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Co-cultured Production of Lignin-Modifying Enzymes with White-Rot Fungi

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Abstract Co-cultivation was a potential strategy in lignocellulolytic biodegradation with producing high activity enzymes due to their synergistic action. The objective of this study was to investigate the rarely understood effects of co-culturing of two white-rot fungi on lignin-modifying enzymes (LMEs) production. Six species, *Bjerkandera adusta*, *Phlebia radiata*, *Pleurotus ostreatus*, *Dichomitus squalens*, *Hypoxylon fragiforme* and *Pleurotus eryngii*, were cultured in pairs to study the production of LMEs. The paired hyphal interaction observed showed that *P. eryngii* is not suitable for co-growth. The use of agar plates containing dye RBBR showed elevated decolourisation at the confrontation zone between mycelia. Laccase was significantly stimulated only in the co-culture of *P. radiata* with *D. squalens* under submerged cultivation; the highest value was measured after 4 days of incubation (120 U mg^{-1}). The improved productions of MnP and LiP were simultaneously observed at the co-culture of *P. ostreatus* and *P. radiata* (MnP=800 nkat L^{-1} after 4 days of incubation; LiP=60 nkat L^{-1} after 7 days of incubation), though it was not a good producer of

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laccase. *P. ostreatus* appeared to possess specific potential to be used in co-cultured production of LMEs. The phenotype of LMEs production was not only dependent on the species used but also regulated by different nutritions available in the culture medium. The present data will provide evidence for illustrating the regulatory roles of C/N on LMEs production under the co-cultures' circumstances.

Keywords LMEs · White-rot fungi · Co-cultivation · Interspecies interaction · Nutrients regulation

Introduction

Lignin removal is a key step for recycling the carbon fixed by land photosynthesis. The lignin polymer is highly recalcitrant towards chemical and biological degradation due to its molecular architecture, where the monomers, coniferyl alcohol, *p*-coumaryl alcohol and sinapyl alcohol form a complex three-dimensional network linked by a variety of ether and carbon–carbon bonds. The explanation to this complex structure is that it is created by a radical polymerisation. The biodegradation of this polymer is therefore an intricate problem, and in woody tissues the situation is further complicated by the fact that the lignin makes the structure so compact that proteins cannot penetrate the cell wall [1]. Ligninolytic microbes have developed a unique strategy to handle lignin degradation based on unspecific one-electron oxidation of the benzenic rings in the different lignin substructures by extracellular oxidative enzymes. Some basidiomycetes (the so-called white-rot fungi) are the most efficient lignin degraders in nature [2]. Lignin-modifying enzymes (LMEs) are considered to be involved in lignin biodegradation, which include oxidative enzymes that catalyse unspecific reactions, laccase (EC 1.10.3.2), lignin peroxidase (LiP, EC 1.11.1.14), manganese peroxidase (MnP, EC 1.11.1.13) and versatile peroxidase (VP, EC 1.11.1.16) [3, 4]. Also, several H_2O_2-generating enzymes such as aryl alcohol oxidase (AAO, EC 1.1.3.7), glyoxal oxidase and pyranose-2 oxidase (EC 1.1.3.10) are regarded as members of white-rot fungal lignin-degrading machinery [5–7]. Recently, peroxidases potentially involved in the degradation of lignin and related aromatic compounds have been classified into enzyme families according to their protein sequences and biochemical properties, and integrated into FOLy (Fungal Oxidative Lignin enzymes) database [8].

Wood rotting white-rot fungi are the most efficient lignin degraders in nature, with a capacity to remove lignin that makes them ideally suited for industrial applications [9, 10]. However, the effects on lignin-degrading abilities of white-rot fungi in mixed cultures have been paid little attention. Experimental evidence suggests that the competition for space and nutrients may result in enhanced degradation of lignin and elevated production of LMEs such as laccase and MnP [11, 12]. Most biotransformations in nature take place by the combination of metabolic pathways from different microorganisms. There are so many examples for the co-existence of different microorganisms observed, such as forest soils, compost piles and mammalian intestines [13, 14]. As recently reviewed by Bader et al. [15], co-culture cultivations can be utilised in the production of foods, food additives, pharmaceuticals, enzymes, fine chemicals, and bioremediation and degradation of lignocelluloses. There are many instances where the utilisation of co-cultures appears to be advantageous over pure cultures because of the potential for synergistic utilisation of the metabolic pathways of all involved strains in a co-culture situation [15].

Most of what is known about lignin biodegradation is from pure culture studies with white-rot basidiomycete fungi. However, little is known about the lignin degradation in complex ecosystems such as insect guts [16]. One of the current approaches in improving the efficiency of biodegradation and bioconversion of agricultural or agro-industrial residues in solid-state fermentation (SSF) is the use of co-cultures or mixed cultures of lignocellulolytic microorganisms. Many of these co-cultures or mixed cultures were more efficient in lignocellulolytic biodegradation with producing high activity enzymes due to their synergistic action [17]. The study by Geib et al. [16] showed that gut microbial communities can efficiently degrade lignin, which provides considerable enthusiasm for bio-prospecting these systems for new lignin-degrading enzymes and systems by means of metagenomic or proteomic approaches. Presently, nevertheless, degradation mechanisms are obtained relatively more in several white-rot fungi when cultivated as pure cultures either in liquid cultures or in solid media; little is known about the biochemistry and enzymology of mixed fungal cultures during lignin degradation. Several attempts have been made to enhance laccase by the fungi co-cultivation strategy. Crowe and Olsson [18] described the induction of laccase production by co-cultivating *Rhizoctonia solani* and *Pseudomonas fluorescens*. Laccase activity in *Trametes versicolor* was increased several times when this fungus was co-cultivated with *Trichoderma harzianum* and other soil fungi or bacteria [19]. Moreover, the induction of new isozymes was observed in dual cultures of *Pleurotus ostreatus* with *Trichoderma longibrachiatum* [20] and *Trametes* sp. AH28-2 with a *Trichoderma* [21]. Laccase was significantly stimulated in the co-culture of *P. ostreatus* with *Ceriporiopsis subvermispora* while manganese peroxidase was stimulated in co-cultures of *P. ostreatus* with *C. subvermispora* or with *Physisporinus rivulosus* [22]. The improved enzyme activities have been reported for co-culture of *P. chrysosporium* and *P. ostreatus* in comparison to the respective monocultures under SSF condition [23]. In contrast, Koroleva et al. [24] reported that the co-cultivation of *C. hirsutus* and *Cerrena maxima* resulted in little or no increases of LMEs activities. The aforementioned examples of possible applications of co-culture processes illustrate the increasing importance of this kind of cultivation in industrial biotechnology. Although some literatures indicate that the co-cultivation can be applied to solid fermentation using lignocellulose resources, the available information on the mechanism of multiple enzymes production and characterisations under liquid cultivation is rather limited. Nowadays, more than 90% of the technical microbial enzymes are produced by submerged fermentation [25]. Some of the advantages of a submerged culture system compared to solid fermentation are the homogeneity of the fermentation system, the presence of industrial equipment and the easement in measurement of process parameters [26]. Thus, this work investigated the co-cultured production phenotypes of three main LMEs under submerged co-cultivation. In this study, six different species, *Bjerkandera adusta*, *Phlebia radiata*, *Pleurotus ostreatus*, *Dichomitus squalens*, *Hypoxylon fragiforme* and *Pleurotus eryngii*, used display selective delignification or exhibit good co-cultivation characteristics. *D. squalens* belongs to the large group of white-rot fungi that express a set of laccases and MnPs [9, 27]. In various studies, it has been shown that different strains of the fungus efficiently degrade both natural lignin and synthetic lignin derivatives [9, 28, 29]. *P. radiata* express a multitude of extracellular LMEs including isozymes of laccases, LiPs and MnPs [9]. Also, H_2O_2-producing glyoxal oxidase (GLOX) has been detected in the cultures of *P. radiata* [30]. *P. eryngii* and *B. adusta* produce a third family of ligninolytic peroxidases called ‘versatile peroxidases’ (VP) [31]. In addition to white-rot basidiomycete, another fungus that had never been tested in the context of biopulping and bio-modification of lignin before—the white-rot ascomycete *Hypoxylon fragiforme*—was also considered in this work.

The main objective is to study laccase, LiP and MnP production phenotypes using this six co-cultivated white-rot fungi growing under shaken condition in comparison to pure fungal cultures, and to elucidate the responses mechanism of LMEs production under nutritional shocks in order to obtain more knowledge of the ligninolytic system.

Materials and Methods

Materials and Instrumentation

All chemicals used as buffers and substrates were commercial products of at least reagent grade, unless otherwise indicated. ABTS [2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)], VA (verotyl alcohol) and Remazol brilliant blue R (RBBR) were obtained from Sigma-Aldrich. Organosolv lignin was kindly supplied by Gerd Unkelbach at Fraunhofer ICT, Germany. Protein analysis reagents were obtained from Biorad, USA. Ultraviolet–visible absorption spectroscopy was performed on a Thermospectronic Photometer (Genesys 10 UV). Micro-detection of laccase enzyme was performed on a Synergy 2 Multi-Mode Microplate Reader (BioTek Instruments, Inc.).

Fungal Strains

B. adusta (DSM 4710), *P. radiata* (DSM 5111), *P. ostreatus* (DSM 11191), *D. squalens* (DSM 9615), *H. fragiforme* (DSM 5012) and *P. eryngii* (DSM 9619) were obtained from the Deutsche Sammlung von Microorganism und Zellkulturen, Braunschweig. The fungi were maintained on 2% (w/v) malt extract (ME) (Biokar) agar slants and stored at 4 °C. Before use, the stored fungi were inoculated on the newly prepared potato dextrose agar (PDA) plates. The inoculated fungi were cultured 7 days at 28 °C for further use.

Culture Conditions and Experimental Design

The paired growth observation was investigated on PDA agar medium plates. The modified Kirk medium (10 g L^{-1} glucose, 2 g L^{-1} peptone, 2 g L^{-1} KH_2PO_4, 0.5 g L^{-1} $MgSO_4{\cdot}7H_2O$, 0.1 g L^{-1} $CaCl_2$, 2 mg L^{-1} thiamine and mineral salts) containing 0.05% (w/v) lignin analogue synthetic dye RBBR as the reporting substrate was used to examine the decolourisation capability of the designed fungi [32].

During the comparison of different fungi and the paired groups, *B. adusta*, *P. radiata*, *P. ostreatus*, *D. squalens*, *H. fragiforme*, *P. eryngii* and the responding co-cultivated combinations were grown in 100-mL Erlenmeyer flasks containing 30 mL medium under 150 rpm shaking speed and at 28 °C. The culture medium used (PG medium) had the following composition (g/L): 3.0 peptone, 10.0 glucose, 0.6 KH_2PO_4, 0.001 $ZnSO_4$, 0.4 K_2HPO_4, 0.0005 $FeSO_4$, 0.05 $MnSO_4$ and 0.5 $MgSO_4$. The pH of the medium was adjusted to 6.0. The culture medium was diluted ten times and 0.1% lignin was added for cultivation investigation before sterilisation. For the inoculation of liquid cultures, fungi were pre-grown on PDA plates for 7 days. Five inoculum plugs (10 mm diameter) were placed on 30 mL of PG medium. After inoculation, the cultivation was carried out in a dark chamber under 140 rpm shaking speed at 28 °C during a total period of 20 days. At various times during cultivation, 1-mL samples were removed and analysed for laccase, MnP and LiP activities, and protein concentration as described below.

As the influences of different C/N ratios on LMEs production phenotype were examined, three ratios of glucose/peptone in diluted PG medium containing the glucose/peptone ratio of 10 g/L:0.3 g/L (designated as PG-A), 10 g/L:30 g/L (designated as PG-B) and 10 g/L:3 g/L (designated as PG-C) were designed. Other media compositions did not change except those specifically pointed out.

Paired Interactions Evaluation and Peroxidase-Producing Selection

Interspecies interactions of the six fungal species, paired in all possible combinations, were investigated by placing 1-cm circular inoculum plugs on PDA plates 3 cm apart. Interactions between opposing mycelia were assessed visually every second day using the classification of Rayner and Boddy [33] and Chi et al. [22].

RBBR is a polymeric dye widely used for screening of lignin-degrading peroxidases. In this study, the dye decolourisation rate and position in monocultures and in various co-cultures was assessed visually on agar plate. Inoculum plugs 5 mm in diameter were placed 30 mm apart on modified Kirk agar medium [32] containing 0.05% (w/v) RBBR. Plates were incubated at 28 °C and decolourisation recorded daily.

Enzyme Activity Determination

Lignin-degrading enzyme activities were assayed from the liquid cultures. Laccase activity was assayed at 3 mL reaction systems. Peroxidases activity assays were conducted in 1-mL reaction mixtures at 25 °C using the extracellular medium of the fungal cultures under low temperature centrifugation. Laccase activity was estimated for 2 weeks and peroxidase activities for at least 3 weeks.

Laccase activity was measured spectrophotometrically by monitoring the oxidation products of ABTS [2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)] (molar extinction coefficient 36,000 M^{-1} cm^{-1}), buffered with 50 mM sodium tartrate (pH 4.5), at 420 nm [34]. Enzyme activity was expressed in units defined as 1 U=1 μmol ABTS oxidised min^{-1}.

Lignin peroxidase (LiP) assay was slightly modified according to Kirk et al. [32]. Oxidation of 3.0 mM veratryl alcohol to veratraldehyde (molar extinction coefficient 9,300 M^{-1} cm^{-1}) in 100 mM sodium tartrate (pH 3.0) was followed at 310 nm. MnP activity was measured by following the formation of Mn^{3+}–tartrate complex (molar extinction coefficient 6,500 M^{-1} cm^{-1}), buffered with 50 mM sodium tartrate (pH 5.0) in the presence of 0.5 mM $MnSO_4$, at 238 nm [35]. Peroxidase reactions were started by addition of 0.1 mM hydrogen peroxide. Enzyme activity was expressed as μkat (10^{-6} mol/s).

Reproducible data were obtained between replicated submerse cultivations of mono and co-cultures. In general, nearly 90% of measured activities deviated less than 15% from the average in duplicate measurements.

Protein Concentration Analysis

The Bio-Rad DC protein assay was a colorimetric method for protein concentration. The reaction is similar to the well-documented Lowry assay [36], but with the following modifications; the reaction reaches 90% of its maximum colour development within 15 min. The detailed microplate assay protocol followed the manufacturer's instructions with bovine serum albumin as the standard. The standard curve was $y=0.0912c+0.0419$ ($y=OD_{570}$, c=protein concentration, mg/mL, $R^2=0.9912$).

Results and Discussion

Hyphal Interactions for Paired Growth and Peroxidase-Producing Capability on Agar Plates

White-rot fungi produce extracellular lignin-modifying enzymes, the best characterised of which are laccase, LiP and MnP. In this study, six different fungi used displayed selective delignification or exhibit good co-cultivation characteristics. As reported previously, many metabolites and enzymes produced by interacting fungi can affect wood decay [22, 37]. The paired growth characterisation of the six species was firstly compared on PDA plates. As a result, agar confrontation studies should be investigated to examine the interaction within the paired species. *P. eryngii* was inhibited by other five corresponding paired fungi. *P. eryngii* showed bad growth when co-cultivated with other five fungi. The co-cultures of *D. squalens* and *B. adusta* with *P. ostreatus* showed co-growth when co-cultivated, but as the incubation time was prolonged, the browning of hyphal apices and emergence of aerial hyphae were observed. *H. fragiforme* mycelium showed resistance to invasion during interactions with *P. eryngii*, *P. radiata* and *B. adusta*. No browning of hyphal apices of *P. radiata* appeared in interaction with *P. ostreatus* and with *D. squalens*, whereas with *H. fragiforme* the hyphal front became strongly pigmented. In the paired combinations with *P. ostreatus*, the interaction mycelia front of the partner became pigmented. The co-cultured combination of *B. adusta* and *D. squalens* grows quickly and no pigmentation was observed at the interface of the co-culture mycelia. More importantly, *P. ostreatus* and *D. squalens* grew very quickly than the corresponding paired fungus.

The pigmentation phenomenon of interacting hyphal fronts possibly derives from melanin biosynthesis and enhanced phenol oxidase activity in the interacting hyphal tip region [38, 39]. As others have reported, pigmentation may be an indicator of free radicals appearance in the hyphal tip region. Scavenged and emitted free radicals in melanin can thus stimulate lignin degradation [40]. In contrast to the pure culture, interactions between the different microorganisms play a critical role in a co-culture. Growth of cells of one strain may be enhanced or inhibited by the activities of other microorganisms present in the medium. The same is also true for the formation of primary and secondary metabolites [41]: it may be a unique characteristic of the co-cultivation processes. However, the exact interaction mechanisms of paired fungi groups need to elucidated with more experimental evidences.

Dye decolourisation has been ascribed by many reports to produce peroxidases by the isolated fungi, namely LiPs, MnPs and laccases [42–46]. Decolourisation of specific dyes, e.g. Remazol Brilliant Blue R (RBBR) and Poly R, has been used as a rapid screening method for detection of ligninolytic ability in fungal strains. As mentioned above, *P. eryngii* was not considered in further experiments. The decolourisation of the selected fungi and the paired groups on the Kirk medium plate containing RBBR was carried out. As shown in Fig. 1, decolourisation of RBBR on the modified Kirk plate enabled the assessment of potential lignin-degrading activity in both spatial and temporal aspects (additionally paired culture screening result on agar plates are summarised in Supplementary Table 1). *H. fragiforme* showed no capability to decolourise RBBR dye in this work. Other four fungi monocultures can degrade RBBR. The degree of decolourisation was clearly stimulated due to mycelial interactions. A bleached area was observed below the hyphal front of *D. squalens* in the modified Kirk medium within 4 days, especially in the interaction with *P. radiata*. Also, the decolourisation by *P. ostreatus* was clearly enhanced in the presence of *P. radiata*.

The strong response by *P. radiata* and *P. ostreatus* against *B. adusta* was seen as clear decolourisation zone in those areas where the hyphal fronts first interact (photos not

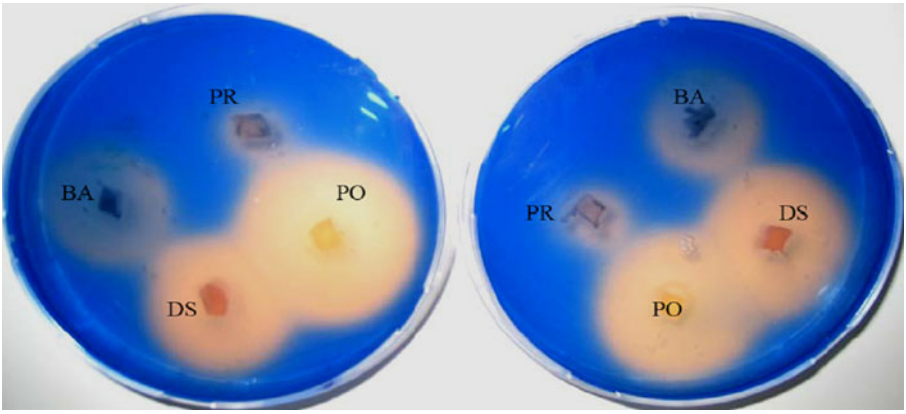


Fig. 1 Decolourisation observations of four selected fungi (PO, DS, PR and BA) on the modified Kirk medium plate containing 0.05% RBBR for 5-day incubation. *PO Pleurotus ostreatus*, *PR Phlebia radiata*, *DS Dichomitus squalens*, *BA Bjerkandera adusta*

shown). Similarly, the interaction of *D. squalens* and *B. adusta* showed no inhibition in contrast to the corresponding fungus. Interestingly, nevertheless, *H. fragiforme* did not exhibit decolourisation ability; this fungus yet stimulated the bleaching below *P. ostreatus* or *B. adusta* hyphae. Chi et al. [22] observed that the co-culture of *C. subvermispora* with *P. ostreatus* significantly stimulates wood decay in comparison to monocultures. Recently, genes were identified, which were differentially expressed during fungal–fungal interactions. One of the identified induced genes is essential for DNA repair [47]. The results indicate that fungal–fungal interactions lead to the release of reactive oxygen substances (ROS) such as hydroxyl radicals that induce DNA damage. In addition to ROS involvement, RBBR dye bleaching may have functioned via peroxidases, especially MnP and VP. Thus, additional studies are required to clarify the involvement of peroxidases and/or ROS in interspecific interactions between white-rot fungi [40].

Comparison of LMEs Production Characterisation

White-rot fungi produce three main extracellular enzymes involved in ligninolysis: laccase, lignin peroxidase and manganese peroxidase. Though not all white-rot fungi do produce all three enzymes, laccase occupies an important place in ligninolysis [48]. As indicated in Table 1, the pure culture of *P. ostreatus*, the co-cultures of *B. adusta* with *P. radiata*, *P. ostreatus* with *P. radiata*, *P. ostreatus* with *D. squalens* and *B. adusta* with *D. squalens* can

Table 1 LMEs production patterns of different white-rot fungi and the corresponding combinations

	PO	DS	BA	PR	HF	BA+ PR	BA+ HF	PR+ DS	PO+ PR	PO+ DS	PO+ HF	BA+ DS	PO+ BA
Lac	+	+	−	+	−	+	−	+	+	+	−	+	−
MnP	+	+	+	+	+	+	+	+	+	+	+	+	+
LiP	+	−	−	−	−	+	−	−	+	+	−	+	+

'+' means the detectable activity using the present method, '−' means no detectable activity, *PO Pleurotus ostreatus*, *PR Phlebia radiata*, *DS Dichomitus squalens*, *BA Bjerkandera adusta*, *HF Hypoxylon fragiforme*

produce the main three enzymes simultaneously. *B. adusta* and *H. fragiforme* did not produce laccase under the submerged cultivation, which only produced one enzyme, MnP. *H. fragiforme* was not the suitable paired fungus for co-culturing production of LMEs, and because of that it produced a low amount of MnP and, moreover, inhibited the expression of LMEs when co-cultivated with the corresponding fungus. As evaluated above on Kirk medium, *H. fragiforme* possibly can increase the decolourisation of *P. ostreatus* and *B. adusta*, whereas under the submerged cultivation, it was toxic to LMEs expression in terms of the present culture conditions.

The courses of laccase production for different combinations and monocultures are summarised in Fig. 2a. Comparison of laccase activity among the designed fungi demonstrated that *P. radiata* is the best producer in terms of volumetric activity, whereas the co-culture of *P. radiata* with *D. squalens* showed the maximum specific activity. Next to the monoculture of *P. radiata* and co-cultures of *P. radiata* and *D. squalens*, the co-culture of *P. ostreatus* and *D. squalens* was another potential combination for laccase production. *D. squalens* co-cultured with *P. ostreatus* or *P. radiata* likely stimulated laccase production when compared to monocultures. Interestingly, the paired group of *P. ostreatus* and *P. radiata* presented the rapid incline of laccase production before 48 h, after which laccase production was slowly decreasing. The present data were well in agreement with the above plate experiments. Furthermore, most of the combinations and monocultures gave the greater laccase production before 168 h culture time. Reappearance of enzyme activity during later stages of lignin degradation might be attributed to fungal autolysis resulting in the release of cell membrane bound or intracellular enzymes in the medium. As reported earlier, *P. radiata* is a good producer of laccase enzyme [49, 50].

Except laccase, MnP was considered as the main enzyme involving into partially mineralisation of a broad spectrum of aromatic substances [51]. Thus, the comparisons of MnP production were examined among the designed fungi. As shown in the results presented in Fig. 2b, the designed fungi all can produce MnP enzyme. *P. ostreatus* produced the highest MnP activity and showed a stable increased expression before the experiment process ended. But the occurring time of the highest activity of MnP produced in co-culture of *P. ostreatus* against *P. radiata* (96 h) was earlier than that of other fungi. The stimulating effect was clearly observed at the co-culture of *P. ostreatus* and *P. radiata* if compared to the monocultures before 120 h culture time. Furthermore, this co-cultured combination kept the stable production of MnP during the cultivation period. In contrast to the monocultures, *B. adusta* and *D. squalens* co-culture can stimulate MnP expression, whereas *D. squalens* clearly inhibited MnP expression by the corresponding fungus, such as *P. ostreatus* or *P. radiata*. Apart from the co-culture of *P. ostreatus* and *P. radiata*, other monocultures and co-cultured combinations appeared to have higher production after 120 h time.

LiP was another key enzyme for non-phenolic compounds degradation. LiP is an enzyme commonly associated with the degradation of lignin by *P. chrysosporium*. It oxidises phenols to phenoxy radicals and non-phenolic aromatics to radical cations [52]. Herein, LiP was compared with different fungi and combinations. As illustrated in Fig. 2c, among the designed fungi, significant LiP activity was detected in *P. ostreatus* close to 240 h culture time employing VA oxidation assay. The comparison of LiP produced by co-cultures presented that the co-cultures of *P. ostreatus* and *P. radiata*, and *B. adusta* and *P. radiata* were beneficial for stable expression of LiP. Interestingly, the monocultures of *B. adusta*, *P. radiata* and *D. squalens* showed no capability for LiP production using VA as the substrate, but their co-cultures (*B. adusta* and *P. radiata*, *B. adusta* and *D. squalens*) can stably produce LiP (Fig. 2c). As identified earlier, *B. adusta* can produce a new enzyme that

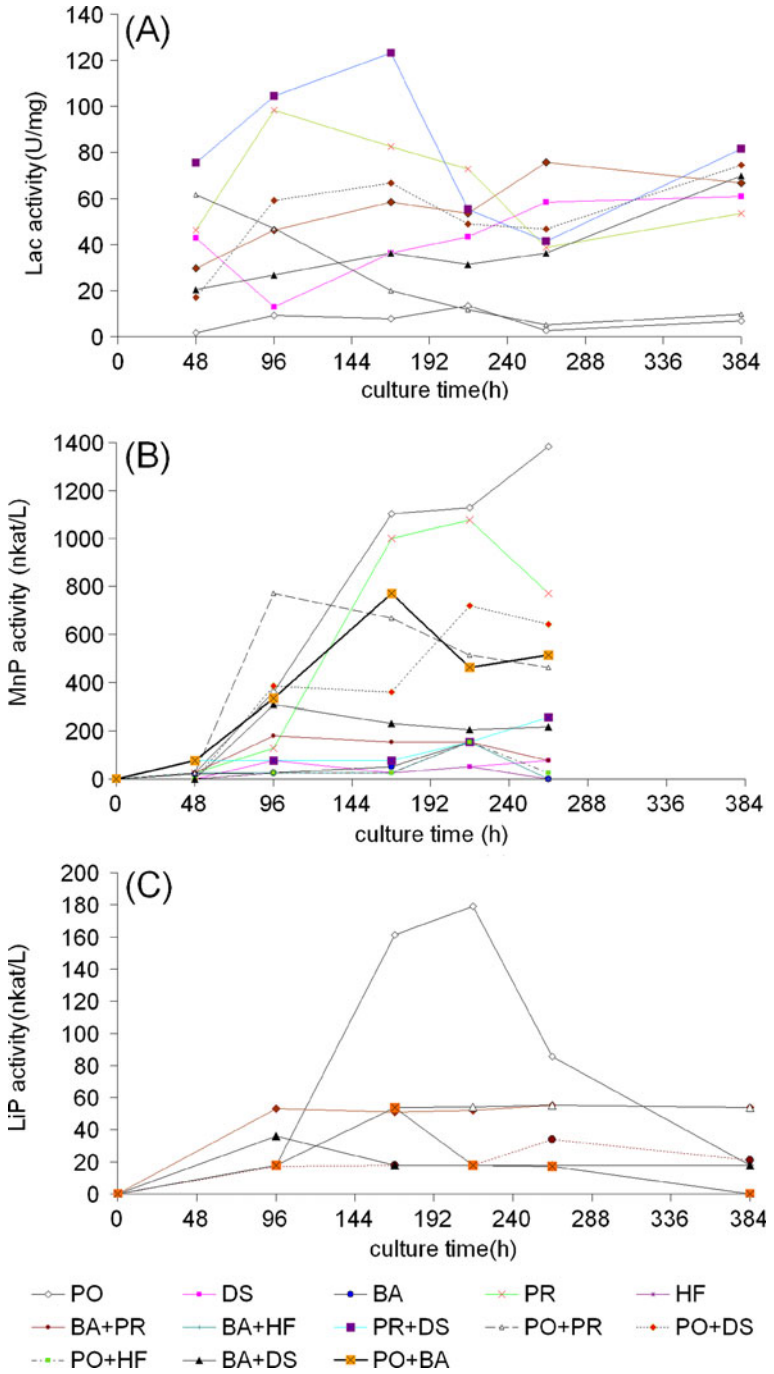


Fig. 2 Comparison of laccase (**a**), MnP (**b**) and LiP (**c**) production among different paired combinations and monocultures. *PO Pleurotus ostreatus*, *PR Phlebia radiata*, *DS Dichomitus squalens*, *BA Bjerkandera adusta*, *HF Hypoxylon fragiforme*

is different from MnP and LiP, named versatile peroxidase (VP), that not only degrades Mn^{2+} to form Mn^{3+} but also oxidises non-phenolic compounds (such as VA) in the absence of Mn^{2+} ions [53]. Thus, it is supposed that *B. adusta* plays a key role in co-cultured production of LiP. Unfortunately, the monoculture of *B. adusta* only detected the expression of MnP enzyme; thus, this surprising phenomenon awaits further elucidation. No LiP production was detected in the co-cultures of *B. adusta* with *H. fragiforme*, *D. squalens* with *P. radiata* and *P. ostreatus* with *H. fragiforme*.

Both LiP and MnP are involved in lignin and xenobiotic degradation [54]. LiP isozymes can directly oxidise a variety of organic substrates. Due to its high redox potential, the preferred substrates for LiP are non-phenolic methoxy-substituted lignin subunits. LiP follows the same reaction pathway as horseradish peroxidase (HRP) and cytochrome *c* peroxidase (CCP) in catalysing the oxidation of substrate by H_2O_2 [55]. Taken together, the findings demonstrate that the co-culture of *P. radiata* and *D. squalens* was the potential alternative for laccase production, and the co-culture of *P. ostreatus* and *P. radiata* was beneficial for MnP and LiP expression. In this study, the purpose was to focus on elucidating the phenotypes of three LMEs (Lac, MnP and LiP) productions under co-culture environment; the respective co-cultures of *P. radiata* against *P. ostreatus* and *D. squalens* was thus initially determined to keep further verification under different culture systems.

As evaluated above, *P. ostreatus*, *P. radiata* and *D. squalens* fungi were selected to examine the metabolism of LMEs production. The large-scale cultivation was employed to verify the effect of co-cultures on the LMEs phenotype. As clearly shown in Fig. 3a, the co-culture of *P. radiata* against *D. squalens* showed stable and higher production of laccase when compared to the monocultures and mixed cultures. The highest activity of laccase was observed at 72 h time for the pair-wise of *P. ostreatus* and *P. radiata*; after that, laccase production rapidly decreased. *P. radiata* presented the same change as that of the co-culture of *P. ostreatus* and *P. radiata.* As compared to the screening results (Fig. 2a), the present data demonstrate that laccase activity detected was generally lower, which imply that oxygen was likely unfavourable for laccase expression.

Peroxidases are involved into lignin polymerisation and depolymerisation. MnP is the most common lignin-modifying peroxidase produced by almost all wood-colonising basidiomycetes causing white rot and various soil-colonising, litter-decomposing fungi [56]. MnP data under large-scale cultivation further indicated that the co-culture of *P. ostreatus* and *P. radiata* was the potential combination (Fig. 3b), which was in accordance with the results from the screening design. The comparison of co-culture of *D. squalens* against *P. ostreatus* and *P. radiata*, respectively, with the monocultures of *P. ostreatus and P. radiata* revealed that *D. squalens* likely played an unfavourable role on MnP expression. Except the monoculture of *P. radiata*, other examined cultures appeared the highest activity of MnP after 10 days of culture time. As LiP enzyme was evaluated (Fig. 3c), the co-culture of *P. radiata* and *D. squalens* showed no detectable LiP. LiP produced by the pure culture of *P. ostreatus*, the co-cultivated *P. ostreatus* and *P. radiata* had the same trend. Before 300 h culture time, the co-culture of *P. ostreatus* and *P. radiata* showed little higher LiP activity than the monoculture of *P. ostreatus*, whereas at the later stage of cultivation, the co-cultured broth of them is clearly best for LiP production. As reported earlier, *P. ostreatus* is a good producer of MnP and VA oxidation peroxidase, which implies that peroxidase produced by *P. ostreatus* was a new enzyme [57]. The mycelial interactions with *P. ostreatus* gave synergistic effects during the degradation of lignin and lignosulphonates with most of the 20 fungal species investigated [11]. In the present work, *P. ostreatus* gave rise to the increasing effect on the corresponding fungus of the mixed cultures for LMEs

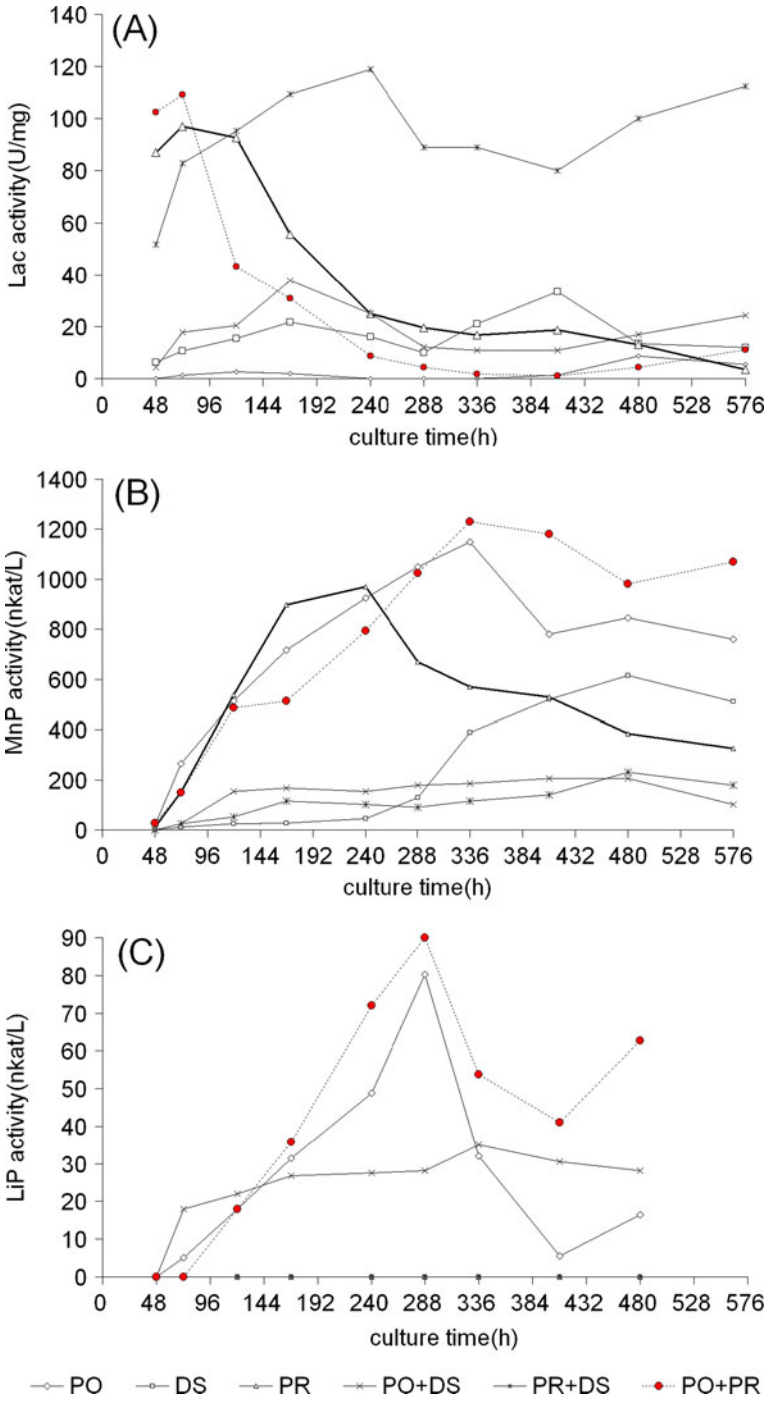

(A)
Lac activity(U/mg)
0
20
40
60
80
100
120
140
0 48 96 144 192 240 288 336 384 432 480 528 576
culture time(h)
(B)
MnP activity(nkat/L)
0
200
400
600
800
1000
1200
1400
0 48 96 144 192 240 288 336 384 432 480 528 576
culture time(h)
(C)
LiP activity(nkat/L)
0
10
20
30
40
50
60
70
80
90
0 48 96 144 192 240 288 336 384 432 480 528 576
culture time(h)
PO
DS
PR
PO+DS
PR+DS
PO+PR

Fig. 3 Laccase (**a**), MnP (**b**) and LiP (**c**) production and comparison of different co-cultures and monocultures under 1 L submerged culture system. Liquid culture consisted of 120 mL culture medium in 1,000-mL flasks containing 0.1% lignin. *PO Pleurotus ostreatus*, *PR Phlebia radiata*, *DS Dichomitus squalens*

production, particularly for peroxidases. Hence, *P. ostreatus* is the potential fungus in the co-cultures cultivation for LMEs production or lignin degradation.

LMEs analysis data mentioned above can be verified in the results obtained from the selective plate experiments. Fungal lignin-degrading systems can be stimulated by interspecific interactions with other white-rot fungi. The exact mechanism of stimulation remains to be studied further in detail. However, the full understanding of this process encourages the use of co-cultivation of white-rot fungi as an improved method for biopulping and for efficient LMEs production.

Optimisation of LMEs Production by Nutritional Conditions

White-rot fungi typically produce multiple isoforms of oxidative enzymes, which are differentially regulated by nutrient limitation and cultivation time [9, 58], especially by nutrition availability. Thus, the main nutritional components were examined to investigate the significance on LMEs production pattern under different co-cultures environment (*D. squalens*, *P. ostreatus* and *P. radiata*). Three kinds of C/N ratios were designed to elucidate the roles of different nutrients on LMEs production pattern in the co-culture situation. Laccase production was compared (Fig. 4a–c). As data summarised in Fig. 4, the low nitrogen was significantly favourable for laccase production by the mixed culture of *P. radiata* and *D. squalens* (Figs. 4a and 5c); moreover, the rich carbon nutrition under nitrogen-limitation conditions efficiently promoted laccase production, as compared to the monocultures. Herein the present results provide the principle approach to optimise laccase yields. Laccase production is not suppressed by a high nutrient content. In many species, the use of nutrient-rich media resulted in higher laccase titres if compared to nutrient-limited media [58]. However, it has been found that *P. ostreatus* is without detectable laccase production in PG medium containing carbon-rich nutrient, but can only be pronouncedly detected under nitrogen- and carbon-limited cultures. At the circumstance of carbon starvation, the supply of nitrogen nutrients enables the significant increase of laccase produced by *P. radiata*, whereas laccase activity in the respective co-cultures of *D. squalens* against *P. radiata* and *P. ostreatus* was evidently decreased. Although the laccase production patterns produced by the monoculture of *P. radiata* and mixed culture of *P. radiata* against *D. squalens* were greatly influenced by different C/N ratios, the effects of *D. squalens* and the paired group of *P. ostreatus* and *P. radiata* have no significant difference in regulating laccase production among the three designed C/N ratios. As well acknowledged, the carbon or nitrogen limitations can be favourable for laccase production. However, the regulatory responses were indeed dependent on species.

Extensive research has been conducted on the physiological onset of lignin degradation. The lignin-degrading system of white-rot fungi is composed of extracellular enzymes, such as ligninolytic peroxidases and H_2O_2-generating oxidases, and low molecular weight co-factors. Nutritional and culture parameters have been investigated which influence the onset of the production of the typical ligninolytic enzymes LiP and MnP [59]. Among them, the investigations with *P. chrysosporium* generally show that lignin degradation only occurs during the secondary metabolism, which is triggered by the depletion of nutrient nitrogen, carbon or sulphur [60, 61]. It is suggested that N-limited growth conditions were natural for fungi since wood contains only low levels of nitrogen [62]. However, recent studies have

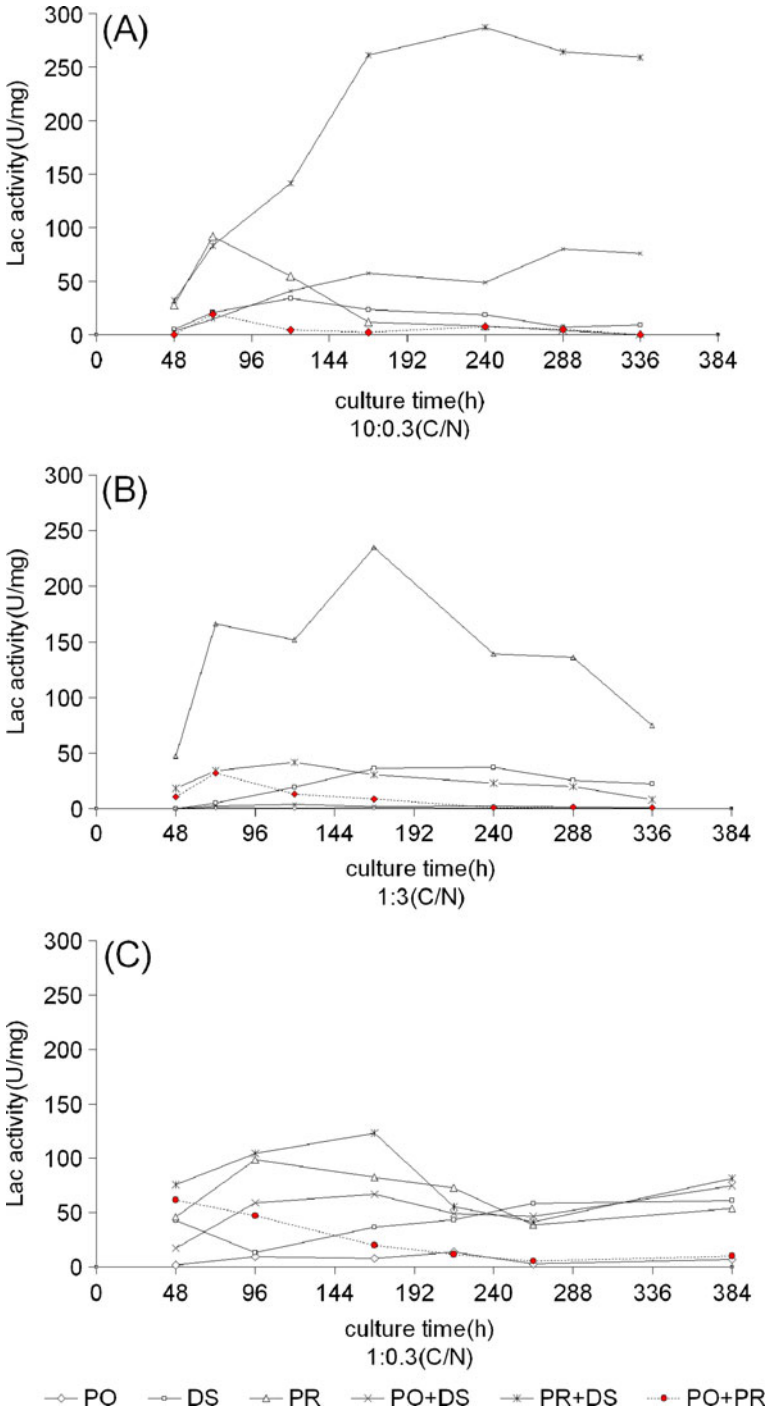
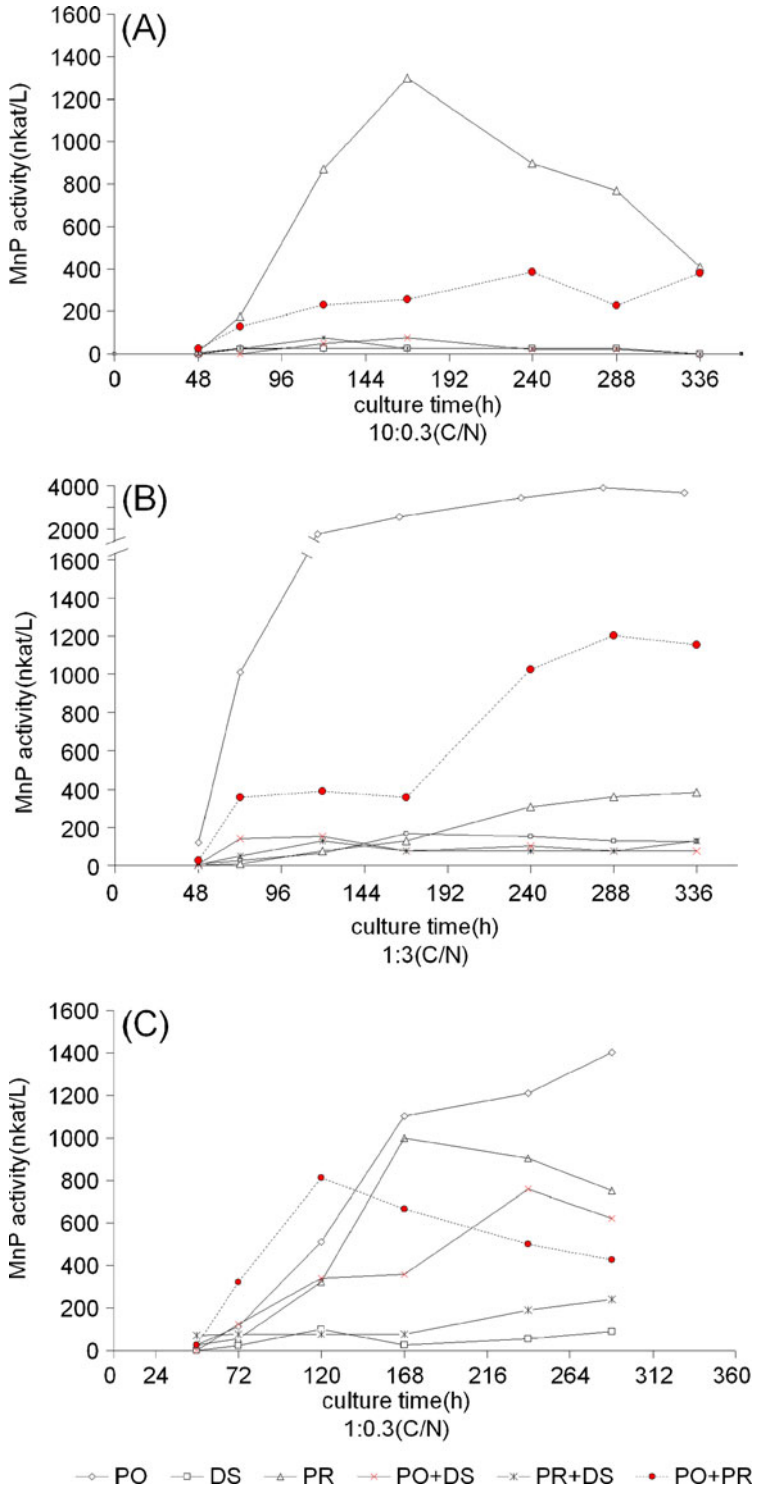

(A)
Lac activity(U/mg)
300
250
200
150
100
50
0
0
48
96
144
192
240
288
336
384
culture time(h)
10:0.3(C/N)
(B)
Lac activity(U/mg)
culture time(h)
1:3(C/N)
(C)
Lac activity(U/mg)
culture time(h)
1:0.3(C/N)
PO
DS
PR
PO+DS
PR+DS
PO+PR

Fig. 4 Responses of laccase production to different C/N ratios for the designed fungi and paired combinations. **a** 1:0.03 (glucose/peptone), **b** 1:3 (glucose/peptone), **c** 1:0.3 (glucose/peptone). *PO Pleurotus ostreatus*, *PR Phlebia radiata*, *DS Dichomitus squalens*

shown that not all white-rot fungal species and strains are N-regulated, such as *B. adusta* ssp. and *Coriolus versicolor* [63, 64]. The expression of the multiple genes encoding LiP and MnP isoenzymes has been reported to be differentially regulated depending on the conditions of nutrient limitation [65]. Thus, the two peroxidases were compared among the designed fungi combinations. Considering the differences of MnP activity presented in Fig. 5a–c, the ratio of C/N played a key role in MnP regulation by *P. ostreatus*; among them the ratio of 1:3 (glucose/peptone) was best for MnP production. The observed MnP activity produced by *P. ostreatus* is rather low at the high ratio of 10:0.3 (glucose/peptone) conditions, which implies that rich carbon nutrition likely gives rise to the inhibitive influence on MnP production. Also, it was reported that the synergistic attack of some white-rot fungi on lignosulphonate is more pronounced on a meager medium than on a carbohydrate-rich one [11]. In contrast, *P. radiata* could produce more MnP under the high C/N ratio conditions than low C/N ratio. As three co-cultures were compared, the co-culture of *P. ostreatus* and *P. radiata* showed better than other co-cultures in response to MnP production. The co-culture of *P. ostreatus* and *P. radiata* was highly favourable for MnP expression in the PG medium with the ratio of 1:0.3 (glucose/peptone) before 120 h culture time, whereas at the later stage, the ratio of 1:3 (glucose/peptone) in PG medium was more useful for MnP production. Though the ratio of C/N did not produce a significant influence on MnP regulation by the monoculture *D. squalens*, MnP produced by the co-culture of *P. ostreatus* and *D. squalens* was pronouncedly controlled by the ratio of C/N. Owing to the reason that the respective monoculture of *P. ostreatus* and *P. radiata* exhibited completely different responses to the shock of C and/or N limitation, thus MnP production in co-cultured situation was not greatly enhanced in contrast with that of the monoculture. MnP is the primary enzyme that is involved in the decomposition of lignin-containing resources in nature, in which the nutrition limitation was the main environmental characteristic.

As for the consideration that LiP was the best for non-phenolic compounds degradation, thus LiP possesses the outstanding perspective in LMEs research and application. In view of LiP production phenotype (Fig. 6), the response of *P. ostreatus* to nitrogen limitation was more pronounced than other fungi and combinations. Furthermore, the pure fungal *P. ostreatus* showed no detectable LiP activity under the carbon-rich medium. Combined with the above described results, peroxidase production (MnP and LiP) was strongly sensitive to the carbon-rich nutrition condition. The pure fungal culture of *P. radiata* or *D. squalens* showed no LiP production under three kinds of culture media, which implies that these fungi at a molecular level did not constitutively express LiP. As LiP comparison presented in Fig. 6, the co-cultivated *P. ostreatus* and *P. radiata* gave more LiP production in the carbon-meager media than that in the carbon-rich medium.

The capability of *P. chrysosporium* for producing LiPs and MnPs makes it a model organism. The major difficulty in using this organism for enzyme production is the instability of its productivity. This is largely due to the poor understanding of the regulatory mechanisms of *P. chrysosporium* responding to different nutrient sources in the culture medium, such as metal elements, detergents, lignin materials etc. [66]. Though different conclusions are derived from the reported studies, most investigations focus on single fungus as the LMEs producing system. As a consequence, the regulation studies on co-cultured production of LMEs are highly required. The general LMEs production pattern was not only dependent on gene expression but also regulated by different nutritional conditions.


(A)
MnP activity(nkat/L)
culture time(h)
10:0.3(C/N)
(B)
MnP activity(nkat/L)
culture time(h)
1:3(C/N)
(C)
MnP activity(nkat/L)
culture time(h)
1:0.3(C/N)
PO
DS
PR
PO+DS
PR+DS
PO+PR

◀ **Fig. 5** Effect of different C/N ratios on MnP production for the designed fungi and paired combinations. **a** 1:0.03 (glucose/peptone), **b** 1:3 (glucose/peptone), **c** 1:0.3 (glucose/peptone). *PO Pleurotus ostreatus*, *PR Phlebia radiata*, *DS Dichomitus squalens*

Considering the above results obtained the general characteristics of LMEs regulation by the availability of nutritional components may be derived in this work. As for the monoculture of *P. ostreatus*, laccase, MnP and LiP were all significantly inhibited by a carbon-rich nutritional environment. Although the pure culture *D. squalens* was not sensitive to C/N variations, the carbon- and nitrogen-meager medium was still beneficial for laccase and MnP production. The responses of laccase and MnP to the shock of carbon and/or nitrogen availability in pure culture *P. radiata* were completely consistent. Laccase produced by *P. radiata* in the nitrogen-rich medium was higher than that in nitrogen-meager condition, but the regulation of MnP by *P. radiata* was mainly dependent on the carbon-rich nutrition.

Whereas the regulation of LMEs by three kinds of co-cultures was assessed, obviously the carbon-rich culturing condition is useful for laccase and MnP expression by the co-cultivated *P. radiata* and *D. squalens*, but without LiP production. The co-culture of *P. ostreatus* and *P. radiata* easily expressed more laccase, MnP and LiP in the carbon-meager media than in the carbon-rich environment. Furthermore, under the carbon-meager environment, the nitrogen limitation produces a beneficial effect on LiP production, except MnP enzyme by co-cultivated *P. ostreatus* and *P. radiata*. To this combination, this characteristic can be applied to selectively control laccase, MnP and LiP productions at different stages in view of the availability of carbon/nitrogen nutrition. The nitrogen-rich nutritional condition was unfavourable for laccase, MnP and LiP production by the co-culture of *P. ostreatus* and *D. squalens*. Under the nitrogen-meager condition, the rich carbon nutrition could produce more laccase by the co-cultivated *P. ostreatus* and *D. squalens*, but which was the opposite to MnP. The present data will provide more evidence for understanding the regulatory roles of C/N or other nutritional components on LMEs production characteristics under the co-culture circumstances. Interestingly, the results obtained seem to provide clues for selective control of LMEs expression under the submerged cultivation.

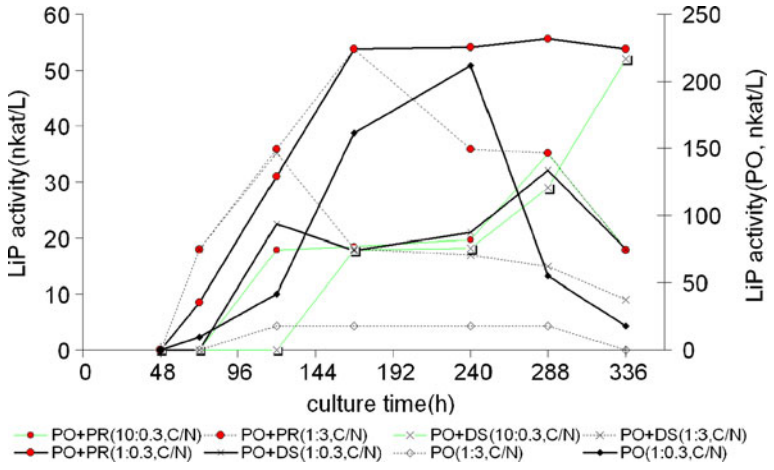


Fig. 6 Effect of different C/N (glucose/peptone) ratios on LiP production by the designed fungi and paired combinations. *PO Pleurotus ostreatus*, *PR Phlebia radiata*, *DS Dichomitus squalens*

Concluding Remarks

In summary, taken together, the results described above suggest that the regulation of LMEs production under the shaken cultivation was species specific as well as dependent on available nutritional components. As compared to the monocultures, the combination of *P. radiata* and *D. squalens* shows the potential to enhance the production of laccase as well as with stable production of MnP. *D. squalens* likely plays a key role in stimulating laccase production through the fungal–fungal interaction. The improved productions of MnP and LiP were simultaneously observed at the co-culture of *P. ostreatus* and *P. radiata*, though this combination was not a good producer of laccase. Taking the good co-growth characteristic and peroxidases-producing capability into account, *P. ostreatus* appears to possess specific potential to be used in co-cultured production of LMEs. LMEs production phenotype was dependent on the species used, furthermore regulated by different nutritions available in the culture system. Laccase production by co-cultivated *P. radiata* and *D. squalens* was strongly regulated by rich carbon nutrients environment. Peroxidases produced by the co-culture of *P. ostreatus* and *P. radiata* was more easily affected by carbon nutrition presented in the culture medium, although the monocultures of *P. ostreatus* and *P. radiata* exhibited different responses to the shock of carbon and/or nitrogen shortage. As the co-cultured combinations were comparatively evaluated, the available carbon nutritions generally regulate peroxidase production. Although the synergistic effect in peroxidase (MnP and LiP) production by the selected co-culturing combinations was not observed, the comparative study of LMEs production will elucidate the interaction mechanism.

The results described in this work imply that whereas a broad regulatory mechanism may control the response of co-cultures to any kind of starvation conditions or environmental conditions, the factors that trigger overproduction of the individual components of the ligninolytic system may differ. Though some interesting and disputing conclusions were obtained, lots of problems are required to be further understood. Future experiments should be carried out to elucidate the interaction mechanism at the interface of co-cultured fungi using efficient approaches, and to characterise these enzymes' physico-chemical and biochemical properties in order to obtain more knowledge of the ligninolytic system. More importantly, the molecular differences of LMEs between the co-cultures and the monocultures are required to make elucidation.

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