Univ. j. zool. Rajshahi. Univ. Vol. 36, 2017 pp. 12-21

http://www.ru.ac.bd/zoology/university-journal-of-zoology-rajshahi-university/

ISSN 1023-6104

Decolourization of synthetic melanoidin by bacteria isolated from sugar mill effluent

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Abstract: Sugar mills play a major role in polluting the water bodies and land by discharging a large amount of waste water as effluent. The waste water contains melanoidin which is responsible for dark brown colour of water, prevents the penetration of sunlight and affect the photosynthetic activity of aquatic plants and also increases COD value. A number of clean up technologies have been put into practice and novel bioremediation approaches for decolourization of melanoidin are being worked out. In this study, the sugar-mill effluent was collected and the physio-chemical properties of the effluent were analyzed. A total of four synthetic melanoidin degrading bacteria *viz*. R1, R2, R3 and R4 were isolated from sugar-mill effluent which were identified as *Exiguobacterium acetylicum* strain QD-3, *Bacillus cereus* strain H3, *Enterobacter cloacae strain CPO 4.14C* and *Enterobacter sp. LY402* by biochemical tests and 16S rDNA gene sequence analysis. The isolates and their consortia were tested for COD and colour removal ability. The highest COD reduction and decolourization percentage which were obtained from the consortium of the four bacterial isolates were about 75% and 60% respectively.

Key words: Melanoidin, Decolourization, Bacteria and Sugar mill effluent

Introduction

Molasses-based distilleries are one of the most polluting industries generating large volumes of high strength wastewater (Satyawali and Balakrishnan, 2007). Sugarcane molasses-based distillery wastewaters disposal into the environment is hazardous and has high pollution potential. High COD, total nitrogen and total phosphate content of the effluent may result in eutrophication of natural water bodies (Kumar *et al*., 1997a, b). The highly colored components of the molasses wastewater reduce sunlight penetration in rivers, lakes or lagoons which in turn decrease both photosynthetic activity and dissolved oxygen concentration affecting aquatic life. Kumar *et al*. (1995) evaluated the toxic effect of distillery effluent on common guppy, *Lesbistes reticulates* and observed remarkable behavioural changes with varying effluent concentration. Kumar and Gopal (2001) reported hematological alterations in fresh water catfish, *Channa punctatus*, exposed to distillery effluents. Saxena and Chauhan (2003) investigated the influence of distillery effluent on oxygen consumption in fresh water fish, *Labeo rohita* and observed that the presence of inorganic and organic salts in the effluent interfered with the respiration in the fish.

Disposal of sugarcane molasses wastewater on land is equally hazardous to the vegetation. It was reported to reduce soil alkalinity and manganese availability, thus inhibiting seed germination (Kumar *et al*., 1997a, b). Wastewater from sugar mills with its high Biological Oxygen Demand (BOD) and Chemical Oxygen Demand (COD) rapidly deplete available oxygen supply when discharged into water bodies endangering fish and other aquatic life and also creates septic conditions, generating foul-smelling hydrogen sulfide, which in turn can precipitate iron and any dissolved salts, turning the water black and highly toxic for aquatic life (ETPI, 2001). Kannan and Upreti (2008) reported highly toxic effects of raw distillery effluent on the growth and germination of *Vigna radiata* seeds even at low concentration of 5% (v/v). Application of distillery effluent to soil without proper monitoring, perilously affects the groundwater quality by altering its physicochemical properties such as color, pH, electrical conductivity, etc. due to leaching down of the organic and inorganic ions (Jain *et al*., 2005). Juwarkar and Dutta (1990) evaluated the impact of application of distillery effluent on soil microflora. Irrigation with raw distillery

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effluent resulted in low overall bacterial and actinomycetes count. Nitrogen fixing bacteria *Rhizobium* and *Azotobacter* reduced considerably. However, population of fungi increased. Anaerobically treated effluent also showed similar reduction as previously mentioned with bacteria but not as much as that of the raw effluent.

Molasses distillery wastewater colorants are mainly polyphenols, melanoidin, alkaline degradation products of hexoses, and caramels. A lot of efforts have been made to remove the colorants including biological methods employing different fungi, bacteria & algae, enzymatic treatment, chemical oxidation, coagulation/ precipitation, oxidation and membrane filtration (Saha *et al*., 2017; Satyawali and Balakrishnan, 2008).

Melanoidins are generated in sugarcane molasses as complex polymer due to non-enzymatic reaction of sugar and amino acid produced browning reactions called Maillard reaction (Adams, *et al*., 2003). These compounds are highly resistant to microbial attack, conventional biological processes such as activated sludge treatment are inefficient to decolorize melanoidin-containing wastewaters, such as molasses wastewaters from distilleries and fermentation industries (Sirianuntapiboon *et al*., 1988). However, microbial decolorization of anaerobically treated effluent reduced the toxic effect which indicated that there is necessity for microbial degradation at secondary or tertiary stage prior to its disposal for environmental safety (Santal *et al*., 2011).The microbial decolorization can be exploited to develop a cost effective, eco-friendly biotechnology package for the treatment of distillery effluent.

Materials and Methods

Sample collection

The sugar mill effluent was collected from the outlet of Harian sugar mill, Rajshahi, Bangladesh and distillery spent wash was collected from the oxidation ponds of Carew and Company alcohol industries at Darshana, Chuadanga, Bangladesh and stored at room temperature in the laboratory. Characterization of the effluent was done for colour, odour, temperature, pH, TDS and COD according to standard methods (APHA, 2002).

Choice of the synthetic substrate

Generally, wastewaters obtained from sugarcane molasses based distilleries have no consistency and uniformity, as the compositions in wastewater such as COD, BOD, chemical elements and color substances vary depending on time, day or season (Mogan, 2002). Moreover, sugarcane molasses also contain other colorants such as phenolic compounds and caramel, whereas melanin is abundant in beet molasses (Godshall, 1999). Hence, synthetic melanoidin was used in this study as an appropriate alternate of natural melanoidin of wastewater to reduce the experimental error.

Preparation of synthetic melanoidin

Synthetic melanoidin, glucose-aspartic-acid (GAA) type was prepared following the method described by Ohmomo *et al.* (1985a), in which 1.0 molar (M) glucose, 1.0M aspartic acid and 0.5M sodium carbonate were dissolved in 1.0L of distilled water and solution was refluxed at 100ºC for 7 hours. Further reaction mixture adjusted to pH 7.0 with 1N NaOH, the solution was filtered through 0.45µm membrane filter. These solutions contained 1,83,000 mg/l COD value.

Strain isolation

All samples were used for isolation of melanoidin decolourizing bacterial cultures by enrichment culture techniques using enrichment medium amended with 5% of synthetic melanoidin GAA (glucose-aspartic-acid) for adaptation of the microorganisms. After serial dilutions (10^{-1} – 10^{-6}), the collected samples were incubated into nutrient agar medium which contained: peptone, 10 gL-1; yeast extract, 5 gL-1; NaCl, 5 gL-1; agar, 15 gL-1 (pH 7 to 7.2) at 28°C by streak plate technique for 24-72 hours. Bacterial colonies that showed a clear decolourization zone around them on nutrient agar medium were picked and individual colony was then reintroduced into 9 ml GPYM broth medium (glucose, 1 gL-1; peptone, 0.3 gL-1 ; yeast extract, 0.2 gL-1; K_2HPO_4, 0.1 gL-1; $MgSO_4.7H_2O$, 0.05 gL-1) containing 5% of respective synthetic melanoidin, incubated to observe melanoidin decolourization by individual bacteria. The strains that achieved the best decolourization were selected for this study.

Microscopic examination and morphological identification

For the identification of the bacteria, morphological characters, microscopic observations, growth characteristics, biochemical tests were performed. The microorganisms were identified using Bergey's Manual of Determinative Bacteriology (Holt *et al.*, 2005).

Identification by 16S rRNA Gene Sequence

Identification of the isolated strain was performed by 16S rDNA sequence analysis. Genomic DNA was extracted from the bacterial cells using Maxwell Blood DNA kit (Model: AS1010, Origin: Promega, USA). The 16S rDNA gene was amplified by PCR using the specific primers, 27F and 1492R which are capable of amplifying 16S from a wide variety of bacterial taxa. The sequence of the forward primer was 16SF 5'- AGA GTT TGA TCM TGG CTC AG -3' (Turner *et. al.,* 1999) and the sequence of the reverse primer was 16SR 5'-CGG TTA CCT TGT TAC GAC TT -3' (Turner *et. al.,* 1999). The PCR amplicons are separated electrophoretically in a 1% agarose gel and visualized after Diamond™ Nucleic Acid Dye (Cat: H1181, Origin: Promega, USA) staining. The PCR products were purified using SV Gel and PCR Clean Up System (Cat: A9281, Origin: Promega, USA) according to the manufacture′s protocol. The total DNA yield and quality were determined spectrophotometrically by NanoDrop 2000 (Thermo Scientific, USA). The sequence analysis was performed using the ABI 3130 genetic analyzer and Big Dye Terminator version 3.1 cycle sequencing kit . The 16S rRNA genes in the Gene Bank by using the NCBI Basic Local Alignment Search Tool (BLASTn) (http://www.ncbi.nih.gov/BLAST). A distance matrix was generated using the Jukes-cantor corrected distance model. The phylogenetic trees were formed using Weighbor (Weighted Neighbor Joining: A likelihood-Based Approach to Distance-Based Phylogeny Reconstruction) with alphabet size 4 and length size 1000 .The 16S rRNA gene sequences were deposited to Genbank.

Decolourization activity test

The isolated strains were inoculated in nutrient broth to study the decolorizing ability of the culture. The synthetic melanoidin was added after sterilization of medium throughout the study. The synthetic melanoidin (5%) was added immediately and incubated under static condition at 28^0C. Aliquot (2 ml) of culture media was withdrawn at different time intervals and centrifuged at 10000 rpm for 10 minute. Decolorization was monitored by measuring the absorbance of the culture at 475 nm (lamda max) using UV-vis Spectrophotometer. All decolorization experiments were performed in triplicates. Abiotic control (without microorganism) was always included in each study. The % decolorization rate was measured (Saratale, 2009) as follows:

$$Dye\ Decolorization(\%) = \frac{Initial\ absorbance - Final\ absorbance}{Initial\ absorbance} \times 100$$

Measurement of COD value

The chemical oxygen demand (COD) test determines the oxygen required for chemical oxidation of organic matter with the help of strong chemical oxidant. The COD is a test, which is used to measure pollution of domestic and industrial waste. The waste is measured in terms of quality of oxygen required for the oxidation of organic matter to produce carbon dioxide and water (Poddar and Sahu, 2015). According to the DoE standard the COD of the effluent should be 200 mg/l. The measurement of COD value was carried out by using HI 93754C-25 HR Reagent kit. HI 83099 COD and multiparameter Bench Photometer was used for colorimetric determination.

Results

Physico-chemical characteristics of sugar mill effluent

In the present study, physico-chemical characteristics of the collected sugar mill effluent was analysed and the results were showed in Table 1.

Table1. chemical analysis of the-Physico collected sugar mill effluent

Serial no.	Parameters	values	Standard by DoE
1	Colour	Dark brown	Colourless
2	Odour	Unpleasant	Odourless
3	pH	4.5	6-9
4	COD (mg/L)	92,000	200
5	Total dissolved solids (mg/L)	9,710	2,100
6	Electro Conductivity (mS/cm)	19.26	1.2

Isolation and characterization of the bacteria

Bacteria were isolated by plating onto an agar solidified nutrient medium. The plates were incubated at 28ºC for 2 days and bacterial colonies were found to grow on the medium.

Table 2(a). Colony morphology of the isolated bacterial strains

Bacterial strains	Colony morphology						
	Colour	**Shape**	**Surface**	**Elevation**	**Edges**	**Opacity**	**Consistency**
R1	Yellow	Circular	Smooth	Raised	Entire	Opaque	Sticky
R2	Creamy white	Circular	Smooth	Raised	Entire	Opaque	Non- Sticky
R3	Creamy white	Circular	Smooth	Raised	Entire	Opaque	Sticky
R4	Creamy white	Circular	Smooth	Raised	Entire	Opaque	Sticky

Table 2(b). Microscopic observations of the isolated bacterial strains

Bacterial strains	Gram characteristic	Shape	Motility
R1	+ve	Rod shaped	Non motile
R2	+ve	Rod shaped	Non motile
R3	-ve	Rod shaped	Motile
R4	-ve	Rod shaped	Motile

Results of microscopic analysis of bacterial cells and their growth characteristics are presented in Table 2 (a) and 2 (b). For biochemical characterization different tests were conducted. The results of biochemical tests of the bacterial isolates are presented in Table 3.

Table 3. Biochemical test results for the isolated bacterial strains

Tests performed	Isolates			
	R1	**R2**	**R3**	**R4**
Triple Sugar Iron (TSI) test	+	-	+	+
Citrate Utilization test	-	-	+	+
Oxidase test	-	-	+	+
Catalase test	-	-	-	-
Sulfide Indole Motility (SIM) test	-	-	+	+
3% KOH test	-	-	+	+
MacConkey Agar	-	-	+	+
Carbohydrate Utilization Test				
Fructose	-	-	-	-
Galactose	+	+	+	+
Maltose	+	+	+	+
Cellulose	-	-	-	-

Identification of the bacteria

Isolated bacterial strains identified by morphological and biochemical tests were subjected to 16S rRNA gene sequence analysis. Analysis of 16S rRNA gene sequences (data not shown here) revealed that the isolates R1, R2, R3 and R4 were similar to *Exiguobacterium acetylicum* strain QD-3, *Bacillus cereus* strain H3,*Enterobacter cloacae* strain CPO 4.14C and *Enterobacter* sp. LY402 respectively.

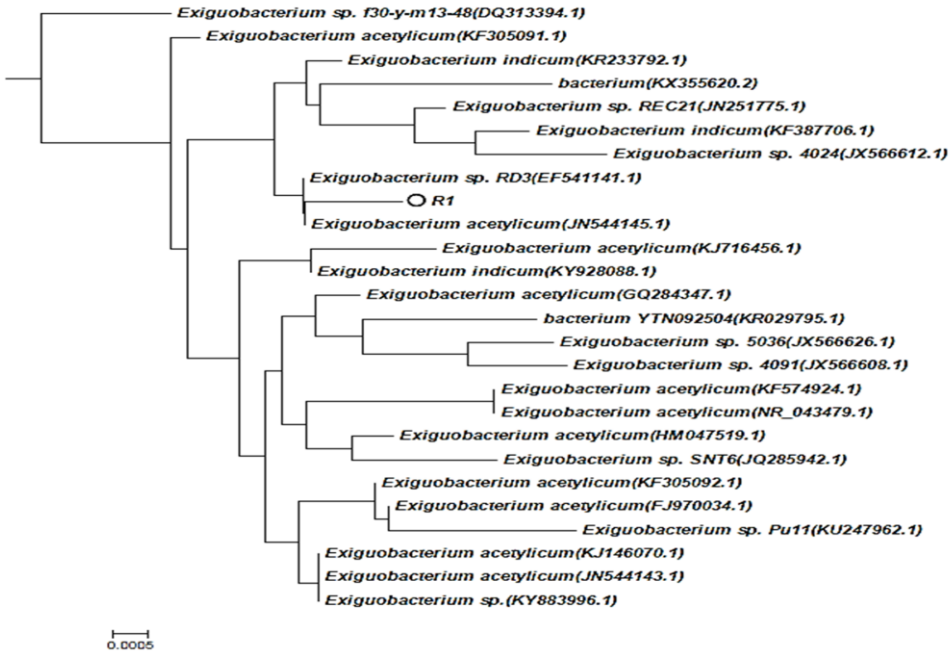

Figure 1 (a). Phylogenetic tree showing the genetic relationship among the cultivated bacteria R1 and reference 16S rDNA sequences from the GenBank based on partial 16S ribosomal RNA gene sequences. Scale bar 0.0005 = 0.05% difference among nucleotide sequence.

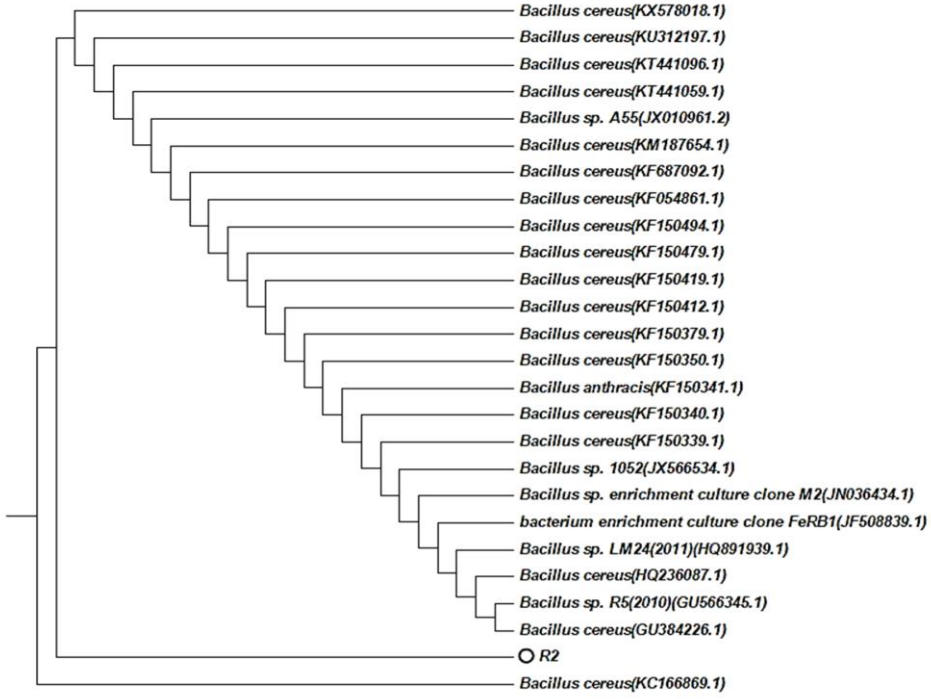

Figure 1(b). Phylogenetic tree showing the genetic relationship among the cultivated bacteria R2 and reference 16S rDNA sequences from the GenBank based on partial 16S ribosomal RNA gene sequences.

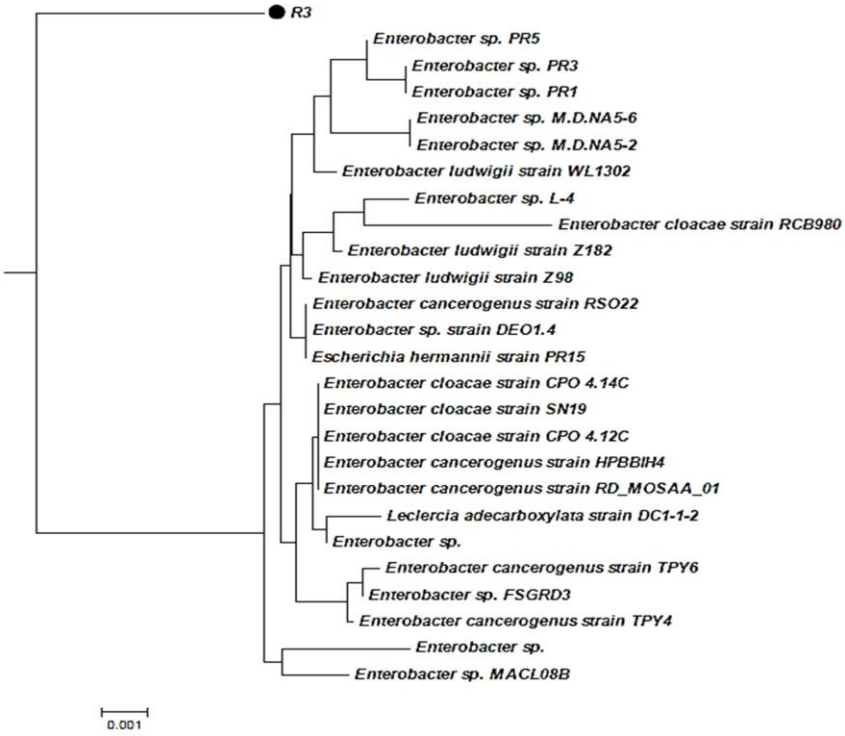

Figure 1(c). Phylogenetic tree showing the genetic relationship among the cultivated bacteria R3 and reference 16S rDNA sequences from the GenBank based on partial 16S ribosomal RNA gene sequences. Scale bar 0.001 = 0.1% difference among nucleotide sequences

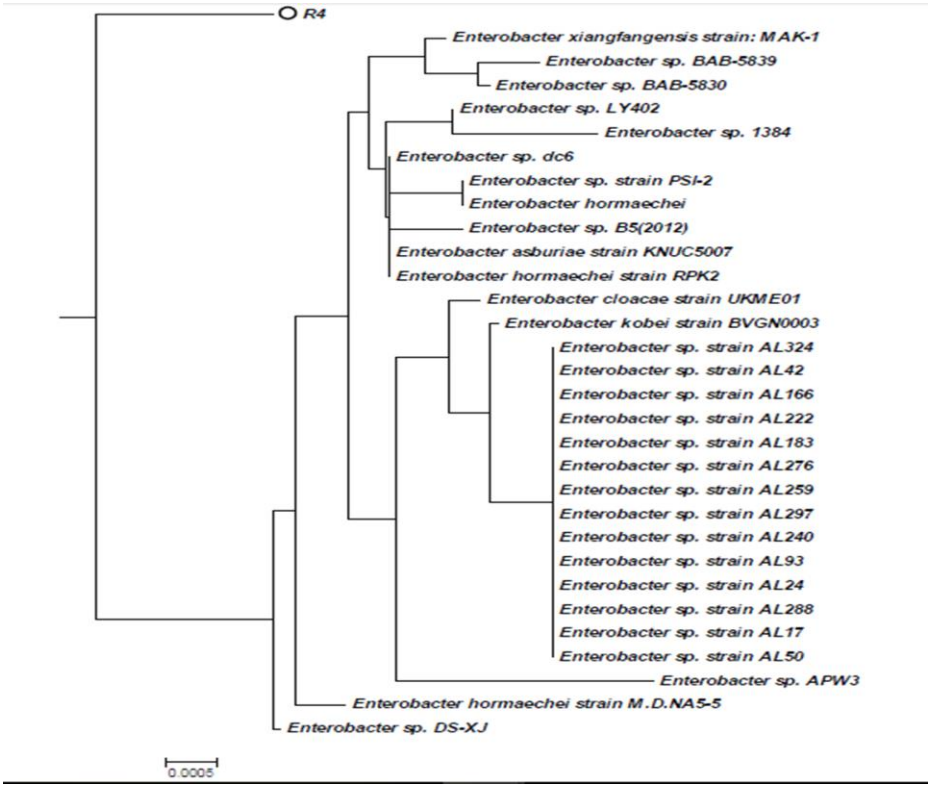

Figure 1(d). Phylogenetic tree showing the genetic relationship among the cultivated bacteria R4 and reference 16S rDNA sequences from the GenBank based on partial 16S ribosomal RNA gene sequences. Scale bar 0.0005 = 0.05% difference among

The homologous identity of the four bacterial isolates were present in table 4. Phylogenetic relationship of identified bacterial isolated to their neighboring strains are shown in figures 1(a), 1(b), 1(c) and 1(d) respectively.

In this experiment, pure individual cultures of R1, R2, R3 and R4 gave dye decolorization percent of 52%, 53%, 58% and 54% respectively while their mixed culture (consortia) gave decolorization percent of 60% (Figure 2).

Table 4. Homologous identity of the bacterial isolates

Tested Strains and accession number	Related bacterial species	Accession number	Max Score	Total Score	Query Cover	Identity (%)
R1	***Exiguobacterium acetylicum* strain QD-3**	FJ970034.1	1406	1406	96%	99%
MH 517397	***Exiguobacterium* sp. REC21**	JN251775.1	1404	1404	97%	99%
R2	***Bacillus cereus* strain H3**	KC166869.1	1415	1415	98%	99%
MH 517398	***Bacillus cereus* strain YB3**	KX578018.1	1413	1413	97%	99%
R3	***Enterobacter cloacae* strain CPO 4.14C**	MF666755.1	2048	2048	95%	98%
MH 517399	***Enterobacter cancerogenus* strain HPBBIH4**	KU605688.1	2048	2048	95%	98%
R4	***Enterobacter* sp. LY402**	DQ659161.1	2533	2533	99%	99%
MK478828	***Enterobacter hormaechei* strain RPK2**	KX980424.1	2527	2527	99%	99%

Decolourization activity by bacterial isolates

Decolorizatiom percentage of synthetic melanoidin by bacterial isolates and their consortium were analyzed and the growth of bacterial isolates on the nutrient medium with 5% synthetic melanoidin (*p*H 7.0 and 28^{0}C) was monitored by UV spectrophotometer at 475 nm.

Efficiency of COD reduction by representative strains

It was observed that the COD of untreated effluents was 92,000 mg/l and the COD value of 5% synthetic melanoidin (GAA) was 45,000 mg/l. After 15 days of incubation maximum COD reduction was 74.54% shown by bacterial consortia (R1+R2+R3+R4). Whereas, 67.59%, 70.89%, 71.35% and 71.21% COD reduction were observed by bacterial isolates R1, R2, R3 and R4 respectively (Figure 3).

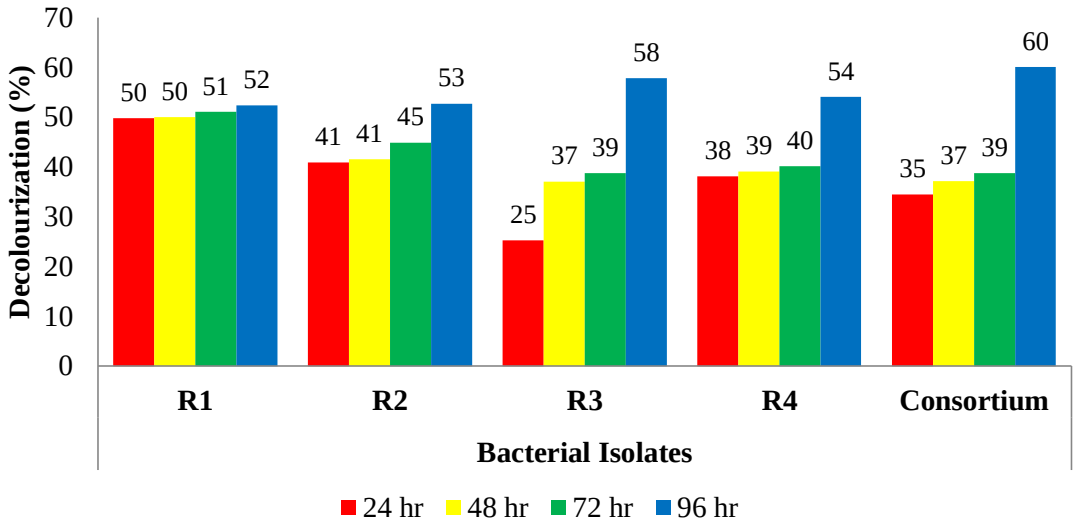

Figure 2. Decolorization by isolated bacteria

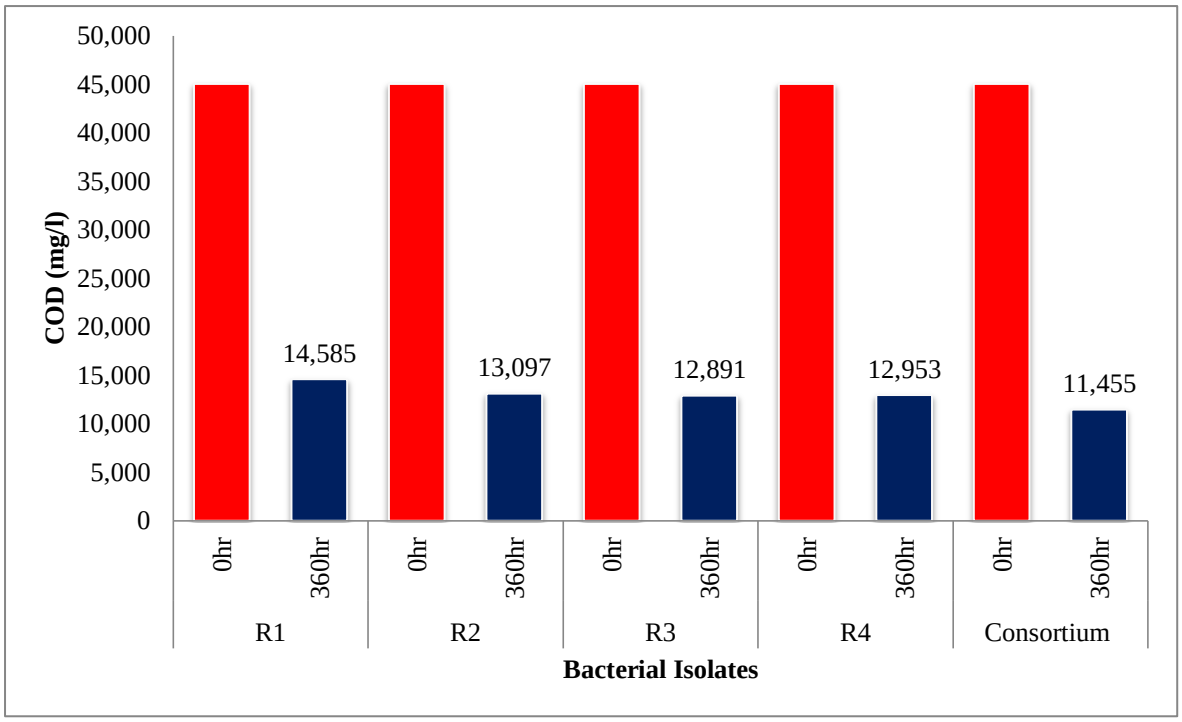

Figure 3. Reduction of COD value by isolated bacteria

Discussion

The main color constituents in cane molasses are melanoidins. Physico-chemocal characterization of melanoidins revealed them to be a complex polymer of Maillard reaction, where melanoidin GAA- polymer of glucose and aspartic acid, was found most recalcitrant to decolourization than other melanoidins. This suggests that chemical complexities of different melanoidins depend on the type of amino acids and sugar moieties reacted at different temperature (Kumar and Chandra, 2006). Similar observations were reported by Ohmomo *et al.* (1985a, 1988) and Hayashi and Namiki (1986) during their studies on synthetic melanoidins.

In present study, four melanoidins degrading bacteria *viz*. R1, R2, R3 and R4 were isolated from sugar mill effluent and identified as *Exiguobacterium acetylicum* strain QD-3, *Bacillus cereus* strain H3, *Enterobacter cloacae strain CPO 4.14C* and *Enterobacter sp. LY402* by 16S rDNA gene sequence analysis.

The identification of culturable bacteria by 16S rDNA based approach showed that the consortium composed of *Exiguobacterium acetylicum* strain QD-3, *Bacillus cereus* strain H3, *Enterobacter cloacae strain CPO 4.14C* and *Enterobacter sp. LY402* decolorise the synthetic melanoidins. In this study, synthetic melanoidin (GAA) was prepared to reduce error due to variations in concentration of melanoidin in different sample of molasses wastewater as well as for its complex composition. Kumar and Chandra (2006) studied on the decolorization of synthetic melanoidins (*i.e*., GGA, GAA, SGA and SAA) by three *Bacillus* isolates *Bacillus thuringiensis* (MTCC 4714), *Bacillus brevis* (MTCC 4716) and *Bacillus sp.* (MTCC 6506).

The decolorization activity (52%, 53%, 58% and 54%) was found after 4 days of incubation under the optimized condition were achieved for cultures R1, R2, R3 and R4 respectively. In this investigation, maximum melanoidin decolorization i.e. 60% was achived by bacterial consortia (R1+R2+R3+R4) shown by in Fig. 2. Similar colour removal was obtained by Sivakumar *et al.* (2006) within 5 days of decolourization of anaerobically digested molasses spent wash by *Bacillus megaterium* SW3, *Bacillus subtilis* SW8 and consortia (SW3 + SW8).

After 15 days of incubation maximum COD reduction was 74.54% shown by bacterial consortia (R1+R2+R3+R4). Whereas, 67.59%,

70.89%, 71.35% and 71.21% COD reduction were observed by bacterial isolates R1, R2, R3 and R4 respectively. Jain *et al.* (2002), studied on degradation of anaerobically digested distillery wastewater by three bacterial strains, *viz. Xanthomonas fragariae, Bacillus megaterium* and *Bacillus cereus* in free and immobilized form, isolated from the activated sludge of a distillery wastewater treatment plan. The strain *Bacillus cereus* showed the maximum efficiency of COD (81 %) and color (75 %) removal out of the three strains. Similar COD reduction was obtained by Sivakumar *et al.* (2006). However, the present study shows that optimal conditions for the maximum decolourisation and COD reduction were 30°C and pH 7.0.

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