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A NOVEL STRAIN OF BACTERIUM, *Arthrobactor* sp. USE FOR DECOLORIZATION OF MELANOIDIN OF DISTILLERY EFFLUENT

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Abstract*: Molasses based sugarcane distillery effluent known as spent wash contain high BOD, COD along with melanoidin pigment. Melanoidin is a Xenobiotic dark brown colour compound, which is formed by the Maillard reaction between amino acid and carbonyl group of sugars. This recalcitrant compound is hazardous to human, animal and microorganisms therefore must be treated before disposal. A novel strain of Arthrobactor is isolated and optimized for decolorization of distillery effluent at various physico-chemical and nutritional levels. This novel bacterium showed maximum decolorization, 84-86.29% at 35-40˚C using 0.1% glucose, 0.1% peptone, pH-6.0 within 48 hours. Hence, this bacterium has potential to degrade the melanoidin of distillery spent wash at industrial level.*
Keywords*: Decolorization, Arthrobacor sp., Melanoidin, Maillard reaction, Xenobiotic, Decolorization*

Introduction: Distilleries can be classified among the most polluting industries producing bulky amount of wastewater known as spentwash [1]. The characteristics of the wastewater produced depend on the feed stock used. Distilleries spent wash contain high Biological Oxygen Demand (BOD), Chemical Oxygen Demand (COD), low pH and toxic substances such as phenols. The major problem in treating distillery spentwash is due to its color, which contains nearly 2% (w/w) of a dark brown recalcitrant compound, melanoidin is 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 ecosystem.

Melanoidins are widely distributed in food drinks and majorly discharged in massive amount by different agro-based industries mainly from cane molasses based distilleries and fermentation industries as ecological contaminants. The chemical structure of melanoidins is still not finally implicit but it is assumed that its chemical structures basically depend on the nature and molar concentration of reacting compounds and reaction conditions as pH, temperature, heating time and solvent system used.

Distillery spentwash disposed to the ecosystem former to treatment is unsafe and can be a chief source of soil and water pollution. It induces toxic substances into water bodies as rivers, lakes and lagoons which badly affect aquatic plants and animals. The dark brown colored nature of the distillery spentwash also leads to the reduction of sunlight penetration in rivers, lakes or lagoons which, in turn, drops oxygenation of the water by photosynthesis and hence becomes damaging to aquatic life [2]. Disposal of distillery spentwash into soil is evenly dangerous, as it reduces soil alkalinity and manganese availability [3]. It also imparts high concentration of heavy metals viz., copper, nickel, silver, cadmium, iron and mercury which are able to inhibit the seed germination [4, 5]. There are several reports that when distillery spentwash applied for irrigation without proper treatment might result in reduction of soil fertility by suppressing the activity of soil microorganisms as nitrogen fixing bacteria *rhizobium* and *azotobacter* [6,7]. Hence, for its secure removal into the ecosystem, pretreatment is required. Usually, wastewater treatment methods can be categorized as physical, chemical and biological methods. Physico-chemical treatment methods involves activated carbon adsorption, sedimentation,

ozonation, coagulation, reverse osmosis, ultrafiltration, flocculation oxidation, electrolysis, membrane filtration and evaporation, but these methods are cost competitive on large scale whereas biological methods are eco-friendly.

In recent years, biological wastewater treatment using microorganisms has attracted the attention of researchers all over the world. Microbial degradation/decolorization of distillery effluents have been found as cost effective and environmental friendly alternative to physic-chemical methods. Various types of microorganisms as bacteria, fungi, and algae have been reported for their potential in decolorization of various industrial effluents including that of distilleries. Bacteria such as *Pseudomonas fluorescens, Pseudomonas putida, Bacillus* sp., *Alkaligenes* sp., *Lactobacillus* sp., have been successfully achieved and thus can be applied as bioremediation techniques. In this paper, *Arthobacter* sp. was isolated from soil sample of agricultural field of Masaudh distillery for biological treatment.

Materials and Methods

Sample Collection: The spentwash was collected aseptically from Masudha sugars and Distillery pvt.ltd, Faizabad, India. The spentwash was centrifuged at 10,000 rpm for 15 min before use to remove the suspended solids and stored at 4°C. The stored distillery spentwash was filtered through (Whatman No: 1) filter paper and was diluted with distilled water. Four different soil samples were collected from different place around the Masaudh Distillery. All samples were collected in sterile polythene bag and keep these samples in refrigerator at 4C.

Isolation, Screening and Identification of Melanoidin Decolorizing Microorganisms: For isolation of melanoidin decolorizing bacteria, one gram soil samples were suspended in 9 ml distilled water. After that, 1ml suspended soil sample were poured in blank plate and then melanoidin containing medium was plated and incubated for 24-48h for proper growth. Prominent bacterial isolates were screened on the basis of maximum clear zone around the bacterial colony on melanoidin containing agar plate. The isolates which showed maximum clear zone around the colony around the colony were selected for further study. After that, bacterial isolates were identified on the basis of biochemical test of Bergies Manual Methods.

Preparation of Mother Culture: Mother culture was prepared by inoculating isolates in melanoidin containing broth at 37^{o}C and incubated for overnight night for proper growth. After proper growth, bacterial growth was measured at 620nm. Distillery spentwash (DSW)

Decolorization Assay: The isolates were inoculated in the melanoidin containing broth and incubated for proper decolorization. After that, the broth was centrifuged at 10,000 rpm for 15 min. The supernatant was read at absorbance maximum (Amax) of the melanoidin i.e. 475 nm. The decolorization yield percentage was expressed as the decrease in the absorbance at 475 nm against initial absorbance at the same wavelength. Uninoculated medium was served as control. The entire assay was performed in triplicates and compared with control. The decolorization efficiency of the isolate was expressed as per following equation:

Decolorization (%) = I – F/I

Where, I = Initial absorbance (Control) and F = Absorbance of decolorized medium broth.

Optimization of Different Physico-chemical and Nutritional Parameters for Melanoidin Decolorization

Effect of pH, Temperature and Time on Melanoidin Decolourization: Optimum pH for the melanoidin decolourisation was determined by inoculating the bacterial isolates in melanoidin containing medium in the pH range of 4.5-8.5 and incubated at 37C. Effect of temperature on the melanoidin decolourization was determined by inoculating the bacterial isolates in the same medium over a temperature range of 35-60^{O}c at optimized pH. Effect of incubation periods on efficient decolorisation was determined by incubating the bacterial isolates in above mentioned medium for 8-96h at optimized pH and temperature.

Optimization of Different Nutritional Parameters: The various process parameters influencing melanoidin decolorization and biomass production were optimized individually and independently of the others, therefore, the optimized conditions were subsequently used in all the experiments in sequential order. For optimization, the basal medium contained glucose0.5%; peptone 0.1 %; KH2Po4 0.05% with 3.5 OD spentwash was used for inoculation with 2% (v/v) of bacterial culture. For the optimal melanoidin decolorization the bacterial isolates may require additional carbon and nitrogen sources with varying concentrations in its growth medium. Therefore, the growth medium was supplemented with the different carbon sources viz. glucose, fructose, maltose, sucrose, starch and nitrogen sources viz. yeast extract, peptone, beef

extract, ammonium sulphate, ammonium nitrate and sodium nitrate. Thereafter, optimized carbon and nitrogen sources were further optimized at different concentration (0.1 to 1%, w/v). The medium was sterilized at 121°Cfor 15 min and incubation was done at 40°C with all the other conditions at the optimal levels.

Results

Isolation, Screening and Identification of Melanoidin Decolourizing Microorganisms: Twenty two bacterial isolates were isolated from four different soil samples. Out of twenty two, three bacterial isolated were selected prominent isolates on the basis of maximum (1-2cm) clear zone around the colony. Maximum clear zone (2cm) observed by bacterial strain B-3 from the soil sample collected from the agriculture field which supplemented with untreated distillery effluent. The isolates B-3 was identified as *Arthobacter* sp. on the basis of biochemical test. All prominent bacterial isolates were maintained on melanoidin agar at 4oC for further studies. *Arthobacter* sp. showed maximum 84% decolorization with 10% molasses spentwash followed by bacterial isolates B-5 and B-17.

Table 1: Different bacteria were isolated from different samples

Different samples from Masaudha distillery	Isolates of Bacteria
Soil sample 1	B-1, B-5, P-1, P-2
Soil sample 2	S-1, S-2, S-3, S-4, S-5
Soil sample 3	A-1, A-2, A-4, A-7, A-8, A-10, A-12, A-15
Soil sample 4	B-3, B-17, B-5, B-19

Table 2: Selected efficient isolates of the bacteria on the molasses Agar by primary screening from different samples

Bacterial isolates	Clear zone (cm)
B-3	2
B-5	1.4
B-17	1

Table 3: Decolorization potential of bacterial isolates and with their morphology and Gram reaction (B-3 is *Arthrobactor*)

Bacterial isolates	Decolouriztion (%)	Morphology	Gram reaction
B-3	79	Rod	Positive
B-5	57	Rod	Negative
B-17	51	Short rod	Positive

Table 4: Effect of 10% molasses medium on melanoidin decolorization by Isolates of bacteria

Media	Decolorization (%)			Biomass production(g/l)		
	B-3	B-5	B-17	B-3	B-5	B-17
Medium 10% molasses media	84	62	53	5.2	3.10	3.2

Optimization of Different Physic-chemical and Nutritional Parameters for Melanoidin Decolorization

Effect of Different Temperature and Incubation Period: In this experiment, maximum decolourisation (84%) was observed at 35C by Arthobacter sp. when compared with other isolates. Bacterial isolate B-5 and B-17 showed maximum decolorization about 73 and 55% respectively. Similarly, reported that *Pseudomonas Fluorescence* [8] showed maximum melanoidin decolorization at 35C. Also reported that isolate SAG1 showed maximum decolorization (66.1 ± 2.1%) at 37C. It was also observed that with the further increase in temperature, the decolorization efficiency of isolates were reduces. Similarly, reported that maximum decolorization was achieved the temperature from 20 _C to 37^0C but further increase in temperature inhibited the decolorization efficiency [9]. However, the optimum temperature for the maximum decolorization activity was reported 35^0C [10,2].

In another set of experiment, effect of different pH on melanoidin decolorization was observed under optimal temperature. *Arthobacter* sp. showed maximum decolorization (84%) within 48 h of incubation period. Bacterial isolate B-5 and B-17 showed maximum decolorization about 63 and 54% respectively. Several authors have reported that bacterial isolates showed maximum melanoidin decolorization within 2 to 8 days of incubation [11, 9, 1]. It was also observed that further increase in incubation time, the decolorization efficiency of isolates were reduces.

Table 5: Effect of different temperature and incubation period on melanoidin decolorization by the bacterial isolates

Temperature (^{o}C)	Decolorization (%)		
	B-3	B-5	B-17
25	43 ± 1.5	35 ± 0.9	20 ± 0.8
30	67 ± 1.3	53 ± 1.7	40 ± 1.0
35	**84 ± 2.3**	73 ± 1.9	55 ± 1.5
40	83 ± 2.1	70 ± 1.4	53 ± 1.2

45	80 ± 1.8	67 ± 1.1	53 ± 1.8
50	78 ± 1.6	66 ± 1.3	52 ± 1.7
Incubation period (h)		**Decolorization (%)**	
	B-3	**B-5**	**B-17**
8	42 ± 1.2	33 ± 1.1	19 ± 1.8
16	54 ± 1.9	36 ± 1.1	29 ± 0.9
24	70 ± 2.2	43 ± 0.8	47 ± 1.3
32	80 ± 1.7	46 ± 1.2	51 ± 1.9
40	82 ± 2.1	51 ± 0.9	54 ± 1.7
48	84 ± 2.6	63 ± 1.6	53 ± 1.5

Effect of pH and Different Carbon Sources on Melanoidin Decolorization: The effect of pH, play a vital role on melanoidin decolorization under different experimental condition. From the results it clears that *Arthobacter* sp. showed maximium melanoidin decolorization (85%) at pH 6.0. Similar, results was also observed by several authors. Further increase or decrease in pH, the decolorization efficiency of isolates were reduces.

The effect of various carbon sources on melanoidin decolourisation was studied under optimal condition. *Arthobacter* sp. showed maximum melanoidin decolorization (86%) in the presence of glucose followed by sucrose and maltose as carbon sources. Similarly, reported that glucose was the best carbon source [11], which utilized by *Bacillus* sp. for maximum degradation of melanoidins and further increase in glucose concentration, increased the bacterial biomass but no change in decolorization level. 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 melanoidin that is complex carbon source [3]. Reported that the enzymatic degradation of melanoidin by Coriolus sp. No. 20 having an intracellular enzyme [12], which required active oxygen molecules and sugars (sorbose as well as glucose) in the reaction mixture, was later identified as sorbose oxidase which oxidize glucose into gluconic acid [6, 13]. The decline in melanoidin decolorization encountered with high sugar concentration in the medium is probably due to inhibition effect to the enzyme like lignolytic activity of laccase enzyme and oxidation activity of the peroxidase [14-19].

Table 6: Effect of pH and different carbon sources on melanoidin decolorization by the bacterial isolates

pH Range		**Decolorization (%)**	
	B-3	**B-5**	**B-17**
4.0	42 ± 1.6	35 ± 1.8	22 ± 0.4
4.5	62 ± 1.9	52 ± 0.9	40 ± 0.8
5.0	76 ± 1.1	60 ± 0.8	42 ± 1.2
5.5	83 ± 2.3	69 ± 0.6	46 ± 1.7
6.0	**85 ± 1.1**	73 ± 1.8	52 ± 1.3
6.5	80 ± 0.9	73 ± 1.4	51 ± 0.9
7.0	72 ± 1.2	67 ± 1.0	50 ± 0.8
Carbon sources (0.5%)		**Decolorization (%)**	
	B-3	**B-5**	**B-17**
Control	85 ± 1.1	73 ± 1.8	52 ± 1.3
Sucrose	83 ± 1.10	75 ± 0.8	55 ± 1.1
Glucose	**86 ± 2.5**	79 ± 1.0	56 ± 1.1
Maltose	83 ± 2.2	72 ± 1.3	53 ± 1.6
Fructose	81 ± 1.7	76 ± 1.5	57 ± 1.6
Starch	77 ± 1.5	71 ± 1.3	52 ± 0.9
Lactose	65 ± 1.1	68 ± 1.8	50 ± 2.0

Effect of Nitrogen Sources on Melanoidin Decolorization: Different nitrogen sources were optimized for melanoidin decolorization. Among different nitrogen sources (organic and inorganic), the highest melanoidin decolorization (86%) was reported with peptone followed by sodium nitrate by *Arthobacter* sp when compared with other isolates (B-5, B-17). Similarly various nitrogen sources were optimized by different workers for melanoidin decolorization, but peptone was the most effective for color removal [20, 6, 21, 22, 19, 11]. Reported that enzymatic systems catalyse degradation of lignin and lignin-like compound during the secondary phase of the metabolic growth in the presence of peptone [23]. Synthesis and secretion of lignin peroxidase or ligninase (LiP) and manganese-dependent peroxidase (MnP) are triggered by nutrient limitations such as carbon and nitrogen sources. At high concentration, there was no significant decolorization due to surplus supplementation of nitrogen which inhibited the growth.

Table 7: Effect of different glucose concentration and different nitrogen sources on melanoidin decolorization by the bacterial isolates

Glucose concentration (%)	Decolorization (%)		
	B-3	**B-5**	**B-17**
Control	85 ± 1.1	73 ± 1.8	52 ± 1.3
0.1	**86 ± 2.7**	76 ± 1.4	57 ± 1.1
0.2	83 ± 1.10	75 ± 0.7	59 ± 1.6
0.3	81 ± 1.11	78 ± 1.0	60 ± 1.7
0.4	80 ± 1.2	79 ± 1.5	64 ± 1.1
0.5	79 ± 1.5	70 ± 0.9	67 ± 1.2
0.6	77 ± 1.5	71 ± 1.4	67 ± 1.4
Nitrogen sources (0.5%)	**Decolorization (%)**		
	B-3	**B-5**	**B-17**
Control	86 ± 2.7	76 ± 1.4	57 ± 1.1
Malt extract	76 ± 1.6	72 ± 1.3	53 ± 0.9
Yeast extract	78 ± 1.2	72 ± 1.5	54 ± 1.8
Beef extract	83 ± 1.2	70 ± 2.0	55 ± 1.2
Peptone	**86 ± 2.7**	75 ± 0.9	57 ± 1.0
$NaNo_3$	84 ± 1.11	73 ± 0.8	48 ± 1.5
$NH_4(SO_4)_2$	79 ± 0.9	72 ± 1.0	50 ± 0.9

Table 8: Effect of different peptone concentration on melanoidin decolorization by the bacterial isolates

Peptone concentration (%)	Decolorization (%)		
	B-3	**B-5**	**B-17**
Control	86± 2.7	76 ± 1.4	57 ± 1.1
0.1	**86± 2.9**	76 ± 1.3	57 ± 0.3
0.2	83± 1.3	77 ± 0.8	59 ± 1.2
0.3	81± 1.7	76 ± 1.4	62 ± 1.5
0.4	79± 2.2	79 ± 1.7	64 ± 1.5
0.5	79± 2.2	70 ± 1.0	65 ± 2.0
0.6	77± 1.0	71 ± 1.2	69 ± 1.0

Figure 1: Effect of different temperature on melanoidin decolorization

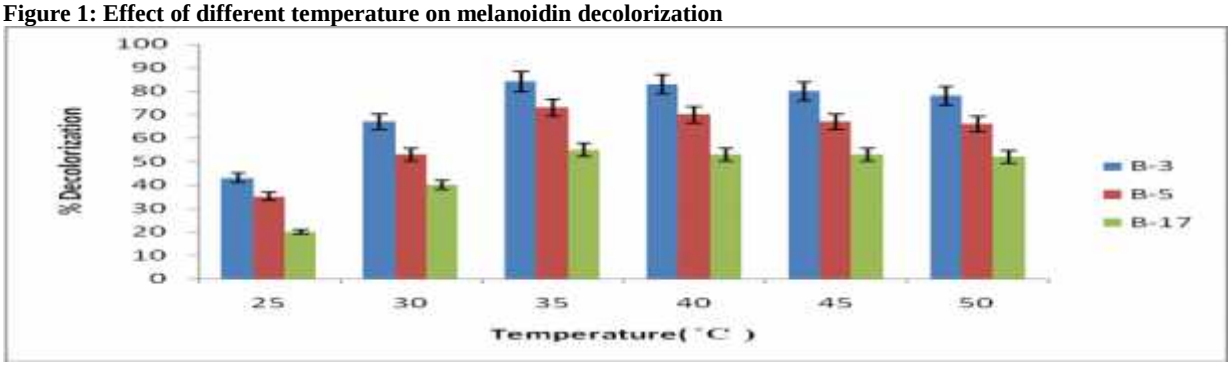
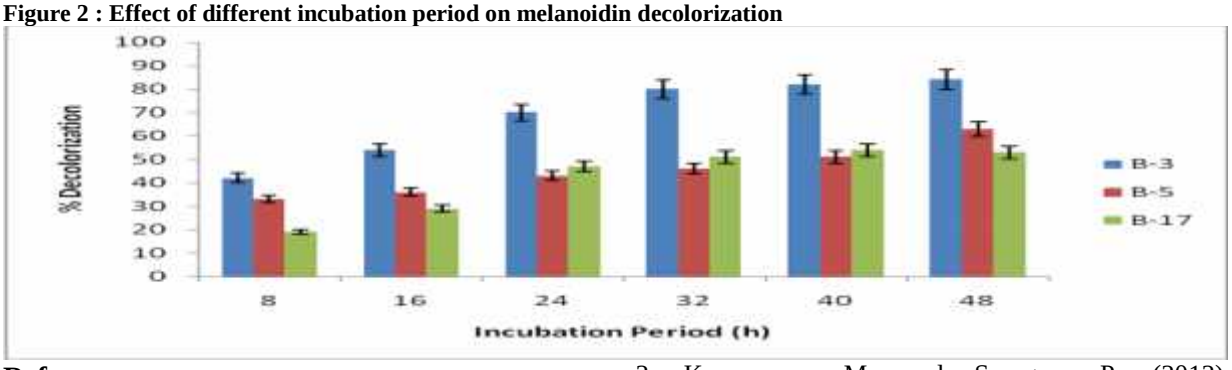


Figure 2 : Effect of different incubation period on melanoidin decolorization



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