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Review

Enzymatic demethylation of lignin for potential biobased polymer applications

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ABSTRACT

Lignin is a highly methylated, recalcitrant biopolymer available aplenty in nature, and is highly heteropolymer in nature, but yet it has been an under-utilized biopolymer. Modifying it chemically, biologically or enzymatically could render it a good candidate for phenol formaldehyde resin or into fine chemicals, fuels, and plastics applications. Lignin demethylation is facilitated by the enzymes called the *O*-demethylases, which are able to strip-off of the $-OCH_3$ group in lignin, that give rise to the more widely accessible phenolic hydroxyls groups. Biological demethylation of lignins can be accomplished by means of the microorganisms, such as the white-rot, soft-rot and brown-rot fungi, besides some species of bacteria. Although the enzymes responsible for the lignin demethylation process have not been identified and purified adequately, it is perhaps possible that the *O*-demethylases, which have the ability to remove the *O*-methyl groups at the C-3 and (or) C-4 positions of the benzyl ring of low molecular weight lignin-like model compounds (LMCs) and lignin makes them the suitable candidate. These LMCs resemble the aromatic moieties inherent in the molecular structure of lignins, such as the vanillate, syringate, and veratrate. Thus, these enzymes are known as vanillate-*O*-demethylases, syringate *O*-demethylases, veratrate *O*-demethylases and Tetrahydrofolate (THF)-dependent *O*-demethylase (LigM), respectively. Whereas, some ligninolytic enzymes are known to cause damage to the structure of lignins (e.g., laccases, manganese-dependent peroxidase and lignin peroxidases). The *O*-demethylase enzymes are believed to be capable of removing the *O*-methyl groups from the lignins without affecting the complex backbone structure of the lignins. The mechanism of action of *O*-demethylases on lignin degradation is still largely unexplored, and their ability to remove the *O*-methyl groups from lignins has not been elucidated sufficiently. In this review, the recent advances made on the molecular approaches in the lignin demethylation (*O*-demethylases and ligninolytic enzymes), degradation and the probable strategies to tone up the lignin quality have been discussed in detail. The demethylation process of lignins by means of enzymes is envisaged to open up new vistas for its application as a biopolymer in various bioprocess and biorefinery process.

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1. Introduction

O-demethylases catalyze the demethylation process of lignin and the lignin-like compounds (LMCs) and cause the strip off of the O-methyl group to release methanol as the end product and renders the lignin more phenolic in nature, which makes it amenable for its application as a bio polymer (Abdelaziz *et al.*, 2016; Rosini *et al.*, 2016; Kohler *et al.*, 2017; Kamimura *et al.*, 2017; Venkatesagowda, 2018; Venkatesagowda and Dekker, 2019). By making suitable modifications in biomass using the green chemistry approaches on native biopolymers, it is possible to generate value-added fine chemicals and bio-materials, such as bioethanol, biobutanol, pharmaceuticals, adhesives, and composites. To this end, chemical alterations of certain biomass-derived polymers, such as cellulose, hemicellulose and lignin are imperative. In nature, the plant biomass is mainly comprised of cellulose, hemicellulose and lignin and makes a heterogeneous polymeric network. Among them, lignin is widely available. Furthermore, the unused plant residue from the biorefinery and the pulp and paper industry is readily available for applications elsewhere. The chemical and enzymatic modification of lignin by means of different approaches, like oxidation, hydroxylation, depolymerization, and polymerization, besides demethylation is pivotal for this purpose. However, the demethylation event renders demethylation process is an important step that generates appreciable amount of the hydroxyl groups in highly recalcitrant lignin in biorefinery process. Lignin demethylation has been studied with several bacteria, brown-rot, white-rot and the soft-rot fungi (Filley *et al.*, 2000; Martinez *et al.*, 2011; Bashtan-Kandybovich *et al.*, 2012; Prabhakaran *et al.*, 2015; Abdelaziz *et al.*, 2016; Rosini *et al.*, 2016; Kohler *et al.*, 2017; Venkatesagowda, 2018). Some of them have the ability to secrete the *O*-demethylases that can demethylate the Kraft lignin (KL) and the lignin-like compounds (LMCs) and to release methanol by a specific cleave-off mode of the O-methyl groups. Lignin demethylation is considered to be a hitherto unknown function of the lignin demethylases. However, adequate information on the purification protocols of this enzyme, for its application on an industrial scale, as a polymer is not available, to date.

The *O*-demethylases, which have the ability to remove the *O*-methyl groups at the C-3 and (or) C-4 positions of the benzyl ring of lignin-like model compounds (LMCs) and lignin. Various fungi have the ability to demethylate LMCs and lignin, which are *Penicillium simplicissimum*, *P. cinnabarinus* A-360 (Buswell *et al.*, (1982), *Chaetomium piluliferum* and *Xerocomus badius* (Paszczyfiski and Trojanowski, 1977; Paszczyinski et al., 1985, 1986), *Gloeoporus* (*Polyporus*) *dichrous* (Kirk and Lorenzo's, 1974), *Phanerochaete chrysosporium* (Ander *et al.*, 1985), *Haplographium* sp., *Hormodendrum* sp., and *Penicillium* sp., (Henderson, 1957), *Aspergillus* sp. and *Galerina autumnalis* (Venkatesagowda, 2018), and brown-rot, *Fomitopsis pinicola* and *Galerina autumnalis*, and a mitosporic *Aspergillus* sp.3 BRI 270 (Venkatesagowda and Dekker, 2019). Also some bacteria can produce *O*-demethylases such as *Nocardia corallina* A81 (Crawford *et al.*, 1973), *Pseudomonas putida* (Hibi *et al.*, 2005), *Rhodococcus jostii* RHA1 (Chen *et al.*, 2012), *Desulfitobacterium hafniense* strain DCB-2 (Studenik *et al.*, 2012), *Rhodococcus erythropolis* (Malarczyk *et al.*, 2011) and *Sphingomonas* sp. SYK6 (Kamimura *et al.*, 2017). These fungal and bacteria able to secrete various *O*-demethylases such as veratrate *O*-demethylase (Paszczyfiski and Trojanowski, 1977; Crawford *et al.*, 1973), vanillate *O*-demethylase (VanAB) (Chen *et al.*, 2012), 3-*O*- or 5-*O*-demethylation process (Tai *et al.*, 1982), vanillate *O*-demethylase (VanA and VanB) ((Hibi *et al.*, 2005; Nishimura *et al.*, 2014a, b), vanillate *O*-demethylase oxidoreductase (Kumar *et al.*, 2016), Rieske monooxygenase VanA and VanB (Lanfranchi *et al.*, 2018), syringate *O*-demethylase (Masai *et al.*, 2004), p-anisate-*O*-demethylase (Cartwright and Buswell, 1967; Bernhardt *et al.*, 1970; Buswell and Mahmood, 1972), NADPH- and the O_2- dependent oxygenases (Bernhardt *et al.*, 1970), oxygen-dependent demethylase (Malarczyk *et al.*, 2011), tetrahydrofolate (THF)-dependent *O*-demethylase (Rosini *et al.*, 2016), DDVA *O*-demethylase (Harada *et al.*, 2017; Kohler *et al.*, 2017; Kamimura *et al.*, 2017), cytochrome P450 aromatic *O*-demethylase (Mallinson *et al.*, 2018) has been reported in literature.

In the literature, the terms demethylation (-O-CH_3) and demethoxylation (-OCH_3) have often been used interchangeably, implying different reaction mechanisms, perhaps adding to the confusion of the reader. Hence, the term demethylation has been used in this review without any ambiguity, only to describe the reactions, where the final product apparently loses a methyl group from an aryl methoxyl complex, regardless of the inherent mechanistic subtlety (i.e., methoxyl substitution). Such demethylation reactions primarily give rise to methanol, formaldehyde or CO_2 (Haider and Trojanowski, 1975; Ander and Eriksson, 1978; Crawford, 1981). Initially, the lignin demethylation process has been observed with ruminants and the insects associated with the gut microbiota (Csonka *et al.*, 1929; Fernandez and Regueiro, 1946; Geib *et al.*, 2008) besides with the naturally decaying organic matter (Bray and Andrews, 1924; Sowden and Atkinson, 1949; Filley *et al.*, 2000; van Bergen *et al.*, 2000; Vane, 2003; Vane *et al.*, 2006; Lucejko *et al.*, 2009; Martinez *et al.*, 2011; Abdelaziz *et al.*, 2016; Kohler *et al.*, 2017; Venkatesagowda, 2018). The wood-rot fungi mainly mediate this process. Significant information pertaining to the biochemistry of these fungi has accumulated, with important advances having been made in understanding the enzymes involved in the lignin decay as mediated by the fungi. In the early 1980's, two ligninolytic enzymes from the white-rot fungus, *Phanerochaete chrysosporium* have been described: lignin peroxidase (LiP) (Tien and Kirk, 1983; Glenn *et al.*, 1983) and manganese-dependent peroxidase (MnP) (Kuwahara *et al.*, 1984) are known to depolymerize lignin by a set of oxidative reactions by making use of hydrogen peroxide (H_2O_2) as a co-substrate. Laccase is another important ligninolytic multi-copper oxidase/oxidoreductase enzyme, first isolated in the 1880's from the Japanese lacquer tree, *Rhus fernicicfera* (Messerschmidt, 1997). The oxidoreductase enzymes, such as laccase, lignin peroxidase, Mn-peroxidase have been implicated in the lignin demethylation process and are believed to generate the vicinal hydroxyl in a polyphenylpropanoid unit (Ibrahima *et al.*, 2011; Paice *et al.*, 1995; Bashtan-Kandybovich *et al.*, 2012; Rashid, *et al.*, 2015; Rashid *et al.*, 2018; Wang *et al.*, 2018). It was not until the mid 1990's that definitive proof of

their involvement in lignin mineralization to methanol was established (Eggert *et al.*, 1997). Lignin mineralization, by way of quinone reduction increases the number of the hydroxyl groups involved and a precondition for MnP and laccase enhance the expression of the enzymes and stimulate the radical systems and the reactive oxygen species (ROS) that are involved in the degradation of the lignin-related dimers, besides lignin (Srebotnik and Hammel, 2000; Hammel *et al.*, 2002).

Methanol production occurs either by removal of the methyl group (demethylation) or of the methoxyl group (demethoxylation) from lignin, while in the aromatic compounds, the same occurs by the extracellular lignin-degrading reactions, or by some intracellular demethylating enzymes of different microbial origins (Fig. 1a and b). However, the intracellular demethylating or demethoxylating enzymes have not been described from the white-rot fungi. The efficacy of white-rot (WR) fungal decay (*P. chrysosporium*) of the spruce lignin has been monitored by the ^{13}C NMR analysis, whereby the demethylation process of the aromatic methoxy groups (Tai *et al.*, 1983) has been elucidated. The MnP and laccase activity of the white-rot fungi-mediated radical systems and the reactive oxygen species' involvement in the demethylation process has also been observed (Srebotnik *et al.*, 1988; Hammel *et al.*, 2002). A mutant utilizes the 3-^{14}C-methoxy labelled veratrate and evolves $^{14}CO_2$ (Ander and Eriksson, 1976). However, several studies adequately suggest that the demethylation reactions primarily produce methanol (Bashtan-Kandybovich *et al.*, 2012; Zou *et al.*, 2015; Venkatesagowda, 2018; Venkatesagowda and Dekker, 2019) only few studies suggest the generation of formaldehyde or CO_2 as the demethylation product (Kamimura et al., 2019; Niemenman *et al.*, 2008).

The brown-rot (BR) wood decay process is, perhaps initiated by the Fenton chemistry involving Fe^{2+} with H_2O_2 for the generation of the hydroxyl anions and radicals that can simultaneously act on the methyl groups of lignin and produce the demethylated lignin as a residue, even while degrading cellulose (Kirk and Adler, 1970; Ander *et al.*, 1988; Agosin *et al.*, 1989; Enoki *et al.*, 1988; Jin et al. 1990a, 1990b; Filley et al., 2000, 2002; Vane *et al.*, 2006; Martínez *et al.*, 2011). Initially it was believed that the white-rot fungi rapidly

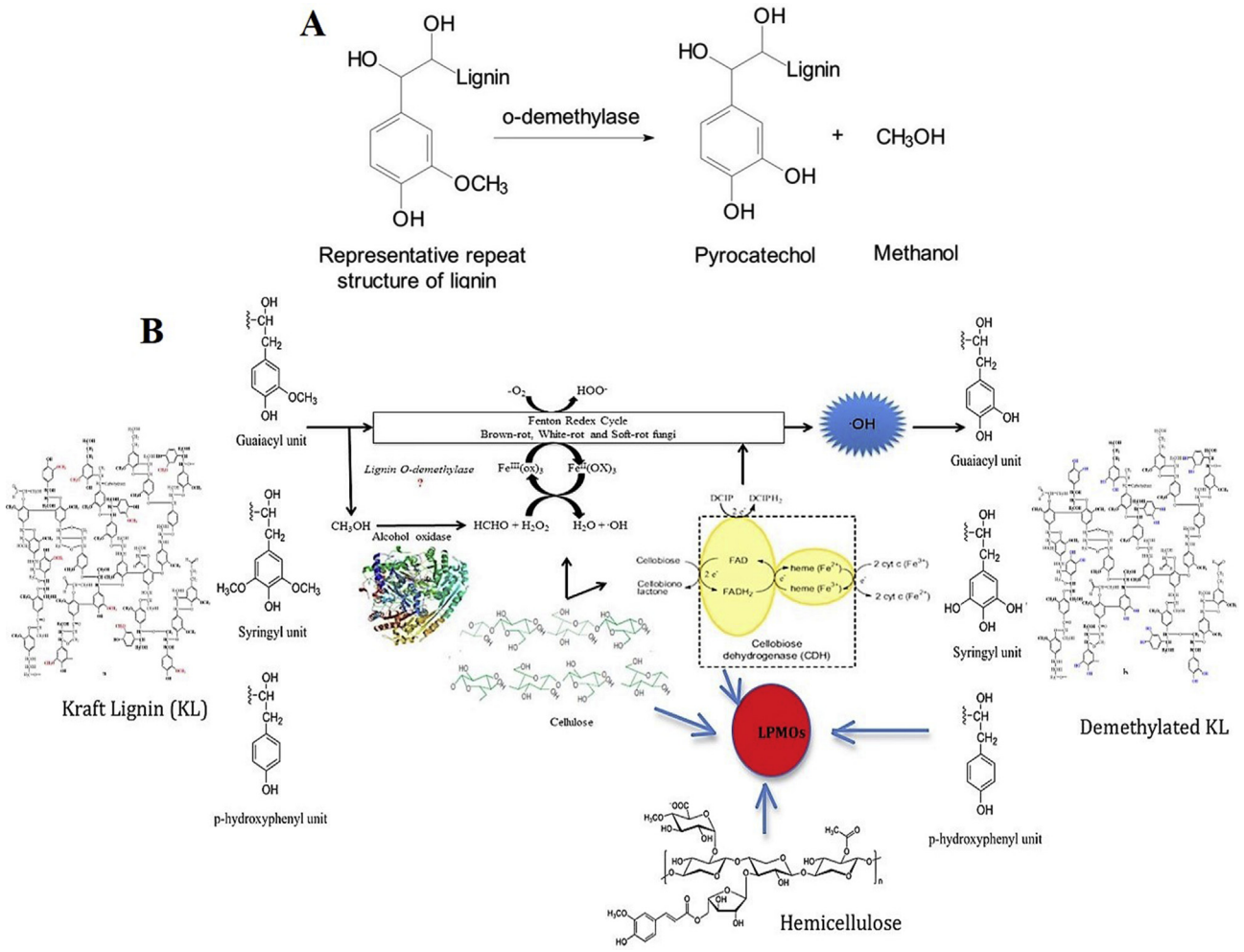

Fig. 1 – a. Enzymatic O-demethylase mediated lignin demethylation that generates methanol and pyrocatechol as by products. Fig. 1b. The possible mechanism of enzymatic fungal demethylation of krfat lignin (KL) (a) KL (b) demethylated KL (Venkatesagowda and Dekker, 2019).

degrade lignin and do not enhance the dihydroxybenzene units. It has also been suggested that the white-rot organisms also rapidly demethylate lignin (Buswell and Cain, 1973). The soft-rot have been investigated for their possible involvement in the demethylation of lignin-like compounds, besides lignin. Several investigators worked on the O-demethylase secreting ability of organisms, purification of the enzyme and the genes involved in the demethylation reactions (Cain, 1980; Masai *et al.*, 2004; Malarczyk *et al.*, 2011; Yoshikata *et al.*, 2014). Several methods have been evolved to analyze the demethylation process by high performance liquid chromatography (HPLC), Fourier transform infrared spectroscopy (FTIR) and the ^{13}P nuclear magnetic resonance (NMR) analyses to establish that *Botryosphaeria rhodina* laccase can demethylate lignin and produces methanol as an end product (Bashtan-Kandybovich *et al.*, 2012). Various approaches have been adopted, so as to detect the demethylation of lignin, including ^{13}C-TMAH thermochemolysis (Suzuki *et al.*, 2006; Daniel et al., 2007; Arantes *et al.*, 2009; Martínez *et al.*, 2009a).

The removal of the methoxyl groups by the laccase enzyme from *Pycnoporus cinnabarinus* leads to a complete damage of the polymer structure (Eggert *et al.*, 1997), and the effect is similar in most free radical-mediated processes investigated to date. The secretomic and transcriptomic studies associated with the BR fungal genome sequence clearly indicate, that the enzyme mainly mediates the degradation process of cellulose and carries out the lignin demethylation process only as a side reaction. The BR genome sequences have displayed that the alcohol oxidase genes are overexpressed and play a pivotal role in bringing about the wood degradation event (Martínez *et al.*, 2009a; Martinez *et al.*, 2011**).** Although nearly 50 % of the available hydroxyl groups in lignin are covered with the methyl groups that are highly unsusceptible to the enzyme action (Kirk, 1975; Ander *et al.*, 1988; Eriksson *et al.*, 1990a, b; Jin *et al.*, 1990a; 1990b; Goodell, 2003), very few wood-rot fungi secrete the enzymes that can demethylate the methoxy groups at the C-3, C4 or C-5 positions in the phenylpropanoid units of lignin, the LMCs and the non-phenolic compounds as well (Fig. 2, Tables 1 and 2). These enzymes are referred to as 3-O-demethylase, 4-O-demethylase and 5-O-demethylase, respectively (Fig. 2). *Gloeoporus dichrous* catalyzes the removal or hydroxylation of 4-O-alkyl groups in the 3,4- alkyl benzoates and an oxidative dealkylation mechanism may also operate (Kirk and Lorenz, 1974). The WR fungi produce extracellular laccase (Bollag and Leonowicz, 1984) and some of them produce LiP that might have an involvement in the

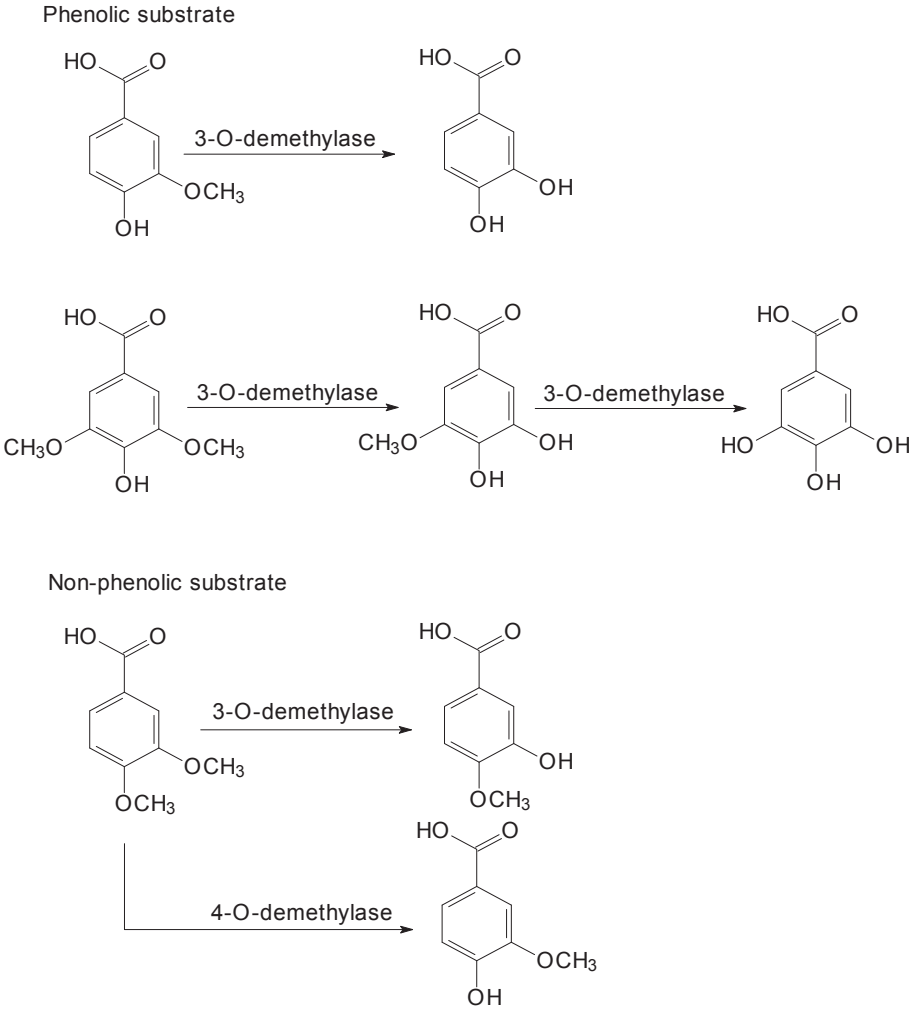

Fig. 2 – Various types of O-demethylase and their enzymatic demethylation reactions on lignin model compounds.

Table 1 – Classification of different types of O-demethylases and their action on methylated lignin and lignin related compounds.

O-demethylases	Substrate	Mode of action	References
Pectin methyl-esterase	Pectin	Catalyses the de-esterification of pectin to acidic pectin with a lower degree of esterification and methanol	Hobson, (1964)
3-O-demethylases	Veratric acid	forms isovanillic acid from veratric acid	Crawford *et al.*, (1973)
Methoxyphenol-O-demethylase			Kalil and Stephens, (1997)
4-methoxybenzoate monooxygenase (O-demethylating)	4-methoxybenzote	demethylate 4-methoxybenzoate into 4-hydroxybenzoate	Bernhardt *et al.*, (1977)
Dichloro-p-nitroanisole O-demethylase	Dichloro-p-nitroanisole		Hultmark *et al.*, (1978)
Anisole O-demethylase	Anisole		Pitout and Thiel, (1978)
Guaiacol glycerol ether O-demethylase	Guaiacol glyceryl ether	Conversion of guaiacol glyceryl ether into Catechol glyceryl ether	Kauert *et al.*, (1980)
2-methoxyestrogen demethylase	2-methoxyestrogen		Hoffman *et al.*, (1980)
4-nitroanisole O-demethylase	4-nitroanisole		Sugiyama *et al.*, (1980)
Veratrate 3-O-demethylase	Veratric acid	Forms isovanillic acid from veratric acid	Malarczyk, 1984
7-methoxycoumarin-O-demethylase	7-methoxycoumarin		Horner *et al.*, (1985)
Methoxyphenamine O-demethylase	Methoxyphenamine		Hawes *et al.*, (1987)
Dextromethorphan O-demethylase	Dextromethorphan		Kronbach *et al.*, (1987)
Scoparone O-demethylase	Scoparone		Kato and Yamamoto, (1993)
Methyl-CpG DNA demethylase			Bhattacharya *et al.*, (1999)
ligX protein, *Sphingomonas paucimobilis*			Sonoki *et al.*, (2000)
Vanillate O-demethylase	vanillate (3-methoxy-4-hydroxybenzoate)	Demethylates vanillate (3-methoxy-4-hydroxybenzoate), syringic or hydroxyvanillic acids to protocatechuate (3,4-dihydroxybenzoate) and corresponding phenolic products	Naidu and Ragsdale, (2001)
Lignin-related biphenyl compound, 5,5′-dehydrodivanillate (DDVA) O-demethylase (LigX)	DDVA	DDVA), to 5-carboxyvanillate (5CVA)	Peng *et al.*, (2002)
Syringate O-demethylase gene (*desA*)		syringate to 3-O-methylgallate (3MGA)	Masai *et al.*, 2004; Kohler *et al.*, 2017
Vanillate/3MGA O-demethylase (*ligM*)	Vanillate and syringate	The O demethylation of Vanillate and syringate are converted into protocatechuate (PCA) and 3-O-methylgallate (3MGA) into gallate	Abe *et al.*, 2005; Rosini *et al.*, 2016; Kohler *et al.*, 2017
Dicamba O-demethylase	2-methoxy-3,6-dichlorobenzoic acid	The conversion of 2-methoxy-3,6-dichlorobenzoic acid to 3, 6-dichlorosalicylic acid	Herman *et al.*, (2005)
manganese-dependent superoxide dismutases (MnSODs)	Organosolv and Kraft lignin	aryl-C and C–C bond oxidative cleavage reactions as well as from O-demethylation activity of Organosolv and Kraft lignin, and lignin model compounds	Rashid *et al.*, (2015)
Pseudomonas Rieske monooxygenase (VanA and VanB)	Vanillic acid	protocatechuic acid from vanillic acid giving 77 % yield of desired product	Lanfranchi *et al.*, (2018)
Laccases, N51002 (L1; white-rot fungus; *Trametes versicolor*) and N51003 (L2), from (*Coprinopsis cinereus*), Novozyme (Denmark)	Kraft lignin	O-demethylation of Kraft lignin, and methanol released as end product	Wang *et al.*, (2018)
Sphingobacterium sp. T2 manganese superoxide dismutase (SpMnSOD)	Lignin	20–40 % increases in phenolic and aliphatic OH content	Rashid *et al.*, (2018)
Cytochrome P_{450} aryl-O-demethylase (aryl-O-demethylation)	guaiacol	Converting guaiacol to catechol	Mallinson *et al.*, (2018)

Table 2 – O-demethylating enzymes from microorganisms and their demethylation lignin products.

O-demethylating enzymes	Lignin demethylating organisms	Demethylation Product	References
O-demethylases			
Tetrahydrofolate-dependent aryl demethylase LigM	*Sphingomonas paucimobilis*	vanillate, 3-O-methylgallate, and syringate	Kohler *et al.*, (2017)
3-O- demethylase	*Pseudomonas aeruginosa*, *P. clzrysosporium*		Ribbons, 1970; Huynh and Crawford, 1985
O-demethylase	*Acetobacterium dehalogenans*, Basiodiomycetes, Ascomycetes	2-methoxyphenol to 1,2-dihydroxybenzene	Engelmann *et al.*, 2001; Haider and Trojanowski 1975
3- and 4-methoxybenzoate demethylases	*Pseudomonas fluorescence*, *Pseudomonas* sp. *Pseudomonas putida*, *Pseudomonas testosterone*, *Cunninghamella bainieri*		(Cartwright and Buswell, 1967); Bernhardt *et al.*, (1975); Ribbons (1971)
Veratrate O-demethylase	*Chaetomium piluliferum*, *Xerocomus badius*		Paszczynski and Trojanowski, (1977)
Tetrahydrofolate-Dependent O-Demethylase	*Sphingomonas paucimobilis* SYK-6	3-O-methylgallate	Masai *et al.*, 1993a; Masai *et al.*, 1993b; Masai *et al.*, 2004; Rosini *et al.*, 2016; Kamimura *et al.*, 2017
DDVA O-demethylase	*Sphingomonas paucimobilis* SYK-6	5-carboxyvanillate	Peng *et al.*, (2002)
Vanillate demethylase	*Pseudomonas*, *Sphingomonas* (*Pseudomonas*) *paucimobilis* SYK-6	3,4-dihydroxybenzoate	Brunel and Davison, (1988); Nishikawa *et al.*, 1998
Syringate O-demethylase	*Sphingomonas* (*Pseudomonas*) *paucimobilis* SYK-6		Nishikawa *et al.*, 1998
peroxidase	*Bjerkandera adusta* CCBAS 930		Korniłłowicz-Kowalska *et al.*, 2008
Laccase like peroxidase	*B. adusta* CCBAS 930		Leonowicz *et al.*, (1999)
Tetrahydrofolate-dependent demethylase	*S. paucimobilis* SYK-6	5-methyl tetrahydrofolate	Masai *et al.*, 2004; Peng *et al.*, 2002
Non-heme iron-dependent demethylase (LigX)	*S. paucimobilis* SYK-6		Sonoki *et al.*, (2000)
Veratrate O-demethylase	*Chaetomium piluliferum*, *Xerocomus badius*		Paszczynski and Trojanowski, (1977)
NAD(P)H-dependent O-demethylase	*Penicillium simplicissimum*	Protocatechuate	Jong *et al.*, (1990)
Alcohol oxidase	*Gloeophyllum trabeum*	Formaldehyde (HCHO) and hydrogen peroxide (H_2O_2)	Filley *et al.*, 2002; Daniel *et al.*, 2007; Martínez *et al.*, 2009a
Methanol oxidase, ligninolytic peroxidases	*P. chrysosporium*	H_2O_2	Ander *et al.*, 1992; Martínez *et al.*, 2005; Vanden Wymelenberg *et al.*, 2010
Oxygen- dependent 4-O-demethylase	*Rhodococcus erythropolis*		Malarczyk *et al.*, (2011)
Syringate O-demethylase gene	*S. paucimobilis* SYK-6	3-O-methylgallate	Masai *et al.*, (2004)
Vanillate/3MGA O-demethylase	*S. paucimobilis* SYK-6	PCA, gallate	Abe *et al.*, (2005)
	Gloeophyllum trabeum, *Poria placenta*	$^{14}CO_2$	Niemenmaa *et al.*, (2008)
Veratric acid O-demethylase	*Nocardia* sp.	Vanillic acid, isovanillic acid, Protocatechuic acid	Malarczyk, (1984)
Pseudomonas Rieske monooxygenase (VanA and VanB)	*Pseudomonas* Rieske and recombinantly coexpressed in *E. coli*.	protocatechuic acid from vanillic acid giving 77 % yield of desired product	Lanfranchi *et al.*, (2018)
Laccase	*Trametes versicolor*, *Phlebia trernellosa*, *Plzanerochaete chrysosporiurn*, *Botryosphaeria rhodina*, *Galerina* sp. HC1, *Cerrena unicolor*, *Sporotrichum pulverulentum*, *Polyporus versicolor*, *Chaetomium piluliferum* C 44, *Stachybotrys chartarum* C 805, *Pleurotus ostreatus*, *Trametes trogii*, commercial laccases, NS51002 (L1; *Trametes versicolor*) and NS51003 (L2; Coprinopsis cinereus),	$^{14}CO_2$, CH_3OH, HCHO and pyrocatechol	Eriksson *et al.*, 1990a, b; Krik and Farrell 1987; Bourbonnais and Paice, 1992; Crestini and Argyropoulos 1998; Reid, 1992; Bashtan-Kandybovich *et al.*, 2012; Bourbonnais and Paice 1992; Bourbonnais and Paice, 1996; Ibrahim *et al.*, 2011; Malarczyk *et al.*, 2009; Ander *et al.*, 1983a; Ander *et al.*, 1983b; Ishihara and Miyazaki 1974; Haider and Trojanowski, 1975; Ai *et al.*, 2015; Wang *et al.*, 2018
Alcohol oxidase	*P. chrysosporiurn*, *P. trernellosa*	CH_3OH	Reid and Deschamps, 1991

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Table 2 (*continued*)

O-demethylating enzymes	Lignin demethylating organisms	Demethylation Product	References
Peroxidase	*Sporotrichum pulverulentum; Chaetomium piluliferum* C 44, *Stachybotrys chartarum* C 805, *Pleurotus ostreatus, Bjerkandera adusta* CCBAS 930	CH_3OH, $^{14}CO_2$	Ander *et al.*, 1983; Haider and Trojanowski, 1975; Kornilłowicz-Kowalska *et al.*, 2008
Manganese Peroxidase,	*Trametes versicolor*, *P. chrysosporium*	CH_3OH, polyguaiacol	Paice *et al.*, 1995, Frick and Crawford, 1983, Frick and Crawford, 1984
Lignin peroxidase	*P. chrysosporium*, *Schtzophyllum commune*, *Polyporus melanopus* and *P. ciliatus*	polyguaiacol, $^{14}CO_2$	Frick and Crawford, 1983, Frick and Crawford, 1984
Manganese-dependent superoxide dismutases (MnSODs)	*Sphingobacterium* sp. T2 (MnSOD1 and MnSOD2)	Demethylated fragments of Organosolv and Kraft lignin, and lignin model compounds	Rashid *et al.*, 2015; Rashid *et al.*, 2018

lignin demethylation process (Kirk *et al.*, 1986; Hatakka, 1994; Hatakka *et al.*, 1983). The laccase -catalyzed demethylation and the generation of ortho-diphenols have been reported by Ander *et al.*, (1983). The demethylation process of lignin occurs extracellularly in BR fungi but in bacteria demethylation process occurs by intracellular action that introduces a hydroxyl group (hydroxylate). The low MW compounds of the methyl group, obtain the hydroxyl oxygen atom arising from O_2 (Cartwright and Buswell, 1967; Ribbons, 1970) and it has been proposed, that the BR fungi transport not only the oxygenase, but also the reducing cofactor to the site of action in the lignin polymer and this occurs extracellularly (Kirk, 1975). The L-glutamate, glucose or glycerol levels decrease the mineralization of the methoxyl-labelled lignin by *G. trabeum*. However, the addition of Fe^{2+} and Mn^{2+} ions raises the mineralization rate by up to 20 % with the synthetic (3-O-$^{14}CH_3$) lignin (Kirk and Adler, 1970). The $^{14}CO_2$ release from 3- or 4-O$^{14}CH_3$ labelled veratrate displays the highest rate at position 4- than at position −3 of the methyl group and demethylates the anisate at position 4-, unlike the vanillate. The methyl groups from these compounds at position 3- have been less reactive to induce demethylation; probably they need a cofactor, such as NADH, H_2O_2 or O_2. Although microbes and enzymes also are capable of releasing CO_2 at a higher rate from the other functional groups in the phenylpropanoid units by the side chain carbon oxidation rather than the $-OCH_3$ groups (Haider and Trojanowski, 1975). The CO_2 release is mainly associated with many other reactions, like oxidation, reduction, and depolymerization of the functional groups in the phenylpropanoid units. Enzymes simultaneously activate the free radical systems to generate the reactive oxygen species (ROS), which are believed to mediate the mineralization process of lignin and the lignin-related compounds (Srebotnik and Hammel, 2000; Hammel *et al.*, 2002). Microorganisms produce methanol by various processes, such as decarboxylation, demethoxylation, besides the quinone reduction events (Buswell and Eriksson, 1979). Among the various wood degrading microorganisms, the bacteria are endowed with the ability to secrete different kinds of ligninolytic enzymes, which motivate various degradation pathways. The demethylation process of lignin or of the non-phenolic compounds requires O_2, CO_2, ATP, NADH and electron donors or acceptors to produce a highly reactive lignin. Some bacteria utilize the DL-Tetrahydrofolate (THF) and ATP for their anaerobic *O*-demethylation of phenylmethylethers under stringent anaerobic conditions (Berman and Frazer, 1992). The demethylation products of formaldehyde were identified in *Pseudomonas fluorescens*. Also to demethylate, vanillate requires NADH and oxygen to generate formaldehyde (Cartwright and Buswell, 1967). This clearly explains that the reaction is catalyzed by *O*-demethylase and not a methoxilate. *Acetobacterium woodii O*-demethylase requires tetrahydrofolate for the methyl group acceptance, which is derived from the aryl-*O*-methyl groups (Berman and Frazer, 1992). *Pseudomonas fluorescens* produces two types of enzymes, namely vanillate-*O*-demethylase from protocatechuate and 3,4-oxygenase. In the demethylating reaction, when added, NADH at 1 mol of oxygen/mole rapidly catalyzes the oxidative demethylation reactions and oxidizes vanillate, while the removal of the methyl group results in the accumulation of the protocatechuate (Cartwright and Buswell, 1969; Cartwright and Buswell, 1967). Later, it has been demonstrated that an oxygen-stable enzyme is responsible for the *O*-demethylation reaction (Kasmi *et al.*, 1994) and that ATP also is required for the *O*-demethylase activity (Berman and Frazer, 1992). Cloning, sequencing, and the active expression of the gene encoding for corrinoid protein from A. *dehalogenans* and other microbial populations (Kaufmann *et al.*, 1997; Kaufmann *et al.*, 1998a; Kaufmann *et al.*, 1998b; Masai *et al.*, 2004; Peng *et al.*, 2002; Sonoki *et al.*, 2000; Kornilłowicz-Kowalska *et al.*, 2008; Shi *et al.*, 2013) have also been worked out. Recently, a Ti-(III)-NTA colorimetric assay was carried out in order to detect the microbial demethylation of lignin and lignin-like compounds under aerobic conditions (Gibson *et al.*, 2014). Microbial demethylation of industrial lignin has been studied with some fungi, including *Absidia cylindrospora*, *Cylindrocladium* sp. and *Aspergillus* sp. by the lignin recovery methods (Akhtar *et al.*, 2009; Zou *et al.*, 2015). Also two extracellular bacterial manganese-dependent superoxide dismutases (Mn SOD 1 and MnSOD 2) from *Sphingobacterium* sp. T2 enzymes have been resulted in catalyzing the degradation of the Organosolv, Kraft lignin and different lignin model substrates that produced different byproducts by means of the aryl-C and C−C bond oxidative cleavage reactions or by the

O-demethylation activity (Rashid *et al.*, 2015). The 54.0 % decreased rapidly lignin-derived compounds by lignin demethylation using *Physisporinus vitreus* were identified using pyrolysis–gas chromatography–mass spectrometry (Py–GC/MS) (Kong *et al.*, 2017). The two Novozyme commercial laccases of N51002 (L1; *Trametes versicolor*) and N51003 (L2; *Coprinopsis cinereus*) using softwood kraft lignin (SKL) in presence of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), 1-hydroxybenzotriazole (HBT), and 2,2,6,6-tetramethylpiperidin-1-yloxy (TEMPO) that generated methanol as O-demethylation product (Wang *et al.*, 2018). As mentioned above, the genes involved in the degradation of OH-DDVA numbering 68 have been decoded. However, the genes for the enzyme involved in the first step of the DDVA degradation, O demethylation, still remain unknown, to date (Yoshikata *et al.*, 2014). According to a recent study undertaken to study the overexpression of the coenzymes, such as the tetrahydrofolate (THF)-dependent O-demethylase LigM from the bacterium, *Sphingobium* sp. strain SYK-6, and the plant methionine synthase MetE enzyme, have been observed to have overexpressed in *E. coli*, wherein the vanillic acid gets converted to the protocatechuic acid (PCA) with a 500-fold decrease in the cofactor utilization (Rosini *et al.*, 2016). Bacteria are known to mediate certain metabolic pathways, thereby generating intermediates, such as vanillate, 3-O-methyl gallate, and syringate, by the action of the tetrahydrofolate-dependent aryl demethylase LigM from *Sphingomonas paucimobilis*. This enzyme is also involved in the downstream aryl modification, ring opening, and mediates the aryl O-demethylation process by LigM (Tables 1 and 3). The enzyme's 1.81 Å crystal structure, it's fold and the canonical folate-binding domain, the LigM's tetrahydrofolate-binding site and the protein–folate interactions, besides it's catalytic tyrosine-dependent reaction mechanism have all been adequately worked out (Kohler *et al.*, 2017). Both vanillate-O-demethylase of VanA and VanB from *Pseudomonas putida* was overexpressed in *Escherichia coli* strain K-12 that converted vanillate into protocatechuate (Hibi *et al.*, 2005). Also operon encoded genes of O-demethylase components of *Desulfitobacterium hafniense* strain DCB-2 were cloned and heterologously expressed using *Escherichia coli* (Studenik *et al.*, 2012). Also vanilliate analogs generated protocatechuate by vanillate demethylase of VanA and VanB in *Streptomyces* sp. NL15-2K were expressed in *Escherichia coli* with recombinant whole cells demethylated vanillate, syringate, m-anisate, and veratrate, whereas, ferulate, vanillin, and guaiacol were not demethylated by *Streptomyces* vanillate demethylase because of *Streptomyces* vanillate demethylase mediated lignin demethylation at meta-position relative to the carboxyl group (Nishimura *et al.*, 2014a, b). Similarly various aryl-methyl ethers oxidative demethylation were mediated by Rieske monooxygenase VanA and VanB of *Pseudomonas* sp. HR199 were recombinantly coexpressed in *E. coli* showed that VanA-VanB system demethylates at meta position of the aromatic ring and the presence of a carboxylic moiety produces 77 % of protocatechuic acid from vanillic acid (Lanfranchi *et al.*, 2018). The recent study on *Sphingobacterium* sp. T2 manganese superoxide dismutase (SpMnSOD) catalyzed aryl-Cα oxidative cleavage, alkene dihydroxylation, alcohol oxidation and demethylation that produces 20–40 % increases in phenolic and aliphatic OH content (Rashid *et al.*, 2018). In another study on Cytochrome P450 aromatic O-demethylase system that efficiently demethylates both guaiacol and various lignin-relevant monomers (Mallinson *et al.*, 2018).

To date, there has been no record of any fungal enzyme that is specifically involved in the O-demethylation reactions by means of a hydrolytic mechanism, having been isolated or described. The LiP, MnP, and the laccases are believed to be capable of removing O-methyl group from lignins; these enzymes are also known to have been involved in inducing appreciable measure of degradation of lignin by way of the less known free-radical mechanism. Microbial demethylation of lignins adequately implies that certain specific lignin demethylation enzymes are involved in the process. In this review, the authors present a comprehensive account of this important but less known arena - the enzymatic demethylation of lignin. The outcome of the studies undertaken with the purified LiP, MnP and the laccases has been presented, perhaps by describing and providing certain rare examples of the fungal demethylation process of lignin and the lignin-like compounds (LMCs). These include, the vanillate (3-methoxy-4-hydroxybenzoate), syringate (3,5-dimethoxy-4-hydroxybenzoate), and veratrate (3,4-dimethoxybenzoate), which resemble the aromatic moieties in the lignin structure. In this review, the recent advances made in the molecular approaches of the lignin demethylation process, besides its degradation and the strategies involved to tone up the quality of lignin have also been given adequate impetus. Given the recent resurgence of interest in lignin modification for commercial purposes, this review provides a valuable collation of data in a potentially profitable, yet less explored area - the biotechnology !!!.

2. Enzymatic demethylation of lignin

The wood-rot fungi are known to employ different basic mechanisms to cleave the methoxyl groups from the polypropanoid units of lignin (Rosini *et al.*, 2016; Kohler *et al.*, 2017). The first mechanism involves a direct hydrolysis of the ether bond of the polypropanoid units, while the second is an enzyme-mediated hydrolysis of the methoxyl groups. However, catecholamine-O-methyltransferase showed that the transfer of the methyl group happens via an S_N2 transition state and provides a strong electrophile, such as Zn^{2+} and perhaps oxygen reverses the usual direction of this reaction. The methyl ether can be converted to an easily hydrolyzed hemiacetal by the oxidation of the methyl group and by the hydrolysis of the hemiacetal yielding a gem diol, which readily gets dehydrated in order to yield formaldehyde as the product. However, three possible basic mechanisms for the demethoxylation of lignin and the lignin-related aromatic compounds have been proposed (Frick and Crawford, 1983): 1) direct hydrolysis, a kind of methyl transferase; 2) a hemiacetal reaction, yielding formaldehyde; and 3) an acetal-forming reaction, yielding methanol; mediated, for instance by laccase or peroxidase. Since *P. cinnabarinus* is a well-known producer of the phenol oxidases, the most probable reaction would be the third alternative. Ander *et al.*, (1983b) have shown that phenol oxidizes the demethoxylate vanillate and

Table 3 – Molecular approaches in demethylation and degradation of lignin.

O-demethylase gene/ protein	Nucleotide sequence/protein sequence	Mode of action	Microorganism	GenBank Accession	Reference
ligXa gene (5,5′-dehydrodivanillate O-demethylase oxygenase subunit)	MLSAEQNDKLARVGPGTPMGELLR RYWHPIGGESEFETKATRPVRLMGED LVLYKDLSGN YGLMDRHCPH RRADMACGMVEADGLRCSYHGWMFDAQGACTEQPFEDTANPKGRYKDKVRIKAYPVR ALGGLLWAYMGPLPAPELPDWEPFSWKNGFRQIVISVLPCNWLQGQENSMDPIHFEWM HANWSKRLRGETGPYGPKHLKIDFREYDYGFTYNRIREDTDETNPLWTIGRACLWPNAMF TGDHFEYRVPIDDETMMSVGWFFTRVPRDAEPYVQESIPVWHGPIKDAQGEWITSHVMN QDFVAWIGQGTISDRTQENLGLSDKGIGMMRRQFLRDMEKISRGEDPKAIIRDPAINKAIPLP TIHRDAVMEGMTAEEIEAGGALHLKRFIFQYGQPEHVLKMQQDAMRISQDNKGYVDA	The O-demethylation of DDVA, leading to the formation of 2,2′,3-trihydroxy-3′ -methoxy-5,5′-dicarboxybiphenyl (OH-DDVA)	*Sphingobium* sp. (strain NBRC 103272/SYK-6)	G2IN04	Masai et al., 2012
ligXc (5,5′-dehydrodivanillate O-demethylase ferredoxin subunit)	MAQLKVVTRDGSLHEFEAPDGYTV MEAIRDQGIDELLAICGGCCSCATCHVFVEEAFLDKLPPLKGDEDDLLDS SDHRQANSRLSCQLPIGPELGGMTVTIAPED	The formation of 2,2′,3-trihydroxy-3′-methoxy-5,5′ -dicarboxybiphenyl (OH-DDVA). LigXc act as an intermediate electron transfer protein between LigXd and LigXa	Sphingobium sp. (strain NBRC103272/SYK-6)	G2IN77	Masai et al., 2012
ligD gene (Cα-dehydrogenase)	MKDFQDQVAFITGGASGAGFGQAKVFGQAGAKIVVADVRAEAVE KAVAELEGLGITAHGIVLDIMDREAYARAADEVEAVFGQAPTLLSNTAGVNSFGPIEK TTYDDFDWIIGVNLNGVINGMVTFVPRMIASGRPGHIVTVSSLGGFMGSALAGPYSAA KAASINLMEGYRQGLEKYGIGVSVCTPANIKSNIAEASRLRPAKYGTSGYVENEESIA SLHSIHQHGLEPEKLAEAIKKGVEDNALYIIPYPEVREGLEKHFQAIIDSVAPMESDP EGARQRVEALMAWGRDRTRVFAEGDKKGA	Oxidizes GGE to MPHPV	*Sphingomonas paucimobilis* SYK-6	BAA01953.1	Masai et al., 1993a; Masai et al., 1993b
Vanillate/3-O-methylgallate O-demethylase (*ligM*)	MSTPTNLEQVLAAGGNTVEMLRNSQIGAYVYPVVAPEFSNWRTEQWAWRNSAVLFD QTHHMVDLYIRGKD ALKLLSDTMINSPKGWEPNKAKQYVPVTPYGHVIGDGIIFYLAEEEFVYVGRAPAANW LMYHAQTGGYNV DIVHDDRSPSRPMGKPVQRISWRFQIQGPKAWDVIEKLHGGTLEKLKFFNMAEMNIAG MKIRTLRHGMAG APGLEIWGPYETQEKARNAILEAGKEFGLIPVGSRAYPSNTLESGWIPSPLPAIYTGDKL KAYREWLPAN SYEASGAIGGSFVSSNIEDYYVNPYEIGYGPFVKFDHDFIGRDALEAIDPATQRKKVTLA WNGDDMAKIY ASLFDTEADAHYKFFDLPLANYANTNADAVLDAAGNVVGMSMFTGYSYNEKRALS LATIDHEIPVGTELT VLWGEENGGTRKTTVEPHKQMAVRAVVSPVPYSVTARETYEGGWRKAAVTA	Vanillate and syringate are converted into protocatechuate (PCA and 3-O-methylgallate (3MGA) into gallate	*Sphingomonas paucimobilis* *Pseudomonas* sp. strain YS-1p; *Rhizobium* sp. strain YS-1r	BAD61059.1; JPYP00000000; JPYQ00000000	Nishikawa et al., 1998; Abe et al., 2005; Prabhakaran et al., 2015; Rosini et al., 2016
LigH10-formyltetrahydrofolate synthetase	MAQGSDIEIAREAKMQNIAEVGAKVGIPQDALLNYGPYKAKLSW DFINSVQGNQDGKLILVTAINPTPAGEGKTTTTVGLADGLNRIGKKTVAALREPSLGP CFGVKGGAAGGGYAQVVPMEDINLHFTGDFHAITSANNLLAALIDNHIYWGNKLGLDP RRIAWRRVLDMNDRALRSIVNSLGGVSNGYPREDGFDITVASEVMAILCLSSDLKDLE RRLGNIHAGYTRERKAVLASELNASGAMTVLLKDALQPNMVQTLENNPVLIHGGPFAN IAHGCNSVLATKTALKIADYVVTEAGFGADLGAEKFFDIKCRKAGLKPSAAVIVATIR ALKMHGGVDKADLGTANPEAVRKGGVNLARHIENVRQFGVPVVVAINQFITDTDEEMA MVKEIAEAAGAEAVLCSHWANGSAGTEELARKVVALAESGSSNFAPLYEDSMPLFEKI DTIAKRIYRATEATADSSVRNKLKGWEADGFGHLPVCMAKTQYSFSTDPALRGAPTDH VVPVRDVILSAGAEFIVAVCGDIMRMPGLPKVPSADFIKLDEQGQIQGLF		*Sphingomonas paucimobilis*	BAD61061.1	Nishikawa et al., 1998; Sonoki et al., 2002; Masai et al., 2004; Abe et al., 2005
desA Syringate O-demethylase	MAKSLQDVLDNAGNAVDFLRNQQTGPNVYPGVPAEYSNWRNEQR AWAKTAVLFNQSYHMVELMVEGPDAFAFLNYLGINSFKNFAPGKAKQWVPVTAEGYVI GDVILFYLAENQFNLVGRAPAIEWAEFHAATGKWNVTLTRDERTALRTDGVRRHYRFQ LQGPNAMAILTDAMGQTPPDLKFFNMADIQIAGKTVGALRHGMAGQPGYELYGPWADY EAVHSALVAAGKNHGLALVGGRAYSSNTLESGWVPSPFPGYLFGEGSADFRKWAGENS YGAKCSIGGSYVPESLEGYGLTPWDIGYGIIVKFDHDFIGKEALEKMANEPHLEKVTL ALDDEDMLRVMSSYFSDSGRAKYFEFPSAVYSMHPYDSVLVDGKHVGVSTWVGYSSNE GKMLTLAMIDPKYAKPGTEVSLLWGEPNGGTSKPTVEPHEQTEIKAVVA PVPYSAVAR TGYADSWRTKKA	tetrahydrofolate-dependent O demethylation ofsyringate	*Sphingomonas paucimobilis*	BAC79257.1	Masai et al., 2004; Masai et al., 2002
DesZ 3-O-methylgallate 3,4-dioxygenase	MAEIVLGIGTSHGPMLVTQTEQWRSRLAFDQSVNHAWRGGSWSY DQLVAERADQNFAAQITPEAMTAHNARCQASLDQLAEIFSEAKIDVAVILGNDQMEIF DERLVPAFSVFYGDTITNYEFPPERMAALPPGINLSVAGYIPSGGAEYAGQPELARSI IAQAMADEFDVAAMKALPKPETPHAFGFVYRRIMRDNPVPSVPVLVNTFYPPNQPTVR RCYEFGKSVLRGIQAWESDARVAVLASGGLTHFVIDEEIDRLFFQAMEDRDIARLADL GEAIFQDGTSELKNWIPLAGMMAELGLDHEILDYVPCYRSEAGTGNAMGFVCWR	meta-cleavage of 3-O-methylgallate	*Sphingomonas paucimobilis*	BAC79261.1	Kasai et al., 2004
metF"5,10-methylenetetrahydrofolate reductase	MATATLDKAALSRLFTDYSLEITPKDVEALENAAHMIPPGTLIS VTFLPGAEYEDRARAAKRIQELGFRPVPHLSARRLIDEADLRTYLDMLKGVIDLKHVF VIAGDPNEPLGIYEDALALIDSGILKEYGIEHCGISGYPEGHPDITDEKLAKAMHDKV ASLKRQGIDYSIMTQFGFDAEPVLEWLKQIRSEGIDGPVRIGLAGPASIKTLLRFAAR CGVGTSAKVVKKYGLSITSLIGSAGPDPVIEDLTPVLGPEHGQVHLHFYPFGGLVKTN EWIVNFKGKQGI		*Sphingomonas paucimobilis*	BAD61060.1	Nishikawa et al., 1998; Sonoki et al., 2002; Masai et al., 2004; Abe et al., 2005
ferA feruloyl-CoA synthetase	MTVEAGVRPQAGARDINRLLRPRSIAIVGASETPGSLGASVLAN LVRNEFPGDIHLVNPKRETISGRPAVPSVDALPEGVDCAILAIPRVAVLDTMRQLAAR KAGAAIIFAAGFAEGGEQGMADQQEIGRIAHEAGIVVEGPNCLGSVNYLDRIPLTFID TDIKAPSPGGVGIVSQSGAMAAVLAVMLESRTLDLTYSVSTGNEAGSGVEDYVEFMIA DEKTRIIAMIVEQFRDPARFLALMDKANAAGKLVVLLHPGKSSAARESAATHTGAMAG DYKLMRAKVERAGVVVAETLEELGDITEIAARCPALPSGGTAVLGESGALKALTLDLA EELELALPTLDDGNAPALRAALPEFVPVSNPLDLTAQGLVDPDMYYRTLAALFEDDRV GTIFAGIIQTNPATIGIKLPPFLKAVRELKATKPVIFGGVDEGADVPADWIEQLRAEG IPYFPTTERALRAIRRLSAAGARDASRTDAAPASVPALASEKGVVVPEYKAKALLAPLG ISFPKGQFAATVEDAIAAAEAIGGPVVMKAQAAALSHKSDAGGVALNLVGAEAIRAAW DKMFADVKRYDASIILDGVLIEAMGARGLELIVGAKNDPQWGPVILAGFGGVTAEILQ DVRLLSPDMTKEAIVAELGKLKQAALLHGYRGSPALDVGAVAELIGQVGRLLRGEPRI QELDLNPVVVYPEGQGAIALDALMLVD		*Sphingomonas paucimobilis*	BAC79255.1	Masai et al., 2004; Masai et al., 2002

ferB feruloyl-CoA hydratase/lyase	MSEENKPVEREEDTVRYEIVNNVAWVYYNRPTKRNAQSPKLNRQ MLKVLTELEFRDDVGVLVLGGEGPAWCAGMDLKEYFRETEAEGLAGTRKAQREAYTWW ERLRWYQKPTIAMVHGWCFGGAYGPLFACDLAFAADEAQFGLSEVNWGILPGGGATKV AVDLMPMRVAMYHAMMGENLSGQDAARYNLVNESMPADQLKARVKQVAETLIQKNWAT VKYTKDAVRRVKEMTYDNAEDYLIRLQEGLNWFDKSDGRHVAMKQFLDDKTFKPGLGH YDKTKTEV		*Sphingomonas paucimobilis*	BAC79254.1	Masai et al., 2004; Masai et al., 2002
FerB2	MSDELTFETVKVTLDDGIAWVALNRPEKRNAMSPTVNREMWVAL DELEIRDDVGVVVLTGEGEAFSAGMDLKEYFRETEGESAIVQARYRRAATDWMWRRLM HYPKPTIAMVNGWCFGGAFTPLVACDLAIAADEATFGLSEINWGIIPAGNVTRAVAQV MNHRDALHYIMTGDPFDGKEAAKMGLVNRSVPLAELRGEVEVLARKLLSKNPTILRHA KSAFKYVERLDWDTSEAMLGALAATAASQDTERGRRKGMTQFLDEKSFKPGLGGYRRED		*Sphingomonas paucimobilis*	BAC79255.1	Masai et al., 2004; Masai et al., 2002
ligW5-carboxyvanillate decarboxylase	MRLIATEEAVTFQPVVDALRAHSRTDDASLDMILVRDVYGDEPA RPAMIGRLSDVTGERLAEMDSNGVDMHLLSLTAPGVQMFDAETGTRLARIANDLMAQT VAANPTRFAGLGTFAPQDPASAAREIERVATQLRLNGLVINSHTNDLYYDDPFFHPVF EAIEASGLALYIHPRAPSKQIDRAFRDYGMNSAIWGYGIETSTNAVRMILSGLFDRFP RLKIVLGHMGEAIPFWLWRLDYMHGNATTFGGAPKLKLKPSEYFRRNFAITTSGVESH AALRYSIEVLGPENVMWAIDYPYQPMAPAVQFIRTAPIPEDVKAMVAGGNAARIFRIT	decarboxylation of 5-carboxyvanillate to yield vanillate, decarboxylase for 5,5′-dehydrodivanillic acidcatabolic pathway	*Sphingomonas paucimobilis*	BAB86295.1	Masai et al., 2002; Peng et al., 1998
orf1putative formaldehyde dehydrogenase	MSHSPSKAAPLLLLPGLICDDRIWAPQAEALGAHQPVTAVAGYG EADSLGAMAEQVLAQAPERFVVVGHSMGGRVALEIIRRAPERVAGLGLISTGIHLPRP GEADGRFALLARGVEQGMDALIDAWLPPMVGEANRSRPGLMDMLWQMCSDAGLDMYER QMRALLARPEVESLLPAIRCPVLVATGADDAWAPPVQHEAIAAAIPGATLTIIPDAGH MVPVEQPAAMTRVLASWLDAAHAAEKDRILNS		*Sphingomonas paucimobilis*	BAC79256.1	Masai et al., 2004; Masai et al., 2002
Orf2putative alkyl salicylate esterase	MASFLMIHGAWHGGWCFERLRRPLEARGHAMAAPDLPGMGGDAR ALAAATLDRWADFAIAQADTLPGPVILCGHSRGGLVVSRAAERAPEAFAALVYVAALL CEDGRSLYDMMGEPQHGGFSDGLSPVADGLGVALSAQAAIPLFYSHCEPQVQAVSAAR LVAEPVRPLGTPVRVTPERFGRVPRHYVECIHDRVLPLATQRAMTAALPCASVTTLDS DHSPFLCVPDALAGALTTIAERTQA		*Sphingomonas paucimobilis*	BAD61058.1	Nishikawa et al., 1998; Sonoki et al., 2002; Masai et al., 2004; Abe et al., 2005
orf3	MIQKVDSSSVKSATRTLDILEFVVGCGRAVSAAEIAAALAIPVS SLSYLLGTLVERGYLTRNGRLHLPGPALARLSAGERSATLAXRVYPVVRSVSLQLNET ASFFVRHGHEMEAITGEVAPQALRYTIEVGRPVPLHAFAAGKAILATFDEAELNAYFA ASRRDAFTAHTLTSEADLRREVAAIARTGTARTIEEYTPGIIGIGRAVISGGQLLGAI SVAIPAARTTPQLEERAIQMLQRAAEALGDR		*Sphingomonas paucimobilis*	BAC79258.1	Masai et al., 2004; Masai et al., 2002
orf4	MAKTFITCAITGASPMPKHPNFPFRPEHVAQEALDAAAAGASII HVHVRNGADGTPSQELEDYRKVVGLIREKNTDVILNVTTGPGCMWFPKSAEEPAVPDM EKTLMFTAERRIEHILELKPDMCTLDICTMNLWGGIAMNLEMIVGKMGTMLQDAGVLT EIECFEAGDFVFADDLMAKGLIPKNSPFTFVLGTKYGLPATPEAMMYSRNQIPRGAHF TGFGVSRHSFPMAAQSVLLGGHMRVGFEDTIYLRKGVLANSNAELVEWGADIVNKLGG EVATPGETREMLGLKG			BAC79259.1	Masai et al., 2004; Masai et al., 2002
orf5	MTARGLTVEKPARAEGPTVRDAVMTLLRDLGMTTLFGNPGSTEL PLFRDMPQDFRYILGLQESVVVGMADGFAQMSGNAAFVNLHSSAGTGHALGNIFTAFR NQTPLVITAGQQARSIFPYDPFLYAERATEFPQPFVKWTCEPARAEDVPLAILRAYHM AMQAPRGPVFVSVPIDDWDRACTPIDVSTVSQDVMASPALIGAAAEALDKARSPVIVA GAGVGRENARAALAKLAEAHKARVFVAPMAARAVFPEDHPLFAGFLPASREAVVSALK GHDLVLVLGAPVFTYHVEGFGPYVPEGASLIQIVDDPSVAARTPVGTAIIGNVADAIA RLSAATSPAPPRAVPDAALPAPNVVGSGTAIEEALLMQQLATLRPKGIIISEEAPSTR GPMHDHFPIRLEDEFMATASGGLGFALPAAVGAAIAQPDRTVLCLLGDGSSMYSIQGL WTAAEMGLDVRFLIVNNGGYAALDQFGALFDIEVVGSKLPGLDFVKIAEGMGVPGARV STVDALDEAIRELFAGKGPRLLEVVVQRPA		*Sphingomonas paucimobilis*	BAC79260.1	Masai et al., 2004; Masai et al., 2002
ligE beta-etherase	AGAGGQALNKGPQTTKMPPRVAEKAD	cleavage of beta-aryl ether	*Sphingomonas paucimobilis*	BAA77215.1	Masai et al., 1991; Masai et al., 1999
ligG glutathione S-transferase homolog	MAEPQELTIYHIPGCPFSERVEIMLELKGLRMKDVEIDISKPRP DWLLAKTGGTTALPLLDVENGESLKESMVILRYLEQRYPEPAVAHPDPFCHAVEGMLA ELAGPFSGAGYRMILNREIGKREEMRAAVDAEFGKVDAFLKRYATGSDFLFDDRFGWA EVAFTPMFKRLWFLDYYEDYEVPANFDRVLRWRAACTAHPAAQYRSKEELLKLYYDYT QGGGNGRIPEGRSISSFSPDVDWRTRPMPPRDKWGHAATDAELGLTR		*Sphingomonas paucimobilis*	BAA77216.1	Masai et al., 1991; Masai et al., 1999
LigX DDVA O-demethylase	MLSAEQNDKLARVGPGTPMGELLRRYWHPIGGESEFETKATRPV RLMGEDLVLYKDLSGNYGLMDRHCPHRRADMACGMVEADGLRCSYHGWMFDAQGACTE QPFEDTANPKGRYKDKVRIKAYPVRAWGAFSGPIWAPPRAQTADWAPFSWKNGFRQIV ISVLPCNWLQGQENSMDPIHFEWMHANWSKRLRGETGPYGPKHLKIDFREYDYGFTYN RIREDTDETNPLWTIGRACLWPNAMFTGDHFEYRVPIDDETMMSVGWFFTPCRAMPSP MCRSPSPSGMARSRTRKASGSPAMMNQDFVAWIGQGNISDRTQENLGLSDKGIGMMRR QFLRDMEKISRGEDPKAIIRHPAINKAIPLPTIHRDAVMEGMTAEEIEAGGALHLKRF IFQYGQPEHVLKMQQDAMRISQDNKGYVDA	oxygenase	*S. paucimobilis*	BAA36168.1	Sonoki et al., 2000
ligZ OH-DDVA oxygenase	MAEIVLGMWTSHGPTLSTTPEQWTLRVSADLKRKHPFRGEEYSF EELVSCAAARSWRRPVPFPSASDARPGAKVPSPTWPTFAAAKIDVAVIMGNDQRELFL EDVTPAITVYLGETIWDQPATPEQAARMPPGIHEAEWGIARPSAGTIPASPSSACMCA KRWCRRLRSGGLQDTARARRPLVERRAHSLGFIYRQIIRDQVVPNLPIIINTFFPPNQ PTARRCFELGRAVGKAIRSWKEDLPVAVFGSGGMSHFVIDEDFDRMFFEALRNRDAET LCAIEDKHLQSGTSELKTWIAAAGALFDTDLKGDVVGYEPCYRSEAGTGTANGFVAWQ		*S. paucimobilis* SYK-6	BAA75884.1	Peng et al., 1998
ligY OH-DDVA meta-cleavage compound hydrolase	MIIDCHGHVSAPVELWAYKASLLAHRGSHGRGGVKVTDEQIIAA AHHKETWPDGHIELLHNHGTDMQLISPRTFQMMNSAKPARVVHWFCEEVNTLIHRQCT LIPEMFIPVAGLPQVAGEPIENVFAEMDRCVSMGFKGFLLNPDPYENGAEEAPPLGDR YWYPLYEKLCELDLPAHIHATGSQSERSPYSLHFINEETIATYNLCTSSVFDDFPQLK VVVSHGGGAIPYQLGRFESQSRRSKHLFSERMAKLYFDTVLYTEGALRLLIETVGPER CLFGSECPGVGSTIDPATGKQMDHIAPFIQKFDFLSDADKKLIFEDNARKVFNLEV		*S. paucimobilis* SYK-6	BAA75885.1	Peng et al., 1998
putative methyl corrinoid protein (xdmD)tetrahydrofolate methyl transferase	LTAKAIAAKDVEFIRNLAIKQAEAGANFIDVCASVDDDIELETM KWLIDIVQDATDVPIAVDSPNVYTCIESMKYCNKPGLFNSVSLEGDKVDVAFKVLADT KWECVALLNSDKGIPKTAKDRLELFTDLMAKCQEYKIDPSRMHIDPLIEMLCTSEDGI TMVTEVIREIKKQYPTIHVTGAVSNISFNLPARRIANQAFAVLAMSAGMDSFILDPLN KDMMGMLFATEAMMGEDEYCMEYIGAFREGIFVK	transfers a methyl group from methylated corrinoid protein to tetrahydrofolate	Acetobacterium dehalogenans	AAQ89568.1	Engelmann et al., 2001

(continued on next page)

Table 3 (continued)

O-demethylase gene/ protein	Nucleotide sequence/protein sequence	Mode of action	Microorganism	GenBank Accession	Reference
putative O-demethylase corrinoid protein (xdmA) genes	MSKIEDVKVLVETGKSKKVAAAVQEALDAGDKAQDILDAMVASM GVVGDKFSSGEIPVPEMLIAAKAMSKGVFVLKPVMAGDGANSLGTGMGTVACGDLHDI GKNLVVMMLESAGFDMVDLGVDVPADKFVEAVKENNNVVLVAC SGLLTTTMPALKEAVQTHKR	methyl group carrier in an O-demethylasereaction	Acetobacterium dehalogenans	AAQ89569.1	Engelmann et al., 2001
4,5-PCD a subunit (LigA)	MSAITVEITTWKNTDLREDLTAHPAGLGFGELSFEDLRREDRTIYA ASSGWVCTLTECGTLVCAC	Protocatechuatemetabolism	*Actinoplanes liguriensis* strain NCIMB41362	ADE10221.1	Boakes et al., 2010
LigOmonooxygenase	MLSVLDQVPVFRGDDPAEAVREAVGLARAAASLGYHRFWIAEHH GSAANACAAPEIVAAAVAGATERIRVGTGGVVLPYYSPLKVAEAFRVLAALYPGRIDL GFGRGRGGPAVMAELLNPTAIATEEAYAEQVGRLLAFLGDARTVSRVSVTPAVQDPPL PWLLGSGVGSARLAGMLGVPFCFAQFIATECPEAIAAYQESFRSSPWLDEPQAMLAL RVLAAGTAEDAEELATGFWMSCTTGWRAQVRPDDDDYRGGVPNLADAQRYTLTEEDLAM RASRPYLQSGTAETVGEEIRRLRKVYDVAEVMLTTNCPGAAAPAPVLRAAGRRARA DRAGVTRVRPARRW				
vanA - Vanillate O-demethylase oxygenase	MICNERMVIYRGAGQRVVAALEDFCPHRGAPLSLGSIQDGK LVCGYHGLVMDCDGRTASMPAQRVQAFPCIRAFPAQERHGFIWVWPGDAALADPALIP HLEWAENPAWAYGGGLYHIACDYRLMIDNLMDLTHETYVHASSIG QKEIDEAPVSTRVEGDRLITGRFMEGILAPFWRAALRGNGLADDVPVDRWQICRF TPPSHVLIEVGVAHAGRGGYDAPADCKASSIVVDFITPETDTSIWYFWGMARSFRPEDNEL TARIREGQGTIFAEDLEMLEQQQRNLLAWPERPLLKLNIDAGGVQSRRIIERLVSAERAA EA QLIGRQA	Vanillate + O_2 + NADH = 3,4-dihydroxybenzoate + NAD^+ + H_2O + formaldehyde	Pseudomonas sp. (strain ATCC 19151)	PRO_000008058	Brunel and Davison, 1988
vanB - Vanillate O-demethylase oxidoreductase	MIEVISAMRLVAQDISLEFVRADGGLLPPVEAGAHVDV HLPGGLIRQY SLWNQPGA QSHYCIGVLKDPASRGGSKAVHENLRVGMRVQISEPRNLLFPLEEGVERSLIFAGGIGITPIL CMAQELAARERQDFELHYCARSTDRAAFEWLKVCDFADHVRFHFDNGPDQQKLNAAAL LAAEAEGTHLYVCGPGGFMGHVLDTAKEQGWADNRLHREYFAAAPNVSADDGSFEVRI HSTGQVLQVPADQTVSQVLDAAGIIVPVSCEQGICGTCITRVVDGEPDHRDFFLTDAEKA KNDQFTPCCSRAKS ACLVLDL	Demethylates vanillate (3-methoxy-4-hydroxybenzoate)' syringic or hydroxyvanillic acids to protocatechuate (3,4-dihydroxybenzoate) and corresponding phenolic products	*Pseudomonas* sp. (strain HR199/DSM 7063); *Colletotrichum gloeosporioides* Nara gc5, *Aspergillus flavus* NRRL3357, *Aspergillus niger* CBS513.88, *Aspergillus flavus* NRRL3357	PRO_0000189403; ELA30307.1 ELA24286.1 EED5488.1 XP_001727834.2 XP_001389618.2, XP_002376160.1	Priefert et al., 1997; Naidu and Ragsdale (2001)

(methoxyhydroquinone) MHQ to yield methanol. Also another study reported that the fungal enzymatic reactions of demethylation, somewhat resembles the bacterial enzyme reactions, in terms of the requirement for a reduced coenzyme (i.e., NADH). However, the bacterial enzyme demethylates the methoxyl group at the meta position to carboxylic acid and the enzyme from the fungus demethylates the para position of veratryl moiety. Besides, the fungal enzyme does not attack the β-ether linkage of such aromatic compounds that have an alpha-ketone group, instead of an alpha-hydroxyl group of the side chain. This fact suggests that the existence of the benzyl hydroxyl group is an essential factor in the structure of the substrate for the reaction of the enzyme (Fukuzumi *et al.*, 1965; Fukuzumi, 1969).

In a potential demethylating fungus, *Cunninghamella bainieri*, Haider and Trojanowski, (1975) reported the demethylation of $O^{14}CH_3$-labelled methoxyphenols. Paszczynski and Trojanowski, (1977) published a list of the *O*-demethylases from the various Ascomycete and Basidiomycete fungi. Also attempted to isolate the *O*-demethylase enzyme from those fungi by means of the affinity chromatography by using the fungal veratrate coupled with AH-Sepharose 4B as the substrate. Approximately, a 20-fold increase in the demethylase activity has been observed with *Xerocomus badiu* demethylase (Table 2). With *Chaetomium piluliferum*, however, over a 300 fold activity has been observed. The partially purified veratrate O-demethylase system acts on various methoxyl-substituted benzoic and cinnamic acid derivatives. The highest affinity is observed with position 4- OCH_3 of the methyl veratrate. *O*-demethylase has shown substrate specificity towards *p*- and *m*-anisate and 3,4-methoxycinnamate and oxygen. NADH has been found to be required for the *O*-demethylase activity of *Chaetomium* and *Xerocomus,* both the organisms displaying the requirement of an optimum pH of 5 and 7 respectively (Paszczynski and Trojanowski, 1977). Recently, two extracellular manganese-dependent superoxide dismutases (MnSOD1 and MnSOD2) from *Sphingobacterium* sp. T2 have been reported to mediate the aryl-C and C–C bond oxidative cleavage reactions and the *O*-demethylation activity on Organosolv, Kraft lignin and the other lignin-like substrates produces several byproducts (Rashid *et al.*, 2015). In a recent study, tetrahydrofolate (THF)-dependent *O*-demethylase (LigM) from *Sphingobium* sp. strain SYK-6 and a plant methionine synthase MetE enzyme co-overexpression in *E. coli* that converts vanillic acid to 5 mM protocatechuic acid (PCA), where a 500-fold decrease in the cofactor has been observed (Rosini *et al.*, 2016).

3. O-demethylase secretion by microorganisms

Lignin degradation and their main degradation products were identified as early as 1920 and the initial interest, for several decades has been on lignin degradation as mediated by oxidation, reduction, polymerization and the depolymerization reactions (Figs 1a, b and 2). The literature available on the bacteria-mediated demethylation of the phenolic and non phenolics compounds has been scanty. Besides, the lignin and LMC demethylation had been largely ignored during the

period from 1800 to around 2000. Later, however, interesting findings with the tetrahydrofolate (THF)-dependent O-demethylase (LigM) identified from the *Sphingobium* sp. SYK-6 prompted further investigation on demethylation of the poly phenylpropanoid units. Lignin and the LMC's demethylating enzymes are found in scores of microorganisms. A major class of these enzymes is the O-demethylases. The brown-rot fungi, primarily produce 3,4- and 4,5-catechols by the demethylation process, most of which are reported to be inhibitory for microbial degradation (Filley *et al.*, 2000; Dekker *et al.*, 2002). A few microbes have been observed to possess the inherent ability to secrete the ligninolytic enzymes that can demethylate lignin. To authenticate this, several studies had been initiated with various organisms, by the use of various methoxyphenols (LMCs) as growth substrates. Demethylation has been observed in various species of *Pseudomonas* (Cartwright and Smith. 1967; Haider *et al.*, 1978), *Nocardia* (Crawford and Crawford, 1980), Basidiomycetes, and Ascomycetes (Haider and Trojanowski, 1975). Specific NAD- and NADP-dependent O-demethylases (Bernhardt *et al.*, 1970) are believed to mediate the demethylation process, some of which have been partially characterized, such as veratrate O-demethylase from *Chaetomium piluliferum* and the anisic acid O-demethylase from *Pseudomonas* sp (Paszczyinski et al., 1985, 1986; Bernhardt *et al.*, 1970). Unfortunately, most of these enzymes have proven to be too difficult to isolate, mainly owing to their oxygen sensitivity and also to their tendency to bind to the cellular membranes (Ander and Eriksson, 1978). The phenolic phenylpropanoid and non-phenolic units are inducible substrates for the O-demethylases (Rann and Cain, 1973). The findings of NADPH- and O_2-dependent enzymes that split the aryl ether linkage from veratryl-glyceryl-β-guaiacyl ether substantiated the importance of NADPH- and the O_2- dependent oxygenases (Bernhardt *et al.*, 1970). However, Buswell and Mahmood, (1972) observed that it is not easy to conclude that the enzyme demethylates veratrate specifically at 4-position of the $-OCH_3$ groups (Fig. 2). Although the ether splitting enzymes are characteristic of the monooxygenases, these enzymes are believed, mainly to cause the depolymerization reactions of lignin and generally are found as intracellular enzymes, and that the spatial contact of these degrading enzyme with lignin would be a difficult proposition, since lignin readily gets incorporated into the plant cell walls. If LiP, MnP or the laccase mediates demethoxylation, then the availability of a free phenolic group is a prerequisite for this to happen. Besides, studies undertaken on the S. *pulverulentum* vanillate hydroxylase have indicated that this enzyme does not effectively decarboxylate the veratrate (Buswell *et al.*, 1982).

The O-demethylases are several categories of enzymes with different demethylating systems and are believed to act with the help of cofactors that mainly strip-off the methoxyl group from various poly phenylpropane units, such as lignin, vanillate, syringate, guaiacol and the non-phenolic units (Kaufmann *et al.*, 1997; Kaufmann *et al.*, 1998a; Kaufmann *et al.*, 1998b; Masai *et al.*, 2007a; Masai *et al.*, 2007b; Sudtachat *et al.*, 2009). Among the demethylation systems, two O-demethylase systems are mainly recognized. The first type is found in association with the aerobic microorganisms, and consists of two proteins: IA type oxygenase and a reductase, both iron-sulfur enzymes containing [2Fe−2S] redox clusters and consume oxygen and a reducing agent in the form of NADH. As a result of demethylation, these enzymes generate formaldehyde, water and NAD^+, besides the demethylated substrate, mainly protocatechuate as the end product (Overhage *et al.*, 1999; Masai *et al.*, 2007a; Masai *et al.*, 2007b). The second one is a tetrahydrofolate (THF)-dependent O-demethylase system that involves several enzymatic components, including the substrate, tetrahydrofolate (THF) or a corrinoid protein as the methyl group acceptor, methyl transferase (transfer the methyl group to THF). Subsequently, the other enzymatic elements regenerate the THF, yielding THF and formic acid, in order to recover this cofactor for further conversion of the methoxylated substrates (Kaufmann *et al.*, 1997; Kaufmann *et al.*, 1998a; Kaufmann *et al.*, 1998b; Masai *et al.*, 2007a; Masai *et al.*, 2007b). There after, it passes through different funneling degradation pathways either intra- or extradiol ring opening as catalyzed by specific dioxygenases, perhaps, by way of the β-ketoadipate pathway. However, the central node in the lignin demethylation systems is the formation of protocatechuic acid, followed by catechol by the action of the O-demethylase (Table 3). The other aromatic phenylpropanoid units may also be involved in inducing the ring cleavage, of the 3-O'Methyl gallate and gallic acid systems as described with *Sphingobium* sp. SYK-6 (Kasai *et al.*, 2004; Masai *et al.*, 2004; Masai *et al.*, 2007a; Masai *et al.*, 2007b; Masai *et al.*, 1999). Normally, the phenolic units' ring cleavage occurs at first, followed by demethylation (Kamimura and Masai, 2014; Masai *et al.*, 1999). The methanogenic microbial communities in the rice paddy field soil were enriched with the lignin-derived methoxylated phenylpropanoid units, such as vanillate, syringate and their intermediates like protocatechuate, catechol, while the gallate acts as the sole carbon and energy sources. Among the various tested organism, *Sporomusa* sp. by the acetogenesis process initially demethylates vanillate and syringate into protocatechuate, gallate, respectively through the O-demethylation process (Kato *et al.*, 2015). The co-overexpression of the enzyme, plant methionine synthase MetE and the tetrahydrofolate (THF)-dependent O-demethylase (LigM; *Sphingobium* sp. strain SYK-6) demethylates vanillic acid and reduces the cofactor utilization by 500 fold (Rosini *et al.*, 2016).

3.1. *Tetrahydrofolate(THF)-dependent O-demethylases*

Tetrahydrofolate-dependent demethylase (ligM; EC 2.1.1.ge) mediates the transfer of methyl moiety from the methoxy functional group in vanillate or 3-O-methylgallate to nitrogen 5 (N_5) of H_4folate, which forming protocatechuate (PCA), or gallate as demethylation products. THF-dependent O-demethylase mediates the reaction of (i) vanillate + tetrahydrofolate = protocatechuate + 5-methyltetrahydrofolate and (ii) 3-O-methylgallate + tetrahydrofolate = gallate + 5-methyltetrahydrofolate. THF-dependent O-demethylase is involved in the lignin degradation process and vanillin and vanillate are important intermediate metabolites of lignin-generated aromatic compounds. The bacterium, *Sphingomonas* sp. SYK6 converts vanillin to vanillate by vanillin dehydrogenase (encoded by ligV) (Kamimura *et al.*,

2017). Vanillate is further converted into protocatechuate by a tetrahydrofolate-dependent demethylase in Sphingomonas sp. SYK6, vanillate/3-O-methylgallate O-demethylase, encoded by ligM (Nishikawa *et al.*, 1998). Among the various wood degrading microorganisms, the bacteria are able to secrete the ligninolytic enzymes, which mediate various degradation pathways (Table 3). The demethylation process of lignin or non-phenolic compounds require either O_2, CO_2, ATP, NADH or some electron donors or acceptors in order to produce a highly reactive lignin molecule. Some bacteria utilize DL-Tetrahydrofolate (THF) and ATP for their anaerobic *O*-demethylation of phenyl methyl ethers (Berman and Frazer, 1992). The *O*-demethylases are several categories of enzymes with different demethylating systems, which act with the help of a cofactor that mainly strips off the methoxyl group from different poly phenylpropanoic units, such as lignin, vanillate, syringate, guaiacol and some non phenolic units (Kaufmann *et al.*, 1997; Kaufmann *et al.*, 1998a; Kaufmann *et al.*, 1998b; Masai *et al.*, 2004; Masai *et al.*, 2007; Sudtachat *et al.*, 2009). Among the demethylation systems, two O-demethylase systems are mainly involved; first type is from the aerobic microorganisms and consists of two proteins: IA type oxygenase and a reductase, both iron-sulfur enzymes containing the [2Fe–2S] redox clusters that consume oxygen and a reducing agent in the form of NADH, which as a result of demethylation generates formaldehyde, water and NAD^+, besides the demethylated substrate, protocatechuate as the end product (Overhage *et al.*, 1999; Masai *et al.*, 2007a; Masai *et al.*, 2007b). The second type is a tetrahydrofolate (THF)-dependent *O*-demethylase system, which is involved in several enzymatic components, including the substrate, tetrahydrofolate (THF) or a corrinoid protein as the methyl group acceptor, methyl transferase (transfer the methyl group to THF), that subsequently regerates THF, thereby yielding THF and formic acid in order to recover this cofactor for further conversion of the methoxylated substrates (Kaufmann *et al.*, 1997; Kaufmann *et al.*, 1998a; Kaufmann *et al.*, 1998b; Masai *et al.*, 2007a; Masai *et al.*, 2007b). Further, it goes through different funneling degradation pathways either intra- or extradiol ring opening as catalyzed by specific dioxygenases, and further may go through the β-ketoadipate pathway. However, the central node in the lignin demethylation systems is formation of protocatechuic acid, followed by catechol by the action of O-demethylase (Tables 1 and 3). Also other aromatic phenylpropanoid units may mediate the ring cleavage of the 3-O'Methyl gallate and the gallic acid systems have been described in *Sphingobium* sp. SYK-6 (Kasai *et al.*, 2004; Masai *et al.*, 2007a; Masai *et al.*, 2007b; Masai *et al.*, 1999). Usually, the phenolic units' ring cleavage occurs followed by demethylation (Kamimura and Masai, 2014; Masai *et al.*, 1999; Kamimura *et al.*, 2017). Another study on the co-overexpression of the coenzymes, such as the tetrahydrofolate (THF)-dependent *O*-demethylase LigM from the bacterium, *Sphingobium* sp. strain SYK-6, and the plant methionine synthase MetE enzyme are overexpressed in *E. coli*, which converts vanillic acid to the protocatechuic acid (PCA) with a 500-fold reduction in the cofactor utilization (Rosini *et al.*, 2016). The bacterium mediates the metabolic pathways and generates the intermediates, such as vanillate, 3-*O*-methyl gallate, and syringate, by the involvement of the tetrahydrofolate-dependent aryl demethylase LigM from *Sphingomonas paucimobilis* (Table 2). This enzyme is also believed to have been involved in the downstream aryl modification, ring opening, and mediates the aryl *O*-demethylation by LigM. Kohler *et al.*, (2017) described its 1.81 Å crystal structure, fold and a canonical folate-binding domain, its LigM's tetrahydrofolate-binding site and the protein–folate interactions, besides its catalytic tyrosine-dependent reaction mechanism.

3.2. *Vanillate O-demethylases*

The vanillate O-demethylase system consists of two types of proteins (i) an oxygenase and (ii) an oxygenase reductase. The first type is the vanillate O-demethylase oxygenases' subunit (vanA; EC:1.14.13.82), also known as the 4-hydroxy-3-methoxybenzoate demethylase, which mainly mediates the demethylation reactions of vanillate + O_2 + NADH = 3,4-dihydroxybenzoate + NAD^+ + H_2O + formaldehyde. Besides, this enzyme belongs to the bacterial ring-hydroxylating dioxygenase alpha sub-unit family. The second type of proteins is the vanillate O-demethylase oxidoreductases (vanB; EC:1.14.13.-), also known as the vanillate degradation ferredoxin-like protein. Vanillate may undergo an initial demethylation to yield protocatechuate as the demethylation product and the responsible enzyme is protocatechuate 3,4-dioxygenase, which is detectable from the mycelial extracts (Buswell *et al.*, 1982). However, several attempts have been made to demonstrate a cell-free demethylase activity but these efforts turned unsuccessful (Table 2 annd 3). However, a veratrate *O*-demethylase, able to demethylate both position 3- and 4- of the methyl groups, has been purified by affinity chromatography using a soft-rot fungus, *Chaetomium piluliferum* (Paszczyfiski and Trojanowski, 1977; Paszczyinski et al., 1985, 1986). The birch wood lignin biodegradation that the white-rot fungus, *P. chrysosporium* mediates, proceeds by way of common reactions, like those that drive the 3-*O*- or 5-*O*-demethylation process (Hibi *et al.*, 2005; Crewford and Olson, 1978; Tai *et al.*, 1982). It is evident from the fact that, three biodegraded lignins have significantly lower methoxyl contents than those of the birch milled wood lignin. Among them, the M-HmWA-2 fraction has extremely low methoxyl content (7.32 %) when compare to that of the birch milled wood lignin (20.52 %). The M-HMWA-2 fraction also has significantly higher oxygen content but lower C and H contents than those of the birch milled wood lignin (Tai *et al.*, 1982). The vanillate *O*-demethylase converts vanillate to protocatechuate (3,4-dihydroxybenzoate) and syringate or hydroxyvanillates to their corresponding phenolic compounds. Acetogenic bacteria use the *O*-methyl group, as a one-carbon growth substrate to carry out the oxidation of a methyl group to CO_2, which provides the six electrons, required for the conversion of the three methyl groups to acetate. Oxidation of one equivalent of CH_3–H_4folate to CO_2 generates H_4folate and six electrons, which drive the synthesis of three equivalents of acetyl coenzyme A (acetyl-CoA) by the Wood-Ljungdahl pathway of acetyl-CoA synthesis by the catalytic action of a three-component aromatic *O*-

demethylase from *M. thermoacetica* (Daniel *et al.*, 1991; Kasmi *et al.*, 1994; Kaufmann *et al.*, 1997; Kaufmann *et al.*, 1998a; Kaufmann *et al.*, 1998b; Meβmer *et al.*, 1993; ten Have and Teunissen, 2001). The extracts from cells grown on dicamba demethylate vanillate to protocatechuate and further decarboxylate the protocatechuate to catechol under standard assay conditions. Similarly, extracts of cells grown in the presence of vanillate could demethylate dicamba to dichlorosalicylate and convert vanillate to protocatechuate and catechol (Table 3). The enzyme from dicamba-grown cells utilizes vanillate much more efficiently than dicamba, as substrate. To conform to the nomenclature used by Krzycki and Thauer to describe the components of the methanogenic methyltransferases involved in demethylation of methylamines, methyl sulfides, and pterins (Sauer and Thauer, 1999) have been designated as the Mtv system, i.e., methyltransferase for vanillate. The reaction catalyzed by the Mtv system of *M. thermoacetica* is most similar to that of the four-component system of *A. dehalogenans* (Kaufmann *et al.*, 1997; Kaufmann *et al.*, 1998a; Kaufmann *et al.*, 1998b). Vanillate *O*-demethylase is presumed to be structurally similar to the ferredoxins, which are the iron-sulfur proteins that facilitate the electron transfer in a range of metabolic reactions (Naidu and Ragsdale, 2001). The vanillate *O*-demethylase from *Moorella thermoacetica* has been characterised as an enzyme with three components viz., MtvA, MtvB and MtvC. The function of MtvB has been established by studying the transfer of the *O*-methyl group of the vanillate to the Co(I) state of B12 to generate methylcobalamin. It has been observed that the protocatechuic acid concentration rises as the vanillate gets consumed, perhaps indicating that the MtvB participates in the initial methyl transfer (Naidu and Ragsdale, 2001). The three components required for the *O*-demethylation of dicamba and vanillate have been identified, isolated and purified to homogeneity. Omission of MtvA, MtvB, or MtvC from the *O*-demethylase reaction mixture results in a complete loss of the enzyme activity. All three-protein components have been observed to be indispensable for the dicamba *O*-demethylase activity (Table 1). Cell extracts that were bubbled with air for 0.5 h and then with nitrogen for 15 min (so that O_2 was not introduced into the assay mixture) have been observed to retain maximum *O*-demethylase activity. Therefore, it has been observed that the reaction itself, which involves Co (I) is O_2 sensitive but not the enzyme. The demethylation reaction appears to require the presence of a reductant in the reaction mixture. Although dithionite reduces Co(II) to Co(I), it appears to interfere with the *O*-demethylase activity. On the other hand, Ti (III) citrate reduces Co (II) and accelerates the O-demethylase reaction. The optimal temperature is 55 °C and the optimal pH is 6.6. As described above, H4folate is required as the acceptor of the methyl group from the vanillate (Naidu and Ragsdale, 2001). Another gene, ligM that is involved in converting the vanillate to protocatechuate has been identified from Sphingomonas sp. SYK6 by way of the functional complementation of *Escherichia coli*. The crude recombinant host-cell filtrates in the presence of tetrahydrofolate have been observed to be able to convert vanillate and 3-O-methylgallate into protocatechuate and gallate respectively (Abe et al., 2005). The vanillate-O-demethylase (VanA and VanB) enzyme from *Pseudomonas putida* that is expressed in *E. coli* strain K-12, has been found to convert effectively, the vanillate into protocatechuate releasing formaldehyde as the by-product in the demethylation process, that further gets converted into formate with glucose as the carbon source for growth. The lack of pgi or zwf genes in the mutant displays a low converting rate but the reverse has been true with the wild strain (Hibi *et al.*, 2005). By the gene disruption method and by measuring the enzyme activities, the gene encoding for the vanillin dehydrogenase (vdh) and vanillate O-demethylase (VanAB) enzymes has been identified for *Rhodococcus jostii* RHA1 (Fig. 2, Tables 2 and 3). With that strain grown on vanillin or vanillate, the gene vanA has been observed to be highly specific and upregulated, but not the gene, vdh as observed during lignin degradation by *Rhodococcus jostii* RHA1 and the other actinomycetes (Chen *et al.*, 2012). The vanillate gets demethylated into protocatechuate by the action of vanillate O-demethylase (VanA and VanB) in the *Streptomyces* sp. NL15-2K that was functionally expressed in the host *Escherichia coli*. With the tested substrates, vanillate O-demethylase displays high specificity to vanilliate syringate, m-anisate, and veratrate, but the veratrate demethylation occurs at the meta-position, relative to the carboxyl group (Nishimura *et al.*, 2014a, b). The draft genome sequence of *Pandoraea* sp. ISTQB, a lignin-degrading beta-proteobacterium revealed that the presence of putative genes has been responsible for the degradation of lignin and the other lignin-derived aromatic compounds. The lignin-degrading enzymes identified have been - DyP-type peroxidases, peroxidases, multicopper oxidases, esterases, coniferyl-alcohol dehydrogenase, coniferyl-aldehyde dehydrogenase, etherases, methyltransferases, and the vanillate O-demethylase oxidoreductase (Kumar *et al.*, 2016). Both vanillate-O-demethylase of VanA and VanB from *Pseudomonas putida* was overexpressed in *Escherichia coli* strain K-12 that converted vanillate into protocatechuate (Hibi *et al.*, 2005). Also operon encoded genes of *O*-demethylase components of *Desulfitobacterium hafniense* strain DCB-2 were cloned and heterologously expressed using *Escherichia coli* (Studenik *et al.*, 2012). Also vanilliate analogs generated protocatechuate by vanillate demethylase of VanA and VanB in *Streptomyces* sp. NL15-2K were expressed in *Escherichia coli* with recombinant whole cells demethylated vanillate, syringate, m-anisate, and veratrate, whereas, ferulate, vanillin, and guaiacol were not demethylated by *Streptomyces* vanillate demethylase because of *Streptomyces* vanillate demethylase mediated lignin demethylation at meta-position relative to the carboxyl group (Nishimura *et al.*, 2014a, b). Similarly various aryl-methyl ethers oxidative demethylation were mediated by Rieske monooxygenase VanA and VanB of *Pseudomonas* sp. HR199 were recombinantly coexpressed in *E. coli* showed that VanA-VanB system demethylates at meta position of the aromatic ring and the presence of a carboxylic moiety produces 77 % of protocatechuic acid from vanillic acid (Lanfranchi *et al.*, 2018) (Tables 2 and 3).

3.3. *Syringate O-demethylases*

Syringate O-demethylase [desA; EC:2.1.1.-] mainly mediates the demethylation of Syringic acid + Tetrahydrofolate <=> 3-O-Methyl Gallate + 5-Methyltetrahydrofolate (Masai *et al.*, 2004; Kamimura *et al.*, 2017). In the lignin degradation process, generation of the vanillate and syringate are important intermediates that can be utilized by *Sphingomonas paucimobilis* SYK-6 along with various lignin-derived biaryls as its sole sources of carbon (Fig. 2, Tables 2 and 3). These intermediates undergo the *O*-demethylation process and give rise to the 3-O-methyl gallate (3MGA), protocatechuate (PCA), respectively and thereafter, perhaps further degradation occurs by the ring cleavage mode (Masai *et al.*, 2004). The *desA* gene, encoding for the enzyme, syringate *O*-demethylase has been successfully isolated (Masai *et al.*, 2004). Substrate specificity of the syringate *O*-demethylase enzyme has been studied by experiments conducted with the intact cells (Tack *et al.*, 1972). Tetrahydrofolate appears to be required for the *O*-demethylation activity of SYK-6 to demethylate vanillate and the syringate intermediates (Nishikawa *et al.*, 1998). The DesA and the anaerobic demethylase systems are essentially different types of the O-demethylases systems, because DesA catalyzes the *O*-demethylation process of syringate and also transfers the methyl moiety to H4folate by a single protein (Fig. 2, Tables 2 and 3). The disruption of desA in SYK-6 causes growth deficiency in the mutant in the presence of syringate, but the mutant has been observed to grow still on vanillate, perhaps suggesting that different enzymes act on different modes to degrade the syringate and vanillate to varying degrees (Nishikawa *et al.*, 1998; Masai *et al.*, 2004). The *O*-demethylation process of the phenyl methyl ethers occurs under anaerobic conditions with *Desulfitobacterium* spp. Also *Desulfitobacterium* could be enriched with the product of the *O*-demethylation of syringate process as a growth-selective process. Adding an external source of the electron acceptor, like 3-chloro-4-hydroxyphenylacetate or thiosulfate, carries out the enrichment (Mingo *et al.*, 2016).

3.4. *Veratrate O-demethylases*

The Veratrate O-demethylases belong to the family of oxidoreductases, also know as 4-methoxybenzoate monooxygenase (O-demethylating; EC 1.14.99.15), which mainly catalyze the chemical reaction that requires 3 substrates for this enzyme's activity, namely 4-methoxybenzoate, an electron acceptor AH_2, and O2, while its 4 products are 4-hydroxybenzoate, formaldehyde, the reduction product A, and water acting on the paired donors, with O_2 functioning as the oxidant. The oxygen that gets incorporated need not be derived from the O miscellaneous (Fig. 2, Tables 2 and 3). The systematic name for this enzyme class is, 4-methoxybenzoate, hydrogen-donor: oxygen oxidoreductase (O-demethylating). The other common names in use currently, include 4-methoxybenzoate 4-monooxygenase (O-demethylating), 4-methoxybenzoate O-demethylase, p-anisic O-demethylase, and piperonylic-4-O-demethylase. This enzyme participates in the 2, 4-dichlorobenzoate degradation (Bernhardt *et al.*, 1977; Paszczynski and Trojanowski, 1977; Twilfer *et al.*, 1981).

4-methoxybenzoate + AH_2 + O_2 —→4-hydroxybenzoate + formaldehyde + A + H_2O

Veratrate *O*-demethylase produces isovanillate by the demethylation or hydroxylation of veratrate, a non-phenolic compound. *Nocardia corallina* A81 converts *p*-anisic acid to *p*-hydroxybenzoic acid and isovanillate (Crawford *et al.*, 1973). This enzyme also demethylates veratrate to a mixture of vanillate and isovanillates. The chloro-substituents present at the aromatic carbons of the methoxylated benzoic acids are believed to inhibit the normal metabolism of these compounds in *Nocardia* spp. Further, the *o*-chloro substituents prevent the demethylation process of the of *p*-anisic acid. The washed whole cells of veratrate-grown *N. corallina* A81 do not appear to bring about any structural changes to the isolated lignin (Crawford *et al.*, 1973).

Cell-free enzymes sometimes are believed to be inactive on the methoxylated substrates, perhaps due to the possibility of air-inactivation of the demethylase activity (Ribbons *et al.*, 1970). The veratrate demethylation and reduction by *P. cinnabarinus* has been a proven one. *P. radiate* catalyzes the veratrate demethylation by the involvement of both the demethylation and decarboxylation reactions resulting in the generation of guaiacol as an end product. However, such products are not detectable with the *P. cinnabarinus* demethylation (Hatakka, 1985; Leonowicz *et al.*, (1991). But guaiacol formation has been observed with a *Streptomyces* sp. during the vanillin degradation (Crawford and Olson, 1978). These veratrate demethylation reactions, as mediated by the veratrate demethylase clearly indicate that the demethylation process does not get catalyzed by the LiP or peroxidase or by the laccase (Leonowicz *et al.*, 1991; Pasczynski and Trojanowski, 1977; Farmer *et al.*, 1959; Minami *et al.*, 1965). The veratrate *O*-demethylase has also been isolated from *Chaetomium piluliferum* and *Xerocomus badius* (Paszczynski and Trojanowski, 1977). Interesting findings have been reported of a microbial demethylase from bacteria, yeasts, and fungi isolated from the paper-mill wastewater grown on veratryl alcohol (Fig. 2, Tables 2 and 3). *Penicillium simplicissimum* oxidizes the veratryl alcohol to veratrate via veratraldehyde. The veratrate-induced cells have been observed to display the *O*-demethylase activity towards veratrate, vanillate and the isovanillic acid (Jong *et al.*, 1990). The specificity of vanillate hydroxylase, isolated from *P. cinnabarinus* is not known, but since the demethoxylation process of veratrate is relatively rapid and formation of the free hydroxyl groups is essential, the phenoloxidase-driven demethoxylation that mediates the oxidative decarboxylation of veratrate may not be fast enough to rid the methoxyl group. Buswell *et al.*, (1982), were unable to identify any intracellular *O*-demethylase activity from the *P. cinnabarinus* A-360 mycelial extracts, owing to the influence of various factors, including the culture conditions, extract preparation and the relative stability of the *O*-demethylases etc. However, Paszczynski and Trojanowski, (1977) partially purified a veratrate *O*-demethylase from two fungi, including a basidiomycete, *Xeromyces badius*, which require NADH and oxygen to mediate the demethylation process and the veratrate demethylation process at both the 4- and 3-OCH_3 positions, also exhibits stronger activity towards the para-methoxyl group (Fig. 2). The study with *P. cinnabarinus* exhibits stronger

activity against the position 4- of the methoxyl group. The 4-demethylation of veratrate, which forms vanillate is one of the most important tangents with lignin degradation in *P. chrysosporium*. In another case, it has been proved that phenol oxidases are not involved in the veratrate demethylation, and this probably points to the possibility of an alternative demethylating system operating. This study provides adequate evidence of a rapid demethylation process of veratrate taking place in the early stages of growth and the to the fact that the regular phenol oxidase activity is non-existent, perhaps suggesting the operation of some other demethylase mechanism. An early report says that lignin demethylation is less studied with fungi. The mycelial extracts of *Chaetomium piliferum* (ascomycete) and *Xerocomus badius* (basidiomycete) are known to give rise to formaldehyde from the veratrate substrate. A fungal O-demethylase enzyme requires both oxygen and NADH to mediate the reaction and the enzyme is highly unstable and also is observed to be sensitive to the molecular oxygen (Paszcynki and Trojanowski, 1977). The soft-rot fungi, however, release high volume of $^{14}CO_2$ from the ^{14}C-labelled methoxyl groups of vanillate-, syringate-, veratric- and ferulate (Buswell *et al.*, 1982; Ander *et al.*, 1984; Haider and Trojanoski, 1975, 1980). The microorganisms can also trigger an alternate degradation pathway, mainly involving the 4-*O*-demethylation process followed by an *O*-ether cleavage event (Fukuzumi, 1969; Kirk, 1975). Initially, demethylation of the lignin-like dimers has received little attention as compared to that of the monomeric compounds, such as veratrate. The phenolic and non-phenolic β-O-4 dimers have been used as substrates in order to rear *Phanerochaete* sp.-K-3, which displays a scanty methanol production (Ander *et al.*, 1985). *Gloeoporus* (*Polyporus*) *dichrous* selectively demethylates the 3,4-alkyl benzoic acids at position at 4 and is proposed to be driven by an oxidative dealkylation mechanism (Kirk and Lorenzo's, 1974) and the vanillate metabolite, methoxy hydroquinone gets demethylated by *P. chrysosporium* (Ander *et al.*, 1983). *Phanerochaete chrysosporium* is believed to possess the ability to liberate methanol from a variety of dimethoxyl aromatic monomers. Interestingly, these alkylated compounds are much less reactive than their corresponding phenols, perhaps suggesting that the free hydroxyl radical is important for their reactivity (Ander *et al.*, 1985).

The number of methoxyl and the hydroxyl functional groups in the phenylpropanoid units also appears to influence the demethylation process, besides the O-demethylase activity. The soft and white-rot fungi release CO_2 from the methoxyl groups and both groups of fungi strongly release CO_2 from the 4-OCH_3-group than from the 3-OCH_3-group of the veratrate. This probably suggests that with respect to the demethylation process, the two methoxyls of the veratrate are not equivalent; similar observations have been made with different groups of organisms. Early studies suggest that a purified *p*-anisate-*O*-demethylase from *Pseudomonas* sp., demethylates the veratrate only at the 4-OCH_3 group, but not at position-3 (Cartwright and Buswell, 1967; Bernhardt *et al.*, 1970; Buswell and Mahmood, 1972). Similar observations made with the soft-rot fungi indicate that the demethylation of the two methoxyl groups of veratrate could be induced upon preincubation with the vanisic or veratrate at position- 4-O but not at position- 3-O. The conversion from methoxy to the hydroxyl groups in the mono methyl benzoic acids was investigated in *Haplographium* sp., *Hormodendrum* sp., and *Penicillium* sp. and in each case, the corresponding hydroxyl-acid has been found in the extract. Only traces of *o*- and *p*-hydroxybenzoic acids are observed, but strong traces of *m*-hydroxybenzoic acid have been noticed (Henderson, 1957). The removal of the methoxyl group, during the conversion of the *m*-methoxybenzoic acid to the *m*-hydroxybenzoic acid and formation of the p-methoxyphenol from *p*-methoxybenzoic acid has been confirmed (Fig. 2, Tables 2 and 3). These results indicate that if the methoxy groups are transferred to another molecule, as appears probable, this molecule does not get accumulated but gets rapidly decomposed. A further possibility perhaps is the release of a volatile compound. Attempts made to trap and identify the volatile compounds, if any, have not been successful (Henderson, 1957). The effect of diluted formaldehyde on the rate of demethylation/re-methylation of veratrate in *Rhodococcus erythropolis* was studied, wherein certain nonlinear changes in the activity of oxygen-dependent demethylase system and the ROS-activated formaldehyde have been observed to have been produced during the course of the veratrate demethylation process (Malarczyk *et al.*, 2011). These trends, perhaps suggest that the demethylation reactions require a 4-*O*-demethylase enzyme and that methylation occurs spontaneously by means of the radicals. It has been well established that the methylation process of the vanillate occurs rapidly. It has also been shown that the fluctuation in the concentration of the veratrate and some changes to the NADH oxidase activity associated with the methylation processes do occur (Malarczyk *et al.*, 2011).

Overall, O-demethylase mediates the initial step in lignin-derived compounds and lignin degradation, which strip off the O-methyl groups from the phenylpropanoid units produces methanol and widely accessible catecholic groups. The demethylation step in *Sphingobium* sp., SYK-6 mediates by secreting the tetrahydrofolate (H_4folate)-dependent *O*-methyltransferase (LigM) generates vanillate/3-*O*-methylgallate and syringate that further O-demethylated to protocatechuate/gallate and 3-*O*-methylgallate or ring cleavage (DesA) (Masai *et al.*, 2004; Abe *et al.*, 2005). Also O-demethylation catalyzes by O-demethylase metabolizes vanillate/3-*O*-methylgallate and syringate that produces 5-methyl-H_4folate through one-carbon metabolism. In addition the crystal structure of tetrahydrofolate (H_4folate)-dependent *O*-methyltransferase (LigM) described and overall the LigM structures similar like T-protein (Harada *et al.*, 2017; Kohler *et al.*, 2017). These studies showed that the overall structure of LigM is similar to T-protein but differs in active site that indicates LigM divergently evolved from T-proteins, which contains suitable active site to mediate O demethylation of vanillate/3-*O*-methylgallate (Fig. 2, Tables 2 and 3). In addition, for LigM activity, it needed Tyr247 hydroxy proton and lone pair of vanillate methoxy oxygen involve in methyl moiety to H4folate transfer (Harada *et al.*, 2017). However, *Sphingobium* sp., SYK-6 is utilizes protocatechuate as corbon source for their growth in presence of methionine only (Masai *et al.*, 2004). As a result O-demethylation generates 5-methyl-H_4folate from vanillate/3-*O*-methylgallate and syringate and

the crystal structure of LigM were described (Harada *et al.*, 2017; Kohler *et al.*, 2017; Kamimura *et al.*, 2017). In the reaction of O-demethylation in lignin-derived biphenyl compound DDVA mainly mediated by a three-component monooxygenase that consist: (i) an oxygenase component (LigXa: SLG_07770), (ii) ferredoxin (LigXc: SLG_08500) and (iii) ferredoxin reductase (LigXd: SLG_21200) (Yoshikata *et al.*, 2014). At the same time, the vanillate demethylation efficacy mediated by *Pseudomonads* using class IA oxygenase VanA and VanB reductase was reported (Brunel and Davison, 1988; Civolani *et al.*, 2000). The *ligXa deduced* amino acid (AA) sequence shows similarities with Rieske-type non-heme iron aromatic-ring hydroxylating oxygenases (RHOs), phenoxybenzoate dioxygenase (PobA) from *Pseudomonas pseudoalcaligenes* POB310, 3-chlorobenzoate 3,4-dioxygenase (CbaA) in *Comamonas testosteroni*BR60 (CbaA), and phthalate 4,5-dioxygenase (Pht3) by *P. putida* (Nomura *et al.*, 1992; Nakatsu *et al.*, 1995; Dehmel *et al.*, 1995). But the 60 % AA sequence of LigXc and LigXd similar with in a three-component O-demethylase of ferredoxin (DdmB) and ferredoxin reductase (DdmA1) in dicamba O-demethylase secreted by *Stenotrophomonas maltophilia* DI-6 (Herman *et al.*, 2005). Whereas, LigXa of AA sequence similar to 16 % of dicamba O-demethylase oxygenase component (DdmC), 39 % of LigXc (CarAcI) and 59 % of LigXd (CarAdI) of *Novosphingobium* sp. KA1 can act as electron transfer components (ETCs) like ferredoxin and ferredoxin reductases (Shintani *et al.*, 2007). However, in FAD prosthetic group of LigXd utilizes NADH as electron donor (Yoshikata *et al.*, 2014). Also the another observation of Fe(II) requires for the activity of DDVA O-demethylase that modified with homotrimer of LigXa, monomer of LigXc and monomer of LigXd (Harada *et al.*, 2017; Kohler *et al.*, 2017; Kamimura *et al.*, 2017). Further the genome searches of seven bacterial strains of *Sphingomonads* SYK-6, *N. aromaticivorans* DSM 12444, *Novosphingobium* sp. PP1Y, *Sphingobium chlorophenolicum* L-1, *Sphingobium japonicum* UT26S, *Sphingomonas wittichii* RW1 and *Sphingopyxis alaskensis* RB2256 resulted in gene encoding of 2–8 genes of ferredoxin, 1–3 genes of ferredoxin reductases with pyr_redox and Reductase_C domains, and 2–4 genes of reductases contains FNR-like domain and presence or absences of fer2 domain (Fig. 2, Tables 2 and 3). Also, these bacterial strains shows one or more *ligXc* ortholog, *ligXd* orthologs with >58 % AA sequence identities. In addition the characterization and sequence identity of with LigXc and LigXd, with electron transfer components of cytochrome P450s in *N. aromaticivorans* DSM 12444 shows 64 % Saro_1477 ferredoxin (Arx) and 60 % of Saro_0216 ferredoxin reductase (ArR) (Bell *et al.*, 2010). These results suggest that LigXc and LigXd orthologs also involves in transfer electrons to oxygenase components of Rieske-type non-heme iron aromatic-ring hydroxylating oxygenases (RHOs) in addition electrons transfer occurs in P450 enzymes (Tables 2 and 3). As a result of genomic sequence of Sphingomonad strains showed less ETC genes and larger number of 7–53 genes of oxygenase component genes but only 1 gene in *S. alaskensis*RB2256. These strains showed P450 genes of 5–18 genes in each but 1 gene in SYK-6. Hence, these few ETCs of RHO and P450 systems and the LigXc and LigXd orthologs plays major role in oxygenase systems of Sphingomonads (Harada *et al.*, 2017; Kohler *et al.*, 2017; Kamimura *et al.*, 2017).

4. Ligninolytic enzymes and demethylation

4.1. *Laccases and Peroxidases*

The multicopper oxidases – laccases mediate the one-electron oxidations of a variety of aromatic compounds, such as phenolics, non-phenolics and xenobiotics. During the oxidation reactions, the laccase enzyme shows a wide range of substrate specificity towards the phenolic type in the detoxification process (Yaropolov *et al.*, 1994; Ishihara and Nishida, 1983; Kersten *et al.*, 1990). Several investigations focused on the potential applications of the laccases in the demethylation of lignin and the LMCs. The findings of Frick and Crawford, (1984) demonstrated the demethylation of [$O^{14}CH_3$]-polyguaiacol with *P. chrysosporium* serving as a model. The laccase enzyme displays activity against catechol or hydroquinone. But the unsupplemented preparations of the enzyme do not show any activity against o-dianisidine. However, when H_2O_2 (0.8 mM) was added to o-dianisidine, it gets more rapidly oxidized, perhaps suggesting, that the enzyme preparation contains a peroxidase, but lacks the needed levels of the substrate, H_2O_2. The demethylation process with poly guaiacol was investigated by including, NADH, NADPH and FAD, or FMN as co-factors, and wherein the enzyme reactions are not observed to have induced any demethylation reactions. However, demethylation gets stimulated by >50 % upon the addition of H_2O_2. A laccase from *Polyporus versicolor* converts more than 1 % of the milled wood lignin methoxyl groups into methanol on a 3 h incubation (Ishihara and Miyazaki, 1974). However, laccase and peroxidase have been observed to demethoxylate lignin (Trojanowski *et al.*, 1966) and methanol released from both the vanillate and MHQ with *S. pulverulentum* (*P. chrysosporium*) (Ander *et al.*, 1983a). A reinvestigation with the methoxyl-labelled vanillate suggests that a 12.5 % methanol is produced by the activity of laccase, while with peroxidase; only a 10.2 % could be recovered (Trojanowski *et al.*, 1966; Ishihara and Miyazaki, 1974; Ander *et al.*, 1980).

Among the various wood-rotting fungi, the white-rot fungi do not appear to display any laccase activity, but with the soft-rot fungi, a mild peroxidase activity is detectable. Some investigators surmise that both the laccase and peroxidase are involved in the lignin degradation process, besides in the demethylation as well as the depolymerization processes (Harkin, 1967; Trojanowski and Leonowicz, 1969; Gierer and Opara, 1973). In the white-rot fungi, the methoxyl groups of vanillic acid (VA), methoxyhydroquinone (MHQ) and the other lignin-related phenols are cleaved by means of laccase and peroxidase and are observed to generate methanol as a demethylation product (Ander *et al.*, 1983).

4.2. *Laccases and lignin demethylation*

Laccases (EC 1.10.3.2) mediates the oxidation of a wide range of phenolic and non-phenolic compounds coupled to the four-electron reduction reaction that generates molecular oxygen to water. Laccase are copper-containing oxidase enzymes that act on phenols and lignin-related compounds and are known to mediate the one-electron oxidations

(Fig. 3). Laccases rapidly catalyze the demethylation reactions of the terminal phenolic units, which is followed by a complete degeneration by means of oxidation, reduction, depolymerization and polymerization reactions within the lignin macromolecule. Laccases acts on the biopolymers of lignin that mainly involve in the demethylation reactions of lignin, simultaneously modifications of cellulose occurs by the action of the radicals generated by the other enzymes, like LiP and MnP that are involved in the laccase-mediated demethylation process. Sometimes, the demethylation reaction requires mediators, such as 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulfonate) (ABTS) or 1-hydroxybenzotriazole (HBT) (Bourbonnais and Paice, 1992). The laccase also catalyze the demethylation process of the phenolic compounds and the non-phenolics as well, besides the LMCs (Eriksson *et al.*, 1990a, b; Krik and Farrell, 1987). It has been reported that in the presence of ABTS or HBT, these enzymes effectively demethylate the kraft pulp (Bourbonnais and Paice, 1992). Laccases catalyze the oxidation of the lignin-related phenol vanillyl glycol resulting in the initial formation of dimers, and the subsequent polymerization reactions release methanol as a byproduct (Lundquist and Kristersson, 1985). The initial step in the degradation by *T. versicolor*, the laccases appear to be involved mainly in the demethylation and hydroxylation reactions, even in the absence of the ABTS and the HBT mediators (Crestini and Argyropoulos, 1998). The laccase-mediated demethylation of the non phenolic β-O-4-linked LMC dimers with *Phlebia tremellosa* suggests that the phenolic products are oxidized by laccase and the α,β-cleavage is catalyzed by the LiP activity. The reaction was studied by measuring the amount of $^{14}CH_3OH$ produced by the demethylation of 1-(3′,4′-dimethoxyphenyl)-2-(o-methoxyphenoxy)-propane-1,3-diol. Demethylation is stimulated by oxygen and is inhibited by several other common metabolic inhibitors, such as cyanide, diethyldithiocarbamate, mercury chloride, acetyltrimethylammonium bromide, and dithiothreitol (Reid, 1992). Laccase is believed to be responsible for the demethylation via release of the methoxyhydroquinone (MHQ) (Higuchi, 1985). The results clearly suggest that dealkylation is a rate-limiting step, and the phenolic products are rapidly attacked by the laccase enzyme for further metabolism and methanol appears to be a direct product of demethylation and that formaldehyde is not a product of the demethylation reaction.

Lacasses and the ABTS mediators catalyze the delignification of various softwood and hardwood kraft pulps. However, the demethylation reaction, as measured by the amount of methanol released correlates better with the initial kappa number of the pulp than with the amount of lignin released. Higher syringyl content of the hardwoods lead to a higher degree of methanol production than that of the softwood pulp with similar lignin quantities. Hardwood contains more free phenolic groups than the softwood, and these were previously demonstrated to be the only groups that get demethylated by laccase-ABTS (Bourbonnais and Paice, 1992, 1996). The demethylation of lignin is a crucial step during lignin degradation and in a recent study on *Galerina* sp., HC1 laccase was put to use, so as to demethylate the Eucalyptus kraft pulp (Ibrahim *et al.*, 2011). Low concentrations of laccase 1 were found to be more efficient in the presence of ABTS (46.7 vs. 21.7 mg/mL and 125 vs. 86.7 mg/mL of methanol released at 0.1 and 0.5 U/mL, respectively). Bourbonnais and Paice (1992, 1996) observed a similar trend in their studies on the laccase-ABTS -mediated demethylation process of both the hardwood and softwood. However, a noticeable correlation between the demethylation rate and the enzyme dose has not been observed (Ibrahim *et al.*, 2011). The highest concentration of methanol obtained has been, 142 mg/L, which corresponds to a 13.7 mg MeOH/g lignin. Although the extent of demethylation observed with 0.1 U/mL laccase 1 is comparable to the levels observed with the other white-rot laccases, the demethylation reaction with laccase 1 appears much faster and to proceed at lower temperatures (Bourbonnais and Paice, 1992; Bourbonnais et al., 1995, 1997; Kim *et al.*, 2002; Huttermann *et al.*, 2001) concluded that the most significant alterations of lignins during treatment with laccase enzymes, include changes that occur during solubilization, demethylation and the phenolic and aliphatic hydroxyl content, besides changes that occur in the molecular weight distribution. Even low doses of ABTS or HBT appear to enhance the laccase demethylation rate of the veratrate in *T. versicolor* and *Cerrena unicolor* (Malarczyk *et al.*, 2009). The degradation products of the kraft

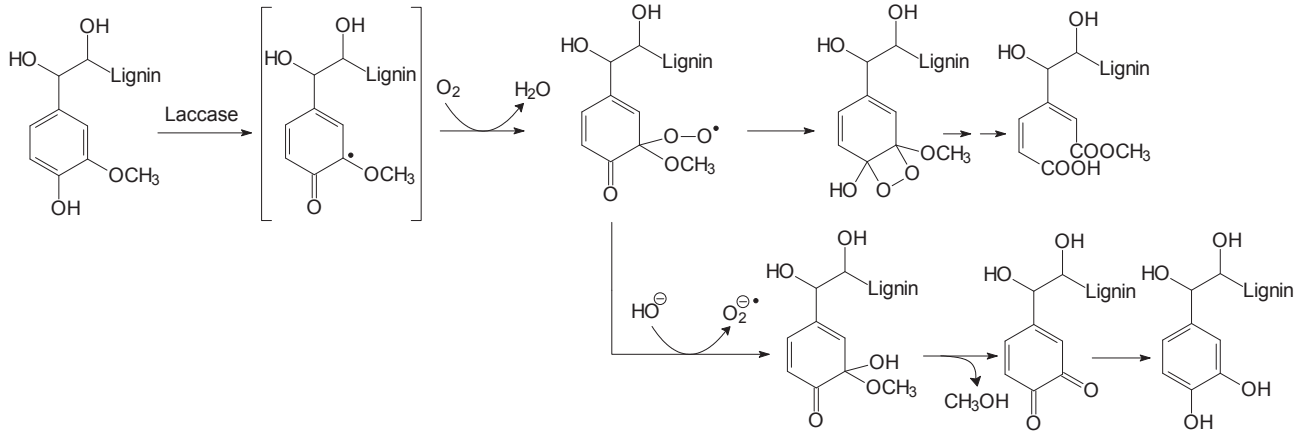

Fig. 3 – Laccase catalyzed lignin demethylation which is generates vicinal diols rich lignin for polymer applications.

pulp lignin containing polyoxometalate (POM) releases veratrate and veratraldehyde, which reveals that a potential demethylation reaction occurs and releases methanol as a demethylation co-product (Bujanovic *et al.*, 2010; Kim *et al.*, 2007). In another study with the kraft lignin and LMCs, it has been observed that the oxidative processes are also involved in demethylation (Hern'andez Fernaud *et al.*, 2006). Trojanowski *et al.*, (1966) and Ishihara and Miyazaki, (1974) reported a laccase and peroxidase-mediated demethylation of the vanillate (Fig. 3). Ander *et al.*, (1983) confirmed by their findings with *S. pulverulentum* by using the methoxyl-labelled vanillate incubated with laccase and a 12.5 % MeOH, that the production occurs only after a 2 h incubation. The milled maple–wood lignin, lignin sulfonate, vanillate, or vanillyl alcohol incubated with laccase from the wood-rot fungus, *Polyporus versicolor*, have been observed to have produced both methanol and formaldehyde (Ishihara and Miyazaki, 1974). Demethylation of the methoxy aromatic acids by *Polyporus versicolor* and *Polystictus sanguineus* has been reported by Farmer *et al.*, 1959; Minami *et al.*, 1965. Peroxidase (with H_2O_2) and laccase in the *Pholiota mutabilis* cultures have been found to be responsible for the demethylation of lignin. LMCs that result form the basis of inhibition (Trojanowski *et al.*, 1966). By the use of horseradish peroxidase (with H_2O_2) with methoxyphenols, it has been observed that a simultaneous occurrence of both demethylation and the ortho quinone formation takes place (Minami *et al.*, 1965). However, Fukuzumi *et al.*, (1969), Cartwright and Smith, (1967) proposed that the *O*-demethylation process occurs in the presence of NADH or NADPH and the required enzymes from *Poria subacida* and *Pseudomonas fluorescens*, respectively (Table 2). In this case, formation of formaldehyde has been adequately proved. Normally, the role of laccase is to rapidly attack the phenylpropanoid units that eliminate hydrogen from the phenolic hydroxyl group that generates the phenoxy radical. This may be subsequently, converted into the ortho quinone, just as in the case of the peroxidase oxidation, perhaps accompanied by the release of methanol. Although it is confirmed that laccases are capable of splitting the methoxyl group from lignin and LMCs, whether laccase plays a main role in the biological decomposition of lignin or not, is still uncertain. A basic scheme has been proposed for the demethylation of the 2.4, 6-trimethoxyphenol by laccase or horseradish peroxidase and H_2O_2. A similar mechanism could, perhaps operate during the reaction of the phenoxyl radical with O_2. In another study, Ishihara and Miyazaki (1974) reported that laccase B purified from *Polyporus versicolor* liberates methanol from the milled wood lignin equivalent to 1.3 % of the original methoxyl groups after a 3 h incubation (Ishihara and miyazaki, 1976).

For fungal enzymes related to the demethylation such as laccase, peroxidase (Trojanoski *et al.*, 1966), and ligninase (Ander and Eriksson, 1985) are believed to mediate the reactions involved in that process. Also proposed that the initial, non-specific one-electron oxidation of lignin, followed by a subsequent breakdown by means of the radical cations provides the necessary mechanism for the demethylation process. By comparing the ability of *Schizophyllum commune* to demethylate DHP-lignin (Dehydrogenation polymer) with that of *Polyporus* spp., it has been observed that no appreciable amounts of laccase, peroxidase or Poly B-411 oxidase could be detected. *S. commune*, however, has been observed to release $^{14}CO_2$ resulting from the demethylation of $O^{14}CH_3$- labelled synthetic lignin-DHP. Even after the addition of H_2O_2, neither laccase nor peroxidases are detectable after 6–7 d of incubation, whereas during this period, maximum rates of DHP demethylation are observed. Interestingly, the rise in the demethylation rate with the culture of *S. commune* correlates with the exhaustion of glucose, indicating, perhaps that further development proceeds by way of secondary metabolism. On the basis of these findings, it appears that the ability of *S. commune* to demethylate lignin may not be due to laccase, peroxidase or ligninase production by the organism. However, the analogous elimination of the methoxyl groups that potentially paves the way for the formation of the ortho-benzoquinone structures has also been adequately established (Gupta and Sehgal, 1979; Gupta *et al.*, 1981; Erickson *et al.*, 1990a, b) on the enzymatic oxidation of the syringyl models. Further, the demethylation process, by way of an enzymatic phenol oxidation has been studied by using 2,4,6-trimethoxyphenol as a model that releases methanol to give rise to the quinone products. The formation of those products has been observed with the other phenol oxidase, laccases, tyrosinases or peroxidases, probably confirming the possible operation of those mechanism that have been suggested for the ortho-quinone formation by the demethylation process (Fig. 3, Table 2). Whereas, the formation of the quinones XI and XII confirms that phenol oxidases are involved in their formation and that the degradation (Ishihara and Miyazaki, 1972) of lignin readily induces demethylation, by means of a free radical coupling mechanism (Erickson *et al.*, 1990a, b). The preponderance of the *p*-quinone XI would seem to suggest that elimination of a para-oriented substituent, even a *p*-methoxy group, is much preferred to elimination of an ortho-methoxyl group. This, however, contradicts the formation of substantial yields of ortho-quinones from the syringyl moieties on oxidation with peroxidase/H_2O_2, as observed with two other LMCs (Erickson *et al.*, 1990a, b). In each of these cases, the para-substituent, i.e., another syringyl group or a t-butyl group (Erickson *et al.*, 1990a, b), is higher in content, thus presumably the coupling process in a para position gets sterically suppressed. In lignin, the bulky groups occur in the para position in higher lignols; formation of the ortho-quinones might be substantial or even preferred. The brown-rot of wood is mainly associated with extensive loss of the methoxyl groups from lignin (Kirk and Adler, 1970), and a portion of the loss can, thus be ascribed to the *o*-quinone formation by the phenol oxidation mechanism. However, a methoxyphenol yields a catechol compound and the orthoxyphenol yields a catechol compound and an ortho quinone compound generated by way of the oxidation by peroxidase and H_2O_2. Presence of a quinone reducing enzyme (Westernmark and Eriksson, 1974) and a ring splitting enzyme (Flaig and Haider, 1961; Cain et al., 1968) in some wood rotting fungi has already been reported. Therefore, the following sequence in the biodegradation of lignin may be conceivable: demethylation (ortho quinone formation) -> reduction (catechol formation) -> ring splitting (aliphatic compound formation) -> metabolic pathway and the process and production of an *ortho*-quinone group in the product has

been proved by converting it into the phenazine derivatives (Ishihara and Ishihara, 1976). The oxidation activity of the separated inducible forms of laccase from *Pholiota mutabilis* has been three times as high, and the demethylating activity twice as high, as that of the constitutive forms. The oxidation/demethylating activity ratio of the constitutive (either extra- or intra-cellular) forms is distinctly lower than that of the inducible forms. It is of considerable interest that the number of moles of methanol released on demethylation of the syringate corresponds to a decrease in the methoxyl groups in the reaction medium. This confirms the scheme of demethylation of syringate, as proposed by Ishihara and Ishihara, (1976).

Both the fungus and a combination of laccase and ABTS, which demethylate the pulp and during the delignification process releases methanol, even as the rate of delignification greatly diminishes (Fig. 3, Table 2). MnP was first discovered in *P. chrysosporium* (Kuwahara *et al.*, 1984; Paszczynski *et al.*, 1985). The MnP and a peroxidase-dependent demethylation and delignification of the kraft pulp have been observed to occur *in vitro*. A partially purified MnP has been noticed to carry out most of the demethylation process. The results shows that for the hardwood pulp, MnP and to a lesser extent, laccase remove the methoxyl groups form the phenolic rings of the residual lignin. But under similar conditions, laccase alone has been observed to be unable to demethylate the pulp. However, as has been previously reported (Bourbonnais and Paice, 1992), laccase alone can cause less demethylation in the Kraft pulp, but in the presence of ABTS, an extensive demethylation takes place. Since demethylation of the kraft pulp by the crude enzyme filtrate of *T. versicolor* is enhanced by Mn (II) and H_2O_2, it has been concluded that most of the *in vivo* demethylation is caused by an MnP -mediated oxidation process.

Investigations on the demethylating ability of laccases or peroxidases revealed interesting observations with *Pholiota mutabilis,* wherein the demethoxylation of lignin and vanillate occurs in the presence of selective inhibitors (Fig. 3, Table 2). Peroxidase and laccase have been actively involved in the demethoxylation process of vanillate and lignin with the methoxyl groups, albeit dwindling (Trojanowski and Hüttermann, 1987; Trojanowski and Leonowicz, 1963; Leonowicz and Trojanowski, 1965). The difference in the degree of demethylation of lignin and vanillate is, probably due to the fact that, in the case of highly polymerized molecules of lignin, only a small quantity of the methoxyl groups is available for the activity of the enzymes involved. A majority of these groups is, probably within the tightly packed heterogeneous molecules. It appears that the highest effect of demethoxylation occurs after the addition of H_2O_2 and a total inhibition, perhaps occurs after the addition of a combination of inhibitors of laccase and peroxidase, such as dequalinium (DECA), hydroxylamine, or sodium azide. The addition of either excess or small amounts of H_2O_2 affects the demethoxylation process. Besides, an excess of H_2O_2 may even cause inactivation of the catalase enzyme (Fig. 3, Table 2). These observations indicate that peroxidase and laccase are sufficiently reactive to drive the demethylation process of the vanillate and lignin (Trojanowski and Hüttermann, 1987). In the culture media of *B. adusta* CCBAS 930, which contains lignin as a growth substrate, the laccase activity has not been detected, which perhaps leads to the conclusion that the laccase-like peroxidase can catalyze the reactions involved in the process of demethylation (Leonowicz *et al.*, 1999). The demethylation process that is associated with a phenol oxidation, probably involves an acid-catalyzed liberation of methanol. Such an acid-catalyzed mechanism provides an explanation for a probable pH-dependence of the demethylation process (Ishihara, 1983; Lundquist and Kristersson, 1985). In short, the extensive oxidation of the phenolic lignin-related phenylpropane compounds, that takes place by the activity of the fungal laccases leads to an appreciable demethylation quantum, besides the generation of the quinones (Bourbonnais and Paice, 1992).

During the bleaching process of the kraft pulp, it is well known that the demethylation of lignin is a key step. The enzyme involved, is also know to demethylate the phenolic lignin sub-structures (Ishihara and Miyazaki, 1974; Lundquist and Kristersson, 1985). The bleaching process of the pulp by the fungus, *T. versicolor* is accompanied by a methanol release, owing to the action of the laccase enzyme, which in the presence of ABTS produces methanol, besides delignifying the pulp (Fig. 3, Table 2). The hardwood kraft pulp incubated with *T. versicolor*, under conditions that induce delignification (kappa reduction), enhances brightening of the pulp as methanol gets accumulated in the solution (Paice *et al.*, 1995). Probably, methanol originates from the demethylation reactions of the pulp, because no methanol is detectable in the absence of pulp. The methanol production has also been previously reported during the lignin biodegradation by *P. chrysosporium* (Ander and Eriksson, 1985; Ander *et al.*, 1985) and also is produced when the $O^{14}CH_3$-1abelled DHP lignin is treated with MnP, LiP or laccase, although the extent of demethoxylation is much less (Ander *et al.*, 1990). The addition of ABTS to the crude or purified enzyme preparations, drastically improves the amount of methanol released, accompanied by a reduction in the kappa number. Methanol release with laccase and ABTS stabilizes, approximately a day after. This perhaps suggests that, under these experimental conditions, lignin in the pulp is first rapidly demethylated, as there has been no significant improvement in the demethylation rate nor a kappa reduction of the pulp after a 5d incubation. The delignification process of the unlabeled pulp is preceded by a substantial demethylation quantum. In the presence of ABTS, the pulp demethylation, as measured by the released methanol has been to the tune of between 53.3 and 69 mg/L (Table 2). In general, pulp demethylation by the laccases, in the presence of 1-hydroxybenzotriazole (HBT) is much lower than that by laccases in the presence of ABTS, except the *G. glossum* laccase, which releases 75 mg/L of methanol in the presence of HBT (Bourbonnais *et al.*, 1997). Laccase alone exerts little effect on the pulp, but when used in the presence of certain mediators, such as ABTS or HBT, laccase drastically influences the demethylation process of the kraft pulp (Bourbonnais and paice, 1992). The Kraft lignin contains significant amounts of condensed structures, such as 5—50, a-5, diphenylmethane, and the stilbene units (Eriksson and Gierer, 1985; Gellerstedt and Lindfors, 1984; Chiang *et al.*, 1990). To date, there has been no literature available that dealt with the behavior of the laccases toward such lignin moieties, despite the fact that their presence is very significant within

the kraft lignin backbone. There have been no studies, either, aimed at elucidating the reactivity of laccase towards the condensed LMCs, in the presence or absence of ABTS and HBT. Besides, the products of the side-chain oxidation, demethoxylation, and hydroxylation and, of the oxidative coupling have also been detected and identified (Crestini and Argyropoulos, 1998). The enzyme is active and rapidly affects lignin and the rate increases more than 4 times in the initial 10 min upon adding 1 mM ABTS which is known to deactivate laccase (Crestini and Argyropoulos, 1998; Bourbonnais *et al.*, 1997; Kim *et al.*, 2002), the degradative reaction gets triggered quickly after 20 min. The amount of methanol formed after a period of 1 h is 0.65 mM, which can subsequently be converted to 21 mg/L of methanol (Kim *et al.*, 2002).

Another important area of activity of laccase is the demethylation of the non-phenolic lignin-like structures (Srebotnik and Hammel, 2000; Bourbonnais and Paice, 1990). At the beginning of this process, non-phenolic lignin particles, rich in the methoxylic compounds, such as veratrate or anisate are degraded by laccase to their respective phenolic compounds, mainly by way of the demethylation process. The influence of various mediator dilutions on the laccase activity was evaluated on the veratrate demethylation, wherein it has been observed that the demethylation process occurs as a rather slow process (Tuomela, 2002). With those dilutions, which prompted high activity, the isoenzymes patterns have been considered to be responsible for the active demethylation processes. Such dilutions of the mediators have been noticed also to induce similar changes in the demethylation abilities of a pure laccase isolated from *Trametes versicolor* (Malarczyk, 2009). The laccase induced by lignin and its low molecular weight derivatives have been shown to display much higher activity than those induced by cadmium, menadione, or other xenobiotics. These findings suggest that the extracellular laccases of *Abortiporus biennis*, *Cerrena unicolor* and *T. versicolor* induced by lignin and its derivatives that demethoxylate the lignin derivatives are much more active than those from the other sources. Laccases isolated and purified with high yield, as constitutive forms from *Abortiporus biennis* and *T. versicolor* display the demethoxylation activity at par, whereas the activity of those from *Cerrena unicolor* have been three times higher (Cho *et al.*, 2006). It has been demonstrated that the demethoxylation of lignin and the methyl phenolic acids is involved in the functioning of a fungal laccases secreted by the white-rot fungus, *Pholiota mutabilis* (Leonowicz and Trojanowski, 1965; Trojanowski *et al.*, 1966; Leonowicz *et al.*, 1979). It has also been found that the laccase activity is highly pH dependent and that the demethoxylation process occurs more rapidly at low pH with laccases isolated from several white-rot fungi (Ishihara 1983; Leonowicz *et al.*, 1984). The optimum demethoxylation process usually occurs at lower pH (Leonowicz *et al.*, 1984), as has been observed with the laccase activity on the lignin-related substances, which display higher activity at pH 4.5 rather than at 6.0. The constitutive forms of laccase are, probably never used for the demethoxylation purpose (Fig. 3, Table 2). However, working with separated and purified constitutive form of laccase from *C. unicolor*, it has been unequivocally stated that this enzyme demethoxylates several of the low molecular weight derivatives of lignin and some other lignin variants. Many investigators suggest that laccases do demethoxylate the lignin and its derivatives. The oxidative/demethoxylation ratio of *C. unicolor* laccase activity has been very similar to that of the *Pholiota mutabilis* laccase (Leonowicz *et al.*, 1979). It is of considerable interest that the high amount of methanol released during the demethoxylation of different lignin-related substances corresponds to a decrease in the number of the methoxyl groups in the test media. This observation seems to lend adequate support to the earlier ones obtained with the *P. mutabilis* laccases (Leonowicz *et al.*, 1979). Several studies revealed that the demethylation of syringate and vanillate is pH-dependent and that the lower pH is more favourable for this process than the higher one (Ishihara and Miyazaki, 1974; Ishihara, 1983; leonowicz *et al.*, 1984).

During demethoxylation, laccase acts on the aryl-O-alkyl bonds. The time and temperature dependency on liberation of the methoxyl groups has been investigated by employing certain methoxy phenolic substrates by the immobilized laccase and it has been shown that the methoxyl groups, primarily decreased with an increasing molecular weight of the substrates. These observations seem to be in agreement with the findings made by Ander *et al.*, (1985). Some investigators measured methanol production resulting from the demethoxylation of lignin-related substances by *P. chrysosporium* and suggested that laccase probably contributes to this process, and that the demethoxylation activity maybe responsible for either H_2O_2 produced by glucose oxidases or the LiP (Kersten *et al.*, 1985). The demethoxylation activity with some water miscible organic solvents displays better demethoxylation activity of laccase in these solvents than with water alone. The lignin model compounds with a free phenolic group are more rapidly oxidized by laccase to the phenoxy radicals. An alternative pathway, involving the 4-O-demethylation process, combined with the O-ether cleavage has been proposed by Fukuzumi (1969); Ishikawa *et al.*, (1963), but further details have not been disclosed. During the metabolism of $^{14}CH_3O$-dimer 1, the labelled volatile metabolite of the $^{14}CH_3O$-dimer 1 has been identified to be methanol. The labelled lignin ($^{14}CH_3O$-KL-O) added to the 24 h old cultures produces 3.25, and 0.26 % release of radioactive CH_3OH and CO_2 within 72 h respectively, significantly more than the 0.71 + 0.06 % as released by the autoclaved cultures. Continued incubation increases the volume of $^{14}CO_2$ at the expense of $^{14}CH_3OH$, but does not seem to raise the total amount of the labelled materials released. The metal chelators, cyanide and diethyldithiocarbamate, mercuric chloride, the cationic detergent, cetyltrimethylammonium bromide and the reducing agent, dithiothreitol, all inhibit the demethylation activity as effectively as that of the autoclaved cultures. The O_2 concentration exerts a major effect on the rate of demethylation. In the cultures flushed with nitrogen, $^{14}CH_3O$-dimer 1 demethylation has been low but measurable and has been 60 % of O_2 by volume in the culture headspace and the same has been sufficient for maximum demethylation to occur. Methanol appears to be the direct product of the demethylation; there has been no evidence for the production of formaldehyde as a first product. The labelled methanol could be released from 4-O-$^{14}CH_3$- dimer 1 by at least two pathways: (i) direct demethylation, and (ii) laccase attack on 1-(3′-hydroxy-4′-methoxypheny1)-2-(0-methoxyphenoxy)-

propane-1,3-diol formed by 3-O-demethylation of $^{14}CH_3O$-dimer 1. Demethylation process of the phenolic compounds by laccases has been a well-established phenomenon (Frick and Crawford, 1983; Ishihara and Miyazaki, 1974; Lundquist and Kristersson, 1985). In either pathway, (i) or (ii), the initial step would be demethylation of a non-phenolic compound that is not a substrate for laccase (Kawai *et al.*, 1987). The formation of labelled non-extractable polymerized products from 4-OCH_3-labelled 1 shows that either 3-O-demethylation of the A ring or demethylation of the B ring takes place in *P. tremellosa* cultures (Table 2). However, the 4-O-methoxyl groups are not found in lignin, although they can apparently be produced by the fungal methylation during lignin biodegradation process (Chen *et al.*, 1982) and it is expected therefore, that the fungal demethylation shows a preference for 3-O-demethylation. Methanol is detectable as a product of metabolism of dimer 1, because further metabolism of methanol to CO_2 gets delayed. Demethylation appears to be an important process in the lignin biodegradation. Both the white-rot and brown rot fungi cause a decrease in the methoxyl content of lignin during wood decay (Kirk and Adler, 1970). A blue laccase was purified from a white-rot fungus, *Trametes trogii*, which shows an increased number of the condensed structures as well as the carbonyl and aliphatic hydroxyl groups, while there has been a fall in the number of the phenolic hydroxyl and methoxy groups (Ai *et al.*, 2015). The two Novozyme commercial laccases of N51002 (L1; *Trametes versicolor*) and N51003 (L2; *Coprinopsis cinereus*) using softwood kraft lignin (SKL) in presence of ABTS, HBT and TEMPO that generated methanol as O-demethylation product (Wang *et al.*, 2018).

4.3. *Peroxidases and lignin demethylation process*

Fungal peroxidases catalyze the demethylation reactions of the o-hydroxymethyl phenols. In a study on the activity of the fungal and horseradish peroxidases on veratrate, it has been observed that the o-dimethoxy compounds are also subject to demethylation by the peroxidases (Trojanowski *et al.*, 1967). Similarly, it has been suggested by Kornił owicz-Kowalska *et al.*, (2008) that the alkaline lignin in *Bjerkandera adusta* CCBAS930 effectively reduces number of the methoxyphenol units, by way of a peroxidase-mediated demethylation. Activity of the pure horseradish peroxidase and H_2O_2 on the demethylation of poly guaiacol and *P. chrysosporium* enables the release of H_2O_2, probably suggesting that the demethylation activity is driven by the peroxidase. *T. versicolor* and *Xanthochroid pini* rapidly bring down the number of the methoxyl groups in a sample containing the veratrate. When a mycelium-free filtrate was incubated with the veratrate, a similar activity follows (Table 2). In a study with specific inhibitors and their efficacy on demethylation by incorporating certain inhibitors, so as to identify the enzyme that is responsible for the demethylation process, the use of specific inhibitors, such as hydroxylamine it has been noticed that the activity of peroxidase gets slowed down. A similar effect has been observed by the action of the horseradish peroxidase on the veratric acid. The products of demethylation have been similar to those with the vanillate and protocatechuic acids (Trojanowski *et al.*, 1967). Trojanowski *et al.*, (1966) first reported that peroxidases from the cultures of the white-rot fungi, *Trametes versicolor* (*Coriolus versicolor*) and *Xantochorus pini*, catalyze the demethylation of varatrate to vanillate and protocatechuic acid in the presence of H_2O_2. However, previous studies by Fukuzumi (1969) indicated that peroxidase is not probably involved in the demethylation reaction. The demethylation of the *p*-methoxy group of the veratryl moiety in the presence of NADH produces a hydroxyl group and formaldehyde (Fukuzumi, 1969). To date, only a few cases of a catalytic effect of peroxidase on the demethylation process are known, namely the demethylation of mesitol, mesidine and 4-methoxy-2,6-dimethylaniline. One of the methyl or the methoxyl group is removed and replaced by a quinone group. The demethylation is accompanied by an action on $-NH_2$ or$-OH$ groups at the *p*-position of the methyl group. Peroxidase catalyzes demethylation of the aromatic compounds containing two $-OCH_3$ groups at *o*-position that generate o-diphenol as a result of the demethylation process. On the other hand (Leonowicz and Trojanowski, 1965), the demethylation also takes place when $-OH$ occupies the o-position of $-OCH_3$ and this proposition lends support to the former hypothesis, that the demethylation process constitutes an important step in the primary reactions of the lignin breakdown (Leonowicz and Trojanowski, 1965).

Laccases and peroxidases release methanol from the syringyl- and guaiacyl-type aromatic rings with a free phenolic hydroxyl radical (Ander *et al.*, 1983a; Ander *et al.*, 1983b; Ander *et al.*, 1985; Ishihara and Miyazaki, 1974; Lundquist and Kristersson, 1985), but not from the non-phenolic rings. Frick and Crawford, 1983, 1984 also observed the activity of an extracellular enzyme in the *P. chrysosporium* culture filtrates that demethylate poly guaiacol and suggesting that this activity may have been due to the presence of LiP and (or) MnP. The demethylation process of the 2-methoxy-3-phenylbenzoic acid is driven by an unstable Mn(II)- and H_2O_2-dependent activity in the extracellular enzyme in the culture media of *P. chrysosporium* (Huynh and Crawford, 1985). The enzyme responsible for the 3-O-demethylation of the veratrate by *P. chrysosporium* appears to be different from that responsible for the 4-O-demethylation (Ander *et al.*, 1985). Being unable to determine whether the demethylating enzyme was intracellular or extracellular it has been concluded that both the mycelium and its extracellular medium are necessary for the demethylation process (Table 2). If the demethylating enzyme is extracellular in nature, it may perhaps require some cofactor or some reagent continuously produced by the mycelium. H_2O_2 does not seem to be a necessary factor to induce the demethylation process and also the added catalase does not appear to affect the demethylation process, either. The demethylation activity seems to be very sensitive to physical disturbances within the organisms, which perhaps suggests that a spatially organized multicomponent system operates within (Ander *et al.*, 1985). The inhibition of demethylation by the metal-complex agents that occurs probably suggests that the demethylating enzyme is a metalloenzyme, or that it could be an indirect effect of a general inhibition of the cellular metabolism. The oxygen concentration affects both the production and the activity of the demethylating enzymes. Flushing with O_2 at intervals of 3–4 d appears to be more favourable for the production of the demethylating enzymes than a routine daily flushing. The

stimulation of demethylation that occurs by increasing the O_2 concentration during the reaction suggests that the demethylation reaction is an oxidative one (Ander *et al.*, 1985).

The pure horseradish peroxidase incubated with 0.5 mM H_2O_2 for the demethylation of poly guaiacol in the buffered media displays a significant demethylase activity with Fe^{2+} concentrations below 1 mM, but the same appears to exert very little effect on the demethylase activity of the ligninolytic cultures. At higher concentrations, Fe^{+2} inhibits the $^{14}CO_2$ generation. *P. chrysosporium* mineralizes the methoxyl label twice as faster than *Lenzites trabea*, but it produces only about a third as much of ethylene from methanol (Crawford *et al.*, 1981; Beauchamp and Fridovich, 1970). Both the singlet oxygen and hydroxyl radical are capable of cleaving the methoxyl groups (-OCH_3) from poly guaiacol, but there has been no convincing evidence that they are involved in the fungal demethylation processes (Frick and Crawford, 1983). Coniferyl alcohol and ferulate appear to give rise to a small amount of methanol over the first 48 h of incubation but the delay in detecting significant methanol levels is probably due to an initial α-; β-cleavage of the propanoid side-chain, occurring prior to the onset of the demethoxylation process. Demethoxylation of many compounds tested gets inhibited by glutamate and the methoxy groups of the veratrate, which are released more or less simultaneously by *P, chrysosporium*. Although, *P, chrysosporium* metabolizes the 3-methoxy group of veratrate to $^{14}CO_2$ slightly faster than the 4-methoxy group, Hatakka, (1985) showed that the white-rot fungus, *Pycnoporus cinnabarinus* evolved $^{14}CO_2$ more rapidly from the 4-methoxy-labelled veratrate than from the 3-methoxy labelled one. An almost complete inhibition by glutamate on the demethoxylation of the 4-methoxy group indicates that this demethoxylation occurs under the strong influence of the ligninolytic system of the fungus, whereas the 3-methoxy group is not so strongly influenced. These results, indicate that the enzyme responsible for the demethoxylation of the lignin-related phenols is a peroxidase. The presence of Glutamate or high amounts of nitrogen in the medium displays a low peroxidase activity lending support, perhaps to the conclusion that peroxidases can cause the demethoxylation of the lignin-related phenols. On addition of a 23 % methoxyhydroquinone (MHQ) to syringate it gets a demethoxylates in the presence of only 2.6 mM H_2O_2 during a 22 h period at pH.2, as compared to that of 4–6 % of acetovanillone and ferulate. This indicates that phenols get demethoxylated by H_2O_2 that is produced by various glucose oxidases. Demethoxylation of 1,4-dimethoxybenzene to methanol by a ligninase (α-β-cleaving enzyme) has been reported (Kersten *et al.*, 1985; Tien and Kirk, 1984). The limited decrease in the S = G values suggests that *L. edodes* causes demethylation of the methoxyl groups (-OCH_3 to −OH). Demethylation of the methoxyl groups attached to the aromatic rings is a replacement by the hydroxyl groups and also occurs when the lignin model compound, (LMC) 5–50 dihydrocresol is treated with MnP extracted from the extracellular fluid of *L. edodes* (Crestini *et al.*, 2000; Vane *et al.*, 2006). It has been proposed that the main pathway for degradation of the LMC vanillate by the white-rot fungus, *Sporotrichum pulverulentum* Nov. involves rapid oxidative decarboxylation of the methoxyhydroquinone (MHQ), a process that is repressed by glucose. Methanol is an early product of the MHQ metabolism in the cultures of *S. pulverulentum*, which gets generated before the $^{14}CO_2$ release from the ^{14}C-ring-labelled vanillate. Methanol is also produced *in vitro* from the vanillate upon the addition of a purified laccase and a peroxidase. Demethoxylation of the MHQ to a demethoxylated quinone is followed by an enzymatic reduction to give rise to the tri-hydroxylated compound, hydroxyquinol, which then undergoes a ring-cleavage with the formation of a ring-cleavage product, maleyl acetate (Ander *et al.*, 1983).

4.4. *Lignin peroxidases (LiP; EC 1.11.1.14) and lignin demethylation*

Lignin peroxidase (LiP) is a heme-containing enzyme that catalyzes H_2O_2-dependent oxidative degradation of lignin. LiP oxidizes the substrates by way of multi-step electron transfers giving rise to some intermediate radicals, such as phenoxy and veratryl alcohol radical cations. These intermediate radicals undergo the subsequent reactions, such as radical couplings, polymerization, and demethylation (Wong, 2009). LiP is able to oxidize the electron-rich aromatic rings without a free phenolic group and does not require mediators for its functioning (Haglund, 1999). It has been suggested that a hydroxyl radical-producing system may be involved in the lignin degradation via LiP. Cellobiose dehydrogenase can transfer electrons from the oligosaccharides to an electron-acceptor, such as Fe^{3+} or O_2, generating the hydroxyl radicals (Table 2). It has been shown that these radicals react with the non-phenolic LMCs, resulting in the demethylation process (ten Have and Teunissen, 2001). Laccases and peroxidases can release methanol from the syringyl- and guaiacyl-type aromatic rings with a free phenolic hydroxyl radical (Ander *et al.*, 1983; Ander *et al.*, 1985; Ishihara and Miyazaki, 1974; Lundquist and Kristersson, 1985), but not from the non-phenolic rings. Frick and Crawford, 1983, 1984 observed an extracellular poly guaiacol demethylation by the enzyme activity within the *P. chrysosporium* culture media and suggest that this activity may be due to LiP. The demethylation process of the 2-methoxy-3-phenylbenzoic acid by a Mn(II)- and H_2O_2-dependent enzyme has been studied with *P. chrysosporium* cultures by Huynh and Crawford, (1985).

4.5. *Manganese-dependent peroxidases and lignin demethylation*

Manganese peroxidase (EC 1.11.1.13; MnP) catalyzes the Mn-dependent reaction $2Mn(II) + 2H{+}{+}H_2O_2 = 2Mn(III) + 2H_2O$. In the enzymatic reaction Mn^{II} interacts with MnP in the presence of H_2O_2 as oxidant, that potentially leads to the formation of Mn^{III}-oxalate complex which is oxidizes lignin. Besides the ligninolytic enzymes, laccases and the MnP from the white-rot fungi display high reactivity during the oxidative functionalization of the lignins. The enzyme, contains one iron protoporphyrin IX as the prosthetic group, and is believed to be able to activate H_2O_2 during the oxidation of Mn(II) to Mn(III), which in turn, after chelation by the organic acids, becomes a freely diffusible oxidizing species. The ultimate oxidation of lignin is carried out by way of generation of the reactive phenoxy radicals by means of a hydrogen abstraction process (Table 2). However, *T. versicolor* bleaches the kraft pulp

brownstock with a concomitant release of methanol. This fungus produces laccase and MnP, but has not been shown to produce LiP. The purified MnP alone affects the demethylation process, besides the delignification process, essentially exhibiting a preference for oxidation of the phenolic lignin sub-structures (Paice *et al.*, 1995). Conversion of the methyl 2-hydroxy-3-phenyl benzoate to the 2-hydroxy-3-phenyl benzoic acid facilitates the identification of an extracellular, aromatic methyl ester esterase produced by this fungus (Frick and Crawford, 1983). The culture filtrates also are observed to retain the activity of the enzyme that converts the 2-methoxy-3-phenyl-benzoic acid (M1) to 2-hydroxy-3-phenyl-benzoic acid. Unconcentrated culture filtrates also demethylate the methyl 2-methoxy-3-phenyl benzoate (M4) leading to the formation of the methyl 2-hydroxyl-3-phenylbenzoate (M3), the reaction turning dependent upon both Mn^{2+} and H_2O_2. A single enzyme, most probably demethylates the substrates (M1 and M4). Thus, *P. chrysosporium* produces an extracellular, aromatic methyl ester esterase and a separate aromatic methoxyl demethylase. That the demethylase and the esterase are separate entities has been adequately confirmed by incorporating both M4 and M3 in a single assay mixture. The compound, M4 gets demethylated in preference to an ester hydrolysis of the compound, M3. The demethylase appears to have a higher affinity for H_2O_2 than does the esterase. Both esterase and demethylase are produced exclusively during the ligninolytic phase of fungal degradation (Kirk *et al.*, 1978; Tien and Kirk, 1984). Perhaps, the demethylase activity observed by Frick and Crawford, (1983) in the poly guaiacol demethylation process is same as that observed with the *Phanerochaete* spp. This enzyme could perhaps be a demethylase, removing the methoxyl groups, while intactly releasing methanol (Frick and Crawford, 1983). Since MnP produces methanol during delignification and initially darkens the pulp, it appears likely that the o-quinones are generated during this period (Paice *et al.*, 1995). Generation of o-quinone, by way of such hypothetical reactions by this enzyme, which contains an iron protoporphyrin IX prosthetic group (Glenn and Gold, 1985) functions by oxidizing Mn(II) to Mn(III), which, in turn, if suitably chelated, becomes a freely diffusible oxidizing species, characterised by a high redox potential (Glenn and Gold, 1985; Glenn et al., 1986; Paszczyinski et al., 1985, 1986; Crestini *et al.*, 2000). Besides, trace amounts of a demethoxylation product 9, have also been realized (0.62 % yield).

5. Molecular approaches in lignin demethylation and degradation processes

Lignin is heteropolymeric in nature and is interlinked with the β-aryl ether, biphenyl, diarylpropane, pinoresinol linkages and various lignin-derived mono aryls, including ferulate, vanillate, and syringate as the sole sources of carbon and energy (Masai *et al.*, 2007a; Masai *et al.*, 2007b; Masai *et al.*, 1993a; Masai *et al.*, 1993b; Abdelaziz *et al.*, 2016; Kohler *et al.*, 2017; Kamimura *et al.*, 2017). However, bacteria are endowed with the ability to degrade the lignin and their dimeric compounds by the operation of certain unique, enzymes and genes, as reported with *Sphingomonas paucimobilis* SYK-6 (Katayama *et al.*, 1987; Masai *et al.*, 1991; Masai *et al.*, 1993a, b; Peng *et al.*, 1999; Sambrook, *et al.*, 1989; Masai *et al.*, 2004; Peng *et al.*, 2002; Masai *et al.*, 2007a; Masai *et al.*, 2007b; Kamimura and Masai, 2014; Yoshikata *et al.*, 2014; Abdelaziz *et al.*, 2016; Kohler *et al.*, 2017; Kamimura *et al.*, 2017). The lignin-related compounds are degraded into vanillate or syringate, which mediate the operation of the protocatechuate 4,5-cleavage pathway (Hara *et al.*, 2000; Masai *et al.*, 1993a; Masai *et al.*, 1993b; Masai *et al.*, 2000; Masai *et al.*, 1999; Noda *et al.*, 1990; Abdelaziz *et al.*, 2016; Kohler *et al.*, 2017). The most abundant linkage in lignin is the β-aryl ether (50–70 %), whose generation is a crucial step in the lignin degradation mechanism (Adler, 1957). Later, *a* gene cluster *ligDFEG*, from *S. paucimobilis* SYK-6, responsible for the degradation of β -aryl ether linkages has been isolated and identified (Tables 1 and 3) (Masai *et al.*, 1991; Masai *et al.*, 1993a; Masai *et al.*, 1993b; Masai *et al.*, 1999; Masai *et al.*, 1989; Masai *et al.*, 1993a, b). The Cα-dehydrogenase (LigD) gene encoded oxidizes a typical model compound of β-aryl ether, guaiacylglycerol-β-guaiacyl ether, into α-(2-methoxyphenoxy)-β-hydroxypropiovanillone (MPHPV). Initially, it has been believed that the MPHPV β-aryl ether linkage is reductively cleaved by the gene products of *ligE* (LigE) and *ligF* (LigF) β-etherases, producing the β-hydroxypropiovanillone (HPV) and guaiacol in the presence of glutathione (Kasai *et al.*, 2005; Masai *et al.*, 1991; Masai *et al.*, 1993a; Masai *et al.*, 1993b). The HPV further degrades it by means of a side chain cleavage and by the vanillate degradation pathway (Tables 1 and 3). The LigE and LigF- deduced amino acid sequences are up to 27 % homologous with the eukaryotic glutathione S-transferases (GSTs), but only with an 18 % identity between LigE and LigF. In addition, another GST gene, *LigG*-deduced amino acid sequence show 20 % identity with those of both LigE and LigF, located just downstream of the *ligE gene* (Masai *et al.*, 1999). However, the *ligG* gene product (LigG) does not show any β-etherase activity, and its actual role remains obscure, to date. The eukaryotes' GST enzymes catalyze the formation of glutathione conjugates from various compounds and GSTs have been classified into 11 categories, based on their sequence similarities (Yanisch-Perron *et al.*, 1985). Although, limited information is available on the bacterial GSTs, many proteobacteria are believed to contain large and widely divergent sets of sequences of the GST genes (Yanisch-Perron *et al.*, 1985). The GST genes play a major role in the β-aryl ether cleavage and initially the O-demethylase acts on the DDVA to generate the 2,2′,3-trihydroxy-3′-methoxy-5,5′-dicarboxy phenyl (OH-DDVA) in *S. paucimobilis* SYK-6 (Katayama *et al.*, 1988; Sonoki *et al.*, 2000), and one of the two aromatic rings in the OH-DDVA gets cleaved by the OH-DDVA dioxygenase (LigZ) and the *met*-ring-cleavage product gets hydrolyzed by LigY (Peng et al., 1998, 1999). The conversion of 5-carboxy vanillate (5CVA) as mediated by LigW and LigW2 decarboxylases and produces vanillate as an intermediate product (Peng *et al.*, 1999; Peng *et al.*, 2002; Peng *et al.*, 2005 and the same further gets degraded into pyruvate and oxaloacetate by way of the protocatechuate (PCA) 4,5-cleavage pathway (Masai *et al.*, 2007a; Masai *et al.*, 2007b; Kamimura and Masai, 2014).

Hitherto, around 68 genes involved in the OH-DDVA degradation have been characterised, but genes for the enzymes involved in the first step of the O- demethylation process in

the OH-DDVA degradation still remain unknown (Yoshikata *et al.*, 2014). *S. paucimobilis* SYK-6 has the ability to degrade the lignin-derived compounds containing guaiacyl (4-hydroxy-3-methoxyphenyl) and syringyl (4-hydroxy-3,5-dimethoxyphenyl) moieties into the vanillate and syringate, respectively (Masai *et al.*, 2007a; Masai *et al.*, 2007b). In lignin demethylation, the vanillate usually gets converted into the protocatechuate by tetrahydrofolate-dependent enzyme, vanillate/3-*O*-methylgallate (3MGA) *O*-demethylase (LigM) (Abe *et al.*, 2005; Abdelaziz *et al.*, 2016; Kohler *et al.*, 2017). The protocatechuate 4,5-dioxygenase (4,5-PCD; LigAB) catalyzes protocatechuate, which further gets degraded to the protocatechuate by the 4,5-cleavage pathway route and produces the 2-pyrone-4,6-dicarboxylate (PDC) and 4-oxalomesaconate (OMA) (Noda *et al.*, 1990; Masai *et al.*, 1999, 2000; Hara *et al.*, 2000, 2003). Whereas, the syringate is converted into 3MGA by the tetrahydrofolate-dependent syringate*O*-demethylase (DesA) (Masai *et al.*, 2004), and thereafter the 3MGA is degraded through three different pathways, in which LigAB, 3MGA 3,4-dioxygenase (DesZ), and LigM and gallate dioxygenase (DesB) participate (Fig. 1a and b) (Kasai *et al.*, 2004, 2005). The amino acid sequence similarities between DesZ, DesB, and LigB (β subunit of LigAB) run up to their identify by 17–27 %, and these enzymes belong to the type II extradiol dioxygenases class (Vaillancourt *et al.*, 2004). The catalytic efficiency (V_{max}/K_m) of LigAB with respect to 3MGA has been only 5 % that of DesZ; however, a similar level of participation of *ligAB* and *desZ* in the 3MGA degradation has been estimated by undertaking the necessary analyses of *ligB* and *desZ* mutants (Kasai *et al.*, 2004). Besides, the DesZ catalyzes 3MGA, which is converted to 4-carboxy-2-hydroxy-6-methoxy-6-oxohexa-2,4-dienoate (CHMOD) and PDC has been demonstrated (Kasai *et al.*, 2004). In recent studies, *Sphingomonas paucimobilis* SYK-6 degrades the syringate to 3-*O*-methyl gallate by the tetrahydrofolate-dependent *O*-demethylase, and also, the DDVA *O*-demethylase catalyzes the conversion of 5,5-dehydrodivanillate (DDVA) into 5-carboxy vanillate. The DDVA *O*-demethylase enzyme plays an important role in the demethylation process (Masai *et al.*, 2004; Peng *et al.*, 2002). *Sphingomonas paucimobilis* SYK-6 has the ability to grow on a wide range of substrates, such as DDVA, syringate, vanillate, and the other dimeric LMCs used as the sole carbon source. The gene responsible for the vanillate and syringate *O*-demethylation has been successfully cloned (Nishikawa *et al.*, 1998). It has also been found that an unrelated non-heme iron-dependent demethylase catalyzes the same demethylation reaction in *Pseudomonas* and *Acinetobacter* (Abe *et al.*, 2005; Brunel and Davison, 1988; Segura *et al.*, 1999). Similar non-heme iron-dependent demethylase activity has been reported with *S. paucimobilis* SYK-6 (Sonoki *et al.*, 2000). The effect of dilute formaldehyde on the rate of demethylation/re-methylation of veratrate using *Rhodococcus erythropolis* has been studied wherein, non-linear changes in the activity of oxygen-dependent demethylase system and the ROS-activated formaldehyde have been observed Malarczyk *et al.*, (2011). These observations suggest that the demethylation process requires a 4-*O*-demethylase enzyme and the methylation seems to occur spontaneously by way of the radicals. It was established that methylation of vanillate occurs rapidly and it has been shown that the increased fluctuations in the concentration of veratrate occur; besides some changes to the NADH oxidase activity associated with the methylation processes occurring (Tables 1 and 3). Further, Shi *et al.*, (2013) undertook the genomic analysis of the kraft lignin degrading enzyme from the beta-proteobacterium, *Cupriavidus basilensis* B-8 and attempted a metabolic reconstruction and to work out a draft genome sequence for *C. basilensis* B-8 and succeeded to that extent. A genomic analysis was carried out based on the catabolic potential of the bacterium against several lignin-derived compounds. Analyses and sequence comparisons predicted the existence of three major metabolic pathways, - (i) β -ketoadipate, (ii) phenol degradation, and (iii) gentisate pathways. In bacteria, the vanillin produced from ferulate gets further converted to protocatechuate by the catalytic action of an aldehyde dehydrogenase (HcaB) and a demethylase (VanAB) (Shi *et al.*, 2013). A mixture of purified LigXa, LigXc, and LigXd convert DDVA into 2,2,3-trihydroxy-3-methoxy-5,5-dicarboxy phenyl in the presence of NADH, indicating that the DDVA *O*-demethylase is a three-component monooxygenase (Table 3). This enzyme requires Fe(II) for its activity and is highly specific for DDVA with a *Km* value of 63.5 μM and *k*cat of 6.1 sec^{-1} (Yoshikata *et al.*, 2014). The lignin-degradation capacity of *Pseudomonas* sp. Strain YS-1p and *Rhizobium* sp. strain YS-1r draft genome analysis revealed that both organisms contain the genes that encode for the enzymes laccase, Dyp-peroxidase, beta-etherase, vanillate *O*-demethylase, feruloyl esterase, carboxyl esterase, and cytochrome P450, and chloroperoxidase. Further, the genes for the aromatic ring-oxidation and ring-cleavage, including the phenol 2-monooxygenase, 4-hydroxybenzoate 3-monooxygenase, catechol 2,3-dioxygenase, protocatechuate 3,4-dioxygenase, and gentisate 1,2-dioxygenase have been detected in lignin and in the degradation of the lignin-derived aromatic compounds (Prabhakaran *et al.*, 2015). Expression of the lignin-degrading enzymes - MnP, laccases, cellobiose dehydrogenase, glutathione reductases and acyl-CoA-dehydrogenase has been studied in *G. lucidum* secretome. An extracellular redox enzyme of cellobiose dehydrogenase that is of the ping-pong type, generates the hydroxyl radicals by reducing Fe^{3+} to Fe^{2+} and O_2 to H_2O_2 and plays an important role in both lignin and cellulose hydrolysis by breaking the beta-ethers, by demethoxylating the aromatic structures in lignin and introducing the hydroxyl groups into the non-phenolic lignin (Manavalan *et al.*, 2012). The ligninolytic enzyme consortium is typically formulated by the high redox-potential oxidoreductases; perhaps, this array of enzymes can be put to use in order to demethylate lignin (Alcalde, 2015).

Recently, two extracellular Mn SODs from *Sphingobacterium* sp. T2 (Mn SOD-1 and Mn SOD-2) were identified and partially purified (Rashid *et al.*, 2015). Interestingly, both enzymes are able to perform the degradation of Organosolv and Kraft lignin, as well as different lignin model substrates, into several compounds (Tables 3 and 4). All the products formed result from the aryl-C and C–C bond oxidative cleavage reactions as well as from the *O*-demethylation activity (Rashid *et al.*, 2015). In another study on co-enzyme overexpression of tetrahydrofolate (THF)-dependent *O*-demethylase, LigM from the bacterium, *Sphingobium* sp. strain SYK-6 and the plant methionine synthase, MetE enzyme in *E. coli* that convert the vanillic

acid to 5 mM protocatechuic acid (PCA) with a 500 fold less utilization of the cofactor (Rosini *et al.*, 2016). Bacteria are known to mediate the metabolic pathways and generate the intermediates, such as vanillate, 3-*O*-methyl gallate, and syringate, by the action of the tetrahydrofolate-dependent, aryl demethylase. LigM from *Sphingomonas paucimobilis* also is believed to be involved in the downstream aryl modification, ring opening, and to mediate the aryl demethylase systems (Tables 3 and 4), and facilitates further modification and ring opening of the single-ring aromatics, vanillate and the 3-*O*-methylgallate, which are common byproducts of the biofuel production (Kohler *et al.*, 2017). The aryl *O*-demethylation by the LigM has its 1.81 Å crystal structure, fold and a canonical folate-binding domain and enables the identification of LigM's tetrahydrofolate-binding site and the protein–folate interactions, besides the catalytic tyrosine-dependent reaction mechanism (Kohler *et al.*, 2017).

6. Methods involved in the determination of lignin demethylation

Biological and enzymatic demethylated lignin and lignin-related compounds are identified by measuring methanol, formaldehyde and CO_2 generated in the demethylation reaction (Tables 1 and 2). Several methods have been evolved in order to detect the demethylation process. The following methods are some of the widely accepted methods to analyze the demethylation reactions:

Table 4 – Lignin demethylation carryout by using microbes, enzymes and chemical methods for various industrial polymer applications.

Lignin source	Microorganisms/ Enzymes/Catalyst	Lignin demethylation	Polymer applications	References
Wheat straw alkali lignin (WSAL)	Lewis acid	-OCH_3 decreased from 0.82 to 0.17	lignin-based phenol formaldehyde adhesives (D-LPF)	Song *et al.*, (2016)
Guaiacyl-type synthetic lignin (GDHP)	1-dodecanethiol (DSH), hydroiodic acid (HI), iodocyclohexane (ICH)	higher tannin-like properties	resins/adhesives	Sawamura *et al.*, (2017)
Softwood kraft lignin	iodocyclohexane in dimethyl formamide (DMF)	lignin demethylation (87 %)		Ferhan *et al.*, (2013)
Softwood kraft lignin	*Aspergillus* sp. and *Galerina autumnalis*		phenol formaldehyde adhesives	Venkatesagowda, (2018)
Alkali Soluble Hardwood lignin	alkaline lignin oxidase MetZyme® LIGNO™, a genetically engineered laccase of bacterial origin.	30 % increase phenolic hydroxyls and 0.47 mmol of MeOH released	bio-based materials	Hämäläinen *et al.*, (2018)
Kraft Lignin	Wood rotting fungi and *B. rhodina* MAMB-05 laccase	methanol released of 0.382 μg/mL	phenol formaldehyde adhesives	Bashtan-Kandybovic *et al.*, (2012)
Guaiacol	Cytochrome P450 aryl-*O*-demethylase	converting guaiacol to catechol		Mallinson *et al.*, (2018)
Lignin-Derived Biphenyl Compound (5,5′-dehydrodivanillate (DDVA))	DDVA *O*-demethylase	produced 2,2′,3-trihydroxy -3′-methoxy-5, 5′-dicarboxybiphenyl		Yoshikata *et al.*, 2014
Vanillate, 3-*O*-methylgallate, and syringate	tetrahydrofolate-dependent aryl demethylase LigM from *Sphingomonas paucimobilis*,	protocatechuate (PCA), or gallate	High value products	Kohler *et al.*, (2017)
Vanillate and syringate	Tetrahydrofolate-Dependent *O*-Demethylase, LigM	protocatechuate (PCA) and 3-*O*-methylgallate (3MGA)	TCA cycle	Abe *et al.*, (2005)
Methyl phenyl ethers	two-component enzymatic system consists of a corrinoid protein and a methyltransferase	catechols	antioxidants, flavoring agents, fragrances, dyes, agrochemicals, and fine chemicals	Farnberger *et al.*, (2018)
Softwood kraft lignin (SKL)	The two laccases, N51002 (L1), prepared from a white-rot fungus (*Trametes versicolor*) and N51003 (L2), from *Coprinopsis cinereus*, were obtained from Novozyme (Denmark)	methanol		Wang *et al.*, (2018)
Softwood kraft lignin/OrganoSolv lignins (OL, poplar, willow, wheat straw, and mixed agricultural wastes)	*Fomitopsis pinicola*, *Galerina autumnalis* and *Aspergillus* sp.	lignin demethylation (30.1 %)	phenol formaldehyde adhesives	Venkatesagowda and Dekker, (2019)

6.1. Methanol assay

The amount of formaldehyde formed, as a consequence of the AO oxidation of methanol produced from the demethylation reactions is determined by reacting it with the **(i)** acetylacetanilide reagent (25 μL 0.59 M acetylacetanilide reagent in DMSO/water, 80/20 v/v). The fluorescence of the product formed is measured at 460 nm (excitation wavelength at 360 nm) (Bashtan-Kandybovich *et al.*, 2012). **(ii)** The lignin demethylated and released by methanol gets oxidized in the presence of the alcohol oxidase and releases 1 μmol equivalent of HCHO and H_2O_2 as the end products. The formaldehyde then reacts with 100 μl of acetyl acetone (0.02 M 2,4-pentanedione in 2 M ammonium acetate and 0.05 M acetic acid). Mildly neutral solutions of acetylacetone and the ammonium salt are allowed to react with HCHO, which gradually develops a yellow color, owing to the synthesis of diacetyldihydrolutidine. The intensity of the yellow color, so formed is measured at 412 nm (Bashtan-Kandybovich *et al.*, 2012; Venkatesagowda and Dekker, 2019).

6.2. Catecholic structure

The presence of the aromatic vicinal diol groups in the low-MW lignin-like molecules and in the lignins are used to detect **(i)** 4-aminoantipyrine (4-AAP) and the demethylated lignin sample is vigorously stirred to ensure oxygen saturation. The resulting red-coloured solution is read at 516 nm. **(ii)** HNO_2 procedure measures the pyrocatecholic content by titrimetry. The demethylated KL (1 mL of Solution B is treated by adding 0.1 mL 10 % $NaNO_2$ (w/v), 0.5 mL 50 % NaOH, and 3.0 mL deionized water and the contents are mixed well. I order to initiate the reaction for a pyrocatechol analysis, a drop of K3 [Fe(CN)6] solution (0.3 %, w/v) is added after which the contents change their color from red to yellow. Solution A in a microburette is then used to titrate the test solution until the solution turns red again (colour was compared to that of the reference) (Bashtan-Kandybovich *et al.*, 2012). **(iii)** The demethylated lignin, assayed with the Ti (III)−Nitriloacetic acid is directly detected by way of the *O*-demethylation activity by measuring the intensity of a yellow complex that develops with the titanium (III) nitrilotriacetate (Ti (III)−NTA) and vicinal diols and the same can be determined by spectrophotometric measurement at 380 nm (Gibson *et al.*, 2014). **(iv)** ^{31}P NMR analysis: the demethylated lignin samples are prepared (Bashtan-Kandybovich *et al.*, 2012) and the content s of the hydroxyl groups is obtained by integration of the following spectral regions: aliphatic hydroxyls (150.0−145.4 ppm), condensed phenolic units (144.4−140.2 ppm), guaiacyl phenolic hydroxyls (140.2−139.0 ppm), pyrocatechol-type hydroxyls (139.0−138.2 ppm), p-hydroxyphenyl phenolic hydroxyls (138.2−137.0 ppm), and carboxylic acids (136.6−133.6 ppm). **(v)**. Demethoxylated products are analyzed by the thin layer chromatography (TLC) with silica gel and acetic acid:chloroform (1:3) as the solvent and H_2SO_4:formalin sprayed to visualize the demethylated products (Lopretti *et al.*, 1998). **(vi)** the *O*-demethylation reaction products are analyzed by the electrospray ionization- MS (ESI-MS) (Abe *et al.*, 2005). **(vii)** O-demethylase activities are determined by measuring the decrease in the substrates - syringate, vanillate, 3MGA, syringaldehyde, vanillin, ferulate, and sinapinic acid by the high performance liquid chromatography (Masai *et al.*, 2004). **(viii)** Measurement of the $^{14}CO_2$ release from the [$O^{14}CH_3$]-labelled vanillate (Hatakka, 1994; Hatakka, 2001). **(ix)** Methoxyl quantification by the acetylation method is done by the use of the proton nuclear magnetic resonance (^1H NMR) (Aberu and Freire, 1995). The ^1H NMR spectra of the acetylated lignins: the ratios can actually be measured from the ^1H NMR spectra of the acetylated lignins. i.e., x = H (aromatic)/H (methoxyl). The ^1H NMR spectral data of a whole range of acetylated lignins are analyzed (aromatic and methoxyl protons occur between 6.4−7.1 ppm and 3.5−4.1 ppm, respectively and the ratio x against the % methoxyl (OCH_3) is plotted (Venkatesagowda and Dekker, 2019). The actual content is arrived at by the classical hydroiodic acid method of the same lignins. The data are analyzed by the linear regression analysis so as to obtain Eq. (1): % OCH_3 = 28.28436 − 19.750047x. The acetylated lignins are then analyzed by the ^1H NMR analysis so as to deduce the x value, from which the % OCH_3 is calculated from Eq. (1). **(x)** Fe^{3+}-catechol complexation method: *O*-demethylation was determined using catechol and ferric ion coordination (catechol-Fe^{3+} complexes) by reduction of Fe^{3+} to Fe^{2+} and formation of mono, bis- and/or tris-catechol-Fe^{3+} complexes has been investigated to identify enzyme that can strip-off O-methyl groups from lignin such as *O*-demethylase by measuring the Fe3+-catechol complexes formed in a spectrophotometer at A547 nm. The standard curve was prepared using different concentrations of (10−100 μmol) pyrocatecholic structures (Venkatesagowda, 2018).

7. Strategies to improve lignin demethylation

Based on the developments that have taken place in the recent times in the biorefinery arena on lignin and authors carryout extensive research on lignin demethylation (Bashtan-Kandybovich *et al.*, 2012; Venkatesagowda, 2018), it has been thought worthwhile, to propose some possible strategies to improve upon the process of demethylation of lignin for its various industrial applications. The US department of energy has been evincing considerable interest in the lignocellulosic biorefinery arena, by lending the needed support to sequence over 80 fungal genomes; the department also provides any additional information available with it pertaining to the lignin demethylation technology. Herewith few strategies to improve lignin demethylations are (i) the first strategy involves evolving adequate protocols to improve upon the functional expression, activity, and stability of the *O*-demethylase enzymes, (ii) facilitating the enzyme to adapt to varied environmental conditions, including some adverse environments, (iii) to make an in-depth study to ascertain the intricacies of the brown-rot fungi-mediated Fenton Reaction, involved in the demethylation process, (iv) to gain knowledge on the substrate specificity of the enzyme, (v) to study the importance of O_2, H_2O_2, the methyl group acceptors, −OH donors, free radicals, NADH, mediators cofactors etc. required for the demethylation process, (vi) to study the overexpression of the vanillin *O*-demethylase genes of VanA and

VanB in *E. coli*, (vii) to study the influence of the ligninolytic enzymes on the *O*-demethylation process, (viii) to manipulate the functioning of the ligninolytic secretomes so as to improve the *O*-demethylation efficacy, (ix) gaining adequate information on the coupling random mutagenesis and the DNA recombination processes, (x) to study protoplast fusion and mutation of the demethylating organisms, (xi) to study the site directed mutagenesis, (xii) evolving bioinformatic computations supported by the sequence databases of the *O*-demethylases, (xiii) To study the evolution mechanism of the enzymes from the microbes for demethylation and (xiv) to schedule a whole genome sequence analysis of the lignin demethylating microorganisms.

8. Conclusions

The demethylation process of lignin has been an industrially important process for synthesis of the value-added products, more importantly without breaking the chemical nature of lignin. Lignin can be chemically modified by the action of effective lignin demethylation enzymes, such as vanillate-*O*-demethylase, syringate *O*-demethylase, veratrate *O*-demethylase, 3-*O* demethylase and 4-*O* demethylase and also by means of certain ligninolytic enzymes like laccase, peroxidase, Mn-peroxidase and lignin peroxidase, all of which can demethylate, oxidize, polymerize or depolymerize and even breakdown the same into dimers, trimers and some low MW phenolic fragments. However, the *O*-demethylase enzymes appear to be the best option to strip off the methyl group (-OCH_3) from the lignin molecules, without altering the complex nature of lignin. Although, the mechanism of the *O*-demethylases involved in lignin degradation has not been fully explored, this review attempts to bring out most of the relevant information pertaining to the lignin demethylation methodology. Besides, this enzyme is considered to be amenable for bringing about a host of chemical alterations to the lignin molecule, so as to making it more valuable from the perspective of its industrial applications. Our research team is actively engaged in research on investigating all about this novel enzyme - "Lignin Demethylase", an enzyme responsible for the demethylation reactions of lignin that we hope, will open up new vistas in the lignin biochemistry, *vis-a-vis* its industrial applications, in the days to come !!!.

Declarations of interest

None.

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Appendix A. Supplementary data

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