Characterization of a cellobiose dehydrogenase in the cellulolytic fungus *Sporotrichum (Chrysosporium) thermophile*

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An extracellular enzyme from culture filtrates of *Sporotrichum (Chrysosporium) thermophile* (A.T.C.C. 42 464) after growth on cellulose or cellobiose was shown to oxidize cellobiose to cellobionic acid *in vitro*. Lactose and cellodextrins were also efficiently oxidized, but the enzyme was not active against most mono- and di-saccharides. Several redox substances could act as electron acceptors, but molecular oxygen, tetrazolium salts and NAD(P) were not reduced. Activity was stimulated up to 2-fold in the presence of 0.05 M-Mg²⁺. The pH optimum of the enzymic reaction was acidic when the activity was tested with dichlorophenol-indophenol or Methylene Blue, but was neutral to alkaline for 3,5-di-*t*-butyl-1,2-benzoquinone or phenazine methosulphate as electron acceptors. As the enzyme was formed inductively in parallel with the endocellulase, its possible function in relation to cellulolysis is discussed.

The catabolism of cellulose by cellulolytic micro-organisms occurs mainly through the concerted action of several hydrolytic enzymes (Wood & McCrae, 1979; Fägarstam & Pettersson, 1980) whose location (free extracellular, cell-associated or intracellular) is a function of the particular organism. Occasionally, additional non-hydrolytic, e.g. oxidative, enzymes have also been claimed to be operative in some instances (Eriksson *et al.*, 1974; Highley, 1980), but little attention has been paid to the role of them in the whole process of cellulose degradation.

A cellobiose:quinone oxidoreductase has been found to occur in two white-rot fungi, namely *Polyporus versicolor* and *Sporotrichum pulverulentum* (Westermarck & Eriksson, 1974a,b, 1975). A similar oxidative enzyme, but unable to react with quinones, has been found in a non-lignolytic *Monilia* species (Dekker, 1980). The enzyme from *Sporotrichum pulverulentum* has been shown to be functionally involved in lignin as well as in cellulose degradation.

It has now been found that an enzyme, catalysing the reactions:

is also formed by the cellulolytic but non-lignolytic fungus *Sporotrichum thermophile*. A preliminary characterization of this enzyme was carried out: (1) by identifying the reaction product, i.e. cellobionic acid; (2) by searching for redox substances to substitute for the unknown natural oxidant (X_{ox.}); (3) by testing the specificity of the enzyme against some mono- and oligo-saccharides; and (4) by studying the optimum biochemical conditions for the reaction.

It should be noted that *Sporotrichum thermophile* belongs phylogenetically to the Ascomycetes (von Klopoteck, 1974), whereas *Sporotrichum pulverulentum* is the imperfect form of the Basidiomycete *Phanerochaete chrysosporium* (Bursdall & Eslyn, 1974). The presence of isofunctional cellobiose-oxidizing enzymes in species as different as those cited, but still related to their common cellulolytic property, raises the question as to whether such enzymes have a main catabolic or rather a regulatory role in cellulolysis.

Materials and methods

Materials

All substances for the preparation of culture media were of analytical or bacteriological purity.

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Cellobiose was purchased from Senn, Dielsdorf, Switzerland. Mannobiose, xylobiose, mannosyl- β 1 $\rightarrow$ 4-glucose and galactosyl- β 1 $\rightarrow$ 4-galactose were from the collection of this Institute. Radioactive cellobiose (asymmetrically labelled with ^{14}C either at the reducing or at the non-reducing glucose moiety) was prepared as described by Canevascini *et al.* (1979).

Organism

The organism studied was a strain of *Sporotrichum (Chrysosporium) thermophile*, A.T.C.C. 42464. For maintenance and conidia production it was grown at 44°C for 1 week on Difco Sabouraud maltose/agar slants or plates. Submerged batch cultures were performed in a complex medium containing Difco Casamino acids, yeast extract, salts and an optional carbon source as previously described (Canevascini *et al.*, 1979). For details of culture techniques, see also Canevascini *et al.* (1981).

Enzyme production and enrichment

Cultures (up to 6 litres in volume) on 1% (w/v) cellulose powder (Whatman CC 31) or on 0.2% cellobiose were set up by inoculating the complete medium with the young mycelium obtained by pre-germination of conidia (usually 8 h incubation at 44°C) in the basic complex medium without sugar carbon. The culture was adequately aerated and incubated at 44°C for 4–8 h, depending on the substrate (cellobiose or cellulose respectively). The cold (ice bath) culture filtrate was subsequently dialysed and concentrated by hollow-fibre (type H1P5, Amicon) ultrafiltration. With this technique the endocellulase yield was usually less than 50%, whereas cellobiose dehydrogenase was recovered almost quantitatively. The concentrated enzyme preparation was in some cases further enriched by stepwise salting-out of the different proteins with $(\text{NH}_4)_2\text{SO}_4$ at 0°C. Although with a great deal of overlapping, endocellulase was recovered between 40 and 60% saturation, whereas cellobiose dehydrogenase was precipitated later (from 60 to 80% saturation).

Analytical methods

Cellobiose dehydrogenase. (i) Photometric determination with dichlorophenol-indophenol as electron acceptor at pH 7.0 and 30°C. The reaction mixture (in a total volume of 3 ml) consisted of phosphate buffer⁻ (Sørensen, $\text{Na}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$), pH 7.0 (0.3 mmol), dichlorophenol-indophenol (0.12 μmol) and cellobiose (1 μmol). The reaction was started by adding enzyme and the change in A_{600} monitored for the first 3 min. The activity was expressed as nmol of dye reduced/min for a given amount of enzyme under the specified conditions.

The applied molar absorption coefficient (ϵ_{600}) for dichlorophenol-indophenol at the working pH was $1.61 \times 10^4 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ (Dawson *et al.*, 1969).

(ii) Photometric determination with 3,5-di-*t*-butyl-1,2-benzoquinone as oxidant. The reaction mixture was the same as that described above, except that 3,5-di-*t*-butyl-1,2-benzoquinone (1 μmol) was substituted for dichlorophenol-indophenol and sufficient ethanol (10%) was added to keep the quinone in solution. The activity was expressed as before in nmol of quinone reduced by applying the experimentally determined molar absorption coefficient (ϵ_{420}) of $1.43 \times 10^3 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$.

(iii) Photometric determination with Methylene Blue. Reduction of Methylene Blue was recorded at different pH values (citric acid/ Na_2HPO_4 buffer) aerobically or anaerobically with Thunberg cuvettes evacuated and flushed with nitrogen. The molar absorption coefficient (ϵ_{668}) applied was $6.3 \times 10^4 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ (Peel, 1972).

(iv) Manometric measurement of cellobiose dehydrogenase activity. Substrate (cellobiose) oxidation was coupled with oxygen consumption by means of phenazine methosulphate, and oxygen uptake was measured by conventional constant-volume manometry. The reaction mixture (in a total volume of 3 ml) consisted of phosphate buffer, pH 7.0 (0.18 mmol), phenazine methosulphate (5 μmol) and cellobiose (10 μmol). The enzyme was added from the side-arm of the Warburg vessel after temperature equilibration at 40°C. The activity was expressed in μmol of O_2 taken up/min for a given amount of enzyme. A good linearity was observed between oxygen consumption and enzyme concentration.

H^+ release during substrate oxidation was monitored manometrically as CO_2 evolution (Quastel, 1957) by carrying out the reaction in bicarbonate buffer under a constant CO_2 atmosphere (Umbreit *et al.*, 1972). The reaction mixture (in a total volume of 3 ml) consisted of NaHCO_3 (6.75 μmol), a suitable electron acceptor (10 μmol) and cellobiose (10 μmol). The enzyme was added from the side-arm after temperature equilibration at 40°C. Activity was expressed as nmol of CO_2 evolved (H^+ formation)/min for a given amount of enzyme at the initial maximal velocity of the reaction.

Endocellulase (EC 3.2.1.4). This was assayed viscometrically with CM-cellulose as previously described (Canevascini *et al.*, 1979). The arbitrarily defined units of activity are related to absolute units by the factor: 1 unit = $0.63 (\pm 0.03) \times 10^{-5} \mu\text{mol}$ of reducing groups liberated/min (Canevascini & Gattlen, 1981).

Glucose oxidase (EC 1.1.3.4) and (similarly) cellobiose oxidase. These were assayed with o-dianisidine and peroxidase as described by Bergmeyer *et al.* (1974).

Laccase (*p*-diphenol oxidase, EC 1.10.3.2) and **peroxidase** (EC 1.11.1.7). These were determined with syringaldazine by the procedure of Petroski *et al.* (1980).

Catalase (EC 1.11.1.6). This was determined as described by Aebi *et al.* (1959).

Other assays. Glucose was specifically determined with the glucose oxidase/peroxidase system (Biochemica Test Combinations; Boehringer, Mannheim, Germany). Gluconate was determined with gluconate kinase and gluconate 6-phosphate dehydrogenase (Möllering & Bergmeyer, 1974).

Protein was determined either directly or after precipitation with trichloroacetic acid (6%, w/v, final concn.) by the Lowry *et al.* (1951) procedure, with bovine serum albumin as standard.

H₂O₂ was determined as described by Aebi *et al.* (1959) with Ti₂(SO₄)₃ and H₂SO₄.

Identification of cellobionic acid

(a) Cellobiose (20 μ mol) was allowed to react with phenazine methosulphate (5 μ mol) and enzyme in the air at 30°C. The extent of reaction was controlled by constant-volume manometry. The reaction mixture was then fractionated by ion-exchange chromatography on a Dowex 50 (H⁺ form) column (10 cm $\times$ 1.2 cm diam.) and an Amberlite IRA 68 (basic form) column (10 cm $\times$ 1.2 cm internal diam.) successively. The acidic product was eluted from the anion exchanger with 5 M-acetic acid (100 ml). The acid fraction was then evaporated to dryness under reduced pressure, redissolved in 1 M-HCl (1 ml) and hydrolysed for 3 h at 100°C. After neutralization with NaOH, the glucose and gluconate were specifically determined with glucose oxidase and gluconate kinase respectively [yields (mean of triplicate analyses): 14.8 μ mol of glucose; 13.3 μ mol of gluconate].

(b) D-[U-¹⁴C]Glucosyl- β 1 $\rightarrow$ 4-glucose (A) or D-glucosyl- β 1 $\rightarrow$ 4-[U-¹⁴C]glucose (B) were allowed to react with a suitable electron acceptor and enzyme. The products were separated by paper electrophoresis in triethylamine buffer (0.1 M, pH 9.2) at 50 V/cm. The spots corresponding to radioactive cellobionic acid were eluted, the solutions were evaporated to dryness and the residues hydrolysed with 1 M-HCl for 3 h at 100°C. The products were again separated by paper electrophoresis. In the experiment in which (A) was used as substrate, components corresponding to radioactive glucose and non-radioactive gluconic acid were obtained, whereas with (B) as the substrate, non-radioactive glucose and radioactive gluconic acid were obtained.

Results

Identity of the reaction product

The oxidative enzyme of *Sporotrichum thermophile* dealt with in the present paper was released in

the culture medium during growth on cellulose or cellobiose in parallel with the endocellulase. The crude enzyme preparation used to characterize the reaction was obtained by ultrafiltration and in some cases further enriched by precipitation with (NH₄)₂SO₄.

On reaction with cellobiose and a suitable electron acceptor, an acidic product was formed. This was shown by carrying out the reaction in a bicarbonate buffer under a fixed CO₂ atmosphere and measuring CO₂ evolution by constant-volume manometry. Table 1 gives the activity, expressed as μ mol of CO₂ evolved (H⁺ formed)/min at the initial maximal velocity for a given amount of enzyme, with different electron acceptors. The reaction was linear with time for the first 20 min, but, owing to uncontrollable small changes in the actual pH of the reaction mixture, a strict proportionality between activity and enzyme concentration could not be obtained.

The identity of the acid formed was determined as follows (see also the Materials and methods section): after completion of the reaction, the mixture was passed successively through a cation- and an anion-exchange resin, the acid product being recovered from the latter by elution with acetic acid. Hydrolysis of the compound thus recovered gave equimolar amounts of glucose and gluconic acid, showing that cellobionic acid had been formed from cellobiose. Further evidence for the oxidation of the reducing glucose residue of cellobiose by the enzyme was obtained by using asymmetrically ¹⁴C-labelled cellobiose as substrate. When D-[U-¹⁴C]glucosyl- β 1 $\rightarrow$ 4-glucose was oxidized by the enzyme and then hydrolysed, only radioactive glucose was obtained; when D-glucosyl- β 1 $\rightarrow$ 4-[U-¹⁴C]glucose was used, only radioactive gluconate was recovered.

Table 1. *H⁺ production (CO₂ evolution) during cellobiose oxidation in a bicarbonate buffer at pH 6.6 with different electron acceptors*

The reaction mixture (in a total volume of 3 ml per Warburg flask) contained 6.75 μ mol of NaHCO₃, 10 μ mol of one of the specified electron acceptors and 10 μ mol of cellobiose. Quinones were kept in solution with ethanol (final concn. 10%, v/v). The enzyme (a concentrated hollow-fibre culture filtrate from a cellulose-grown culture) was added from the side arm after temperature equilibration at 40°C under a CO₂/N₂ (1:19) atmosphere.

Electron acceptor	CO ₂ evolved (μ mol $\cdot$ min ⁻¹)
Phenazine methosulphate	5.1–7.3
Potassium ferricyanide	0.5–1.2
<i>p</i> -Benzoquinone	4.2–4.7
3,5-Di- <i>t</i> -butyl-1,2-benzoquinone	3.1–3.6
4-Anilino-5-methoxybenzoquinone	3.0–3.4

Electron acceptors

Molecular oxygen could not directly be used by the enzyme as an oxidizing agent. This applies to different enzyme preparations obtained after growth on cellobiose or cellulose. Evidence for the lack of reactivity with molecular oxygen was sought either directly in a Warburg respirometer or with a Clark oxygen electrode or indirectly by estimating the hypothetical H_2O_2 formed during the aerobic incubation of enzyme and cellobiose. In all these experiments, interference by endogenous catalase was eliminated either by cyanide inhibition or by adding external catalase. Similarly, no glucose oxidase activity was found in any of the extracellular enzyme preparations tested, irrespective of whether the organism had been previously grown on cellulose, cellobiose or glucose. Additionally, no evidence could be found for laccase or peroxidase activity.

As shown in Fig. 1, cellobiose oxidation could be efficiently coupled with oxygen reduction by means of the autoxidizable electron acceptor phenazine methosulphate. As expected, with added cyanide (8.3 mM) the reaction rates were maximal and twice that with an excess of catalase. With no additives, oxygen uptake was intermediate (between 70 and 80% of the maximal theoretical value, in many experiments performed with different enzyme preparations), showing that phenazine methosulphate, at the concentration used (1.6 mM), did not seriously affect endogenous catalase. The activity of the latter was quite high: specific catalase activities of up to $3.5 \mu\text{mol/min per mg}$ of total protein were not uncommon in the various concentrated enzyme samples.

Other redox substances capable of being linked to cellobiose oxidation are listed in Tables 1 and 2. No reaction was, however, observed with triphenyl-tetrazolium salts as oxidant, nor with NAD or NADP. At a neutral pH and under anaerobic conditions, Methylene Blue reduction was hardly detectable with an amount of enzyme equivalent to that used in the routine photometric enzyme assay with dichlorophenol-indophenol (see the Materials and methods section). Methylene Blue was, however, readily reduced at a pH below 6.0.

Specificity of the reaction with respect to the oxidizable substrate

Several mono- and di-saccharides were tested as potential substrates for the cellobiose-oxidizing enzyme of *Sporotrichum thermophile*. Table 3 lists all of the disaccharides and oligosaccharides which have been found to be oxidized with either dichlorophenol-indophenol or 3,5-di-*t*-butyl-1,2-benzoquinone as oxidizing agents. No reaction was, however, observed with the following substances: mannose, galactose, fructose, xylose, arabinose, sucrose, isomaltose, laminaribiose, gentiobiose, tre-

halose, sophorose, mannosyl- β 1 $\rightarrow$ 4-glucose, galactosyl- β 1 $\rightarrow$ 4-galactose, raffinose, glucosamine, gluconic and glucuronic acid, lactitol, maltitol, cellobitol. Glucose oxidation was minimal; maltose, mannobiose and xylobiose were only slightly attacked. The xylobiose sample used (isolated by charcoal chromatography from a partial acid hydrolysate of birch xylan) was contaminated with some cellobiose; in spite of this, the linear behaviour of the reaction with respect to time was consistent with a definite oxidation of this disaccharide.

Biochemical reaction parameters

Depending on the electron acceptor used, cellobiose oxidation was found to be very sensitive to pH changes. With dichlorophenol-indophenol (Fig. 2a), Methylene Blue (Fig. 2b) as well as with ferri-cyanide (Fig. 2c), the reaction rates were faster at an

Fig. 1. Cellobiose oxidation and oxygen consumption via autoxidation of reduced phenazine methosulphate. Oxygen uptake was monitored manometrically in a Warburg respirometer at 40°C and in an atmosphere of air. The main compartment of each Warburg flask contained, in a total volume of 3 ml: 0.25 mmol of Sørensen's phosphate buffer, pH 7.0; 5 nmol of phenazine methosulphate; and 25 nmol of cellobiose. Addition of KCN (25 nmol) was optional. The reaction was started by pouring in the enzyme [a concentrated [60%-satd.-(NH_4) $_2\text{SO}_4$ precipitate] culture filtrate from a cellulose culture] from the side arm. Additional catalase [from bovine liver (Sigma); 4 units] was optional. Control experiments, without enzyme, cellobiose or phenazine methosulphate respectively, were negative.

Table 2. *Specificity of cellobiose dehydrogenase with respect to the electron acceptor*

The reaction mixture contained, in a total volume of 3 ml: 0.15 mmol of pipes (1,4-piperazinediethanesulphonic acid)/NaOH buffer, pH 6.6; 1 μ mol of cellobiose; an appropriate amount of a particular electron acceptor to be tested (as given in the Table) and enzyme (different enzyme preparations were assayed). Detailed analytical procedures are given in the cited references. The efficiency of the cellobiose oxidation with various electron acceptors at their corresponding pH optima were as follows: for an activity corresponding to 100 nmol of dichlorophenol-indophenol reduced/min, 121 nmol of 3,5-di-*t*-butyl-1,2-benzoquinone, 175 nmol of ferricyanide, 215 nmol of phenazine methosulphate and 11 nmol of Methylene Blue were reduced.

Electron acceptor	Concn. (mm)	Reduction	Reference for analytical procedure
Dichlorophenol-indophenol	0.04	+	Peel (1972)
3,5-Di- <i>t</i> -butyl-1,2-benzoquinone	0.3	+	Westermarck & Eriksson (1974a,b)
<i>p</i> -Benzoquinone	0.3	+	Westermarck & Eriksson (1974a,b)
Potassium ferricyanide	0.8	+	Symons & Burgoyne (1966)
Fe ³⁺ cations (in the presence of 4 mm- <i>o</i> -phenanthroline)	0.5	+	Weber <i>et al.</i> (1956)
Cytochrome <i>c</i>	0.05	+	Singer & Cremona (1966)
Methylene Blue*	0.01	+	Quastel (1957)
Triphenyltetrazolium chloride	0.8	—	Brodie & Gots (1951)
NAD or NADP	0.2	—	Sadoff (1966)

* Methylene Blue reduction was slow at pH 6.6; see Fig. 2a.

Table 3. *Specificity of cellobiose dehydrogenase with respect to various oxidizable substrates*

The actual concentration of the different substrates in the reaction mixture was 0.33 M, except for xylobiose, whose concentration was only approximate. All values are means of duplicates. Results obtained with enzyme samples of different cultures are given in parentheses.

Substrate	Electron acceptor	Relative activity (%) (on a molar basis)	
		2,6-Dichlorophenol-indophenol	Di- <i>t</i> -butyl-1,2-benzoquinone
Cellobiose	...	100	100
Cellotriose		98 (93–98)	—
Cellotetraose		92 (93–109)	100
Lactose		87 (87–96)	94
Glucosyl- β 1 $\rightarrow$ 4-mannose		81 (81–85)	—
Maltose		9 (6–9)	—
Mannobiose		17 (16–17)	—
Xylobiose*		38 (32–38)	20
Glucose		2 (1–3)	—

* T.l.c. indicated traces of cellobiose.

acidic pH, whereas with 3,5-di-*t*-butyl-1,2-benzoquinone (Fig. 2d) as well as with both stable radicals Wurster's Blue and 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) (see Duine *et al.*, 1978) (results not shown) or with phenazine methosulphate (Fig. 2e), the reaction rates increased with increasing pH up to alkaline values. The effect of pH is strikingly seen in Fig. 2(c), where ferricyanide reduction was recorded in a bicarbonate solution whose buffering capacity was limited by H⁺ liberated during the reaction. Cellobiose oxidation was intermediate with dichlorophenol-indophenol as electron acceptor; it was maximal with phenazine methosulphate and minimal with Methylene Blue. In this context it should be pointed out that, as a rule, the activity of the enzyme determined photometrically with dichlorophenol-indophenol was measured at pH 7.0, to avoid possible interference by some

β -glucosidase that is always present, although in small amounts, in the different enzyme samples; the efficiency of the reaction was thus lowered to about 50%. At pH 7.0 and with dichlorophenol-indophenol (0.04 mm) as oxidant, the apparent K_m for cellobiose oxidation was $6.9(\pm 0.3) \times 10^{-6}$ M.

The reaction rate was also variably affected in different buffers when the reaction was carried out with dichlorophenol-indophenol at pH 7.0. Tris buffer, despite its high pK_a (unsuitable for buffering at pH 7.0, an H⁺-liberating reaction) was the most favourable buffer, whereas the reaction rate was lowered to less than 50% with some zwitterionic buffers such as Mes [4-(2-morpholine-ethanesulphonic acid), Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] or Tes (2-[2-hydroxy-1,1-bis-(hydroxymethyl)ethyl]amino)ethanesulphonic acid). Surprisingly the reaction was unaffected by the buf-

Fig. 2. *pH-dependence of cellobiose oxidation with different electron acceptors*

Enzymic cellobiose oxidation was performed in the following buffer solutions: (a), (b) and (e): McIlvaine citrate/phosphate buffer for the pH range 3.0–8.0; (e) Tris/HCl buffer for pH values between 7.0 and 9.0; (c) bicarbonate buffer in an atmosphere of CO_2/N_2 (1 : 19). (a), (b) and (d) were photometric determinations; (c) and (e) were manometric (constant-volume) determinations of CO_2 evolution and oxygen consumption respectively. Symbols: (a), ●, 530nm; ○, 600nm; (b), ●, aerobic; ○, anaerobic; (e) ●, phosphate/citrate buffer; ○, Tris/HCl buffer.

Table 4. *Effect of various buffers on enzymic cellobiose oxidation*

Various enzyme preparations were tested for their activity at 30°C in the usual way with the two electron acceptors as indicated. Activity is expressed relative to that obtained in Tris/HCl buffer. No significant decrease in pH was observed during the reaction.

Buffer at pH 7.0	pK_a at 25°C	Electron acceptor	Relative activity (%)	
			Dichlorophenol-indophenol	3,5-Di-t-butyl-1,2-benzoquinone
Phosphate	(7.21)	...	77–78	100–101
Hepes	(7.55)		27–28	103–105
Mes	(6.15)		44–46	105–111
Tes	(7.50)		28–30	98–102
Tris	(8.30)		100	100
Triethanolamine	(7.76)		70–72	85–87
Pipes	(6.80)		70–76	94–96

fer composition when carried out with 3,5-di-*t*-butyl-1,2-benzoquinone (Table 4).

Discussion

Biochemical properties of cellobiose dehydrogenase

The present investigation deals with the preliminary biochemical characterization of an extra-cellular oxidoreductase produced by *Sporotrichum thermophile* (A.T.C.C. 42464) during growth on cellulose or cellobiose, i.e. under conditions triggering cellulolysis in this organism (Canevascini *et al.*, 1979). This enzyme oxidizes cellobiose, as well as other cello-oligosaccharides and also some other disaccharides, to the corresponding aldonic acids by means of a few oxidizing agents, but not with molecular oxygen. It thus corresponds to the definition of a dehydrogenase. This enzyme is isofunctional with a cellobiose:quinone oxidoreductase first discovered in some white-rot fungi (Westermarck & Eriksson, 1974b) and also with a cellobiose dehydrogenase more recently reported to occur in culture filtrates of a *Monilia* species (Dekker, 1980). These organisms, although taxonomically distinct, are physiologically related by their common property of being cellulolytic. Such oxidative enzymes acting on cellobiose and cello-dextrins may therefore belong to the enzymic equipment used for the catabolism of cellulose.

On the basis of present knowledge, the cellobiose dehydrogenase from the organisms thus cited, although markedly similar with respect to their sugar substrate specificity (with only minor differences) differ in some other properties. The enzyme from *Monilia* does not react with benzoquinones (Dekker, 1980), for which the dehydrogenases from both *Sporotrichum* species have a high affinity. The enzyme from *Sporotrichum thermophile* is able to use a wide spectrum of redox substances, including various quinones, Methylene Blue, Fe³⁺ ions, phenazine methosulphate, as well as the stable free radicals Wurster's Blue and 2,2'-azinobis-(3-ethyl-benzothiazoline-6-sulphonic acid). Molecular oxygen can be used as a final electron acceptor with catalytic amounts of phenazine methosulphate. This could possibly occur with the two enzymes from *Sporotrichum pulverulentum* and *Monilia*, as phenazine methosulphate is known to react with most dehydrogenases (Singer & Kearney, 1957). The apparent K_m for cellobiose oxidation with dichlorophenol-indophenol was $6.9 \times 10^{-6} \text{ M}$ and is thus similar in magnitude to that reported from the enzyme from *Monilia* ($12.2 \times 10^{-6} \text{ M}$; Dekker, 1980). Although the present study was not primarily devoted to enzyme production and regulation, it may be noted for comparative purposes that the absolute activity in culture filtrates after approx. 10h growth on cellulose varied between 30

and 35 nmol of cellobiose oxidized/min per ml of culture fluid with dichlorophenol-indophenol at pH 7.0 and 30°C. This corresponds to about 100 nmol of 3,5-di-*t*-butyl-1,2-benzoquinone at the same pH value, thus showing an enzyme yield slightly superior to that recorded for both *Sporotrichum pulverulentum* (Westermarck & Eriksson, 1974b) and *Monilia* sp. (Dekker, 1980).

With respect to pH optimum, the enzyme from *Sporotrichum thermophile* was distinctly different from that of *Sporotrichum pulverulentum* in showing maximal activity for quinone and phenazine methosulphate reduction over a neutral-to-basic pH range. An alkaline pH optimum was also observed with Wurster's Blue and 2,2'-azinobis-(3-ethyl-benzothiazoline-6-sulphonic acid) as artificial electron acceptors (results not shown). A similar, but sharper, pH profile has also been observed for the methanol dehydrogenase of *Hyphomicrobium* X when tested with the same two redox carriers (Duine *et al.*, 1978). With dichlorophenol-indophenol, Methylene Blue and ferricyanide, however, the pH optimum was clearly in the acidic range. This was particularly marked for Methylene Blue, which was not reduced (anaerobically) at pH values above 6.0. Such behaviour may be ascribed to a pH-dependent electron delocalization on quinones and phenazine methosulphate.

The observed regulation of the enzyme activity by the buffer type, when dichlorophenol-indophenol was used as oxidizing agent, was not predictable, but clearly some zwitterionic buffers were inhibiting. On the other hand, the stimulation recorded with Tris was not due to a decrease in pH. The observed buffer side effects are possibly due to interactions with the oxidizing agent used rather than with the enzyme itself, as the reaction carried out with the benzoquinone was completely unaffected.

Metabolic role of cellobiose dehydrogenase

From the evidence presented here, no definite statements can be made on the function of this enzyme (possibly more than one enzyme) in *Sporotrichum thermophile*. The absence of any cellobiose oxidase, glucose oxidase, laccase and peroxidase activity may be taken as evidence that this organism is also physiologically different from the white-rot fungus *Sporotrichum pulverulentum*. In fact *Sporotrichum thermophile* has been found to be unable to degrade lignin under conditions that allow lignin degradation with *Sporotrichum pulverulentum* (Th. Haltmeier, personal communication), and therefore there is no reason to ascribe to the cellobiose-oxidizing enzyme of this organism a functional role in ligninolysis. But it may have a regulatory role on the activity of some cellulases, particularly exocellobiohydrolases, by alleviating them from cellobiose feedback inhibition (Sternberg *et al.*, 1977). In

most cellulolytic fungi such a function is usually ascribed to the β -glucosidase component, which in turn is almost totally absent from the culture filtrate of *Sporotrichum thermophile* (Canevascini & Meyer, 1979). Another possible function of cellobiose dehydrogenase in this organism (and likely in *Monilia*) may be in the metabolism of cellobiose itself, as this could be catabolized via oxidation rather than by intracellular hydrolysis. This view is corroborated by some experiments, not reported here, which are indicative of cellobionic acid being assimilated by *Sporotrichum thermophile*, although the enzymes involved in its catabolism are not yet known. The identity of the cellobiose-oxidizing agent (electron acceptor) *in vivo*, for all the organisms thus cited, is unknown. Clearly the intermediate reduction of a quinoid compound formed by laccase oxidation of some lignin degradation products (Eriksson, 1978) can be operative only in white-rot fungi. Alternatively, phenolic compounds may be produced by the organism itself (Shimada *et al.*, 1981) and subsequently oxidized by laccase or peroxidase. For the present organisms, however, this latter mechanism as yet lacks experimental support. Nevertheless, it is conceivable, also on the basis of reports on oxidoreductases in some bacteria (Hauge, 1961; Hayaishi, 1966), that the final oxidizing agent *in vivo* is molecular oxygen. The indirect oxygen reduction with phenazine methosulphate may be considered as a model of a short redox chain functionally associated with some cellular surface structure (membrane), starting with a specific dehydrogenase and ending with a component (e.g. a cytochrome) readily reacting with oxygen. The soluble free enzyme described here would then be the first member of this chain. This hypothesis is supported by the fact that in *Sporotrichum pulverulentum* an additional oxidase, isofunctional in respect of the sugar substrate with the previously reported cellobiose dehydrogenase, has been found and characterized as a flavocytochrome complex (Ayers *et al.*, 1978). This aspect of the problem, i.e. the nature, localization and function of cellobiose dehydrogenase in *Sporotrichum thermophile*, as well as the metabolic fate of cellobiose and cellobionic acid, requires further investigation.

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