of each strain containing cell density 3×10^4 cells ml^{-1} was used for preparation of different bacterial composition. The *P. mirabilis*:*Bacillus* sp.:*R. planticola*:*E. sakazakii* were taken in the various combination i.e. in the ratio of 1:1:1:1, 4:2:3:1, 3:2:1:1 and 4:3:2:1, among them the ratio 4:3:2:1 showed maximum MM decolourisation and all the bacterial strains in this ratio was growing approximately in equal number hence this ratio was chosen for further study. Further, in order to verify the bacterial inoculum size for effective colour removal by optimized inoculum ratio (4:3:2:1), different inoculum size (3, 5, 7 and 9%) was inoculated in the MM containing MS medium.

Nutrient optimization

For nutrient optimization MS medium containing 1,000 ppm MM was studied. Further, above culture was used to investigate the effects of different carbon sources (glucose, galactose, sucrose, fructose, mannose, ribose, xylose and arabinose) as well as nitrogen sources (yeast extracts, beef extract, peptone, sodium nitrate, ammonium nitrate and ammonium chloride) on MM degradation. Each nutrient was supplemented individually at final concentration in range of 0.1 to 1.5% and 0.1 to 0.5% for carbon and nitrogen, respectively as an additive to the MS medium. The optimum concentration for carbon and nitrogen was noted at 1.0 and 0.1%, respectively.

Optimization of environmental factors

The effect of various environmental parameters such as temperature (20–60°C), pH (6–8) and shaking speed (100–250 rpm) on MM degradation was also studied. The pH of the media was adjusted with 1 N NaOH and HCl.

Optimized media composition

The MS medium consisting: glucose (1%), peptone (0.1%), K_2HPO_4 (0.1%) and $MgSO_4 \cdot 7H_2O$ (0.05%) in double distilled water showed good bacterial growth. This medium was designated as GPM and used throughout the study.

Bacterial degradation of MM at optimized conditions

MM degradation and decolourisation

The degradation experiments were carried out in triplicate in 250 ml Erlenmeyer flasks containing 100 ml of sterile GPM broth supplemented with MM (1,000 ppm). The flasks were inoculated with 7% (v/v) overnight grown mixed bacterial culture comprising *P. mirabilis*, *Bacillus* sp., *R. planticola* and *E. sakazakii* in the optimized ratio of 4:3:2:1 and incubated at 35°C under shaking flask condition (Innova 4230 Refrigerated Incubator shaker, New Brunswick, USA) for 240 h. The flask containing 100 ml of sterile GPM broth supplemented with MM (1,000 ppm) without inoculums was considered as control. The degradation of MM was monitored spectrophotometrically (Techcomp, UV-2300 spectrophotometer, Korea) in terms of bacterial growth and decrease of the colour intensity at 620 and 475 nm, respectively (Kumar and Chandra 2006; Bharagava et al. 2009).

Bacterial growth pattern during MM degradation

To observe the performance of individual strain in mixed conditions during MM degradation, morphologically distinct colonies of different strains *P. mirabilis*, *Bacillus* sp., *R. planticola* and *E. sakazakii* were counted periodically to evaluate the colony forming unit (CFU) of potential strains with reduction of pollutants.

MnP and laccase activity during MM decolourisation

To measure the MnP and laccase activity, the culture supernatant was obtained by centrifugation at 10,000 rpm for 15 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, five milliliter 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 at every minute by measuring the increase in absorbance at 610 nm. One milliliter of reaction mixture was taken from above solution and 40 µl 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 U 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 25°C for 2 h. The absorbance was read at 450 nm using spectrophotometer (Techcomp, UV-2300 spectrophotometer, Korea) after 2 h when reaction time completed. Laccase activity has been expressed as unit ml^{-1} (U ml^{-1}).

Adsorption evaluation during decolourisation process

This study was carried out to verify whether the decolourisation observed was due to biological or non-biological activity. The living and autoclaved cells of bacterial consortium with cell concentration at 7% (v/v) were added into 250 ml Erlenmeyer flask containing GPM medium amended with 1,000 ppm of MM. The flasks were incubated at 35°C for 240 h. The samples were withdrawn at every 24 h interval and then centrifuged at 10,000 rpm for 15 min. The supernatants were read for the OD at 475 nm using spectrophotometer. Additionally, to confirm the MM decolourisation occurred either through biological activity or adsorption mechanism, NaOH extraction method was also adopted (Sirianuntapiboon et al. 2004a). The cell pellets of both living cell and autoclaved cell after centrifugation were resuspended with equal volume of 0.1 M NaOH to extract colour substances adsorbed to cell surface.

Thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) analysis of bacterial degraded MM

To assess the degradability of MM, the TLC was done by two ways. In first way colourant available in control and bacterial treated MM were detected in the supernatant after centrifugation at 10,000 rpm for 15 min, while in other ways the phenolics present in control and degraded MM were also extracted by ethyl acetate at acidic condition (pH < 2) as described previously (Bharagava and Chandra 2009). For extraction of phenolic compounds, 25 ml of bacterial degraded and control (without bacterial inoculum) MM was acidified (pH 2.0) with 37% aqueous hydrochloric solution and then mixed with equal volume of ethyl acetate, the mixture was shaked continuously for 4 h 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. Further, the concentrated samples were spotted on silica gel pre-coated chromatographic plate column (120 mesh; 75 × 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, 230 V-2A, UK). However, the extracted compounds were separated through the solvent comprising toluene (90):methanol (16):ethyl acetate (8). The changes in extracted compounds were observed under UV light. Further, the degradation of MM was confirmed through HPLC analysis. The bacterial decolourised and control samples of MM was analyzed by Waters, 515 HPLC system equipped with reverse phase column C-18 (250 mm × 4.6, particle size 5 µm) at 27°C and 2487 UV/Vis detector via millennium software. The control and degraded sample (20 µl) was injected into HPLC and monitored at wavelength 290 nm to assess the degradation of colourant. 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).

GC–MS analysis of phenolics compounds extracted from bacterial degraded and un-degraded MM

The dry residue obtained from liquid–liquid extraction method using ethyl acetate was derivatised with trimethylsilyl (TMS) as described by Minuti et al. (2006). 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 60°C 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 × 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°C (5 min); 50–300°C (10°C min^{-1}, hold time: 5 min). The transfer line and ion source temperatures were maintained at 200 and 250°C, respectively. A solvent delay of 3.0 min was selected. The chromatographic run time was maintained up to 33.84 min. In full-scan mode, the electron ionization 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).

Statistical analysis

All assays were performed in triplicate and compared with respective uninoculated control. Tukey's test (Ott 1984) using the Graph Pad software (Graph Pad Software, San Diego, CA) was used for statistical analysis.

Results and discussion

Physico-chemical properties of MM

The detail physico-chemical properties of MM extracted from BMDS in control and bacterial degraded sample (i.e. 5 and 10 days) has been shown in Table 1. This indicated MM contribute colour, BOD and COD to BMDS. The presence of sulphate, phenols and metals during physico-chemical analysis revealed that the MM has binding property with these compounds present in BMDS. Similar observations were also reported for post methanated distillery effluent by Bharagava and Chandra (2010).

Table 1 Physico-chemical characteristics of MM before and after bacterial treatment

Parameters	Control (MM)	Bacterial treated MM (5 days)	Bacterial treated MM (10 days)	Reduction (%)
pH	8.20 ± 0.41	5.50 ± 0.17*	7.00 ± 0.21*	14.63
Colour	35000 ± 768	15000 ± 320*	8750 ± 166*	75.00
COD	36000 ± 834	22800 ± 453*	10440 ± 231*	71.00
BOD	15200 ± 312	7000 ± 165*	4256 ± 78.00*	72.00
TSS	26842 ± 437	10456 ± 204*	2684 ± 54.32*	90.00
TDS	12654 ± 321	5174 ± 176*	1138 ± 33.65*	91.00
TS	39496 ± 765	15630 ± 210*	3742 ± 87.24*	90.00
Phenol	180 ± 2.56	83.56 ± 0.34*	46.80 ± 0.68*	74.00
Sulphate	2457 ± 73.71	714 ± 23.34*	196 ± 5.63*	92.00
Phosphate	1433 ± 34.65	1426 ± 33.00^{ns}	243 ± 6.23*	83.00
Potassium	54.00 ± 2.12	50.00 ± 2.45^{ns}	44.00 ± 2.20*	18.00
Chloride	750.00 ± 32.13	150 ± 3.21*	500 ± 12.80*	33.00
Sodium	420.00 ± 5.32	220 ± 4.65*	280 ± 5.76*	33.00
Copper	1.64 ± 0.07	0.19 ± 0.00*	0.019 ± 0.00*	98.00
Cadmium	0.013 ± 0.00	0.009 ± 0.00*	0.002 ± 0.00*	84.00
Zinc	10.52 ± 0.21	1.21 ± 0.04*	0.65 ± 0.02*	93.00
Iron	40.70 ± 0.31	35.40 ± 1.42*	8.78 ± 0.26*	78.00

All values are means of three replicate ($n = 3$) ± SD in mg l^{-1} except colour (Co–Pt) and pH. *COD* chemical oxygen demand, *BOD* biological oxygen demand, *TSS* total suspended solid, *TDS* total dissolved solid, *TS* total solid. ANOVA was analyzed within row of each parameter * significant ($p < 0.001$), *ns* non significant ($p > 0.05$)

Optimization of culture conditions for MM decolourisation

The decolourisation of MM by bacterial consortium in presence of different carbon sources in MS medium, revealed that glucose supported maximum decolourisation (60%) and COD reduction (49%) along with bacterial growth (Fig. 1a) within 10 days of bacterial treatment. It was also observed that the degradation of MM improved with increase in glucose concentration from 0.5 to 1% (w/v), while further increase in glucose concentrations did not improve degradation rather than the bacterial growth and biomass production. The other carbon sources significantly reduces the MM decolourisation in the following order, maltose (glucose + glucose) > lactose (glucose + galactose) > sucrose (glucose + fructose) > galactose > fructose > mannose (Fig. 1a). The patterns of sugar utilization again support the preferential use of glucose by these strains, because sugars containing glucose as monomer unit were found to be fairly good.

The results of the effect of various organic and inorganic nitrogen sources at a concentration of 0.1% (w/v) showed that the supplementation of nitrogen sources enhances the MM decolourisation. In this study, the effect of different organic and inorganic nitrogen sources on colour and COD reduction showed optimal in presence of 0.1% peptone (62, 53%) in MS

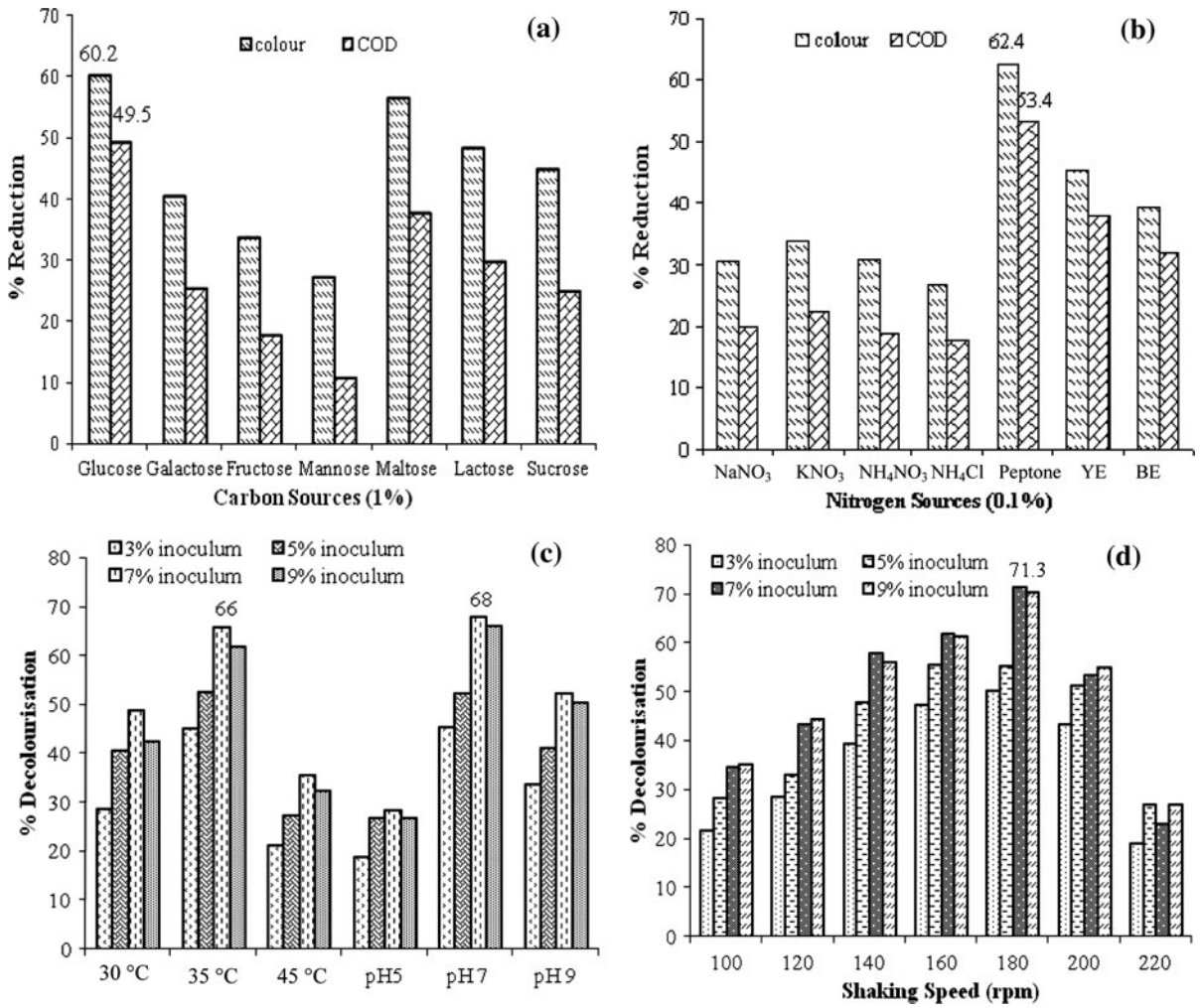

Fig. 1 Showing optimization of bacterial decolourisation of MM; effect of different carbon sources (**a**), effect of different nitrogen sources (**b**) on colour and COD reduction, effect of pH and temperature at different inoculum size (**c**) and effect of shaking speed at different inoculum size (**d**) *YE* yeast extract, *BE* beef extract

media followed by yeast extract (45, 38%) and beef extract (39, 32%). However, >0.1% (w/v) organic nitrogen showed inhibitory effect. The comparative observation between organic and inorganic nitrogen sources also revealed that inorganic nitrogen was inhibitory for MM decolourisation (Fig. 1b). However, Mohana et al. (2007) reported organic nitrogen at 0.05% (w/v) concentration showed suppressive effect on decolourisation and degradation of anaerobically treated distillery effluent. This indicated that the nitrogen available for bacterial growth in MM degradation is different than anaerobically treated distillery effluent.

Result showed that 48 and 66% decolourisation was achieved when the bacterial consortium was grown at 30 and 35°C, respectively. This was dropped to about 35% when consortium incubated at 45°C (Fig. 1c). These results are in agreement with Boer et al. (2006) who stated that temperature affects the rates of microbial metabolism of melanoidin. A range of pH (6–8) was used to examine the effect of pH on the degradation process (Fig. 1c). Maximum degradation of MM (68%) was recorded at pH 7.00 ± 0.1 and pH higher or lower than 7.00 adversely affected the bacterial growth and degradation capability of the bacterial consortium (Fig. 1c). Melanoidin solubility depends on pH; it is less soluble in acidic pH than in alkaline pH (Pena et al. 1996).

It was also observed that the decolourisation increased with increase in inoculum size of selected consortium ratio (4:3:2:1). Maximum 71% decolourisation was observed at 7% (v/v) inoculum size. Further, increase in the inoculum size did not improve the decolourisation (Fig. 1d). Sirianuntapiboon et al. (2004b) also reported similar observation for the relationship between inoculum size and decolourisation of molasses wastewater by acetogenic bacteria.

Further, in order to investigate the optimal shaking speed for MM decolourisation, 7% of bacterial consortium was incubated at variable shaking speed (100–220 rpm). The shaking speeds from 100–180 rpm were effective in enhancing MM degradation and the optimum decolourisation (71%) was observed at 180 rpm. But, higher shaking speed above 180 rpm suppressed the decolourisation process within 240 h (Fig. 1d). The optimum decolourisation of MM was estimated to be 71% at 180 rpm which subsequently decreased to 53 and 22%, at 200 and 220 rpm, respectively (Fig. 1d). This might be due to mechanical injury of bacterial cell at high speed.

The effect of incubation time for MM decolourisation by bacterial consortium showed simultaneous increase of cell biomass and decolourisation process with incubation time.

Bacterial growth pattern and MM decolourisation assay

To investigate the compatibility pattern of different bacteria in consortium during MM decolourisation, CFU of each bacterium was evaluated. Each bacterium showed rapid growth in consortium up to 72 h of incubation in GPM media at 180 rpm as shown in Fig. 2. This might be due to abundant availability of glucose in media as supplementary carbon source at initial stage. Further, incubation showed gradual increase of CFU up to 168 h led to decrease in colour (Fig. 2a, b). This indicated exhaust of available glucose in the media, where bacteria utilized the melanoidin as

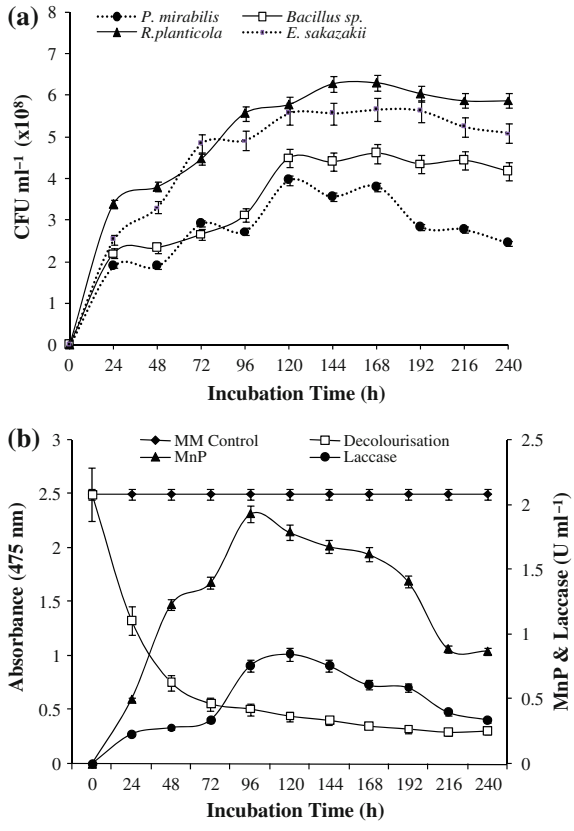

Fig. 2 Growth pattern of each potential strains in consortium at optimized ratio (**a**), MnP and laccase activity during MM degradation (**b**)

carbon and nitrogen source. Furthermore, incubation up to 240 h did not affect the process. This either might be due to the formation of such metabolites which is either not utilized by bacteria or creates such environment which is not conducive for bacterial growth. The CFU count of *P. mirabilis* and *Bacillus* sp. showed less at initial phase of incubation (up to 72 h). However, the CFU count was found to be highest for *R. planticola* followed by *E. sakazakii* > *Bacillus* sp. > *P. mirabilis* after 72 h (Fig. 2a). The differential CFU count of different bacteria in the study revealed the potentiality of strain for MM degradation and COD reduction. The carbon and nitrogen starvation in culture media initiated the bacterial utilization of melanoidin pigment as nitrogen and probably a carbon source which supported the metabolic activity at later stage (Kalavathi et al. 2001). The bacterial consortium at optimized conditions (GPM media amended with 1000 ppm MM, pH 7.0, 35 ± 1°C and 180 rpm) reduced the colour (75%) and COD (71%) significantly along with other parameters at 10 days bacterial growth (Table 1).

Initially during MM degradation the pH of media was reduced up to 4.6, but after 96 h of bacterial growth the pH was gradually increased. The increased pH of media might be due to potentiality of bacterial consortium for MM utilization and enzyme activity; this induced the solubility and utilization of melanoidin which favored the mineralization of MM.

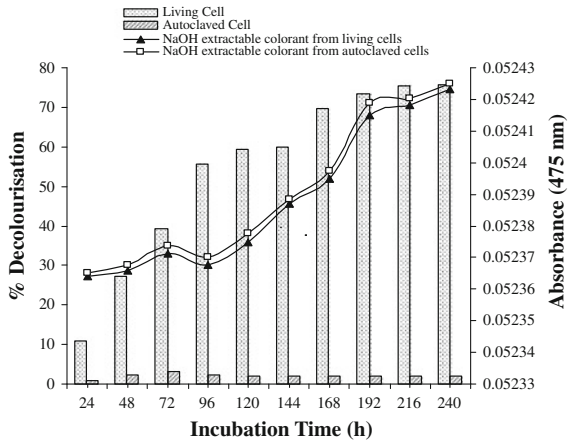

Fig. 3 Comparative adsorption and biological degradation of MM during bacterial decolourisation

Changes in enzyme activity during MM degradation and adsorption study

The MnP and laccase activity in the culture supernatant showed maximum 1.93 and 0.85 U ml^{-1} at 96 and 120 h, respectively of bacterial growth (Fig. 2b). While, further bacterial incubation showed gradual decrease of both enzyme activities (Fig. 2b). The enzyme activity showed direct co-relation with the MM decolourisation (decrease in absorbance at 475 nm). The induction of MnP in culture supernatant was noted at 48 h of bacterial growth and remains active up to 192 h, while the laccase induction was observed at 96 h and its activity also declined after 192 h (Fig. 2b). This indicated the initial role of MnP in MM degradation. The induction of MnP for decolourisation of melanoidin has also been reported by various workers in fungus (Raghukumar et al.

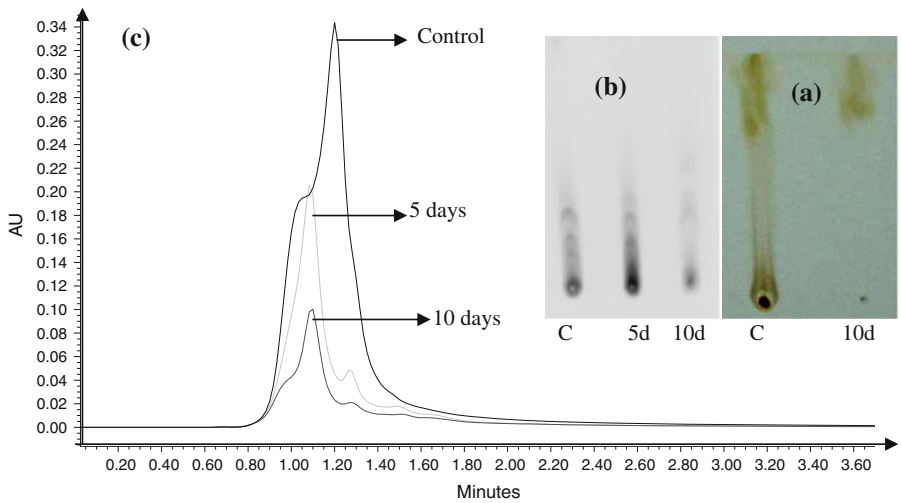

Fig. 4 TLC analysis of bacterial degraded MM along with control under visible light (**a**), compounds detected in culture supernatant without extraction), UV light (**b**), compounds extracted with ethyl acetate from culture supernatant) and periodic HPLC analysis of degraded MM samples along with control at different time interval (A_{290} nm) (**c**). *C* control, *d* days

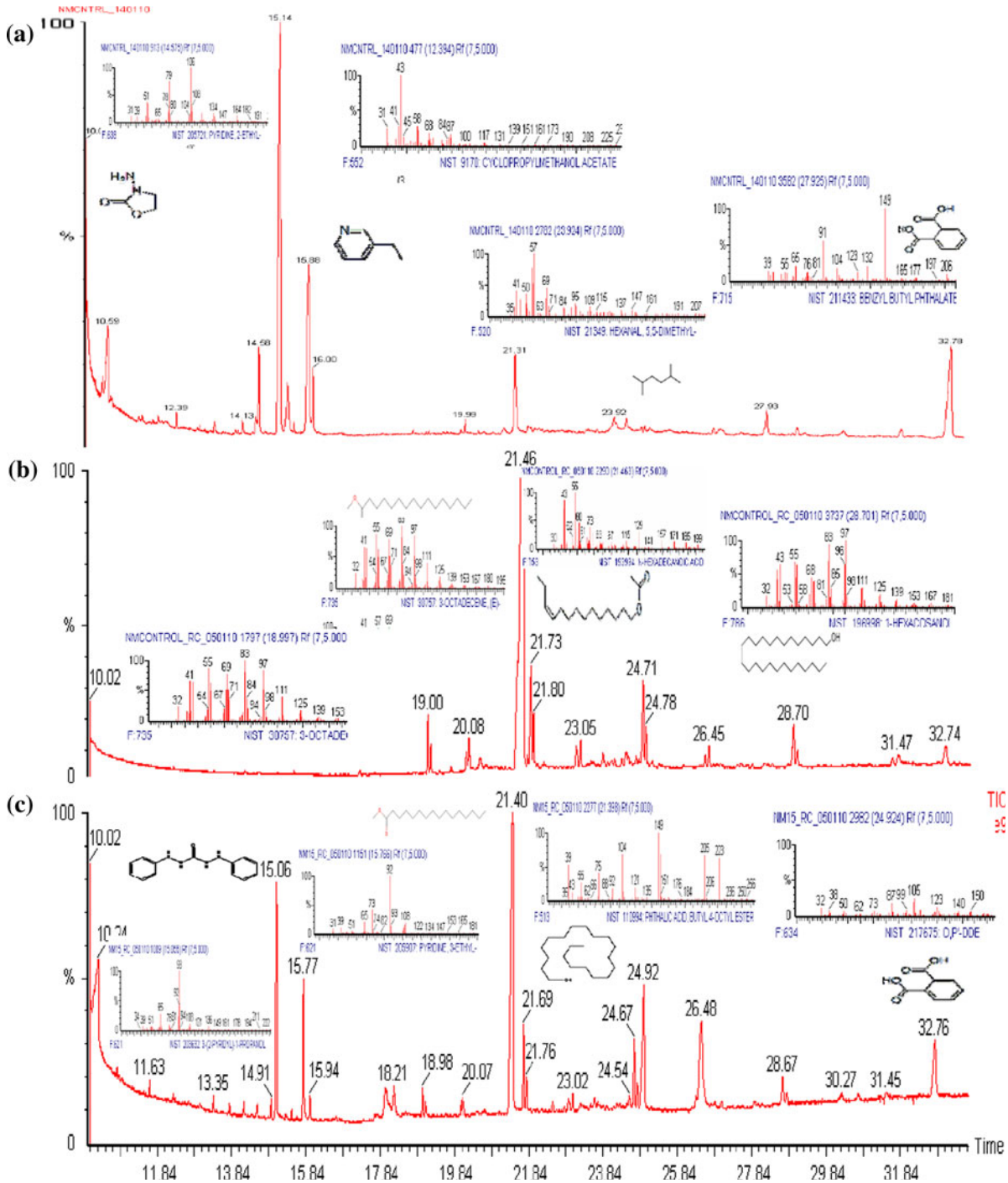

Fig. 5 GC–MS analysis of phenolics extracted from MM after 5 days (**b**), 10 days (**c**) of bacterial treatment along with control (**a**)

2004; Pant and Adholeya 2007). Recently, laccase has also been reported for decolourisation of melanoidin (Bharagava et al. 2009; Sangeeta et al. 2011).

Additionally, to confirm that the MM decolourisation occurred by biological activity but not adsorption mechanism, It showed that the experiment with

Table 2 Identified compounds in control and bacterial degraded MM

S no.	RT	Compounds	Control	Degraded	
				5 days	10 days
1.	10.59	3-Amino-2-oxazolidinone	+	−	−
2.	12.39	Cyclopropylmethanol acetate	+	−	−
3.	14.13	4-Pyridinecarboxlic acid	+	−	−
4.	14.58	2-Ethylpyridine	+	−	−
5.	15.06	3-(2-Pyridyl)-1-propanol	−	−	+
6.	15.14	*n*-Methyl-2-nitro-3-pyridinamine	+	−	−
7.	15.31	Pyridine,2-methyl-1-oxide	+	−	−
8.	15.77	3-Ethylpyridine	−	−	+
9.	15.88	3-Ethylpyridine	+	−	−
10.	15.94	Nicotinic acid, propyl ester	−	−	+
11.	16.00	Isonicotinyl formaldoxime	+	−	−
12.	19.00	3-Octadecene	−	+	−
13.	19.99	Phthalic acid	+	−	−
14.	21.40	Phthalic acid, butyl-4-octyl ester	−	−	+
15.	21.46	*n*-Hexadecanoic acid	−	+	−
16.	21.69	1-Eicosanol	−	−	+
17.	21.73	13-Tetradecen-1-*O*-acetate	−	+	−
18.	23.92	5,5-Dimethyl hexane	+	−	−
19.	24.71	Unknown	−	+	−
20.	24.92	*O*,*P*′-DDE	−	−	+
21.	26.45	Unknown	−	+	−
22.	26.48	Hexadecamethyl octasiloxane	−	−	+
23.	27.93	Benzyl butyl phthalate	+	−	−
24.	28.70	1-Hexacosanol	−	+	−
25.	32.76	Phthalic acid, dodecyl octyl ester	−	−	+
26.	32.78	1,2-Benzenedicarboxylic acid	+	−	−

autoclaved cell of consortium, exhibited no MM decolourisation after incubation up to 240 h (Fig. 3). In contrast, 75% MM was decolourised by living cell of consortium (Fig. 3). Further, NaOH extraction method showed that the final fraction of NaOH extractable colour substances were negligible (Fig. 3). The comparative data of extracted colourant from living and autoclave cell showed that living activity rather than adsorption. These results clearly indicated that the decolourisation of MM was due to biological mechanism.

Characterization of metabolic products through HPLC and GC–MS analysis during MM decolourisation

The TLC analysis of bacterial degraded sample showed diminishing of band observed under visible as well as in UV light as compared to control (Fig. 4a, b). This indicated that both high and low molecular weight compounds present in MM were degraded simultaneously by bacterial consortium. Therefore, a trailing of compounds observed in control was totally finished and only very faint band was appeared after bacterial treatment indicated degradation of MM (Fig. 4a, b). The HPLC analysis of bacterial degraded MM has shown the periodic reduction in peak area compared to control (Fig. 4c) indicated that decreased in colour intensity might be largely attributed to the bacterial degradation of colour containing compounds. In addition, the bacterial degraded MM also showed minor shifting of peak compared to control from RT 1.20 to 1.05 min, indicated the biodegradation of higher molecular weight compounds into lower molecular weight compounds by these bacterial

consortium at 5th days of bacterial growth. This subsequently showed further reduction of absorption peak area in 10 days bacterial treated sample. This observation was also supported by TLC. Further, the metabolites characterization through GC–MS analysis in ethyl acetate extracted sample showed less peaks in control compared to bacterial degraded MM at 5 and 10 days (Fig. 5). Few prominent peaks were noted in control at RT 14.58–16.00 min, these were characterized as 2-ethylpyridine (RT = 14.58 min), *n*-methyl-2-nitro-3-pyridinamine (RT = 15.14 min), pyridine, 2-methyl-1-oxide (RT = 15.31 min), 3-ethylpyridine (RT = 15.88), isonicotinyl formaldoxime (RT = 16.00 min) (Fig. 5a, Table 2). Besides, 3-amino-2-oxazolidinone (RT = 10.59 min) and 1,2-benzenedicarboxylic acid (RT = 32.78 min) were also detected in control sample but cyclopropylmethanol acetate (RT = 12.39), 4-pyridinecarboxlic acid (RT = 14.13 min), phthalic acid (RT = 19.99 min) and benzyl butyl phthalate (RT = 27.93 min) as trace compounds might be present in melanoidin or generated in control during processing (Fig. 5a; Table 2). However, several new peaks from RT 19.00–24.00 min appeared after 5 days of bacterial treatment (Fig. 5b), indicated the formation of numerous metabolic products. These metabolic products were identified as 3-octadecene (RT = 19.00 min), *n*-hexadecanoic acid (RT = 21.46 min) and 13-tetradecen-1-*O*-acetate (RT = 21.73 min) using the NIST mass database (Fig. 5b; Table 2). Whereas, the disappearance of peak at RT 14.13–16.00 min of control after 5 days bacterial treated sample might be due to utilization of these colourant as nutrient by bacterial consortium. Besides, bacterial treated MM showed more peaks at 5 days, this indicated the formation of simpler molecules from the complex MM. The degradation of phenolics compounds from the distillery effluent after bacterial degradation was observed previously (Bharagava and Chandra 2009).

However, new metabolic products were generated at RT 15.06, 15.94, 21.40, 21.69, 24.92, 26.48 and 32.76 min after 10 days bacterial degradation of MM (Fig. 5c; Table 2). The 3-(2-pyridyl)-1-propanol (RT = 15.06 min) and phthalic acid, butyl-4-octyl ester (RT = 21.40) as major products were generated after 10 days of bacterial treatment, which are metabolic products of organic complex present in original sample. Similar product has also been reported as melanoidin product of distillery waste after fungal treatment (Gonzalez et al. 2000). The compounds 3-(2-pyridyl)-1-propanol (RT = 15.06 min), 3-ethyl-pyridine (RT = 15.77 min), nicotinic acid, propyl ester (RT = 15.94 min), phthalic acid, butyl-4-octyl ester (RT = 21.40 min), 1-eicosanol (RT = 21.69 min), O,P′-DDE (RT = 24.92 min), hexadecamethyl octa-siloxane (RT = 26.48 min) and phthalic acid, dodecyl octyl ester (RT = 32.76 min) were also found in significant amount in the decolourised MM (Fig. 5c; Table 2).

Conclusion

This study concluded that the supplementary glucose as carbon and peptone as nitrogen were essential for bacterial degradation and decolourisation of MM. The comparative physico-chemical detection of different products in isopropanol extracted sample of MM revealed that phenol, sulphate, metals and melanoidin form complex in complex BMDS. This study also revealed that the growth and decolourisation of MM involved MnP and laccase enzyme, which completely metabolized the MM rather than adsorption. Hence, the developed bacterial consortium could be high prospect for decolourisation of melanoidin containing sugarcane molasses based distillery effluent for environmental safety.

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