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REVIEW

Microbial decolorization of spentwash: a review

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Abstract Spentwash is one of the most complex and cumbersome wastewater with very high BOD, COD and other organic and inorganic toxic constituents. It is dark brown colored and difficult to treat by normal biological process such as activated sludge or anaerobic lagooning. The color is due to the presence of melanoidins, caramels and other polymers. These compounds have anti oxidant properties which render them toxic to microorganisms. Spentwash disposal into the environment is hazardous and has a considerable pollution potential. It affects the aesthetic merit. Its decolorization by physical or chemical methods have been investigated and were found unsuitable. In the recent past, increasing attention has been directed towards utilizing microbial activity for decolorization of spentwash. This review reveals various groups of microorganisms which have potential in spentwash decolorization. The role of enzymes in decolorization and the microbial degradation of individual compounds imparting color to spentwash are also discussed.

Keywords Decolorization · Spentwash · Melanoidin · Caramel · Marine fungi · Molasses pigment

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Introduction

Alcohol industry is one of the major agro-based industries, which utilize molasses as raw material for the production of rectified spirit. In addition to rectified spirit, distilleries also produce ethanol, which can be mixed with diesel and used as biofuel, which helps in reducing import of crude oil thereby saving foreign exchange. At present, 295 distilleries are operating in the country which have an installed capacity for production of 3,198 million liters of ethanol per annum [1]. Expecting a substantial increase in the requirement of ethanol, a large number of distilleries would be installed shortly and the production capacity is likely to climb up as the demand for ethanol increases.

Distilleries generate spentwash as waste water @ 8–15 L for every liter of alcohol produced [2]. Spentwash has dark brown color and an objectionable odor. Its dark brown color is due to the presence of brown polymers called melanoidins which are formed by the Maillard amino carbonyl reaction [3]. Spentwash is believed to resemble humic acids in its properties [4]. These compounds are highly recalcitrant and have antioxidant properties, which render them toxic to many microorganisms, typically present in wastewater treatment processes [5].

The raw spentwash is acidic in nature [6] and has a high BOD in the range of 40,000 to 60,000 ppm and COD of 1, 00,000 to 1, 20,000 ppm [7, 8] with the total solids exceeding 10 per cent by weight. Hence, it is treated anaerobically in huge methane reactors to recover methane to be used as a fuel in boilers [9]. Organic compounds present in vinasse are of humic in nature [10], similar to those in soil, except that fulvic acid predominated over humic acid. Spentwash is a rich source of plant nutrients. Biomethanated spentwash has the elemental concentration of total N: 21,000 ppm, P:

4,000 ppm, K: 20,000 ppm, Ca: 32,000 ppm, Mg: 18,000 ppm and Na: 14,000 ppm [11].

Treatment methods for wastewater

Waste treatment methods aim at the removal of unwanted compounds in wastewater for safe discharge into environment. This can be achieved by using physical, chemical and biological methods either alone or in combination. Physical treatment methods such as screening, sedimentation and skimming remove floating objects. Chemical treatment methods such as precipitation, pH adjustment, coagulation etc., remove toxic materials and colloidal impurities [12]. Coloring compounds are more difficult to treat because of their synthetic origin and complex aromatic molecular structures. Such structures resist fading on exposure to water, light or oxidizing agents, and this render them more stable and less amenable to biodegradation [13].

Decolorization of distillery spentwash by biological systems

Physical or chemical methods of decolorization are invariably cost intensive and can not be employed in industries. Hence, in recent years, the importance of biological wastewater treatment systems has attracted the attention of workers the world over and has helped in developing efficient low cost waste treatment systems. Increased attention has been directed towards utilization of microbial activity for the mineralization and decolorization of spentwash [14–17]. Diverse microorganisms involved in decolorization of spentwash are furnished in Table 1.

Decolorization by bacteria

Pioneering work on spentwash decolorizarion by bacteria was done by [16]. They observed that two aerobic bacterial isolates LA-1 and D-2 brought about maximum decolorization (36.5% and 32.5%) and COD reduction (41% and 39%) under optimized conditions in eight days. Ramachandra [18] identified four bacterial species, predominantly growing in spentwash, as *Pseudomonas stutzeri, P. acidovorans, Enterbacter* sp. *and Alcaligenes eutrophus.* Decolorization was observed by them up to 60 per cent, with a substantial reduction in BOD and COD as well. In a screening experiment [19] observed *Bacillus* strain MD-32 as the best strain which decolorized 35.5 per cent of molasses pigment within 20 days at 55 [0]C under anaerobic conditions. However, no decolorization activity was observed when it was cultivated aerobically. Three bacterial strains (TA2, TA4 and SA), when mixed, showed up to 60 per cent reduction in color of spentwash at pH 6.5, temperature 35 [0]C and at 1 per cent inoculum level (w/v), when supplemented with molasses as an additional carbon and energy source [20]. Sharma et al [21] isolated several lactic acid bacteria from anaerobically digested molasses spentwash (ADMSW). Some of the isolates decolorized ADMSW (25% v/v) by 70 per cent and reduced COD by 60 per cent. Jagroop Dahiya et al [22] reported that *Pseudomonas fluorescens,* isolated from soil samples contaminated with molasses, decolorized molasses wastewater up to 76 per cent under unsterile conditions in four days at 30 [0]C. Ghosh et al. [23] described the feasibility of using *Pseudomonas putida* for decolorizing spentwash. The organism had the ability to extracellularly convert glucose into gluconic acid and hydrogen peroxide, using glucose oxidase. The hydrogen peroxide produced by this enzymatic oxidation decolorized spentwash by oxidizing melanoidins. Decolorization experiments were also conducted by immobilizing *Pseudomonas putida* in calcium alginate beads which showed more than two folds greater decolorizing ability than free cells. A strain of acetogenic bacterium i.e., NOBP103 showed the highest decolorization yield (79%) when cultivated at 30 [0]C for five days in molasses pigments medium provided with glucose (3%) as the carbon source and yeast extract (0.5%) as the nitrogen source [24]. However, without nutrient supplements, the decolorization capacity of bacteria was drastically reduced.

A bacterial consortium DMC comprising of three bacterial strains was selected on the basis of rapid effluent decolorization and degradation [25]. It exhibited 67 per cent decolorization within 24 h and 51 per cent COD reduction within 72 h when incubated at 37 [0]C under static condition. The organisms were identified as *Pseudomonas aeruginosa PA01, Stenotrophomonas maltophila and Proteus mirabilis* by the 16S rDNA analysis. Chavan et al [26] also screened several bacteria for their ability to decolorize spentwash and selected *Pseudomonas* sp. for degradation studies. They found that spentwash dilution up to 10 per cent and addition of externally added carbon source was essential for decolorization. The maximum decolorization up to 56 and 32 per cent reduction in COD of spentwash could be achieved within 72 h.

From *Phragmites australis* growing in spentwash contaminated site, rhizosphere bacteria were isolated and characterized [27]. As many as 15 isolates were identified based on the 16 S rRNA sequencing. All the 15 isolates grew on effluent and reduced color by 75.50 per cent. Concomitantly, there was a reduction in BOD, COD, phenol, sulfate and heavy metals as well.

Table 1 Microorganisms capable of decolorizing distillery spent wash

Microorganism		Additional carbon source	Extent of decolorization(%)	Reference
A.	Bacteria			
1.	*Pseudomonas sturzeri*	Glucose	Up to 60.00	Ramachandra, 1993
2.	*P. acidovorans*	Glucose		
3.	*Enterobacter sp.*	Glucose		
4.	*Alcaligenes eutrophus*	Glucose		
5.	*Bacillus sp.*	Glucose	35.5	Nakazima Kambe *et al.,* 1999
6.	*Pseudomonas sp.*	Molasses	60.00	Asthana and Ramachandra, 1999
7.	Lactic acid bacteria	Molasses	70.00	Sharma *et al.,* 2000
8.	*P. fluorescens*	Glucose	76.00	Jagroop Dahiya *et al.,* 2001
9.	*Acetogenic bacterium N0BP103*	Glucose	70.00	Sirianuntapiboon *et al.,* 2004
10.	*P. aeruginosa*	Glucose	67.00	Sarayu *et al.,* 2005
11.	*Stenotrophomonas*			
12.	*maltopilaProteus mirabilis*			
13.	*Pseudomonas sp.*	Glucose	56.00	Chavan *et al.,* 2006
B.	Fungi			
1.	*Aspergillus G-2-6*	Glycerol	75.00	Ohmomo *et al.,* 1985
2.	Mycelia sterilia	Glucose	93.00	Sirianuntapiboon *et al.,* 1988
3.	*Coriolus versicolor*	Glucose	71.0053.50	Kumar *et al.,* 1998
4.	*Phanerochaete chrysosporium*			
5.	*Aspergillus niger*	Glucose	80.00	Dhamankar and Patil, 2001
6.	*Flavodon flavus*	Sucrose	80.00	Raghukumar and Rivonkar, 2001
7.	*Aspergillus niveus*	Sugarcane bagasse	37.00	Angayarkanni *et al.,* 2003
8.	*Coriolus versicolor*	Cotton stalk	62.00	Kahraman and Yesilada, 2003
9.	*Funalia trogii*			
10.	*Pleurotus pulmonarius*			
C.	Yeasts			
1.	*Citeromyces sp.*	Glucose	75.00	Sirianuntapiboon *et al.,* 2004
D.	Cyanobacteria			
1.	*Oscillotoria boryana*	Glucose	60.00	Kalavathi *et al.,* 2001

Decolorization by fungi

As many as 228 filamentous fungi, isolated from Thailand soils were tested for their ability to decolorize molasses pigments [28]. Out of these, nine strains belonging to the class Deuteromycetes showed decolorization yields of more than 50 per cent and differed in their requirements of pH, carbon and nitrogen sources for maximum decolorization. Mycelia sterilia D90 was found to be the most potent strain with a decolorization yield of about 93 per cent at the end of eight days. Its optimum requirements were glucose (2.5%) as C source and yeast extract as N source, with the ideal pH being 6.0. Subsequently, Sirianuntapiboon et al. [28] reported that this strain also caused about 80 per cent decrease in BOD when glucose (2.5%), $NaNO_3$ (0.2%), KH_2PO_4 (0.1%) and $MgSO_4.7H_2O$ (0.05%) were added as nutrients to molasses wastewater. However, in the absence of these nutrients, decolorization yield was only 17.5 per cent.

Aspergilli have been implicated in decolorization. Miranda et al. [29] observed 25 per cent color removal by *Aspergillus niger* without phosphate addition. But, this was increased to 68 per cent when potassium phosphate (1.0 g/l) was added. Batch processes showed a maximal color elimination of 69 per cent when $MgSO_4$, KH_2PO_4, NH_4NO_3 and a carbon source were added to wastewater. Dhamankar and Patil [30] examined *Aspergillus niger* VM2 for its ability to decolorize spentwash. The optimum fungal growth and decolorization occurred at pH 4.5 and 30 [0]C in presence of carbon and nitrogen sources. Maximum color removal was up to 80 per cent with 86.4 per cent reduction in COD. *Aspergillus niveus*, a litter degrading fungus was used by Angayarkanni et al [31] for the treatment of distillery

effluent using paddy straw, sugarcane bagasse, molasses and sucrose as carbon source for growth of the fungus in the effluent. In presence of sugarcane bagasse at 1% (w/v) concentration, the fungus resulted in the maximum removal of color (37%) and COD (91.68%). Shayegan et al. [32] used a strain of *Aspergillus* for decolorization of distillery spentwash in a sequential Plackett–Burmann design in three stages. Maximum decolorization and COD reduction (84% each) were obtained under optimized conditions. By implementing the optimum values in a pilot scale of an activated sludge system, a continuous process for decolorization was conducted.

Several scientists evaluated white rot fungi for decolorization of spentwash. Four white rot fungi were screened for their ability to decolorize and bioremediate anaerobically digested molasses spentwash (DMSW) [33]. *Coriolus versicolor* and *Phanerochaete chrysosporium* showed the ability to decolorize and reduce COD of diluted DMSW (12.5% v/v). Both cultures required an additional carbon source to carry out decolorization. Maximum decolorization (71.5% and 53.5%) and COD reduction (90% and 70%) were achieved in 6.25 per cent (v/v) DMSW medium by *C. versicolor and P. chrysosporium,* respectively.

The sensation in spentwash decolorization in India was created by [34]. They isolated a basidiomycete fungus *Flavodon flavus* from decomposing sea grass leaves from a coral lagoon in one of the Lakshadweep Islands in the Arabian Sea. This strain decolorized spentwash by 80 per cent in eight days of incubation, even when used at a concentration of 50 per cent. Decolorization was the highest when glucose or sucrose was used as the carbon source in the low nitrogen medium. In subsequent studies, Raghukumar et al. [35] immobilized this fungus to improve decolorization yields. Polyurethane foam–immobilized fungus decolorized 10 per cent diluted MSW by 73 per cent in seven days. The immobilized fungus could be effectively used for a minimum of three cycles repeatedly to decolorize MSW. Besides decolorization, the fungus also removed the toxicity of spentwah. Toxicity bioassay of the fungus-treated spent wash using an estuarine fish *Oreochromis mossambicus* showed no liver damage in contrast to untreated effluent, which showed moderate liver damage. Benzo(a)pyrene, a polycyclic aromatic hydrocarbon (PAH) present in spentwash, which is one of the causes of toxicity of spentwash, was also decreased by 68 per cent in five days upon treatment with the fungus. The isolate of *Flavodon flavus has been* deposited in the ARS Patent Culture Collection, USDA, Illinois, on June 19, 2000 under the accession number NRRL 30302.

Naik [36] isolated many marine fungi from decaying plant samples collected from mangroves and selected efficient spentwash decolorizing isolates. Out of these, *Pleurotus* sp. KU3 was promising with 79.7 per cent decolorization of 30 per cent spentwash under optimized conditions. Miyata et al. [37] observed that the addition of Mn (II) to the pretreated heat treatment liquor (HTL) caused a further increase in the decolorization efficiency of *Coriolus hirsutus* and a marked increase in the manganese peroxidase (MnP) activity. Consequently, the increase in manganese independent peroxidase (MIP) and manganese peroxidase (MnP) activities were considered to play an important role in the enhanced ability of *C. hirsutus* to decolorize HTL. Molasses wastewater was decolorized and its COD reduced in static cultivation using *Coriolus versicolour, Funalia trogii, Phanerochaete chrysosporium* and *Pleurotus pulmonarius* [38]. The addition of cotton stalks augmented decolorizing and COD removing capability of these fungi.

Decolorization by other microorganisms

Very few studies on decolorization by yeasts have been reported. And some of them have mentioned biosorption as the major mechanism of decolorization [39,40]. Sirianuntapiboon et al. [41] screened 205 yeast strains isolated from Thai fruit samples. *Citeromyces* sp WR-43-6 was found promising with color, chemical and biological oxygen demand removal efficiencies of 75 per cent, almost 100 and 76 per cent, respectively. In a periodical feeding system, it showed an almost constant decolorization yield of 60 to 70 per cent over eight days feeding of 10 per cent fresh medium. In a replacement culture system also, it resulted in a constant decolorization yield (about 75%) during four times replacement.

Kalavathi et al. [42] worked on the metabolism of melanoidin pigment by a marine filamentous, non-heterocystous cyanobacterium *Oscillotoria boryana* BDU 92181. This cyanobacterium used melanoidin as the nitrogen and carbon source leading to decolorization. The organism decolorized pure melanoidin pigment (0.1% w/v) by about 75 per cent and crude pigment in the distillery effluent (5% v/v) by about 60 per cent in three days. The mechanism of color removal is postulated to be due to the production of hydrogen peroxide, hydroxyl ions and molecular oxygen, released by the cyanobacterium during photosynthesis.

Role of enzymes in effluent decolorization

A large number of enzymes from a variety of different plants and microorganisms have been reported to play an important role in an array of waste treatment applications. Several studies regarding degradation of spentwash using basidiomycetes have also suggested participation of

at least one laccase enzyme in fungi belonging to *Trametes* genus.

The role of enzymes other than laccase or peroxidases in the decolorization of melanoidins by *Trametes* strain was reported during the 1980's. Several reports claimed that the intracellular sugar-oxidase type enzymes (sorbose-oxidase or glucose oxidase) had melanoidin decolorizing activities. It was suggested that melanoidins were decolourized by the active oxygen (O_2:H_2O_2) produced by the reaction with sugar oxidases [43]. Ohmomo et al. [44] used *Coriolus vessicolor* Ps4a, which decolorized molasses wastewater up to 80 per cent in darkness under optimum conditions. Decolorization activity involved two types of intracellular enzymes sugar dependent and sugar independent enzymes. Kelley and Reddy [45] identified the glucose oxidase activity (GOX) as the primary source of hydrogen peroxidase production in liginolytic cultures of *Phanerochaete chrysosporium*. Lee et al. [46] investigated on the dye decolorizing peroxidase by cultivating *Geotrichum condidum* Dec 1 using molasses as the carbon source. Components in the molasses medium stimulated production of the decolorizing peroxidase. The involvement of glucose oxidase (GOX) activity in decolorization by the promising organisms *Aspergillus sp., Plerotus sp.,* and *Pseudomonas fluorescens* was tested [38]. All the strains produced gluconic acid, as evidenced through TLC.

Recently, D'souza et al. [46] reported 100 per cent decolorization of 10 per cent spentwash by a marine fungal isolate whose laccase production increased several folds in the presence of phenolic and non-phenolic inducers. Enzymatic decolorization of molasses medium has also been tried using *Phanearochaete chrysosporium* [47]. Under stationary cultivation conditions, none of the strains decolorized molasses nor produced enzymes like lignin peroxidase, manganese peroxidase and laccase. All of them could produce lignin peroxidase and manganese peroxidase when cultivated in flat bottom glass bottles under stationary cultivation conditions.

Colorants in distillery effluent

Phenolics (tannic and humic acids from the feedstock), melanoidins from Mailard reaction of sugars (carbohydrates) with proteins (amino groups), caramels from overheated sugars and furfurals from acid hydrolysis mainly contribute to the dark brown color of spentwash [48].

During heat treatment, the Maillard reaction (non-enzymatic reaction) takes place accompanied by formation of a class of compounds known as Maillard products. The reaction proceeds effectively at >50 [0]C and is favored at pH 4 to 7 [49]. Melanoidins are one of the final products of the Maillard reaction. They are complex compounds with their structures not fully understood. Hayase *et al*. [50] confirmed the presence of olefinic linkages and conjugated enamines of the chromophores in melanoidin. For the melanoidins formed from carbohydrates and amino acids, a new model of a basic melanoidin skeleton mainly built up from amino branched sugar degradation products was suggested by Cammerrer et al [51]. Recently, the empirical formula of melanoidin has been suggested as $C_{17–18}H_{26–27}O_{10}N$. The molecular weight distribution is between 5000 and 40,000. It consists of acidic, polymeric and highly dispersed colloids, which are negatively charged due to the dissociation of carboxylic acids and phenolic groups [52]. The basic structure of melanoidin is given in Fig. 1.

Caramel is formed by caramelization process. Caramelization occurs when sugars are heated in the absence of nitrogen containing compounds. During a caramelization reaction, the sugar initially undergoes dehydration and then condensation or polymerization into complex molecules. Lighly colored, pleasant tasting caramel flavors are produced during initial stages, but as the reaction continues, more high molecular weight bodies are produced which are more bitter [53].

Patil and Dhamankar [54] quantified colorants in molasses and spentwash samples. Molasses samples from 14 sugar factories and spentwash samples from 12 distilleries were analyzed for melanoidin, alkaline degradation product and caramel contents. The average values were 2.6, 10.4 and 13.9 percent in molasses and 1.3, 4.15 and 5.70 percent in spentwash respectively.

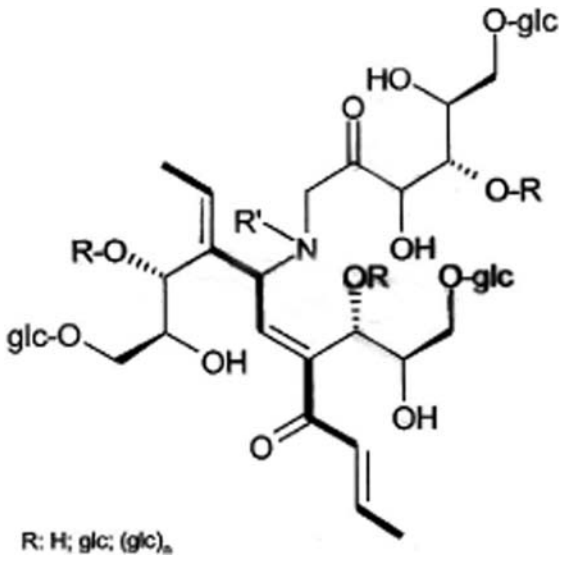

Fig. 1 Basic structure of melanoidin

Biodegradation of individual colorants in distillery spentwash

Degradation of melanoidins by fungi was initiated as early as in 1987 by Ivarson and Benzing-Pardie [4], who observed slow decomposition of synthetic melanoidins (both unlabelled and U-14 labelled) when inoculated with a soil suspension and incubated in Warburg vessels for 30 days. Then, Ohmomo *et al*. [55] isolated and screened several fungi for their ability to decolorize melanoidin in the tropical zone of Japan. Out of these, *Aspergillus* G-2-6 was the most active strain with 75 per cent decolorization, when cultivated on a glycerol-peptone medium at 45 [0]C for three days with shaking. In successive decolorization studies also, the strain exhibited more than 60 per cent of the melanoidin decolorizing activity, thus showing stability of the strain [56]. In the studies on continuous decolorization of melonoidin solution in a jar fermentor, the strain resulted in an almost constant decolorization yield of about 70 per cent.

Singh and Nigam [57] worked on growth of a strictly anaerobic bacterium on furfural, a coloring component present in distillery spentwash. The bacterium was isolated from a continuous fermentor culture which converted the organic constituents of sulfite evaporator condensate to methane and CO_2. This isolate could degrade furfural as the sole source of carbon and energy. The furfural isolate was tentatively identified as *Desulfovibrio* sp. strain F-1.

Dhamankar and Patil [30] observed that *Aspergillus niger* VM2 possessed the ability to degrade individual coloring compounds like melanoidin, caramel and alkaline degradation products in the range 21 to 22 per cent each. Gel permeation chromatographic studies of the biologically treated effluent on sephadex G-25 showed slight reduction in molecular weights of different species of melanoidin, caramel and alkaline degradation products with accumulation of a new lower molecular weight species.

One of the coloring compounds from the distillery effluent was purified by thin layer chromatography and adsorption chromatography, and its structure was elucidated by the application of the spectroscopic techniques viz., UV, NMR, IR and Mass spectroscopic analysis [23].

Several bacterial isolates were evaluated for the ability to degrade melanoidin and a strain of *Pseudomonas* sp. was selected [26]. Spectrophotometric and HPLC analysis of the treated effluent confirmed biodegradation of melanoidin pigments by the isolate. The decolorization of synthetic melanoidins by three *Bacillus* isolates (*Bacillus thuringiensis* (MTCC 4714), *Bacillus brevis* (MTCC 4716) and *Bacillus* sp. (MTCC 6506) was studied [58]. Significant reduction in the values of physicochemical parameters was noticed along with the decolorization of melanoidins. The mixed culture decolorized melanoidins much more effectively. The addition of 1 per cent glucose as a supplementary carbon source was essential for co-metabolism of melanoidin complex.

Naik [36] isolated marine fungi and selected efficient spentwash decolorizing isolates. Out of 97 promising isolates, *Aspergillus* sp. K1 was the best melanoidin (77%) as well as caramel (54%) degrader. Degradation of melanoidin and caramel was confirmed by UV and IR spectral analysis.

Conclusion

It can, thus, be concluded that microbial decolorization of spentwash holds promise and can be exploited to develop a cost effective, eco-friendly biotechnology package for the treatment of spentwash. Genetic improvement of strains can be explored in future for improving their decolorization efficiency. However, the need to supplement an additional carbon source and dilute biomethanated spentwash are two major features which have to be addressed in future research.

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