International Journal of Education and Science Research Review

Volume-1, Issue-2 April- 2014 ISSN 2348-6457

Decolorization of melanoidin pigment through Bacillus subtilis Pseudomonas aeruginosa, their consortium and optimizing the effect of carbon source

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ABSTRACT
Industrial pollution has been and continues to be a major factor causing the degradation of the environment around us, affecting the water we use, the air we breathe and the soil we live on. In whole world every year the total production of distillery products is less than the production of distillery effluents. Distillery effluent is perilous and hazardous to human race by polluting the water bodies and soil. Distillery spent wash is the unwanted residual liquid waste generated during alcohol production and pollution caused by it is one of the most critical environmental issue. The distillery wastewater with its characteristic unpleasant odor poses a serious threat to the water quality in several regions around the globe. Distillery effluent containing high Biological Oxygen Demand (BOD) and Chemical Oxygen Demand (COD) along with melanoidin, a color compound generally produced by "Millard reaction". A number of clean up technologies have been put into practice and novel bioremediation approaches for treatment of distillery spent wash are being worked out. Potential microbial (anaerobic and aerobic) as well as physicochemical processes as feasible remediation technologies to combat environmental pollution are being explored. In this research work we have used bacterial strains (Bacillus subtilis, Pseudomonas aeruginosa) and their consortia for decolorization of distillery effluent. During the study, role of carbon source i.e. glucose was also studied for decolorization. From the results it was observed that a maximum color reduction was achieved by bacterial consortium i.e 84.45% when supplied by additional carbon source (glucose 1%) on day 3, wheresas 55.45% and 51.18 % color reduction was shown by Bacillus subtilis and Pseudomonas aeruginosa respectively.

Keywords: Bacillus subtilis, Pseudomonas aeruginosa, Bioremediation, Distillery spent wash, Decolorization, Melanodin, Consortia.

Introduction

Distillery effluent from sugarcane molasses leads to an environmental pollution due to its large volume and the presence of dark brown colored compounds, known as melanoidins. The distillery units use sugarcane molasses as a preferred raw material because of its easy and large scale availability. There are about 579 sugar mills and 319 distilleries in India. The wastewater released from distilleries is known as spent wash, which is highly acidic in nature (Agarwal, R; et al ,2010). Alcohol is produced from molasses by two types of fermentation processes, Praj type and Alfa Laval distillation. Currently, about $40.72*10^{10}$ l of spentwash is generated annually from distilleries alone in India (Saha et al., 2005; Pant and Adholeya, 2007; Belkacemi et al., 2000; Dahiya et al., 2001; Sangave and Pandit, 2006). Alcohol distilleries are rated as one of the 17 most polluting industries and generate large volumes of high strength wastewater that is of

serious environmental concern. The spentwash is acidic (pH 3.94 - 4.30) dark brown liquid with high BOD (45000–100000 mg l^{-1}) and COD (90000– 210000 mg l^{-1}), and emits obnoxious odour. Although it does not contain toxic substances, its discharge without any treatment brings about immediate discolouration and depletion of dissolved oxygen in the receiving water streams, posing serious threat to the aquatic flora and fauna (Mane et al., 2006). The production and characteristics of spent wash is highly variable and dependent on feed stocks and various aspects of the ethanol production process (Durate et al., 1997). In soil, it inhibits seed germination and reduces soil alkalinity as well as manganese availability. Low pH values in a water bodies impair recreational uses of water and effect aquatic life. The molasses spent wash (MSW) is a potential water pollutant in two ways. First, the highly coloured nature of MSW can block out sun light from rivers and streams thus reducing oxygenation of the water by photosynthesis and hence becomes detrimental to aquatic life. Secondly, it has a high pollution load which would result in eutrophication of contaminated water sources (FitzGibbon et al., 1998). The first reason is due to the presence of water soluble recalcitrant colouring compound called melanoidin (Evershed et al., 1997). Melanoidin are condensation product of sugar and amino acids produced by nonenzymatic browning reactions called maillard reactions (Plavsic et al., 2006). Melanodin is a dark brown to black coloured natural recalcitrant compound degraded by specific microorganisms having ability to produce mono and dioxygenases peroxidases, phenoxidases and laccases, are mainly responsible for degradation of complex aromatic hydrocarbons like color compound.

The physical and chemical methods of industrial effluent treatment processes remove organic pollutants at low level; they are highly selective to the range of pollutants removed and prohibitively expensive. Flocculation treatment and physicochemical treatment such as ozonation (Kim et al., 1985) and activated carbon adsorption have been accomplished, but these methods are not economically feasible on large scale due to cost limitation. It is also difficult to treat it by conventional biological treatment methods because melanoidins have antioxidant properties that render them toxic to aquaticmacro and microorganisms, whereas biological decolourization by using fungi such as Coriolus, Aspergillus, Phanerochaete and certain bacterial species such as Bacillus, Alkaligenes and Lactobacillus (Kumar and Chandra, 2006; Kumar et al., 1997; Ohmomo et al., 1987, 1985; Aoshima et al., 1985 and Yadav and Chandra, 2013) have been successfully achieved and thus can be applied as a bioremediation technique.

A number of biological processes such as bioadsorption and biodegradation have been reported having prospective application in color removal from spentwash (Ohmomo et al., 1987; Kumar et al., 1997; Kumar and Chandra, 2006; Plavsic et al., 2006; Pant and Adholeya, 2007; Nwuche and Ugoji, 2008; 2010). A wide variety of aerobic microorganisms capable of decolorizing spent wash those having lignolytic activity with enzymatic potential for Bioremediation has been extensively studied. The major enzymes like Lignin Peroxidase (LiP), Manganese Peroxidase (MnP) and Laccase are the key lignin degrading enzymes with great potential in industrial applications.

In this research study, we focused on the decolorisation ability of Bacillus subtilis, Pseudomonas aeruginosa and their consortia that resulted in bioremedation of effluent and optimizing the effect of carbon source.

Material and Method

Collection of sample:

Distillery spent wash was collected from Shamli Sugar Industry and stored at 4^0C. Characterization of the effluent was done for colour.

Characterization of wastes (5%):

Characterization of spent wash has been done after anaerobic and aerobic treatment. During alcohol production some amount of acetic acid and lactic acid are also formed in the fermented broth that's why the pH of spent wash (distilled water OD alcohol broth) is 5.3 (acidic) Anaerobic Process involves hydrolysis and production of fatty acids. In order that methanogensis is promoted, the system is buffered by the release of NH_4^+ ions. The pH thus, gradually reverts to near neutral condition. The process continues during aerobic treatment also. The pH of final effluent reaches a value of 8.3.

For biological treatment with bacterial strain (Bacillus subtilis, Pseudomonas aeruginosa and their consortia) the effluent used in present study is diluted upto 5% and measurement of Colour was done at different time intervals (0, 3, 5, 8 and 11 days). The experimental results of Colour are plotted against time.

Bacterial Treatment:

A lyophilized culture of Bacillus subtilis (ATCC 6933) and Pseudomonas aeruginosa (ATCC 9027) collected from the IMTEC Chandigarh. The organism was revived on the NAM broth and agar plate under aseptic conditions. The plates and the broth were incubated for 4-5 days.

Extra metabolite source:

From the literature enough evidence is available to indicate that the fungus requires additional extra metabolites source, if the material is to be graded as recalcitrant (Ravikumar, et al 2011). In the present study 1% glucose was used as an extra metabolite. It is also based on previous study and their results (Akthar and Mohan 1995, Yadav and Chandra, 2013).

Decolourisation Studies:

Bacillus subtilis and Pseudomonas aeruginosa was inoculated in 5% spent wash in 250 ml flask and incubated at 30^0 C to study the decolourisation ability. Dilution of spent wash was necessary to reduce the level of toxicity in spent wash which otherwise inhibit or reduce the growth of bacteria. In this study we designed four sets of experiments:

E1- 5% spent wash+Bacillus subtilis

E2- 5% spent wash+Bacillus subtilis+ Glucose (1%)

E3- 5% spent wash+ Pseudomonas aeruginosa

E4- 5% spent wash+ Pseudomonas aeruginosa+ Glucose (1%)

E5- 5% spent wash+Bacillus subtilis+ Pseudomonas aeruginosa

E6- 5% spent wash+Bacillus subtilis+ Pseudomonas aeruginosa+ Glucose (1%)

After two days of interval 10 ml aliquot was withdrawn for assaying decolourisation. The decolourisation was calculated by decrease in optical density of supernatant of treated effluent. Colours of the sample were measured spectrophotometrically. Optimum wavelength λ_{max} at 475 nm was calculated.

RESULTS:

Alcohol distilleries are highly water intensive units generating large volumes of high strength wastewater that poses a serious environmental concern. Hence, the present investigation was carried out with the objective of studying the decolourization efficiency of bacterial strains i.e. Bacillus subtilis, Pseudomonas aeruginosa and their consortium with higher efficiencies for the

degradation of individual colorants in distillery spentwash. The results obtained from this investigation are discussed below (Table. 1).

Table 1. Effect of different experiment at 0, 3, 5, 8 and 11th day with optimization treatments

	DAY 0		DAY 3		DAY 5		DAY 8		DAY 11	
Experiment	OD (Average ± Std Dev)	% Reduction	OD (Average ± Std Dev)	% Reduction	OD (Average ± Std Dev)	% Reduction	OD (Average ± Std Dev)	% Reduction	OD (Average ± Std Dev)	% Reduction
E1	2.174 ±0.14	0.00	2.083 ±0.07	3.89	2.417 ±0.06	-11.52	2.5 ±0.00	-15.37	2.5 ±0.00	-15.37
E3	2.174 ±0.14	0.00	1.365 ±0.06	37.03	1.101 ±0.05	49.18	0.966 ±0.05	55.45	1.047 ±0.05	51.72
E5	2.174 ±0.14	0.00	1.31 ±0.05	39.55	2.485 ±0.02	-14.66	2.5 ±0.00	-15.37	2.5 ±0.00	-15.37
E7	2.174 ±0.14	0.00	1.447 ±0.02	33.23	1.354 ±0.03	37.53	1.143 ±0.05	47.27	1.058 ±0.05	51.18
E9	2.174 ±0.14	0.00	1.072 ±0.08	50.53	1.119 ±0.03	48.38	1.461 ±0.03	32.59	1.628 ±0.02	24.86
E11	2.174 ±0.14	0.00	0.337 ±0.02	84.45	0.976 ±0.02	54.95	1.045 ±0.05	51.78	1.333 ±0.45	38.50

Effect of Bacillus subtilis, Pseudomonas aeruginosa and their consortia:

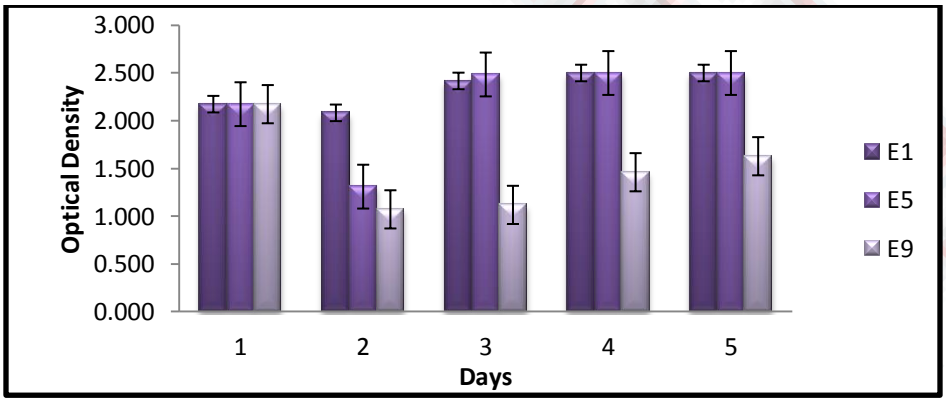

Figure 1 Effect of bacterial strains and their consortia in absence of carbon source.

In present study, the percentage reduction of coloration by two microorganisms alone in respect to consortium shows less result. Here, Bacillus subtilis shows 3% Pseudomonas aeruginosa shows 39.55%, and the Consortium of both microorganism shows 50.53% when no extra carbon source is provided. (Fig.1)

Effect of Bacillus subtilis, Pseudomonas aeruginosa and their consortia in presence of carbon source:
After addition of glucose as carbon source reduction reach upto 84.45% on day 3, by bacterial consortium, whereas Bacillus subtilis shows 55.45%, Pseudomonas aeruginosa shows 51.18% shown in figure 2, similar results were also observed Soni Tiwari et al, 2012.

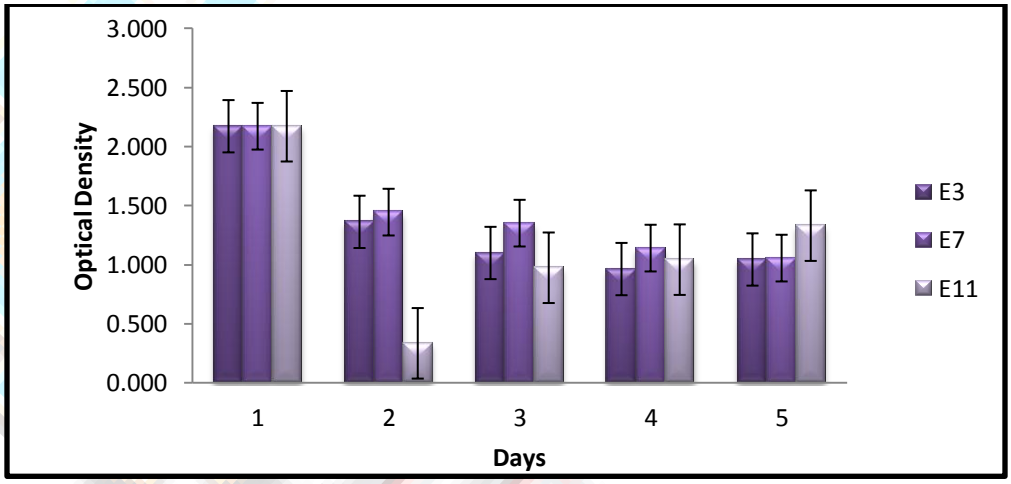

Figure 2 Effect of bacterial strains and their consortia in presence of carbon source.

Discussion:

The bacteria have capability to purify the effluent by consumption of organic substances, thus, reduction in color, COD and BOD. In this investigation, maximum melanoidin decolorization i.e. 84.45% is achived by bacterial consortia shown by in Table 1 was observed when glucose as carbon source at the level of 1% is added during treatment. 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 spentwash that is complex carbon source (Kumar et al., 1997). Ohmomo et al. (1987) reported that glucose was the best carbon source, which utilized by Aspergillus fumigatus G-2-6 for maximum degradation of melanoidins and further increase in glucose concentration, increased the mycelial biomass but no change in decolorization level. Watanabe et al. (1982) have reported that the enzymatic degradation of melanoidin by Coriolus sp. No. 20 having an intracellular enzyme, 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 (Miyata et al., 2000; D'souza et al., 2006). 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 peroxidases (Raghukumar and Rivonkar, 2001; Guimaraes et al., 2005; Pant et al., 2008; Jiranuntipon et al., 2008; Zhao et al., 2010; Ravikumar et al., 2011).

The thermotolerant (Soni et al; 2012) strain of Bacillus subtilis has ability to decolorized melanoidin at wide range of temperature and Pseudomonas aeruginosa also show remarkable color reduction in the presence of little amount of carbon source within a very short incubation period, but consortia of both strains shows better results with and without carbon souce. Therefore, it is beneficial at industrial level and economical level for treatment of distillery effluent. Microbial decolorization of distillery stillage therefore shows great promise as a cost-effective, environmentally safe biotechnology for the treatment of high-strength industrial wastewater.

Acknowledgement

The authors are thankful to the management of ITS Paramedical college for providing them necessary facilities and support to carry out this work.

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IJESRR

Volume-1, Issue-1 February 2014 ISSN 2348-6457