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Decolourization of Distillery Waste Water – Role of Microbes and their Potential Oxidative Enzymes (Review)

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ABSTRACT: Detoxification and decolourization of industrial wastewater and distillery effluent is acquiring its importance in terms of environmental and aesthetic point of view. The characteristic of dark brown appearance of distillery wastewater is mainly due to the high molecular weight organic compounds called melanoidin. Studies dealing with pure culture of bacteria, fungi, and yeast and their oxidative enzymes (peroxidase, laccase) in decolourization of industrial wastewater to develop a better understanding of the phenomenon of microbial decolourization. This review paper presents an overview of the characteristics of the distillery wastewater in terms of its toxicity and as a pollutant and biological treatment using microbial enzymes.

Keywords: Distillery effluent; Decolourization; Melanoidin; Biosorption; Bioaccumulation; Oxidative enzymes.

INTRODUCTION

The limited availability of Non-renewable energy resources and variability in crude oil and natural gas prices worldwide has increased the demand in the production of ethanol from agricultural materials for use as an alternative fuel, industrial solvent and in beverages. In India, this demand is projected to increase because of newly framed legislation for mixing 5% ethanol with petrol and predicted to increase 10% in near future [1]. In India, there are a number of large-scale distilleries integrated with sugar mills. The waste products from sugar mill comprise of baggasse, pressmud and molasses. In molasses-based distilleries, 13 to 15 L of wastewater is generated for per liter of ethanol produced [2]. In 1999, there were 285 distilleries in India producing 2.7 x 10^9 L of alcohol and generating 4 x 10^{10} L of wastewater each year [3] but today a total of 319 distilleries, producing 3.25 x 10^9 L of alcohol and generating 40.4 x 10^{10} L of wastewater annually [4]. Wine distilleries produce large volumes of liquid waste called wine distillery wastewater, which is the aqueous by-product of the distillation of ethanol, wine and some waste biological material [5,6,7]. A high volume of wastewater is produced in these industries; figures range from 2 L per liter of wine produced [8,9] to 20 L per liter of ethanol produced [6].

2. Characteristics of Distillery wastewater

Generally the effluents from molasses based distilleries contain large amounts of dark brown coloured molasses spent wash (MSW). Because of its acidic pH, dark brown colour, High ash content, high percentage of dissolved organic and inorganic matter, MSW is one of the most difficult waste products to dispose [10] Table 1. The biochemical Oxygen demand (BOD) and chemical oxygen demand (COD) of MSW range Between 35,000–50,000 and 100,000–150,000 mg L^{-1}, respectively [17]. Various studies conducted in South Africa, the COD of wine distillery wastewater ranged from 20 g/L to 30 g/L (18), while [19] most of the studies on distillery wastewater reported concentration of COD range between 22 and 48 g/L. Distillery wastewaters contain phenolic compounds, mainly gallic acid, *p*-coumaric acid and gentisic acid, which impart high Antibacterial activity [7, 20, 21]. Organic acids such as lactic acid (29% v/v), tartaric acid (27% v/v), succinic acid (26% v/v), acetic acid (10% v/v) and malic acid (8% v/v) also documented in distillery waste water [22]. Apart from these distillery waste water also contains soluble proteins[7,21].

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Table 1. Chemical characteristics of distillery wastewaters

Parameter	Type of wastewater					
	Distillery wastewater [11]	WDW [12]	Vinasse [13]	Raw spent wash [14]	Molasses wastewater [15]	Lees stillage [16]
pH	3.0- 4.1	3.53- 5.4	4.4-4.2	5.2	3.8	
Alkalinity (meq/l)	-	30.8- 62.4	-	2	6000	9.86
EC	346	-	-	2530	-	-
Phenol (mg/l)	-	29 - 474	477	-	450	-
VFA (g/l)	1.6	1.01 - 6	-	-	8.5	0.248
COD_T (g/l)	100 - 120	3.1 - 40	-	37.5	80.5	-
COD_S (mg/l)	-	7.6 - 16	97.5	-	-	-
BOD_5 (mg/l)	30	0.21- 8.0	42.23	-	-	20
TOC (mg/l)	-	2.5- 6.0	36.28	-	-	-
VS (g/l)	50	7.340- 25.4	-	-	79	-
VSS (g/l)	2.8	1.2-2.8	-	-	2.5	0.086
TS (g/l)	51.5 - 100	11.4 - 32	3.9	2.82	109	68
TSS (g/l)	-	2.4- 5.0	-	-	-	-
MS (g/l)	-	6.6	-	-	30	-
MSS (mg/l)	-	900	100	-	1100	-
TN (g/l)	-	0.1 - 64	-	2.02	1.8	1.53
NH_{4+} (mg/l)	-	140	-	125 400	-	45.1
NO_{3-} (mg/l)	4900	-	-	-	-	-
TP (g/l)	-	0.24- 65.7	-	0.24	-	4.28
PO_{43-} (mg/l)	-	130 - 350	-	139	-	-

Table 2. Bacteria employed for the decolourization of distillery effluent

S.no.	Name	Comments	Colour Removal (%)	Reference
1	*Acetobacter acetii*	The organism required sugar especially, glucose and fructose for decolourization of MWWs	76.4	[50]
2	*Bacillus smithii*	Decolourization occurred at 55 °C in 20 days under anaerobic conditions in presence of peptone or yeast extract as supplemental nutrient. Strain could not use MWW as sole carbon source	35.5	[51]
3	*Bacillus thuringiensis*	Addition of 1% glucose as a supplementary carbon source was necessary	22	[52]
4	*Lactobacillus hilgardii*	Immobilized cells of the heterofermentative lactic acid bacterium decolourized 40% of the melanoidins solution within 4 days aerobically	40	[53]
5	*Pseudomonas aeruginosa*	The three strains were part of a consortium which decolourized the anaerobically digested spent wash in presence of basal salts and glucose	67	[54]
6	*Pseudomonas fluorescens*	This decolourization was obtained with cellulose carrier coated with collagen. Reuse of decolourized cells reduced the decolourization efficiency	94	[55]
7	*Pseudomonas putida*	The organism needed glucose as a carbon source, to produce hydrogen peroxide which reduced the colour	60	[56]
8	*Xanthomonas fragariae*	All the three strains needed glucose as carbon source and NH4Cl as nitrogen source. The decolourization efficiency of free cells was better than immobilized cells	76	[57]

Table 3. Fungi employed for the decolourization of Distillery effluent

S.no.	Name	Comments	Colour Removal (%)	Reference
1	*Aspergillus fumigatus* G-2-6	Thermophilic strain tried for molasses wastewater decolourization but colouring compounds hardly degraded	56	[66]
2	Aspergillus niger	Maximum colour removal was obtained when MgSO4, KH2PO4, NH4NO3 and a carbon source was added to wastewater	63	[67]
3	*Aspergillus niger* UM2	Decolourization was more by immobilized fungus and it was able to decolourize up to 50% of initial effluent concentrations	80	[68]
4	*Aspergillus niveus*	The fungus could use sugarcane bagasse as carbon source and required other nutrients for decolourization	56	[69]
5	Aspergillus oryzae Y-2-32	The thermophilic strain adsorbed lower molecular weight fractions of melanoidin and required sugars for growth	75	[60]
6	*Aspergillus*-UB2	This was with diluted wastewater with optimum values of supplemented materials	75	[70]
7	*Citeromyces* sp. WR-43-	Organism required glucose, Sodium nitrate and KH2PO4 for maximal decolourization	68.91	[71]
8	*Coriolus* sp. no. 20	First strain for the application of its ability to remove melanoidins from MWW, showed decolourization activity in 0.5% melanoidin when sorbose or glucose was added as carbon source	80	[72]
9	*Coriolus hirsutus*	A large amount of glucose was required for colour removal but addition of peptone reduced the decolourizing ability of the fungus	80	[73]
10	*Coriolus hirsutus* IFO4917	Melanoidins present in heat treatment liquor were subjected to sequencing batch decolourization by the immobilized fungal cells	45	[74]
11	*Coriolus versicolor*	Cotton stalks were added as additional carbon source which stimulated the decolourization activity of all fungi in 30% vinasses	63	[75]
12	*Coriolus versicolor* sp no. 20	10% diluted spent wash was used with glucose @ 2% added as carbon source	34.5	[76]
13	*Coriolus versicolor* Ps4a	Two types of enzymes, sugar-dependent and sugar-independent, were found to be responsible for melanoidin decolourizing activity	80	[77]
14	*Flavodon flavus*	MSW was decolourized using a marine basidiomycete fungus. It also removed 68% benzo(a)pyrene, a PAH found in MSW	80	[78,79]
15	*Geotrichum candidum*	Fungus immobilized on polyurethane foam showed stable decolourization of molasses in repeated-batch cultivation	70	[80]
16	Marine Basidiomycete NIOCC # 2a	Experiment was carried out at 10% diluted spent wash	100	[81]
17	*Mycelia sterilia*	Organism required glucose for the decolourizing activity	93	[82]
18	*Penicillium* sp	All fungi produced decolourization from first day of incubation, with maximum being shown byP. decumbens at fourth day with a reduction of 70% of the phenolic content of the wastewater	30	[83]
19	*Phanerochaete chrysosporium*	Sugar refinery effluent was treated in a RBC using polyurethane foam and scouring web as suppor	55	[84]
20	*Phanerochaete chrysosporium* JAG-40	This organism decolourized synthetic and natural melanoidins when the medium was supplemented with glucose and peptone	80	[85]
21	6Phanerochaete chrysosporium NCIM 1073 NCIM 1106 NCIM 1197	Molasses medium decolourization was checked in stationary and submerged cultivation conditions	0 82 76	[86]
22	*Pycnoporus coccineus*	Immobilized mycelia removed 50% more colour than free mycelia	60	[87]
23	*Rhizoctonia* sp. D- 90	Mechanism of decolourization of melanoidin involved absorption of the melanoidin pigment by the cells as a macromolecule and its intracellular accumulation in the cytoplasm and around the cell membrane as a melanoidin complex, which was then gradually decolourized by intracellular enzymes	90	[88]
24	*Trametes* sp. I-62	No colour observed associated with either fungal mycelium or polysaccharides secreted by the fungus and therefore colour removal was attributed to fungal degradation and not to a simple physical binding	73	[89]

Table 4. Microbes and their specific enzymes in wastewater dye decolourization

Organisms	Dye and concentration	Percent removal/ Time	Mechanism	Reference
Acetobacter acetii	Paper mill bleach effluent	76.4	Sugar oxidase	[50]
Coriolussp.No. 20	Melanoidin (0.5%)	80(14d)	Activeoxygen	[72]
Halosarpheia ratnagiriensis	Paper mill bleach effluent	85 (14 d)	Lignin enzymes	[103]
Lactobacillus hilgardii	Pulp bleach effluent(40v/v)	40	Sugar oxidase	[53]
Merulius tremellosus	Pulp bleach effluent(40v/v)	50(14d)	Peroxidase	[105]
P.chrysosporium	Olive mill wastewater	70(10)	Ligninperoxidase	[106]
P.chrysosporium	Kraft bleach plant E1 Stage effluent	70(5.8d)	Ligninperoxidase	[107]
P.chrysosporium	Pulp bleach effluent(40v/v)	7614d)	Peroxidase	[105]
P.chrysosporium	Alkali extraction stage bleach effluent	90(3 d)	Lignin peroxidase	(108)
Phlebia radiata	Pulp bleach effluent 40v/v)	76(14d)	Peroxidase	[105]
Pseudomonas putida	Paper mill bleach effluent	60	Sugar oxidase	[56]
Pycnoporus cinnabarinus	Pigment plant effluent	90(3d)	Extracellularoxidases	[109]
Schizophyllum commune	Bagasse-based pulp mill effluent	80±90 (2 d)	Lignin enzymes	[110]
Sordaria fumicola	Paper mill bleach effluent	55 (14 d)	Lignin enzymes	[103]
Trichoderma sp.	Hardwood extraction effluent	85(3d)	Ligninolytic enzymes	[111]
Wood rotting fungus (unidentified)	Cotton bleaching effluent (20±50%)	81.5±43.8 (5 d)	Manganese peroxidase	[112]

Table 5.Native and synthetic mediators in LME systems

Mediator	Organism (enzyme)	Reference
	Native mediators	
Mn3 +	Phanerochaete chrysosporium (MnP)	[122]
Organic acids (malonate, oxalate, etc.)	Armillaria mellea, Fomes annosus, Pleurotus ostreatus, Phanerochaete chrysosporium, Phlebia radiata, Cenporiopsis subvermispora, Nematoloma frowardii (LiP, MnP)	[123] [145]
Veratryl alcohol	Phanerochaete chrysosporium (LiP)	[146]
3-Hydroxyanthranilic acid (3-HAA)	Pycnoporus cinnabarinus (Lac)	[147,148]
2-Chloro-1,4 dimethoxybenzene (2Cl-14DMB)	Trametes versicolor (LiP)	[149]
Synthetic mediators		
1-Hydroxybenzotriazole (1-HBT)	Trametes versicolor, Trametes villosa, Pycnoporus cinnabarinus, Botrytis cinerea, Myceliophthora thermophila, Coriolopsis gallica, Pleurotus ostreatus various organisms (Lac)	[150.151,152,153]
Violuric acid	Trametes villosa, Pycnoporus cinnabarinus, Botrytis cinerea, Myceliophthora thermophila (Lac)	[152]
2,2V-Azinobis (3-ethylbenzthiazoline-6-sulfonate) (ABTS)	Trametes versicolor, Coriolopsis gallica, Pleurotus ostreatus, various organisms (Lac)	[150,151.153]

3. Pollution and toxicity profile of distillery effluent

The MSW is a potential water pollutant in two ways. Firstly, MSW block out sunlight from entering the bottom layers of rivers and streams, thus reducing oxygenation of the water by blocking photosynthesis and becomes detrimental to aquatic life. Secondly, it has a high pollution load which leads to the eutrophication of water courses [23]. Due to the presence of putriciable organics like skatole, indole and other sulphur compounds, the MSW that is disposed in canals or rivers produces obnoxious smell [24]. Undiluted effluent has toxic effect on fishes and other aquatic organisms. The estimated LC50 for distillery spent wash was found to be 0.5% using a bio-toxicity study on

fresh water fish *Cyprinus carpio* var. *communis* [24]. The respiratory process in *C. carpio* under distillery effluent stress was affected resulting in a shift towards anaerobiosis at organ level during sublethal intoxication. Soil pollution and acidification are the other pollution issues caused by spent wash and it is reported to inhibit seed germination, reduce soil alkalinity, cause soil manganese deficiency and damage agricultural crops [25,26].

Wine distillery wastewaters are acidic and their high organic content can cause considerable environmental pollution [20,7]. Problems that are encountered during the biological treatment of wine distillery wastewater is because of high toxicity and the inhibition of biodegradation due to the presence of polyphenolic compounds [27], these waters also demonstrates the antibacterial activity reported in the earlier literature [20]. Polyphenol concentrations in some distillery wastewaters vary considerably and can range from 29 to 474 PPM [12] which is responsible for strong inhibitory effects on microbial activity and must be removed during wastewater treatment, owing to the environmental and public health risks they pose. Humans exposed to phenol at 1300 PPM of concentration exhibit significant increases in diarrhoea, dark urine, mouth sores and burning of the mouth [28]. Wine distillery wastewaters have also been Characterized for heavy metals, viz. iron and zinc, metal ions such as Ca^{2+}, K^{+} and Na^{+}[29,30,11] and sulphates [29,30]. High concentrations of these constituents [9, 30], plus other nutrients such as nitrate and phosphate, make the possible discharge of wine distillery wastewaters into water bodies problematic, as they cause eutrophication and other adverse environmental effects[20, 28,31].

4. Colorants in distillery wastewaters

During anaerobic treatments and due to repolymerization, the brown pigment in the molasses wastewater is hardly degraded and also increased during the conventional treatments. The brown colour of the effluent is due to the presence of phenolics (tannic and humic acids) from the feedstock, melanoidins from Maillard reaction of sugars (carbohydrates) with proteins (amino groups), caramels from overheated sugars, and furfurals from acid hydrolysis [32]. At above 50^{o}C the Maillard reaction (non enzymatic reaction) takes place effectively and favored at acidic pH (4–7) [33]. Complex compounds namely, melanoidins are one of the final products of the Maillard reaction.

Melanoidin is one of the biopolymers that is hardly decomposed by microorganisms and is widely distributed in nature. Melanoidins have antioxidant properties, which render them toxic to aquatic micro and macroorganisms [34]. Melanoidins, or related formation products can occur in different processes of beverage manufacture, such as heat concentrated juices and musts, beers or wines [35]. From studies using ^{13}C and ^{15}N CP-NMR spectrometry, [36] confirmed the presence of olefinic linkages and conjugated enamines which were suggested to be important for the structure of the chromophores in melanoidin. For 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 [37]. A blue pigment (Blue-M1, $C_{27}H_{31}N_4O_{13}$) was isolated from the reaction mixture of D-xylose and glycine in 60% ethanol stored at 26.5^{o}C for 48 h (or 2 ^{o}C for 96 h) under nitrogen, whose chemical character was comparable to that of a nondialyzable melanoidin preparation obtained from the reaction mixture of D-xylose and butylamine neutralized with acetic acid in methanol incubated at 50 ^{o}C for 7 days. Recently, the empirical formula of melanoidin has been suggested as $C_{17\text{-}18}H_{26\text{-}27}O_{10}N$. 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 [38].

5. Mechanism involved in decolourization of distillery effluents

Generally a dye can be described as a colored substance with an affinity to the substrate to which it is applied. Dyes are believed to be toxic and carcinogenic or prepared from other known carcinogens [39]. The discharge of these dye stuffs from industries into rivers and lakes results in a reduced dissolved oxygen concentration causing anoxic conditions, which subsequently affect aerobic organisms [40]. In recent years, research attention has been focused on biological methods for the treatment of effluents, some of which are in the process of commercialization [41]. There are three principle advantages of biological technologies for the removal of pollutants; first, biological processes can be carried out in situ at the contaminated site; Second, bioprocess technologies are usually environmentally benign (no secondary pollution) and third, they are cost effective. Of the different biological methods, bioaccumulation and biosorption have been demonstrated to possess good potential to replace conventional methods for the removal of dyes/metals [42,43].

5.1. Biosorption

Biosorption can be defined as the passive uptake of toxicants by dead/inactive biological materials or by materials derived from biological sources. Due to a number of metabolism-independent processes that essentially take place in the cell wall, where the mechanisms responsible for the pollutant uptake will differ according to the biomass type. These mechanisms are based on the use of dead biomass. Biosorption, which may be one or combination of ion exchange, complexation, coordination, adsorption, electrostatic interaction, chelation and microprecipitation [44,45].

5.2. Bioaccumulation

Bioaccumulation is defined as the phenomenon of living cells. To be precise, bioaccumulation can be defined as the uptake of toxicants by living cells. The toxicant can transport into the cell, accumulate intracellularly, across the cell membrane and through the cell metabolic cycle [43]. Since biosorption has significant advantages over bioaccumulation [46]. This review will be focused on biosorption mechanism as standalone technique in decolorization of distillery effluents.

5.3. Mechanism of biosorption

As the cells outer layer, where the solutes can be deposited on the surface or within the cell, the bacterial cell wall is the first component that comes into contact with metal ions/dyes. Since the mode of solute uptake by dead/inactive cells is extracellular, the chemical functional groups of the cell wall play vital roles in biosorption. Usually the bacterial cell wall is composed of compounds of several functional groups , including carboxyl, phosphonate, amine and hydroxyl groups [46]. Negative polarity and surplus nature of these compounds makes the carboxyl groups to actively participate in the binding of metal cations. Generally the dye molecules exist as dye cations in solutions, which are attracted towards carboxyl and other negatively charged groups. This phenomenon was well documented in *Streptomyces pilosus* by [47] as the carboxyl groups of the cell wall peptidoglycan were responsible for the binding of copper.

In acidic environment, amine groups were protonated and effectively attracted negatively charged chromate ions via electrostatic interaction [48] this was confirmed by Vijayaraghavan and Yun [49] as the amine groups of *C. glutamicum* electrostatically attracted reactive dye anions. In general, increase in the pH proportionately increases the overall negative charge on the surface of cells until all the relevant functional groups are deprotonated, which favors the electrochemical attraction and adsorption of cations. Anions would be expected to interact more strongly with cells with increasing concentration of positive charges, due to the protonation of functional groups at lower pH values. Fungi on the otherhand utilizes phenolic compounds in the dye as carbon and energy source [48].

6. Decolourization of distillery effluent by bacteria

Microbial treatments employing pure bacterial culture have been reported frequently in past and recent years Table 2. Bacterial strains were isolated from sewage and acclimatized on increasing concentrations of distillery waste were able to reduce COD by 80% in 4–5 days without any aeration [58]. The major products left after treatment were biomass, carbon dioxide and volatile acids. An air bubble column reactor with activated sludge carrying self adapted microbial population in both free and immobilized on polyurethane particles was used for treating aerobic winery wastewater. The highest COD removal rate was with free activated sludge in the bubble column reactor[59]. The most prominent bacterial species isolated from the reactor was *Pseudomonas* while *Bacillus* was isolated mostly from colonized carriers. *Pseudomonas fluorescens*, decolourizes melanoidin wastewater (MWW) up to 76% under non-sterile conditions and up to 90% in sterile samples [55]. The difference in decolourization might be due to the fact that melanoidin stability varies with pH and temperature and at higher temperature during sterilization melanoidin-pigments decompose to low molecular weight compounds [60]. Under low oxygen condition, *Lactobacillus hilgardii* immobilized on Ca-alginate gel decolourized melanoidin solution very effectively [53].

Acetogenic bacteria are capable of oxidative decomposition of melanoidins. Biodegradation of potato slops (distillation residue) by a mixed population of bacteria under thermophilic conditions up to 60 ^{o}C was achieved [61]. A COD removal of 77% was achieved under non-optimal conditions. Marine cyanobacteria such as *Oscillatoria boryna* have also been reported to degrade melanoidin due to production of H_2O_2, hydroxyl, perhydroxyl and active oxygen radicals, resulting in the decolourization of the effluent [62] 96%, 81% and 26% decolorization of distillery effluent through bioflocculation by *Oscillatoria* sp., *Lyngbya* sp. and *Synechocystis* sp. respectively was also reported [63]. Distillery spent wash, despite carrying high organic load contains little readily available carbon. Isolation of bacterial strains capable of degrading recalcitrant compounds of anaerobically digested spent wash from soil of effluent discharge site was reported by [64]. These were *Pseudomonas, Enterobacter, Stenotrophomonas, Aeromonas, Acinetobacter* and *Klebsiella* all of which could carry out degradation of some component of spent wash. Maximum 44% COD reduction was achieved using these bacterial strains either singly or collectively. An acetogenic bacterium was used to obtain a decolourization yield of 76.4% under optimal nutrient conditions [50]. However, this value was only 7.3%, by using anaerobic pond. Also, it required sugar, especially glucose and fructose for decolourization of MWWs. The decolourization activity might be due to a sugar oxidase.

7. Decolourization of distillery effluent by fungi

In recent years, several basidiomycetes and ascomycetes type fungi have been used in the decolourization of natural and synthetic melanoidins in connection with colour reduction of wastewaters from distilleries. The aim of fungal treatment is to purify the effluent by consumption of organic substances, thus, reducing its COD and BOD, and at the same time to obtain

some valuable product, such as fungal biomass for protein-rich animal feed or some specific fungal metabolite. Filamentous fungi have lower sensitivity to variations in temperature, pH, nutrients and aeration and have lower nucleic acid content in the biomass [65]. Several fungi have been investigated for their ability to decolourise melanoidins and MSW Table 3. *Coriolus* sp. no.20, in class basidiomycetes was the first strain to decolourize MWW in the presence of sorbose and glucose [72]. At the same time glucose was also required for enhancing decolourization as the peroxidases require H_2O_2, which is generated by glucose oxidation, to decolourize melanoidin.

Coriolus versicolour Ps4a was used for the decolourization of MWW And obtained 80% decolourization in darkness under optimum conditions [77]. Later, autoclaved mycelium of *Aspergillus oryzae* Y-2-32 that adsorbed lower weight fractions of melanoidin and degree of adsorption was influenced by the kind of sugars used for cultivation [60]. Phenolic compounds present in the wine distilleries imparts high inhibitory and anti-bacterial activity which slows down the anaerobic digestion process. *Geotrichum candidum* partial elimination of these phenolics compounds [20]. *Rhizoctonia* sp. D-90 decolourized molasses melanoidin medium and a synthetic melanoidin medium by 87.5% and 84.5% respectively, under experimental growth conditions. Electron microscopy revealed that the mycelia absorbed melanoidin pigment, which was in the form of electron dense material in the cytoplasm. However, melanoidin could be eluted from the mycelia by washing in the solution of NaOH and the relative amount of melanoidin eluted from the mycelia increased with increase in the concentration of NaOH [88].

Colour elimination from MSW using *Aspergillus niger* was studied [63]. Under optimal nutrient concentration 83% of the total colour removed was eliminated biologically and 17% by adsorption on the mycelium. Microbial decolourization of melanoidins is due to two decomposition mechanisms; in the first the smaller molecular weight melanoidins are attacked and in the second the larger molecular weight melanoidins are attacked [57]. Under nutrient limiting conditions, fungal cells generally cannot remain active during a long-term cultivation. Therefore, the continuous-culture method is not practical and the semi-batch or repeated-batch method can be an alternative for long-term cultivation. The immobilization of the fungus on a solid support is an appropriate means for controlling the thickness of the biofilm. The immobilization of the fungus offers advantages such as short retention time, easy recovery of the cells and increased activity. Furthermore, in the presence of the foam matrix, pellet size is restricted by the size and the physical properties of the foam [80]. An inhibitory effect of organic nitrogen on melanoidin decolourization by fungus *Coriolus hirsutus* [73]. Absence of additional nitrogen could not inhibit activity of fungus *C. versicolour* sp no. 20 considerably, as significant decolourization and COD reduction occurred even in the absence of it [76]. Colour removal from distillery effluent using a marine fungus, *Flavodon flavus* has been reported [78]. This fungus was more effective in decolourizing raw MSW than was the molasses wastewater collected either after anaerobic treatment or after aerobic treatment. The oxygen demand of the fungus was quite high. *P. chrysosporium* JAG-40 decolourized synthetic and natural melanoidins present in spentwash up to 80% [85]. The larger molecular weight fractions of melanoidin were decolourized rapidly, while the small molecular weight fractions remained in solution and were metabolized slowly. Also, the decolourization was less in sterilized spentwash than in non sterile solution. This observation is completely opposite of the one when *Pseudomonas fluorescens* was used by same authors [55]. Molasses decolourization in semi solid state (SSS) cultivation by fungi *C. versicolour, Funalia trogii, P. chrysosporium* and *Pleurotus pulmonarius* with cotton stalks being used as additional source of carbon [75]. *C. versicolour* decolourized 48% of 30% diluted vinasse without any additional carbon source which increased to 71% on addition of cotton stalks. *Aspergillus niveus*, a litter degrading fungi was used by [69] for the treatment of distillery effluent using paddy straw, sugarcane bagasse, molasses and sucrose as carbon source for growth of fungus in the effluent. Sugarcane bagasse at 1% (w/v) concentration resulted in maximum removal of colour (37%) and COD (91.68%). The decrease in colour removal in this study might be due to the fact that the effluent taken for study was alkaline (pH 9.0) and the melanoidins responsible for colour were more soluble in the alkaline pH. In the acidic pH, the melanoidins might be precipitated and removed easily.

An *Aspergillus* species isolated from the soil for decolourization of anaerobically digested (UASB) and aerobically treated distillery wastewater [70]. With diluted wastewater at optimum values of supplemented materials 75% decolourization was achieved which reduced to 40% on using undiluted wastewater. It was suggested that decolourization by fungi takes place due to the destruction of coloured molecules and partially because of sorption phenomena. A longer aeration period causes the adsorbed colour molecules to be released as a result of endogenous respiration and cell death, hence reducing decolourization efficiency.

7.1. Decolorization of textile dyes and effluents by White rot fungi

White rot fungi are the most intensively studied dye-decolorizing microorganisms. As stated earlier, thanks to their nonspecific LME these fungi are able to transform a wide range of organic compounds. Primarily, the decolorization of sulfonated polymeric dyes was used to assay ligninolytic activities [94,95] and to assess the biodegradation capabilities of WRF [96,97]. Uptake effects or dye sorption by WRF mycelia without real degradation are

generally minimal [94]. These effects are, rather, seen in applications of non-WRF, such as *Aspergillus niger*, whose (dead) biomass could be used as an adsorbent [98,99] and serve as part of a technical solution in water pollution control. The involvement of individual LME in decolorization has been confirmed using in vitro (cell-free) LME systems from WRF culture supernatants. LME-producing profiles vary. For instance, Lac was the main enzyme involved in dye decolorization by cultures of *Phlebia tremellosa* [100, 101] and by *Pleurotus sajorcaju* [102].

8. Decolourization of distillery effluent by yeast

Yeast, *Citeromyces* was used for treating MWW and high and stable removal efficiencies in both colour intensity and organic matter were obtained. However, the semi-pilot and pilot-scale experiments are to be tested for checking the stability of *Citeromyces* sp. [71]. Microorganisms associated with a rotating biological contactor (RBC) treating winery wastewater was studied [90]. One of the yeast isolates was able to reduce the COD of synthetic wastewater by 95% and 46% within 24 h under aerated and non-aerated conditions, respectively. Two flocculant strains of yeast, *Hansenula fabianii* and *Hansenula anomala* was used for the treatment of wastewater from beet molasses-spirits production and achieved 25.9% and 28.5% removal of TOC respectively from wastewater without dilution [91]. Dilution of wastewater was not favourable for practical treatment of wastewater due to the longer treatment time and higher energy cost.

Color removal from MSW using terrestrial white-rot fungi was shown to be Mn-P dependent in *Phanerochaete chrysosporium* [92] and laccase dependent in *Trametes versicolor* [93]. The process was sorbose oxidase and glucose oxidase-dependent in mitosporic fungi *Aspergillus fumigatus* [67] and *A. oryzae* [60] and in the basidiomycete *Coriolus* sp. No. 20 [72]. It was demonstrated that MnP-independent decolorization of MSW by the marine-derived fungus NIOCC #312 which decolourized 60% of MSW when added at 50% concentration in seawater medium. There was a direct co-relation between concentration of glucose oxidase and decolorization of MSW [78]. As previously discussed in this review, that decolorization was dependent on glucose oxidase levels in the culture medium. like bacterial decolourization, it was suggested that H2O2 produced by glucose oxidase act as a bleaching agent. It was further demonstrated that marine fungi are capable of decolorizing MSW effectively in the presence of seawater of 15-34 ppt salinity [81]. Studies to understand the mechanism of decolorization of MSW in marine fungi merit further attention.

9. Potential decolourizing oxidative enzymes

For living cells, the major decolorization mechanism in biodegradation is the production of lignin modifying enzymes (LME), laccase, manganese peroxidase (MnP) and lignin peroxidase (LiP) to mineralize synthetic lignin or dyes [103,104] Table 4. However, the relative contributions of LiP, MnP and laccase to the decolorization of dyes may be different for each organism.

Lignin-modifying enzymes are essential for lignin degradation, however for lignin mineralization they often combine with other processes involving oxidative enzymes. An older concept of ligninolysis reemerges, enzymatic ‘‘combustion’’ [113]. By extension, this enzyme-assisted process is applicable to the degradation of many other recalcitrant molecules including dyes [94,114,115]. The main LME are oxidoreductases, i.e., two types of peroxidases, LiP and MnP and a phenoloxidase, Laccase.

9.1. Manganese peroxidases (MnP)

The most common ligninolytic peroxidases produced by almost all white-rot basidiomycetes and by various litter decomposing fungi are manganese peroxidases (MnP). These are glycosylated glycoproteins [116] with an iron protoporphyrin IX (heme) prosthetic group [117], molecular weights between 32 and 62.5 kDa [118] and are secreted in multiple isoforms [119,120]. MnP preferentially oxidize Mn^{2+} into Mn^{3+}[121], which is stabilized by chelators such as oxalic acid [122], itself also excreted by the fungi [123,124]. Chelated Mn^{3+} acts as a highly reactive (up to 1510 mV in H_2O, [125] low molecular weight, diffusible redox-mediator. Thus, MnP are able to oxidize and depolymerize their natural substrate, i.e., lignin as well as recalcitrant xenobiotics such as nitroaminotoluenes [126,127] and dyes [128].

9.2. Lignin peroxidases (LiP)

Lignin peroxidases (LiP) catalyze the oxidation of nonphenolic aromatic lignin moieties and similar compounds. LiP have been used to mineralize a variety of recalcitrant aromatic compounds, such as three and four ring PAHs [129], polychlorinated biphenyls [130] and dyes [131]. The extracellular N-glycosylated LiP with molecular masses between 38 and 47 kDa contain heme in the active site and show a classical peroxidase mechanism [132]. Lignin peroxidase requires H_2O_2 as the co-substrate as well as the presence of a mediator like veratryl alcohol to degrade lignin and other phenolic compounds. Here H_2O_2 gets reduced to H_2O by gaining an electron from LiP (which itself gets oxidized). The oxidized LiP then returns to its native reduced state by gaining an electron from veratryl alcohol and oxidizing it to veratryl aldehyde. Veratryl aldehyde then gets reduced back to veratryl alcohol by gaining an electron from lignin or analogous structures such as xenobiotic pollutants. [133].LiP catalyze several oxidations in the side chains of lignin and related compounds [124] by one-electron abstraction to form reactive radicals [134]. Also the cleavage of aromatic ring structures has been reported [135].

9.3. Versatile peroxidases (VP)

A third group of peroxidases, versatile peroxidases (VP), has been recently recognized, that can be regarded as hybrid between MnP and LiP, since they can oxidize not only Mn^{2+} but also phenolic and nonphenolic aromatic compounds including dyes. VP have been described in species of *Pleurotus* and *Bjerkandera* [128]. A novel enzyme which can utilize both veratryl alcohol and Mn^{2+}, versatile peroxidase has been recently described as a new family of ligninolytic peroxidases[136]. The most noteworthy aspect of versatile peroxidase (VP) is that it combines the substrate-specificity characteristics of LiP, MnP as well as cytochrome *c* peroxidase [137]. In this way, it is able to oxidize a variety of (high and low redox potential) substrates including Mn^{2+}, phenolic and non-phenolic lignin dimers, veratryl alcohol, dimethoxybenzenes, different types of dyes, substituted phenols and hydroquinones [138]. It has an Mn^{-}binding site similar to MnP and an exposed tryptophan residue homologous to that involved in veratryl alcohol oxidation by LiP. It issuggested that the catalytic properties of the new peroxidase is due to a hybrid molecular architecture combining different substrate-binding and oxidation sites [139].

9.4. Laccases

Laccase is a benzenediol:oxygen oxidoreductase (a multi-copper enzyme), present across the kingdoms [140]. This multi-copper oxidase has the ability to oxidize phenolic compounds and aromatic dyes [141,142,143]. Laccases catalyze the oxidation of a variety of aromatic hydrogen donors with the concomitant reduction of oxygen to water. Unlike peroxidases, it does not contain heme as the cofactor but copper. Neither does it require H_2O_2 as the co-substrate but rather molecular oxygen. Laccase often sports a high degree of glycosylation, which confers a degree of self resistance to attack by proteases [144]. The downside however, is that the redox potential although varying between different laccase isozymes, cannot be compared with that of the presence of mediators like veratryl alcohol and Mn^{2+}, their presence increases the effective range of substrates, which can be degraded by laccase.

10. Native and synthetic mediators of LME for decolourization activity

Examples of native as well as synthetic mediators are given in Table 5. They could be involved in the LME-catalyzed generation of reactive radical moieties from a variety of lignin-like substrates, but also in the formation of reactive oxygen species (ROS) which either directly or indirectly could attack lignin or xenobiotic molecules [154,155,156].

Organic acids, excreted by several fungal organisms, chelate and stabilize Mn^{3+}. MnP was found to simultaneously decompose organic acids (such as malonate) oxidatively and oxidize Mn^{2+} to Mn^{3+} even in the absence of H_2O_2. Thus, organic acids are postulated to be the origin of carbon-centered radicals (acetic acid radicals, COOH–C S H_2), peroxyl radicals (COOH–CH_2OOS), superoxide (O_2^{-}), formate radicals (CO_2^{-}). Such radicals could be a source of peroxides, which can be used by MnP as substrates instead of H_2O_2. Consequently, even fungi obviously lacking H_2O_2-generating oxidases could be efficient lignin-degraders [118] and, by extension, useful in the degradation of xenobiotics such as dyes.

On the other hand organic acids (e.g., oxalate) chelate cations including Fe^{2+} [157], therefore such acids are indirectly involved in the regulation of Fenton's reaction due to regulation of Fe^{2+} concentration [158), which supplies fungal degradation reactions with hydronium ions (H_3O^{+}) and hydroxyl radicals (HO, HO^{-}). Recent evidence strongly suggests the involvement of formyl and superoxide free radicals in the in vitro mineralization of recalcitrant nitroaminoaromatic molecules by MnP or by its biomimetic analog Mn(III)/oxalate/O_2) [156].

In vitro degradation of lignin and other recalcitrant molecules by MnPs is considerably enhanced in the presence of thiols [reduced glutathione (GSH), cysteine (Cys)]. Thiols were shown to promote the attack of the aromatic ring of veratryl alcohol and nonphenolic h–O–4 lignin model dimers [159,160]. Although fungal secretion of reduced thiols is unlikely, thiolic peptides released during partial cell lysis might be a source of thiol mediators [118]. Both MnP and chelated Mn(III) catalyze the oxidation of GSH to reactive free glutathionyl radical GS, whose production correlates with the mineralization of recalcitrant aminonitroaromatic compounds [161.162].

Veratryl alcohol (VA, 3,4-dimethoxy benzyl alcohol), a secondary metabolite of several WRF(163), after its oxidation to the VA cation radical (VA^{+}) by LiP, acts as a mediator for the degradation of lignin [164]. However, due to the short life span of VA^{+} long-distance charge transfers are not likely to occur. Mediating properties of VA could be enhanced if the radical is somehow complexed to the LiP [165]. Nevertheless, LiP is stimulated by VA probably by protecting the enzyme against the damaging effect of H_2O_2 [166]. 3-Hydroxyanthranilic acid (3-HAA) was the first natural mediator for laccases described. This mediator enables a laccase-catalyzed oxidation of nonphenolic lignin model dimers [147]. To delignify kraft pulp by laccase a number of synthetic mediators have been tested. For instance, using 2,2' -azinobis-(3-ethylbenzthiazoline- 6-sulphonate) (ABTS) laccases are able to attack nonphenolic lignin model compounds and to delignify kraft pulp [167]. The discovery of 1- hydroxybenzotriazole (HBT), an effective laccase mediator in pulp processing [168] lead to a new class of mediators with NOH as the functional group, which is oxidized to a reactive radical (R–NO). These mediators [e.g., 4-hydroxy-3-nitroso-1-naphthalenesulfonic acid (HNNS), 1-nitroso-2-naphthol-3,6-disulfonic acid (NNDS), and Remazol brilliant blue (RBB)] have been shown to support delignification reactions by laccases [169].

11. Conclusion

Biological method of decolourizing the effluent waste water is gaining its momentum in the arena of wastewater treatment methods. The hinderances with this technique were the recycling ability of the microbial mass used for decolourization and narrow specificity towards the dyes. Isolation and characterization strain improvement will be the initial step in establishing efficient biological decolourization systems. Gene amplification and expression in appropriate hosts could be promising for abundant production and affordable price of effluent treatment as is already the case with laccases used commercially in the pulp and paper industry. Further potential benefits of genetically improved LME could be extended substrate range, catalytic activity and stability for industrial application of LME. This is not surprising, given the structural similarity of most commercially important dyes to lignin (sub) structures amenable to transformation by LME. It is clear that LME play significant roles in dye metabolism by WRF [170]. The influence or not of the substitution pattern on the dye mineralization rates is a matter of controversy [115,171], though it is clear that dye decolorization is not equivalent to dye mineralization. There is a definite gap in our current knowledge of decolorization and, even more so, of mineralization mechanisms. With a lack of insight concerning potentially toxic albeit colorless accumulating intermediates, our capacity to evaluate the true technical potential of WRF and their LME remains incomplete. With continuous research and full scale field level studies will tremendously improve the sector of wastewater treatment in coming future.

12. References

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