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Full Length Research Paper

Ligninolytic enzyme activities in mycelium of some wild and commercial mushrooms

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Lignin is probably one of the most recalcitrant compounds synthesized by plants. This compound is degraded by few microorganisms. White-rot fungi have been extensively studied due to its powerful ligninolytic enzymes. In this study, ligninolytic enzyme activities of different fungal species (six commercial and 13 wild) were investigated in solid and liquid culture media. It was postulated that, among the wild strains, only *Pleurotus ostreatus*-1 (MCC45), *P. ostreatus*-2 (MCC40), *Pleurotus eryngii*-1 (MCC25) and *P. eryngii*-2 (MCC26), and commercial strains *P. ostreatus*, *P. sajor-caju*, *P. eryngii* presented lignin peroxidase (LiP) activity. All enzymes tested in this study were not determined in *Rigidoporus ulmarius, Gloeophyllum trabeum* and *Tricholoma caligatum* as well as commercial strain *Pleurotus citrinopileatus*. Therefore, the results of the present study allow us to conclude that wild *P. ostreatus* and *P. eryngii* are good candidates for scale-up ligninolytic enzyme production.

Key words: Basidiomycetes, enzymatic activity, lignocellulose.

INTRODUCTION

Lignocellulose is the predominant component of woody plants and dead plant materials and the most abundant biomass on earth. White-rot fungi (Basidiomycetes) produce various extracellular enzymes, such as laccase (Lac), manganese peroxidase (MnP) and lignin peroxidase (LiP), which are involved in the degradation of lignin and their natural lignocellulosic materials (Nagai et al., 2007). This ligninolytic system of white-rot fungi is also directly involved in the degradation of various xenobiotic compounds and dyes (Hofrichter, 2002; Songulashvili et al., 2007). Besides white-rot fungi, some microorganisms can also degrade and decolorize wide range of recalcitrant organic compounds (Hammel et al., 1985; Gopinath et al., 2005). Through intensive study of ligninolytic fungi, it has been determined that these organisms produce extracellular enzymes with very low substrate specificity, enabling them to mineralize a wide range of highly recalcitrant organopollutants that are structurally similar to lignin (Hofrichter, 2002).

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The potential application of ligninolytic enzymes in biotechnology has stimulated the investigation of their production with the purpose of selecting promising enzyme producers and increasing of their yield. The understanding of physiological mechanisms regulating enzyme synthesis in lignocellulose bioconversion could be useful for improving the technological process of edible and medicinal mushroom production (Songulashvili et al., 2007).

Laccase (benzenediol: oxygen oxidoreductases, EC 1.10.3.2) belong to the family of multicopper that catalyzes the oxidation of various aromatic substances. Catalytic properties and broad substrate specificity of laccase have a great potential in varied industrial applications (Edens et al., 1999; O'Malley et al., 1993; Givaudan et al., 1993). Although Lac were isolated and reported from bacteria (Thurston, 1994), most Lac were reported and studied from fungal organisms (Hernandez et al., 1994; Perumal and Kalaichelvan, 1996; Vasconcelos et al., 2001; Zhang et al., 2006; Mechichi et al., 2006). LiP appears to be a key enzyme in the oxidation of nonphenolic phenylpropanoid units which lead to polymer fragmentation (Hammel et al., 1985). MnP, one of the important enzymes of fungi, oxidizes Mn^{2+} to Mn^{3+}, which

in turn may attack phenolic structures in lignin as long as it is stabilized by suitable metal chelators secreted by fungi (Zhang et al., 2006).

In order to get more effective results in biodegradation of any pollutants, biopulping in paper industries and the other industrial applications by fungal strains, various fungal organisms must initially be investigated for Lac, MnP and LiP activity and their quantitative enzyme production must be determined. On the other hand, it is more important to elucidate different enzyme activities in wild organisms for applied and environmental science. In field researches made in Mediterranean region of Turkey, with an aim to establish the current fungarium, many macro fungi were isolated and identified from nature. The aim of the present study was to investigate the determination of qualitative and quantitative production of Lac, MnP and LiP in both commercial and wild fungal strains, collected from nature by using two different protocols.

MATERIALS AND METHODS

Organisms

In this study, we used mycelia that belong to 19 mushroom species (six commercial and 13 wild) to determine their ligninolytic activities. Commercial strains were obtained from a local spawn producer, Agromycel company Denizli, Turkey (www.agromantar.com). The commercial mushroom species were *Pleurotus ostreatus* (Jacq.) *Pleurotus Kumm*. (MCC16), *Pleurotus sajor-caju* (Fr.) Singer (MCC29), *Pleurotus citrinopileatus* Singer (MCC23), *Pleurotus eryngii* (DC.) Gillet (MCC58), *Pleurotus djamor* (Rumph. Ex Fr.) Boedijn (MCC15) and *Lentinula edodes* (Berk.) Pegler (MCC55).

Wild basidiocarps used in this study were collected from the southwestern part of Turkey in the context of a project (project code 104T236). The wild mushroom species were *Agaricus romagnesii* Wasser (MCC28), *Coprinus comatus* (O.F. Müll.) Pers. (MCC35), *Gloeophyllum trabeum* (Pers.) Murrill (MCC05), *Meripilus giganteus* (Pers.) P. Karst. (MCC08), *Paxillus involutus* (Batsch) Fr. (MCC37), *P. eryngii*-1 (MCC25), *P. eryngii*-2 (MCC26), *P. ostreatus*-1 (MCC45), *P. ostreatus*-2 (MCC40), *P. ostreatus*-3 (MCC41), *P. ostreatus*-4 (MCC07), *Rigidoporus ulmarius* (Sowerby) Imazeki (MCC32) and *Tricholoma caligatum* (Viv.) Ricken (MCC50). *A. romagnesii* and *C. comatus* were distributed in anthropically disturbed localities (gardens, parks and open areas of villages) and pastureland nearby forest areas respectively. *P. involutus* also was found in garden, parks as well as in coniferous forest. *G. trabeum* was widely distributed on death bark of coniferous (*Picea*, *Pinus*, etc.) and deciduous trees (*Acer*, *Quercus*, *Birch*, etc.). *M. giganteus* is a parasite organism. It can be especially encountered on beech tree and other hard wood. *T. caligatum* is an ectomycorrhizal mushroom, living mutualistically with the roots of coniferous trees. *R. ulmarius* was grown on bottom of deciduous trees, especially elm. Wild types of *P. eryngii* were collected on poplar trees during the field research in Turkey.

Mycelia obtained from fruit bodies of mushrooms by tissue culture method and were transferred on Potato Dextrose Agar. All strains were deposited in the Mushroom Culture Collection (MCC) of the Department of Bioengineering, Ege University, Turkey.

Qualitative determination of ligninolytic enzyme activity on solid media

The ability of the fungal strains to produce extracellular 2,2'-azinobis 3-thylbenzothiazoline-6-sulphonic acid (ABTS, Sigma) and guaiacol (Sigma) oxidizing activity was performed in Petri dishes (90 mm diameter) with 15 ml of G.A.E. (Glucose Asparagine) agar medium (Dhoulib et al. 2005) containing per liter of medium: 10 g of glucose, 1 g of L-asparagine, 0.5 g of yeast extract, 0.5 g of K_2HPO_4, 0.5 g of $MgSO_4.H_2O$, 0.01 g of $FeSO_4.H_2O$ and 15 g of agar-agar. Guaiacol or ABTS were added to G.A.E. agar medium (pH 5.0) at the concentration of 5 mM and 0.35 g/L, respectively (Bains et al., 2003; Kalmis et al., 2007). Plates without these chromogenic substrates were used as control. For each test, 90 mm diameter plates were inoculated at the center using a 6 mm in diameter cylindrical plug of mycelium and incubated at 27°C for 21 days (Kiiskinen et al., 2004). The experiment was performed in triplicate for each culture.

Quantitative determination of ligninolytic enzyme activity on liquid media

The quantitative determination of Lac, LiP and MnP activities, ABTS and guaiacol oxidizing strains were grown in 500 ml Erlenmeyer flasks containing 100 ml of G.A.E. medium without agar. Each flask was inoculated with four cylindrical plugs (6 mm in diameter) of active mycelia from previously cultured in G.A.E. agar and incubated at 27°C, 150 rpm for 14 days. Samples were taken at regular intervals and centrifuged at 11000 rpm, +4°C for 10 min. The supernatants were used to measure enzyme activities in a spectrophotometer (Silva et al., 2005; Revankar and Lele, 2006).

Ligninolytic enzyme assays

Extracellular laccase (EC 1.10.3.2) production was measured by assaying the oxidation of 5 mmol l^{-1} ABTS in 100 mmol l^{-1} Glycine-HCl (pH 3.0) at 420 nm and using an extinction coefficient of 36000 M^{-1} cm^{-1} (Silva et al., 2005).

Lignin peroxidase (EC 1.11.1.14) activity was evaluated by UV spectrometry of the veratryl aldehyde produced (ε_{310}= 9300 M^{-1} cm^{-1}) during veratryl alcohol oxidation. The reactive mixture contained 375 µL sodium tartrate buffer 0.33 M pH 3.0, 125 µL veratryl alcohol 4 mM, 50 µL hydrogen peroxide 10 mM, 450 µL distilled water and 250 µL culture medium for a final volume of 1250 µL (Kuwahara et al., 1984).

Manganese peroxidase (EC 1.11.1.13) activity was measured by 2,6-dimethoxyphenol (2,6-DMP; ε_{469} = 27 500 M^{-1} cm^{-1}) oxidation (Leonowicz et al., 1999). For all enzymes under evaluation, one activity unit was defined as the amount of enzyme necessary to oxidize 1 µmol of substrate per minute. All activities determined in this study were expressed as U/l.

Statistical analysis

Similarity (%) of organisms were statistically analyzed with the MINITAB Release 13.20 program, in relation to their ligninolytic enzyme activities were analyzed by the multivariate cluster analysis according to the data obtained from liquid media assays.

RESULTS AND DISCUSSION

Screening of lignin degrading strains on solid media

Macrofungi that produce laccases have been screened for either on solid media containing colored indicator compounds that enable the visual detection of laccase production or with liquid cultivations monitored with enzyme

Table 1. Plate assay for laccase production (Ø mm).

Organisms	Green halo zone formation (ABTS)	Reddish-brown zone formation (Guaiacol)	Control (No substrate)
Commercial			
Pleurotus ostreatus (PO)	47	39 ± 2	60 ± 3
P. sajor-caju (PS)	62 ± 2	43 ± 2	75 ± 2
P. citrinopileatus (PC)	ND	ND	90
P. eryngii (PE)	29 ± 1	39 ± 1	40 ± 1
P. djamor (PD)	14	35 ± 3	20 ± 1
Lentinula edodes (LE)	11 ± 1	6 ± 1	20 ± 1
Wild			
Agaricus romagnesii (AR)	ND	ND	45 ± 1
Coprinus comatus (CC)	29 ± 4	23	50 ± 3
Gloeophyllum trabeum (GT)	ND	ND	60
Meripilus giganteus (MG)	20 ± 4	13 ± 2	30 ± 2
Paxillus involutus (PI)	20	5	50
Pleurotus eryngii (PE-1)	30 ± 1	35 ± 1	35 ± 1
P. eryngii-2 (PE-2)	33 ± 2	41 ± 1	34
Pleurotus ostreatus-1 (PO-1)	36	40	70
P. ostreatus-2 (PO-2)	38 ± 1	42 ± 1	60 ± 1
P. ostreatus-3 (PO-3)	34 ± 3	43 ± 1	47 ± 2
P. ostreatus-4 (PO-4)	45	46 ± 1	60 ± 3
Rigidoporus ulmarius (RU)	ND	ND	65 ± 3
Tricholoma caligatum (TC)	ND	ND	67 ± 2

All values are media of three replications ± standard error.
ND: Not detected.

activity measurements. The use of colored indicators is generally simpler as no sample handling and measurement is required. As laccases oxidize various types of substrates, several different compounds have been used as indicators for laccase production. The traditional screening reagents tannic and gallic acid have nowadays mostly been replaced with synthetic phenolic reagents, such as guaiacol, syringaldazine and 2,2'-azino-bis 3-thylbenzothiazoline-6-sulphonic acid (ABTS) (Szklarz and Leonowicz, 1986; Niku-Paavola et al., 1988).

The chromogen ABTS is a very sensitive substrate that allows a rapid screening of fungal strains producing extracellular ABTS-oxidising enzymes by means of a color reaction (Stajic et al., 2006). Colonies that showed green halo on GAE medium containing ABTS that exceeded the colony diameter were considered as ABTS-oxidizing activity producers.

Enzymatic activities of 19 fungal strains were detected to screen their ligninolytic enzymes by using primary screening method on solid medium. The solid cultures inoculated by the fungal strains on GAE medium containing ABTS have yielded 15 positive strains which exhibited green halo formation after 7 days of incubation. *P. citrinopileatus*, *G. trabeum*, *A. romagnesii*, *R. ulmarius* and *T. caligatum* exhibited no green halo during the incubation period. The diameter of the halo and the color intensity indicating a positive extracellular oxidoreductase secretion from mycelium was used to screen the level of ligninolytic enzyme production of each strain (Table 1).

Screening of ligninolytic enzyme activities in liquid media

The highest level of Lac activity in commercial strains was found in *P. eryngii* (62.39 ± 0.21 U/l) at 27°C for 14 days, while lowest level of this enzyme was determined in *P. ostreatus* (1.68 ± 0.04 U/l) at the same incubation period (Table 2). For the wild species, highest levels of this enzyme activity was found in *P. ostreatus*-4 (941.66 ± 1.67 U/l), *P. eryngii*-1 (162.5 ± 1.66), *P. ostreatus*-1 (119.41 ± 0.25 U/l) and *P. eryngii*-2 (109.50 ± 0.59), respectively. Stajic et al. (2006) detected lowest Lac activity in medium containing 10 g/l glucose at the level of 24 ± 13 U/l for *P. eryngii* and 45.4 ± 5.8 U/l for *P. ostreatus*.

The highest level of MnP activity in commercial strains was found in *P. eryngii* (165.67 ± 0.22 U/l) at 27°C for 14 days, while lowest level of this enzyme was determined in *L. edodes* (3.30 ± 0.02 U/l) at the same incubation period

Table 2. Maximum ligninolytic enzyme production in liquid culture.

Organisms	Lac activity (U/l)	MnP activity (U/l)	LiP activity (U/l)
Commercial			
Pleurotus ostreatus (PO)	62.39 ± 0.21 (7)	165.67 ± 0.22 (14)	0.91 ± 0.05 (12)
P. sajor-caju (PS)	31.75 ± 0.41 (4)	67.58 ± 0.14 (14)	17.84 ± 0.11 (14)
P. citrinopileatus (PC)	1.68 ± 0.04 (4)	5.01 ± 0.02 (6)	0.69 ± 0.06 (12)
P. eryngii (PE)	1.96 ± 0.06 (5)	4.14 ± 0.06 (14)	ND
P. djamor (PD)	ND	ND	ND
Lentinula edodes (LE)	3.75 ± 0.16 (14)	3.30 ± 0.02 (14)	ND
Wild			
Agaricus romagnesii (AR)	ND	25.67 ± 0.07 (14)	ND
Coprinus comatus (CC)	2.18 ± 0.05 (12)	23.85 ± 0.04 (12)	ND
Gloeophyllum trabeum (GT)	ND	ND	ND
Meripilus giganteus (MG)	7.30 ± 0.03 (7)	20.00 ± 0.09 (8)	ND
Paxillus involutus (PI)	3.83 ± 0.08 (7)	ND	ND
Pleurotus eryngii (PE-1)	162.5 ± 1.66 (8)	267.63 ± 0.55 (10)	11.12 ± 0.17 (12)
P. eryngii-2 (PE-2)	109.50 ± 0.59 (8)	71.18 ± 0.09 (14)	9.40 ± 0.27 (14)
Pleurotus ostreatus-1 (PO-1)	119.41 ± 0.25 (8)	23.85 ± 0.15 (14)	6.02 ± 0.16 (14)
P. ostreatus-2 (PO-2)	19.00 ± 0.16 (10)	27.45 ± 0.18 (12)	2.63 ± 0.05 (14)
P. ostreatus-3 (PO-3)	2.20 ± 0.03 (8)	29.03 ± 0.17 (12)	ND
P. ostreatus-4 (PO-4)	941.66 ± 1.67 (12)	78.63±0.13 (14)	7.41±0.11 (14)
Rigidoporus ulmarius (RU)	ND	ND	ND
Tricholoma caligatum (TC)	ND	ND	ND

All values are media of three replications ± standard error.
ND: Not determined, Lac: laccase; MnP: manganese peroxidase; LiP: lignin peroxidase.
Numbers between parentheses show the day when the maximum enzyme was detected.

(Table 2). For the wild species, the highest level of this enzyme activity was found in *P. eryngii*-1 (267.63 ± 0.55).

Commercial strain *P. sajor-caju* used in our study showed lower laccase (31.75 U/l) and higher MnP (67.58 U/l) activity compared to Lac (43.00 U/l) and MnP (22.00 U/l) activity values previously reported by Jaouani et al. (2003). Lac activity of *P. djamor* was also very low. Similarly, Lac activities of *P. djamor* strains IE 121 and IE 218 were not statistically significant (Pelaez et al., 1995). Weak Lac and MnP activities were also detected in *L. edodes*. It has been reported that two laccases were purified from *L. edodes* (Elisashvili et al., 2001). In contrast, a recent study on indicated that *L. edodes* has no LiP and only weak MnP activity (Mikiashvili et al., 2004).

Lac activity was found in wild strains *C. comatus, M. giganteus, P. involutus, P. ostreatus and P. eryngii*. *C. comatus*, *M. giganteus*, strains of *P. ostreatus* and *P. eryngii* were shown to be manganese peroxidase producers. *M. giganteus* has high enzyme activity (laccase, peroxidase, tyrosinase and polyphenol oxidase). To our knowledge, this is the first presentation of manganese peroxidase activity in *A. romagnesii*, but neither Lac nor LiP activities were observed. Among the wild strains, only *P. ostreatus*-1, *P. ostreatus*-2, *P. ostreatus*-4, *P. eryngii*-1 and *P. eryngii*-2 presented LiP activity.

Statistical analysis results of Lac activity assay in liquid media showed that *P. ostreatus*-4 was significantly different than other organisms (Figure 1). According to MnP activity results, *P. eryngii* and *P. eryngii*-1 have similar activity level of MnP and these mushrooms are different from others (Figure 2). In Figure 3, *P. sajor-caju* has different LiP activity level than others and similarity (%) found to be between *P. eryngii*-1 and *P. eryngii*-2, and *P.ostreatus*-1 and *P. ostreatus*-4.

The extracellular enzymes LiP, MnP and Lac are thought to be involved in lignin degradation by white-rot fungi, which produce different combinations of these three extracellular enzymes. White-rot fungi and their ligninolytic enzymes have the potential to degrade xenobiotic compounds by partial degradation or complete mineralization. In general Lac and MnP are more widely distributed among white-rot fungi than LiP (Rothschild et al., 2002).

In conclusion, nature is the biggest source to find out organisms containing various properties as well as the above mentioned enzymes. This study emphasizes the need to explore more organisms to evaluate the real potential of fungi producing ligninolytic enzymes. Target enzyme synthesis may play an important role in the deve-

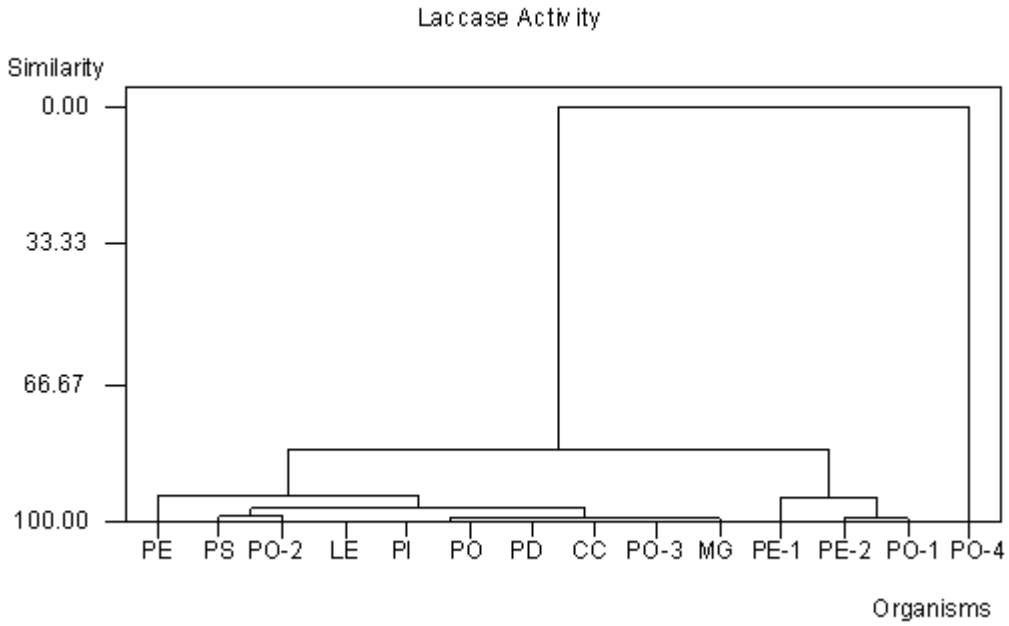

Figure 1. Similarity (%) of organisms in relation to their laccase activities. See Table 1 for abbreviations.

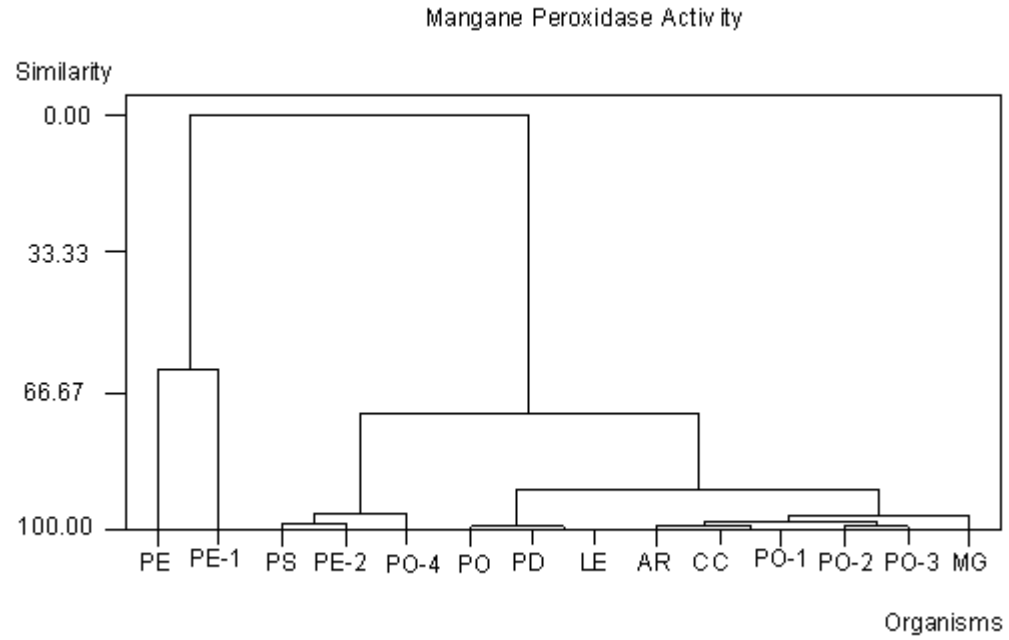

Figure 2. Similarity (%) of organisms in relation to their MnP activities. See Table 1 for abbreviations.

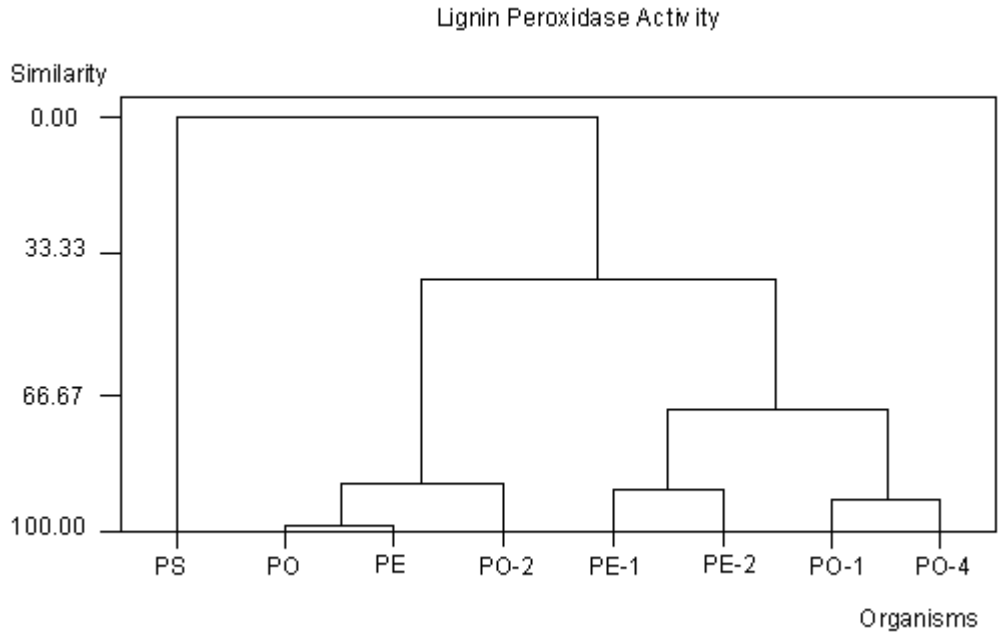

Figure 3. Similarity (%) of organisms in relation to their LiP activities. See Table 1 for abbreviations.

lopment of an efficient technology. The results of the present study allow us to conclude that wild *P. ostreatus* and *P. eryngii* are good candidates for scale-up ligninolytic enzyme production.

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