

Influence of vanillin on the production of cellulolytic and xylanolytic enzymes from a wood-rotting fungus, *Coriolus versicolor*

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To examine the influence of a phenolic compound on the production of cellulolytic and xylanolytic enzymes of a wood-rotting fungus *Coriolus versicolor*, a two-dimensional map of enzyme activity was constructed with various concentrations of cellobiose and vanillin. The productions of CMCase, xylanase, β -glucosidase, and β -xylosidase increased with higher cellobiose concentration and were markedly enhanced by addition of vanillin. Higher ratio of vanillin/cellobiose activated the production of these enzymes. Only acetyl esterase, which is not actively produced at the ligninolytic stage of *C. versicolor*, was inhibited by the monolignol vanillin. As the presence of vanillin is considered to approximate conditions of wood decay more closely than its absence, the present result demonstrates that addition of vanillin, a phenolic compound, enhanced the production of cellulolytic and xylanolytic enzymes for wood cell wall degradation.

Key Words—cellulase; *Coriolus versicolor*; induction; vanillin; xylanase.

Wood-rotting fungi can degrade the wood component polymers, such as cellulose, hemicellulose and lignin, and are the focus of studies on the utilization of lignocellulose resources. Highly active cellulolytic and hemicellulolytic enzymes produced by wood-rotting fungi have been studied from various aspects. However, the production of these enzymes has been investigated only on a single substrate, such as cellulose or xylan. Considering that wood-rotting fungi inhabit wood, which is a complex of cellulose, hemicellulose and lignin, the investigation of wood-rotting fungi on a single substrate is not suitable for examining the essential physiology of fungi. In particular, it is unclear what effect lignin, a polyphenol component of wood cell wall, has on wood-rotting fungi. It is known that lignin-like phenolic compounds induce ligninolytic enzyme production in white-rot fungi (Fåraeus, 1954; Łoberzewski and Trojanowski, 1979; Haars and Hüttermann, 1983; Faison and Kirk, 1985). On the other hand, these phenolic compounds can inhibit or reduce the production of cellulolytic and hemicellulolytic enzymes in various microorganisms (Vohra et al., 1980; Sharma et al., 1985; Martin and Akin, 1988). Phenolic compounds generally inhibit the growth of these microorganisms. But white-rot fungi can detoxify these phenolics by phenol-oxidizing enzymes, and their growth is hardly inhibited. Furthermore, phenolic compounds are generally present in the process of wood decay being produced in large amounts during lignin degradation by ligninolytic enzymes. In this study, to estimate the action of wood-rotting fungi during wood decay, the influence of addition of a monolignol, vanillin, was exa-

mined on the production of cellulolytic and hemicellulolytic enzymes.

Materials and Methods

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

Basal medium used in this study was according to Kirk et al. (1978) except for the addition of 10 mM ammonium tartrate as N-source. Incubation was carried out in a 30 ml of liquid medium of 100-ml Erlenmeyer flasks at 28°C under stationary condition.

Screening of substrates inducing cellulolytic and xylanolytic enzymes Substrates inducing cellulolytic and xylanolytic enzymes were screened by the addition of 0.01% of various carbon sources in liquid culture. Carbon sources acting as inductive substrates were as follows: glucose, cellobiose, cellooligomer prepared with H₂SO₄ according to Pereira et al. (1988), FUNACEL-SF (Funakoshi), carboxymethyl cellulose sodium salt (CMC-Na) (Nakalai tesq.), hydroxyethyl cellulose (Nakalai tesq.), soluble starch, xylose, xylooligomer prepared from Japanese beech (*Fagus crenata* Blume) xylan according to Roy and Timell (1968), xylan prepared from Japanese beech (*F. crenata*).

Influence of vanillin on the enzyme production Vanillin was added into a liquid culture containing cellobiose as an induction substrate. Cellobiose concentration was 0.01, 0.05, 0.1, and 0.2% (w/v), and that of vanillin was

0, 1, 10, 100, and 1000 μM .

Enzyme assays After incubation for 2 wk, enzyme assays were performed by using culture filtrate.

Avicelase, endo-1,4- β -glucanase (CMCase), and endo-1,4- β -xylanase activities were assayed by using microcrystalline cellulose FUNACEL-SF, CMC-Na, and alkali-extracted xylan from a Japanese beech (*F. crenata*) as substrates, respectively. Enzyme activities were measured by determining the amounts of reducing ends formed by the Somogyi-Nelson method as reported previously (Tsujiyama and Nakano, 1996). One unit of enzyme activity is defined as the amount of enzyme that releases 1 μmol of reducing sugar (expressed as glucose) per hour.

Glycosidase activities (β -D-glucopyranosidase and β -D-xylopyranosidase) were assayed by using *p*-nitrophenyl glycoside substrates as reported previously (Tsujiyama et al., 1992). One unit of enzyme activity is defined as the amount of enzyme that releases 1 μmol of *p*-nitrophenol per min.

Acetyl esterase activity was measured spectrophotometrically using *p*-nitrophenyl acetate according to Biely (1985). One unit of enzyme activity is defined as the amount of enzyme that releases 1 μmol of *p*-nitrophenol per second.

Results and Discussion

Screening of substrates inducing cellulolytic and xylanolytic enzymes Substrates were tested at 0.01% (w/v) concentration to avoid the influence of byproducts. Cellobiose, celooligomer, and FUNACEL induced CMCase and β -glucosidase, while none of the substrates induced Avicelase at detectable levels (data not shown). In par-

ticular, cellobiose markedly induced CMCase production. Among xylanolytic enzymes, endo-xylanase was induced by cellobiose, celooligomer, and xylooligomer. From this result, cellobiose was used as a substrate for the induction of cellulolytic and xylanolytic enzymes to examine the influence of vanillin.

Influence of vanillin on mycelial growth Dry mycelium weight is shown in Fig. 1. It is clear that mycelium weight depended on the concentration of cellobiose; it increased as the concentration of cellobiose was increased. Vanillin concentration had little influence on

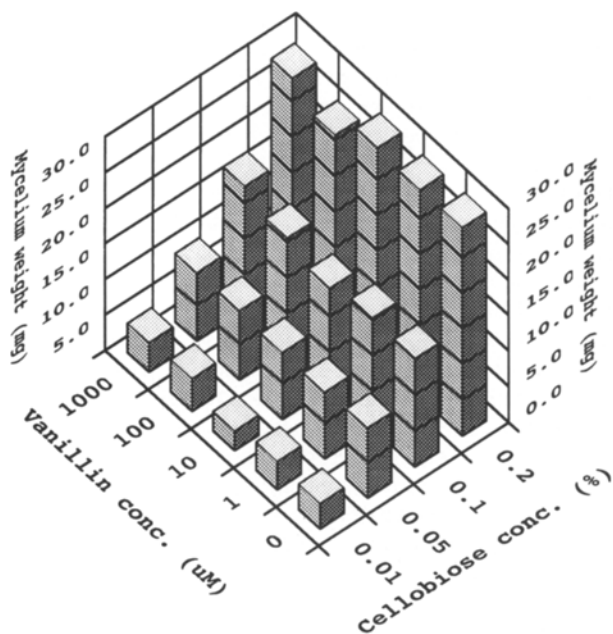


Fig. 1. Effects of cellobiose and vanillin on dry mycelium weight of *Coriolus versicolor* after 2 wk.

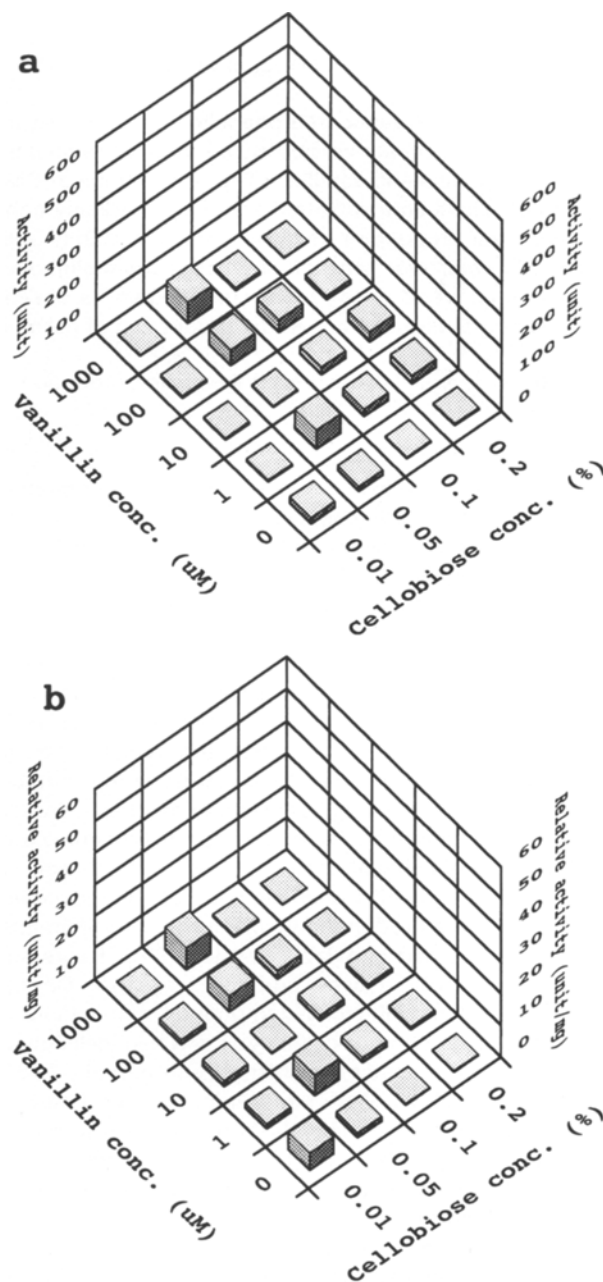


Fig. 2. Effects of cellobiose and vanillin on Avicelase production by *Coriolus versicolor* after 2 wk. a: Total activity, b: Relative activity.

the mycelial growth.

Influence of vanillin on the enzyme production Figures 2-4 show the production of cellulases (Avicelase and CMCase) and endo-xylanase from *C. versicolor* after 2 wk. Avicelase was not induced significantly by cellobiose or vanillin (Fig. 2). As Avicelase was not detected in the screening test culture containing other cellulosic and xylanic substrates, this enzyme might be not extracellular but cell-associated. Productions of CMCase and xylanase were induced by increasing cellobiose concentration, and were enhanced by the in-

crease of vanillin concentration (Figs. 3a, 4a). However, from the activity per unit mycelium weight was not greatly enhanced by cellobiose, whereas addition of vanillin had a greater effect on enzyme production (Figs. 3b, 4b). At the same concentration level of cellobiose, higher concentration of vanillin accelerated production of these enzymes. Furthermore, Figs. 3b, 4b demonstrate that a high ratio of vanillin/cellobiose brought about high enzyme production and seemed to be inductive.

Figures 5 and 6 show the production of glycosidases (β -glucosidase and β -xylosidase), which revealed similar

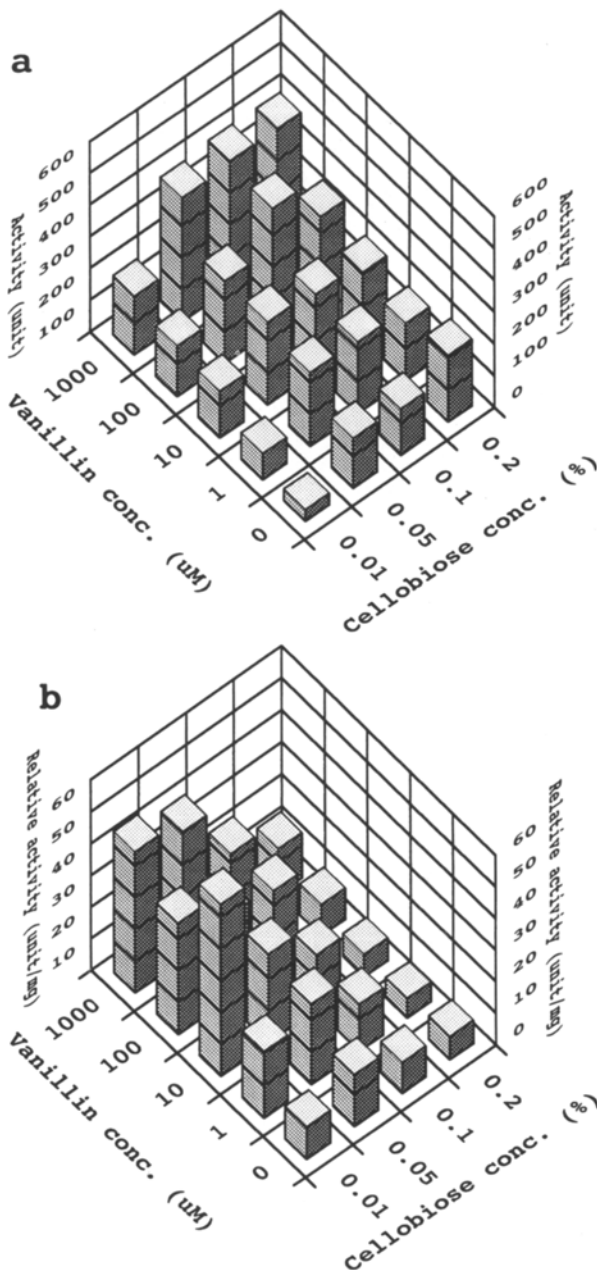


Fig. 3. Effects of cellobiose and vanillin on CMCase production by *Coriolus versicolor* after 2 wk. a: Total activity, b: Relative activity.

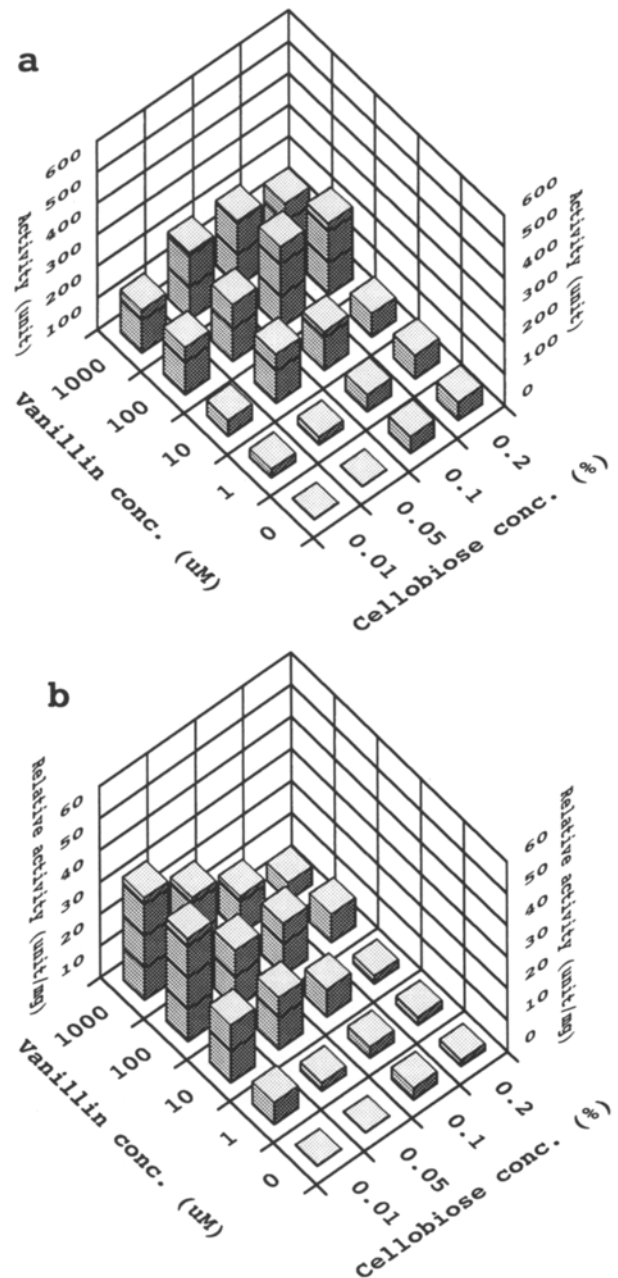


Fig. 4. Effects of cellobiose and vanillin on endo-xylanase production by *Coriolus versicolor* after 2 wk. a: Total activity, b: Relative activity.

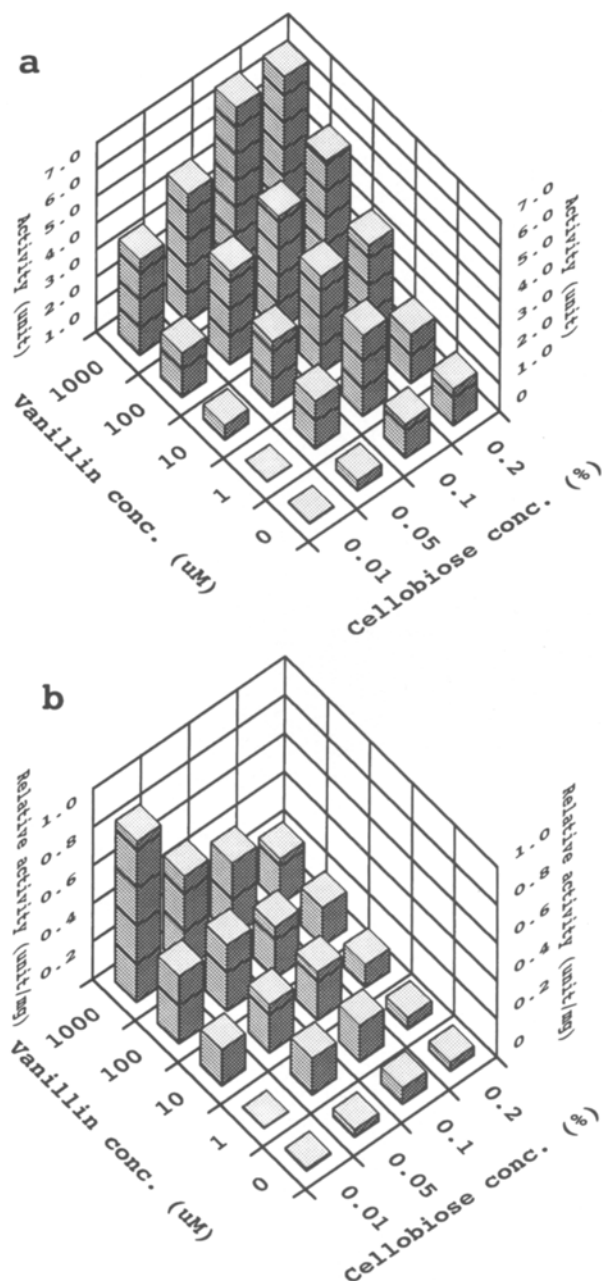


Fig. 5. Effects of cellobiose and vanillin on β -glucosidase production by *Coriolus versicolor* after 2 wk. a: Total activity, b: Relative activity.

patterns to those of CMCase and xylanase. From Figs. 5a and 6a, enzyme production was also induced by cellobiose and further enhanced by addition of vanillin. Enzyme productions per unit weight of mycelium increased with higher ratio of vanillin/cellobiose (Figs. 5b, 6b), like those of CMCase and xylanase.

In earlier reports on the effect of phenolic compounds on the production of cellulolytic and hemicellulolytic enzymes (Vohra et al., 1980; Sharma et al., 1985; Martin and Akin, 1988; Ikegaya et al., 1993), remarkable enhancement was only in the case of Shiitake [*Lentinus*

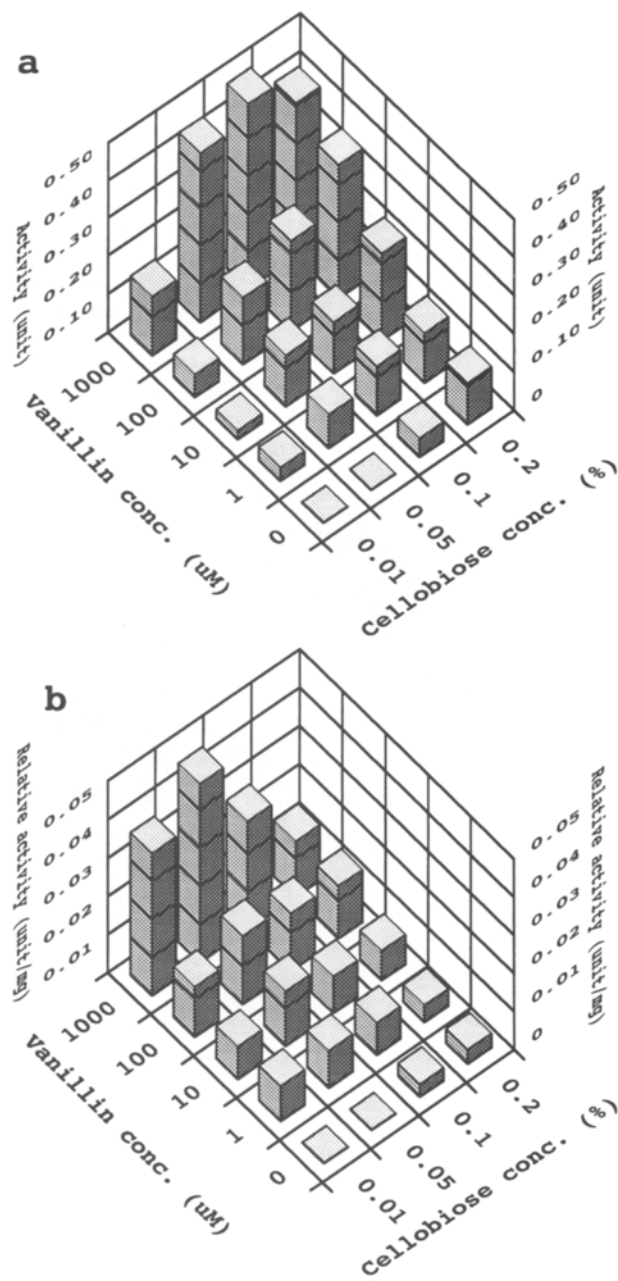


Fig. 6. Effects of cellobiose and vanillin on β -xylosidase production by *Coriolus versicolor* after 2 wk. a: Total activity, b: Relative activity.

edodes (Berk.) Pegler], which produced highly active CMCase in the presence of some phenolic compounds (Ikegaya et al., 1993). The present result shows that addition of phenolic compounds enhanced production of cellulolytic and xylanolytic enzymes, not only CMCase. The acceleration of enzyme production by phenolic compounds may be characteristic of wood-rotting fungi.

The enhancement of cellulolytic and xylanolytic enzyme productions by phenolic compounds would be for the decomposition of wood cell wall. Wood cell wall is composed of cellulose, hemicellulose, and lignin, so cel-

lulolytic and xylanolytic enzymes are essential to increase the accessibility of ligninolytic enzymes to lignin. As *C. versicolor* produced cellulolytic and xylanolytic enzymes actively at the ligninolytic stage (Tsujiyama et al., 1998), *C. versicolor* is deduced to be stimulated by addition of vanillin to produce these enzymes for lignin degradation.

Acetyl esterase is one of the components in the xylanolytic system (Biely et al., 1986). Acetyl esterase production was also enhanced as cellobiose concentration increased (Fig. 7a). However, in the contrast to the

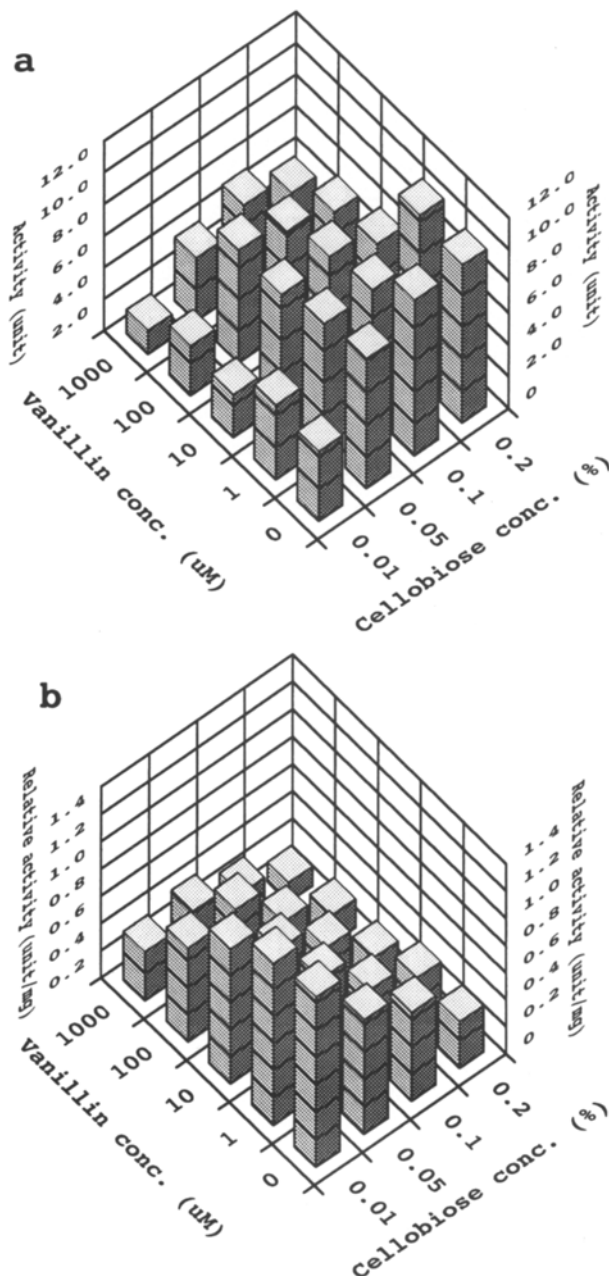


Fig. 7. Effects of cellobiose and vanillin on acetyl esterase production by *Coriolus versicolor* after 2 wk. **a**: Total activity, **b**: Relative activity.

above results, esterase production was inhibited by the addition of vanillin. This indicates that acetyl esterase production was reduced in the presence of lignin monomer. Activity per unit weight of mycelium demonstrates that cellobiose does not induce acetyl esterase, and that vanillin inhibited the acetyl esterase production (Fig. 7b). As reported previously (Tsujiyama et al., 1998), wood decay by *C. versicolor* involved two stages, and acetyl esterase production was not active in the ligninolytic stage. The present result demonstrates that addition of vanillin inhibited acetyl esterase production, suggesting that acetyl esterase, which participates in xylan degradation, would not be produced actively in the presence of phenolic compounds, similarly to the ligninolytic stage.

During decay of wood by white-rot fungi, formation of low-molecular weight phenolic compounds is inevitable. The reason why ligninolytic enzymes can be induced by these phenolics is that these enzymes detoxify the phenolic compounds by polymerization or decomposition. Furthermore, in the presence of cellobiose, *C. versicolor* can detoxify phenolics by cellobiose:quinone oxidoreductase (Westermarck and Eriksson, 1974) and by transglucosylation with β -glucosidase (Kondo and Imaura, 1988, 1989; Kondo et al., 1990). In both cases, cellobiose participates in detoxification of vanillin, so the effect of vanillin on mycelial activity was markedly observed at the high ratio of vanillin/cellobiose. Therefore, undetoxified vanillin might affect the mycelial activity, but have little effect on the mycelial growth. The mechanism of activation remains unknown. It is possible that glucoside produced by the transglucosylation of β -glucosidase might induce these enzymes. If this is so, the presence of phenolics would be concluded to activate the fungus.

The presence of vanillin approximates the condition of wood decay more closely than its absence. The present result demonstrates that *C. versicolor* produces the wood-decaying enzymes more actively and effectively during wood decay than during incubation on cellulose alone. In other words, in the presence of vanillin, the fungus becomes active as during wood decay. Therefore, fermentation of lignocellulose materials needs to be studied in the light of the effect on fungal physiology of the presence of lignin and phenolic compounds, rather than by culture on cellulose or xylan alone.

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