



DEGRADATION AND DETOXIFICATION OF DISTILLERY WASTEWATER POLLUTANTS BY *BACILLUS MEGATERIUM* SP. FOR ENVIRONMENTAL SAFETY

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Abstract:

The present study revealed that distillery wastewater is a complex in nature because it contains organic, inorganic and other coloring compounds. Wastewater discharged also contains high pH, biological oxygen demand, chemical oxygen demand, total solids, phenolics and toxic heavy metals. When untreated/partially treated DWW discharged into the environment, it causes serious ecotoxicological and health threats. In water bodies, it reduces the penetration power of sun light causing a reduction in photosynthetic activity and depletion in dissolved oxygen (DO) content. In present study revealed that the bacterial strain *Bacillus megaterium* sp. (MF967441) was found effective to decolorize DWW upto 63.26% in presence of glucose (0.5%) and peptone (0.1%) at pH 7.0 and temperature 35 °C in axenic culture conditions.

Key Words: Distillery Wastewater, Melanoidins, Decolorization & *Bacillus Megaterium* Sp.

1. Introduction:

The bioremediation process of industrial waste can be making more efficient by using ligninolytic enzymes like laccase which is obtained from fungus, bacteria, higher plant, insect and also in lichen etc. (Chandra and Chowdhary, 2015). In aerobic process various types of microorganisms are involved such as bacteria, fungi, actinomycetes etc. are used due to their inherent capacity to breakdown a variety of complex compounds for degradation/decolorization of toxic and recalcitrant compounds present in various industrial wastes for environmental safety (Gonzalez et al., 2000; Chowdhary et al., 2017; Chowdhary et al., 2018). Distillery wastewater is the waste product released after the distillation of ethyl alcohol from molasses. As it is well known that wastewater generated from distillery industry causes adverse effect on environment due to alkaline nature (pH 7.5-8.5) and high biological oxygen demand (BOD), chemical oxygen demand (COD) and total dissolve solid (TDS) etc. (Chowdhary et al., 2018). The effluents from molasses based distilleries contain large amounts of dark brown colored molasses spent wash (MSW). In India about 319 distilleries in which, capacity of alcohol production of one unit is about 3.25×10^9 l and releasing 40.40×10^{10} l of wastewater annually (Bharagava et al., 2009). An average molasses based distillery generates 15 L of spent wash L⁻¹ of alcohol produced (Beltran et al., 2001). DWW is hazardous for the aquatic fauna and flora because its colored component reduces photosynthetic activity and depletes dissolved oxygen in water bodies (Pal and Yadav, 2012). In distillery wastewater melanoidins is one of the major pollutants causing serious health problem and also adverse effect on environment. Melanoidins are dark brown to black colored recalcitrant compounds (Wang et al. 2011; Arimi et al. 2014; Arimi et al. 2015).

Molasses spent wash is hazardous to aquatic fauna and flora because its colored compounds reduces photosynthetic activity and depletes dissolved oxygen in water bodies (Kumar et al., 1997). The physico-chemical methods suggested for treatment of spent wash are not efficient for decolorization (Jain et al., 2002). Biological methods like anaerobic digestion treatment reduce BOD load of the spent wash, but substantial amount of organic and inorganic components and dark brown color requires secondary treatment process. However, fate and extent of toxicity of anaerobically treated distillery effluent still remains unknown in the environment (Yadav and Chandra, 2012).

Thus the purpose of this study was to identification, characterization of bacteria and degradation /detoxification of distillery wastewater pollutants, which were generated at various steps during alcohol production process. Efforts therefore have to be made provide some specific treatment employing advanced treatment processes (physico-chemical, biological or biphasic treatment) to improve the quality of distillery wastewater before discharging in the environment.

Microbial treatments employing pure bacterial culture have been reported frequently in past and recent years. Bacterial degradation/decolorization of industrial wastes is an eco-friendly and cost effective alternative to chemical decomposition process of wastes minimization.

2. Materials and Methods:

2.1 Collection of DWW Sample: The distillery wastewater used in this study was collected in pre-sterilized plastic Carboy container (capacity 10 L) from the anaerobic digestion Effluent Treatment Plant (ETP) of M/s Unnao Distilleries and Breweries (26° 33'15.92"N, 80° 30' 38" E), Uttar Pradesh, India (Figure 1). The collected

wastewater samples were brought to laboratory, and stored at stored at 4 °C. Further, the collected sample were used in bacterial isolation and degradation/decolorization of distillery wastewater pollutants.

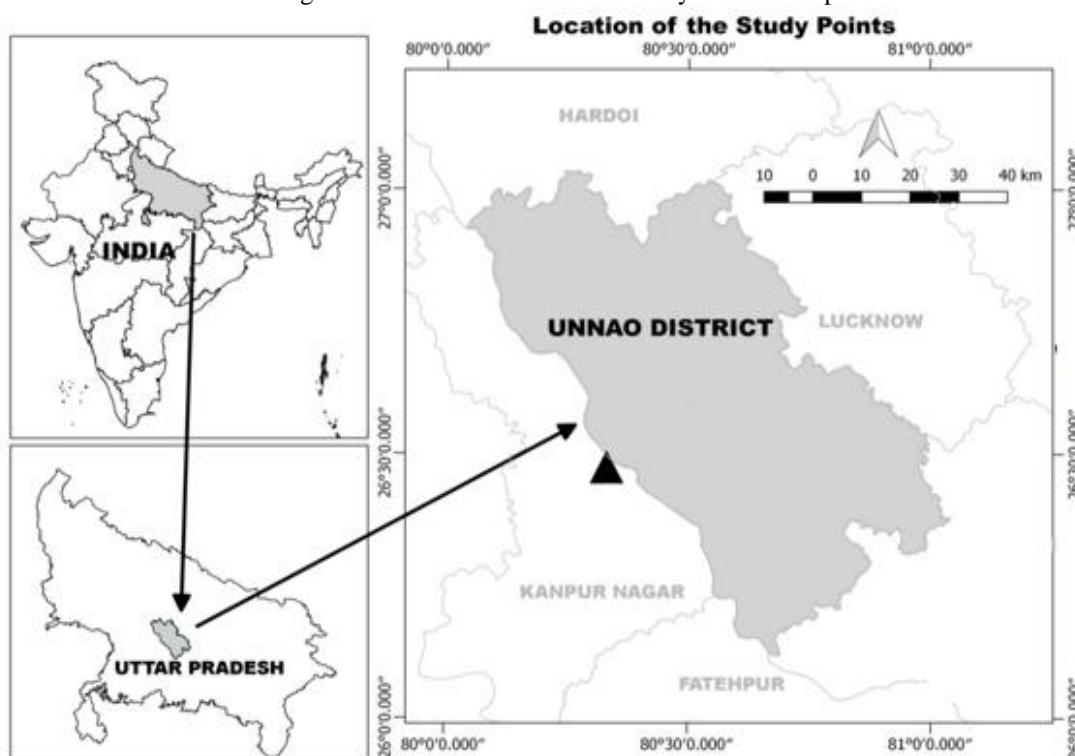


Figure 1: Collection of wastewater and sludge sample from the anaerobic digestion Effluent Treatment Plant (ETP) of M/s Unnao Distilleries and Breweries

2.2 Media Composition: In this study, a modified medium containing glucose (0.5%), peptone (0.1%), K_2HPO_4 (0.1%) and $MgSO_4 \cdot 7H_2O$ (0.05%) in double distilled water was used. To this medium, DWW was added to obtain optical density 2.8 at 475 nm and pH was adjusted at 7.3 ± 0.1

2.3 Nutrient Enrichment Technique, Isolation Purification of Distillery Wastewater Degrading Bacteria: A fraction of sludge/soil sample (5 g) was added to an Erlenmeyer flask (250 mL) containing 50 mL of autoclaved modified GPYM media with optical density 2.8 at 475 nm and pH was adjusted at 7.3 ± 0.1 . followed by the incubation at 32 ± 1 °C for seven days for enrichment (Bharagava et al., 2009; Ghosh et al., 2002). Further, the bacteria with distillery wastewater decolorizing or degrading ability were isolated from enrichment culture by serial dilution method and purified on GPYM agar plate. In addition, purity of each bacterial strains were checked by microscopic method.

2.4 Screening of Isolated Bacterial Strains: Screening of isolated bacterial strain was done by two way screening process. First, in initial step of screening the pollutants degrading bacterial isolates was done by growing in Mineral Salt Medium (MSM) amended with different concentrations of distillery waste water at pH 7.5, temperature 37°C for 144 h incubation period (Pfennig and Lippert, 1966). The bacterial isolates growing at higher concentration of distillery wastewater was selected as the most potential isolates for further studies. Further, these bacterial strains were also strike on different concentration of melanoidins amended agar plate. In second step of screening process, the isolated bacterial strains capable to degraded melanoidins compounds were further screened on modified GPYM agar plate amended with melanoidin 32 ± 1 °C by nutrient enrichment techniques on basis of their growth performance and manganese peroxidase (MnP) activity (Bharagava et al., 2009). The isolated bacterial strains were grown on GPYM agar plate amendment with extracted melanoidins with optical density 2.8 at 475 nm, 1.0% agar and 0.1% phenol red (w/v) (Bharagava et al., 2010) to visualize the manganese peroxidase activity. Further, the streaked plates were incubated at 32 ± 1 °C for 24-120 h. The bacterial strains showing maximum or fast growth and production of manganese peroxidase were used in further experiments.

2.5 Biochemical and Molecular Characterization of Bacterial Isolates: The bacterial strains DS3 and DS5 were characterized morphological and biochemically as per the methods described in Cowan and Steel's Manual for Identification of Medical Bacteria (Barrow and Feltham 1993).

2.5.1 PCR Amplification and Cloning of 16 S rDNA Gene: About 5 µL of genomic DNA was used to amplify the 16S rDNA gene using universal eubacterial primers (27F) 5'-AGAGTTTGATCMTGGCTCAG-3' and (1492R) 5'-CGGTTACCTTGTACGACTT-3' (Narde et al. 2004) and a 1,500 bp product was amplified. The reaction mixture contained 100 ng template, 1x PCR buffer, 200 µM of each dNTP, 3.0 mM $MgCl_2$, 25

pmol of primer, and 2.5 units of Amplitaq DNA polymerase (Perkin Elmer) in a final reaction volume of 50 μ L. The thermocycling steps (Veriti® 96-Well Thermal Cycler, Applied Biosystems, USA) used were as 30 cycles of denaturation at 94 °C for 1 min, followed by annealing at 45 °C for 1 min and extension at 72 °C for 2 min.

The PCR amplified 16S rDNA gene product were electrophoresed through 1.2% (w/v) agarose gel in 1X TAE buffer using 1 Kb DNA ladder (Merk, Biosciences, India) as molecular weight marker and visualised by staining with ethidium bromide (EtBr). The PCR products were gel purified using gel extraction kit (Merk, Biosciences, India) and sequenced using primer 27F. The restriction digestion of purified plasmid DNA made with 10 units of EcoR1 for 2 h at 50 °C in 1X reaction buffer containing 60 mM Tris Tris-Cl, pH 7.9, 1.5 M NaCl, and 60 mM MgCl₂ has shown the presence of an expected ~ 1.5 kb insert when subjected to agarose gel electrophoresis.

2.5.2 16S rDNA Gene Sequencing and Phylogenetic Tree Analysis: The PCR products were gel purified using gel extraction kit (Merk, Biosciences, India) and sequenced using primer 27F and 1492R as described in above section. The PCR amplified product i.e. 16S rDNA gene were gel purified and used as template in sequencing reactions using primer 27 F, carried out using an ABI PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, USA). The partial sequences obtained were subjected to BLAST analysis using the online option available at www.ncbi.nlm.nih.gov/BLAST (Altschul et al., 1997) suggesting the identity of bacterial isolates. The phylogenetic tree was constructed by using the neighbour-joining method using Kimura-2-parameter distances in MEGA-4 software and the 16S rDNA sequences obtained was also deposited in GenBank databases under the accession number MF182113, MF967441, and MF182114 for bacterium DS3, DS4 and DS5, respectively.

2.6 Degradation and Detoxification of Distillery Wastewater Pollutants by Axenic Culture Conditions: The degradation of DWW pollutants was carried out in triplicate in 250 mL Erlenmeyer flasks containing 100 mL of sterile modified medium amended with DWW pollutants (optical density 2.8 at 475 nm). The flasks were inoculated with 1% (v/v) of activated bacterial cultures grown for 48 h in DWW amended medium in axenic and mixed conditions and incubated at 37 °C in incubator shaker at 75 rpm (Innova 4230, New Brunswick, USA) for six consecutive days. The degradation/decolorization of DWW pollutants was measured by spectrophotometer (Evolution 201, Australia) in terms of bacterial growth and reduction in color intensity at 620 and 475 nm, respectively (Bharagava and Chandra, 2009).

3. Result and Discussion:

3.1 Isolation and Screening of Bacterial Strains: From the collected distillery wastewater and sludge sample ten (09) morphologically distinct bacterial strains (DS, DS1, DS2, DS3, DS4, DS5, DS6, DS7, and DS8) were isolated and purified by the repeated plate streak method on distillery wastewater amended GPYM agar plates. Initially screening the pollutants degrading bacterial isolates was done by growing in Mineral Salt Medium (MSM) amended with different concentrations of distillery wastewater at pH 7.5, temperature 37 °C for 144 h incubation period (Pfennig and Lippert, 1966). The bacterial strain DS1, DS3, DS4, and DS5 were found higher resistance capability and grow at maximum concentration (500-2500 ppm) of distillery wastewater amended MSM agar plates (Table 1) and was selected as the most potential isolates for further studies. Further, among ten isolated bacterial strains, four bacterial strain DS1, DS3, DS4, and DS5 have shown rapid growth and high MnP activity on phenol red containing GPYM agar plates amended with distillery wastewater in terms of change in color from deep orange to yellow within 48 h incubation periods.

Table 1: Maximum tolerance limit of isolated bacterial strains on modified MSM media amended with different Melanoidin (extracted from distillery effluent) at different concentration

S.No	Bacterial Strains	Distillery Effluent (Melanoidin Containing)				
		Melanoidin (ppm)				
		500	1000	1500	2000	2500
1.	DS	+	-	-	-	-
2.	DS1	+	+	+	+	+
3.	DS2	+	+	-	-	-
4.	DS3	+	+	+	+	+
5.	DS4	+	+	+	+	+
6.	DS5	+	+	+	+	+
7.	DS6	+	-	-	-	-
8.	DS7	+	-	-	-	-
9.	DS8	+	-	-	-	-
10.	DS9	+	+	-	-	-

DS: distillery Sludge; (+) presence of growth; (-) absence of growth

In this study, bacterial strain DS5 showed normal growth, but moderate MnP activity on phenol red containing modified GPYM agar plate amended with distillery wastewater. Modified GPYM agar plates amended with distillery wastewater and phenol red has been used as indicator of MnP activity (Table 2).

Further, these bacterial strains were used for the initial biochemical identification such as morphological /microscopic study.

Table 2: Manganese peroxidase (MnP) activity of potential bacterial strains after different time incubation

S.No	Bacterial Strains	Enzymes	Time Incubation (h)				
			24	48	72	96	120
1.	DS1	MnP	*	***	***	**	*
2.	DS2	MnP	-	**	**	*	-
3.	DS3	MnP	**	***	****	***	**
4.	DS4	MnP	**	***	***	*	*
5.	DS5	MnP	*	*	**	**	-

**** Very high enzyme activity; ** moderate enzyme activity; * slower enzyme activity; * very slow enzyme activity; - no enzyme activity

3.2 Biochemical and Molecular Characterization of Bacterial Isolates: This study revealed that bacterial strains DS3 and DS4 were identified as gram positive and cell was coccus and rod shape, respectively whereas DS5 were identified as gram negative and rod shape (Table 3).

Table 3: Morphological characteristics of isolated bacterial strains from distillery wastes

S.No	Characteristics	Bacterial strains		
		DS3	DS4	DS5
1.	Color	White	Creamy white	Creamy white
2.	Gram stain	+ve	+ve	-ve
3.	Cell shape	coccus	rod	rod
4.	Surface texture	Normal	rough	Normal
5.	Margin	Entire	Irregular	Entire
6.	Elevation	Convex	Flat	Flat

Bacterial strains DS3 and DS5 were giving negative test for catalase, sorbitol whereas DS4 gives positive test but both the strains i.e. DS3 and DS5 have shown the positive test for ornithine utilization. Further, DS3, DS4, and DS5 have shown the negative test for citrate utilization, H₂S, lactose fermentation test. Whereas these strains i.e. DS3, DS4, and DS5 have shown the positive test for urease test but showed variable test for the lysine utilization. The result of biochemical identification shown in Table 4.

Further, in this study based on the 16S rDNA sequence similarity, bacterial strains DS4 was identified as *Bacillus megaterium* sp. with accession number MF967441 (Fig. 2) (Bharagava et al., 2009; Bharagava and Chandra, 2010).

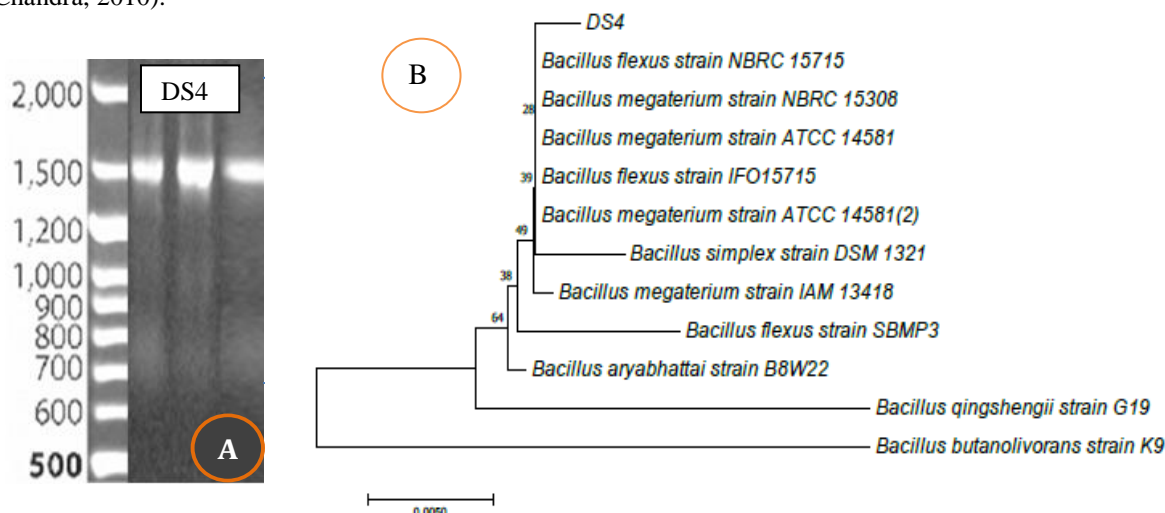


Figure 2: (A) PCR amplification of 16S rRNA gene of isolated bacteria (DS4); and Neighbor-joining tree showing the phylogenetic position of isolated bacteria DS4 with closest related species based on the 16S rRNA gene sequences. The GenBank accession number for each bacterium used in the analysis is shown in parenthesis before the species name.

Table 4: Secondary biochemical results of isolated potential bacterial strains for initial identification.

S.No	Biochemical Test	Bacterial Strains		
		DS3	DS4	DS5
1.	Catalase	+ve	+ve	+ve
2.	Indole Test	+ve	-ve	-ve

3.	Amylase Test	+ve	+ve	-ve
4.	Citrate Utilization	-ve	-ve	-ve
5.	Lysine Utilization	V	V	V
6.	Ornithine Utilization	+ve	V	+ve
7.	Urease	+ve	+ve	+ve
8.	Phenylalanine Deamination	V	-ve	-ve
9.	Nitrate Reduction	+ve	-ve	-ve
10.	H ₂ S Production	-ve	-ve	-ve
11.	Glucose	+ve	+ve	-ve
12.	Adonitol	-ve	-ve	-ve
13.	Lactose	-ve	-ve	-ve
14.	Arabinose	-ve	V	-ve
15.	Sorbitol	-ve	+ve	-ve

(+ve): positive; -ve: (negative); V: variables results

3.3 Degradation and Detoxification of Distillery Wastewater by Bacteria DS4 (*Bacillus megaterium* sp): In this study, the bacterial strain DS4 was found effective to decolorize DWW upto 63.26% in axenic culture conditions with considerable reduction in BOD, COD values, total solids, sulfates, phosphates and phenolic content. During decolorization experiments, an increase in optical density (OD) for bacterial growth was observed indicating the fast growth, which reached to optimum at 120 h of incubation time (Figure 3).

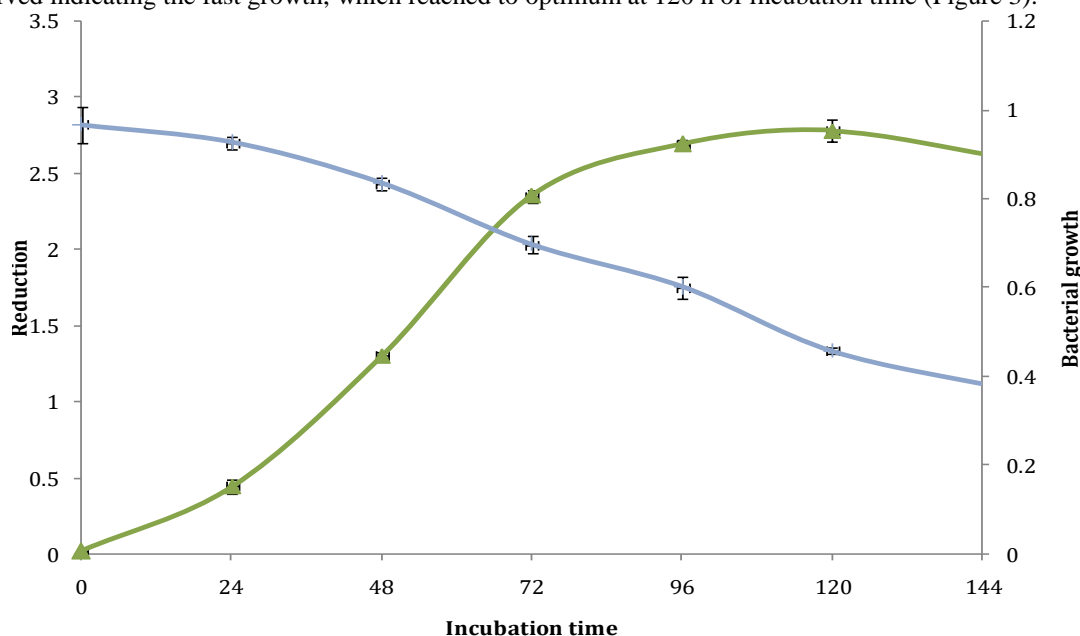


Figure 3: Decolorization graph of distillery wastewater by axenic culture strains DS4 (*Bacillus megaterium* sp.)

However, initially at 24 h of incubation period, slow reduction in color intensity was observed, which might be due to the utilization of glucose (as primary nutrient source) by bacteria for their establishment and subsequently utilization of DWW pollutants as carbon, nitrogen, and energy source (Bharagava and Chandra, 2010). After it, the reduction in color intensity was increased up to 120 h of incubation period, which might be due to the utilization of DWW pollutants as carbon, nitrogen, and energy source.

4. Conclusion:

The present study concluded that distillery wastes (wastewater and sludge) have many potential bacterial strains are present. Initially nine bacterial strains (DS, DS1-DS8) were isolated by nutrient enrichment technique, among of these four bacterial strains DS1, DS3, DS4, and DS5 screened on the basis of their growth on different concentration of DWW and manganese peroxidase activity shown on phenol red containing GPYM agar plates amended with DWW. Finally, out of four bacterial strains DS4 was selected as most potential for DWW pollutants decolorization/degradation. The bacterial strain *Bacillus megaterium* sp. was found effective to decolorize DWW upto 63.26% in axenic culture at optimized conditions. Thus, efforts have to be made either to control the wastewater quality or provide some effective treatment process applying recent/advanced treatment methods to improve the distillery wastewater quality for sustainable development.

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