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Effect of heavy metals and phenol on bacterial decolourisation and COD reduction of sucrose-aspartic acid Maillard product

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Abstract

Melanodins are amino-carbonyl complex, predominantly present in sugarcane molasses based distillery wastewater as major source of colourant. The microbial decolourisation of melanoidin is a challenge due to its binding property with other co-pollutants of distillery waste. Results revealed that the presence of Zn^{2+} (2.00–20.00 mg/L) in melanoidin solution (1200 mg/L) stimulated the bacterial growth and sucrose-aspartic acid Maillard product (SAA) decolourisation as compared to control, while Fe^{3+} and Mn^{2+} at the same concentration inhibited the process. However, the presence of phenol (100 mg/L) along with Zn^{2+}, Fe^{3+} and Mn^{2+} suppressed the bacterial growth, SAA decolourisation and MnP activity. The shrinkage and reduced number of bacterial cell count at higher concentration of heavy metals in presence of phenol was also observed under scanning electron microscope.

Key words: degradation; manganese peroxidase; melanoidin

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Introduction

Melanoidin are generated in sugarcane molasses as complex polymer due to non-enzymatic reaction between amino acid and sugars at high temperature during sugar manufacturing (Chandra et al., 2008). These are major colourants present in sugarcane molasses based distillery effluent. It contributes high biological oxygen demand (BOD, 5000 mg/L) and chemical oxygen demand (COD, 25,000 mg/L). Besides, distillery effluent after biomethanation contains high amount of phenolics (510 mg/L) and heavy metals (Pant and Adholeya, 2007; Bharagava et al., 2008). Sugarcane molasses based distillery effluent content nearly 2% melanoidin along with mixture of heavy metals and phenolics as major source of environmental pollution due to its complex nature. Because, melanoidin have binding tendency with heavy metals, hence this makes effluent more complexe (Migo et al., 1997). When it is discharged to the surface water, it reduces the sunlight penetration in the river, lakes or lagoons. This leads to decreased photosynthesis and dissolved oxygen of aquatic resources. Similarly, its disposal on soil is equally detrimental causing inhibition of seed germination and depletion of vegetation by addition of recalcitrant pollutants (Chandra et al., 2004, 2008).

Recently, decolourisation of molasses melanoidin by bacteria has been reported, due to their versatile nutritional behaviour (Kumar and Chandra, 2006; Bharagava et al., 2009). The degradation and decolourisation of melanoidin by fungus and bacteria are mediated due to prevalence of manganese peroxidase (MnP) as decolourising enzyme (Raghukumar et al., 2004; Bharagava et al., 2009). Assessment of bacterial degradation of different model melanoidins with screened bacteria showed variable degradability (Kumar and Chandra, 2006; Bharagava et al., 2009). But, most of studies have been confined to capability of bacteria for melanoidin decolourisation. Moreover, the assessment based on single pollutant exposure enable us to acquire fundamental knowledge about individual pollutant under carefully controlled condition, which do not reflect real-world exposures. Though, the individual effect of heavy metals and phenol have been reported on bacterial growth and degradation of some environmental pollutants (Yamaoka et al., 2002; Hong et al., 2007; Nweke et al., 2007, de Lima et al., 2007), but the combined effect of phenol and heavy metals on bioremediation of any polymer present in industrial waste is unknown. The biological decolourisation of molasses based distillery effluent is still major challenge. The knowl-

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edge on the effect of heavy metals and phenolics on bacterial decolourisation of melanoidin is not reported so far. Therefore, the knowledge of this would be helpful to understand the mechanism of distillery effluent decolourisation. Hence, present study has been focused on effect of Fe^{3+}, Zn^{2+}, Mn^{2+} and phenol on sucrose-aspartic acid Maillard product (SAA) decolourisation by bacterial consortium comprising *Bacillus* sp. (IITRM7; FJ581030), *Raoultella planticola* (IITRM15; GU329705) and *Enterobacter sakazakii* (IITRM16, FJ581031) with ligninolytic activity capable for decolourisation of melanoidins (Yadav et al., 2011). The sucrose and aspartic acid are predominantly present in sugarcane juice which plays a major role in synthesis of melanoidin during sugar manufacturing process.

1 Materials and methods

1.1 Preparation of synthetic melanoidin and its physico-chemical analysis

The synthetic melanoidin used in this study was prepared as described earlier (Chandra et al., 2004). The melanoidin produced in this reaction process were designated as SAA. The degradation and decolourisation of SAA in the presence of different heavy metals and phenol was assessed with physico-chemical changes. The analysis of different physico-chemical parameters was accomplished as described in standard methods for examination of water and wastewater (APHA, 2005).

1.2 Screening of phenol concentration for SAA decolourisation by bacterial consortium

The variable concentration of phenol (50, 100, 200 and 300 mg/L) was added in glucose-peptone-yeast extract-mineral salt (GPYM) broth to investigate the phenol tolerance limit of bacterial consortium. The phenol concentration showing optimum bacterial growth at 100 mg/L was chosen for further study. The bacterial growth was investigated in 250 mL Erlenmeyer flask containing 100 mL of sterile SAA (1200 mg/L) amended with GPYM broth as reported earlier (Chandra et al., 2004). The flasks were inoculated with 3% (*V/V*) of bacterial consortium consisting mixture of three pre-isolated bacterial strains, i.e., *Bacillus* sp., *R. planticola* and *E. sakazakii* in equal volume (1.00%, *V/V*, each) having cell density 3.30×10^4, 3.50×10^4 and 3.80×10^4 cells/mL, respectively. Further, this was incubated at 180 r/min and (37 ± 1)°C in shaking flask condition incubated at temp controlled shaker (Kuhner, Switzerland) for twelve consecutive days. The bacterial growth and SAA decolourisation was monitored spectrophotometrically (UV-2300 Spectrophotometer, Techcomp, Korea) by taking absorbance at 620 and 475 nm, respectively against the initial absorbance at the same wavelength (Chandra et al., 2004). Subsequently, the viable cells of *Bacillus* sp., *R. planticola* and *E. sakazakii* from day 6 and 12 of bacterial degraded samples were counted using plate spreading method on nutrient agar after 24 and 48 hr incubation.

1.3 Experimental design of SAA decolourisation study in presence of heavy metals and phenol

To study the effect of phenol and heavy metals on SAA decolourisation, two set of experiments were conducted as detail given in **Table 1** to generate the simulated conditions of effluent. The stock solution of Zn^{2+}, Fe^{3+} and Mn^{2+} were prepared by using their respective salts $ZnCl_2$, $FeCl_3$ and $MnCl_2$. In addition, the bacterial inoculum was added in the flasks as mentioned in the above section and incubated it for decolourisation study. The permissible limit of each metal was chosen according to the prescribed standard of US EPA (1986). These metals were selected due to their occurrence in post methanated distillery effluent (Chandra et al., 2008). All the experiments were done without maintaining pH to obtain simulated condition of effluent. All the above treatments were replicated three times.

1.4 MnP activity and comparative SAA degradation study through high performance liquid chromatography

The supernatant received after the biomass separation from bacterial degraded melanoidin were used for the assessment of MnP activity. The MnP activity was measured by oxidation of phenol red as described by Arora et al. (2002). The degradation and decolourisation of SAA in the presence of phenol and heavy metals were assessed with high performance liquid chromatography (HPLC). The samples were analyzed by using a Waters, 515 HPLC systems equipped with reverse phase column C-18 (150 mm × 4.6 mm, particle size 5 μm) at 27°C and 2487 UV/V is detector via millennium software. Samples (20 μL) were injected followed by implementation of HPLC grade water (100%) at the flow rate of 1.00 mL/min. The mobile phase consisted HPLC grade acetonitrile:water at 70:30 (*V/V*) ratio and the detection wavelength was set at 290 nm to monitor the degradation of SAA.

1.5 Scanning electron microscopy

For scanning electron microscopy (SEM) analysis, degraded sample was centrifuged (6500 ×*g*) to separate the bacterial cells for 20 min. The pellets were washed thrice with distilled water to remove the culture medium contents. Subsequently, the bacterial cells were fixed, dehydrated according to the method described previously (Yadav et al., 2011) Then dried cells were mounted on the metal stubs with colloidal sliver paste and coated with a thin conductive film of gold in a sputtering coater and examined under SEM (LEO 435 VP, LEO Electron Microscopy Ltd., USA).

Table 1 Experimental details and growth pattern of potential bacterial consortium in SAA amended GPYM media

Sample	Content (mg/L)	Growth	Sample	Content (mg/L)	Growth
CK	SAA (1200)	++++			
1st set of experiment			**2nd set of experiment**		
S1	SAA + Zn (2.00)	++++	S21	SAA + Phenol + Zn (2.00)	+++
S2	SAA + Zn (10.00)	++++	S22	SAA + phenol + Zn (10.00)	++
S3	SAA + Zn (20.00)	+++	S23	SAA + phenol + Zn (20.00)	++
S4	SAA + Zn (30.00)	+	S24	SAA + phenol + Zn (30.00)	- -
S5	SAA + Zn (40.00)	- -	S25	SAA + phenol + Zn (40.00)	- -
S6	SAA + Fe (2.00)	++	S26	SAA + phenol + Fe (2.00)	++
S7	SAA + Fe (10.00)	++	S27	SAA + phenol + Fe (10.00)	- -
S8	SAA + Fe (20.00)	+	S28	SAA + phenol + Fe (20.00)	- -
S9	SAA + Fe (30.00)	- -	S29	SAA + phenol + Fe (30.00)	- -
S10	SAA + Fe (40.00)	- -	S30	SAA + phenol + Fe (40.00)	- -
S11	SAA + Mn (0.20)	+++	S31	SAA + phenol + Mn (0.20)	+++
S12	SAA + Mn (1.00)	+++	S32	SAA + phenol + Mn (1.00)	+++
S13	SAA + Mn (2.00)	+++	S33	SAA + phenol + Mn (2.00)	++
S14	SAA + Mn (3.00)	++	S34	SAA + phenol + Mn (3.00)	+
S15	SAA + Mn (4.00)	++	S35	SAA + phenol + Mn (4.00)	+
S16	SAA + Zn (2.00) + Fe (2.00) + Mn (0.20)	+++	S36	SAA + phenol + Zn (2.00) + Fe (2.00) + Mn (0.20)	++
S17	SAA + Zn (10.00) + Fe (10.0) + Mn (1.00)	+++	S37	SAA + phenol + Zn (10.00) + Fe (10.0) + Mn (1.00)	++
S18	SAA + Zn (20.00) + Fe (20.0) + Mn (2.00)	++	S38	SAA + phenol + Zn (20.00) + Fe (20.0) + Mn (2.00)	+
S19	SAA + Zn (30.00) + Fe (30.0) + Mn (3.00)	- -	S39	SAA + phenol + Zn (30.00) + Fe (30.0) + Mn (3.00)	- -
S20	SAA + Zn (40.00) + Fe (40.0) + Mn (4.00)	- -	S40	SAA + phenol + Zn (40.00) + Fe (40.0) + Mn (4.00)	- -

++++: luxuriant growth; +++: moderate growth; ++: slow growth; +: very slow growth; -: no growth. CK: control.

1.6 Statistical analysis

One-way analysis of variance (ANOVA) and Tukey's test were employed to test the data variability and validity of the results using the Graph Pad software (San Diego, CA) (Ott, 1984).

2 Results and discussion

2.1 Changes in physico-chemical parameters of SAA containing media in presence of heavy metals and phenol

The SAA containing GPYM media showed variation in physico-chemical properties due to the addition of heavy metals and phenol (**Table 2**). The addition of Fe^{3+} contributed more colour in aqueous solution of SAA as compared to Zn^{2+} and Mn^{2+} even at permissible limit of its disposal. The SAA amended GPYM media containing permissible limit of Zn^{2+} (S1), Fe^{3+} (S6) and Mn^{2+} (S11) showed significant decolourisation after bacterial treatment. However, Fe^{3+} concentration (>15 times of permissible limit) started precipitation. Similar observations were also noted with Mn^{2+} and Zn^{2+} but less precipitation. Sample S16 showed significant decolourisation and COD reduction after bacterial treatment. This showed that the developed bacteria having potential to tolerate selected metals even in mixed condition. The used bacterial consortium was unable to decolourise the SAA solution containing >15 times higher permissible concentration of Zn^{2+} and Fe^{3+} (S5, S10) and COD and BOD reduction was not noted (**Table 2**). Furthermore, in samples S21–S40 BOD (11,000–49,010 mg/L), COD (26,000–120,000 mg/L) and colour (6250–19,532 Co-Pt) was significantly increased (**Table 2**). The chromogenic activity of iron with melanoidin has also been reported (Morales et al., 2005). The addition of phenol with heavy metals in SAA solution (set 2) increased the colour as compared to set 1 where phenol was absent except Mn^{2+}. The order of metals for colour contribution with SAA was noted mixed metals > Fe^{3+} > Mn^{2+} > Zn^{2+} (**Table 2**). It appears that the observed effect followed the schulz-hardy rule which stated that trivalent is more effective than divalent and monovalent (Feron and Gronten, 2002).

2.2 Effects on bacterial cell count and SAA decolourisation

The Zn^{2+} concentration up to 10 times permissible limit (S1–S3) was found stimulatory for the bacterial growth and decolourisation as compared to control (only SAA amended GPYM medium) (**Figs. 1 and 2**). Further, higher concentration of Zn^{2+} at 20 times of permissible limit (S5) showed inhibitory effect. The stimulatory effect might be attributed due to the use of zinc as trace element by bacterial consortium.

In contrast, even permissible concentration of Fe^{3+} (S6) in SAA solution inhibited the bacterial growth and decolourisation process as compared to control (**Figs. 1 and 2**). The bacterial cell count was also inhibited by Fe^{3+} >16 times as compared to Zn^{2+} at the same concentration (**Fig. 1**). Further, increases of Fe^{3+} concentration up to 5 times of the permissible limit (S7) suppressed the bacterial growth. The addition of Fe^{3+} prolonged the lag phase of bacterial cell growth, but this gradually attained the growth cycle, which indicated that the potentiality of bacterial consortium. However, only few bacterial cells could appear at 10 times higher concentration of permissible

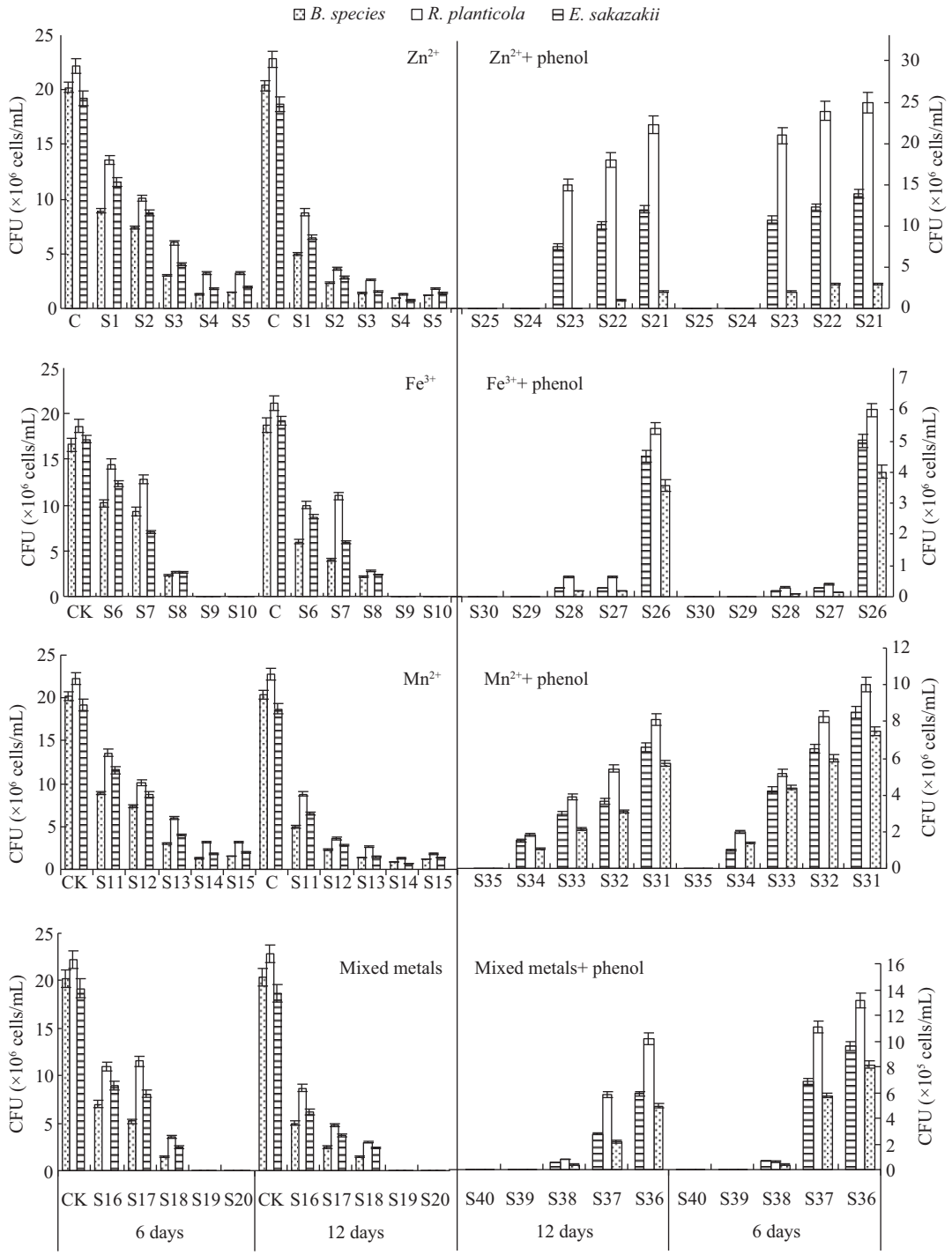

Fig. 1 CFU of potential bacterial strains in SAA containing GPYM media amended with Zn^{2+}, Fe^{3+}, Mn^{2+} separately and in mixed conditions with and without phenol (100 mg/L) at different time incubation (6 and 12 days).

limit of Fe^{3+} with SAA (S8). Furthermore, increased Fe^{3+} concentrations (S9 and S10) ceased the bacterial growth and increased the colour as compared to control even after bacterial treatment (**Figs. 1 and 2**). Similarly, the presence of Mn in SAA solution (S11–S15) also inhibited the bacterial growth and decolourisation process (**Figs. 1 and 2**). But, bacterial consortium showed growth as well as decolourisation up to 20 fold higher concentration of Mn. Besides, all three metals in mixed condition (S16–S20) with SAA solution contributed more inhibition for bacterial growth with prolonged lag phase as compared to samples S1–S15 (**Fig. 1**).

While, Zn^{2+} with phenol (S21–S25) inhibited the bacterial growth as well as decolourisation process as compared to Zn^{2+} without phenol (S1–S5) even at permissible concentration (**Figs. 1 and 2**). The bacterial consortium

Table 2 Heavy metals and phenol effect on physico-chemical parameters of SAA, before and after bacterial treatment

Sample	Before bacterial treatment			After bacterial treatment		
	Colour	COD (mg/L)	BOD (mg/L)	Colour	COD (mg/L)	BOD (mg/L)
Control	6250 ± 250	26000 ± 763	11000 ± 278	1875 ± 56	5720 ± 175	3840 ± 143
1st set of experiment						
S1	6256 ± 135^{ns}	27000 ± 292^{ns}	12000 ± 276*	1438 ± 22*	5130 ± 143^{ns}	2520 ± 78*
S2	6458 ± 175^{ns}	28318 ± 763*	13000 ± 236*	1808 ± 45*	10477 ± 265*	4550 ± 96^{ns}
S3	6876 ± 87*	30138 ± 683*	14653 ± 254*	1994 ± 76*	17178 ± 176*	7619 ± 134*
S4	8450 ± 164*	32132 ± 753*	15932 ± 232^{ns}	5915 ± 72*	25705 ± 543*	13064 ± 260*
S5	8575 ± 213*	36053 ± 413*	16381 ± 234^{ns}	8575 ± 93*	36000 ± 487*	16300 ± 365*
S6	6810 ± 231^{ns}	29786 ± 432*	14321 ± 324*	2941 ± 65*	11318 ± 387*	5585 ± 155*
S7	7590 ± 172*	32321 ± 462*	15861 ± 453^{ns}	3795 ± 76*	16806 ± 386*	7296 ± 98*
S8	8696 ± 241*	36123 ± 653*	17621 ± 245*	6087 ± 132*	24924 ± 435*	12334 ± 435*
S9	9688 ± 251*	43144 ± 532*	21673 ± 654*	10172 ± 176*	43200 ± 655*	21760 ± 675*
S10	9770 ± 231*	47893 ± 671*	23521 ± 547*	10551 ± 198*	47893 ± 677*	23678 ± 643*
S11	6340 ± 165^{ns}	28321 ± 437*	13321 ± 432*	2193 ± 57^{ns}	7646 ± 102*	3863 ± 87^{ns}
S12	6586 ± 186^{ns}	30143 ± 671*	14732 ± 124*	2733 ± 65*	12961 ± 333*	6776 ± 134*
S13	6950 ± 152*	33543 ± 436*	16321 ± 546^{ns}	3780 ± 56*	19119 ± 432*	10608 ± 156*
S14	8540 ± 173*	36231 ± 487*	19535 ± 243*	6405 ± 87*	27897 ± 542*	15628 ± 264*
S15	8832 ± 231*	41132 ± 586*	21111 ± 254*	7065 ± 145*	33728 ± 765*	17099 ± 276*
S16	6656 ± 152^{ns}	30532 ± 432*	14931 ± 343^{ns}	3061 ± 124*	26715 ± 463*	11383 ± 228*
S17	7980 ± 265*	34512 ± 453*	16317 ± 234^{ns}	4628 ± 87*	25884 ± 528*	12440 ± 289*
S18	9050 ± 270*	45532 ± 598*	23498 ± 432*	8552 ± 165*	33347 ± 666*	20969 ± 365*
S19	12320 ± 300*	70133 ± 975*	30132 ± 432*	12320 ± 164*	69599 ± 876*	29763 ± 376*
S20	14890 ± 362*	98543 ± 908*	42383 ± 654*	14890 ± 253*	97891 ± 954*	41353 ± 564*
2nd set of experiment						
S21	6678 ± 154^{ns}	30145 ± 652*	14321 ± 342*	2637 ± 65^{ns}	7837 ± 365^{ns}	4009 ± 87^{ns}
S22	6924 ± 134^{ns}	32154 ± 476*	15893 ± 432^{ns}	3081 ± 76*	13183 ± 487*	6357 ± 154*
S23	7212 ± 165*	34873 ± 587*	16891 ± 432^{ns}	4543 ± 57*	23016 ± 465*	11485 ± 243*
S24	8721 ± 198*	36238 ± 654*	17243 ± 453^{ns}	8721 ± 265*	36238 ± 685*	17243 ± 265*
S25	8932 ± 203*	38183 ± 415*	19983 ± 342*	8932 ± 276*	38183 ± 489*	19983 ± 354*
S26	7315 ± 214*	33453 ± 563*	16321 ± 187^{ns}	3950 ± 367*	13046 ± 355*	6691 ± 786*
S27	7917 ± 376*	36830 ± 654*	18981 ± 275*	8233 ± 187*	36830 ± 473*	18981 ± 387*
S28	8374 ± 434*	39170 ± 584*	19761 ± 453*	8960 ± 187*	40170 ± 642*	19761 ± 342*
S29	9912 ± 254*	46453 ± 543*	23811 ± 453*	10903 ± 254*	46553 ± 657*	23934 ± 435*
S30	10543 ± 354*	50501 ± 675*	24921 ± 309*	11808 ± 322*	50501 ± 854*	25921 ± 465*
S31	6874 ± 187^{ns}	32000 ± 682*	15721 ± 423^{ns}	3100 ± 243*	2130 ± 45*	4087 ± 87*
S32	7289 ± 234*	34321 ± 653*	16883 ± 325^{ns}	3879 ± 176*	2915 ± 55*	8441 ± 183^{ns}
S33	7474 ± 265*	36123 ± 542*	17932 ± 324*	4529 ± 165*	4484 ± 64^{ns}	12373 ± 276*
S34	9232 ± 278*	42762 ± 543*	19981 ± 435*	7754 ± 186*	7754 ± 87^{ns}	17383 ± 365*
S35	9412 ± 185*	48672 ± 587*	20123 ± 654*	9412 ± 187*	8094 ± 176^{ns}	17909 ± 276*
S36	6815 ± 563^{ns}	34325 ± 875*	16561 ± 345^{ns}	4388 ± 154*	3475 ± 78^{ns}	13070 ± 298*
S37	8232 ± 275*	58000 ± 865*	21678 ± 654*	6132 ± 186*	5614 ± 165^{ns}	18671 ± 354*
S38	9443 ± 287*	70000 ± 867*	32516 ± 764*	9443 ± 254*	68000 ± 875*	31516 ± 576*
S39	16312 ± 345*	90321 ± 980*	41220 ± 783*	16312 ± 376*	90321 ± 999*	41220 ± 875*
S40	19532 ± 333*	120000 ± 3212*	49010 ± 876*	19532 ± 453*	120000 ± 3212*	49010 ± 876*

All the values are mean of three replicates (n = 3) ± SD. Significance levels: * $p < 0.05$; ^{ns} $p > 0.05$. COD: chemical oxygen demand; BOD: biological oxygen demand.

showed 30% decolourisation in presence of Zn^{2+} even upto 15 times higher concentration of permissible level. However, in presence of phenol (100 mg/L) bacterial consortium could decolourise SAA only up to 37% at 10 times permissible concentration of Zn^{2+} (**Fig. 2**). But, bacteria could not grow at further higher concentration of Zn^{2+} with phenol. Phenol (100 mg/L) along with Fe^{3+} even at low concentration (S26) drastically suppressed the bacterial growth and decolourisation (46%) (**Figs. 1 and 2**). Result also revealed that the Fe^{3+} along with phenol above than 5 times of permissible limit (S27–S30) completely ceased the bacterial growth and increased the colour of media (**Fig. 2**). Bacterial consortium at 20 times of permissible limit for Mn^{2+}, with and without phenol (S15 and S35) showed decolourisation 20% and 14%, respectively (**Fig. 2**). In previous study the heavy metals toxicity along with phenol for bacterial growth and pollutants degradation has also been reported (Nakamura and Sawada, 2000). However, the presence of phenol with mixed metal solution (S36–S40) increased the complexity of melanoidin and reduced the pH (pH 4.00); this further suppressed the bacterial growth and concomitantly increased the BOD and COD (**Fig. 1 and Table 2**). Consequently, heavy metals with phenol (set 2) reduced the bacterial cell count and SAA decolourisation as compared to solution containing only heavy metals (set 1). This indicated that the multi-metals makes more complex environment with melanoidin in presence of phenol for bacterial growth and degradation. The comparative Colony Forming Unit (CFU) revealed that *R. planticola* was most potential followed by

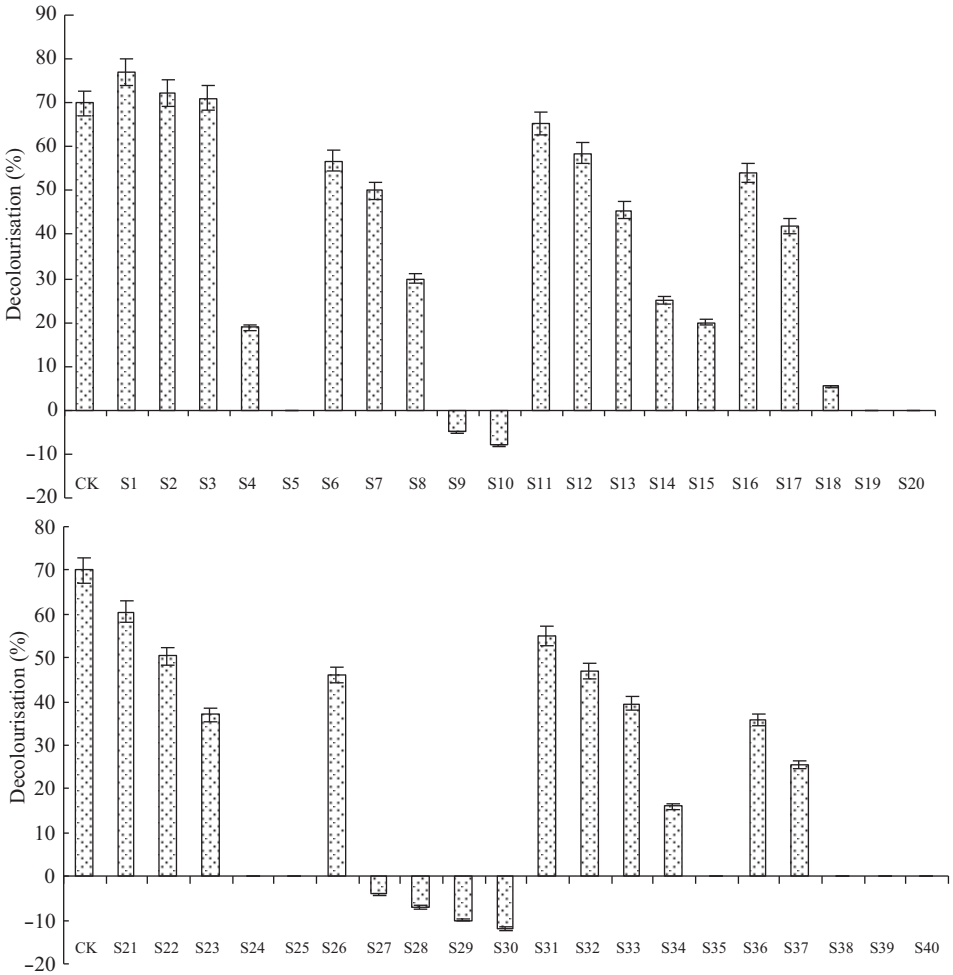

Fig. 2 Effect of heavy metals and phenol on bacterial SAA decolourisation.

E. sakazakii and *Bacillus* sp. (**Fig. 1**). This indicated that the order of potentiality of different bacterial strains for decolourisation of melanoidins in presence of heavy metals and phenol.

The decrease of decolourisation activity was directly proportional to increase of Zn^{2+}, Fe^{3+} and Mn^{2+} concentration, therefore they showed linear correlation (R^2: 0.9457–0.9832) (**Fig. 3**). While, the presence of phenol (100 mg/L) with metals in SAA severely dropped (R^2: 0.4587–0.9538) the SAA decolourisation activity in set 2 experiment. Hence, decolourisation and metals concentrations in the presence of phenol did not follow the linearity (R^2: 0.4584).

The melanoidin peak in HPLC analysis also showed maximum reduction in presence of Zn^{2+} (S1) followed by SAA alone than S21 after bacterial treatment, this supported the above observation (**Fig. 4a**).

However, the higher concentration of Zn^{2+} (>15.00 mg/L) in SAA containing culture media increased absorption peak compared to SAA degraded peak. But, the presence of Zn^{2+}, Fe^{3+} and Mn^{2+} in mixed condition even at lower concentration (S16) after bacterial treatment showed very less reduction in melanoidin absorption peak during HPLC analysis indicated the low decolourisation (**Fig. 4b**). But, the addition of phenol in mixed metals conditions (S36) showed shifting of absorption peak at higher side this indicated the formation of new compounds, which do not showed reduction in absorption peak rather than slight sifting towards lower side as compared to S16 after bacterial treatment (**Fig. 4c**). This explains the complexation of SAA with metals and phenol. Our findings corroborated with earlier observation of metal binding with melanoidin (Feron and Groten, 2002), but the comparative study between metals, phenol and melanoidin is a new observation.

2.3 Effects of heavy metals and phenol on MnP enzyme activity

The effect on MnP activity was noted at permissible concentration of metals with and without phenol in SAA amended GPYM solution as shown in **Fig. 5a**. The permissible concentration of zinc (S1) showed stimulatory effect on MnP activity as compared to control. The maximum enzyme activity was noted at day 4 of bacterial growth and enzyme activities remains constant up to day 9. However, the enzyme activity was declined during subsequent

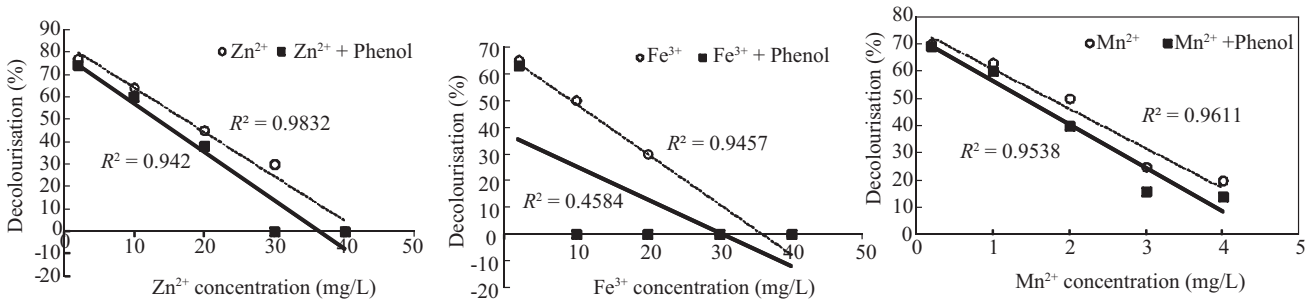

Fig. 3 Linear relations between different concentrations of various metal (Zn^{2+}, Fe^{3+} and Mn^{2+}) and mean decolourization value.

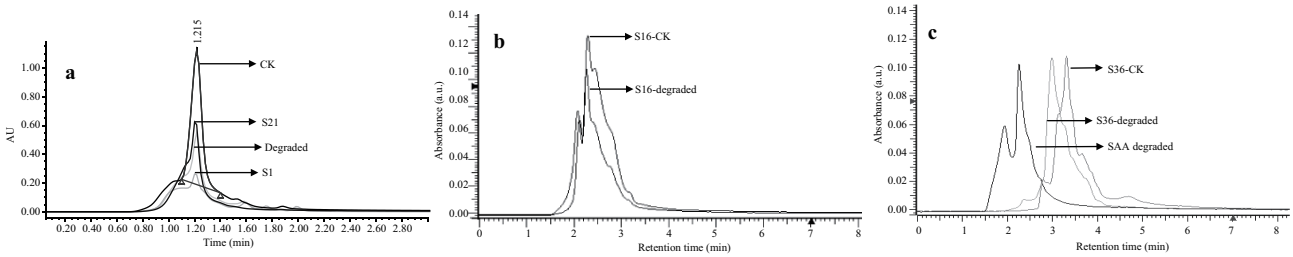

Fig. 4 HPLC chromatogram of SAA degradation in presence of Zn^{2+} (a), mixed metals at permissible concentration (b) and phenol + mixed metals at permissible concentration (c).

incubation. The presence of Fe^{3+} (S6) suppressed the MnP activity for melanoidin decolourisation and it was lower than the presence of Mn^{2+} in melanoidin solution (S11). The order of enzyme induction by different metals was Zn^{2+}> Mn^{2+} > Fe^{3+} > metals in mix condition. However, the presence of phenol along with these metals (set 2) showed inhibitory effect on enzyme activity as compared to control and set 1 (**Fig. 5a**). The mechanism for the bacterial enzyme inhibition might be due to the changes in the molecular structure of the enzyme caused by the inhibitors, i.e., phenol and heavy metals (Shen et al., 2006; Nweke et al., 2007). For the heavy metals, it is assumed that either they bind to sulfhydrile group of the active site or organic chemicals may denature the entire protein structure (Silva et al., 2007).

In addition, the intensity of purified MnP band in SDS-PAGE coincided to the pattern of enzyme activity (**Fig. 5b**). The MnP band intensity was found in order of S1 > CK > S11 > S21 > S6> S31 > S26 > S16 > S36.

2.4 Morphological changes of potential bacterial strains under SEM during SAA degradation in the presence of heavy metals and phenol

The bacterial consortium effectively showed SAA decolourisation in GPYM media with normal cell morphology as shown in Fig. 6a. However, an adverse morphological change on bacterial cells was observed under SEM at higher concentration of heavy metals with and without phenol (**Fig. 6b–e**). The Zn^{2+}, Fe^{3+} and Mn^{2+} in mixed condition at permissible limit (S16) showed favourable growth for *R. planticola* followed by *E. sakazakii* and *Bacillus* sp. Therefore, *R. planticola* appeared dense under SEM observation and cell morphology becomes flat (**Fig. 6b**). But, the further addition of phenol (100 mg/L) with constant metals concentration (S36) showed the drastic cell size reduction (**Fig. 6c**), indicated the toxic effect of phenol (100 mg/L) with heavy metals even at permissible concentration.

The bacterial morphological shrinkage at adverse conditions has also been previously noted (Kaletunç et al., 2004). The ten fold higher concentration of mix metals in media pronounced the shrinkage of bacterial cell morphology (S18) (**Fig. 6d**). This indicated that higher concentration of heavy metals in mixed condition is more toxic to bacterial growth. Furthermore, it was also observed that phenol (100 mg/L) along with 10 fold higher mixed metals concentration (S38) showed more shrinkage and clumped bacterial cells of *R. planticola*, while *Bacillus* sp. was observed very few with thick cell wall (**Fig. 6e**). This might be due to polysaccharides covering around the cell wall which is adoptive feature of bacteria in adverse conditions. The shrinkage of bacterial cell size, secretion of polysaccharides and clumping in adverse conditions has also been reported previously (Kaletunç et al., 2004; Xie et al., 2010).

3 Conclusions

The findings concluded that lower concentration of zinc stimulated bacterial growth and SAA decolourisation. However, Fe^{3+} showed inhibitory effect on bacterial growth even at permissible limit. Further, it was observed that presence of these metals along with SAA contributed the colour, COD, BOD and TS. While, the combination of phenol along with Fe^{3+} in SAA solution, further increased the colour intensity and pollution parameters. But, the

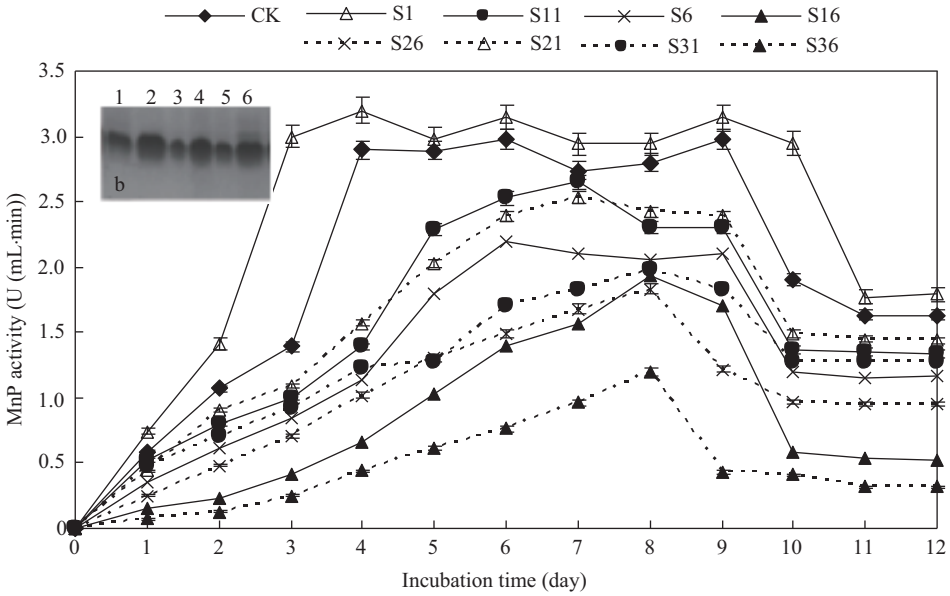

Fig. 5 MnP activity of potential bacterial consortium in SAA containing GPYM media amended with metals, with and without phenol (a). Insert figure (b) shows purified MnP. lane 1: Ladder; line 2: standard; lane 3: CK; lane 4: S1; lane 5: S6; lane 6: S11.

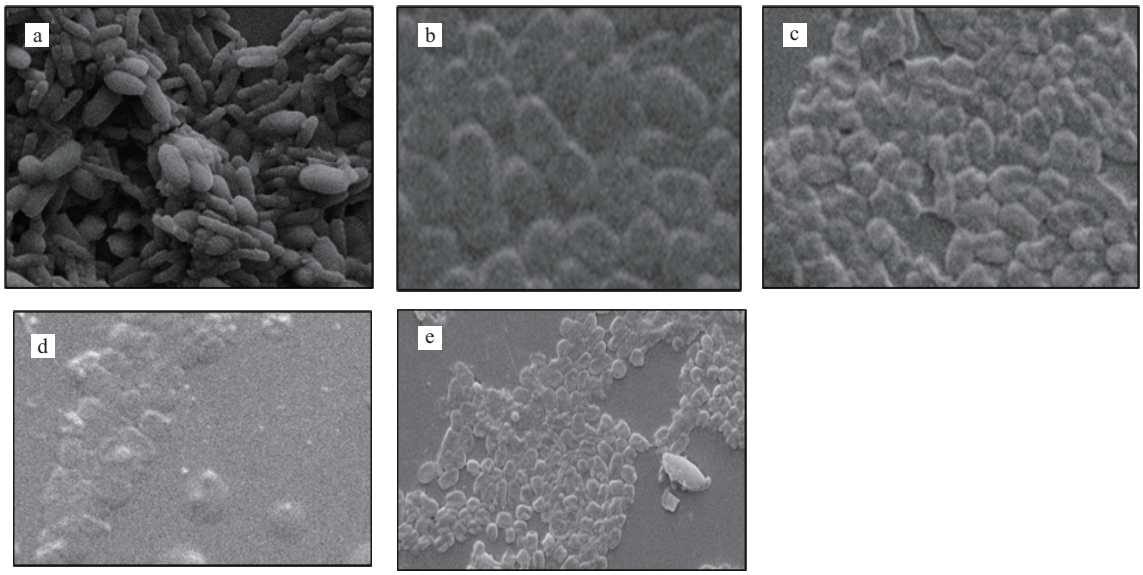

Fig. 6 Scanning Electron Micrograph (SEM) depicting the effect of heavy metals with and without phenol (100 mg/L) during SAA decolourisation at a magnification 5000×. (a) bacterial strains under untreated condition, (b) S16, (c) S36, (d) S18, (e) S38. *shows *Bacillus* sp. with thick cell wall.

bacterial decolourisation of SAA in presence of metals alone showed reduction in melanoidin absorption peak indicated depolymerisation along with slight decrease in pH. While, presence of phenol in these solution inhibited the bacterial growth and enzyme activity and do not show direct reduction of peak also. The order of bacterial dominancy was noted as *R. planticola* > *E. sakazakii* > *Bacillus* sp. However, the shrinkage and reduced number of bacterial cells at higher concentration of heavy metals along with phenol was noted under SEM. Hence, the study concluded that the presence of heavy metals and phenol in melanoidin containing industrial wastewater contributed colour and toxicity, which inhibited the microbial degradation process.

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