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RESEARCH ARTICLE

EFFECT OF PH ON MELANOIDIN EXTRACTION FROM POST METHANATED DISTILLERY EFFLUENT (PMDE) AND ITS DECOLORIZATION BY POTENTIAL BACTERIAL CONSORTIUM

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ABSTRACT

Melanodins are amino-carbonyl complex, predominantly present in sugarcane molasses based distillery wastewater as major source of colourant. The indiscriminate discharge of post methanated distillery effluent (PMDE) from distilleries is a major source of soil and water pollution which directly or indirectly affects the animals and human beings. Adequate treatment is therefore imperative before the effluent is discharged. The effect of pH on melanoidin extraction from PMDE and its decolorization by a developed potential consortium of *Proteus mirabilis* (IITRM5; FJ581028), *Bacillus sp.* (IITRM7; FJ581030*)*, *Raoultella planticola* (IITRM15; GU329705) and *Enterobacter sakazakii* (IITRM16, FJ581031) was studied. Melanoidin was extracted with isopropanol at different pH. The most effective pH for melanoidin extraction was noted at pH 11.0 (2.87%, w/v) from PMDE of color range 80000-85000 Co-Pt with high BOD (29120±10.00 mg/l) and COD (58018.33±23.63 mg/l). Subsequently, the maximum de-colorization (70.00%) of PMDE was noted in the presence of glucose (1%, w/v), peptone (0.05%) at pH 7.0 and minimum at pH 11.0 (11.66%, w/v) within 168 h bacterial incubation. Further, the results also revealed that the pH directly affected melanoidin extraction and bacterial de-colorization of PMDE. The cultures of these strains have better color removal ability after melanoidin extraction and thus can be utilized for bioremediation of PMDE affected sites.

INTRODUCTION

Current population explosion globally urges enlargement of industrial sectors resulting in pollution of water, air and soil. As prevention of pollution is not possible, control of pollution can be practiced effectively. The discharge of pollutants into the environment from various industries possesses a threat to living organisms resulting in a great environmental stress. One such industry of rapid development is the distillery industry. There are about 330 distilleries in India with total installed capacity is about 3500 million liters of alcohol production, generating huge volume of wastewater (Hati *et al.*, 2007). Dark brown color of wastewater of distilleries known as spentwash is mainly due to the presence of compounds such as melanoidins, caramel, furfural etc. The main problem in treating PMDE is its color, which contains nearly 2% (w/w) of a dark brown recalcitrant pigment, melanoidin. Melanoidin is known as a natural browning polymer, produced by the "Maillard reaction" between amino and carbonyl groups of organic matters and is closely related to humic substances in the natural environment (Wedzicha and Kaputo, 1992). These highly colored components lead to a reduction of sunlight penetration in rivers, lakes or lagoons which, in turn, decrease both photosynthetic activity and dissolved oxygen concentration causing harm to aquatic life. Disposal on land is equally hazardous causing a reduction in soil pH, inhibition of seed germination. Since, spentwash is a high strength wastewater; it is generally subjected to anaerobic digestion in huge methane reactors to convert organics into methane, which is used as a fuel. The biomethanated spentwash is then treated aerobically in activated sludge treatment plants to further reduce BOD/COD and used for composting pressmud, a byproduct of sugar mills. The color of spentwash persists even after biomethanation this effluent is known as post methanated distillery effluent (PMDE). Decolorization of PMDE by Chemical methods is expensive and generate large amount of sludge, causing secondary pollution (Kawamura, 1987). Hence, in the recent past, increased attention has been focused on the microorganisms capable for decolorization and mineralization of distillery effluent. Miranda *et al.* studied the effect of pH on color removal from molasses wastewater by *Aspergillus niger* and reported the optimal pH 5.0 (Miranda *et al.*, 1996). The effect of pH on removal of lignin and decolorization of pulp paper mill effluent by three marine fungi has been studied and found pH 4.5 to be effective (Raghukumar *et al.*, 1996). Similarly, the optimum pH for growth and decolorization of pulp paper mill effluent by *Schizophyllum commune* was noted between 4 to 5 (Belsare and Prasad, 1988). Though, several fungi and bacteria have also been implicated for decolorization of molasses melanoidin. Where, various basidiomycetous fungi such as *Coriolus*, *Phanerochaete* and deuteromyceteous fungi have showed promising result for decolorization of melanoidin (Sirianuntapiboon *et al.*, 1988; FitzGibbon *et al.*, 1995). However, due to slow growth and

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requirement of acidic pH for fungal growth, restrict its application at large scale. Therefore, some attempts have been made by using facultative anaerobic bacteria and aerobic bacteria for decolorization of melanoidin. The results for color removal from effluent have been noted between 32 to 65 percent (Kumar *et al*., 1997; Chandra and Singh, 2000). Further, attempts by using immobilized aerobic bacteria have increased the color removal efficiency from 65 to 76 percent. Further, the effects of various nutritional factors have also been observed on decolorization of melanoidin by Sirianuntapiboon *et al* .,(2004). But, the effect of pH on melanoidin-extraction and PMDE decolorization is not reported so far. Therefore, the objective of this study was to investigate the effect of pH on melanoidin extraction followed by decolorization of PMDE.

MATERIALS AND METHODS

Melanoidin extraction from PMDE at different pH and Physico-chemical characterization

PMDE with pH 8.3 was collected from M/s Unnao distilleries Ltd, Unnao, Uttar Pradesh, India. Melanoidin was extracted from PMDE with isopropanol as per the method described by Sangeeta and Chandra (2012). Briefly, 1:1 mixture (final volume 200 ml) of isopropanol and filter distillery effluent was taken in a separating funnel (500 ml). Shook well and kept for setting of precipitated melanoidin at bottom of funnel. While, the upper layer of separating funnel contains only remaining part of PMDE. This layer was taken to extract further melanoidin by addition of isopropanol. Lastly the precipitated melanoidin was separated in sterile beaker. The isopropanol was evaporated from melanoidin and air-dried in a hot air oven at temperature of 55 °C to calculate net weight of extracted melanoidin. The extraction of Melanoidin was done at variable pH 5.0 – 11.0 with fresh PMDE to observe the effect of pH on melanoidin extraction. The pH of the PMDE was adjusted using either 1 mol/l NaOH or HNO_3. Further, the physico-chemical parameters (pH, BOD, COD, total dissolved solids, sulfates, phosphates and phenolics etc.) of remaining PMDE after melanoidin extraction were measured according to the standard methods for the examination of water and wastewaters to observed the change in their properties (APHA, 2005).

Nutrient optimization for potential consortium for optimum bacterial growth and decolorization studies

Further, the developed bacterial consortium of strians *Proteus mirabilis* (IITRM5; FJ581028), *Bacillus sp.* (IITRM7; FJ581030), *Raoultella planticola* (IITRM15; GU329705) and *Enterobacter sakazakii* (IITRM16, FJ581031) (Sangeeta and Chandra, 2012) 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 bacterial growth and decolorization of PMDE before and after melanoidin extraction. Each nutrient was supplemented individually at final concentration in range of 0.1 to 1.5% and 0.05 to 0.5% for carbon and nitrogen, respectively as an additive to 0.1% K_2HPO_4 and 0.05% $MgSO_4.7H_2O$. These medium was designated as GPM media. The optimum concentration for carbon and nitrogen was noted at 1.0 and 0.05%, respectively. The decolorization experiments were carried out by the inoculation of 1.1 x 10^6 cells/ml of mixed bacterial inoculums of *Proteus mirabilis* (IITRM5; FJ581028), *Bacillus sp.* (IITRM7; FJ581030), *Raoultella planticola* (IITRM15; GU329705) and *Enterobacter sakazakii* (IITRM16, FJ581031) of log phase in 250 ml Erlenmeyer flasks containing 150 ml of 50% diluted melanoidin-extracted PMDE of different pH consisting of (w/v): 1% glucose, 0.05% peptone, 0.1% K_2HPO_4 and 0.05% $MgSO_4.7H_2O$ under shaking flask conditions (150 rev/min, Innova 4230 Refrigerated Incubator shaker, New Brunswick, U.S.A.). A separate set of uninoculated flasks was maintained in parallel as control. Experiments were performed in triplicate and samples were withdrawn at regular 24 h intervals for pH and decolorization measurements.

Decolorization Assay

PMDE decolorization was measured as the decrease in color density at 475 nm after 10 X dilutions with sterile 0.1 mol/l acetate buffer (pH 7.0) as adopted by Sangeeta *et al.*, (2011). Decolorization yield was expressed as the degree of decrease in the absorbance at 475 nm against initial absorbance at the same wavelength using a UV-Visible spectrophotometer (GBC Cintra-40, Australia). Bacterial uninoculated flask containing GPM medium was treated as control.

Statistical analysis

To test the data for significance, we performed one-way ANOVA ($p < 0.05$) between columns of different parameters. We used Tukey's test using the Graph Pad software (GraphPad Software, San Diego, Calif.) (Ott, 1984).

RESULTS AND DISCUSSION

Physico-chemical analysis of different stages of PMDE

The physico-chemical analysis of PMDE (BE) showed very high color (85000 Co-Pt), COD (58018 mg/l) and BOD (29120 mg/l) along with other pollution parameters (Table 1). This showed the complex nature of PMDE. PMDE when treated with bacterial consortium (BET) has not shown any significant reduction in physico-chemical properties. However, extraction of melanoidin from PMDE (AE) significantly reduced the all pollution parameters (Table 1). Furthermore, bacterial treatment of melanoidin extracted PMDE (AET) rapidly enhanced the reduction of color and other pollution parameters (Table 1). This might be due to less melanoidin concentration in PMDE which might be degraded by bacterial consortium. The melanoidin concentration dependent color reduction of PMDE has also previously reported (Sangeeta and Chandra, 2012).

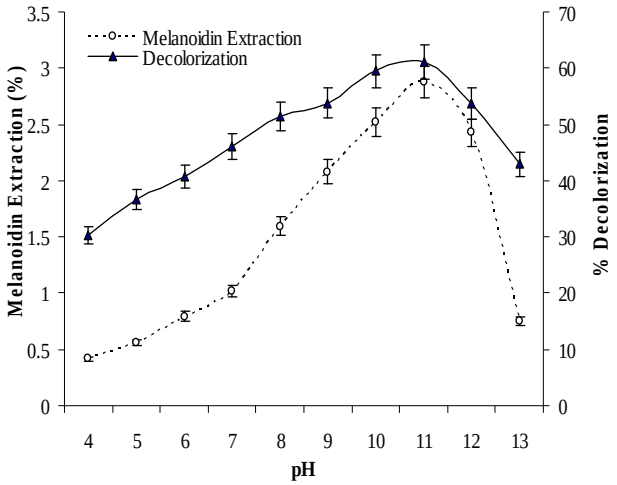

Fig. 1 Melanoidin extraction and decolorization of PMDE at different pH

Table 1 Physico-chemical analysis in post methanated distillery effluent (PMDE) after melanoidin extraction and bacterial treatment

Parameters	PMDE without melanoidin extraction (Control)		PMDE after melanoidin extraction at pH:7	
	BE	BET	AE	AET
PH	8.35±0.25	7.00±0.50 ^{ns}	6.97±0.06	7.00±0.03 ^{ns}
Color	85000.33±30.00	80511.05^{d}±15.12	37530.00±27.84	22549.90^{d}±12.00
COD	58018.33±23.63	50037.78^{d}±24.29	26108.25±30.00	12726.23^{d}±22.94
BOD	29120.00±10.00	27911.77^{d}±10.35	12560.00±15.28	8648.00^{d}±17.31
TS	15150.67±15.37	13070.82^{d}±8.56	6153.33±10.41	3324.37^{d}±6.50
TDS	12002.00±22.52	10690.37^{d}±21.64	5226.67±25.17	2116.87^{d}±17.00
TSS	3148.67±22.55	2380.45^{d}±20.21	926.66±26.51	1207.50^{d}±21.57
Sulphate	20732.33±28.57	19138.40^{d}±18.01	8860.07±21.20	4629.52^{d}±18.14
Phenol	775.66±16.93	513.15^{d}±12.16	567.06±15.73	379.87^{d}±9.26
Nitrate	1840.67±14.01	1173.27^{d}±11.45	1186.03±10.16	128.49^{d}±9.17
Chloride	1946.67±25.17	1892.15^{d}±26.02	918.05±16.43	477.17^{d}±17.61
Ammonium	1055.00±26.46	923.49^{d}±23.67	363.00±20.66	37.90^{d}±12.34
Sodium	5129.67±32.25	4166.14^{d}±27.38	2237.05±27.78	46.38^{d}±21.00
Melanoidin	11720.00±22.91	973.09^{d}±20.16	2892.76±20.26	913.17^{d}±8.33.

All Values are mean (n=3)± S.D.; values are reported in mg/l except pH and color; BE=before melanoidin extraction, BET=bacterial treatment of PMDE before melanoidin extraction, AE=after melanoidin extraction, AET=Bacterial treatment of PMDE after melanoidin extraction; ANOVA, (No-significant) a > 0.05, *p < 0.05 (less significant, b); **p < 0.01 (significant, c); ***p < 0.001 (highly significant, d) between means in a column

Effect of Melanoidin extraction on reduction of color of PMDE

Melanoidin extraction by isopropanol at different pH showed variable results. Maximum extraction was noted at pH 11.0 (2.87% w/v), while the least was observed at pH 4.0 (0.41% w/v). The extraction of melanoidin resulted into increase decolorization of distillery effluent (Fig. 1). The PMDE sample without melanoidin extraction does not show any significant reduction in physico-chemical parameters even after bacterial treatment (Table 1). However, the PMDE sample after melanoidin extraction showed significant reduction of pollution parameters (Table 1). This showed the color contribution of melanoidin in PMDE. It was maximum at pH 11.0 (61.10%) and minimum at pH 4.0 (30.40%) thereby causing extraction of more melanoidin-pigment at pH > 7.0.

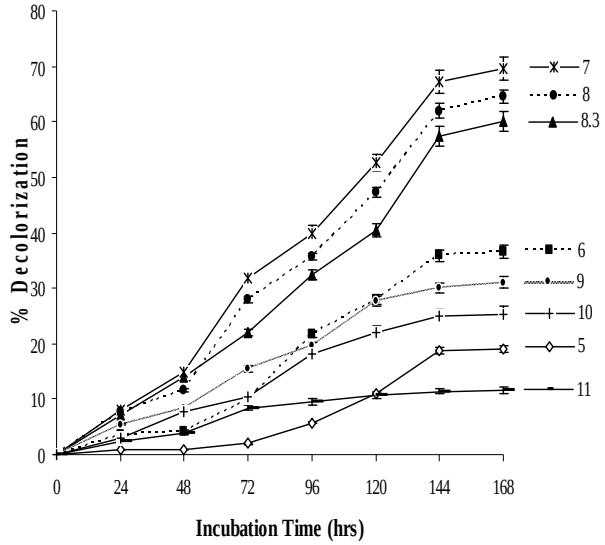

Fig. 2 Bacterial decolorization of PMDE after melanoidin extraction (AET) at different pH

The removal of melanoidins from effluent showed direct correlation between melanoidin extraction and decolorization of effluent from pH 4 to 11 (Fig. 1). However, the melanoidin extraction above pH 11.00 declined and color was increased (Fig. 1). This indicated that melanoidin was as major coloring constituent of PMDE.

Since the lowering of pH causes oxidation so it might be possible that oxidative decomposition of melanoidin could have occurred thereby leading to decrease in the color intensity of distillery effluent at low pH 4.0 - 6.0. Whereas, reduction at alkaline pH may have resulted in increased color intensity as during reduction -OH group is released which is one of the most effective auxochrome that results into increase in color intensity at basic pH (Bahl and Bahl, 1988).

Bacterial decolorization of PMDE after extraction of melanoidin at different pH

The higher decolorization of PMDE by bacterial consortium after melanoidin extraction might be due to co-metabolism in the presence of supplementary carbon and nitrogen sources. Similar observations by some workers on *Bacillus* and other bacteria have also been demonstrated under different environmental conditions (Kumar *et al*., 1997; Nakajima-Kambe *et al.,* 1999). The addition of external carbon (glucose) source emphasized the necessity for a readily available carbon source in the medium as comparatively very less decolorization was observed in the absence of supplementary carbon source (data not shown). The increased decolorization after addition of glucose as external carbon indicated the major role played by sugar-oxidases and peroxidases in melanoidin decolorization as some studies have linked glucose supplementation to the intracellular hydrogen-peroxide (H_2O_2) producing sugar-oxidase enzymes (Hayase *et al.*, 1984; Ohmomo *et al.*, 1985). The H_2O_2 produced, by the action of peroxidases, oxidizes melanoidin thus causing its decolorization (Hayase *et al.*, 1984; Miyata *et al.*, 1998). However, little is known about aforesaid degrading enzyme of bacterial origin. Further, studies are needed on the decolorization mechanism and decolorizing enzyme of bacterial origin. The decolorization of distillery effluent using developed bacterial consortium at different pH showed a wide range of suitable pH (6.8 – 8.0). However, the isolates showed high activity in neutral (pH 7.0) to slightly alkaline pH 8.0 (Fig. 2).The degree of decolorization decreased with both increase (pH > 8.0) and decrease (pH < 7.0) in the pH-values. At very low pH 4.0 and at high pH 12.0 and 13.0 the organisms were unable to grow and multiply. The maximum decolorization was obtained at pH 7.0 (70.00%) while pH 11.0 showed the least decolorization (11.5%) followed by pH 5.0 (19.1%) (Fig. 2). The original distillery effluent had pH 8.3 and it

showed 50.34% decolorization at 35 °C under aerobic conditions. Statistical analysis between columns of different parameters mostly showed highly significant.

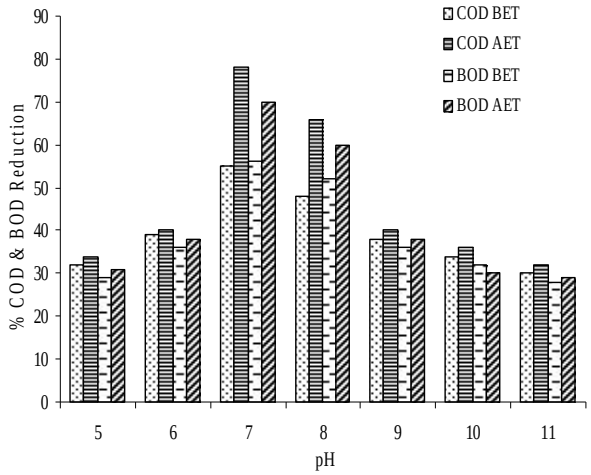

Fig. 3 Reduction of COD and BOD after bacterial treatment of PMDE before and after Melanoidin extraction. BET= bacterial treatment of PMDE before melanoidin extraction, AET= bacterial treatment of PMDE after melanoidin extraction

Similarly, reduction in COD and BOD of PMDE after bacterial treatment was found suitable at neutral pH 7.0 (Fig. 3). Moreover, at less or more pH then 7.00 reductions of COD and BOD were inhibited except pH 8.0. Interestingly it was also noted that bacterial treatment enhanced decolorization and reduction of other pollution parameters of PMDE after extraction of melanoidin at all pH (Fig. 2 and Fig. 3).

CONCLUSIONS

Distillery industry contributes to one of the major industrial pollution with generation of large amount of melanoidin containing distillery effluent. Melanoidin is animo-carbonyl complex compounds formed during sugar processing contribute the colour. Effluent originating from distilleries leads to extensive soil and water pollution due to their dark colour and high COD and BOD. Elimination of pollutants and colour from distillery effluent is becoming increasingly important from environmental and aesthetic point of view. Due to the large volumes of effluent and presence of certain recalcitrant compounds, the treatment of this stream is rather challenging by conventional methods. Extraction of melanoidin before bacterial treatment of distillery effluent would be an effective technique for the decolorization of PMDE for its safe disposal into nearby soil and aquatic ecosystem. Bacterial treated distillery effluent followed by melanoidin extraction results in 70% reduction in colour thus reducing the pollution load. Further, 77.67% of sulphate reduced in the effluent, during which the COD also reduced to 78%. The colour of the distillery effluent was reduced using the cultures of *Proteus mirabilis* (IITRM5; FJ581028), *Bacillus sp.* (IITRM7; FJ581030*)*, *Raoultella planticola* (IITRM15; GU329705) and *Enterobacter sakazakii* (IITRM16, FJ581031). This bacterial consortium was found to degrade more effectively turning dark blackish solution to light brownish colour after extraction of melanoidin at pH 7.0 within a period of 168 h thus reducing the pollution load of soil and aquatic life.

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