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ISOLATION AND CHARACTERIZATION OF MELANOIDINS DEGRADING BACTERIA FROM SUGAR-MILL EFFLUENT

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ABSTRACT ■ Melanoidins are major coloring and polluting constituents in distillery wastewaters which have caused substantial environmental pollution in Bangladesh. Hence, this study was designed for isolation and characterization of melanoidins degrading bacteria from sugar-mill effluent. A total of five melanoidins degrading bacteria viz. Z1, Z2, Z3, Z4 and Z5were isolated from sugar-mill effluent and identifiedas *Exiguobacteriumacetylicum* strain QD-3,*Bacillus cereus* strain H3, *Enterobacter* sp. PR1, *Pseudomonasaeruginosa* strain AU09 and *Bacillusmegaterium* strain Jz11 by biochemical tests and 16S rRNA gene sequence analysis. The isolates were tested for Chmeical Oxygen Demand (COD) and colour removal ability. The highest reduction of COD (93%) and removal of colour (60%) were obtained by the treatment with bacterial isolate*Bacillus cereus* strain H3. Spectrophotometric and COD analyses of treated effluent demonstrated that decrease in colour intensity might be largely attributed to the degradation of melanoidins by isolated bacteria.

Key words: Distillery effluent, melanoidins, bacteria, biodegradation

INTRODUCTION

Sugar industry plays an important role in the economy of Bangladesh by the way of farming and creation of employment. The by-products of sugar mills are also used as raw materials in different industry. However, sugarcane molasses-based distilleries are one of the most polluting industries generating large volume of wastewater having a serious environmental concern (Bezuneh, 2016). Main recalcitrant compound present in distillery effluent is melanoidin which is responsible for the dark brown colour of effluent.Dark brown colour hinders photosynthesis by blocking sunlight and is therefore deleterious to aquatic life (Bezuneh, 2016 and Agarwal*et al*., 2010). It also causes reduction in soil alkalinity and manganese availability and inhibition of seed germination and seedling growth (Agarwall and Pandey, 1994; Pandey*et al*., 2008).The dark brown colour of effluent is mainly due to the formation of polymer melanoidin by a non-enzymatic browning reaction called Millard reactions (Chandra *et al*., 2008; Martins and Van, 2005). Melanoidins are highly recalcitrant and have antioxidant properties which cause toxicity to the microbial flora (Rani and Saharan, 2010; Kitts *et al*., 1993).

Distillery effluents contain high concentration

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of heavy metals *viz*. copper, nickel, silver, cadmium, iron, lead, and mercury and colour pigments (Pandey*et al*., 2008; Rahman*et al*., 2002). These polluted effluents are thrown into the canals, streams or rivers where they deteriorate the quality of water, making the water unfit for irrigation purpose and for the use of animals(Mohanta *et al*., 2010). The harmful effects of effluents and waste products from distillery industries have been reported by Kumar *et al*. (1995), Pandey*et al*. (2008), Rahman*et al*. (2002),Matkar and Gangotri (2003), andSaxena and Chauhan (2003).

Distillery effluents must be treated before it is disposed into the environments which help to minimize the adverse effect posed by the effluents (Bezunes, 2016). Physico-chemical methods of controlling or mitigating distillery wastewater are less effective, more cumbersome, time consuming and expensive than biological methods and can results in formation of harmful by-products (Boopathy and Senthilkumar, 2014). Microorganisms play an important role in bioremediation process and have proven as an efficient, low cost and environmental friendly alternative to conventional methods (Ruhi, *et al.,* 2017; Rahman *et al.,* 2019; Nasrin *et al.,* 2019). Different types of microorganisms as bacteria, fungi and algae have been reported for their potential in degradation and decolorization of various industrial effluents including that of distilleries. The aim of the present study was to isolate indigenous bacteria from sugar mill effluent which able to degrade or decolorize melanoidins and COD reduction of the distillery effluent was studied.

METHODS

Sample collection

The Sugar mill effluent was collected from the outlet of Harian sugar mill, Rajshahi, Bangladesh and distillery spentwash was collected from the oxidation ponds of Carew and Company alcohol industries atDarshana,Chuadanga,Bangladesh andstored 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).

Isolation and characterization of the microbs from sugar mill effluent

Sample of effluents were used as sources of inocula for the isolation of microorganisms capable of degrading effluents. Sample of effluents were suspended onto 250 ml Erlenmeyer flaks containing 100 ml of mineral salts (MS) medium, which was incubated for 2 days at 37°C and subjected to shaking at 120 rpm on an orbital shaker. Control flasks without inoculates were also prepared and incubated at 37°C with an orbital shaker. The cultures that were found to turbid after a period of 0 up to 2 days were used as inocula in subsequent experiments.

Microorganisms which were putatively capable of degrading purified melanoidins in the culture plate were isolated from enrichment cultures by plating out on minimal salts agar medium with glucose (1%) as extra carbon source. The plates were incubated for 2 days at 37°C. Single colonies growing this medium were isolated and stored for the further use.

Screening of isolates for degradation of melanoidins

The isolated bacterial strains were screened for their efficiency to remove COD and colour from distillery waste water. For this, 200μl broth culture of each isolates were taken onto 250 ml Erlenmeyer flaks separately containing 40 ml of mineral salts (MS) medium with 10ml sample of spent wash (COD=92,000mg/l), which were kept at 28°Con a rotary shaker at 120 rpm for 10 days.Every 12 hours interval,

sample was taken from each flask and filtered with membrane for assaying COD and colour removal efficiencies.

Microscopic examination and identification of bacterial cells

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

Identification by 16S rRNA Gene Sequence

Genomic DNA of bacterial isolates wasisolated according to Mohanta *et al*. (2012). Gene fragments specific for the highly variable region of the bacterial 16S rRNA gene was amplified by PCR using universal PCR primer as described by Loffler*et al*. (2000) (Sigma, USA) in a thermal cycler (MJ Research Inc., Watertown, USA). The sequence of the forward universal primer was 16SF 5'-GAGTTTGATCCTGGCTCAG-3' and the sequence of the reverse primer was 16SR 5'-GAAAGGAGGTGATCCAGCC-3'. The PCR products were subjected to 1% agarose gel electrophoresis, stained with ethidium bromide and visualized on a UV transilluminator for the presence of about 1500 bp PCR products. Amplified 16S rRNA gene PCR products were purified using StrataPrep PCR purification kit (Stratagene, USA) according to the manufacturer's protocol. Sequencing reactions were carried out using ABI-Prism Big dye terminator cycle sequencing ready reaction kit and the PCR products were purified by a standard protocol. The purified cycle sequenced products were analyzed with an ABIPrism 310 genetic analyzer. The chromatogram sequencing files were edited using Chromas 2.32. The homology of the 16S rRNA gene sequences was checked with the 16S rRNA gene sequences of other organisms that had already been submitted to GenBank database using the BLASTN (http://www.ncbi.nih.gov/BLAST) algorithm (Accession Number Z1:MH517397, Z2 :MH517398, Z3: MH517399, Z4: MH517400 and Z5: MH236183).

Effects of temperature and pH on bacterial growth

Temperature and pH influence bacterial growth. For the effect of pH, culture medium (nutrient broth, Hi-media) was adjusted to pH 6.0, 7.0, and 8.0. Incubation temperature was varied at 28, 37 and 42ºC. Bacterial cell density of liquid cultures was determined by measuring optical density at 660 nm with a photoelectric colorimeter (AE-11M, Erma Inc., Tokyo) following the procedures described by Mohanta *et al*. (2012).

Bioremediation of sugar mill effluent by bacterial isolates

The sugar mill effluent was bio-remediated by using the bacterial isolates. The bacterial suspension of 24 hours fresh cultures of bacterial isolates was used for bioremediation studies. The bacterial suspensions were prepared in saline solution (0.89% sodium chloride). A loopful of culture was incubated in the saline (100ml) and incubated at 37 ºC for 24 hours (12). After that, the bacterial suspensions (100ml/L) were inoculated into the sugar mill effluent and incubated at room temperature. The physio-chemical characteristics of the bacterially bioremediated effluent was analyzed by using standard methods.

Phylogenetic Analysis

The phylogenetic trees were constructed by the pairwise alignments of all the strains and the related species using Neighbor-Joining algorithms (Saitou and Nei, 1987) using the Jukes-cantor model in NCBI website (http://

www.ncbi.nlm.nih.gov/blast/treeview/treeView.cgi?). Downloads the guide tree into a text file in Newick format which is recognized by a phylogenetic software named Mega VI software (version 6.0) (Tamura *et al.*, 2013).

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.

Isolation and identification of the bacteria

Bacteria were isolated by plating onto an agar solidified MS medium. Results of microscopic analysis of bacterial cells and their growth characteristics are presented in Table 2(a) and 2 (b) while the biochemical and antibiotic sensitivity tests of the bacteria are presented in Table 3, 4 respectively. Isolated bacterial strains were identified by both morphological and biochemical tests and this is further confirmed by 16S rRNA gene sequence analysis. Analysis of 16S rRNA gene sequences (data not shown here) revealed that the isolates similar to *Exiguobacterium acetylicum* strain QD-3,*Bacillus cereus* strain H3 ,*Enterobacter* sp. PR1, *Pseudomonas aeruginosa* strain AU09 and *Bacillus megaterium* strain Jz11. The phylogenetic positions of all isolates within different subgroups were investigated by comparing their 16S rDNA sequences to those representatives of various genera. Three different groups can be seen from the tree: *Exiguobacteriumacetylicum* strain QD-3 (Fig.7), *Bacillus cereus* strain H3 (Fig.6),*Enterobacter* sp. PR1 (Fig.5), *Pseudomonas aeruginosa* strain AU09 (Fig.8) and *Bacillus megaterium* strain Jz11 (Fig.4). The distance was indicated at the branches and its nodes.

Effect of temperature and p^H on bacterial growth:

To determine the effect of temperature and pH of growth medium on the growth rate of the bacteria was tested a series of investigation. The results of the investigations are presented in Fig. 1. The optimumpH for the growth of the isolates was 7.0 and growth rate was moderately low in other pH value 6.0 and 8.0.The optimum temperature for the growth of isolates was found to be 37^0C and growth rate was moderately low in other temperature *viz*. 28^0C and 42^0C (Fig. 2).

Reduction of COD and decolourizationof the effluent by bacterial isolates:

Reduction of chemical oxygen demand (COD) and decolourization of the sugar mill effluent bybacterial isolates were studied in the present research.The highest COD and colour removal was obtained from the bacterial isolate*Bacillus cereus* strain H3 about 93% and 60% respectivelyshown in Fig. 3(a) and 3(b).

Table 1. Physico-chemical analysis of the 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	-

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

Bacterialstrains	Gram characteristic	Shape	Motility
Z1	+ve	Coccus	Non motile
Z2	+ve	Coccus	Non motile
Z3	-ve	Coccus	Motile
Z4	-ve	Coccus	Non motile
Z5	+ve	Coccus	Non motile

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

Bacterial strains	Colony morphology						
	Colour	**Shape**	**Surface**	**Elevation**	**Edges**	**Opacity**	**Consistency**
Z1	Yellow	Circular	Smooth	Raised	Entire	Opaque	Sticky
Z2	Creamy white	Circular	Smooth	Raised	Entire	Opaque	Non-sticky
Z3	Creamy white	Circular	Smooth	Raised	Entire	Opaque	Sticky
Z4	Shiny	Circular	Smooth	Raised	Entire	Opaque	Non-sticky
Z5	Creamy white	Circular	Smooth	Raised	Entire	Opaque	Non-sticky

Table 3. Biochemical test results for the isolated bacterial strains

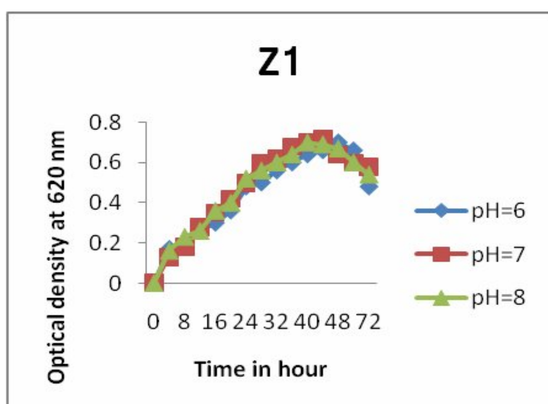
Tests performed	**Isolates**				
	Z1	**Z2**	**Z3**	**Z4**	**Z5**
Triple sugar iron (TSI) test	+	-	+	-	-
Citrate utilization test	-	-	+	+	-
Oxidase test	-	-	+	-	-
Catalase test	-	-	-	-	-
Sulfide indole motility (SIM) test	-	-	+	-	-
Methyl red test	-	-	-	-	-
MacConkeyagar test	-	-	+	+	-
3% KOH test	-	-	+	+	-
Voges-Proskauer (VP) test	+	+	+	+	+
Carbohydrate utilization tests					
Fructose	+	+	+	-	+
Galactose	+	-	+	-	+
Lactose	-	-	-	+	+
Cellulose	-	-	-	-	-
Sucrose	+	+	+	-	+
Glucose	+	+	+	-	+
Maltose	+	-	+	-	+

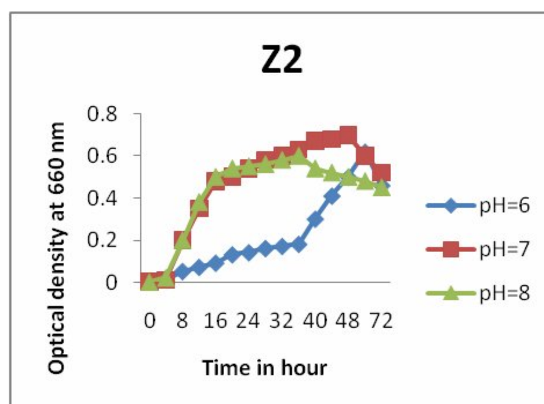
"+" sign indicate growth of the bacteria while "-" sign indicate no growth

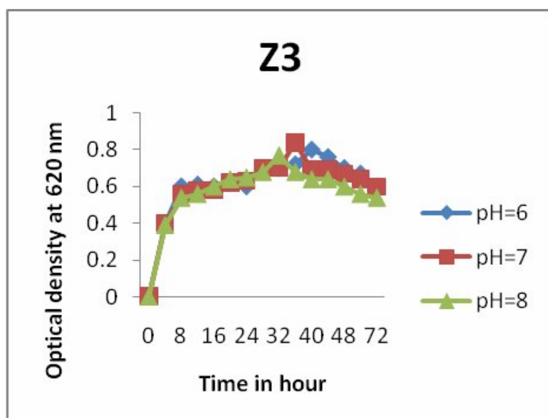
Table 4. Antibiotic sensitivity tests

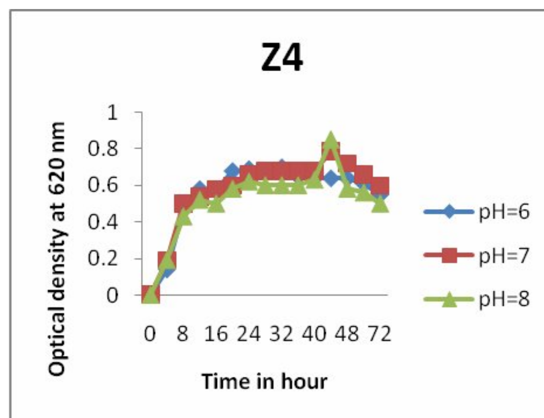
Antibiotic discs	Z1 Disc distance (mm)	Z2 Disc distance (mm)	Z3 Disc distance (mm)	Z4 Disc distance (mm)	Z5 Disc distance (mm)
Amoxycilin (30 µg)	30 (S)	9 (R)	7 (R)	7 (R)	24 (S)
Azithromycin (15 µg)	14 (I)	9 (R)	18 (S)	7 (R)	7 (R)
Cephradine (25 µg)	18 (S)	8 (R)	5 (R)	7 (R)	29 (S)
Ciprofloxacin (5µg)	22 (S)	7 (R)	30 (S)	32 (S)	30 (S)
Cefalexin (30 µg)	18 (S)	8 (R)	7 (R)	7 (R)	25 (S)
Erythromycin (15 µg)	24 (S)	7 (R)	10 (R)	8 (R)	28 (S)
Gentamicin (10 µg)	15 (I)	8 (R)	19 (S)	20 (S)	20 (S)
Kanamycin (30 µg)	15 (I)	8 (R)	15 (I)	7 (R)	28 (S)
Neomycin (30 µg)	14 (I)	8 (R)	14 (I)	14 (I)	24 (S)
Rifampicin (5 µg)	24 (S)	10 (R)	9 (R)	7 (R)	22 (S)
Streptomycin (10 µg)	12 (I)	7 (R)	20 (S)	18(S)	8(R)
Tetracycline (30 µg)	22 (S)	7 (R)	19 (S)	15(I)	30 (S)

(5-10 mm) =Resistant to antibiotics (R); (10-15 mm) = Intermediateresistance (I);
(15-20) = Sensitive to antibiotics (S)









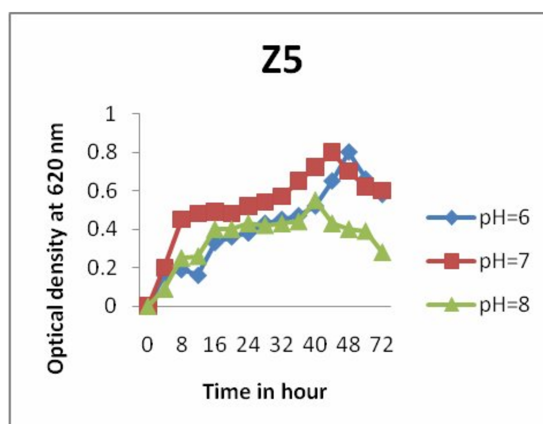
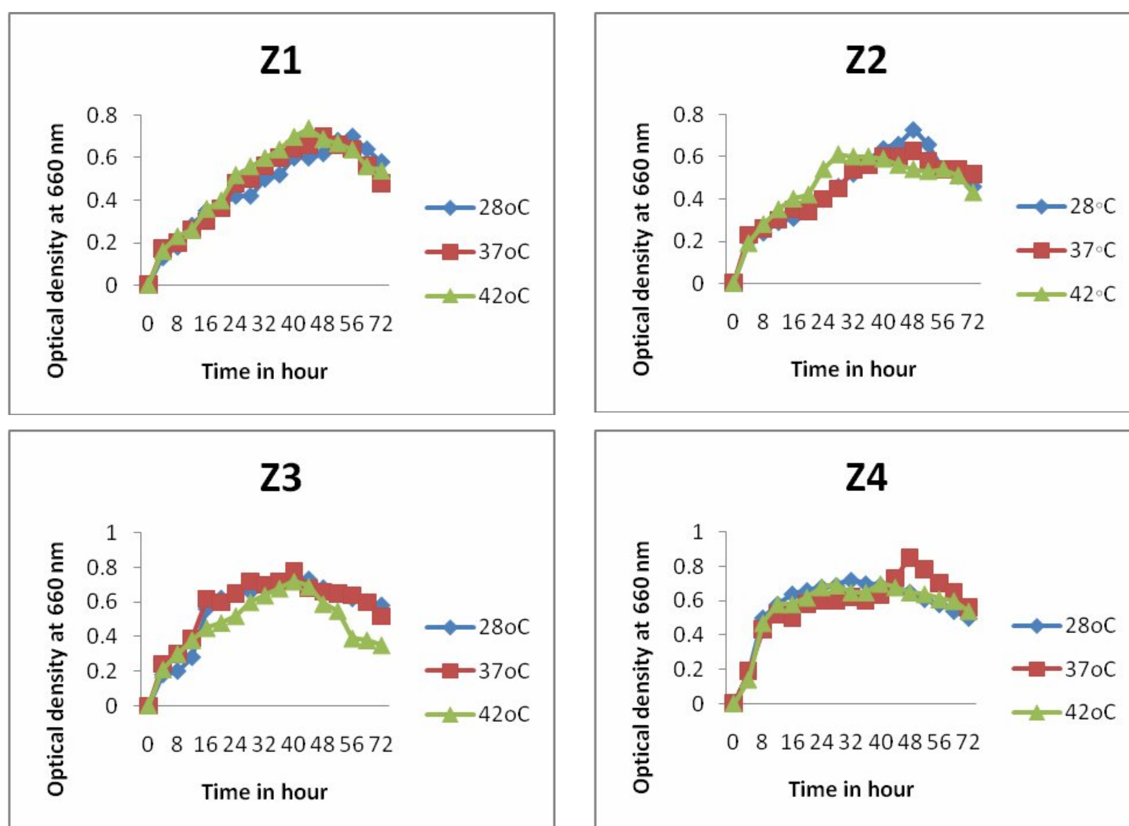


Fig. 1: Effects of pH on bacterial growth. For the effect of pH, culture medium (nutrient broth, Hi-medium, India) was adjusted to pH 6.0, 7.0 and 8.0. Then, the media were inoculated and incubated for 72 hours at 37ºC. During incubation, bacterial cell density of liquid cultures was determined by measuring optical density at 620 nm with photoelectric colorimeter (AE-11M, ERMA INC, TOKYO) at every 8 hours interval.



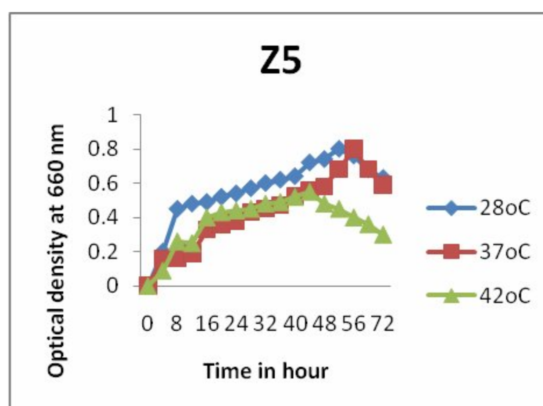
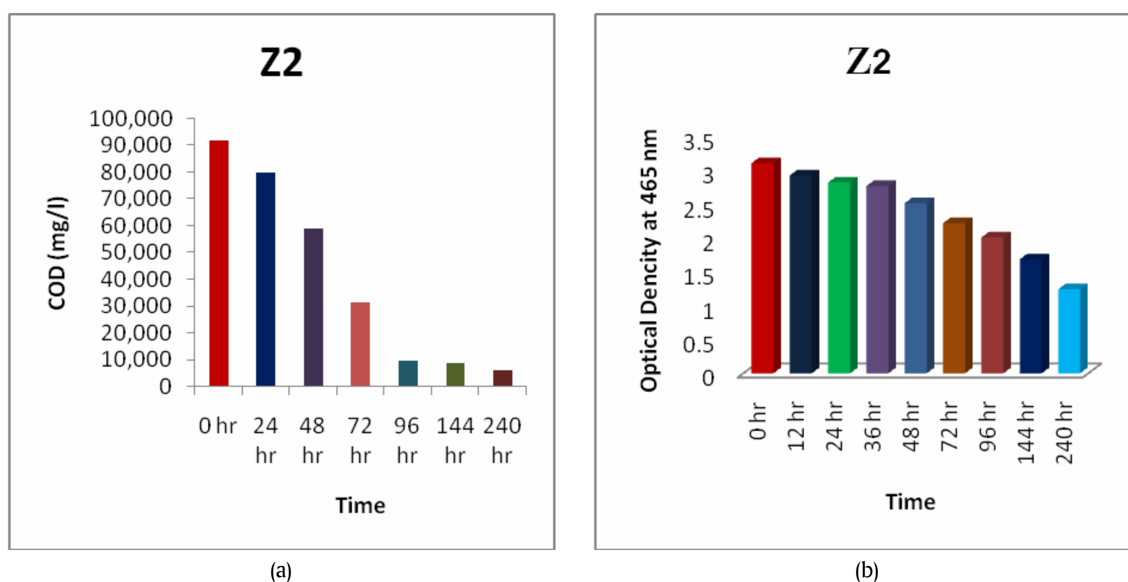


Fig.2: Effects of temperature on bacterial growth. For the effect of temperature, culture medium (nutrient broth, Hi-medium, India) was adjusted to 28 ºC, 37 ºC and 42 ºC. Then, the media were inoculated and incubated for 72 hours at 37ºC. During incubation, bacterial cell density of liquid cultures was determined by measuring optical density at 620 nm with photoelectric colorimeter (AE-11M, ERMA INC, TOKYO) at every 8 hours interval



(a) (b)

Fig. 3(a). Reduction of COD by Z2. For the reduction of COD, culture medium (nutrient broth, Hi-medium, India) inoculated and incubated for 240 hours at 37ºC. During incubation, the COD range of the treated effluent measured by COD meter at every 24 hours interval; **3(b)Decolourization of the effluent by Z2.**Colour removal was monitored by measuring the decrease in colour density at 465 nm wavelength using a UV- Spectrophotometerat every 12 hours interval.

DISCUSSION

Molasses-based distilleries are one of the most polluting industries generating large volumes of high strength wastewater containing dark brown colored compounds melanoidins. In this study, five melanoidins degrading bacteria were identified from sugar-mill effluent and characterized as*Exiguobacteriumacetylicum* strain QD-3,*Bacillus cereus* strain H3,*Enterobacter* sp. PR1, *Pseudomonas aeruginosa* strain AU09 and *Bacillus megaterium* strain Jz11 by biochemical

Fig.4: Phylogenetic tree showing the genetic relationship among the cultivated bacteria 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.

tests and 16S rRNA gene sequence analysis. Melanoidins degrading bacteria have also been isolated from distillery effluents by several researchers (Kumar *et al.*, 1997; Kumar *et al.*, 2008; Kumar and Chandra, 2006 and Jain *et al.*, 2002; Bharagava*et al.*, 2009, Ruhi *et al.*, 2017). During the present investigation it was observed that the bacterial isolate *Bacillus cereus* strain H3 showed approximately60%decolorizationand 93% COD reduction activity at optimum condition.A similar study achieved by Chavan*et al.* (2006) showed that *Pseudomonas* sp. coulddecolorize spent wash up to 56% and 63% reduction in COD of the spent wash after 72 h treatment.Jain *et al.* (2002) isolated three bacterial strains from the activated sludge of a distillery effluent identified as a *Bacillus megaterium*, *B. cereus* and *B. fragairae* which were found to remove colour and COD from the distillery effluent in the range of 38-58 and 55-68%, respectively.

Tiwari*et al.* (2012) reported that melanoidins decolorizing bacterial strains*viz*. *Bacillus subtilis*, *B. cereus* and *Pseudomonas aeruginosa*were isolated from distillery wastewater contaminated soil. Among which *B. subtilis* showed maximum decolorization 85% at 45ºC in the presence of little amount of carbon (0.1%,w/v) and nitrogen sources (0.1%, w/v) within a very short incubation period 24 h. Bacillus cereus and Pseudomonas sp. showed 73 and 69% decolorization, respectively under optimum conditions.

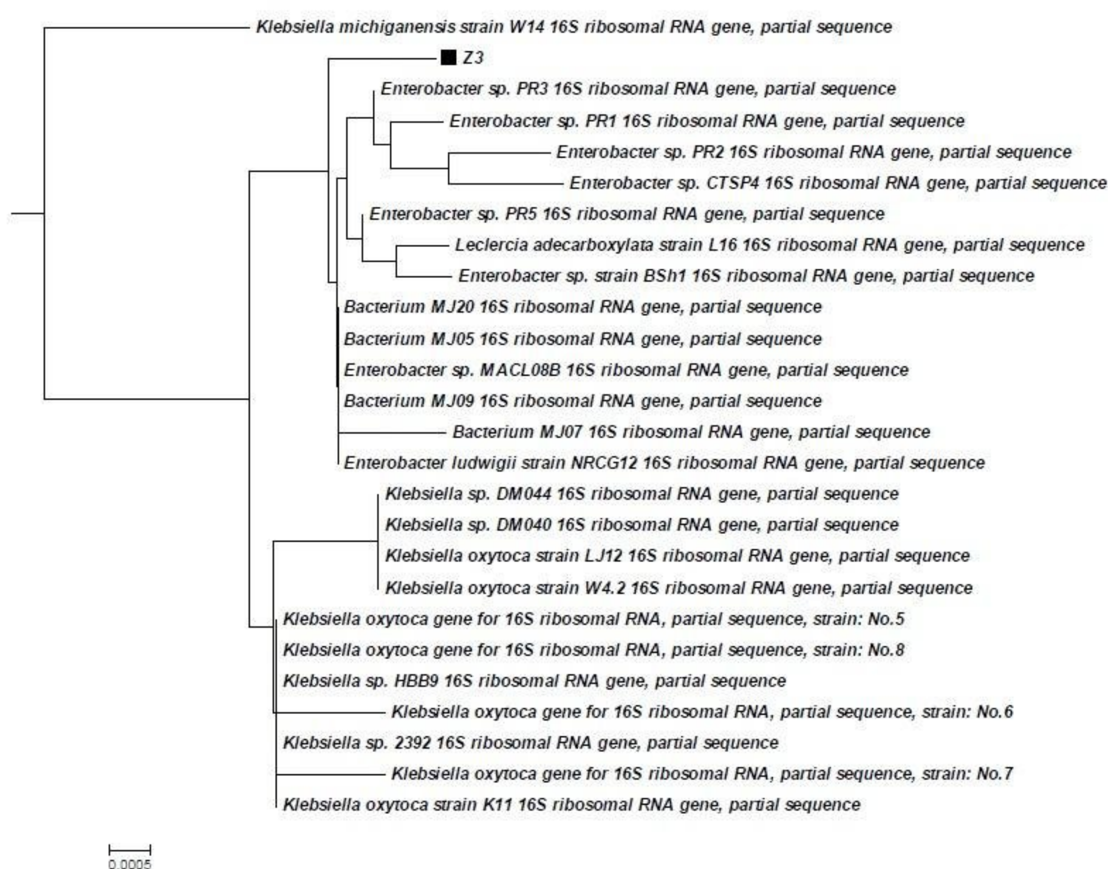


Fig.5: Phylogenetic tree showing the genetic relationship among the cultivated bacteria and reference 16S rDNA sequences from the GenBank based on partial 16S ribosomal RNA gene sequences. Scale bar 0.0005 = 0.05% difference among nucleotide sequences.

According to reports from different investigations the various strains of *Bacillus* sp. showed an average decolorization 75-81%, COD 80-85% and BOD 85-95% removal efficiency under optimum conditions (Chavan*et al*., 2006; Kumar *et al*., 2008; Dahiya*et al*., 2001).

The growth of the isolated bacteria and toxic pollutants decolorization were dependent on P^H and temperature. Optimum pH and temperature for the growth of the isolates were found to be 7 and 37 ºC, respectively. Optimum P^H and temperature for growth of spent wash degrading bacteria was reported at ranging from 6.8-7.2 and temperature range of 30-35°C was found to be suitable for activity of the isolate (Chavan*et al*., 2006; Saha *et al*., 2017). It was recovered that the optimum temperature for the best growth of isolates was found to be 37°C and growth rate was moderately low in other temperature *viz*. 28°C and 42°C. So, 37°C temperature is the most suitable temperature for the decolorization of spent wash effluents.It has been reported that under aerobic condition Bacillus sp. has been decolourize molasses wastewater upto 35.5% at 55°C temperature (Nakajima *et al*., 1999).

The bioremediation of distillery wastewater could be depending media containing carbon

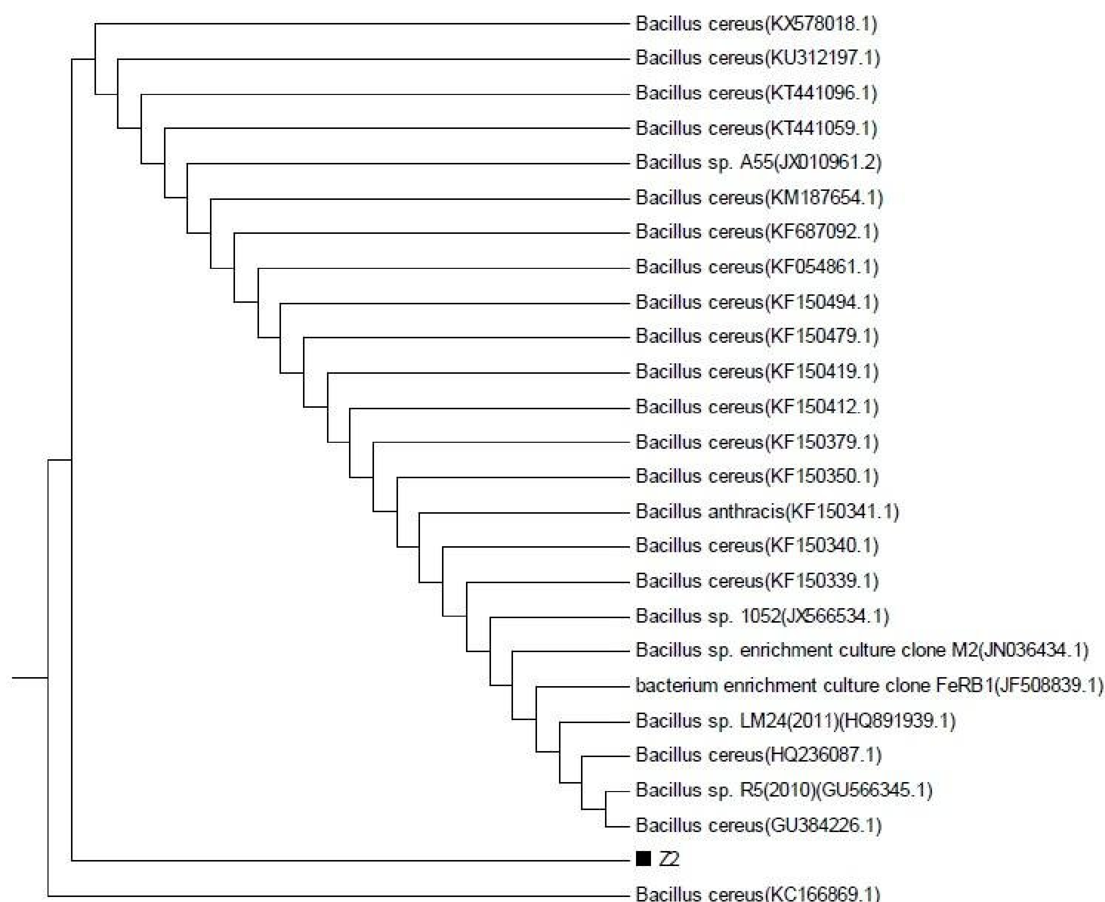


Fig. 6: Phylogenetic tree showing the genetic relationship among the cultivated bacteria and reference 16S rDNA sequences from the GenBank based on partial 16S ribosomal RNA gene sequences. Scale bar 0.0005 = 0.05% difference among nucleotide sequences.

source.In the present study, it was also observed that when glucose as carbon source at level of 1% added during treatment the decolorization and COD reduction rate was increased. This effect can be explained that during initial phase of growth, organism utilizes easily available carbon sources added to the medium and then starts to degrade spent wash that is complex carbon source (Kumar *et al*., 1997). Reduction in the content of melanoidins may be attributed to their bacterial degradation in the presence of supplementary carbon and nitrogen sources through co-metabolism (Kumar and Chandra, 2006). Ohmomo*et al*. (1987) reported that glucose was the best carbon source, which utilized by *Aspergillusfumigatus* G-2-6 for maximum degradation of melanoidins and further increase in glucose concentration, increased the mycelial biomass but no change in decolorization level.Soni*et al*.(2012) reported that the thermotolerantstrain of *Bacillussubtilis* has ability to decolorized melanoidin at wide range of temperature and *Pseudomonas aeruginosa* also show remarkable color reduction in the presence of little

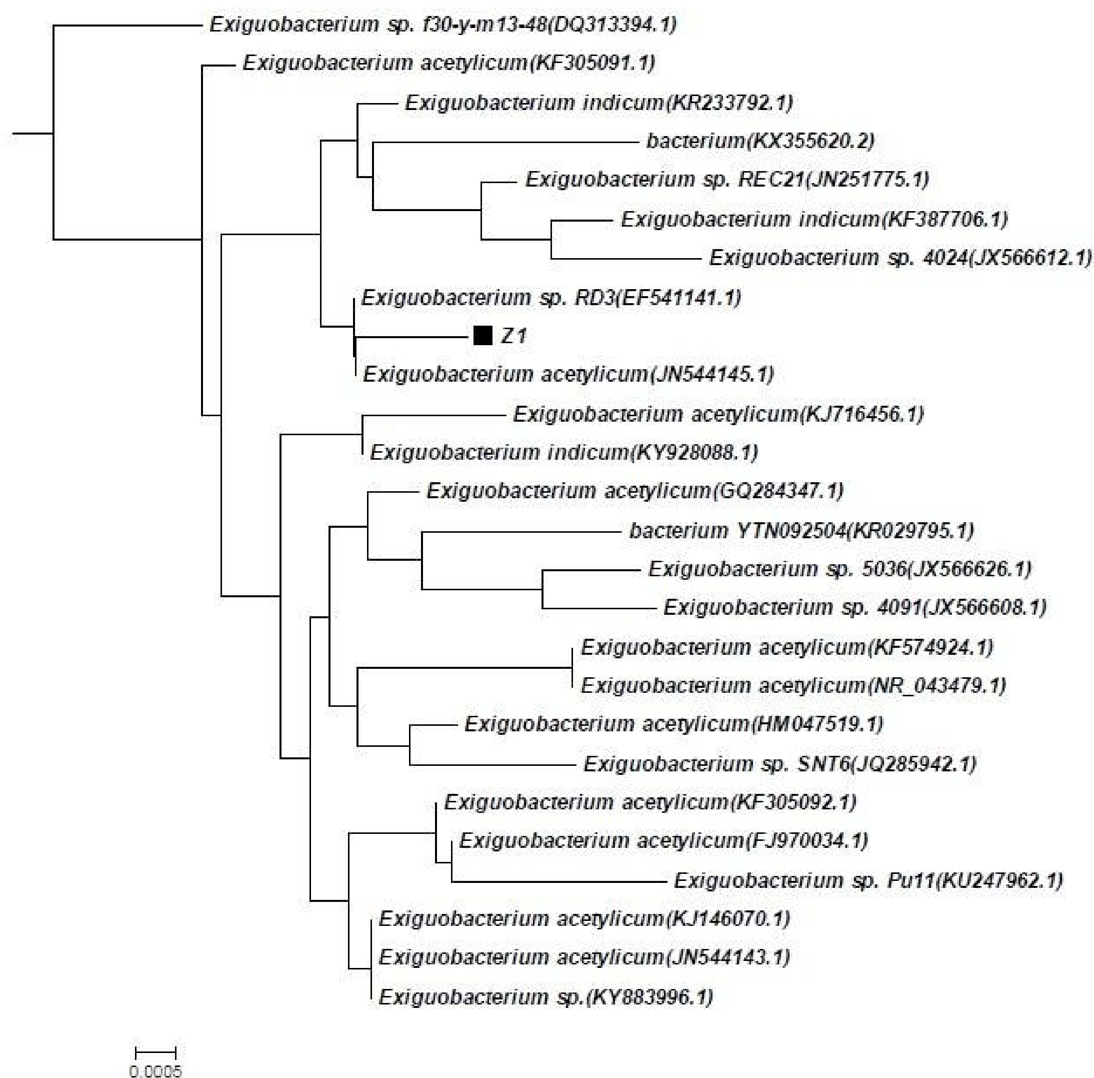


Fig.7: Phylogenetic tree showing the genetic relationship among the cultivated bacteria and reference 16S rDNA sequences from the GenBank based on partial 16S ribosomal RNA gene sequences. Scale bar 0.0005 = 0.05% difference among nucleotide sequences.

amount of carbon source within a very short incubation period, but consortia of both strains shows better results with and without carbon souce.

The antibiotic resistance pattern is generally used for strain / identification in ecological studies. It is evident from the present investigation that Z2were resistant to amoxycilin, azithromycin, cephradine, ciprofloxacin, cephalexin, erythromycin, gentamicin, kanamycin, neomycin, rifampicin, streptomycin and tetracycline while Z1 and Z5 were found to be sensitive to those antibiotics except azithromycin and streptomycin. Z4showed resistance against amoxycilin, azithromycin, cephradine, cephalexin, erythromycin, kanamycin, rifampicin and

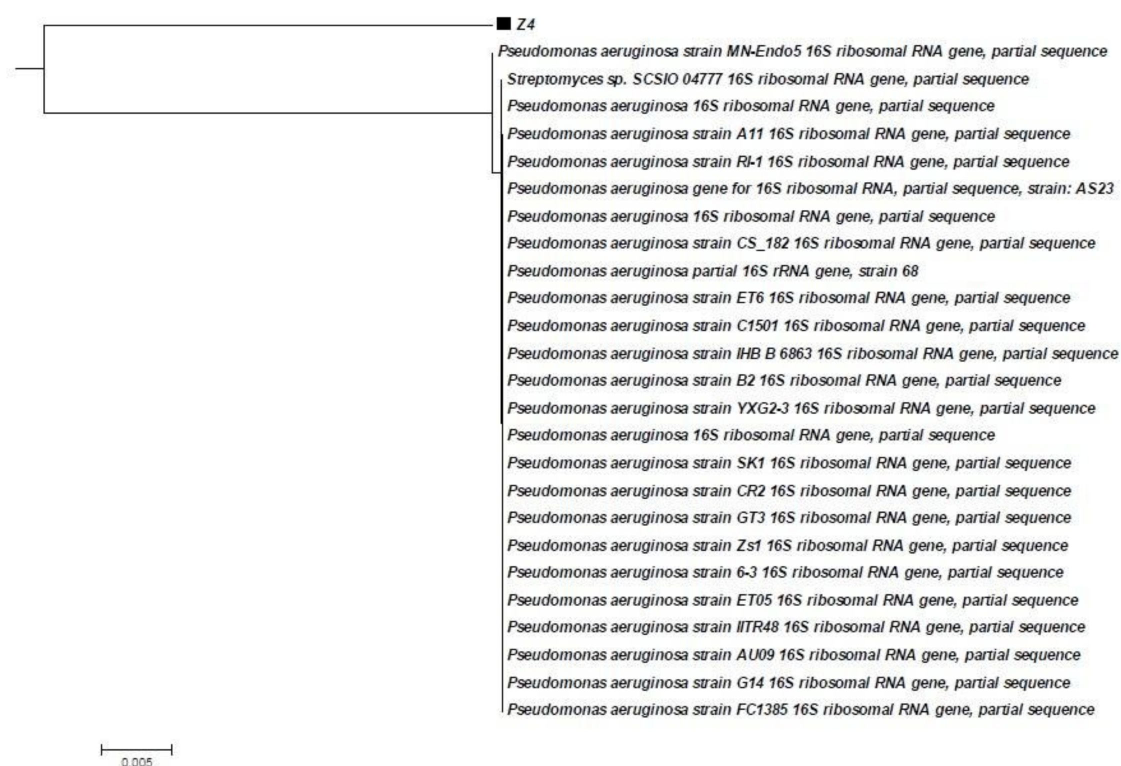


Fig.8: Phylogenetic tree showing the genetic relationship among the cultivated bacteria and reference 16S rDNA sequences from the GenBank based on partial 16S ribosomal RNA gene sequences. Scale bar 0.0005 = 0.05% difference among nucleotide sequences.

Z3 showed resistance against amoxycilin, cephradine, cephalexin, erythromycin, kanamycin, neomycin and rifampicin. Mechanisms of resistance by microorganisms include microbial surface sorption, enzymatic transformation, and perception by oxidation/reduction reaction and biosynthesis of metal binding proteins (Srinath*et al*., 2002; Zoubilis*et al*., 2004). Bacterial isolates capable of decolorizing melanoidin is probable due to the presence of the novel catabolic enzymes. Various forms of intracellular and extracellular enzymes as laccases, manganese, peroxidase, lignin peroxidase, sugar oxidase such as sorbose oxidase have been reported to show melanoidin degradation activity (Sirianuntapiboon and Chairattanawan, 1998). Watanabe *et al*. (1982) purified enzymes from *Coriolus* sp. No.20 which was identified as sorbose oxidase and involved in melanoidin degradation activity. It was suggested that melanoidins were decolorized by the active oxygen such as hydrogen peroxide species produced by the enzymatic oxidation reaction with sugar oxidase in the presence of sugar such as glucose, maltose, sucrose, lactose, sorbose, galactose and xylose as a substrate (Watanabe *et al*., 1982; Pant and Adholeya, 2007).

In the present study, *Bacillus cereus* strain H3, an indigenous isolate was found to be more

efficient in decolorizing of spent wash along with melanoidin degradation in comparison to earlier reports for bacterial decolorization. This approach can be further exploited to develop a cost-effective, eco-friendly biotechnology package for the treatment of distillery spent wash. Alternative bioremediation strategies using engineered strains also offer great promise. There are many benefits to be derived from the successful application of recombinant DNA techniques to evolve microbes that disseminate polluting xenobiotic. Also, the current revolution in genetic engineering is sure to have an impact on biodegradation technology, and in time the catabolic potential of microbes will be realized. However, decolourization efficacy of the isolated bacteria was studied only for melanoidins but their efficacy to degrade many other compounds of sugar-mill effluent remained obscured. Likewise, degradation efficacy of the isolated bacteria was tasted in small-scale in lab which do not ensure their similar degradation capacity in large-scale in industrial bioreactor. Notably, the higher rate of degradation of melanodins by the isolated bacteria does not confirm the higher detoxification rate of sugar-mill effluents. Hence, future study should be focused on detoxification of melanoidins along with other compounds of sugar-mill effluent in large scale so that the isolated bacteria could be considered as efficient microbial agents for using in an industrial bioreactor to neutralize the sugar-mill effluents.

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