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Induction and Characterization of a Cellobiose Dehydrogenase Produced by a Species of *Monilia*

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A species of the imperfect fungus *Monilia* produced cellobiose dehydrogenase extracellularly when grown on cellulose. The inducible enzyme was both bound to the mycelium and released into the growth medium. The enzyme showed a high degree of specificity for cellobiose, but also oxidized lactose and 4-β-glucosylmannose. The specificity of the electron acceptor was restricted to those compounds having a redox potential of +0·22 V. *p*-Benzoquinone and several other quinones, however, were not reduced. Oxygen was not consumed nor was hydrogen peroxide produced by cellobiose dehydrogenase oxidation of cellobiose. The enzyme had a molecular weight of 48000 and an isoelectric point of 5·3 to 5·5. A new zymogram technique was developed for the detection of cellobiose dehydrogenase in polyacrylamide gels following electrophoresis and isoelectric focusing.

INTRODUCTION

The enzyme cellobiose dehydrogenase (cellobiose:quinone oxidoreductase) was recently discovered by Westermark & Eriksson (1974*a*, *b*, 1975) to be produced extracellularly by the white-rot fungi *Polyporus versicolor* and *Chrysosporium lignorum* (*Sporotrichum pulverulentum*) when grown on cellulosic substrates. Cellobiose dehydrogenase catalyses the oxidation of cellobiose to cellobiono-γ-lactone in the presence of a suitable hydrogen, or electron, acceptor. The *S. pulverulentum* enzyme was capable of utilizing some lignin degradation products as electron acceptors, e.g. certain quinones and phenoxy radicals formed by the action of phenol oxidases on lignin. This enzyme may therefore play an important role in the degradation of cellulose and lignin by wood-rotting fungi (Westermark & Ericksson, 1975; Eriksson, 1978). The enzyme from *S. pulverulentum* has recently been purified to homogeneity and its properties have been studied (Westermark & Eriksson, 1975). It is a flavoprotein containing FAD as the prosthetic group and is specific for cellobiose and other cello-oligosaccharides, but will not oxidize cellulose. Its role is therefore to facilitate the biodegradation of cellulose by exo- and endo-cellulases by alleviating end-product inhibition of cellulase activity by cellobiose.

Cellobiose dehydrogenase activity has also been detected in the thermophilic white-rot fungus *Sporotrichum thermophile* when grown on cellulose. It possessed similar properties to those of the *S. pulverulentum* enzyme (Canevascini & Meier, 1978) but the natural electron acceptor was not identified.

The present paper examines the induction when grown on cellulosic substrates of extracellular-released and mycelial-bound cellobiose dehydrogenases produced by a species of the imperfect fungus *Monilia*. Some properties of the enzyme and a zymogram method for its detection on polyacrylamide gels are also reported.

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METHODS

Fungus and culture medium. A *Monilia* (Pers. ex Fries) species, tentatively identified as *Monilia sitophilia* (Mont.) Sacc., was isolated from decomposing sugar cane bagasse on solid Czapek Dox medium containing Whatman no. 1 filter paper. The culture isolate was purified on agar (Difco-Bacto) medium containing Czapek salts and phosphoric acid-swollen cellulose, i.e. Walseth cellulose (Walseth, 1952), and maintained on potato-dextrose agar at 4 °C. The organism was cultured in liquid medium containing 1 % (w/v) carbon source as described by Mandels & Reese (1957) and Reese & Maguire (1971). Cell-free culture fluid was obtained by centrifugation (20000 *g*, 30 min). In a typical experiment, 100 ml medium (in a 250 ml Erlenmeyer flask) was inoculated with $1{\cdot}5 \times 10^6$ spores and incubated at 28 °C and 120 rev. min^{-1} in a New Brunswick G-25 shaker. Conidia were washed from potato-dextrose agar plates using water containing 0·2 % (w/v) Tween 80, and filtered through a no. 3 porosity sintered glass filter.

Biochemicals. Most of the enzymes and substrates used were purchased from commercial sources. Glucono-1,5-lactone, cellobiose, carboxymethylcellulose, galactomannan (locust bean), xylan (larchwood), cytochrome *c* (horse heart), ubiquinone, vitamin K_3, vitamin E, peroxidase (horse radish), *o*-dianisidine dihydrochloride, NAD^+, $NADP^+$ and molecular weight marker proteins (cytochrome *c*, trypsinogen and ovalbumin) were obtained from Sigma. Sophorose monohydrate was purchased from Carl Roth, Karlsruhe, West Germany. 4-Methylumbelliferyl-β-D-cellobiopyranoside was supplied by Koch-Light, and Avicel, a microcrystalline cellulose preparation, was purchased from Merck. 4-*O*-β-D-Glucopyranosyl-β-D-mannopyranose and 4-*O*-β-D-mannopyranosyl-β-D-glucopyranose were gifts from Professor K. Kato of the Department of Agricultural Chemistry, Gifu University, Gifu, Japan.

Isolation and preparation of intracellular and mycelial enzymes. Mycelium grown in submerged culture was harvested by filtration on a glass-fibre filter paper (Whatman GF/A) and washed three times with 50 ml volumes of 100 mM-citrate/phosphate buffer (pH 7·0) containing 0·02 % (w/v) sodium azide. The washings were discarded. The mycelium was then removed from the filter paper with a spatula, suspended in 10 ml of the same buffer at 4 °C and ground in a chilled mortar using acid-washed sand as an abrasive. The ground mycelial suspension was then filtered and the filtrate (i.e. the cytosol) was collected and used as the source of intracellular enzymes. The disrupted mycelium retained on the filter was washed three times with 50 ml volumes of the same buffer. The filter paper and macerated mycelium were then removed from the funnel and ground in a mortar with 10 ml of buffer. The resultant suspension which contained glass-fibre filter paper, sand and macerated mycelium was used as the mycelial enzyme preparation. This method of obtaining the extracellular, intracellular and mycelial enzymes was more convenient, when processing large numbers of flasks each containing a different growth substrate, than the more usual but time-consuming centrifugation procedure. By using the above method it was possible to process a batch within 20 to 30 min.

Assays. Soluble protein content was assayed by a modification of the Lowry method as described by Hartree (1972), using bovine serum albumin as the standard. Mycelial protein content was determined by a modified Lowry method incorporating sodium dodecyl sulphate (SDS) (Sandermann & Strominger, 1972). The procedure for solubilizing mycelial protein consisted of heating mycelial suspension (0·25 ml) with SDS (5 %, w/v; 0·25 ml) for 20 min at 100 °C. A separate protein standard curve using bovine serum albumin as the standard was calibrated for this procedure.

Cellobiose dehydrogenase activity was assayed by measuring the decrease in absorbance of 2,6-dichlorophenolindophenol (DCPIP) spectrophotometrically at 600 nm. A typical assay mixture consisted of DCPIP (0·05 ml, 1·25 mM in citrate/phosphate buffer, 100 mM, pH 6·6), cellobiose (1·0 ml, 2·5 mM in the same buffer), glucono-1,5-lactone (0·05 ml, 20 mM in buffer) and enzyme (0·05 ml) in a glass cuvette at 37 °C in a Beckman 25 recording spectrophotometer equipped with a thermostatted cuvette holder. Decrease in absorbance was usually determined over a 4 min interval. Results are expressed as μmol DCPIP reduced min^{-1} (ml enzyme)$^{-1}$. Glucono-1,5-lactone was added to the substrate reactants at a final concentration of 1 mM which totally inhibited the strong β-D-glucosidase activity present. In experiments using electron acceptors other than DCPIP, the assay procedure was similar (using the various electron acceptors at 1 mg ml^{-1}) and the decrease in absorbance (i.e. enzyme activity) was monitored at their respective absorption maxima.

In assaying for mycelial-bound cellobiose dehydrogenase activity, mycelial suspension (0·25 ml) was added to the reaction substrates and incubated at 37 °C. After 10 min, enzyme activity was terminated by the addition of 100 mM-mercuric chloride solution (5 μl). The inactivated suspension was then centrifuged at 48000 *g* for 10 min and the absorbance of the supernatant was measured at 600 nm. A reference blank in which mycelial suspension was replaced by buffer was used as a control. This did not differ significantly from a boiled enzyme blank. Results are expressed as μmol DCPIP reduced min^{-1} (mg protein)$^{-1}$.

Hydrogen peroxide was measured colorimetrically by a modification of the glucose oxidase method (Dekker & Richards, 1971) for the determination of D-glucose. The reaction mixture, which contained

cellobiose (10 mM, 0·10 ml), glucono-1,5-lactone (20 mM, 0·05 ml in citrate/phosphate buffer, 100 mM, pH 6·6), peroxidase–chromogen reagent [1·0 ml of a solution containing peroxidase (3 units) and *o*-dianisidine dihydrochloride (0·1 mg) in 0·5 M-Tris/HCl/glycerol buffer, pH 7·0] and enzyme (0·50 ml), was incubated at 40 °C for 1 h. Enzyme activity was terminated by the addition of 5 M-HCl (4·0 ml) and the absorbance was measured at 540 nm.

Oxygen uptake was determined in a Yellow Springs model 55 Oxygen Monitor (Yellow Springs Instrument Co., Ohio, U.S.A.).

Gel filtration. Extracellular fluid was concentrated 10-fold by ultrafiltration using a Diaflo PM-10 membrane (Amicon Corp., Lexington, Mass., U.S.A.). The retentate (7·0 ml) was applied to a column (2·6 × 60 cm) of Sepharose 4B equilibrated in 100 mM-citrate/phosphate buffer (pH 6·6) and eluted with the same buffer. Fractions of 3 ml were collected and assayed for enzyme activity. Fractions containing cellobiose dehydrogenase activity were combined, concentrated by ultrafiltration and applied to a molecular weight-calibrated column (2·6 × 94·5 cm) of Biogel P-100 equilibrated in 50 mM-potassium phosphate buffer (pH 7·0).

Polyacrylamide gel electrophoresis and isoelectric focusing. Slab polyacrylamide gel electrophoresis and analytical isoelectric focusing in polyacrylamide gel were carried out using a Multiphor 2117 unit (LKB-Produkter, Bromma, Sweden) as described in LKB Application Notes 250 and 306. Polyacrylamide gel Ampholine plates of pH 3·5 to 9·5 were purchased from LKB. Discontinuous polyacrylamide gel electrophoresis was performed by the method of Davis (1964). Proteins within the gels were detected by staining with Coomassie Brilliant Blue R-250.

Zymogram for the detection of cellobiose dehydrogenase. Cellobiose dehydrogenase activity was detected within polyacrylamide gels by a zymogram technique in which the gel, following electrophoresis or isoelectric focusing, was incubated with substrate at room temperature, or 37 °C. The reaction mixture consisted of DCPIP (10 mM, 4·0 ml), cellobiose (2·5 mM, 40·0 ml) and glucono-1,5-lactone (20 mM, 2·0 ml in citrate/phosphate buffer, 100 mM, pH 6·6).

RESULTS

Production of cellobiose dehydrogenase

The Monilia cellobiose dehydrogenase appeared to be an inducible enzyme released into the extracellular medium. Support for the conclusion that the enzyme is bound to the mycelium and subsequently released was obtained while assaying for mycelial-bound cellobiose dehydrogenase. It was found that reduction of DCPIP continued even after all mycelium had been removed from the reaction mixture by high speed centrifugation. Some cellobiose dehydrogenase activity was also detected in the intracellular fraction but activity was very low and it is interpreted as enzyme released from the disrupted mycelium during the extraction procedure.

The ability of various substrates to support cellobiose dehydrogenase production is shown in Table 1. Cellulosic substrates were the strongest inducers of the enzyme. Celluloses that were more susceptible to attack by exo- and endo-cellulases, e.g. amorphous cellulose and carboxymethylcellulose, were less effective inducers. Xylan, which differs from cellulose only in the configuration of the substituent on carbon-5 of the glucopyranoside ring, was, like carboxymethylcellulose, capable of inducing the mycelial-bound enzyme, but no cellobiose dehydrogenase activity could be detected in the extracellular fluid. The same was true for D-glucose and the disaccharides, lactose and cellobiose. With these substrates however, the enzyme was present in much lower amounts than when the fungus was grown on cellulose. Even at the highest concentration tested (0·5 %, w/v) there was no repression of cellobiose dehydrogenase synthesis by these sugars. Addition of the surfactant Tween 80 to the nutrient medium had no significant effect on the release of mycelial-bound enzyme. Likewise the plant peptone, phytone, failed to induce higher yields of cellobiose dehydrogenase than proteose peptone (Table 1).

The time course of Avicel-grown Monilia cellobiose dehydrogenase formation is shown in Fig. 1. Maximum activity of both released and mycelial-bound enzyme occurred after 6 to 8 d growth.

Table 1. *Production of cellobiose dehydrogenase by the Monilia species grown for 6 d on various carbon sources*

Carbon source (1 %, w/v)	Cellobiose dehydrogenase activity*	
	Extracellular-released	Mycelial-bound
None	0	0
Cotton	29·7	ND
Avicel + phytone†	13·5	15·1
Avicel	12·5	11·9
Avicel + Tween 80	12·0	11·8
Bagasse	10·0	15·8
Amorphous cellulose‡	5·0	8·8
Carboxymethylcellulose§	0	3·3
Xylan‖	0	8·1
Galactomannan	0	ND
Cellobiose§	0	3·6
Cellobiose§ + Tween 80	0	3·1
Lactose§	0	4·1
Glucose	0	3·2

ND, Not determined.

* Extracellular-released enzyme activity is expressed as μmol DCPIP reduced min^{-1} (ml enzyme)$^{-1}$ and mycelial-bound activity as μmol DCPIP reduced min^{-1} (mg protein)$^{-1}$.

† Replaced protease peptone in the nutrient medium.

‡ Phosphoric acid-swollen Avicel (i.e. Walseth cellulose).

§ Present in the nutrient medium at 0·5 % (w/v); experiments using 0·05 and 0·10 % (w/v) gave similar results.

‖ Purified by extraction with $Ba(OH)_2$ to remove a glucomannan contaminant.

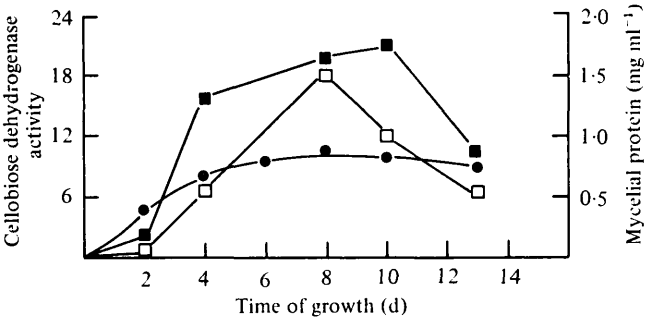

Fig. 1. Production of cellobiose dehydrogenase by the *Monilia* species grown on Avicel: □, extracellular-released enzyme activity [expressed as μmol DCPIP reduced min^{-1} (ml enzyme)$^{-1}$]; ■, mycelial-bound enzyme activity [expressed as μmol DCPIP reduced min^{-1} (mg protein)$^{-1}$]; ●, mycelial protein content.

Characterization of the extracellular-released cellobiose dehydrogenase

The relative rates of oxidation of cellobiose and other saccharides by cellobiose dehydrogenase are shown in Table 2. Glucono-1,5-lactone was added to the reaction mixture to inhibit the strong extracellular β-D-glucosidase activity which would otherwise hydrolyse cellobiose. Glucono-1,5-lactone itself did not affect the activity of cellobiose dehydrogenase or inhibit the oxidation of cellobiose. The enzyme showed a high degree of specificity for cellobiose and cello-oligosaccharides. The disaccharides lactose and 4-glucosyl-β-D-mannose were also oxidized, but to a lesser extent than cellobiose, while sophorose, maltose, sucrose, melibiose, raffinose, glucose, mannose, xylose and glucono-1,5-lactone did not serve as substrates. The specificity of the hydrogen, or electron, acceptor appeared to be restricted to those compounds having a standard redox potential (E_0') of around +0·22 V. Ferricyanide, despite having an E_0' value of +0·36 V, could still accept electrons arising from

Table 2. *Substrate and electron acceptor specificities of extracellular-released cellobiose dehydrogenase from 7 d cultures of the Monilia species grown on Avicel*

Substrate	Relative activity
Cellobiose	100
4-Methylumbelliferyl-β-D-cellobioside	70
Cello-oligosaccharides (DP* 3 to 6)	67
Lactose	60
4-Glucosyl-β-D-mannose	47
4-Mannosyl-β-D-glucose	0

Electron acceptor	Redox potential (V)	Relative activity†
2,6-Dichlorophenolindophenol	+0·22	100
Phenol blue	+0·22	37
Potassium ferricyanide	+0·36	5
p-Benzoquinone	+0·28	0

* DP, Degree of polymerization.
† On a molar concentration basis.

the oxidation of cellobiose by cellobiose dehydrogenase. Among the artificial electron acceptors listed in Table 2, DCPIP was the most effective. Cytochrome *c* (E_0' +0·22 V) was also reduced. However, several other electron acceptors, e.g. methylene blue (E_0' +0·01 V), NAD^+ and $NADP^+$ (E_0' −0·32 V), coumarin (1,2-benzopyrone), vitamin E (D-α-tocopheryl acetate), failed to accept electrons arising from enzymic oxidation of cellobiose. None of the quinones investigated, e.g. 2,3-dichloro-5,6-dicyano-*p*-benzoquinone, ubiquinone (coenzyme Q_{10}, E_0' +0·10 V), vitamin K_3 (menadione), 9,10-anthraquinone and 1,2,5,8-tetrahydroxy-9,10-anthraquinone (quinalizarin), could act as electron or hydrogen acceptors even though *p*-benzoquinone/hydroquinone has an E_0' of +0·28 V. The natural electron acceptor in the Monilia system has not yet been identified.

During oxidation of cellobiose by cellobiose dehydrogenase, oxygen was not consumed indicating that molecular oxygen could not serve as a hydrogen acceptor, nor was hydrogen peroxide generated.

The pH optimum for Monilia cellobiose dehydrogenase was between 4·0 and 4·5. In determining the pH optimum, reduction of DCPIP was followed spectrophotometrically at 520 nm for buffers of pH < 5·0 and at 600 nm for those of pH > 5·0. Using commercially available polyacrylamide gel Ampholine plates for the zymogram method of detecting cellobiose dehydrogenase (see below), the isoelectric point was found to lie between pH 5·3 and 5·5. The reduction of DCPIP was linear with respect to time over the first 10 min after which time all available DCPIP had been reduced. The apparent Michaelis constant (K_m) of cellobiose dehydrogenase determined at pH 6·6 was 12·2 μM with respect to cellobiose and 0·08 mM with respect to DCPIP (see Fig. 2). There was no inhibition of cellobiose dehydrogenase activity at high concentrations of cellobiose (Fig. 2*a*).

Fractionation of cellobiose dehydrogenase by gel permeation chromatography on Sepharose 4B and Biogel P-100 revealed the presence of only one enzyme species which eluted as a single symmetrical peak of molecular weight 48000 on Biogel P-100. The absence of isoenzymes was confirmed by polyacrylamide gel electrophoresis and isoelectric focusing.

Zymogram method of detecting cellobiose dehydrogenase in polyacrylamide gel

Following polyacrylamide gel electrophoresis and isoelectric focusing cellobiose dehydrogenase was detected by placing the gels in a Petri dish containing cellobiose, DCPIP and glucono-1,5-lactone in buffer (preferably at pH > 5·0) and incubating at room temperature, or 37 °C. Within 10 to 15 min the gels absorbed DCPIP which made them appear

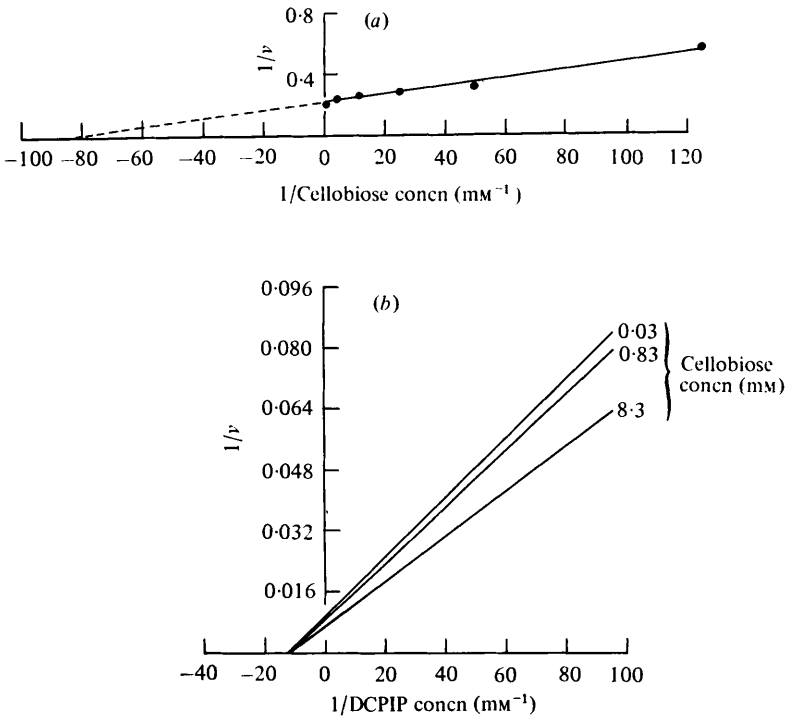

Fig. 2. Lineweaver–Burk plots showing (*a*) the concentration dependence of Monilia cellobiose dehydrogenase activity on cellobiose as substrate, and (*b*) the effect of concentration of DCPIP at different concentrations of cellobiose on Monilia cellobiose dehydrogenase activity. The *Monilia* species was grown on cotton for 6 d, and the extracellular-released enzyme was used in these studies. Enzyme activities (v) are expressed as μmol DCPIP reduced min^{-1} (ml enzyme)$^{-1}$.

blue except for a colourless band where DCPIP had been reduced as a result of cellobiose dehydrogenase activity. The zymogram technique worked equally well with disc gels, gel slabs and polyacrylamide gels containing pH ampholytes. When electrophoresis was performed at pH > 5·0, the dye incorporated into the gel appeared blue. At pH < 5·0, or when acid pH ampholytes were present, the gels appeared red, but this effect did not mask decoloration of the gel where cellobiose dehydrogenase activity was present. Visual detection of enzyme, however, was greatly facilitated by adjusting the pH of the reaction mixture by the addition of solid K_2HPO_4, which kept the DCPIP in the oxidized (i.e. blue) form.

DISCUSSION

The cellobiose dehydrogenase produced by this *Monilia* species is similar in many respects to the enzymes from *Chrysosporium lignorum* (*Sporotrichum pulverulentum*), *Polyporus versicolor* (Westermark & Eriksson, 1974*a*, *b*, 1975) and *Sporotrichum thermophile* (Canesvascini & Meier, 1978). The enzyme is extracellular (mycelial-bound in *S. thermophile* and the *Monilia* species) and highest activities resulted when the fungi were grown on cellulosic substrates that are less susceptible to attack by exo- and endo-cellulases, e.g. cellulose powder, Avicel and cotton. This phenomenon has also been observed in the induction of extracellular β-D-glucosidases in the *Monilia* species (unpublished results) and *Phanerochaete chrysosporium* (Smith & Gold, 1979). Glucose and cellobiose also induced

cellobiose dehydrogenase in these fungi but the activities were much lower than when the fungi were grown on cellulose.

Properties of the crude Monilia cellobiose dehydrogenase were very similar to those reported for *S. pulverulentum* (Westermark & Eriksson, 1974*b*, 1975) and *S. thermophile* (Canevascini & Meier, 1978). In all cases the enzymes showed a high degree of specificity for cellobiose and cello-oligosaccharides and they were also capable of oxidizing lactose (4-β-galactosylglucose) and 4-β-glucosylmannose, but to a lesser extent. However, neither glucose nor mannose was oxidized. Hydrogen peroxide was not produced nor was molecular oxygen consumed during cellobiose oxidation, indicating a mechanism markedly different from the oxidation of glucose and galactose by glucose and galactose oxidases, respectively (Sharon, 1975). Reduction of the artificial electron acceptor DCPIP occurred concomitantly with the oxidation of cellobiose during catalysis by cellobiose dehydrogenase. A natural electron acceptor for the enzyme produced by *S. pulverulentum* has been identified as certain lignin degradation products (quinones and phenoxy radicals) by Westermark & Eriksson (1974*a*, *b*, 1975), and in white-rot fungi this enzyme has been implicated in the breakdown of cellulose and lignin (Eriksson, 1978). The natural electron acceptor for the Monilia enzyme has not been identified but since the Monilia species is non-ligninolytic (unpublished results) the role of cellobiose dehydrogenase in this organism must be different from that proposed by Eriksson (1978) for *S. pulverulentum*.

In its natural environment the extracellular enzyme might be involved in the reduction of lignin degradation products through a coupled reaction with the oxidation of cellobiose. This *Monilia* species is strongly cellulolytic (unpublished results) and is likely to inhabit the same environment as lignin-degrading fungi. Thus, quinones or phenoxy radicals produced by the action of wood-rot fungal laccases or phenol oxidases on lignin (an exo-cellular process) might accept the electrons produced on oxidation of cellobiose by the *Monilia* species, which is also an exo-cellular reaction. However, when this fungus is cultured on cellulose alone another mechanism must be operative in which electrons arising from cellulose oxidation by cellobiose dehydrogenase are transferred to an acceptor, the nature of which is still unknown.

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