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Rogalski, J.

Polish society of microbiologists
1991

Acta Microbiologica Polonica. 1991. 40: 221-234

http://hdl.handle.net/1975/908

Acta Microbiologica Polonica
1991, Vol. 40, 3/4, 221–234

Production of Laccase, Lignin Peroxidase and Manganese-dependent Peroxidase by Various Strains of *Trametes versicolor* Depending on Culture Conditions

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Abstract

The production of laccase, lignin peroxidase (LiP) and manganese-dependent peroxidase (MnP) was studied in four strains of *Trametes (Coriolus, Polystictus) versicolor* by applying different concentrations of carbon and nitrogen in the basal culture medium usually adopted for lignin biodegradation studies. The media were supplemented either with veratric acid or veratryl alcohol. We found that the appearance of these extracellular enzymes greatly depended on the aromatic compound added and the type of medium used. In each strain of *T. versicolor* a different combination of carbon and nitrogen concentrations together with veratric acid or veratryl alcohol was required to provide significant production of laccase, LiP or MnP. For the production of these extracellular ligninolytic enzymes in individual strains of *T. versicolor* appropriate culture conditions should be selected.

Introduction

Bioconversion of lignin is one of the most important processes occurring in the carbon cycle of the biosphere. In spite of that, the pathways of lignin degradation have not yet been completely elucidated. The white-rot fungi *Trametes (Coriolus, Polyporus) versicolor, Phanerochaete chrysosporium* and *Phlebia radiata* are among the most efficient lignin-degrading microorganisms (Cowling, 1961; Ander and Eriksson, 1977; Kirk and Chang, 1975; Lindquist *et al.*, 1977; Hatakka and Uusi-Rauva, 1983). However, little is known about the mechanisms and function of the enzymes involved in the degradation process. Almost all studies concerning enzymatic degradation of lignin have utilized relatively low molecular weight model compounds instead of using macromolecular lignin. Three extracellular enzymes cause changes in lignin or lignin model compounds: laccase, lignin peroxidase (LiP) and manganese-dependent peroxidase (MnP) (Kirk and Farell, 1987). *T. versicolor* produces all these enzymes

(Dodson *et al.*, 1986; Waldner *et al.*, 1988; Jönsson *et al.*, 1987; Ishikara, 1980; Johansson and Nyman, 1987), and the same refers to *P. radiata* (Waldner *et al.*, 1988; Hatakka and Tervilä-Wilo, 1985; Niku-Paavola *et al.*, 1989; Hatakka *et al.*, 1989; Niku-Paavola *et al.*, 1988; Lundel *et al.*, 1991). *P. chrysosporium* which has been by far the most popular fungus in lignin biodegradation research, produces only LiP and MnP, not laccase (Kirk and Farell, 1987; Waldner *et al.*, 1988; Glenn *at al.*, 1983; Tien and Kirk, 1983; Paszczyński *et al.*, 1986; Kuwahara *et al.*, 1984).

Although *T. versicolor* is the "oldest" of all these fungi in the history of biological lignin degradation, the occurrence of LiP and MnP in that fungus has been discovered relatively late (Dodson *et al.*, 1986; Waldner *et al.*, 1988; Jönsson *et al.*, 1987; Johansson and Nyman, 1987). Typical for the occurrence of lignin-modifying enzymes in *T. versicolor* seems to be large variability between strains of *T. versicolor* which may reflect inherent heterogeneity of this species. The most popular strains studied by previous researchers probably were less LiP producing strains since they often were screened and optimized for high laccase production. Similarly, culture conditions may have affected the occurrence of these enzymes. For example, the production of laccase of *T. versicolor* is stimulated either by starvation (Grabbe *et al.*, 1967; Haider and Grabbe, 1967) or by some phenolic compounds added to the growth medium (Haars and Huttermann, 1983; Leonowicz and Trojanowski, 1978). Culture conditions are also important for the production of lignin-modifying enzymes by other fungi. The ligninolytic activity of *P. chrysosporium* can only be seen after the primary growth phase when carbon, nitrogen or sulphur limitation occurs (Keyser *et al.*, 1978; Jeffries *et al.*, 1981). Aromatic substances such as veratryl alcohol are added to the medium as stimulators (Leisola *et al.*, 1984; Harvey *et al.*, 1986; Haemmerli *et al.*, 1986). In *P. radiata* veratric acid stimulates the production of laccase, LiP, and MnP (Lundel *et al.*, 1990). Veratryl alcohol, the secondary metabolite involved in lignin degrading systems, increases the production of LiP in *P. chysosporium* (Kirk and Farell, 1987; Leisola *et al.*, 1984) and in *P. radiata* (Hatakka and Tervilä-Wilo, 1985; Niku--Pauvola *et al.*, 1989; Hatakka *et al.*, 1989).

In order to elucidate how much the production of laccase, LiP and MnP depends on the strain, the culture medium and the aromatic compound added, we examined four strains of *Trametes versicolor* from our collection on fungi. Fungi were cultivated under conditions usually adopted for lignin biodegradation studies and using stimulators known to enhance the degradation of lignin. According to many earlier authors *T. versicolor* is considered one of the most efficient degraders of lignin (Crawford, 1981). The aim of this work was to select the most efficient strain of *T. versicolor* on the basis of the production of lignolytic enzymes and also to select the most suitable culture medium for this strain.

Experimental

Materials and Methods

Aromatic compounds. Veratric acid (3,4-dimethoxybenzyl acid) and veratryl alcohol (3,4-dimethoxybenzyl alcohol) were obtained from Fluka (Buchs, Switzerland). Veratryl alcohol was distilled before use according to (Kirk and Farell, 1987).

Organisms. *Coriolus versicolor* (L. ex Fr.) Quel No. 2, *Trametes versicolor* (L. ex Fr.) Pil. No. 7 (ATCC 44308), *Polystictus versicolor* (L. ex Fr.) Fr. No. 9 and *Trametes versicolor* (L. ex Fr.) No. 20 were obtained from the collection of fungi at the Department of Biochemistry, University of M. Curie Skłodowska, Lublin, Poland. The organisms were kept on 2% (wt/vol) malt agar slants according to (Hatakka and Uusi-Rauva, 1983).

Culture conditions. To prepare inoculum, pieces of agar (ca. 0.5 cm^2) were cut and grown in ADMS medium (Hatakka and Uusi-Rauva, 1983) containing 2 mM nitrogen (LN) and 56 nM glucose in nonagitated 250 ml conical flasks on the surface of 50 ml of the medium for 10 days at 28°C. The fully grown mycelial mat was collected and homogenized. The stationary cultures, after inoculation with 4% (vol/vol) homogentae, were cultivated in 100 ml conical flasks each containing 10 ml of ADMS medium with appropriate concentration of nitrogen and glucose at 28°C. At the beginning of the third day of incubation veratric acid or veratryl alcohol were added to the flasks to the final concentration of 1.0 mM.

Enzyme activities. Four parallel culture flasks were daily collected and their culture fluids were separately filtered through Whatman No4. filter paper. A Shimadzu 160A programmable spectrophotometer was used to assay enzyme activities in these culture filtrates. Laccase activity was monitored by the oxidation of syringaldazine (4-hydroxy-3,5-dimethoxybenzaldehyde azine, EGA-Chemie) (Leonowicz and Grzywnowicz, 1981) at 525 nm in 0.1 M citrate-phosphate buffer (Lundell *et al.*, 1990; Lundell *et al.*, 1991). The veratryl alcohol oxidation method (Tien and Kirk, 1984) was used for the determination of LiP activity (Niku-Paavola *et al.*, 1989; Lundell *et al.*, 1990; Lundell *et al.*, 1991). Activity of LiP and laccase are expressed in nanokatals (*i.e.* nanomoles of substrate oxidized in one second) per liter of culture filtrate. MnP activity was assayed by the phenol-red method (Glenn and Gold, 1985) modified by omitting NaOH supplement and measuring the absorbance change at 520 nm for 5 min at 30°C (Lundell *et al.*, 1991). MnP activity is expressed in absorbance change at 520 nm per minute per liter.

Results and Discussion

The basal ADMS-LN medium which is good for lignin degradation by *P. chrysosporium* and *P. radiata* and supports moderate lignin degradation by *Polyporus versicolor* (Hatakka and Uusi-Rauva, 1983), was not suitable for the production of ligninolytic enzymes by *T. versicolor* strain No. 7 (Leonowicz *et al.*, 1991). However, this strain of *T. versicolor* produces both constitutive and inducible types of laccase (Bollag and Leonowicz, 1984). Furthermore, this fungus starts to produce laccase relatively late, not earlier than after two weeks cultivation (Bollab and Leonowicz, 1984; Trojanowski and Leonowicz, 1969). The production of laccase also increases in the presence of an aromatic compound but usually requires low glucose and high nitrogen concentrations in the medium (Bollag and Leonowicz, 1984; Trojanowski

and Leonowicz, 1969), *i.e.* conditions other than those applied in this work. Laccase production (Grabbe *et al.*, 1967) as well as the degradation of ^{14}C-(RING)-lignin of poplar wood to $^{14}CO^2$ and water soluble products by *T. versicolor* (Hatakka and Uusi-Rauva, 1983) are strongly repressed by the adition of glucose or cellulose. This type of inhibition is not maybe the same as the previously described suppresive effect of extra nutrient nitrogen in *P. chrysosporium* (Fenn *et al.*, 1981; Jeffries *et al.*, 1981; Kirk *et al.*, 1978; Buswell *et al.*, 1984).

In this work we studied the influence of 1mM veratric acid and veratryl alcohol supplement on the production of laccase by four strains of *T. versicolor* which were cultivated on the basal ADMS-LN medium under various carbon and nitrogen concentrations (Figs. 1 and 2). Both compounds are non-phenolic and not substrates for laccase. In the case of veratric acid, the basal medium (2mM N, 56 mM glucose, Fig. 1a) allowed laccase production only by strain No. 20. the highest laccase activities, 400-800 nkat l^{-1}, were obtained in ten days by strains No. 20 and No. 2 under different nitrogen or carbon limited conditions (Fig. 1a-g). The strain No. 2 produced the highest laccase activities in a very diluted medium (Fig. 1g), *i.e.* under starvation conditions. On these media other strains, Nos. 7 and 9, showed low laccase activities (Fig. 1a-g).

Veratryl alcohol added to the medium in the same concentration (1mM) as veratric acid was, enhanced laccase production in all four strains of *T. versicolor* (Fig. 2a-g). However, the increased laccase activity occurred under different carbon and nitrogen concentrations in each strain. Two strains, Nos. 9 and 20, produced the highest laccase activities when cultivated in the basal medium (Fig. 2a). The best producer of laccase was strain No. 20 in which laccase activities reached ca. 1000 nkat l^{-1} (Fig. 2a, d,e). Noteworthy was also the early appearance of laccase and two distinct maxima which may indicate the occurrence of two types of laccase in this strain (Fig. 2a). At the lowest nitrogen concentrations and in the most diluted medium, *i.e.* with 0.2 mM N, the strain No. 2 was again superior compared with the other strains which could not produce laccase under low nutrient conditions (Fig. 2f-g). Thus, this strain strongly responded to the starvation conditions (cf. Grabbe *et al.*, 1967; Haider and Grabe, 1967).

Production of other extracellular ligninolytic enzymes, LiP and MnP, by the four strains of *T. versicolor* is shown in Figs. 3–6. LiP activities were relatively low compared with laccase activities and reached not more than 80 nkat l^{-1} (Figs. 3 and 4). Addition of either veratric acid or veratryl alcohol stimulated the production of LiP in almost all strains. However, the stimulating effect also depended on the strain and on the culture medium.

Veratric acid stimulated LiP production provided that there was relatively high concentration of nitrogen present (Fig. 3a-e). When the lowest nitrogen (0.2 mM N) concentrations together with a low carbon source concentration (1.12

mM or 5.6 mM glucose) were applied there was no LiP production at all (Fig. 3f--g). All four strains produced LiP when 1 mM N and 28 mM glucose were used (Fig. 3c). In every case the level of LiP activity remained relatively low (<100 nkat l^{-1}) and veratryl alcohol enhanced LiP production only slightly more than veratric acid did (Figs. 3,4). However, addition of veratryl alcohol stimulated some LiP production also under conditions of the lowest nitrogen and carbon concentrations (Fig. 4f-g), which did not occur when veratric acid was used (Fig. 3f-g). Veratric acid is most probably reduced to veratryl alcohol *via* veratraldehyde (cf. Lundell *et al.*, 1991), and the effect of the medium composition may affect this reduction, for example being dependent on the avilable nutrient nitrogen or glucose i the medium.

The LiP activities in our work were at the similar level (20 nkat l^{-1}) which was obtained by Waldner *et al.* (1986 and 1988) in *T. versicolor* ATCC 42530 when it was cultivated in a medium containing 2.4 mM N, 56 mM glucose, and veratryl alcohol. Other authors studying *Coriolus versicolor* 28-A, PRL (Dodson *et al.*, 1986; Dodson *et al.*, 1987) and *Trametes versicolor* PRL 572 (Jönsson *et al.*, 1987) under conditions of 5 mM nitrogen and 12.3 mM glucose obtained 6 U ml^{-1} and 100 U l^{-1} of LiP activity, respectively, *i.e.* about 600 and 10 nkat l^{-1}. These authors have used much higher amounts of veratryl alcohol (10 mM). These and also our results indicated that the concentrations of nitrogen and glucose together with the type of the added aromatic compound affected LiP production but in all cases the level of LiP activity was very low compared with *P. chrysosporium* (Kirk and Farrell, 1987) and *Phlebia radiata* (Lundell *et al.*, 1991). In our work, all four strains responded to the varying amounts of nitrogen and carbon in a rather similar way compared with the results in laccase production when much larger variations between the strains occurred. Thus, the capacity to produce laccase seemed to be more strain-dependent than LiP production (Figs. 1-4).

The production of MnP by *T. versicolor* is shown in Figs. 5 and 6. When veratric acid was added to the cultures, MnP activity was detected only in two media: traces of activity with 2 mM N and 28 mM glucose (Fig. 5b, strains Nos. 7 and 9) and slightly more with 1 mM N and 5.6 mM glucose (Fig. 5d, strains Nos. 2, 7 and 9). Other media did not support the production of MnP activity at all (Fig. 5). When veratryl alcohol was used MnP activity appeared only with the lowest nitrogen and carbon concentrations (Fig. 6e-g). Under these conditions strains Nos. 2 and 20 showed some MnP activity. Our results agree with those of Waldner *et al.* (1988) for *C. versicolor* 28-A, PRL and Johansson and Nyman (1987) for T. versicolor PRL 572. However, these authors used a very high concentration of veratryl alcohol [10 mM according to (Johansson and Nyman, 1987) as a stimulator which excludes exact comparison with our results.

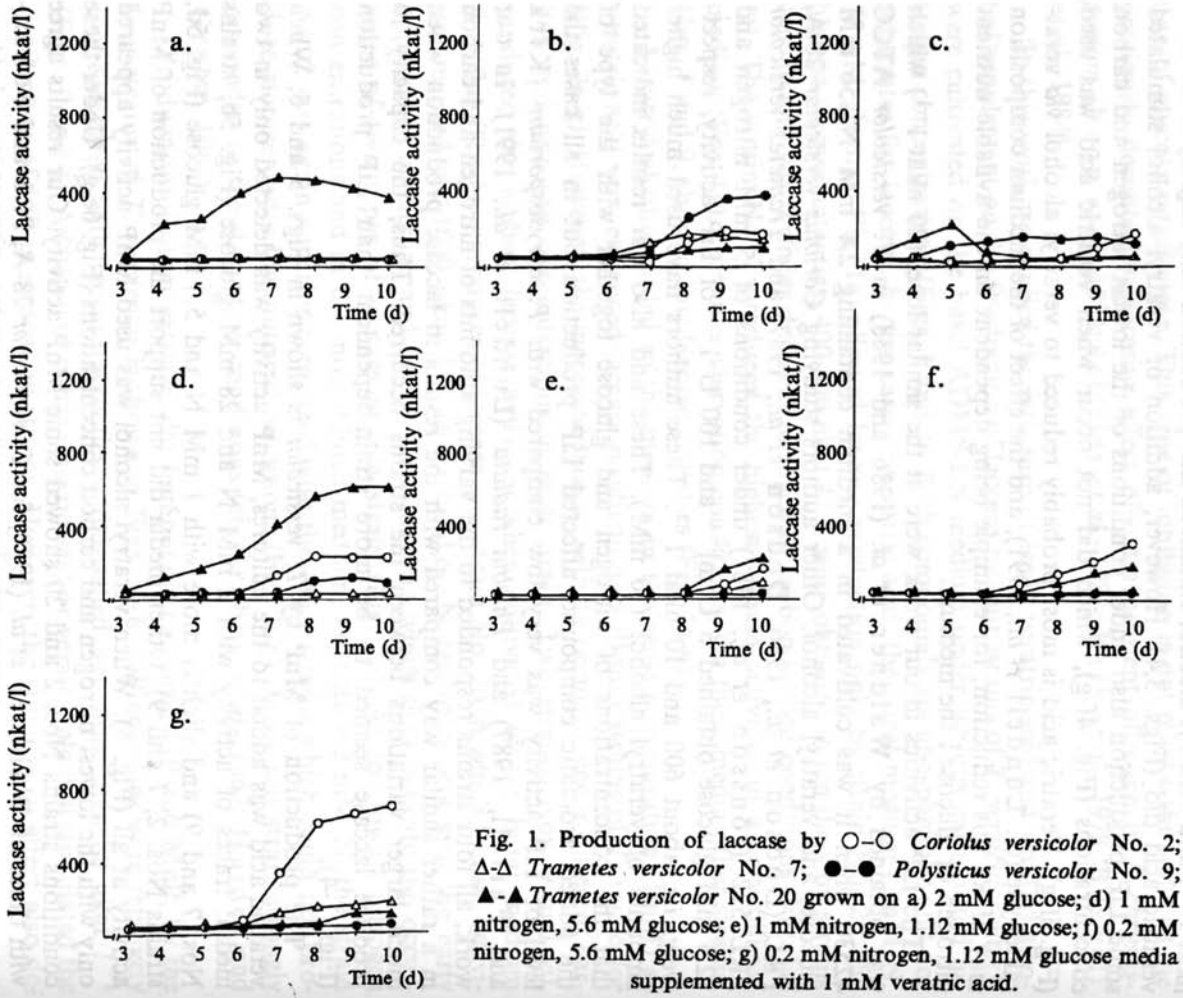

Fig. 1. Production of laccase by ○–○ *Coriolus versicolor* No. 2; Δ-Δ *Trametes versicolor* No. 7; ●–● *Polysticus versicolor* No. 9; ▲-▲ *Trametes versicolor* No. 20 grown on a) 2 mM glucose; d) 1 mM nitrogen, 5.6 mM glucose; e) 1 mM nitrogen, 1.12 mM glucose; f) 0.2 mM nitrogen, 5.6 mM glucose; g) 0.2 mM nitrogen, 1.12 mM glucose media supplemented with 1 mM veratric acid.

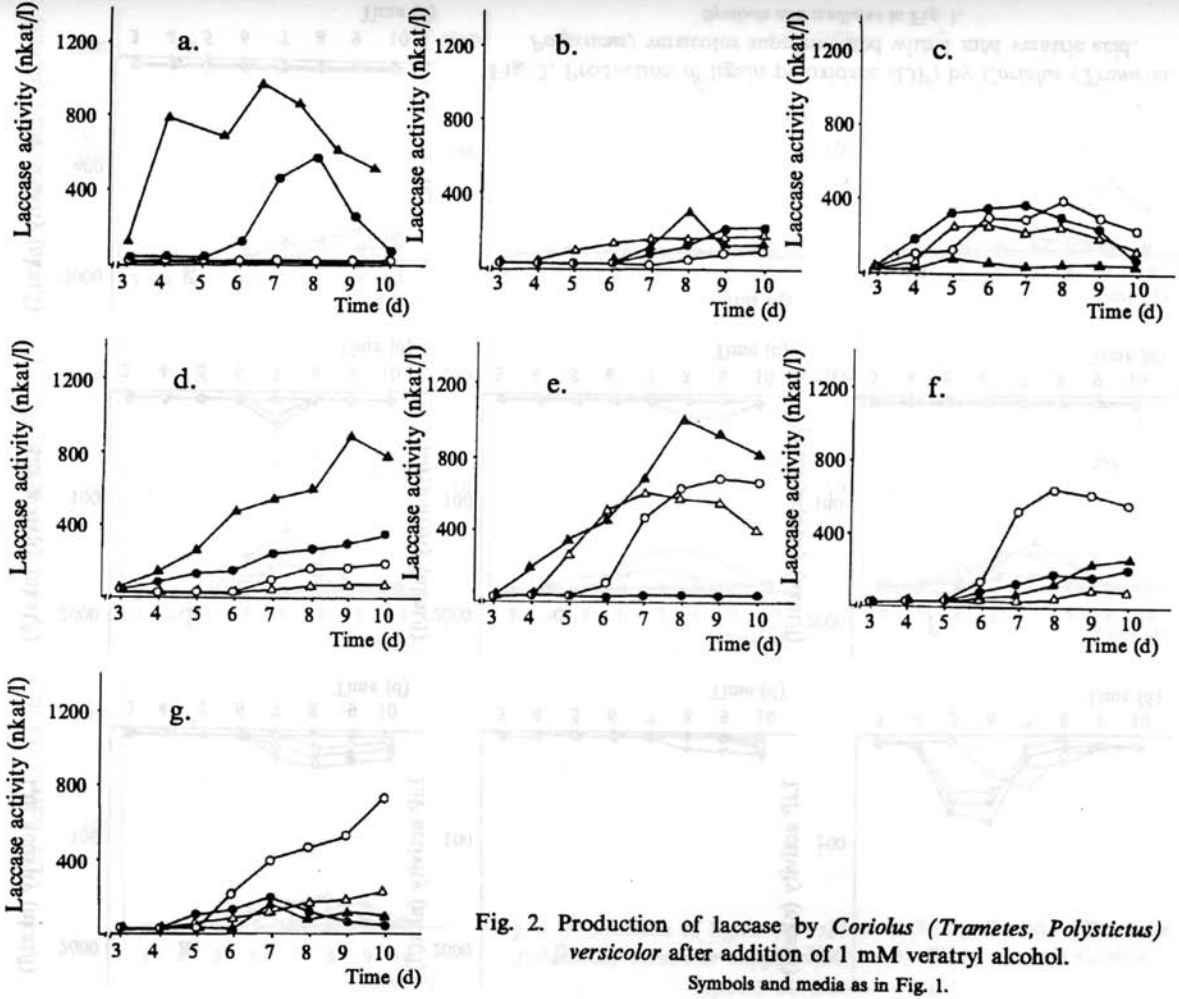

Fig. 2. Production of laccase by *Coriolus (Trametes, Polystictus) versicolor* after addition of 1 mM veratryl alcohol.
Symbols and media as in Fig. 1.

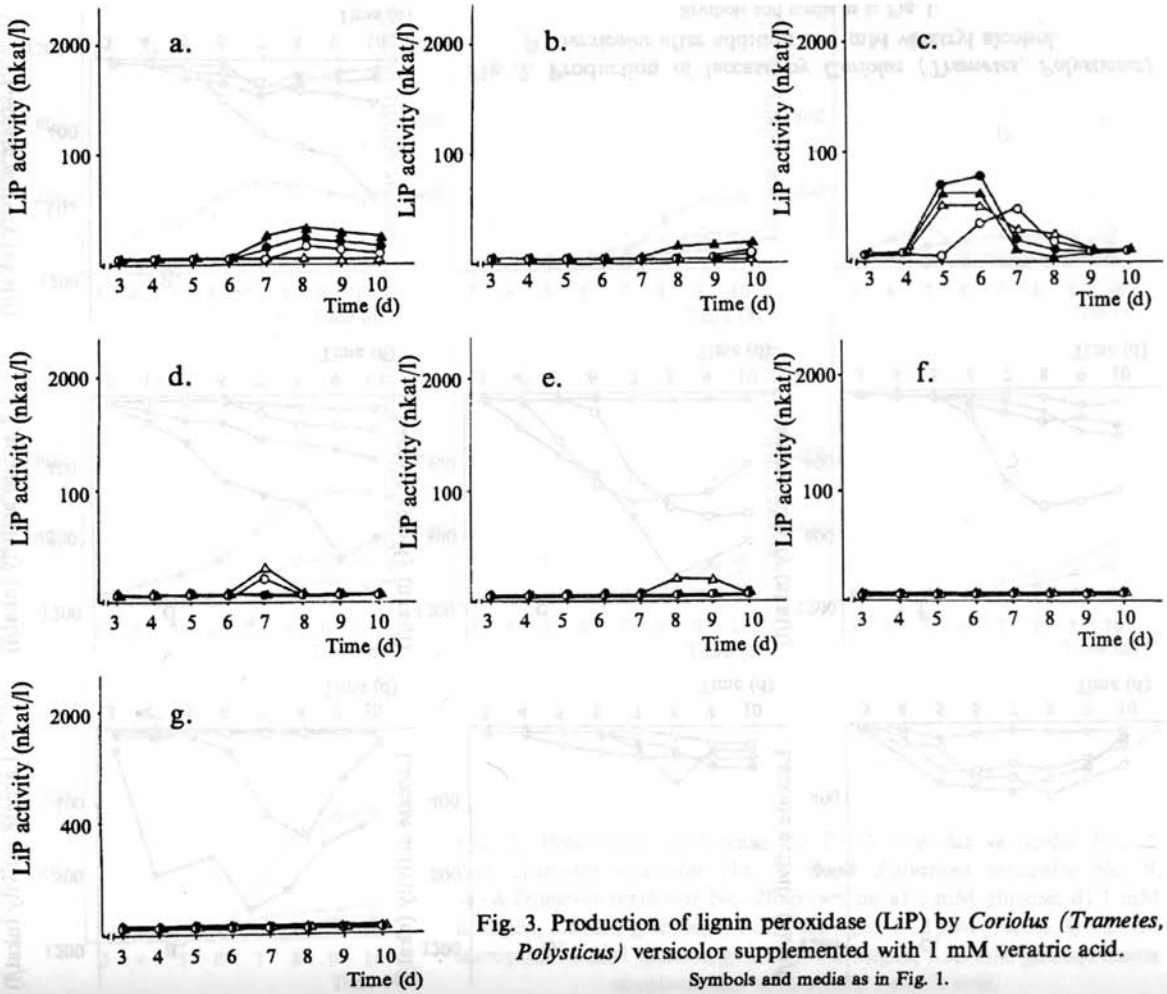

Fig. 3. Production of lignin peroxidase (LiP) by *Coriolus (Trametes, Polysticus)* versicolor supplemented with 1 mM veratric acid.
Symbols and media as in Fig. 1.

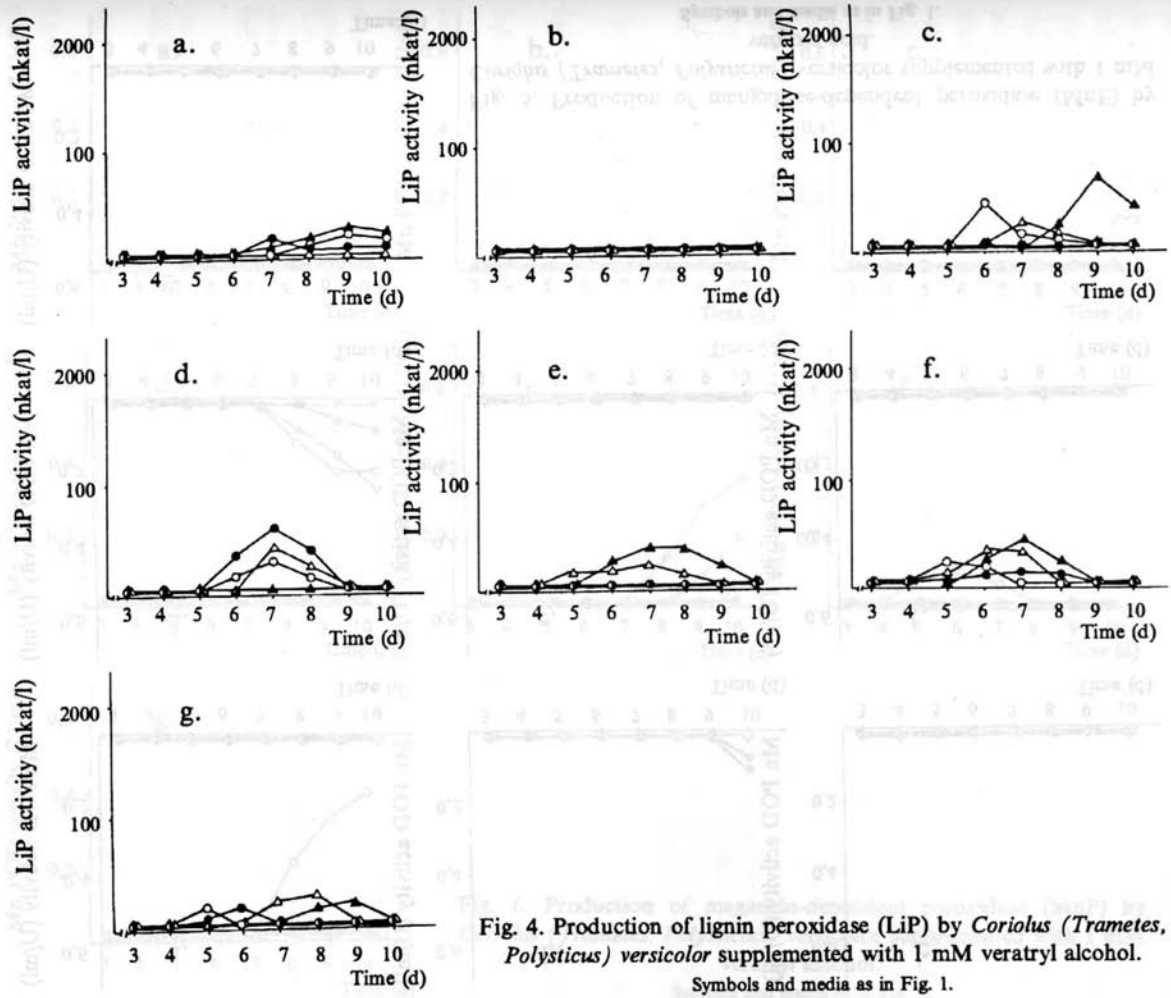

Fig. 4. Production of lignin peroxidase (LiP) by *Coriolus (Trametes, Polysticus) versicolor* supplemented with 1 mM veratryl alcohol.
Symbols and media as in Fig. 1.

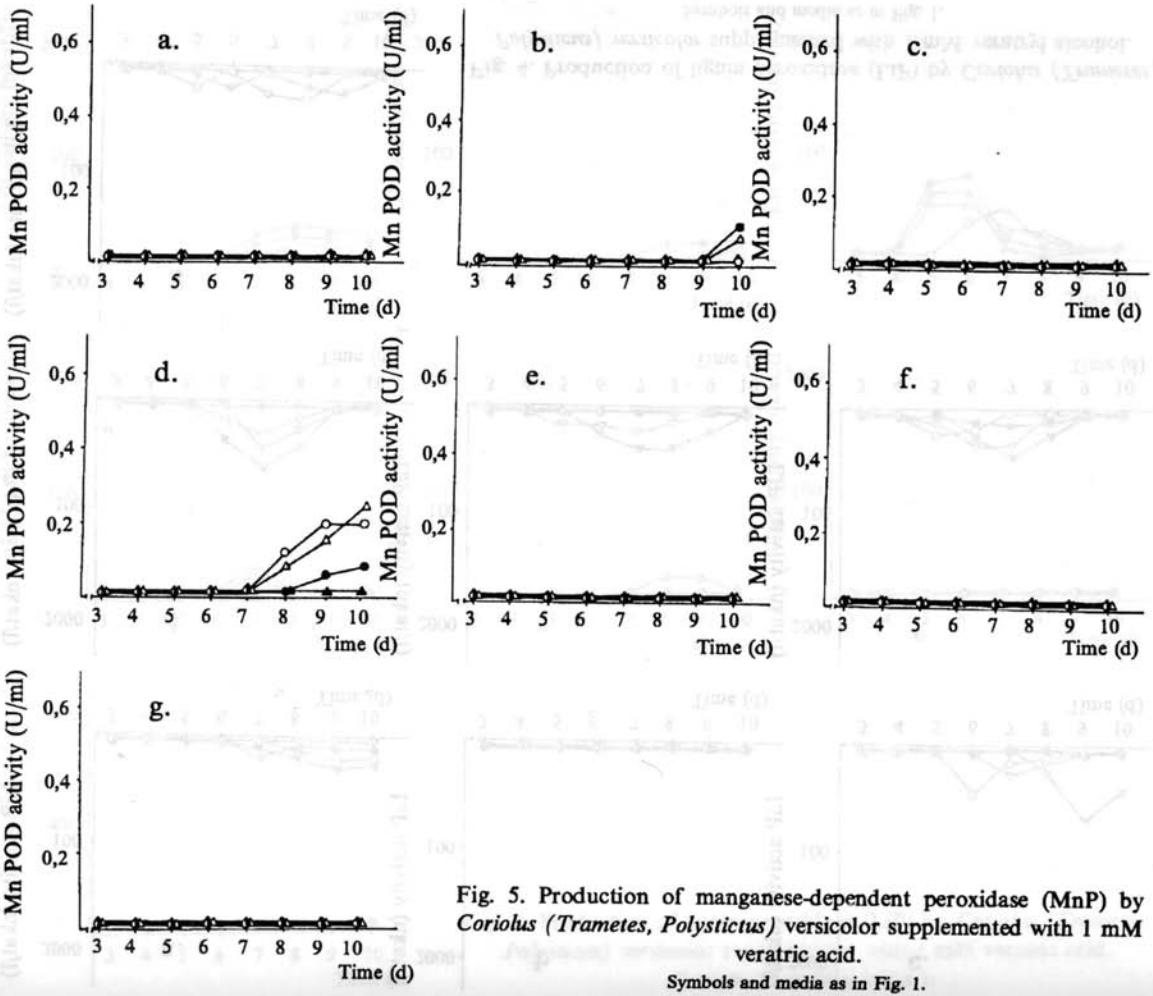

Fig. 5. Production of manganese-dependent peroxidase (MnP) by *Coriolus (Trametes, Polystictus)* versicolor supplemented with 1 mM veratric acid.

Symbols and media as in Fig. 1.

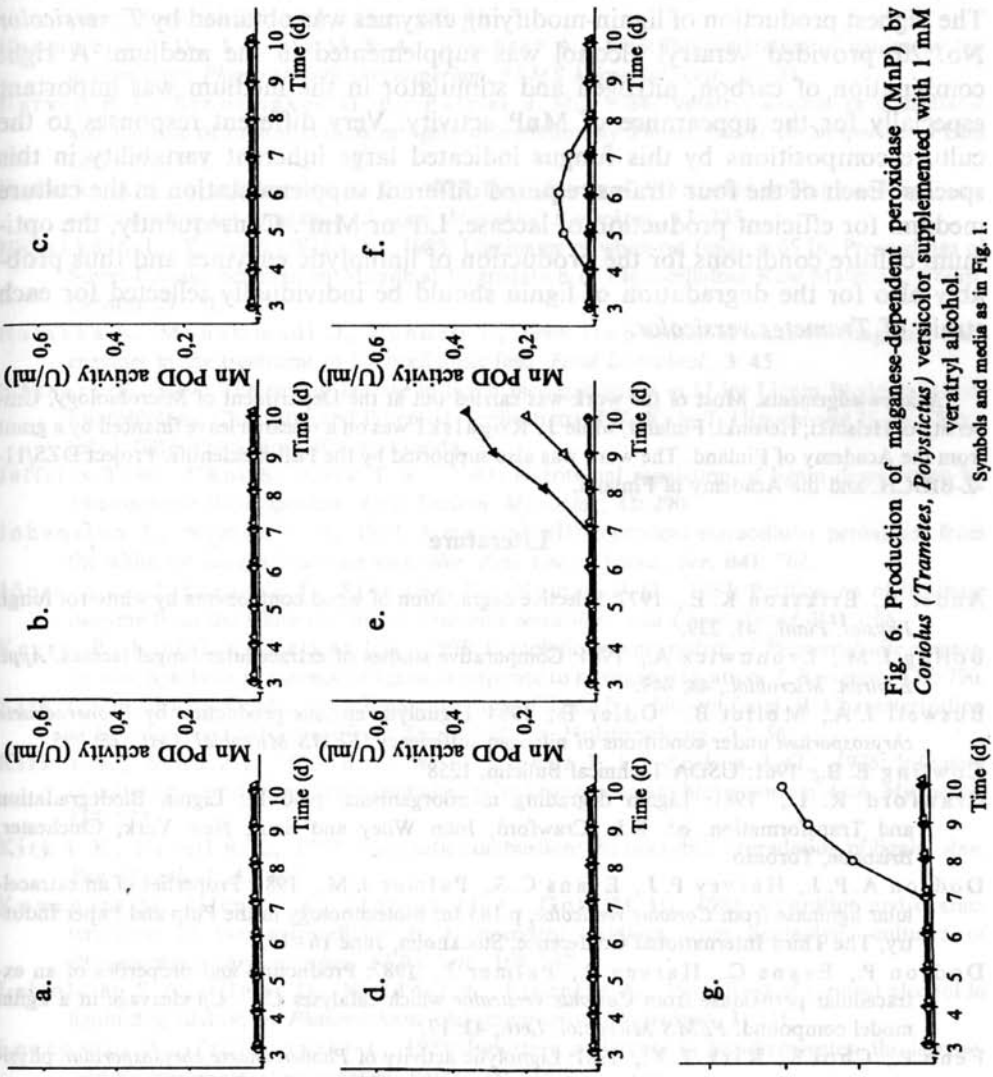

Fig. 6. Production of maganese-dependent peroxidase (MnP) by *Coriolus (Trametes, Polystictus)* versicolor supplemented with 1 mM veratryl alcohol.
Symbols and media as in Fig. 1.

In conclusion, our results show that all four strains of *T. versicolor* produced laccase, lignin peroxidase (LiP) and manganese-dependent peroxida (MnP) when suitable culture conditions were used. Varying amounts of nitrogen and carbon sources and two lignin-related aromatic compounds as stimulators were tested. The highest production of lignin-modifying enzymes was obtained by *T. versicolor* No. 20, provided veratryl alcohol was supplemented in the medium. A right combination of carbon, nitrogen and stimulator in the medium was important especially for the appearance of MnP activity. Very different responses to the culture compositions by this fungus indicated large inherent variability in this species. Each of the four strains required different supplementation in the culture medium for efficient production of laccase, LiP or MnP. Consequently, the optimum culture conditions for the production of lininolytic enzymes and thus probably also for the degradation of lignin should be individually sellected for each strain of *Trametes versicolor*.

Acknowledgements. Most of the work was carried out at the Department of Microbiology, University of Helsinki, Helsinki, Finland, while J. Rogalski was on a research leave financed by a grant from the Academy of Finland. The work was also supported by the Polish Scientific Project DZS/11-Z-BIOCH. and the Academy of Finland.

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