Chapter 4
Environmental Health Hazards of Post-Methanated Distillery Effluent and Its Biodegradation and Decolorization

Sangeeta Yadav and Ram Chandra

Contents

Abstract Anaerobically digested distillery effluent is a mixture of complex organic and inorganic pollutants which is composed of several plant sterols which do not only affect the water quality but also aquatic flora and fauna. Research has revealed the adverse effects of post-methanated distillery effluent (PMDE) on the seed germination and plant growth of *Phaseolus mungo* even at lower concentrations. Studies have also showed the adverse effect on soil fertility by inhibiting the nitrogen-fixing bacteria and root nodulation. The major colorant of distillery effluent is melanoidin, reaction product of amino-carbonyl compounds at elevated temperature in the sugar industries and distilleries due to condensation reaction. Due to its high solubility in aquatic ecosystem and negative charge, it makes complexation with all the humic substances and heavy metals in the environment. Therefore, the decolorization and degradation of PMDE is still a global challenge due to its complexity. The physical, chemical, and biological techniques have been attempted for

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its detoxification and color removal but still warranted for its feasible application. Manganese peroxidase (MnP) and laccase have been reported as key enzymes from fungi and bacteria. During the degradation process of PMDE, different metabolic products through GC-MS/MS analysis have also been characterized. The integration of bacterial treatment with constructed wetland plant treatment (*Phragmites communis*, *Typha angustifolia*, and *Cyperus esculentus*) technique has been reported recently as an effective approach for decolorization and degradation of PMDE. The major challenge of PMDE biodegradation and decolorization is its high total dissolved solids (TDS) containing complex organic pollutants including heavy metals. The high TDS is a result of precipitation of metal sulfides during anaerobic digestion of distillery spentwash due to complexation of heavy metals and sulfates which impose inhibitory effects on the microorganisms, consequently inhibiting the biodegradation process. Several complex organic pollutants present in PMDE have been also reported as endocrine-disrupting chemicals (EDCs) which directly affect the aquatic and terrestrial ecosystems.

Keywords Degradation · Decolorization · Ligninolytic enzymes · Metabolites · Phytoremediation · Post-methanated distillery effluent

Introduction

The disposal of wastewater generated from sugarcane molasses-based alcohol industries is a global challenge and environmental threat for sustainable development. There is production of huge amount of wastewater, i.e., 90 m^3 of effluent from per kilo liter of alcohol production. However, wastewater discharge standard fixed by Environmental Protection Agency (EPA) is 12 m^3 per kilo liter of alcohol produced (EPA 2008). Hence it is more than seven times higher discharge in comparison to the prescribed standard. Moreover, the wastewater remains very dark, viscous, and chemically complex even after secondary treatment which causes water and soil pollution due to its indiscriminate disposal in the environment. Therefore, alcohol industries are rated among the 17 most polluting industries in India. Currently around 397 distilleries are operating in India. In international scenario two countries, i.e., the United States and Brazil, are playing leading role for production of ethanol as fuel. However, the countries following them and rising very fast are India, China, Canada, European, Argentina, Colombia, and Thailand. Moreover, the world's total production of alcohol from cane molasses is more than 13 million m^3/annum (Khairnar et al. 2013)

In alcohol industries, major wastewater is generated during distillation process, where only 5–12% of ethanol is recovered, and 88–95% volume of wastewater is generated from fermented molasses which is known as spentwash. The spentwash remains acidic in nature and contains a variety of recalcitrant coloring compounds such as melanoidins, phenolics, metal sulfide, and methylated complex organics that contribute toxicity and color to distillery effluent. The pH of spentwash increases from 4.5 to 8.5 during the anaerobic treatment process which is finally

known as post-methanated distillery effluent (PMDE). Sugarcane molasses contains high level of caramelized sugar, heavy metals, sulfates, and phenolics along with the fermentable sugar. But, during the fermentation process, the fermentable sugar gets converted into ethyl alcohol, leaving the complex, nondegradable organic residues as spent after distillation process. The water soluble hemicelluloses, proteins, gums, nonsugars, and organic compounds present in sugarcane juice also come into the spentwash in their original or transformed forms. Besides this, there is a high chemical oxygen demand (COD) and biological oxygen demand (BOD) of the spentwash (Chandra et al. 2008a). During conventional secondary treatment, i.e., anaerobic digestion of spentwash, phenolics and butyric acid are produced by reducing sugars, and methane is emitted. Phenolics, sulfides, and heavy metals are left which acted as inhibitors for biodegradation of PMDE during the tertiary treatment process (Borja et al. 1993). The heavy metals also form complexes with sulfides, and these complexes result in toxicity of the effluent (Krishnanand et al. 1993; Kumar and Chandra 2004). Apart from this, distillery wastewater also contains nitrogen (1660–4200 mg/l), phosphorus (225–3038 mg/l), and potassium (9600–17,475 mg/l) as nutrients that can lead to the eutrophication of water bodies. Indiscriminate disposal or application of this effluent on crop plants causes health hazards for environment, humans, and animals. Chandra et al. (2009) have reported accumulation and distribution pattern of metals in wheat (*Triticum aestivum* L.) and Indian mustard (*Brassica campestris* L.) irrigated with mixed effluent of distilleries and tanneries. The study revealed that these plants have accumulated metals in different parts beyond the permissible limit as suggested by Food and Agriculture Organization of the United Nations (FAO)/World Health Organization (WHO) when treated with mixed effluent of distillery and tannery. Maximum accumulation was of Fe, i.e., 340 mg/kg in wheat's root and 560 mg/kg in mustard's leaves, followed by Mn and Zn in the order root > shoot > leaves > seeds. Maximum increase in photosynthetic pigment and protein content was observed between 30–60 days and 60–90 days of plant growth, respectively. Another study with distillery sludge-amended soil (10, 20, 40, 60, 80, and 100%) showed 10% (w/w) sludge was favorable for *Phaseolus mungo*, while above 10% sludge concentration showed inhibitory effect. Soil with 10% (w/w) distillery sludge induced the growth of root, shoot, number of leaves, biomass, photosynthetic pigment, protein, and starch, while 20% (w/w) sludge-amended soil had variable effects on *P. mungo*. Concentrations >40% reduced all the growth parameters, viz., root length, shoot length, number of leaves, biomass, photosynthetic pigment, and protein content, of *P. mungo* (Chandra et al. 2008b). Hence, prior to discharge of effluent into the environment, its treatment is mandatory. Though various wastewater treatment approaches including physical, chemical, and biological have been reported, but the feasible and economic technology has to be developed. Recently the Central Pollution Control Board (CPCB) has recommended two technologies, namely, (1) concentration and incineration and (2) concentration and biocomposting, to achieve zero discharge. But, these technologies are still not operative due to cost feasibility. Complete chemical properties of PMDE and their degradation process are still to be understood. In the present review the available

knowledge on the subject for the awareness and ignition of scientific society to manage this challenging problem has been discussed.

Physico-chemical Properties of PMDE

The spentwash generated after the distillation in industries is highly acidic in nature and has a variety of recalcitrant coloring compounds such as melanoidins, phenolics, and metal sulfides that are mainly responsible for the dark color of this effluent. The bad smell of effluent might be due to the presence of indole, skatole, and other sulfur compounds. Moreover, due to the presence of high amount of organic pollutants, most of the distilleries generate biogas from these by anaerobic digestion. In this process organic substances are converted into methane-rich biogas, which is utilized as fuel. This biogas normally contains 55–65% methane gas, which is well-recognized fuel gas with minimum air pollution potential. The main steps involved for methane production from spentwash are (1) hydrolysis, (2) acidogenesis, (3) acetogenesis, and (4) methanation (Fig. 4.1). In anaerobic treatment process hydrogen sulfide (H_2S) gas is also produced as a result of the reduction of oxidized sulfur compounds, and the resulting wastewater is known as PMDE. Sulfides bind

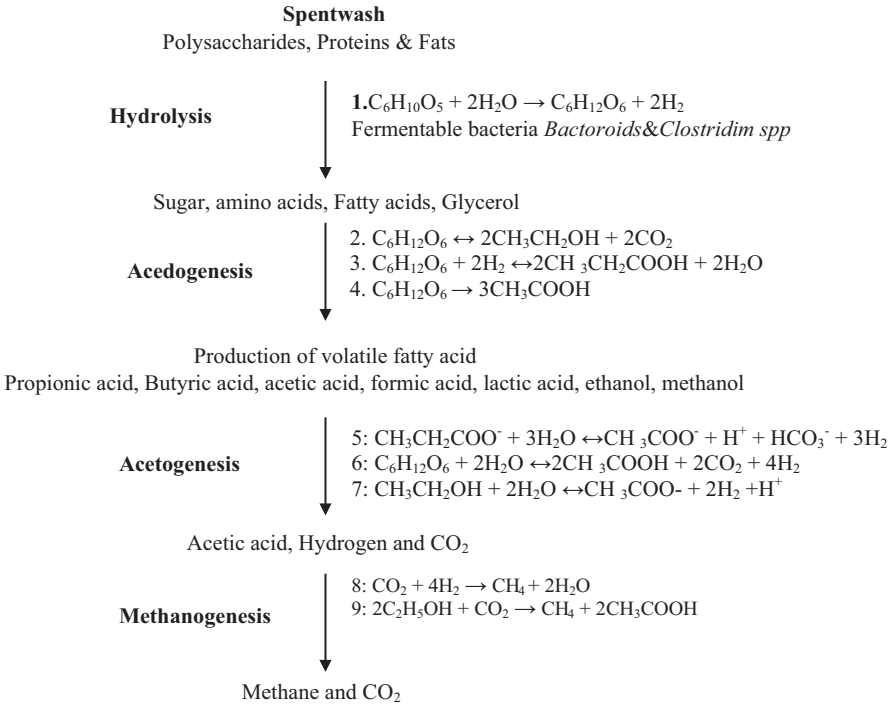

Fig. 4.1 Steps of biomethanation for generation of methane from spentwash

Table 4.1 Physico-chemical properties of spentwash and post-methanated distillery effluent (PMDE)

Physico-chemical properties	Spentwash	PMDE	Permissible limits
Color (Co-pt)	Brown (1,50,000)	Dark brown (70,000)	Not clear
Odor	Jaggery smell	Mild sulfur smell	Not clear
pH	3.90 ± 0.25	8.3 ± 0.31	5–9
TS	103,084 ± 5.50	34,317 ± 455	2200
TDS	77,776 ± 3768	20,022 ± 438	2100
TSS	25,308 ± 1201	14,276 ± 16.00	100
COD	90,000 ± 231	58,018 ± 185	120
BOD	42,000 ± 123	29,120 ± 265	40
Chloride	2200 ± 105	1300 ± 60.50	1500
Phenol	4.20 ± 1.80	1.65 ± 0.76	0.50
Sulfate	5760 ± 260	13,656 ± 21.23	1500
Phosphate	5.36 ± 0.17	1.16 ± 0.15	Not clear
Total nitrogen	2800 ± 130	568 ± 23.00	25
Total organic carbon	25,368 ± 1.06	10,904 ± 0.34	Not clear
Metals			
Cd	0.020 ± 0.00	2.281 ± 0.07	0.010
Cr	0.192 ± 0.01	0.40 ± 0.01	0.050
Fe	6.312 ± 0.21	84.01 ± 1.98	2.000
Ni	0.171 ± 0.01	1.241 ± 0.04	0.100
Cu	0.961 ± 0.00	0.955 ± 0.02	0.500
Pb	0.945 ± 0.00	4.446 ± 0.06	0.050
Zn	2.012 ± 0.00	4.631 ± 0.11	2.000
Mg	0.214 ± 0.00	2.112 ± 0.05	0.200

Chandra et al. (2004) and Chandra and Srivastava (2004)
All the values are in mg/l, except color, odor, and pH

with heavy metals present in effluent and form a colloidal solution of metal sulfides, which results in dark black color of PMDE (Chandra et al. 2008a). The PMDE is a complex, cumbersome, and highly toxic waste, which has very high BOD, COD, color, sulfate, phenol, total suspended solids (TSS), TDS, and various heavy metals (Table 4.1). The dark color of effluent is deleterious to aquatic life because it hinders the photosynthesis by blocking sunlight. The increasingly stringent environmental laws are forcing the distilleries to improve the existing treatment processes and explore suitable methods for effluent management.

Major Colorants in PMDE

Color-contributing substances present in distilleries are generated and concentrated during the processing of sugarcane juice, while some are added during the processing of sugar. The wastewater generated from alcoholic fermentation has a large

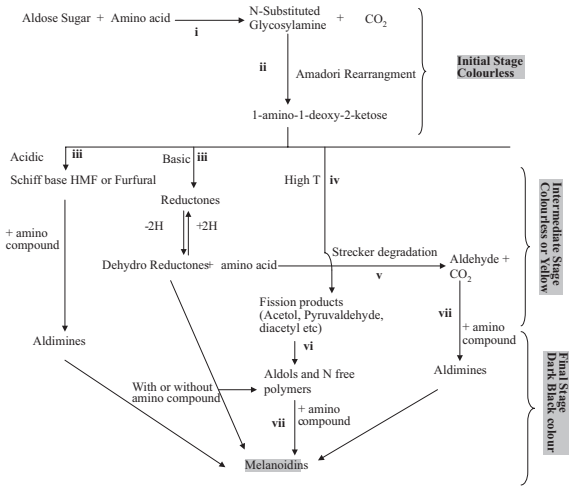

Fig. 4.2 Nonenzymatic browning reaction. (Based on Hodge 1953)

amount of melanoidin (i.e., brown pigment). The major colorant is synthesized as per Maillard reaction due to reaction of sucrose or reducing sugar with mixture of amino acids present in sugarcane juice (glycine, aspartic acid, histidine, lysine, tryptophan, and arginine) at high temperature during concentration of sugar juice. Though the reaction is complicated which forms mixture of compounds at various stages, but to understand the process it can be simplified into three stages as given in Fig. 4.2 (Hodge 1953).

1. *Initial stage*: Products formed during this stage are colorless which show no absorption in ultraviolet range. This stage involves the following two reactions:

   Reaction i: Sugar-amine condensation
   Reaction ii: Amadori rearrangement

2. *Intermediate stage*: Products formed during this stage are colorless or yellow which show strong absorption in the ultraviolet range. This stage involves the following reactions:

   Reaction iii: Sugar dehydration
   Reaction iv: Sugar fragmentation
   Reaction v: Amino acid degradation (Strecker degradation)

3. *Final Stage*: Products formed during this stage are highly colored. This stage involves the following reactions:

   Reaction vi: Aldol condensation
   Reaction vii: Aldehyde-amine condensation and formation of heterocyclic nitrogen compounds

Table 4.2 Major colorants present in post-methanated distillery effluent (PMDE) and their characteristics

Colorant	Characteristics
Melanoidin	Browning reaction products of sugar and amino acids with high molecular weight
Caramel	Process colorant, thermal degradation products of sugar, high molecular weight with low net charge
Phenolics	Plant pigment, low molecular weight may be attached to polysaccharides, pH sensitive, darker at high pH, pale yellow to orange color, react with iron to produce very dark color, may dimerize or oxidize to form darker color
Alkaline degradation products of fructose	Process colorants, reddish to dark brown in color, low molecular weight up to polymeric depending on degree of degradation
Sulfides	Process colorant, toxic to microorganisms and creating foul smell, and have strong binding tendency with metals
Heavy metals	Process colorants, toxic to microorganisms, animals, and humans

The coloring compounds present in spentwash are not degraded by the conventional treatment process and even be increased during the anaerobic treatment process in methane reactors due to the re-polymerization of compound. Melanoidins from overheated sugars, furfurals from acid hydrolysis, and humic and tannic acids from feedstock mainly contribute to the color of the effluent (Chandra et al. 2008a). However, heating of glucose and fructose at acidic or basic conditions leads to the degradation reaction forming the highly reactive intermediate compounds, which undergo condensation and polymerization reactions forming more complex and colored polymers known as melanoidins (Table 4.2). The colorants can be divided into enzymatic colorants such as melanins and nonenzymatic colorants such as melanoidins. Around 6% of melanoidins are present in sugarcane molasses that impart colors to the effluent, and molasses is raw material of distilleries. High concentration of sulfate is also present in sugarcane molasses, which is added during the cleaning of sugar crystals. The high level of sulfate can lead to production of sulfides during the anaerobic digestion of distillery spentwash, which is precipitated out along with the existing metals as metallic sulfides in PMDE, consequently increasing the total solids (TS) contents of the distillery effluent. In addition to these color-contributing components, during the heat treatment process, the Maillard reaction is accompanied by the formation of a class of compounds known as Maillard reaction products (MRPs) in the different fractions of distillery wastewater. The suspended solid fraction of distillery effluent comprises hydroxypropanone, methylbenzene phenol, methylphenol, p-chloroanisole, methylbenzaldehyde, indole, and methylindole. Most of the compounds have been reported as pyrolysis products of different amino acids as methylbenzene from phenylalanine, indole and methylindole from tryptophan, and finally phenol and methylphenol from the tyrosine amino acid (Bharagava and Chandra 2010; Gonzalez et al. 2000).

The presence of all these compounds in distillery wastewater could be derived from the amino acids present in original molasses or from the rest of the yeast cells that remain in distillery effluent after alcoholic distillation process. Despite hydroxypropanone has been reported as a dehydration product of glycerol and typical carbohydrate pyrolysis product, glycerol represents 5–6% of the dry weight of vinasse which is also a raw material of distilleries industries. The existence of carbohydrate-derived compounds such as furfuryl alcohol and 2,3-dihydro-5-methylfuran-2-one or lignin-derived moieties such as 4-vinylguaiacol and syringol could be ascribed to residual sugarcane fragments present in the suspended solids fraction of distillery effluent. Moreover, 4-vinylguaiacol has been described as a pyrolysis product of ferulic acid, one of the two cinnamic acids present in the vegetal cell wall of gramineous plants as is the case of sugarcane (*Saccharum officinarum* L.). The polysaccharide-free fraction of distillery effluent contains hydroxypropane and 2,5-dimethylfuran along with some other minor carbohydrate pyrolysis products such as 2-hydroxymethylfuran and 2,4-dimethylfuran, 1-hexadecanol, and palmitic acid. Moreover, the polysaccharide-rich fraction of distillery effluent has major portion of hydroxypropanone, 2,5-dimethylfuran, 2-hydroxymethylfuran, 2,4-dimethylfuran, 2,3-dihydro-5-methylfuran-2-one, and 5-methyl-2-furfuraldehyde (Gonzalez et al. 2000; Bharagava and Chandra 2010). The presence of all these types of toxic compounds in distillery wastewater indicated that there is an urgent need to develop an environment-friendly and cost-effective treatment technique, which can remove/degrade/detoxify all these toxic compounds from distillery effluent, so that it can be safely disposed into environment.

Environmental Pollution and Toxicity Profile of PMDE

PMDE turns more dark and viscous with alkaline pH than original spentwash during the anaerobic treatment process in industries. The increase in pH from 4 to 8.5 in methane reactor during the anaerobic digestion process is mainly due to the oxidation of organic acids to carbon dioxide (CO_2), which react with basic compounds present in PMDE and generate carbonates and bicarbonates resulting in the increase of pH. The highly colored PMDE is the major source of soil as well as water pollution. PMDE causes water pollution in two ways. First, the highly colored (1,50,0000–1,80,000 co-pt) nature of PMDE blocks out the sunlight penetration in rivers and streams, reducing the photosynthesis leading to the reduction in oxygenation of which becomes detrimental for aquatic life. Secondly, due to the high organic load, i.e., high COD and BOD, it causes eutrophication. In addition, due to the presence of putriciable organics like indole, skatole, and other sulfur compounds, the PMDE that is disposed in aquatic resources also produce bad smell.

The organic and inorganic ions added in water and agricultural land through PMDE pose a serious threat to the soil and groundwater (Table 4.3), if applied without proper dilution/treatment (Jain et al. 2005; Kaushik et al. 2005). The agricultural crops growing on such metal-contaminated soil may accumulate high

Table 4.3 Effect of higher concentration of PMDE on soil and water properties

Properties	Toxicity effect
Soil	
pH	Acidify the soil with spentwash application and decrease the productivity
Electric conductivity	Increase electricity and results in salinization of the soil which is important cause of lower productivity
Organic carbon	Heavy infield transport machinery is most commonly associated with soil compaction problems. Soil compaction decreases porosity and water infiltration rate, restricting the rooting ability of the crop
Total phosphorous	Stunted plant growth decreases the plant's ability to uptake zinc, decreases the plant's ability to uptake iron
Total nitrogen	Increase acidity of soil
Total potassium	Deleterious effects on soil as well as on crops, since very high exchangeable K can also cause dispersion of soil. Higher anionic and cationic concentration can decrease the bulk density as well as water holding capacity of soil by reducing the porosity in clay soil due to deflocculation of clay particles which occurs in the presence of higher Na content, and the consequent effects are seen in the cation exchange capacity in the soil which adversely affects the seed germination and plant growth
Total sodium	Change the electric conductivity, soil dispersion affecting soil permeability and aeration, which causes hindrance to seed germination
Total calcium	Change the electric conductivity
Heavy metals (iron zinc, cadmium, copper)	Inhibit the biodegradation of organic contaminants, adverse effects on the soil microbial diversity, plant growth, and genetic variation
Microbial parameters	*Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Enterobacter* spp., *Klebsiella* spp., *Proteus* spp., and *Bacillus* spp., while the fungi isolated were *Aspergillus* spp., *Rhizopus* spp., and *Penicillium* spp.
Soil enzyme (phosphatase, dehydrogenase, urease)	Soil enzyme activity is increased and found to be more than the recommended NPK and farmyard manure
Water	
pH	The pH of water affects the solubility of chemicals. Therefore, the availability of these chemicals to aquatic organisms is affected. As acidity of water increases by application of spentwash, most metals and other toxic compounds become more soluble and more toxic which decrease the productivity
COD	Increase organic content leading to depletion of oxygen and reduction of dissolve oxygen (DO) which creates anaerobic conditions. Anaerobic condition is deleterious to higher aquatic life
BOD	Fish and other aquatic organisms may not survive under very high level of BOD
Total phosphorous	Eutrophication
Total nitrogen	Increase eutrophication
Total potassium	Increase eutrophication
Heavy metals	Act as inhibitor for many biological treatment processes, recalcitrant and persistence in nature

amount of toxic metals which is excessive enough to cause clinical problems to both humans and animals (Rattan et al. 2005). Wastewater treatment inhibited seed germination, reduced soil alkalinity and soil manganese deficiency leading to damage to agricultural crops. Bharagava et al. (2008) have reported the effect of different concentrations of distillery effluent irrigation on morphological and physiological parameters of Indian mustard (*Brassica nigra* L) at different time intervals. During this study it was found that distillery effluent beyond 50% (v/v) concentration significantly decreases the plant growth parameters (root, shoot length, number of leaves, chlorophyll, and protein content) in tested plants in respect to control. However, the maximum increase in root length, shoot length, number of leaves, and total dry weight were recorded in mustard plants treated with 50% (v/v) distillery effluent. Moreover, the mustard plants treated with 75% (v/v) distillery effluent have shown the vigorous growth initially. But later on reduced growth and delayed flowering and fruiting were also observed as compared to control plants. Lower concentration of distillery effluent which supported the plant growth parameters might be due to the induction of plant growth hormones. While higher concentration of effluent (>50%) suppresses the hormone activities, it has been also reported that higher distillery effluent content acts as inhibitor for plant growth hormone(s) (auxin and gibberellins), which are mainly required for the growth and development of plants (Subramani et al. 1997)

The reduction in plant growth parameters at higher concentration of distillery effluent might be also due to the entrance of toxic heavy metals into the protoplasm resulting in the loss of intermediate metabolites, which are essential for further growth and development of plants. Further, Chandra et al. (2008b) have also observed the effect of distillery sludge amendments in soil (10, 20, 40, 60, 80, and 100%) and found that 10% (w/w) sludge was favorable for *P. mungo*, while >10% was inhibitory for plant growth (Fig. 4.3). Greater than 40% concentrations of sludge-amended soil reduced all the growth parameters, viz., root length, shoot length, number of leaves, biomass, photosynthetic pigment, protein, and starch, of *P. mungo*.

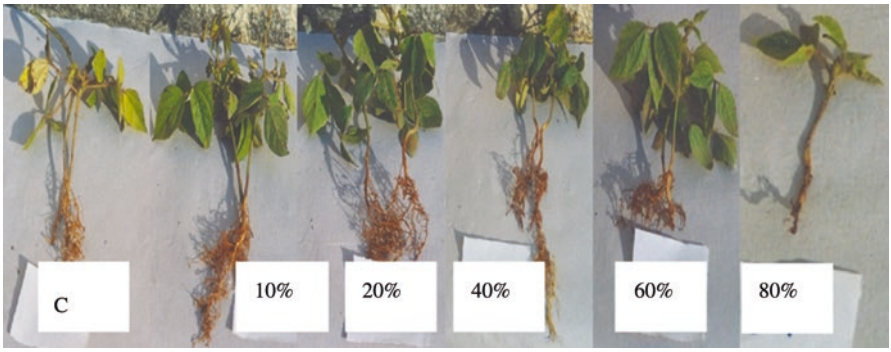

Fig. 4.3 Morphological effect of distillery sludge amendment soils at different concentrations (10–80%) on the growth of root, shoot, and leaves of *P. mungo* after 60 days vs. the control

Disposal of undiluted and untreated PMDE in water bodies has toxic effects on fishes and other aquatic organisms. Ramakritinan et al. (2005) have estimated LC_{50} for distillery spentwash at 0.5% using a bio-toxicity study on freshwater fish *Cyprinus carpio* var. communis. They reported that the respiratory process in *C. carpio* under the PMDE stress is severely affected resulting in a shift toward the anaerobiosis at organ level during sublethal intoxication. The sulfide toxicity of PMDE toward microorganisms is also well documented as sulfide has strong tendency of metal binding which contributed more toxicity and color to the PMDE (Krishnanand et al. 1993). Several studies have reported that PMDE inhibits the microbial growth by sequestering the ammonia, amino acid, and peptide, while melanoidins inhibit growth by cross-linking with polypeptide chain and sequestering essential multivalent metal cations (Silvan et al. 2006). In vitro studies have also revealed that melanoidins (the major color-contributing component in distillery effluent) have mutagenic, carcinogenic, and cytotoxic effects in humans and animals (Silvan et al. 2006). The excessive glycation has also been reported to cause destruction of essential amino acids, decreased digestibility, inactivation of enzymes, inhibition of regulatory molecule binding sites, cross-linking of the glycated extracellular matrix, decreased susceptibility to proteolysis, abnormality of nucleic acid functions, altered macromolecular recognition, and endocytosis as well as increased immunogenicity (Silvan et al. 2006), which are involved in the development of diseases such as diabetes mellitus, cardiovascular complications, and Alzheimer's disease (Silvan et al. 2006; Chandra et al. 2008a).

Since melanoidins are equally harmful to plants, animals, and humans, hence the nutritional and physiological effects of melanoidins have been widely investigated. Lee et al. (1977) showed the effect of non-dialyzable melanoidins prepared from reduction sugars and amino acids on the rats. Results showed that growth was depressed and diarrhea was induced in rats feeding on diets containing melanoidin. The high molecular weight part of non-dialyzable melanoidins was hardly digested or absorbed, and long-term feeding on it did not affect the growth of rats, while the low molecular weight components of melanoidins have depressing effects on protein digestion and absorption.

Over the last decades, toxicity profile of distillery effluent was mainly focused on the persistent organic pollutants (POPs) present in effluent which are not removed during the secondary treatment by the industries. Among these POPs most of the compounds come under the endocrine-disrupting chemicals (EDCs) on which there is need for further research. Table 4.4 presents some EDCs on the basis of authoritative reports (TEDX 2011). EDCs are complex mixtures of compounds that are persistent and bioaccumulative or less persistent and not bioaccumulative. EDCs were mainly associated with nuclear hormone receptors and affected their functions. Due to the EDCs mostly androgen, estrogen, thyroid, and progesterone are affected. Androgen receptors are proteins which are found in the cytoplasm that specifically bind to androgens, which are responsible for male characteristics. Estrogen receptors are also cytoplasm proteins that bind to estrogens, which are most responsible for female characteristics. While thyroid receptors which are usually found in the nucleus regulate DNA transcription and regulate the metabolism. Similarly,

Table 4.4 List of detected organic pollutants present in PMDE screened under EDC compounds

S.No.	Organic compounds present in PMDE	Toxicity	References
1.	4-Methylphenol (p-cresol)	Irritation and burning of the skin, eyes, mouth, and throat; abdominal pain and vomiting, heart damage, anemia, liver and kidney damage, facial paralysis, coma and death	Camarero et al. (1999) and Gonzalez et al. (2000)
2.	4-Methoxyvinylbenzene	Not clear	Camarero et al. (1999)
3.	1,2-Dimethoxybenzene (veratrole)	Pneumoconiosis	Camarero et al. (1999)
4.	Nitroacetone phenone	Group D carcinogenic	Bharagava and Chandra (2010) and Sangeeta et al. (2011)
5.	3,4-Dimethoxytoluene	Carcinogenic	Gonzalez et al. (2000)
6.	p-Anisaldehyde	Neurotoxicant	Camarero et al. (1999)
7.	1-Methoxy-4-(2-propenyl) benzene	Not clear	Camarero et al. (1999)
8.	1,2,3-Trimethoxybenzene	Carcinogenic effects, hazardous in case of inhalation (lung irritant)	Camarero et al. (1999)
9.	1,2-Dimethoxy-4-ethylbenzene	Allergic reactions in certain sensitive individuals, carcinogenic effects	Camarero et al. (1999)
10.	1,2-Dimethoxy-4-vinylbenzene		Camarero et al. (1999)
11.	p-Chloroanisole	Liver and kidney damage, carcinogenic, reproductive damage	Bharagava and Chandra (2010) and Sangeeta et al. (2011)
12.	1,2,3-Trimethoxy-5-methylbenzene s, 1,3,5-tri-tert-Butylbenzene (standard)	Skin or sensory organ toxicants	Gonzalez et al. (2000)
13.	Cis-1,2-Dimethoxy-4-(1-propenyl)benzene	Cause cancer or mutations, inhalation of vapors may cause drowsiness and dizziness, hepatotoxic	Gonzalez et al. (2000) and Sangeeta et al. (2011)
14.	Trans-1,2-Dimethoxy-4-(1-propenyl)benzene	Do	Camarero et al. (1999)
15.	1,2,3-Trimethoxy-5-vinylbenzene	Do	Camarero et al. (1999)
16.	1,2,3-Trimethoxy-5-(2-propenyl) benzene	Do	Camarero et al. (1999)
17.	1,2-Dimethoxy-4-(2-ketopropyl) benzene	Do	Camarero et al. (1999)
18.	Cis-1,2,3-Trimethoxy-5-(1-propenyl)benzene	Do	Camarero et al. (1999)
19.	2,3-dimethyl pyrazine	Irritating to the eyes, respiratory system, and skin	Bharagava and Chandra (2010) and Sangeeta et al. (2011)

(continued)

Table 4.4 (continued)

S.No.	Organic compounds present in PMDE	Toxicity	References
20.	Trans-1,2,3-Trimethoxy-4-(1-propenyl)benzene	Anticoagulant effect	Gonzalez et al. (2000)
21.	Methyl 4-methoxycinnamate	Not clear	Camarero et al. (1999)
22.	3,4,5-Trimethoxyacetophenone	Group D carcinogenic	Camarero et al. (1999)
23.	Methyl 3,4,5-trimethoxybenzoate	Carcinogenic, reproductive and developmental toxicity, neurotoxicity and acute toxicity	Camarero et al. (1999)
24.	Methyl 3,4-dimethoxycinnamate	Not clear	Camarero et al. (1999)
25.	4-Vinylphenol	Pneumotoxicity and hepatotoxicity	Camarero et al. (1999)
26.	1,2-Dimethoxy-4-(2-propenyl) benzene	Persistence and bioaccumulation potential	Camarero et al. (1999)
27.	3,4,5-Trimethoxybenzaldehyde	Carcinogenic, reproductive and developmental toxicity, neurotoxicity, and acute toxicity	Camarero et al. (1999)
28.	2,3-Dihydro-5-methylfuran		Bharagava and Chandra (2010) and Sangeeta et al. (2011)

TEDX (2011)

progesterone receptors are specific proteins found in or on cells of progesterone target tissues that specifically combine with progesterone, which control the menstrual cycle, pregnancy, and embryogenesis; the cytosol progesterone-receptor complex then associates with the nucleic acids to initiate protein synthesis. EDCs can mimic the natural hormone, fooling the body to give overresponse against stimulus and responding at inappropriate times; block the effects of the hormone from certain receptors or directly stimulate or inhibit the endocrine system; and cause overproduction or underproduction of hormones (Kandarakis et al. 2009). Several pollutants of distillery waste act as EDCs, and their exposure may have a much wider impact on the body's endocrine system, including health problems, i.e., nonreproductive cancers, immune effects, metabolic effects, obesity, diabetes, brain development, cardiovascular disease, and behavior. Many metals and metalloids also work as endocrine disruptors. Metal exposure can target all five steroid receptor pathways (progesterone, estrogen, testosterone, corticosteroids, and mineralocorticoids) and also receptors for retinoic acid, thyroid, and peroxisome proliferators. Some EDCs are persistent in the environment and bioaccumulate through food webs to high concentrations in wildlife and humans and can be transferred to the developing fetus and the newborn through the placenta or breast milk, respectively, and cause abnormalities. Hence, their remediation from the distillery wastewater is mandatory and very challenging for the researchers.

Bacterial Treatment of PMDE

Microorganisms including bacteria, fungi, and actinomycetes due to their versatile inherent properties to metabolize a variety of complex compounds have been widely utilized for biodegradation purposes. Degradation and detoxification of industrial wastes by microbes is an environment-friendly and cost-competitive method for industrial waste cleanup. During industrial wastewater treatment process, the utility of microbes largely depends on the nutrient requirement, enzymatic setup, environment conditions, as well as the nature and chemical structure of recalcitrant compounds (Chandra et al. 2007; Mohana et al. 2007). However, most of the researchers have reported the microbial degradation of distillery effluent by using the various fungal strains at laboratory scale, but the application of fungal strains for the treatment of wastewater at industrial scale is not practically possible because of slow growth rate of fungus and their less stability under stress environment such as higher pH, low oxygen, and high pollution load. Hence, bacteria appear to be more effective than fungi for the bioremediation of environmental pollutants due to their broad environmental adaptability and biochemical versatility (Chandra et al. 2007; Bharagava et al. 2009).

Many workers have reported several aerobic bacterial strains for the degradation and decolorization of PMDE (Table 4.5). Bharagava et al. (2009) isolated *Bacillus* sp., *Bacillus licheniformis*, and *Alcaligenes* sp. from distillery effluent-contaminated soil and found that these bacterial strains were capable for 52, 49, and 59% decolorization of natural melanoidins, respectively, in axenic condition, while in mixed condition these showed 70% decolorization in the presence of glucose (1.0%) and peptone (0.1%) at pH 7.0 and temperature 37 °C. Further, Ghosh et al. (2004) have also isolated *Acinetobacter*, *Enterobacter*, *Pseudomonas*, *Klebsiella*, and *Stenotrophomonas* from the distillery effluent contaminated soil which showed degradation of recalcitrant compounds from PMDE with 44% COD reduction. Sangeeta et al. (2011) have also isolated and characterized the potential MnP producing *Bacillus*, *Raoultella planticola*, and *Enterobacter sakazakii*. The consortium of these bacteria showed maximum 60% decolorization of synthetic melanoidin (SAA-MP; 2400 mg/l) at optimized nutrient, pH (7.0 ± 0.2), shaking speed (180 rpm), and temperature (35 ± 2 °C) after 144 h incubation. The addition of D-xylose at stationary stage enhanced the decolorization from 60 to 75% along with reduction of BOD and COD. Jain et al. (2002) isolated three bacterial strains, *B. megaterium*, *B. cereus*, and *B. fragariae*, from the activated sludge of distillery effluent, and these strains showed 38–58% color and 55–68% COD reduction from distillery effluent. Sirianuntapiboon et al. (2004) isolated acetogenic bacterial strains from juice and vegetable samples that were found effective to decolorize 76% molasses pigment medium and 73–76% anaerobically treated distillery effluent within 5 days when media was supplemented with glucose and nitrogen sources. Patel et al. (2001) have reported *Oscillatoria* sp., *Lyngbya* sp., and *Synechocystis* sp. for 96, 81, and 26% decolorization of distillery waste, respectively. Dahiya et al. (2001) have also reported *Pseudomonas fluorescens* from the reactor liquid and

Table 4.5 Different bacterial strains reported for the decolorization of PMDE

Bacterial strains	Decolorization conditions	Decolorization efficiency (%)	References
Lactobacillus hilgardii	Immobilized cells of the heterofermentative lactic acid bacterium decolorized 40% of the melanoidins solution within 4 days aerobically	40	Ohmomo et al. (1988)
Bacillus cereus, *B. megaterium*, and *B. smithii*	Decolorization occurred at 55 °C in 20 days under anaerobic conditions in the presence of peptone or yeast extract as supplemental nutrient. Strains could not use MWW as sole carbon source	76, 82 and 35.5, respectively	Kambe et al. (1999)
Acinetobacter sp., *Bacillus* sp., *P. paucimobilis, Pseudomonas* sp., *Acinetobacter* sp., and *Agrobacterium radiobacter*	These entire organisms were isolated from air bubble column reactor belonged to species of the genus *Bacillus* were used for treating winery wastewater after 6 months of operation	Not checked in this study	Petruccioli et al. (2002)
Acetobacter aceti	The organism required sugar, especially, glucose and fructose, for decolorization of MWWs	76.40	Sirianuntapiboon et al. (2004)
Pseudomonas fluorescens	Decolorization was obtained with cellulose carrier coated with collagen. Reuse of decolorization cells reduced the color	94	Dahiya et al. (2001)
Pseudomonas putida	The organism used glucose as a carbon source and produce hydrogen peroxide with reduction of color	60	Ghosh et al. (2002)
Xanthomonas fragariae, *B. megaterium*, and *B. cereus*	These three strains needed glucose as carbon and NH_4Cl as nitrogen source. Decolorization efficiency was found better in free cells as compared to immobilized cells	76	Jain et al. (2002)
B. brevis	The three strains were part of a consortium which decolorized the anaerobically digested spentwash in the presence of basal salts and glucose	22	Kumar and Chandra (2004)
Pseudomonas aeruginosa		27.4	
B. thuringiensis		67	Kumar and Chandra (2004)
Enterobacter, *Pseudomonas*, *Stenotrophomonas*, *Klebsiella*, and *Acinetobacter*	All were isolated from the soil of effluent discharge site, and addition of 1% glucose was necessary for decolorization	44	Ghosh et al. (2004)

(continued)

Table 4.5 (continued)

Bacterial strains	Decolorization conditions	Decolorization efficiency (%)	References
B. licheniformis, *Bacillus* sp., and *Alcaligenes faecalis*	All require the presence of glucose (1.0%) and peptone (0.1%) in medium and pH 7.0 and temperature 37 °C	70	Bharagava et al. (2009)
Bacillus sp., *Raoultella planticola*, and *Enterobacter sakazakii*	The three strains have potential to decolorized the synthetic melanoidin, and decolorization was enhanced by addition of D-xylose at stationary stage of growth	60 and 75, respectively	Sangeeta et al. (2011)
Proteus mirabilis, *Bacillus* sp., *Raoultella planticola*, and *Enterobacter sakazakii*	They decolorized the molasses melanoidin and PMDE after extraction of melanoidin	75 and 70, respectively	Sangeeta and Chandra (2012, 2013)

found that this bacterial strain was capable to decolorize melanoidin-containing wastewater up to 90 and 76% under sterile and non-sterile conditions, respectively. Marine cyanobacteria such as *Oscillatoria boryna* have potential to produce H_2O_2, hydroxyl, perhydroxyl, and active oxygen radicals for degradation of melanoidin (Kalavathi et al. 2001). The decolorization of molasses wastewater by immobilized cells of *P. fluorescence* on porous cellular carrier was undertaken achieving 76% decolorization in 24 hrs incubation at optimized conditions. *Bacillus* sp. has been reported to decolorize molasses wastewater by up to 36% under aerobic conditions within 20 days at 55 °C (Kambe et al. 1999). Kumar and Viswanathan (1991) isolated bacterial strains from sewage and acclimatized on increasing concentration of distillery waste. These strains reduced 80% COD within 4–5 days without any aeration, and the major products left after the effluent treatment were biomass, carbon dioxide, and volatile acids. Ohmomo et al. (1988) have used *Lactobacillus hilgardii* immobilized cells on calcium alginate for the decolorization of melanoidin under continuous supply of small amount of oxygen resulting in 40% decolorization of PMDE. Moreover, the differences in decolorization of PMDE might be due to the fact that melanoidin's stability varies with pH and temperature. At higher temperature, during the sterilization process, melanoidin pigment decomposes into low molecular weight compounds, while during the anaerobic treatment process, the pH of distillery spentwash increases from 4.5 to 8.5 during which melanoidins undergo complexation process forming complex which can't be easily degraded during the bacterial treatment of distillery effluents.

Recent Approaches for the Treatment of PMDE

The sequential bioreactor treatment of wastewater has become promising over the conventional treatment techniques to remove the complex mixture of pollutants from distillery wastewater. The bacterial pre-treatment of distillery effluent enhanced the phytoremediation process of distillery effluent (Kumar and Chandra 2004). In this study it was found that the recalcitrant compounds present in effluent that were ameliorated by *Bacillus thuringiensis* is through utilization of complex compounds. Therefore, foster phytoremediation of heavy metals was observed from the PMDE. Enzymatic pre-treatment for the enhancement of biodegradability of distillery wastewater has also been reported by Sangave and Pandit (2006a). The cellulase (enzymatic hydrolysis) followed by aerobic oxidation was investigated in this study for the treatment of distillery spentwash. The rate of anaerobic oxidation enhanced by two- to three fold in the pre-treatment sample as compared to the untreated sample when the pH during the pre-treatment step was maintained at a value of 4.8. Similarly, two fold increase in the aerobic oxidation rate was also observed when effluent was pre-treated with the enzyme without any pH control (effluent pH 3.8). This might be due to the enzymatic pre-treatment transforming the complex and large pollutant molecules into simpler biologically assailable smaller molecules. Sangave and Pandit (2006b) have also reported the role of the ultrasound and enzyme assisted biodegradation of distillery wastewater. Efficiency of these two techniques was analyzed by subjecting the effluent to subsequent aerobic biological oxidation (AO). The distillery effluent when treated with ultrasound (US) alone for 30 min, COD reduction was found best after 2 h during the aerobic oxidation step (US+AO). However, the 50 U of enzyme was used alone at pH 4.8 for 24 h, which yielded better COD removal as compared to untreated effluent, while US followed by enzymatic pre-treatment resulted in maximum COD removal efficiency during the aerobic oxidation (US+E + AO) process as compared to the other combinations tested for the treatment of distillery effluent. Moreover, the application of constructed wetland at pilot scale for the treatment of industrial wastewaters was also reported by various authors. Chandra et al. (2008c) reported that the integration of bacterial pre-treatment of PMDE with *Typha angustata* enhanced the reduction of heavy metals, i.e., Cr (36–58%), Cd (34–62%), Fe (33–51%),Cu (33–54%), Ni (36–60%), Mn (36–83%), Zn (32–54%), and Pb (33–60%) in comparison with control lacking this pre-treatment step. In addition to the removal of heavy metals, other physico-chemical parameters, viz, color, COD, BOD, total nitrogen, and phenol, were also reduced significantly by 98%, 99%, 99%, 94%, and 82%, respectively, at 30% effluent after 7 days of free water surface flow treatment process. This indicated that the bacterial pre-treatment of PMDE followed by phytoremediation will significantly boast the treatment process of PMDE. Chen et al. (2006) used four different wetland plant species including floating plants (*Pistia stratiotes*, *Ipomea aquatica*) and emergent plants (*Phragmites communis*, *Typha orientalis*) for the removal of COD, BOD, and suspended solids from industrial wastewater and found that the system with a 50 HRT (hydraulic

retention time) (feed rate 0.4 m^3/day) and vesicles ceramic bioballs as the media was effective to remove 61% COD, 89% BOD and 81% suspended solid (SS), 35% total phosphate (TP), and 56% NH_3-N.

The use of distillery effluent for agriculture has been advocated due to the presences of high amount of nitrogen and phosphorous. But its indiscriminate application may create adverse effects on crop and contamination of groundwater. Kaushik et al. (2005) reported that long-term application of PMDE causes significant increase in total organic carbon (TOC), total kjeldahl nitrogen (TKN), potassium, and phosphorus along with accumulation of high concentration of sodium ions (Na^+) in soil. Moreover, the short-term application of 50% PMDE along with bioamendments proved most useful in improving the properties of sodic soil and also favored the successful germination and improving seedling growth of pearl millet. Similar observations were recorded by Chandra et al. (2004) during a study on the impact of pre-treatment and PMDE irrigation on soil microflora and growth of *Phaseolus aureus* in pot-grown experiment. They concluded that lower concentration of raw distillery effluent (1–5%) and PMDE (1–10%) stimulated the growth of *P. aureus* and soil microflora, except soil bacteria (inhibited by all the concentrations of raw effluent). However, higher concentration (raw effluent, 10–20%, and PMDE, 15–20%) showed toxicity. Similar toxicity pattern was also observed by Sangeeta and Chandra (2006) on *Vicia faba* where they reported that the sludge of PMDE also contains high quantity of organic and inorganic pollutants. The sludge-amended soil above 10% (W/V) concentration causes adverse effects on legume crops. The sludge concentration above 50% causes toxicity to plants and even inhibits the seed germination. Further, the continuous irrigation with PMDE affects the underground water quality by increasing 40% TDS with salt (Jain et al., 2005). A study by Hati et al. (2007) on soil properties and crop yield on a vertisol in India shows increase in organic carbon and salinity. The study supported the judicious application of distillery effluent as an amendment to the agricultural field can be considered as an available option for its safe disposal into the environment.

PMDE Treatment with Consortium

During the last two decades experiments were restricted only to use the axenic culture and immobilized culture on solid supports for the degradation of toxic compounds. Recently, immobilized consortia of two or more selected bacterial strains were employed (Kowalska et al. 1998). The use of bacterial consortium for the treatment of distillery wastewater is highly effective because mixed bacterial culture comprise of many strains which are very helpful in co-metabolism employing different sets of enzymes. The pH and temperature play a very important role for optimum activity of enzymes. In co-metabolism, the metabolites or intermediates produced by one bacterial strain are utilized by other bacterial strains as source of nutrient and energy (Chandra et al. 2007; Bharagava and Chandra 2010). Some authors have also reported the use of jet loop reactors (JLR) for the aerobic treatment

of winery wastewater. Petruccioli et al. (2002) used a JLR with working volume of 15 dm^3 for the aerobic treatment of winery wastewater, and they recorded more than 90% COD removal efficiency with an organic load of the final effluents ranging between 0.11 and 0.3 kg COD m^{-3} at much diluted concentration of effluent. Eusibio et al. (2004) have also reported the operation of a JLR for more than 1 year treating winery wastewater collected in different seasons and achieved 80% COD removal efficiency at diluted concentration of distillery effluent. They also reported that JLR have higher oxygen transfer rate at lower energy costs. Hence the use of mixed bacterial culture for the treatment of industrial wastewater is more effective compared to a single pure bacterial strain.

Phytoremediation Approach for Treatment of PMDE

Phytoremediation is a low-cost technique which is very useful to remediate sites contaminated with recalcitrant toxic compounds and heavy metals. Phytoremediation takes advantage of plant nutrient utilization processes, transpires water through leaves, and acts as transformation system to metabolize organic compounds. They may also absorb, sequester, and bioaccumulate toxic compounds. Billore et al. (2001) used *Phragmites kharka* in a constructed wetland for the treatment of wastewater from the same industry and obtained 36% removal of TKN and 48% removal of TSS. Detoxification of distillery effluent through *B. thuringiensis* (MTCC 4714) enhanced phytoremediation potential of *Spirodela polyrrhiza* (Kumar and Chandra 2004).

Recently, a comparative heavy metal accumulation pattern and ultrastructural changes were studied in *P. communis*, *T. angustifolia*, and *C. esculentus* in both in situ and ex situ conditions (Chandra and Sangeeta 2011; Sangeeta and Chandra 2011). In situ study showed direct correlation of bioaccumulation with the metal content in sediments. Both macrophytes *T. angustifolia* and *C. esculentus* were observed as root accumulators for Fe, Cr, Pb, Cu, and Cd, while ex situ condition showed the maximum accumulation of all tested heavy metals in *P. communis* followed by *T. angustifolia* and *C. esculentus*. Results revealed that *P. communis* and *T. angustifolia* had more potential for tested metals than *C. esculentus*. Similarly, *Potamogeton pectinatus* has also been reported for the bioaccumulation of heavy metals from distillery effluent (Singh et al. 2005), and an increase in effluent concentration greatly reduced the biomass of plant with maximum accumulation of Fe being recorded in plants growing at 100% effluent concentration. In another study, Trivedy and Nikate (2000) have employed *Typha latifolia* for the treatment of distillery effluent in a constructed wetland treatment system resulting in 78% and 47% reduction in COD and BOD, respectively, within a period of 10 days. Using a combined treatment with *Lemna minuscula* and *Chlorella vulgaris*, Valderrama et al. (2002) have achieved 52% color removal from distillery effluent. The microalgal treatment removed nutrients and organic matter from wastewater and produced oxygen for other organisms. The microphyte removed organic matter and eliminated the microalgae from treated wastewater. However, despite the potential

of aquatic macrophytes in cleaning wastewater, the use of these plants in designing a low-cost treatment system is still at experimental stage and is considered to be a potentially important area of environmental management.

Role of Enzymes in PMDE Decolorization

Although the enzymatic process related with detoxification and decolorization of melanoidins and PMDE is yet to be completely understood, it seems greatly connected with fungal ligninolytic mechanisms. The white-rot fungi have complex enzymatic systems which are nonspecific, extracellular, and work under nutrient-limiting conditions. A large number of enzymes from different microorganisms and plants have been reported for the treatment of wastewater discharge from various industries. Important amongst these are ligninolytic enzymes, i.e., manganese peroxidase (MnP), lignin peroxidase (LiP), and laccase reported by several workers for degradation and detoxification of PMDE and synthetic and molasses melanoidin (Ghosh et al. 2002; Petruccioli et al. 2002; Sangeeta et al. 2011; Sangeeta and Chandra, 2012; Sangeeta and Chandra 2013). The catalytic cycle of MnP also resembles with other heme peroxidases, i.e., horseradish peroxidase (HRP) and lignin peroxidase and includes the native ferric enzyme as well as the reactive intermediates compound I and compound II (Fig. 4.4). In contrast to other peroxidases, MnP uses Mn^{2+} as the preferred substrate as electron donor. The catalytic cycle of peroxidases is starting with the reaction of native ferric enzyme (Fe^{3+}-P (porphyrin)) with H_2O_2 yielding compound 1. Compound 1 is a complex of high-valent oxo-iron and porphyrin cation radical (Fe^{4+} = O, P). Compound 1 is reducing a substrate (RH) and yielding a radical cation ($R^{\cdot}$) and one-electron-oxidized enzyme intermediate compound II (Fe^{4+} = O, P). A single one-electron oxidation of a substrate molecule returns the enzyme to the native ferric state, completing the catalytic cycle. Similarly MnP activity is initiated by binding of H_2O_2 or organic peroxide to the native ferric enzyme and formation of an iron-peroxide complex. Subsequent cleavage of the peroxide oxygen-oxygen bond requires a two-electron transfer from the heme resulting in formation of MnP compound I, which is a Fe^{4+}-oxo-porphyrin-radical complex. Afterwards, the

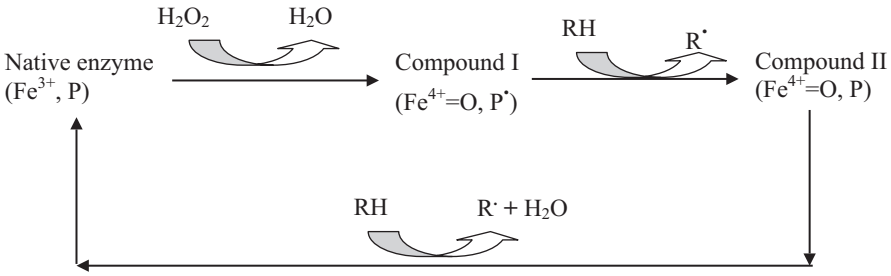

Fig. 4.4 Catalytic cycle of peroxidases

dioxygen bond is heterolytically cleaved and one water molecule expelled. Subsequent reduction proceeds through MnP compound II (Fe^{4+}-oxo-porphyrin complex). A monochelated Mn^{2+} ion acts as one-electron donor for this porphyrin intermediate and is oxidized to Mn^{3+}. The reduction of compound II proceeds in a similar way, and another Mn^{3+} is formed from Mn^{2+} thereby leading to generation of native enzyme and release of the second water molecule. The Mn^{3+} formed is stabilized by organic acids such as oxalate and acts in turn as a low-molecular mass, diffusible redox mediator that attacks organic molecules nonspecifically via hydrogen and one-electron abstraction.

Laccases are common enzymes in nature and are found widely in plants and fungi as well as in some bacteria and insects. Different types of laccases are secreted by different organisms, but they all catalyze polymerization or depolymerization processes including phenols, polyphenols, anilines, and even certain inorganic compounds by a one-electron transfer mechanism. Laccase is a multicopper blue oxidase which reduces oxygen molecules leading to oxidation of broad range of organic substrates. The laccase is a dimeric or tetrameric glycoprotein usually containing per monomer four copper (Cu) atoms bound to three redox sites (Type 1, Type 2 and Type 3 Cu pair). The Type 2 and Type 3 Cu sites are close together and form a trinuclear center that is involved in the catalytic mechanism of the enzyme. The molecular mass of the monomer ranges from about 50 to 100 kDa with acidic isoelectric point around pH 4.0. For the catalytic activity a minimum of four Cu atoms per active protein unit are needed. To function, laccase depends on Cu atoms distributed among the three different binding sites. Cu atoms play an essential role in the catalytic mechanism. There are three major steps in laccase catalysis. The Type 1 Cu is reduced by a reducing substrate, which therefore is oxidized. The electron is then transferred internally from Type 1 Cu to a trinuclear cluster made up of the Type 2 and Type 3 Cu atoms. The O_2 molecule is reduced to water at the trinuclear cluster. The O_2 molecule binds to the trinuclear cluster for asymmetric activation, and it is postulated that the O_2 binding pocket appears to restrict the access of oxidizing agents other than O_2. H_2O_2 is not detected outside of laccase during steady-state laccase catalysis indicating that a four-electron reduction of O_2 to water is occurring. A one-electron substrate oxidation is coupled to a four-electron reduction of oxygen.

Several reports claimed that intracellular sugar-oxidase-type enzymes (sorbose-oxidase or glucose-oxidase) also had melanoidins-decolorizing activities. It was suggested that melanoidins were decolorized by the active oxygen (O_2; H_2O_2) produced by the reaction with sugar oxidases. Ohmomo et al. (1985) used *Coriolus versicolor* Ps4a, which decolorized molasses wastewater by 80% in darkness under optimum conditions. Decolorization activity involved two types of intracellular enzymes, i.e., sugar-dependent and independent. One of these enzymes required no sugar and oxygen for their activity and could decolorize molasses wastewater up to 20% in darkness and 11–17% of synthetic melanoidins. Thus, the participation of these H_2O_2 producing enzymes as a part of the complex enzymatic system for melanoidin degradation by fungi should be taken into account while designing any treatment strategy. Color removal of synthetic melanoidin by *Coriolus hirsutus* involved the participation of peroxidases (MnP and MIP) and the extracellular H_2O_2

produced by glucose-oxidase, without disregard of a partial participation of fungal laccase. Mansur et al. (1997) obtained a maximum decolorization of around 60% by fungus *Trametes* sp. I-62 after 8th day incubation. Here effluent was added at a final concentration of 20% (v/v) after 5 days of fungal growth, the time at which high levels of laccase activity were detected in the extracellular mycelium. The white-rot basidiomycete *Trametes versicolor* is an active degrader of humic acids as well as of melanoidins. Melanoidins mineralizing 47 KDa extracellular proteins corresponding to the major mineralizing enzyme system from *T. versicolor* was isolated by Dehorter and Blondeau (1993). This Mn^{2+}-dependent enzyme system required oxygen and was described to be as peroxidase. Some studies have identified the lignin degradation-related enzymes participating in the melanoidin decolorization. Intracellular H_2O_2 producing sugar oxidases have been isolated from *Coriolus* strains. Also, *C. hirsutus* have been reported to produce enzymes that catalyze melanoidins decolorization directly without additions of sugar and O_2. Miyata et al. (1998) used *C. hirsutus* pellets to decolorize a melanoidin-containing medium. It was elucidated that extracellular H_2O_2 and two extracellular peroxidases, a manganese-independent peroxidase (MIP) and manganese peroxidase (MnP), were involved in decolorization activity. Lee et al. (2000) investigated the dye-decolorizing peroxidase by cultivating *Geotrichum candidum* Dec1 using molasses as a carbon source. Components in the molasses medium stimulated the production of decolorizing peroxidase but inhibited the decolorizing activity of the purified enzyme. D'souza et al. (2006) reported 100% decolorization of 10% spentwash by a marine fungal isolate whose laccase production increased several folds in the presence of phenolic and non-phenolic compounds. A combined treatment technique consisting of enzyme followed by aerobic biological oxidation was investigated by Sangave and Pandit (2006a) for the treatment of spentwash. It was suggested that enzymatic pre-treatment of the distillery effluent leads to in situ formation of the hydrolysis products, which have different physical properties and are easier to assimilate than the parent pollutant molecules by the microorganisms, leading to faster aerobic oxidation even at low biomass. In another study, Sangave and Pandit (2006b) used ultrasound combined with the use of an enzyme as pre-treatment technique for treatment of distillery wastewater. The combination of the ultrasound and enzyme also reported significant COD removal efficiencies as compared to the processes when they were used as stand-alone treatment techniques. Panicker et al. (2015) have showed the possibility of using immobilized enzyme to enhance the biodegradability of the distillery spentwash. The investigations so far can be seen as an initial step toward solving the problem. Moreover, most of these microbial decolorization studies required effluent dilution for optimal activity. While, using microorganisms use of media supplement pose extra burden on overall effluent treatment process. Further, the emerging treatment methods like enzymatic treatment have technological advantages and yet are in its infancy, requiring economic considerations in order to apply on the plant scale. Hence, there is need of suitable technique to treat distillery effluent. Furthermore, capital and operating costs of the available physico-chemical and biological treatment processes of distillery waste stream are inevitably high thus making these processes less lucrative to the industry.

Metabolites Produced During the Bacterial Treatment of PMDE

The study of metabolites produced during the degradation process is very essential to explore the degradation pathway of any pollutant as well as to develop the effective bioremediation techniques. The structural characterization of Maillard reaction products (MRPs) has proved to be rather challenging due to extreme complexity of MRPs that are known to range from small molecules to extremely large polymers. There are many techniques available which are used for characterization of large, complex pollutants and metabolites/intermediate compounds, produced during degradation process (Table 4.6). By using these techniques, some authors have studied the chemical nature of melanoidins and reported that these are complex polymers consisting of repeating units of furans and/or paroles, which are produced during the advance stages of Maillard reaction

Table 4.6 Analytical techniques used for the analysis of Maillard reaction products (MRPs)/ melanoidin

Maillard reaction products (MRPs)	Analytical techniques
Amadori compounds (direct analysis)	Column chromatography, HPLC differential refractometry detection, HPLC involving derivatization, HPAEC coupled electrochemical and/or DAD, FAB-MS, ESI coupled HPLC and EC, EC coupled MS, MALDI-TOF, NMR, LC-MS, NBT, ELISA
Indirect analysis (2-FM-AA)	Immunoblotting (lactosylated proteins), ion-pair RP-HPLC, CEC UV detection
Unreactive lysine	Colorimetric and fluorimetric methods
Advanced Maillard reaction products	FAST
General AGEs	HPLC-DAD
CML	RP-HPLC, RP-HPLC o-phthalaldehyde pre-column derivatization, GC-MS, ELISA
Pyrraline	Amino acid analysis with PAD, RP-HPLC
Cross-linking products lysine dimmers	LC-MS with ESI
Arginine-lysine	LC-MS with ESI, ion-exchange chromatography
Other amino acid derivatives, argpyrimidine	HPLC-coupled GC-MS
OMA	ELISA
PIO	RP-HPLC/LC-ESI-TOF-MS/NMR
Pyrazinones	HPLC with UV and fluorescence detection
Lysine amnioreduction	HPLC-DAD
Final stage MRPs	
General melanoidins	HPLC, NMR, MS, UV, IR spectrometry
Pronyl-L-lysine	GC-MS chemical ionization

Jones et al. (1998), Silvan et al. (2006) and Shen et al. (2007)

and remain linked by polycondensation reactions (Tressl and Wondrak 1998; Gonzalez et al. 2000; Bharagava and Chandra 2010). Hofmann (1998) also reported that MRPs are low molecular weight colored compounds, which cross-link to proteins via amino groups of lysine or arginine and polymerized through aldol-type condensation reactions to produce high molecular weight colored MRPs. The chemical structure of these MRPs is mainly built with sugar degradation products, which largely depends on the starting materials as well as on the reaction conditions (Cammerer and Kroh 1995). Some authors have also reported that the electro spray ionization mass spectrum (ESI-MS) infrared (IR)^{1}H NMR and LC-MS-MS analysis of untreated distillery wastewater has shown the presence of dihydroxyconiferyl alcohol ($C_{10}H_{14}O_3$), 2,2′-bifuran-5-carboxylic acid ($C_9H_6O_4$), 2-nitroacetophenone ($C_8H_7NO_3$), p-chloroanisole (C_7H_7ClO), 2,3-dimethyl pyrazine ($C_6H_8N_2$), 2-methylhexane (C_7H_{16}), methyl benzene (C_7H_8),1,2- dihydro-5 methylfuran (C_5H_8O),3-pyrroline (C_4H_7N), and acetic acid ($C_2H_4O_2$), while in treated distillery wastewater, compounds 2-nitroacetophenone ($C_8H_7NO_3$), p-chloroanisole (C_7H_7ClO), 2,2-bifuran($C_8H_6O_2$), indole (C_8H_7N), 2-methylhexane (C_7H_{16}), and 2,3-dihydro-5-methylfuran (C_5H_8O) were reported (Gonzalez et al. 2000; Bharagava and Chandra 2010). Further, most of the detected metabolic products were reported to as degradation products of pyrroles/furans, which remains present in distillery wastewater.

Challenges for the Degradation and Decolorization of PMDE

The major problem of PMDE is its complex nature due to the presence of recalcitrant constituents that inhibit the growth of microbes during the effluent treatment process in industries. The major coloring constituent, i.e., melanoidins, has a net negative charge and, thus, forms large complex molecules with different heavy metal ions (Cu^{2+}, Cr^{3+}, Fe^{3+}, Zn^{2+}, Pb^{2+}, etc.) in acidic medium and gets precipitated. Besides melanoidins, distillery effluent contains various phenolic compounds such as gallic acids, p-coumaric acid, gentisic acid, and 3–4 dimethoxy 2 (1,1-hydroxyethyl)α-naphthol (Borja et al. 1993; Bharagava et al. 2006). These compounds have inhibitory and antimicrobial activity reducing the anaerobic digestion process of distillery effluent in methane reactors. Further, the sulfates and heavy metals present in PMDE get reduced into black-colored precipitate of metal sulfides, which also act as competitive inhibitor for sulfate-reducing bacteria (SRB) and non-SRB (McCartney and Oleszkiewicz 1991) leading to the inhibition of methanogenesis process. In addition, the presence of EDCs makes effluent more toxic, and complete knowledge of these is lacking.

Thus, it becomes very essential to study the effect of sulfides and metals along with phenolics as well as other compounds on decolorization and the detoxification of PMDE. Similarly complete knowledge of EDCs is mandatory before biological treatment and discharge of effluent in the environment.

Future Prospects

Alcohol production and distilleries are one important commodity and industry world over. But, the huge generation of complex wastewater is still a challenge for its safe disposal and pollution prevention for sustainable development. Therefore, the detailed characterization and biodegradation of complex pollutants present in PMDE is a priority need of researches and industries. The potential microorganisms including bacteria, fungi, and cyanobacteria may be optimized for the attenuation of the complex pollutants with the enzymatic activity (MnP and laccase). Further, the potential wetland plants which enhance the biodegradation may be integrated for degradation of PMDE. The detoxified and decolorized PMDE may be recycled for agricultural practices and green vegetation development around the industrial and urban areas. In addition, generated sludge and biomass in developed treatment technology can be formulated for biocomposting as organic manure for the sustainable development and pollution prevention of aquatic resources.

Conclusion

PMDE is generated after methanogenesis of sugarcane molasses-based spentwash which contains dark color, high TDS due to the presence of complex mixture of organic and inorganic pollutants. The complex organic and inorganic pollutants inhibit biodegradation. Therefore, the degradation and decolorization of PMDE is still a challenge for researchers and industries. Thus the complete knowledge regarding the nature of organic and inorganic pollutants present in PMDE is essential. Further, its biodegradation in valuable products is also essential. The reported research knowledge is still inadequate. The intergradations of bacterial treatment with phytoremediation may be effective and feasible techniques for treatment of this complex wastewater.

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