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Effect of vanillin on the production of wood-decomposing enzymes from a wood-rotting fungus, *Coriolus versicolor*

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Abstract To examine the effect of vanillin on the production of the wood-decomposing enzymes of a wood-rotting fungus, vanillin was added as a model of lignin-related phenols to *Coriolus versicolor* cultures containing cellulosic and xylan substrates. Among five conditions tested, cellobiose alone was the most effective inducer of cellulolytic and xylanolytic enzymes. Addition of vanillin enhanced the effect of cellobiose on enzyme production. However, vanillin did not act as greatly in other cultures, except for cellobiose. Analytical isoelectric focusing and active staining of *endo*-β-1,4-glucanase demonstrated that isozyme patterns in the presence of vanillin were the same as those in absence of vanillin, indicating that vanillin does not induce novel isozymes but rather enhances enzyme production. On the other hand, vanillin, which enhanced production of phenol-oxidizing enzymes, was not always determined in all cultures, suggesting that the action of vanillin depends on the kinds of carbohydrates. Therefore, the effect of a monolignol vanillin on enzyme production was associated with coexistent carbohydrates.

Key words Cellulase · *Coriolus versicolor* · Vanillin · Wood-rotting fungus · Xylanase

Introduction

Wood-rotting fungi inhabit wood and degrade wood component polymers. Among them, white-rot fungi can degrade lignin as well as cellulose and hemicelluloses. Lignin, which is one of the plant cell wall components, is a three-dimensional aromatic polymer. Various phenolic acids and aldehydes are produced during biodegradation of lignin by white-rot fungi. Generally, these lignin-related phenols inhibit growth of microorganisms (Martin and Akin 1988; Sharma et al. 1985; Váradi 1972; Vohra et al. 1980). However, there have been several reports about the enhancement of growth of wood-rotting fungi (Inaba et al. 1979, 1980; Kawamura et al. 1983; Shuen and Buswell 1992; Ikegaya et al. 1993) and the acceleration of fruit-body formation of *Lentinus edodes* (Kawamura et al. 1983; Ikegaya and Goto 1988). Based on these reports, lignin-related phenols could have physiological activity on wood-rotting fungi, although the exact mechanism is not known.

Furthermore, phenol-oxidizing enzymes from white-rot fungi, such as laccase and peroxidase, are induced by the presence of lignins or phenols (Fåraeus 1954; Łoberzewski and Trojanowski 1979; Haars and Hüttermann 1983; Faison and Kirk 1985; Sethuraman et al. 1998). This phenomenon has been reported even for botryosphaeriaceous fungi that have ligninolytic activity (Dekker and Barbosa 2001; Vasconcelos et al. 2001). Also, *endo*-1,4-β-glucanase (CMCase) production of *L. edodes* is enhanced by phenolic compounds (Ikegaya et al. 1993), and the production of cellulolytic and xylanolytic enzymes increased depending on the concentration of vanillin in *Coriolus versicolor* (Tsujiyama et al. 2000). These authors reported that lignin-related phenols stimulated enzyme production to accelerate wood decomposition, not only of lignin but also for cellulose and hemicelluloses.

Lignin-related phenols are a possibility as an activator of wood-rotting fungi for effective lignocellulose fermentation, biomass conversion, and increased yields of edible mushrooms. Because these compounds are natural but not synthetic, they would allow development of applications of wood-rotting fungi without environmental pollution or poisonous residues in the mycelium. In this study, to obtain basic information about the role of lignin-related phenols in the production of cellulolytic and xylanolytic enzymes as well as phenol-oxidizing ones, the white-rot fungus *C. versicolor* was incubated in culture containing cellobiose or xylan or both. The effect of vanillin was investigated by assay of enzyme production and zymogram analysis.

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Materials and methods

Organism and incubation methods

An angiosperm-preferential white-rot fungus, *C. versicolor* Quél (L: Fr) (IFO 30340), was obtained from the Institute for Fermentation, Osaka, Japan (IFO).

The basal medium used in this study was according to Kirk et al. (1978), except for the addition of 10 mM ammonium tartrate as a nitrogen source. Carbon sources used in this study were as follows: cellobiose, a water-soluble xylan prepared from Japanese beech (*Fagus crenata* Blume) according to Browning (1967), glucose, and xylose. Concentrations of the carbon source and vanillin were 0.2% (w/v) and 1 mM, respectively. Cellobiose and xylan were combined at 0.1% (w/v) each.

Preincubation was carried out on an agar culture containing the basal medium and 1.0% (w/v) glucose for 1 week. Mycelium was inoculated into 30 ml liquid medium in 100-ml Erlenmeyer flasks using a cork borer (ϕ, 9 mm) and incubated at 28°C at stationary culture conditions. For each set of culture conditions, three or four replicate flasks were prepared.

Mycelium weight

After incubation, the culture medium was filtered with a preweighed filter paper, which was boiled for 5 min to remove agar and substrate sugars. After drying, the filter paper was weighed, and mycelium weight was calculated before and after filtration.

Enzyme assays

Enzyme assays were performed using the culture filtrate. Activities of *endo*-1,4-β-glucanase (CMCase), *endo*-1,4-β-xylanase, β-glucosidase, β-xylosidase, and acetyl esterase activities were assayed as reported previously (Tsujiyama et al. 2000). Phenol-oxidizing enzyme activities, of laccase and Mn peroxidase, were assayed as reported previously (Tsujiyama et al. 1992).

Residual phenolic compound analysis

After incubation, culture media was acidified with 4 N HCl solution and the residual phenolic compounds were extracted with ethyl acetate. Recovered phenolic compounds were dried and trimethylsilylated with *N*,*O*-bis (trimethylsilyl) acetamide (50 μl) at 100°C for 10 min. The sample was analyzed by gas-liquid chromatography (GLC) using a GL Science GC353B gas chromatograph on a DB-5 capillary column (25 m × 0.25 mm i.d.) (Shimadzu, Tokyo, Japan). The GLC conditions were as follows: injection temperature, 280°C; oven temperature, 180°C for 10 min, then programmed at 5°C/min to 250°C and 5 min at 250°C; with a flame ionization detector (FID).

Isoelectric focusing and detection of *endo*-1,4-β-glucanase isozymes

Isoelectric focusing was performed in 5% polyacrylamide gels of 0.4-mm thickness prepared on glass plates with a flatbed apparatus (Bio-Rad, Hercules, CA, USA). Enzyme solutions with and without vanillin containing 6.05 and 5.35 units *endo*-1,4-β-glucanase activity, respectively, were focused for 1.5 h in the pH range 4–6. After electrofocusing, the detection of isozymes was carried out according to MacKenzie and Williams (1984). Isoelectric points of isozymes were estimated using a pI marker kit (Bio-Rad).

Results

Mycelium growth

The dry mycelium weight of *C. versicolor* is shown in Fig. 1. Growth in glucose substrates was greater than that in those with xylose. To examine the effect of vanillin on growth of *C. versicolor*, dry mycelium weight is represented in the same figure. As shown in Fig. 1, although the addition of vanillin slightly accelerated mycelium growth of *C. versicolor* in the cultures containing xylan (136% of the control) on the seventh day, growth was not always enhanced in any culture. Phenolic compounds generally inhibit growth of bacteria (Martin and Akin 1988) and fungi, including some wood-rotting fungi (Sharma et al. 1985; Váradi 1972; Vohra et al. 1980), but vanillin is not toxic for *C. versicolor*.

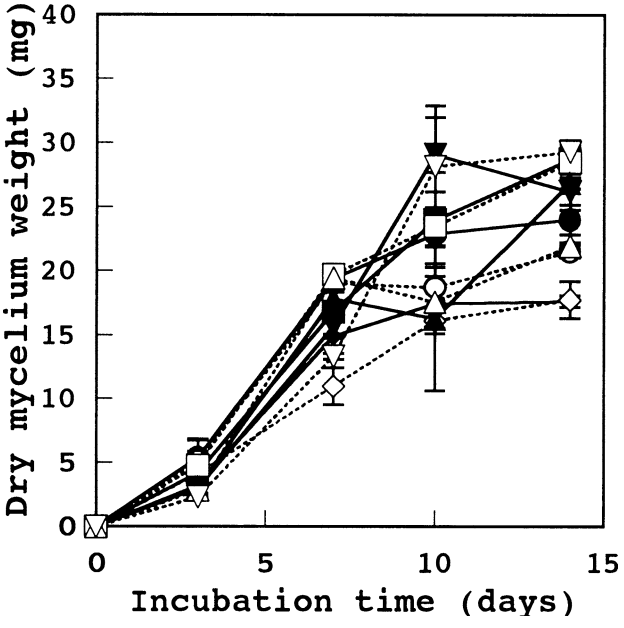

Fig. 1. Dry mycelium weight of *Coriolus versicolor*. ▽, glucose; ▼, glucose + vanillin; △, xylose; ▲, xylose + vanillin; □, cellobiose; ■, cellobiose + vanillin; ○, cellobiose and xylan; ●, cellobiose and xylan + vanillin; ◇, xylan; ◆, xylan + vanillin

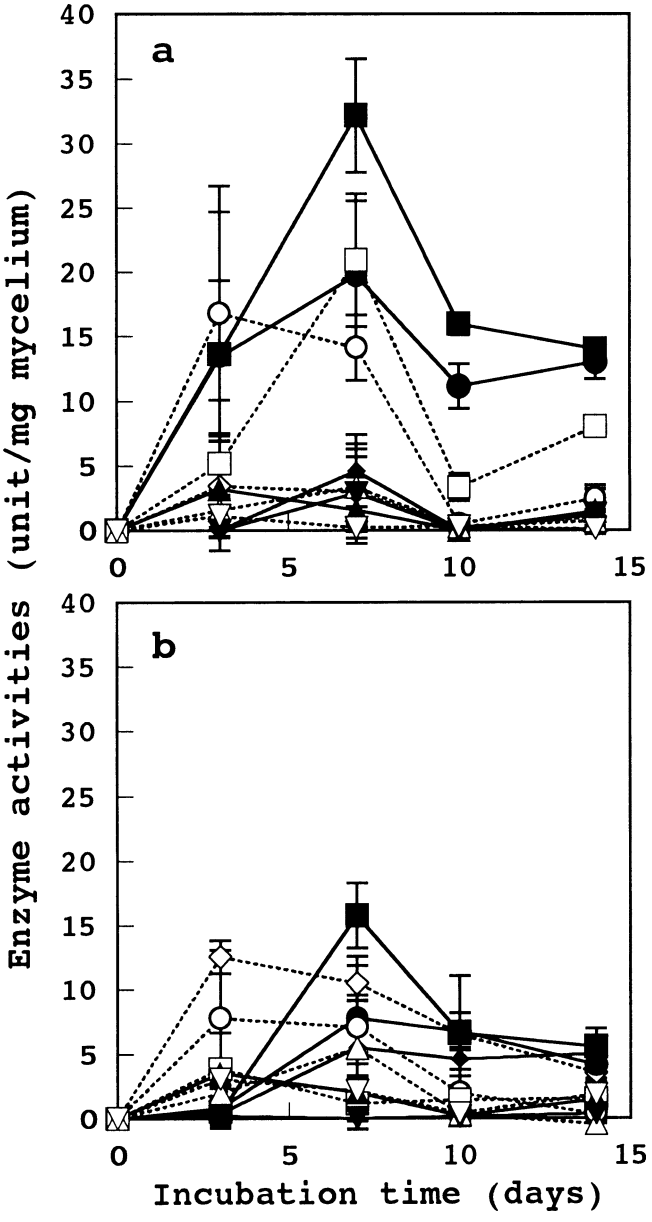

Fig. 2. *endo*-1,4-β-Glucanase (CMCase) and xylanase production by *Coriolus versicolor*. **a** CMCase; **b** *endo*-xylanase (symbols as in Fig. 1)

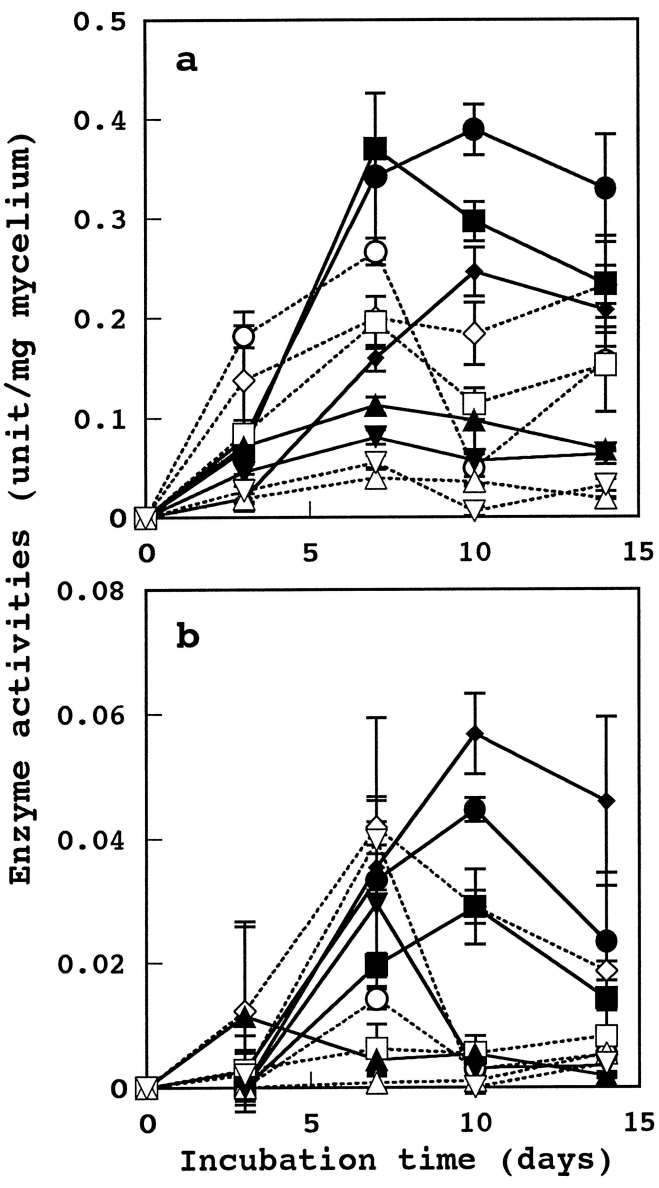

Fig. 3. Glycosidase production by *Coriolus versicolor*. **a** β-Glucosidase, **b** β-xylosidase (symbols as in Fig. 1)

Enzyme production in various cultures

Enzyme activity was described as specific activity per mycelium weight, not as total activity, to clearly represent the effect of vanillin on the fungi. Figure 2 shows the production of CMCase and endo-xylanase by *C. versicolor*. Greater activities of CMCase were produced in the cultures containing cellobiose than in any other culture (Fig. 2a). In the cellobiose cultures, addition of vanillin enhanced CMCase production to a level 154% of the control without vanillin on day 7, and thereafter maintained enzyme production higher than that of the control. In cellobiose-xylan culture, the effect of vanillin was not observed at the beginning of incubation, but enzyme production continued with the addition of vanillin after 10 days. Extracellular xylanase production was determined in the culture containing cellobiose or xylan (Fig. 2b). In the xylan culture, xylanase production started within 3 days but was inhibited in the presence of vanillin. However, in the cellobiose culture, production of xylanase was markedly enhanced by the addition of vanillin. This tendency was also observed in cellobiose-xylan culture; xylanase activity at the culture without vanillin was higher than that with vanillin at the beginning of incubation, but after 10 days xylanase production continued in the presence of vanillin. This result probably occurred because xylanase was initially induced by xylan, but thereafter its production was enhanced by combination with cellobiose and vanillin. Thus, both CMCase and xylanase activities were markedly enhanced by addition of vanillin to the cellobiose-containing cultures.

β-Glucosidase activity was greater in the cultures containing cellobiose or xylan or both (Fig. 3a). The effect of

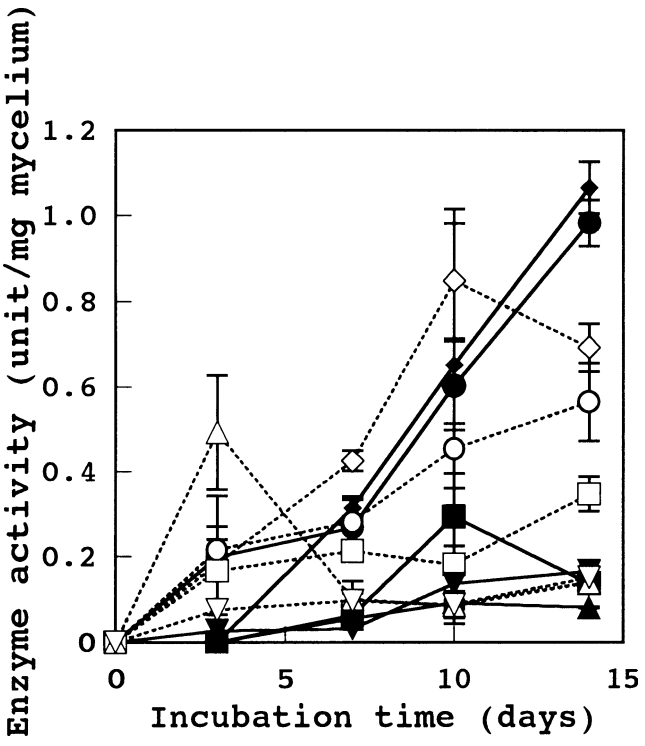

Fig. 4. Acetyl esterase production by *Coriolus versicolor* (symbols as in Fig. 1)

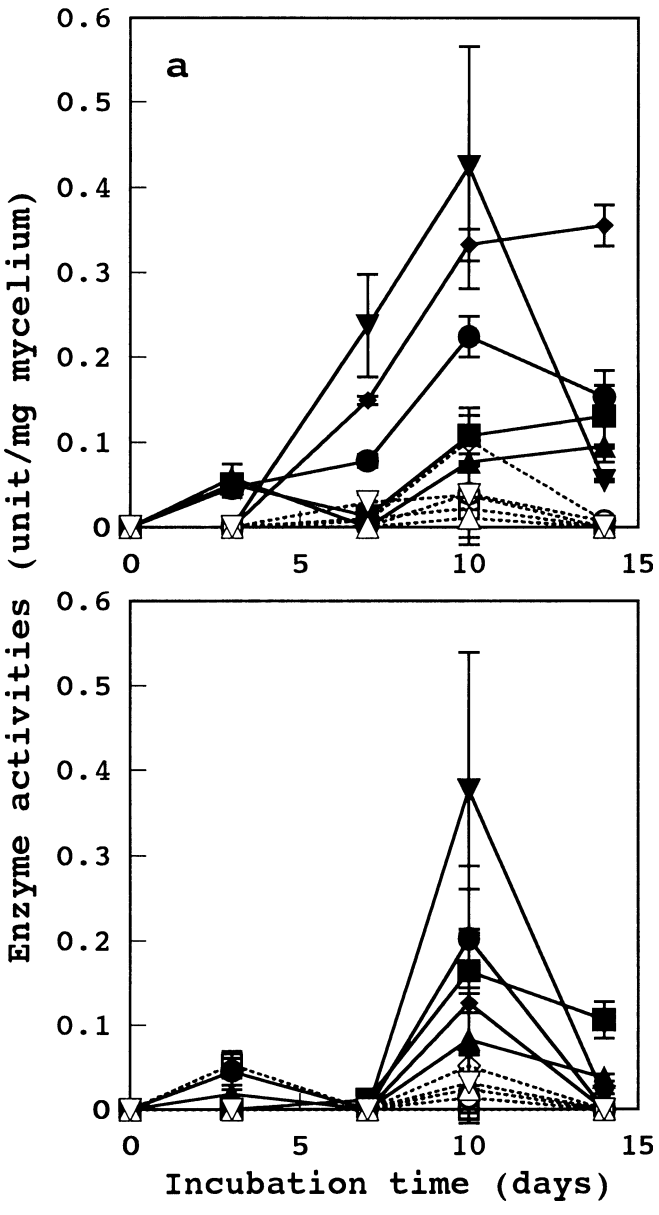

Fig. 5. Phenol-oxidizing enzyme production by *Coriolus versicolor*. **a** Laccase; **b** Mn-dependent peroxidase (symbols as in Fig. 1)

vanillin was observed in the cellobiose-containing cultures (cellobiose alone and combination with cellobiose and xylan). In both cultures, β-glucosidase production increased on the seventh day and continued at the higher level until 14 days with the addition of vanillin compared to the absence of vanillin. On the other hand, vanillin did not remarkably enhance β-glucosidase production at the beginning of incubation, although β-glucosidase production in xylan culture was more active than in those with monosaccharides. This result suggests that vanillin can enhance β-glucosidase activity by combination with cellobiose. Extracellular β-xylosidase production was notably lower than that of β-glucosidase, but relatively higher than that in xylan-containing cultures (Fig. 3b). Enhancement of enzyme production by vanillin was noted in the culture containing xylan, and also in the cellobiose culture.

Acetyl esterase, one of the xylanolytic enzymes, was detected at a high level in the cultures containing xylan during incubation and in the xylose culture at 3 days (Fig. 4). However, enhancement of its production by vanillin was observed only in the cellobiose-xylan culture. In the cultures containing xylan or cellobiose alone, its effect was not clear. A synergetic effect occurred with the combination of cellobiose and xylan.

Activity of the phenol-oxidizing enzymes, laccase and Mn-dependent peroxidase, was detected (Fig. 5). Both enzymes, especially laccase, were produced in greater amounts by the addition of vanillin than in the control culture. In earlier reports, lignin and phenolic compounds enhanced production of these enzymes in white-rot fungi (Fåraeus 1954; Łoberzewski and Trojanowski 1979; Haars and Hüttermann 1983; Faison and Kirk 1985; Sethuraman et al. 1998). In this incubation test, vanillin induced laccase and Mn peroxidase of *C. versicolor* in all culture conditions, but did not have as great effect in cellobiose-containing cultures (Fig. 5a). Rather, laccase (Fig. 5a) production was higher in xylan-containing cultures than that in cellobiose-containing cultures, and Mn peroxidase was very high in glucose culture with vanillin. Probably cellobiose would disturb the effect of vanillin on phenol-oxidizing enzyme production or promote the metabolism of vanillin before vanillin could act.

Cellulolytic and xylanolytic enzymes from *C. versicolor* were induced by cellobiose and xylan, not by monosaccharides such as glucose and xylose. However, the production of these enzymes was enhanced by the addition of vanillin,

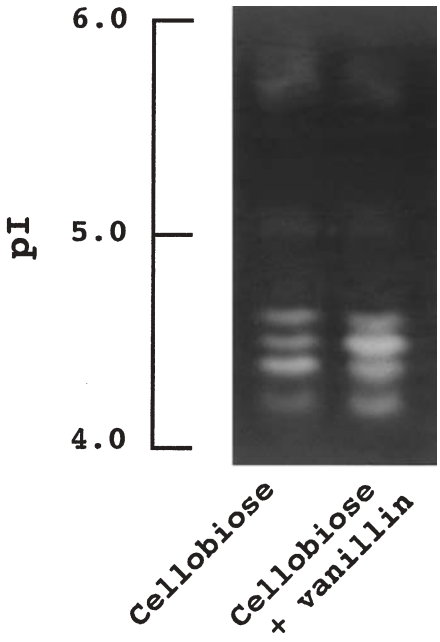

Fig. 6. Isozyme patterns of endo-1,4-β-glucanase from *Coriolus versicolor* after 2 weeks

as was that of phenol-oxidizing enzymes. Note that this function of vanillin was markedly observed in the cellobiose-containing cultures, in which the activity of phenol-oxidizing enzymes was not so high. Thus, from these results, the combination of vanillin and cellobiose could function effectively in the production of cellulolytic and xylanolytic enzymes.

Isozyme pattern of endo-β-glucanase

To examine the role of vanillin on cellulolytic enzyme production, the isozyme pattern of endo-β-glucanase was assayed with isoelectric focusing and active staining. After 2 weeks incubation, when total enzyme activity was 2.80 times higher in the presence of vanillin than in the absence of vanillin, isozyme patterns of endo-β-1,4-glucanase were determined (Fig. 6). In this study, no isozyme was observed at the range of pI 6–10, so analysis was performed at pI 4–6. In Fig. 6, lanes 1 and 2 showed similar isozyme patterns; four bands were observed in the acidic region and one band was detected at around pI 6. This result shows that the addition of vanillin did not induce any novel isozyme but enhanced the quantitative production of endo-β-glucanase (see Fig. 2a). As cellobiose is an inducible substrate of endo-β-glucanase of this fungus, the addition of vanillin enhanced the effect of induction by cellobiose.

Discussion

Many researchers have reported the effect of lignin-like phenols on phenol-oxidizing enzyme production. This study also showed that a monolignol vanillin could induce phenol-oxidizing enzymes, and furthermore demonstrated that it enhanced the production of cellulolytic and xylanolytic enzymes in the presence of cellobiose or xylan or both. Although vanillin has been reported to enhance the cellulolytic and xylanolytic enzyme production of *C. versicolor* depending on its concentration (Tsujiyama et al. 2000), this phenomenon was not observed in the cultures containing monosaccharides, indicating that coexistent carbohydrates participated in enzyme production.

It remains a question whether vanillin itself or its metabolites acted. From the results of GC analysis, vanillin added in culture decreased to 2.22% on the third day and to only 0.6% on the seventh day. Vanillyl alcohol and vanillic acid, which were formed during metabolism, might act on enzyme production. However, the amount of vanillyl alcohol reached a maximum on the tenth day but was only 0.14% of the initial amount of vanillin, and vanillic acid was not detected during incubation. In the previous study, enzyme production of *C. versicolor* was investigated during the degradation of lignin–carbohydrate complex (LCC). Enzyme production was active after 10 days incubation, although released monolignols were metabolized completely within 7 days (Tsujiyama et al. 1992; unpublished data). Thus, *C. versicolor* metabolized or modified released monolignols immediately but produced various wood-decomposing enzymes thereafter. The retardation of enzyme production after addition of phenols was also observed in *L. edodes* (Ikegaya et al. 1993). These results suggest that a small amount of released phenols did not act on fungi directly but were converted into other compounds to cause enzyme production. In this study, enhancement of cellulolytic and xylanolytic enzyme production was more markedly observed in cellobiose and polysaccharides than in monosaccharides. The results suggest that these cellobiose and polysaccharides participated in the enzyme production accompanied with vanillin. In earlier reports about *C. versicolor*, Westermark and Eriksson (1974), Kondo and Imamura (1988), and Kondo et al. (1990) showed that the products, cellobiose-δ-lactone and vanillyl β-D-glucoside, were formed during incubation with vanillin and cellobiose. These products would be the key inducer of wood-decomposing enzymes, and another modified materials might be an activator. Further investigation is required to examine not only the direct effect of monolignols, such as vanillin, but also the products in the coexistence of oligo- and polysaccharides.

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