Coal depolymerizing activity of lignolytic enzymes

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ABSTRACT

Present chemical industry is based on oil reserves which are depleting. One possible alternative is the use of coal for the generation of chemicals. In order to use coal for generation of chemicals, high molecular weight coal fractions should be converted into low molecular weight coal fractions. Since coal has chemical composition similar to lignin, lignolytic micro-organisms and lignolytic enzymes are expected to depolymerize coal. Keeping these points in view, work has been initiated on depolymerisation studies on coal using humic acid as a model of coal and lignolytic fungi namely *Gleophyllum striatum* MTCC-1117, *Hexagona tenuis* MTCC-1119, *Lenzites betulina* MTCC-1183, *Lenzites sepiaria* MTCC-1170*, Loweporus lividus* MTCC-1178, *Pycnoporus sanguineus* MTCC-137, *Pleurotus sajor caju* MTCC-141, *Phellinus linteus* MTCC-1175, *Coriolopsis floccose* MTCC-1177, *Trametes versicolor* MTCC-138, *Phanerochaete chrysosporium* MTCC-1803, *Volvariella valvacca* MTCC-957, *Cladosporium herbarum* MTCC-346, *Heterobasidion annosum* MTCC-146,*Pestalotia bicolor* MTCC-372, *Phanerochaete chrysosporium* MTCC-787,*Pleurotus ostreotus* MTCC-142, *Daedalea flavida* MTCC-145, *Abortiporus biennis* MTCC-1176, *Fomes durissimus* MTCC-1173, *Agaricus compestris* MTCC-972, *Trametes hirsute* MTCC-136, *Trametes hirsute* MTCC-1171, *Tricothesium polybrochum* MTCC-376, *Phanerochaete chrysosporium* MTCC-1801, *Pleurotus sapidus* MTCC-1807, *Pleurotus fossulatus* MTCC-1800 and *Polyporus velutinus* MTCC-1813. An extensive study on depolymerisation of coal by indigenous lignolytic fungi and their lignolytic enzymes is being done with a view to identify efficient micro-organisms and efficient enzymes.

Keywords: Lignin peroxidases, manganese peroxidases, laccases, metalloenzymes, lignolytic enzymes

INTRODUCTION

Studies on depolymerisation of coal have been initiated by German [1-3] and Australian [4-6] scientists but the scientific knowledge for developing technologies for depolymerisation of coal is still insufficient. Moreover such studies have not been initiated in India. Keeping these points in view, we have initiated depolymerisation studies on coal using humic acid as a model of coal, lignolytic fungi and lignin peroxidase produced by it [7]. In order to have extensive studies on depolymerisation of coal by indigenous lignolytic fungi and their lignolytic enzymes is being communicated in this paper with a view to identify efficient micro-organisms and efficient enzymes. Lignolytic enzymes constitute a group of oxido-reductases that are highly specialized in polymerization as well as in the degradation of lignin. These enzymes are mostly produced by white rot fungi and litter-decomposing fungi. The three main lignin modifying enzymes are lignin peroxidase, Manganese peroxidase and laccases. White rot fungi contains all three enzymes and is therefore able to breakdown and mineralize several environmental pollutants into non-toxic forms.

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Lignin peroxidase

The lignin decomposing basidiomycetes white rot fungi secrete a heme protein, lignin peroxidase (LiP) [E.C.1.11.1.14] which in presence of H_2O_2 degrades lignin and lignin model compounds [8]. Different steps involved in the catalysis by lignin peroxidase are as follows:

$$\text{LiP } (Fe^{3+})\ P + H_2O_2 \rightarrow \text{LiP-I } (Fe^{4+}\text{-O})\ P^{+\cdot} + H_2O \quad \text{(I)}$$
$$\text{LiP-I } (Fe^{4+}\text{-O})\ P^{+\cdot} + R \rightarrow \text{LiP-II } (Fe^{4+}\text{- O})P + R^{\cdot} \quad \text{(II)}$$
$$\text{LiP-II } (Fe^{4+}\text{-O})\ P + R + 2H^{+} \rightarrow \text{LiP } (Fe^{3+})P + R^{\cdot} + H_2O \quad \text{(III)}$$

where RH is the organic substrate and P is porphyrin. LiP compound I (LiP-I) carries both oxidizing equivalents of H_2O_2, one as an oxyferryl (Fe^{4+}-O) center and one as a porphyrin π cation radical ($P^{+\bullet}$), whereas LiP compound II (LiP-II) carries only one oxidizing equivalent. The substrate RH is oxidized by compound I to an aryl cation radical which in subsequent nonenzymatic reactions yields the final products.

Manganese peroxidase

Manganese peroxidase, MnP [E.C.1.11.1.13] is a heme containing enzyme [9]. It has been shown to be present in the culture filtrates of a number of fungal strains [10-13]. The catalytic cycle of Mn-peroxidase resembles those of other heme peroxidases such as horseradish peroxidase [14] and lignin peroxidase [15,16] and includes the native ferric enzyme as well as the reactive intermediates compound I and compound II. The catalytic cycle can be shown as follows:

$$MnP + H_2O_2 \rightarrow MnP\ (I) + H_2O \quad \text{(I)}$$
$$MnP\ (I) + Mn^{II} \rightarrow MnP\ (II) + Mn^{III} \quad \text{(II)}$$
$$MnP\ (II) + Mn^{II} \rightarrow MnP + Mn^{III} + H_2O \quad \text{(III)}$$

where H_2O_2 oxidizes the enzyme by two electrons to form MnP compound (I) which is oxyferryl porphyrin cation radical $[Fe^{4+}=O\ P]^{+\cdot}$. Mn (II) or phenolic compounds can serve as reductants for the MnP compound (I) and form MnP compound (II) which is an oxyferryl chemical species $[Fe^{4+}=O\ P]$, one electron oxidized form of the enzyme. For the reduction of MnP compound (II) to the enzyme, Mn (II) is absolutely essential [17,18].

Laccases

Laccase [E.C. 1.10.3.2] belongs to copper containing oxidases and catalyzes the four electron reduction of molecular oxygen to water. It is a glycoprotein which contains four copper atoms per monomer distributed in three redox sites [19] termed as type1 (T1) or blue Cu, type2 (T2) or normal Cu and Type3 (T3) or coupled binuclear Cu. Type 2 and type 3 coppers form a trinuclear cluster. The organic substrate is oxidized by one electron at the active site of the laccase generating a reaction radical which further reacts non-enzymatically. The electron is received at type1 Cu and is shuttled to the trinuclear cluster where oxygen is reduced to water.

MATERIALS AND METHODS

Fungal strains

The fungal strains namely *Gleophyllum striatum* MTCC-1117, *Hexagona tenuis* MTCC-1119, *Lenzites betulina* MTCC-1183, *Lenzites sepiaria* MTCC-1170, *Loweporus lividus* MTCC-1178, *Pycnoporus sanguineus* MTCC-137, *Pleurotus sajor caju* MTCC-141, *Phellinus linteus* MTCC-1175, *Coriolopsis floccose* MTCC-1177, *Trametes versicolor* MTCC-138,*Phanerochaete chrysosporium* MTCC-1803, *Volvariella valvacca* MTCC- 957, *Cladosporium herbarum* MTCC-346, *Heterobasidion annosum* MTCC-146, *Pestalotia bicolor* MTCC-372, *Phanerochaete chrysosporium* MTCC-787, *Pleurotus ostreotus* MTCC-142, *Daedalea flavida* MTCC-145, *Abortiporus biennis* MTCC-1176, *Fomes durissimus* MTCC- 1173, *Agaricus compestris* MTCC-972, *Trametes hirsute* MTCC-136, *Trametes hirsute* MTCC-1171, *Tricothesium polybrochum* MTCC-376, *Phanerochaete chrysosporium* MTCC-1801, *Pleurotus sapidus* MTCC-1807, *Pleurotus fossulatus* MTCC-1800 and *Polyporus velutinus* MTCC-1813 were procured from Microbial Type Culture Collection Center (MTCC) and Gene Bank, Institute of Microbial Technology, Chandigarh and were maintained on agar slants. The medium used for agar slants for the fungal strains consisted of growth medium no. 65 which consists of malt extract (20.0g) and agar (20.0g) dissolved in double distilled water (1.0L).

Chemicals

Veratryl alcohol (3,4-dimethoxy benzyl alcohol) and Humic acid was from Aldrich (Wisconsin, USA). Dimethyl succinate and nitrilotriacetate were from Sigma Chemical Co; (St. Louis USA). All other chemicals were either obtained from CDH (Delhi) or Loba Chemie (Mumbai) and used without further purification.

Enzyme assay

The lignin peroxidase activity has been assayed [20] by monitoring the formation of veratraldehyde spectrophotometrically at λ=310 nm using veratryl alcohol as a substrate with UV/VIS spectrophotometer Hitachi (Japan) model U-2000, which was fitted with electronic temperature control unit. Molar extinction coefficient value 9300 $M^{-1}cm^{-1}$ for veratraldehyde was used to calculate the enzyme unit. The activity of Mn-peroxidase was determined by monitoring the absorbance change at λ=240 nm due to formation of Mn (II) lactate and using $MnSO_4$ as the substrate [20]. The activity of laccase was analyzed using DMP (2,6-Dimethoxyphenol) as the substrates [21]. The assay solution of 1 ml consisted of the substrate DMP containing 1mM 2, 6-dimethoxyphenol in 50 mM sodium malonate buffer, pH 4.5 at 37°C. The reaction was monitored by measuring the absorbance change at λ=468 nm and using the molar extinction coefficient value of 49.6 mM^{-1} Cm^{-1}. One unit of the enzyme was defined as the amount of enzyme, which converts one μ mole of substrate to product under the standard assay condition. The least count of absorbance measurement was 0.001 absorbance unit.

Coal depolymerization activity

The coal depolymerising activity of the purified enzyme was assessed by measuring the decrease of absorbance at 450nm and the increase of absorbance at 360nm when the enzyme along with H_2O_2 was added to the humic acid solution [22]. Screening of the fungi for coal depolymerisation was performed in petridishes containing Tein and Kirk medium [20], modified Kirk and Czapek-Dox

agar medium [22]. Tein and Kirk medium contained (per liter) humic acid (1g), glucose (10 g), malt extract (10 g), peptone (2 g), yeast extract (2 g), L-asparagine (1 g), KH_2PO_4 (2 g), $MgSO_4.7H_2O$ (1 g), thiamine-HCl (1 mg) and agar (20 g). Modified Kirk medium contained (per liter) humic acid (1g), glucose (0.2g), yeast extract (0.05g), 2,2–dimethyl succinate (2.2g), ammonium tartrate (0.5g), KH_2PO_4 (2g), $MgSO_4.7H_2O$ (0.5g), $CaCl_2$ (0.1g), agar agar (28g). Czapek Dox agar medium consisted (per liter) of humic acid (1g), glucose (0.2g), yeast extract (0.05g), $NaNO_3$ (0.5g), K_2HPO_4 (1g), $MgSO_4.7H_2O$, KCl (0.5g), $FeSO_4.7H_2O$, agar-agar (18g). The plates were inoculated with mycelia and were incubated at 25° C in the dark for two weeks. Decolonization of the dark brown agar around the fungal growth area was observed periodically. A pellet of diameter 1 cm taken from the decolorized zone was dissolved in 1ml of double distilled water, filtered through millex-GS 0.22m Millipore filter unit and the filtrate was used for the assay of lignin peroxidase, Mn-peroxidase and laccase.

RESULTS AND DISCUSSION

It has been observed that decolourization of humic acid by *Gleophyllum striatum* MTCC-1117, *Hexagona tenuis* MTCC-1119 takes place in modified Kirk medium containing humic acid (1g/L) [23,24]. Figure 1 shows the coal depolymerising activity assayed by measuring (a) decrease of absorbance at 450nm and (b) increase of absorbance at 360nm. Reaction mixture consisted of 200µl of humic acid, 100 µl of H_2O_2 (0.4 mM) 200 µl of 50mM of sodium tartrate buffer pH 2.5, 450 µl of double distilled water and maintained at 25°C. The reaction was started by the addition of 50µl of the enzyme solution. Sixteen indigenous lignolytic fungal strains namely *Polyporus biennis* MTCC-1176, *Pestalotia bicolor* MTCC-372, *Heterobasidion annosum* MTCC-146, *Pleurotus ostreatus* MTCC-142, *Gloephyllum striatum* MTCC-1117, *Loweporus lividus* MTCC-1178, *Pleurotus sajor caju* MTCC-141, *Fomes durissimus* MTCC-1173, *Hexagona tenuis* MTCC-1119, *Gloephyllum sepiarium* MTCC-1170, *Pycnoporus sanguineus* MTCC-137, *Xylaria polymorpha* MTCC-1100, *Tremetes hirsuta* MTCC-1171, *Lenzites betulina* MTCC-1183, *Phellinus linteus* MTCC-1175 and *Daedalea flavida* MTCC-145 have been shown to depolymerise coal in the plate assay method as stated above. Table 1 shows the lists of fungal strains having different responsible enzymes for depolymerisation of humic acid as a model of coal.

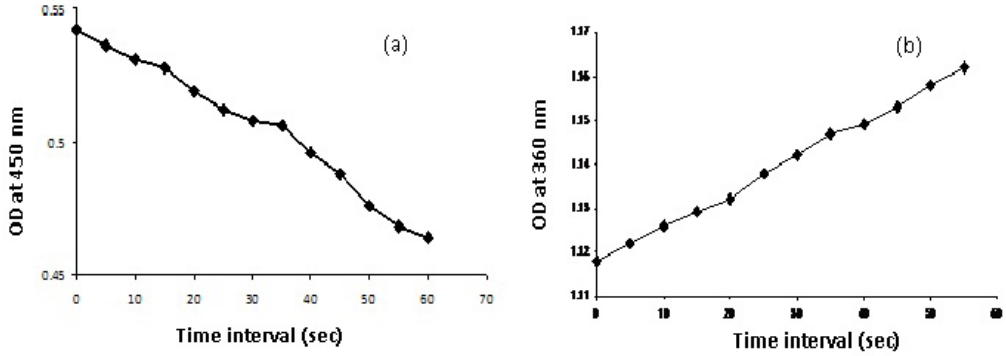

Figure 1. Coal depolymerising activity assayed by measuring (a) decrease of absorbance at 450nm and (b) increase of absorbance at 360nm.

For production of lignolytic enzyme, the fungal strain was grown in a medium containing 10 g glucose, 1.32 g ammonium tartrate 0.2 g KH_2PO_4, 50 mg $MgSO_4.7H_2O$, 10 mg $CaCl_2$, 10 µg thiamine per litre and 1 ml of a solution containing per liter 3 g $MgSO_4.7H_2O$, 0.5 g $MnSO_4.H_2O$, 1

g NaCl, 100 mg $FeSO_4.7H_2O$, 185 mg $CoCl_2.6H_2O$, 80 mg $CaCl_2$, 180 mg $ZnSO_4.7H_2O$, 10 mg $CuSO_4.5H_2O$, 10 mg $AlK(SO_4)_2$, 10 mg H_3BO_3, 12 mg $Na_2MoO_4.2H_2O$ and 1.5 g nitrilotriacetate. The pH of basal medium was adjusted to 4.5 with 20 mM dimethyl succinate. Enzyme was prepared by growing the fungi in 25ml sterilized culture media in 100ml flask X 30 containing veratryl alcohol in one set of experiment and humic acid in other set of experiment as substrates.

Table 1. Lists of fungal strains with enzyme activity.

Name of fungus	Laccase activity	Mn-peroxidase activity	Lignin peroxidase activity
Polyporus biennis MTCC-1176			+
Pestalotia bicolor MTCC-372			+
Heterobasidion annosum MTCC-146			+
Pleurotus ostreatus MTCC-142			+
Gloephyllum striatum MTCC-1117	+		+
Loweporus lividus MTCC-1178	+		+
Pleurotus sajor caju MTCC-141	+		+
Fomes durissimus MTCC-1173	+	+	
Hexagona tenuis MTCC-1119	+		+
Gloephyllum sepiarium MTCC-1170			+
Pycnoporus sanguineus MTCC-137		+	+
Xylaria polymorpha MTCC-1100	+		
Tremetes hirsuta MTCC-1171	+		
Lenzites betulina MTCC-1183		+	+
Phellinus linteus MTCC-1175		+	+
Daedalea flavida MTCC-145	+		

(+) indicate depolymerisation of humic acid.

Table 2. Production of lignin peroxidase in presence of veratryl alcohol and humic acid as inducer with maximum production day.

Name of fungus	Lignin peroxidase Maximum level of activity (IU/ml) without inducer	Lignin peroxidase Maximum level of activity (IU/ml) in presence of veratryl alcohol as inducer	Lignin peroxidase Maximum level of activity (IU/ml) in presence of humic acid as inducer with maximum production day
MTCC-1176	0.020	0.090	1.36 (4^{th} day)
MTCC-372	0.035	0.120	1.00 (4^{th} day)
MTCC-146	0.023	0.058	0.74 (4^{th} day)
MTCC-142	0.135	1.230	4.72 (4^{th} day)
MTCC-1117	0.052	0.280	1.82 (4^{th} day)
MTCC-1178	0.027	0.985	2.20 (4^{th} day)
MTCC-141	0.126	0.420	1.29 (4^{th} day)
MTCC-1119	0.015	0.042	0.194 (5^{th} day)
MTCC-1170	0.025	0.091	0.58 (4^{th} day)
MTCC-137	0.185	0.230	2.20 (4^{th} day)

Flasks were inoculated with mycelia of 1cm diameter under aseptic condition and the fungal culture was grown under stationary culture condition at 30°C in a incubator. Table 2 shows the lignin peroxidase maximum level of activity (IU/ml) without inducers, in presence of veratryl alcohol as inducer and in presence of humic acid as inducer with maximum production day. It

shows that humic acid is best inducer for the production of lignin peroxidase enzyme. Table 3 shows the laccase maximum level of activity (IU/ml) without inducers and in presence of humic acid as inducer with maximum production day. It also shows that humic acid is best inducer for the production of laccase enzyme. Table 4 shows the Mn-peroxidase maximum level of activity (IU/ml) without inducers and in presence of humic acid as inducer with maximum production day. It also shows that humic acid is best inducer for the production of Mn-peroxidase enzyme. Thus it has been concluded that in order to use coal for generation of chemicals, high molecular weight coal fractions should be converted into low molecular weight coal fractions. These lignolytic fungi converts high molecular weight coal fractions to low molecular weight coal fractions efficiently and ecofriendly. Humic acid has been found to be good inducer for the production of lignolytic enzymes in their liquid growth culture medium.

Table 3. Production of laccases in presence of veratryl alcohol and humic acid as inducer with maximum production day.

Name of fungus	Laccase maximum level of activity (IU/ml) without inducer	Laccase maximum level of activity (IU/ml) in presence of humic acid as inducer with maximum production day
MTCC-1117	0.012	1.22 (4^{th} day)
MTCC-1178	0.032	1. 53 (4^{th} day)
MTCC-141	0.023	0.78 (4^{th} day)
MTCC-1173	0.015	1.76 (4^{th} day)
MTCC-1119	0.073	1.63 (4^{th} day)
MTCC-1100	0.083	1.92 (4^{th} day)
MTCC-1171	0.016	0.46 (4^{th} day)
MTCC-145	0.004	0.26 (5^{th} day)

Table 4. Production of Mn-peroxidases in presence of veratryl alcohol and humic acid as inducer with maximum production day.

Name of fungus	Mn-peroxidase maximum level of activity (IU/ml) without inducer	Mn-peroxidase maximum level of activity (IU/ml) in presence of humic acid as inducer with maximum production day
MTCC-1173	0.034	1.45 (5^{th} day)
MTCC-137	0.008	1.15 (6^{th} day)
MTCC-1183	0.017	1.80 (5^{th} day)
MTCC-1175	0.023	0.68 (5^{th} day)

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REFERENCES

[1] Hafrichter M, Fritsche W. Appl.Microbiol Biotechnol. 1996, 46:220-225.
[2] Hafrichter M, Fritsche W. Appl.Microbiol Biotechnol. 1997, 47:419-424.
[3] Hafrichter M, Fritsche W. Appl. Microbiol Biotechnol. 1997, 47:566-571.

[4] Holker U, Ludwig S, Scheel T, et al. Appl. Microbiol. Biotechnol.1999, 52:57-59.
[5] Ralph JP, Catcheside DEA. Appl. Microbiol Biotechnol. 1998, 49:438-444.
[6] Ralph JP, Catcheside DEA. Appl. Microbiol Biotechnol. 1999, 52:70-77.
[7] Yadav M, Yadav P, Yadav KDS. Biochemistry (Moscow) 2009, 10:1125-1131.
[8] Steven LE, Recta RA, Wariishi H, et al. Proc Natl Acad Sci USA 1993, 90: 750-754.
[9] Kuwahara M, Glenn JK, Morgan MA, et al. FEBS Letters 1984, 169: 247-250.
[10] Hatakka A. FEMS Microbiology Review 1994, 13:125-135.
[11] Pelaez F, Martinez MJ, Martinez AT. Mycological Research 1995, 99:37-42.
[12] Silva EM, Martins SF, Milagres AMF. Bioresource Technology 2008, 99:2471-2475.
[13] Tanaka H, Koike K, Itakura S, et al. Enzyme and Microbiol Technology 2009, 45:384-390.
[14] Dunford HB. In Peroxidases in Chemistry and Biology, Everse J, Everse KE, Grisham MB (ed.), CRC Press, Boca Raton, USA, 1991, 1-24.
[15] Wariishi H, Huang J, Dunford, HB, et al. J. Biol. Chem. 1991, 266:20694-20699.
[16] Kirk TK, Cullen D. In Environmentally friendly technologies for the pulp and paper industry, Young RA, Akthar M (ed.), John Wiley & Sons, New York, 1998, 273-307.
[17] Glenn JK, Akileshwaran L, Gold MH. Arch. Biochem. Biophys, 1986, 251:688-696.
[18] Wariishi H, Valli K, Gold MH. J. Biol. Chem. 1992, 267:23688-23695.
[19] Ralph JP, Catcheside DEA. Appl. Microbiol Biotechnol. 1996, 46:226-232.
[20] Tien M, Kirk TK. Methods Enzymol. 1988, 161:238.
[21] Coll MP, Fernandez- Abalos JM, Villomueva JR, et al. Appl. Environ. Microbiol. 1993, 59:2607-2613.
[22] Hafrichter M, Fritsche W. Appl. Microb. Biotechnol. 1996, 46:220-225.
[23] Yadav M, Sharma JK, Singh SK, et al. Environmental Technology 2011, 32:1287-1294.
[24] Yadav M, Yadav KS, Singh SK, et al. Indian Journal of Chemistry (B) 2010, 49B:489-494.