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UNIVERSITY OF NATURAL RESOURCES AND APPLIED LIFE SCIENCES,
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Department of Food Sciences and Technologies
Institute of Food Biotechnology

Induction of cellobiose dehydrogenase in the selectively delignifying fungus *Ceriporiopsis subvermispora*

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DANIEL KRACHER

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ABSTRACT

Cellobiose dehydrogenase (CDH) is an extracellular flavocytochrome that is produced by wood-degrading fungi along with other ligninolytic enzymes. It is supposed to play a decisive role in the degradation of lignocellulosic biomass and could therefore support the production of biorenewables and biofuels. Recently, CDH was found in the white rot fungus *Ceriporiopsis subvermispora*, which exhibits the distinctive feature of selective lignin degradation while leaving the valuable cellulose almost untouched. In this thesis, CDH production in *C. subvermispora* was studied with respect to varying culture conditions, like pH, temperature, nutrient concentration and inducing compounds. The optimal medium for CDH production contained 20 g L^{-1} α-cellulose, 10 g L^{-1} yeast extract and 10 g L^{-1} peptone and highest CDH activity was found at a cultivation temperature of 25°C and an initial pH value of 5.5. To achieve optimal growth it is important that the fungus itself lowers the pH to the optimal milieu (pH<5.0). Additionally, it was shown that manganese sulfate in a concentration of 0.5 mM accelerated the growth of the fungus and increased the production of CDH by 20 %. Furthermore, elevated levels of extracellular exo- and endoglucanases were measured in the presence of manganese, suggesting a highly important role of the heavy metal in wood degradation. Another feature of all CDHs is their strong adsorbance to cellulose. It was found that the binding of CDH to cellulose is highly dependent on pH. The binding isotherm of CDH to α-cellulose was determined and the dissociation constant was calculated with 0.67 µM. Due to this strong adsorption to cellulose, CDH could, so far, only be detected after a long growth period. Employing a new assay procedure, the activity of cellulose-bound CDH was measured and significant amounts of the enzyme were found to be already produced at early stages of cultivation.

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List of Abbreviations

Å	Ångstrom
A_{420}	absorption at 420 nm
AIEX	anion exchange chromatography
°C	degree Celsius
CDH	cellobiose dehydrogenase
C. s.	Ceriporiopsis subvermispora
CV	column volume
cyt *c*	cytochrome *c*
Da	Dalton
DCIP	2,6-dichloroindophenol
DEAE	diethylaminoethyl
DNSA	dinitrosalicylic acid
FAD	flavin adenine dinucleotide
HIC	hydrophobic interaction chromatography
h	hour
k_{cat}/K_M	catalytic efficiency
kDa	kilo Dalton
min	minute
nm	nanometer
PDA	potato dextrose agar
rpm	revolutions per minute
SDS	sodium dodecyl sulfate
s	second
U	Unit [µmol min^{-1}]

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1 INTRODUCTION

1.1 Cellobiose dehydrogenase

Cellobiose dehydrogenase (CDH; EC1.1.99.18; cellobiose (acceptor) 1-oxidoreductase) was first discovered and isolated in 1974 from the wood degrading fungus *Phanerochaete chrysosporium* by Westermark and Eriksson (1974). The enzyme was originally termed cellobiose:quinone oxidoreductase (CBQR) due to its ability of quinone reduction (Westermark and Eriksson, 1974), but later it was found that the enzyme was a FAD containing degradation product of cellobiose dehydrogenase (Cameron, 2001).

1.2 General characteristics

CDH is an extracellular flavocytochrome that is produced by several wood degrading basidiomycetes, ascomycetes and some plant pathogenic fungi when grown on cellulose (Zamocky *et al.* 2006). CDH is a monomeric enzyme that consists of a N-terminal cytochrome domain with a *b*-type haem as cofactor and a C-terminal flavin domain with FAD as cofactor (Bao *et al.* 1993). Both domains are connected by a protease sensitive polypeptide linker comprised of 15 amino-acids. The catalytic activity is restricted to the flavin domain. So far no appropriate crystals of the whole CDH could be obtained and therefore detailed information regarding the 3 dimensional structure is not available. However, both subunits could be crystallised separately (Hallberg *et al.* 2000, Hallberg *et al.* 2002) as shown in Figure 1. Additional structure analysis was done by small-angle X-ray scattering, and the enzyme appears to be „cigar shaped“ with a width of approximately 50 Å and a length of 180 Å (Lehrner *et al.* 1996).

The molecular mass of all known CDHs varies from 79,600 Da (for *Grifola frondosa* CDH) to 115,000 Da (for *Coniophora puteana* CDH) (Zamocky *et al.* 2006). CDH is a glycoprotein with a varying degree of glycosylation from 2 % (Schou *et al.* 1998) up to 15 % (Baminger *et al.* 2001) of total CDH protein. Due to this high degree of glycosylation CDH is a highly stable enzyme and was reported to retain full activity between pH 3 and pH 10 even when incubated for 24 hours. The highest stability was observed between pH 3 and 5. Additionally, CDH was reported to be resistant to temperatures of up to 60°C for 1 hour (Bao *et al.* 1993), therefore it is regarded as an interesting biocatalyst.

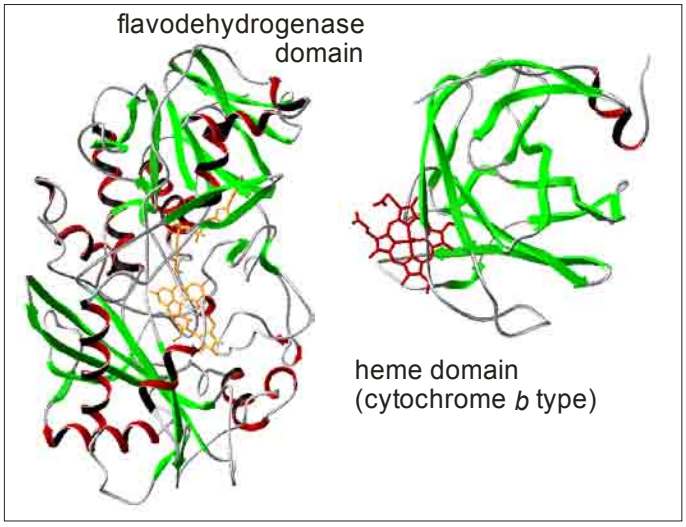

FIGURE 1: Crystal structure of the flavin domain and the haem domain of *Phanerochaete chrysosporium* CDH. The haem *b* and FAD are highlighted in stick drawings.

The dimeric subunit of cellulose, β-D-cellobiose, is regarded as the natural substrate of CDH. Higher cello-oligosaccharides were also shown to be *in vivo* substrates, although decreased catalytic efficiencies (k_{cat}/K_m) were observed (Hai *et al.* 2002). In general, CDH shows a strong preference to β-1,4-linked substrates like cellobiose, lactose or mannobiose (Henriksson et al 1998).

1.3 Catalytic mechanism

Like all dehydrogenases, CDH exhibits an oxidative and a reductive half reaction. During the reductive half-reaction, cellobiose is oxidised at the C1 position to cellobionolactone, which spontaneously hydrolyses to cellobionic acid (Morpeth, 1985) in bulky solution. The catalytic mechanism during the reductive half reaction works such that two electrons and two protons are transferred from the substrate to the FAD cofactor. The oxidative half reaction proceeds by electron transfer to either a two-electron acceptor or two equivalents of a one electron acceptor. Normally, the reduction of these electron acceptors takes place at the flavin domain but one electron acceptors can be also reduced by the haem domain due to the ability of CDH to shuttle electrons from the flavin domain to the haem domain via the so called interdomain electron transfer (Zamocky *et al.* 2006). The *in vivo* electron acceptor, however, remains to be

discovered (Morpeth, 1985; Henriksson *et al.* 1993). Concerning the catalytic role of the haem domain, two general models have been introduced (Henriksson, 2000). The electron chain model states that electrons are conveyed one by one from the reduced FAD to the haem domain, where oxidation of a one electron acceptor occurs. According to the electron sink model, one-electron acceptors are oxidised at the FAD domain, whereas the haem domain acts as an electron storage, providing proper reaction conditions for the oxidative half reaction. However, Igarashi *et al.* (2002) found that after oxidation of cellobiose further conversion of the flavin and subsequent reduction of haem exhibited almost the same reaction speed for any given substrate concentration, implying that both steps take part in a concomitant electron transfer reaction. Furthermore, mutation of Phe166, which is located in the cytochrome domain and approaches a propionat of haem, resulted in a 2.5 times slower oxidation rate of cyt *c* by CDH (Igarashi *et al.*, 2002). Thus the electron chain model is currently the favoured model of cyt *c* oxidation by CDH.

1.4 Cellulose binding

Despite its non-hydrolytic activity, CDH very specifically binds to cellulose (Renganathan *et al.* 1990), but not to xylan, mannan, starch or chitin (Henriksson *et al.* 1997; Temp and Eggert, 1999). The bound CDH is still catalytically active and it was shown that the binding site is located within the flavin domain (Henriksson *et al.* 1991). Further analysis on a genetic level showed that CDH from basidiomycetes (Class I CDH) lack a specific binding domain and adhere to α-cellulose supposedly by hydrophobic interaction. Ascomycete CDH (Class II CDH) contains a type-1 carbohydrate-binding module (CBM1) which facilitates binding (Zamocky *et al.* 2004).

1.5 Biological functions

To date, the biological role of CDH is not known with certainty. Kremer and Wood (1992) proposed the theory of the generation of hydroxyl radicals in a Fenton type reaction, which received great attention in literature. Upon oxidation of cellobiose, the electrons are shuttled from the FAD to the haem domain and transferred to a Fe(III)-complex (e.g. oxalate). In the subsequent Fenton-type reaction (i)

(i) $$Fe(II) + H_2O_2 \rightarrow Fe(III) + OH^- + OH^{\bullet}$$

hydroxyl radicals, which are believed to interact randomly with lignocellulose, are generated. *In vitro* experiments showed, that carboxymethyl cellulose, xylan and lignin can be depolymerized in the presence of CDH, cellobiose, hydrogen peroxide and Fe(III) (Henriksson, 1995). A similar effect of this system was observed when insoluble cellulose in the form of kraft pulp was treated with CDH (Mansfield *et al.* 1997). Furthermore, Ander *et al.* (1996) demonstrated, that the nodes of pulp fibres (short segments that represent „weak points“) became swollen in the presence of CDH. The authors suggest a synergetic mechanism with cellulases, which could penetrate cellulose upon swelling.

A possible role of CDH in lignin degradation was suggested by Roy *et al.* (1994). Thus, CDH could increase the turnover number of manganese dependent peroxidase (MnP) by solubilisation of complexed $Mn(IV)O_2$. MnP is believed to play a highly important role in lignin degradation by oxidizing Mn(II) to reactive Mn(III)-species that perform oxidation of phenolic lignin compounds. Hilden *et al.* (2000) furthered the theory by reporting that MnP is able to oxidise also a non-phenolic model compound of lignin after pre-treatment with CDH. Hydroxyl radicals generated by CDH were shown to demethoxylate and hydroxylate the compound, causing the conversion into a phenolic structure.

1.6 CDH from the basidiomycete *Ceriporiopsis subvermispora*

Recently, CDH was discovered in the white-rot fungus *C. subvermispora* (Harreither *et al.* 2009). Unlike other model organisms, such as *P. chrysosporium* and *T. versicolor*, *C. subvermispora* is one of the few selective delignifiers, leaving the cellulosic components of wood almost untouched (Fackler *et al.* 2007). A complex array of extracellular and oxidizing enzymes like MnP and laccase, which provide an extracellular pathway for lignin degradation (Rüttiman *et al.* 1992), are responsible for the specific decomposition of lignin. To date, 7 isoforms of MnP and 4 isoforms of laccase have been identified in this fungus (Lobos *et al.* 1994, Lobos *et al.* 1998). Investigation of crude extracts derived during a biopulping process revealed a high level of manganese peroxidase, whereas laccases were of minor significance

(de Souza-Cruz *et al.* 2004). *Cs*CDH differs from other white rot CDHs by the most acidic pH optimum found so far and the highest substrate specificity towards β-1,4 linked carbohydrates such as cellobiose and lactose (Harreither *et al.* 2009).

1.7 Applications

The most promising applications of *Cs*CDH in the field of biotechnology and food technology are:

Use in the pulp- and paper industry:

The application of CDH in the pulp and paper industry was suggested by Stapleton and Dobson (2003). In the presence of complexed iron, CDH could bleach pulp and support the degradation of lignocellulosic biomass (Henriksson *et al.* 1995). Previously, CDHs from the white rot fungus *Schizophyllum commune* (Fang *et al.* 1999) and the soft rot and thermophilic fungus *Humicola insolens* (Schou *et al.* 1994) were reported to aid the delignification and bleaching of kraft pulp. A major drawback of the CDH system may be the unspecific action of hydroxyl radicals, which are also likely to degrade the valuable cellulose (Mansfield *et al.* 1997). Therefore the application of whole microorganisms, like the specific delignifier *C. subvermispora*, seems to be a promising attempt.

Bioremediation:

Aromatic waste products like polyacrylate and polyacrylamide, which are harmful to the environment, were shown to be depolymerized in cultures of *P. chrysosporium* (Stahl *et al.* 2000). Since the presence of iron increased the removal of these compounds, a Fenton type reaction involving the generation of hydroxl radicals was suggested as main mechanism for depolymerization.

Biocatalyst for production of lactobionic acid:

Baminger *et al.* (2001a) and Ludwig (2004) employed CDH for the production of lactobionic acid. Conversion of lactose to lactobionic acid was shown to be efficient, without generating any by-products or residual substrates. The oxidation of the cheap raw material lactose to lactobionic acid has several biotechnological applications. This aldonic acid can be used as

stabilizing agent for preservation of organs before transplantation, interferon preparations and to enhance the solubility of interferon antibiotics. In food technology it can be used to reduce the souring and ripening time of cheese and yoghurt, to improve the taste perception of sour, to eliminate the bitterness and enhance the flavour (Ludwig, 2004).

Use in amperometric sensors and biofuel cells:

CDH has shown very interesting properties in the field of bioelectrochemistry and is used as biocomponent in amperometric biosensors and bioanodes in biofuel cells (Gorton *et al.* 1999, Stoica *et al.* 2006, Coman *et al.* 2008, Tasca *et al.* 2008a, Tasca *et al.* 2008b, Tasca *et al.* 2009). A very promising application of *Cs*CDH is in so called third generation biosensors. Third generation biosensors utilize enzymes as biological recognition elements which are able to directly communicate with the electrode. The key benefit of biosensors based on direct electron transfer is the absence of any mediator. Therefore third generation biosensors exhibit higher selectivity due to the simple reaction sequence and consequently less interfering reactions. A major advantage of *Cs*CDH over other enzymes could be its high specificity for β-1,4 linked substrates. Therefore, this CDH is a perfect bioelement in a third generation lactose- or cellobiose-biosensor, as the already employed CDHs from *Trametes villosa* and *Phanerochaete sordida* (Stoica *et al.* 2006).

2 MATERIALS

2.1 Chemicals

All fine- and bulk-chemicals as well as bases, acids and detergents were of analytical grade and purchased from Roth (Vienna, Austria), VWR (Vienna, Austria) and Sigma-Aldrich (Fluka) (Vienna Austria).

2.2 Organism

The strain *Ceriporiopsis subvermispora* FP-90031 was obtained from the Center for Mycology, USDA Forest Products Laboratory (Madison, Wisconsin, USA).

2.3 Media

2.3.1 Potato dextrose agar

Component	Concentration
Potato extract	4 g L^{-1}
Glucose	20 g L^{-1}
Agar-agar	15 g L^{-1}

2.3.2 Standard liquid cultivation medium of *C. subvermispora*

Component	Concentration
α-Cellulose	20 g L^{-1}
Yeast extract	10 g L^{-1}
Peptone from meat	10 g L^{-1}
$MgSO_4 \cdot 7H_2O$	1 g L^{-1}
Trace element solution	0.3 mL L^{-1}

The medium was adjusted to the required pH with H_3PO_4 or NaOH.

2.3.3 Cultivation medium for the stirred bioreactor

Component	Concentration
Cellulose (microcrystalline)	30 g L^{-1}
Yeast extract	30 g L^{-1}
$MgSO_4 \cdot 7H_2O$	1 g L^{-1}
Trace element solution	0.3 mL L^{-1}

The medium was adjusted with H_3PO_4 to pH 5.4 prior to sterilization.

2.3.4 Trace element solution

Component	Concentration*
$ZnSO_4 \cdot 7H_2O$	1.0 g L^{-1}
$MnCl_2 \cdot 4H_2O$	0.3 g L^{-1}
H_3BO_3	3.0 g L^{-1}
$CoCl_2 \cdot 6H_2O$	2.0 g L^{-1}
$CuSO_4 \cdot 5H_2O$	0.1 g L^{-1}
$NiCl_2 \cdot 6H_2O$	0.2 g L^{-1}
H_2SO_4 conc.	4.0 mL L^{-1}

*Sachslehner et al. 1997

2.3.5 Storage solution for CDH (2 x concentrated)

Component	Concentration
Bentoine	1 M
Glycerol	20%

2.3.6 Equipment

Instrument	Company	Application
Stirred bioreactor, 50 L vessel	Applikon	Large-scale fermentation
Äkta Explorer system	GE Healthcare	Protein purification
Äkta Purifier system	GE Healthcare	Protein purification
UV/VIS Photospectrometer Lambda 35	Perkin Elmer	Cyt *c*, DCIP assay
Diode array photometer Agilent 8453	Agilent Techn.	Cyt *c* assay
Spectrophotometer Beckman DU-62	Beckman	Protein concentration, D.N.S.A. assay
Centrifuge, Sorvall (RC 26 Plus, MC 12V, Evolution RC)	Du Pont	Several centrifugation steps
Ultracentrifuge, Beckman L8-55	Beckman	Several centrifugation steps
Omnifuge 2.0 RS	Heraeus	Several centrifugation steps
Eppendorf Centrifuge 5415R	INULA	Several centrifugation steps
Laboratory Autoclave, Certoclave EL 10L	Kelomat	Sterilisation
Autoclave, Varioclave 500, 135L	H+P Labortechnik	Sterilisation
Sterile bench HS-P 12/2 (LF-Werkbank)	Heraeus	all aseptic steps
pH- and conductivity instruments	WTW and Orion	fermentation, buffer preparation, samples
Membrane vacuum pump CVC 2	Vacuubrand	filtration of buffers, fermentation broth
Culture orbital shaker	SZT	Small scale fermentation
SDS-PAGE: Hoefer Mighty Small II SE250	Amersham Bioscien.	Protein purity
Ultrafiltration polysulfone membran	Pall Corporation	Concentration and desalting
Shaker Infors Unitron	Infors	Small scale fermentation
Shaker HAT	Infors	Small scale fermentation
Thermomixer compact	Eppendorf	Binding isotherm
Sonicator	Ultrasonic	Homogenisation prior to CDH activity assay
Balances	Sartorius	Media preparations

3 METHODS

3.1 Cultivation of *C. subvermispora* on solid medium

The fungal strain *C. subvermispora* FP-90031 was continuously subcultered from the internal strain collection of the Division of Food Biotechnology (BOKU, Vienna). It was grown on potato dextrose agar (PDA) petri dishes placed upside-down at 25°C until the surface of the plate was covered with mycelium (4-5 days). The freshly overgrown plates were either used for the inoculation of liquid cultures or sealed with parafilm and stored at 4°C until further use. The plates were continuously subcultered every 4-5 weeks under aseptic conditions by transferring agar plugs of approx. 1 cm^2 from mature cultures onto new PDA plates.

3.2 Cultivation of *C. subvermispora* in liquid medium

All experiments were performed in 300 mL unbaffled flasks containing 100 mL liquid medium. The pre-cultures for the 30 L scale cultivation were grown in 1 L flasks containing 300 mL liquid medium.

The medium, containing α-cellulose as insoluble substrate, was autoclaved in the flask for 20 minutes at 121°C. For all experiments conducted in shaking flasks, α-cellulose was used as main carbon source, whereas microcrystalline cellulose was used in the 30 L cultivation.

Only freshly overgrown Petri-dishes were used for the inoculation of the shaking flask cultures. Agar plugs with a surface of approximately 1 cm^2 were used for the inoculation of 100 mL medium. The agar was aseptically sliced to small pieces with a scalpel and directly transferred into the flask. These were continuously shaken (eccentricity, 2.5 cm) at 110 rpm.

3.3 Sampling

For sampling, autoclaved and cut 1 mL pipette tips with an orifice of approx. 3 mm were used in order to allow the pipetting of the viscous fungal broth. Previous to all measurements, the sample was clarified at 13,200 rpm for 5 minutes to obtain a mycelium free supernatant.

3.4 Optimization of the cultivation medium of *C. subvermispora*

In order to determine the optimal conditions for CDH production, *C. subvermispora* was grown at 3 different temperatures (25°, 30°C and 35°C) and 4 different initial pH values (4.0, 5.5, 7.0 and 8.5). In addition the concentration effects of α-cellulose, peptone and yeast extract was investigated. At the optimized cultivation conditions the impact of various inducers on the production of CDH was tested.

3.5 Cultivation of *C. subvermispora* in a stirred bioreactor

Cultivation of *C. subvermispora* was performed at 30 L scale in a stirred bioreactor. The dissolved oxygen saturation, temperature and pH value were monitored *via* probes and the data were recorded frequently. Before sterilization and inoculation, all technical instruments were attached to the fermenter and calibrated according to the manuals.

The medium, except for the microcrystalline cellulose, was autoclaved inside the fermenter. Therefore, 5 litres concentrated fermentation medium (calculated for 30 L) were diluted with 13.5 L reversed osmotic water and 3 mL anti-foam was added. For reasons of security, a pressure test with air (1 bar overpressure was maintained for 3 minutes) was performed before hot steam sterilization (121°C, 20 min) was applied.

Since microcrystalline cellulose is likely to block the sparger, it was autoclaved separately in two bottles, each containing 5 L tap water and 450 g cellulose. The bottles were connected to the fermenter previous to autoclaving by two push valves and microcrystalline cellulose was transferred into the fermenter after sterilization. Additionally, an autoclaved bottle containing 1 L 20 % phosphoric acid was connected to the fermenter to allow the regulation of the pH-value.

A total of 1.5 L inoculation medium (5%, v/v) was transferred aseptically into the fermenter through a septum. Therefore, 5 pre-culture flasks, each containing 300 mL medium, were grown for 5 days in the standard cultivation medium.

The temperature was set to 30°C and the stirrer speed was 150 rpm (three Rushton turbines; tip speed, 0.79 m s^{-1}). The aeration rate was adjusted to 15 vvm (L air L^{-1} medium min^{-1}). Initially, the pH was allowed to float freely. Later, an infection occurred and the pH was titrated to 4.35 until the end of the cultivation to reduce bacterial growth. Samples were taken every 24 hours through a sample valve, which was sterilized for 15 minutes with hot steam

before and after use.

3.6 Purification of CDH

Prior to the following purification steps, mycelium and residual solid compounds of the fermentation broth were removed by centrifugation (6,000 × g for 20 minutes). The supernatant was further cleared by vacuum filtration employing 120 mm cellulose filterpapers (cut off < 8 µm). No activity was lost during this step.

3.6.1 Cross-flow filtration

Concentration and desalting of the fermentation broth was run with a cross-flow hollow-fibre module with a cut-off of 10,000 Da. The process was performed overnight until the volume was reduced to 2.5 L and the conductivity below 5 mS cm^{-1}, respectively.

3.6.2 Anion exchange chromatography (AIEX)

The initial purification step was performed with a weak anion exchange column with a volume of 500 mL employing the Äkta Explorer system (GE Healthcare; Buckinghamshire, United Kingdom). The specifications of the gel and the purification conditions are shown in Table 1. Adsorption at 420 nm (haem domain), 450 nm (flavin domain) and 280 nm (total protein) was constantly monitored along with the conductivity and the pH value.

The sample solution was applied on the already equilibrated column (buffer A) with a flow rate of 20 mL min^{-1}. Afterwards, the column was washed with buffer A until constant values for the absorptions of the UV lamp and a constant conductivity were measured. The flow-through was constantly checked for CDH activity employing the cyt *c* assay.

CDH was eluted within 5 column volumes with a linear salt gradient from 0-0.5 M NaCl (0-100 % buffer B). The fractions were collected manually, judging the adsorption at 420 nm and 450 nm and the red colour of CDH (haem domain). From each fraction CDH activity (cyt *c* assay and DCIP) and level of extracellular protein (Bradford assay) were determined. Fractions with similar specific activity were pooled.

TABLE 1: Conditions and specifications for AIEX chromatography	
Column material	diethylaminoethyl (DEAE) sepharose
Column volume	500 mL
Buffer A	Sodium acetate buffer 50 mM, pH=5.5
Buffer B	Sodium acetate buffer 50 mM, pH=5.5 with 0.5 NaCl
Gradient	0-100% buffer B

After the purification, the column was cleaned with 0.5 M NaOH followed by 0.5 M acetic acid and was stored on 20% ethanol.

3.6.3 Hydrophobic interaction chromatography (HIC)

For subsequent HIC, phenyl-source bedmaterial derived from GE Healthcare was employed. Conditions of the purification and characteristics of the columns are given in Table 2. In contrast to the purification based on anion exchange, the protein is eluted with decreasing salt concentration.

The fractions from the AIEX were cleared by ultracentrifugation (30,000 rpm, 30 min) and mixed with a solution of saturated ammonium sulphate to a final concentration of 20 % $(NH_4)_2SO_4$. Sodium chloride was added until the conductivity of the sample was equal to the conductivity of buffer A (~110 mS cm^{-1}).

CDH was eluted within 2.5 column volumes with linear gradient from 0-100 % buffer B. Again, samples were manually collected judging from the adsorption at 420 nm and 450 nm and the red colour of CDH. Enzyme activity (cyt *c* assay, DCIP) was tested along with the level of extracellular protein (Bradford assay) and fractions with similar specific activity were pooled.

TABLE 2. Conditions and specifications for HIC chromatography

Column material	Phenyl source
Column volume	70 mL and 20 mL
Buffer A	Sodium acetate buffer 50 mM, pH=5.5; 0.2 M NaCl; 20% ammonium sulfate
Buffer B	Sodium acetate buffer 20 mM, pH=5.5
Gradient	0-100% B

Additionally, a prepacked 1 mL RESOURCE HIC column was used previous to deglycosylation of the enzyme (specifications given in Table 3).

TABLE 3: Conditions and specifications for HIC chromatography with the 1 mL RESOURCE column

Column material	Monodisperse polystyrene cross-linked with divinyl benzene
Bedsize volume	1 mL
Buffer A	Sodium acetate buffer 50 mM, pH=5.5; 0.2 M NaCl; 20% ammonium sulfate
Buffer B	Sodium acetate buffer 20 mM, pH=5.5
Gradient	0 -100 % B

All columns were cleaned with 0.5 M NaOH followed by 0.5 M acetic acid and stored in 20 % ethanol.

3.6.4 Ultrafiltration

The samples derived from the HIC were further concentrated and desalted by ultrafiltration employing an Amicon Ultra-15 centrifugal filter with a cut-off of 10,000 Da. The samples were concentrated at 4,000 g.

3.7 Deglycosylation of CDH

For subsequent determination of the crystal structure of CDH (currently performed at the Royal Institute of Technology, Stockholm, Sweden) glycostructures had to be removed from the enzyme. The concentrated sample was therefore mixed with Endo-β-N-acetylglucosaminidase H (EndoHf) and α-mannosidase in concentrations of 1000 U mg^{-1} CDH and 0.02 mg mg^{-1} CDH, respectively. A total of 4.8 mg CDH was used for the procedure. The added activity is given in Table 4.

TABLE 4: Reaction mix for deglycosylation of 4.8 mg CDH

Enzyme	concentration	used concentration
EndoHf	0.02 mg mg^{-1}	0.086 mg
α-mannosidase	1000 U mg^{-1}	4800 U

The sample was desalted by using an Amicon Ultra-15 Cell (Millipore; Billerica, Massachusetts, USA) and refilled with 50 mM sodium citrate buffer, pH 5.5. The concentration step was repeated and the sample with a total volume of 0.5 mL was mixed with

a solution of 10 mM zinc chloride in a ratio of 1:8. After adding the deglycosylating enzymes, the sample was incubated for 12 h at room temperature under constant agitation (150 rpm).

3.8 CDH activity assay using cytochrome *c*

The CDH activity in crude extracts or partially purified fractions was determined with the cytochrome *c* (cyt *c)* assay. Upon oxidation of the electron donor lactose, the one electron acceptor cyt *c* is reduced by CDH. Reduction of this one-electron acceptor requires the presence of the haem domain in order to achieve electron flow, thus the cyt *c* assay detects only the holoenzyme.

For CDH measurements the assay described by Canevascini *et al.* (1991) was slightly modified with respect to the different requirements of *Cs*CDH. A sodium acetate buffer was used in a concentration of 100 mM (stock solution) and the pH was adjusted to 4.0. In order to avoid substrate inhibition, the natural substrate of CDH, cellobiose, was replaced by lactose. The pipetting scheme is given in Table 5.

TABLE 5: Pipetting protocol of the cyt *c* activity assay.

Component	Concentration	Volume
Cytochrome *c* (in distilled water)	1 mM	20 µL
Lactose solution	300 mM	100 µL
Sodium-acetate buffer, pH 4.0	100 mM	860 µL
Sample	0 – 0.3 U	20 µL

The reduction of cyt *c* was followed spectrophotometrically at 550 nm (ε_{550}=19.6 mM^{-1} cm^{-1}) for 120 seconds.

3.9 CDH activity assay using 2,6-dichloroindophenol

Another reference method for the determination of CDH activity employs the two-electron acceptor 2,6-dichloroindophenol (DCIP) (Baminger *et al.* 2001). Thereby, the CDH holoenzyme and the CBQ (FAD-containing) fragment can be detected. Oxidation of the substrate lactose is accompanied by the concurrent reduction of the two-electron acceptor DCIP. As a result, the initially dark-blue DCIP solution is steadily decolourized, which was measured spectrophotometrically over a period of 120 s at 520 nm (ε_{520}=6.80 mM^{-1} cm^{-1}). The

decrease in the absorbance of DCIP is directly proportional to the actual enzyme activity. In order to omit the disturbing influence of enzymes such as laccase, which immediately reoxidizes CDH, sodium fluoride as irreversible laccase inhibitor was added to the assay. Again, the *in vivo* substrate cellobiose was replaced by lactose. The composition of the reaction mixture is shown in Table 6.

TABLE 6: Pipetting scheme for the DCIP assay.

Component	Concentration	Volume
2,6-Dichloroindophenol (DCIP)	0.3 mM	100 µL
Lactose	30 mM	100 µL
Sodium-fluoride	4 mM	20 µL
Sodium-acetate buffer, pH 4.0	100 mM	760 µL
Sample	0 - 1 U	20 µL

3.10 Determination of the activity of cellulolytic enzymes with the 3,5-dinitrosalicylic acid assay

In the presence of reducing sugars 3,5-Dinitrosalicylic acid (DNSA) is reduced to 3-Amino-5-nitrosalicyic acid and the carbonyl group of the sugar is oxidized to a carboxyl group. The overall reaction scheme is given in Figure 2. As a result, the colour of the solution changes from bright-yellow to red-brown and the absorption maximum is shifted to 575 nm. The activity of different cellulolytic enzymes can be estimated by measuring the quantity of the produced sugar (overview in Table 7) (Miller 1956).

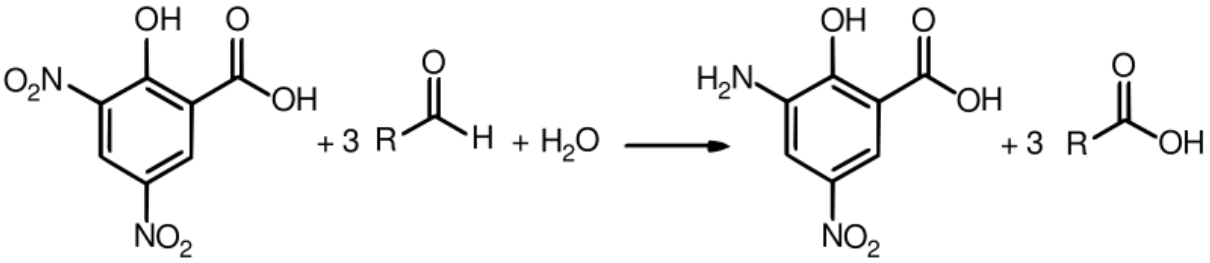

FIGURE 2: Reduction of 3,5-Dinitrosalicylic acid in the presence of reducing sugars.

TABLE 7: Possible enyme assays employing DNSA

Enzyme	Substrate	Cleavage product
Xylanase	Xylan	Xylose
Mannanase	Mannan	Mannose
Endoglucanase	Carboxymethyl cellulose	Glucose
Exoglucanase	Cellulose	Glucose

In the case of the model-compound carboxymethyl cellulose (provided as sodium salt) only endoglucanases can act on the cellulose due to the chemically modified ends of the molecules. The corresponding substrates were incubated with the culture supernatant and the quantity of the released sugar was determined. Enzyme activity was calculated from the incubation time, the sugar concentration and the sample dilution.

A 1% DNSA stock solution (Table 8) was prepared one day before utilization. All compounds were diluted in deionized water, stored in a light protected brown flask at 4°C and were continuously stirred.

TABLE 8: Composition of the DNSA stock solution

Compound	Concentration [g L^{-1}]
3,5-Dinitrosalicylic acid	10.0
Sodium sulfite	0.5
Sodium hydoxide	10.0
Phenol	2.0

Sample solution (600 µL) was added to 600 µL of the DNSA reagent in a 2 mL reaction-tube. The mixture was heated at 90°C for 15 minutes to develop an amber-like colour.

After incubation 200 µL of a 40 % potassium-sodium-tartrate solution (Rochelle-salt) were immediately added in order to stabilize the colour. After chilling the sample on ice for 5 minutes the absorbance at 575 nm was recorded with a Beckman Coulter DU800 UV/Vis spectrophotometer. The assay yields reliable values within a sugar concentration of 0.2 – 1.0 g L^{-1}. For the following experiments (determination of the activity of exoglucanases and endoglucanases) a glucose calibration curve was generated. One unit of enzymatic activity was defined as the amount of enzyme that releases 1 µmol of glucose per min under the assay conditions.

3.11 Determination of the protein concentration

Protein concentrations were determined according to Bradford (1976). A pre-fabricated dye solution (Biorad, Hercules, California, USA) was used for all protein determinations. One mL of the Bradford stock solution was mixed with 20 µL of the appropriately diluted sample. The sample was thoroughly mixed with a plastic spatula and incubated for 15 minutes at room temperature. The absorbance was recorded with a Beckman Coulter DU800 UV/Vis spectrophotometer at 595 nm. The sample concentration was calculated with a calibration curve in the range of 0.1 – 1.0 mg mL^{-1} with bovine serum albumin (BSA) as reference protein.

3.12 SDS-Polyacrylamid Gel Electrophoresis

The purity of the protein fraction after deglycosylation was investigated with a sodium dodecyl sulfate polyacrylamid gel electrophoresis (SDS-PAGE) according to Laemmli (1970). For the separating gel, SDS and all solutions except for TEMED and the APS solution were pre-mixed as described in Table 9 and degassed for 20 minutes with a vacuum pump.

TABLE 9: Composition of an SDS-Gel (two 1 mm Gels)

Description	Separating gel	Stacking gel
Monomer concentration	10 % T; 2.7 % C	5 % T; 2.7 % C
Bis-acrylamid stock solution (40 %)	2.97 mL	0.62 mL
4x resolving buffer (pH 8.8)	3 mL	-
4x stacking buffer (pH 6.8)	-	0.62 mL
10 % SDS	0.12 mL	0.05 mL
Dest. H_2O	5.79 mL	3.60 mL
10 % APS (freshly prepared)	0.12 mL	0.05 mL
TEMED	5 µL	5 µL
Total	12 mL	4.95 mL

After degassing, TEMED and APS were added to the solution under constant stirring and the gel was immediately transferred to the vertical electrophoresis apparatus. The gel was covered with a water saturated 2-butanol solution in order to level the surface of the gel. After 30 minutes, the 2-butanol solution was removed completely and the stacking gel was cast after the addition of TEMED and APS solution. It was placed on top of the separating gel and a slot

comb was immediately put into the gel. After another 30 minutes, the gel was ready to use. The sample solution was diluted with distilled H_2O to a final protein concentration of 5 mg mL^{-1}. Seven µL of the sample were mixed together with 7 µL SDS containing Laemmli buffer and heated to 95°C for three minutes. 10 µL of the mixture were loaded onto the gel along with 5 µL of a molecular weight standard (BioRad Precision Plus Protein™, unstained). Electrophoresis was run at 200 V and 30 mA until the blue bands reached the opposite side of the gel (approximately 120 min).

Acrylamid/Bis (30 % T, 2.67 % C, 1:37.5)	146.0 g Acrylamide + 4.0 g Bis acrylamide dissolved in 500 mL H_2O and filtrated storage in darkness at 4 °C
4x resolving buffer (1.5 M Tris-HCl, pH 8.8)	54.45 g Tris-base + 120 mL H_2O adjusted to pH 8.8 with 6 N HCl and filled up to 300 mL with H_2O
4x stacking buffer (0.5 M Tris-HCl, pH 6.8)	6.00 g Tris-base + 60 mL H_2O adjusted to pH 6.8 with 6 N HCl and filled up to 100 mL with H_2O
10 % ammonium sulphate (APS)	50 mg ammonium sulphate 500 µL H_2O
TEMED	N,N,N',N'-Tetramethyl-ethylendiamine
1x running buffer pH 8.3	160 mL 5x running buffer pH 8.3 640 mL H_2O
5x running buffer pH 8.3	15.15 g Tris-base + 72 g glycine + 5 g SDS filled up to 1000 mL with H_2O storage at 4 °C, before use dissolved 1:5
Stock for 2xSB	2.0 mL 0.5 M Tris-HCl pH 6.8 1.6 mL glycerine (20 %) 3.3 mL 15 % SDS (6 %)

	0.4 mL 0.5 % (w/v) bromphenolblue
2x sample buffer	900 µL stock-2x-SB 100 µL β-mercaptoethanol
10 % (w/v) SDS	1.0 g SDS in 10 mL H_2O

3.13 pH-Dependent binding of CDH to α-cellulose

Purified CDH was incubated in a 1 % α-cellulose (w/v) solution of varying pH values for 45 minutes at 30°C. The used buffers and their corresponding pH values are summarized in Table 10.

Table 10: Buffer systems for binding study

Buffer system	Range	Molarity
Sodium citrate	2.5 – 3.5	50 mM
Sodium acetate	3.5 – 5.5	50 mM
Sodium phosphate	5.5 – 8.0	50 mM

The reaction mixture was continuously shaken at 800 rpm to guarantee a homogeneous distribution of the cellulose. The adsorption was stopped by centrifugation (13,200 rpm, 3 minutes) and the CDH activity of the supernatant was measured with the cyt *c* assay.

3.14 Binding isotherm of CDH to α-cellulose

The binding studies were performed with purified CDH at room temperature in 50 mM sodium acetate buffer, pH 3.5 under constant agitation on a thermomixer (900 rpm). CDH was incubated with 5 mg mL^{-1} α-cellulose for 120 minutes. The adsorption was stopped by centrifugation (13,200 rpm, 3 min) and the supernatant was tested for residual CDH activity with the cyt *c* assay and for total protein concentration with the Bradford assay.

3.15 Measurement of cellulose-bound CDH

The measurement of α-cellulose bound CDH involved the following steps: (i) sample taking, (ii) centrifugation and washing of the pellet, (iii) resuspension/homogenisation by sonication and (iv) activity assay in a stirred cuvette.

Previous to the procedure, the weight of the required reaction tubes was determined for later calculations. Samples were taken aseptically from the culture broth with cut and autoclaved pipette tips in order to avoid the blocking of the tip by fungal pellets. Immediately before sampling, the flask was gently shaken to guarantee the homogeneity of the culture. Then 1 mL of the broth was transferred into a 2 mL reaction tube with known weight and the supernatant was removed by centrifugation (13,200 rpm, 5 minutes). The insoluble components of the pellet were resuspended in 50 mM sodium acetate buffer (pH 4.0) and shortly mixed by a vortex-mixer. The supernatant was removed by centrifugation and the supernatant was tested for CDH activity employing the cyt *c* assay. In case of measurable activity, the washing step was repeated as described above unless CDH was not detectable anymore.

Then the supernatant was removed again and the wet-weight of the sample was determined. One mL of 50 mM sodium acetate buffer (pH 4.0) was added and the pellet was resuspended again. Sonication of the sample was performed in order to achieve approximate homogeneity. Therefore the power output of the sonicator was set to 80 percent and the sample was treated with 5 consecutive pulses. Immediately after the sonication step the sample was chilled on ice for at least 1 minute. The procedure was repeated until good suspension of the sample. Since CDH proofed to be very resistant to sonication, the procedure was repeated up to 5 times.

For the activity measurement, an Agilent 8453 diode array photometer was used. 1.9 mL cyt *c* solution (as described above in the cyt *c* assay) was pipetted in a 3 mL quartz cuvette and stirred with a small magnetic stirrer. Immediately before the sample solution pipetted, the reaction tube was briefly mixed in order to homogenise the suspension. The reaction was started by adding 100 µL of the sample solution and the reaction was followed over a period of 160 seconds. Due to the high concentration of α-cellulose and fungal biomass, an immediate increase in absorption was observed as soon as the sample was added. When the background exceeded an absorption of 1, half of the sample solution was used.

To achieve similar conditions throughout all measurements, the sample was added 5 seconds after starting the data acquisition and the section from 10 to 140 seconds was selected for

calculation of the adsorption units.

After the measurement, the supernatant of the sample solution was removed by centrifugation (13,200 rpm, 5 minutes) and the pellet was dried overnight in order to determine the dry mass of the pellet. Activity of the sample was calculated as U g^{-1} dry biomass.

Samples were measured in triplicates at least. However, in samples derived from cultures older than 14 days, the mycelium could hardly be homogenized with the sonication step and the background was too disturbing to obtain reliable data.

4 RESULTS

4.1 Optimisation of the cultivation medium of *C. subvermispora*

In order to determine the optimal conditions for the cultivation of *C. subvermispora*, the standard liquid medium (see 2.3.3) was varied in the concentration of yeast extract and α-cellulose. Peptone from meat was supplied as additional nitrogen source. Furthermore, the effect of different temperatures and various initial pH values on CDH production was examined. All experiments were carried out in duplicates at least.

4.1.1 Effect of varying temperatures and initial pH values

As can be seen in Table 11, the growth of *C. subvermispora* is crucially influenced by both the cultivation temperature and the initial pH-value. Regarding the CDH activity, high or low initial pH-values (8.5 and 4.0) resulted in low CDH activity.

TABLE 11: Temperature and pH dependence of CDH production

Temperature [°C]	Initial pH [-]	Volumetric activity [U L^{-1}]	Volumetric activity [%]	Cultivation time [days]
25	8.5	11	5	18
25	7.0	146	67	18
25	5.5	219	100	18
25	4.0	0	0	18
30	8.5	0	0	16
30	7.0	22	10	16
30	5.5	163	74	16
30	4.0	21	10	16
35	8.5	0	0	16
35	7.0	0	0	16
35	5.5	0	0	16
35	4.0	0	0	16

The highest CDH activity was observed at a temperature of 25°C and a pH value of 5.5. At 30°C the growth of the fungus was slightly accelerated, but the maximum CDH activity was lower. No growth at all could be observed at 35°C.

The time-course of a typical cultivation of *C. subvermispora* in a shaking flask is given in Figure 3. All cultivations were performed at 25°C and pH 5.5 unless otherwise stated.

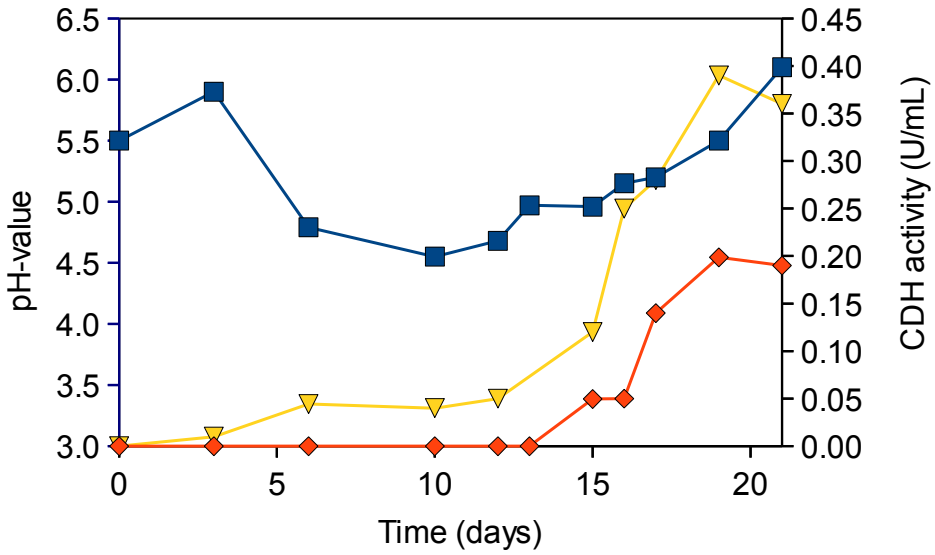

FIGURE 3: Cultivation of *C. subvermispora* in shaking flasks. The pH (blue line, squares), extracellular protein level (yellow line, triangles) and CDH activity (red line, diamonds) were constantly measured.

4.1.2 Variation in the concentration of α-cellulose, peptone and yeast extract

The different media compositions, highest CDH activities and the corresponding cultivation time is shown in Table 12.

The highest activity was measured with the standard cultivation medium and a peak activity of 235 U L^{-1} was reached after 19 days. However, the flasks supplied with 40 g L^{-1} cellulose still exhibited a pH value of 4.40 when the experiment was terminated, indicating that the culture was still viable. When grown on 30 g L^{-1} α-cellulose, cultivation time was extended by approx. 1 week. In cultures grown on 5 g L^{-1} cellulose oscillating levels of CDH were observed. After 13 days an activity of 64 U L^{-1} was measured, on day 16 no CDH activity was detectable. A second maximum of 116 U L^{-1} was determined after 21 days. Cultures lacking either yeast extract or peptone generally exhibited lower CDH activities and the cultivation time was significantly elongated.

TABLE 12: Variation in the concentration of cellulose, yeast extract and peptone

Cellulose [g L^{-1}]	Yeast extract [g L^{-1}]	Peptone [g L^{-1}]	Volumetric activity [U L^{-1}]	Cultivation time [days]
20	10	10	235	19
5	10	10	116	21
10	10	10	121	20
30	10	10	233	28
40	10	10	11	28
20	10	0	171	21
20	0	10	153	28
20	20	0	171	18
20	0	20	215	24
20	20	20	221	18
20	40	0	189	24
20	0	40	187	24

4.1.3 Macroscopic and microscopic assessment of growth

It was observed that the colour of the culture broth changed from bright-yellow after inoculation to a yellowish-green tone in the exponential growth phase and finally became brownish-green in later phases of cultivation when the highest CDH activity was measured. Within the first week of cultivation the formation of pellets was observed (Figure 4 A). In the following cultivation phase when the pH decreased again, the surface of the pellets changed to a “fluffy” structure and finally disintegrated (Figure 4 B). In the latter stages of cultivation the fungal biomass was homogeneously distributed in the shaking flask (Figure 4 C).

FIGURE 4: Appearance of *C. subvermispora* mycelium in shaking flasks on day 4 (A), day 14 (B) and day 19 (C).

On a microscopic level the amount of mycelium in the first stages of cultivation was low. On day 4, hardly any hyphae could be spotted and the α-cellulose was left untouched (see Figure 5 A). When the pH decreased, the amount of mycelium in the medium increased rapidly,

reaching a maximum at the end of the exponential growth phase. All cellulose fibres were enclosed from the fungal hyphae and degraded (Figure 5 B).

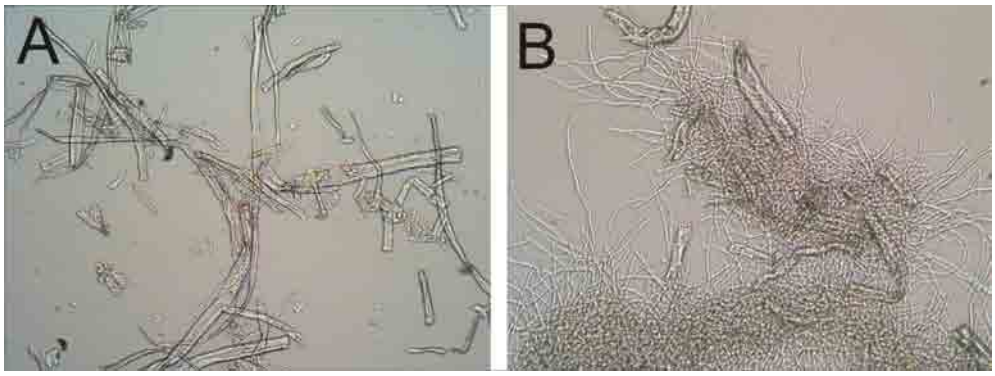

FIGURE 5: Microscopic evaluation of fungal growth on day 4 (A) and day 16 (B).

A quantification method for fungal growth based on the decrease in the length of the cellulose-fibres and the increase in hyphae length was assessed. But due to the inhomogeneity of the α-cellulose fibres and unavoidable errors during sampling, this approach failed by lack of reproducibility.

Cultures grown on an initial pH value of 8.5 exhibited the typical pellet formation, but the degree of pellet-disintegration was generally low and the colour changed only slightly towards a brownish shade. Pellet formation was also observed at initial pH values of 4.0. The change in colour into a green-yellow tone was apparent and hyphae could be spotted in the medium. Judging from the macroscopic and microscopic observations, adaption of the fungus to this low pH value seems to be possible, although growth was significantly slowed down. When no trace element solution was added to the medium, the fungus grew to large pellets and CDH production was significantly lower (see Figure 6).

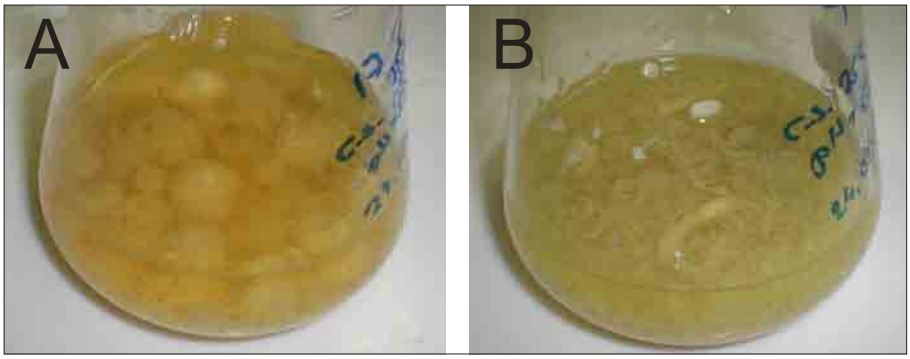

FIGURE 6: In cultures of *C. subvermispora* without trace element solution (TES) the fungus grew to large pellets with a diameter of approx. 0.5-1.0 cm (A), whereas the mycelium in the standard cultivation medium with TES is distributed rather homogeneously (B). Also, the colour of the TES-lacking culture appeared to be yellowish and was much lighter in colour.

As a consequence the trace element solution was added to all flasks in the further cultivation experiments.

4.2 Cultivation in a stirred bioreactor and purification of CDH

During the cultivation of *C. subvermispora* in a 30 L stirred bioreactor, a microbial infection occurred, as verified by microscopic inspection. The infection appeared to be rod-like bacteria which exhibited resistance to the antibiotics ampicillin, kanamycin and chloramphenicol (data not shown). As a consequence, the pH value was automatically adjusted to and maintained at 4.35 with sterilized 20 % phosphoric acid in order to suppress bacterial growth. Due to the infection the fermentation process was elongated to 12 days (see Figure 7).

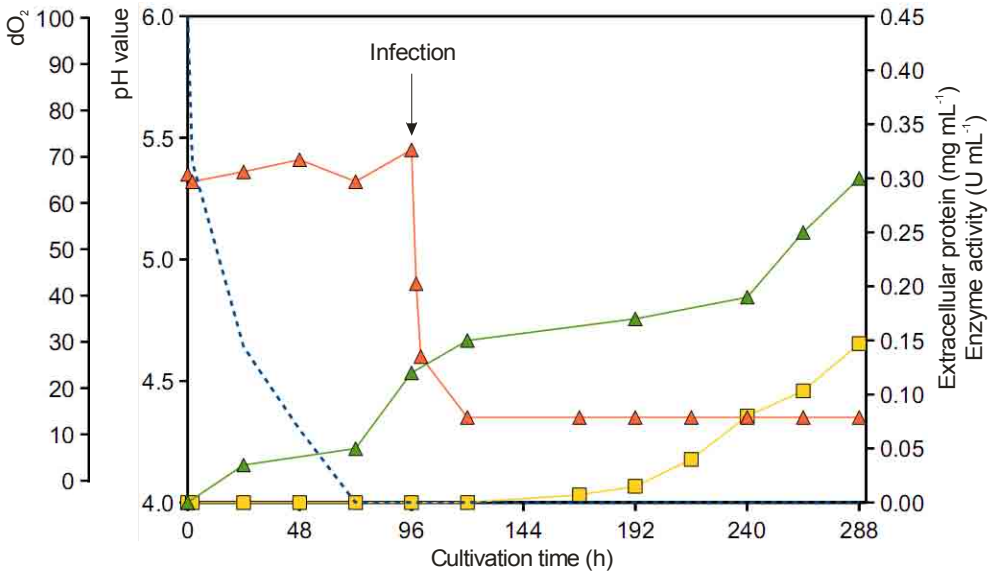

FIGURE 7: Cultivation of *C. subvermispora* at 30 L scale. Extracellular protein (green line) and enzyme activity (yellow line) were measured daily. Oxygen saturation (dashed line) was measured in-line. The pH value (red line) was initially allowed to float freely; when an infection occurred it was lowered to and automatically maintained at 4.35.

Despite the infection, activity in the crude extract was as high as 147 U L^{-1} when the fermentation was terminated and the whole fermentation yielded a total of 3403 U. The overall purification scheme is given in Table 13.

TABLE 13: Purification of CDH from *C. subvermispora*

Purification step	Total protein [mg]	Total activity [U]	Spec. Act. [U mg^{-1}]	Yield [%]	Purification [fold]
Culture supernatant	7081	3403	0.48	100	1.0
DEAE-Sepharose	1620	2152	1.33	63	2.8
Mono Q source	24	433	18	20	13.6

During the hydrophobic interaction chromatography about $^{2}/_{3}$ of the enzyme activity was lost for unknown reasons. Only a minor quantity could be eluted from a 20 mL column, and purification completely failed with a 70 mL phenyl source column. The columns were washed with 10 % PEG and HQ-water, but none of the eluted fractions exhibited any CDH activity. When the column finally was washed with 0.5 M NaOH, the eluted fraction showed a high adsorption at 420 nm and was bright yellow in colour, which could indicate denatured CDH. The remaining fraction of partially purified enzyme was therefore purified by a strong anion exchanger (Mono Q source) with a bedsize of 5 mL.

4.3 Deglycosylation of CDH

A fraction of the purified enzyme, intended for subsequent crystallisation and structure determination, was additionally deglycosylated and further purified. The fraction contained approximately 260 U and was pre-purified with a 1 ml phenyl source column, deglycosylated and finally applied on a 5 mL Mono Q source. In contrast to previous purification steps based on hydrophobic interaction, the 1 mL RESOURCE column worked faultless and a total of 239 U could be recovered. The fractions exhibiting the highest specific activities were pooled to a single fraction of 154 U with a specific activity of 32 U mg^{-1}.

Glycosylation was performed overnight at room temperature in a light protected reaction tube (see Table 4). About 25 % of the enzyme was lost during this step, and after the strong interaction chromatography step a total of 84 U with a specific activity of 28 U mg^{-1} were obtained. The homogeneity of the fraction was confirmed with a denaturing SDS-gel (see Figure 8).

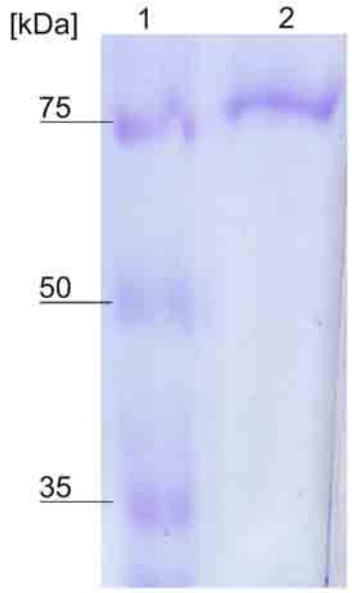

FIGURE 8: SDS PAGE analysis. Lane 1, molecular mass marker; lane 2, deglycosylated and purified *C.s.*CDH.

4.4 CDH induction by manganese sulfate

4.4.1 Induction studies

Components listed in Table 14 were added to liquid cultures of *C. subvermispora* to investigate the inducing effect on CDH production. Table 14 summarizes the induction experiments.

TABLE 14: Induction studies of CDH

Compound	Initial concentration	highest CDH activity [U mL^{-1}]	CDH activity [% blank]
Negative control	-	201.3	100
Manganese(II)sulfate	0.1 mM	241.0	120
Indulin AT	200 mg/L	97.1	48
Indulin AT	100 mg/L	165.0	82
Guaicol	0.5 mM	105.2	52
Vanillin AT	1 mM	173.8	86
Gallic acid	1 mM	124.6	62
Catechol	0.2 mM	199.0	99
Humic acid	100 mg/L	203.4	101
Copper(II)sulfate	0.1 mM	87.2	43
Manganese oxalate	100 µM	160.6	80
Manganese acetate	100 µM	150.9	75
Iron(II)sulfate	1 mM	129.9	65
Iron(III)nitrate	100 µM	148.1	74
Iron(III)chloride	100 µM	183.5	91
Coniferyl alkohol	1 mM	179.7	89
Veratryl alcohol	1 mM	147.1	73
H_2O_2	1 mM	195.5	97
Lithium chloride	100 mg/L	205.0	102
Humic acid	20 mg/L	178.0	88
Atrazin	5 mg/L	130.7	65
2,4-Dinitrophenol	5 mg/L	199.1	99

In manganese sulfate supplemented cultures CDH activity was increased by approx. 20 % and the peak activity was reached three days earlier, when compared to negative controls. Humic acid, iron chloride, lithium chloride, H_2O_2 and 2,4-dinitrophenol had no effect on the overall CDH production. Indulin AT, guiacol, copper sulfate, iron sulfate and atrazin showed a suppressing effect.

4.4.2 Determination of the optimal manganese sulfate concentration

To elucidate the inducing effect of manganese sulfate, a cultivation with varying concentrations of this heavy metal was performed (see Table 15).

TABLE 15: Induction of CDH with manganese(II)sulfate

$MnSO_4$ [mM]	CDH activity [U mL^{-1}]	
	15 days	17 days
2.0	84	21
1.0	165	67
0.5	187	102
0.2	146	87
0.0	121	154

The inducing effect of manganese sulfate up to a concentration of 1 mM could be confirmed and the optimal concentration for CDH production was found to be 0.5 mM. Higher concentrations lowered the formation of CDH. The duration of the cultivation was significantly accelerated and the total activity of CDH was increased by approx. 18 %.

4.4.3 Effect of manganese sulfate on the production of endo- and exoglucanases

The concentration of total extracellular exoglucanases and endoglucanases was determined with a DNSA-based assay. The results are displayed in Figure 9 and Figure 10.

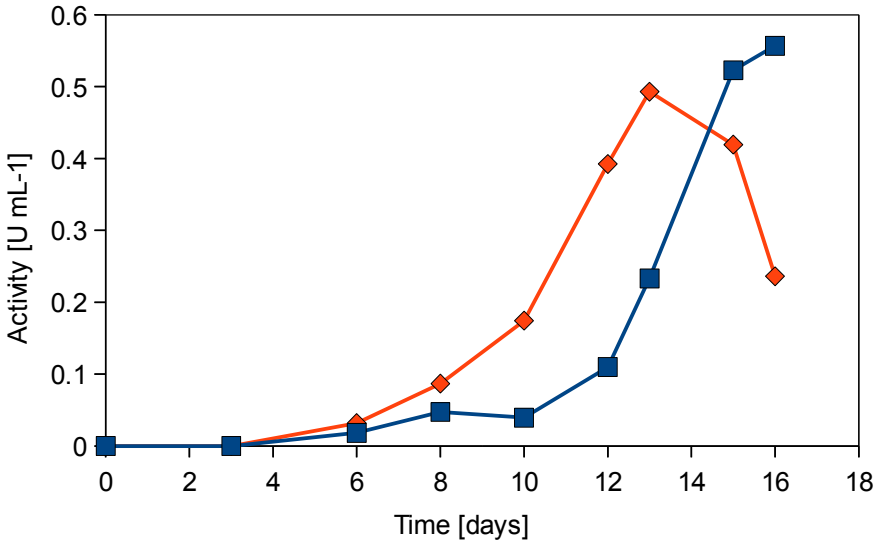

FIGURE 9: Activity of exoglucanases in cultures of *C. subvermispora* supplied with manganese sulfate (red line, diamonds) in comparison with a negative control (blue line, squares).

When compared to negative controls, the level of extracellular cellulases was considerably higher in manganese supplied cultures for most of the cultivation time. A maximum activity of 49 U L^{-1} after 13 days was followed by an immediate decrease in cellulase activity.

For the negative controls a delayed production of cellulases was observed, and the activity at the end of the cultivation was found to be 56 U L^{-1}.

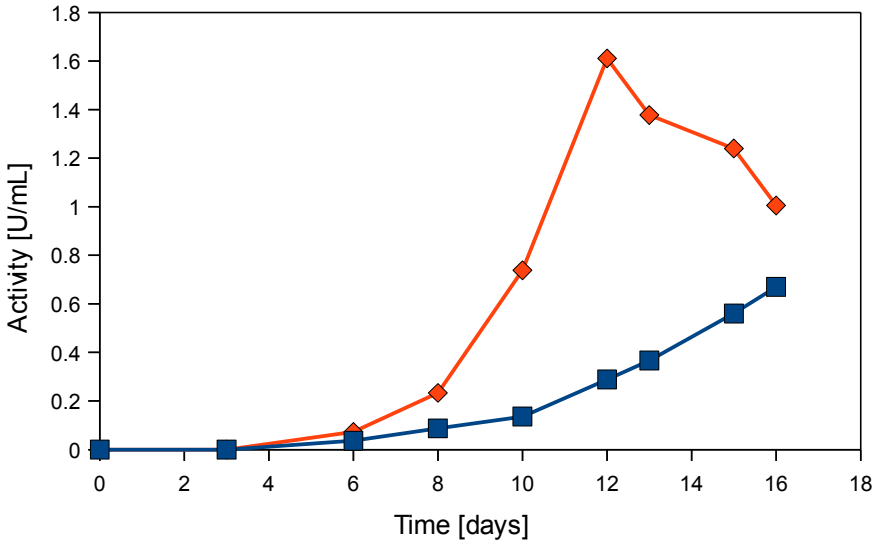

FIGURE 10: Activity of endoglucanases in cultures of *C. subvermispora* supplied with manganese sulfate (red line, diamonds) in comparison with a negative control (blue line, squares).

The level of endoglucanases was significantly increased in the presence of manganese sulfate. A peak activity of 1610 U L^{-1} was reached after 12 days.

4.5 pH-Dependent binding of CDH to α-cellulose

The binding behaviour of CDH towards α-cellulose was investigated in respect to the pH. The pH profile is shown in Figure 11. Three different buffer systems were used in order to cover a broad range of pH. It was observed that CDH was immediately denatured at pH values below 2.5, therefore the data were excluded.

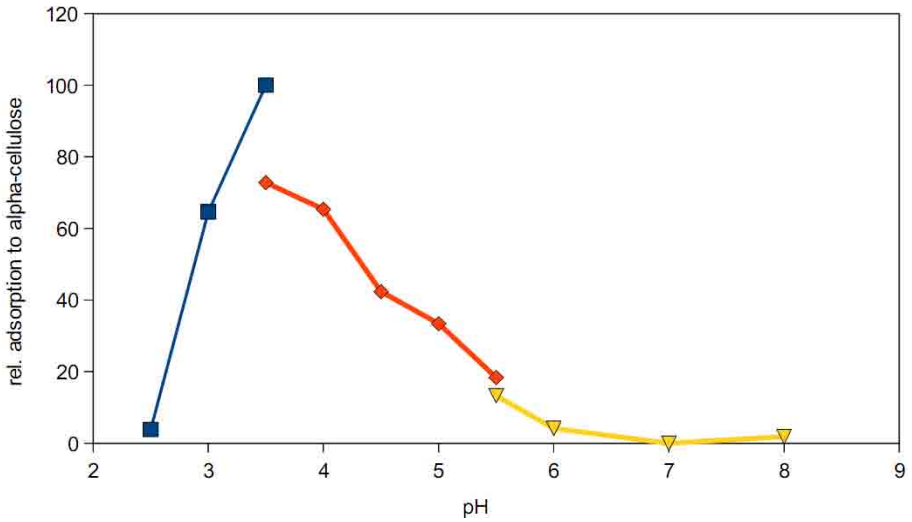

FIGURE 11: pH-dependent binding of CDH to α-cellulose. Sodium citrate buffer (blue line, squares), sodium acetate buffer (red line, diamonds) and sodium phosphate buffer (yellow line, triangles).

The highest binding was observed at pH 3.5 in citrate buffered solution. By increasing the pH to a value of 5.5 a linear decrease in CDH binding can be seen. By increasing the pH to values above 6 CDH is not able to bind to α-cellulose anymore. Regarding the used buffer salts, citrate buffer had a positive influence on the binding behaviour.

4.6 Binding isotherm of CDH to α-cellulose

In Figure 12 the binding isotherm of CDH to α-cellulose is shown. In this graph the residual concentration of CDH is plotted against the concentration of bound CDH per gram α-cellulose. The data were evaluated with the program SigmaPlot and fitted to a one-binding site model. The dissociation constant (K_d) was found to be 0.64 µM and the calculated maximum binding capacity (K_B) was 0.12 µM CDH g^{-1} α-cellulose.

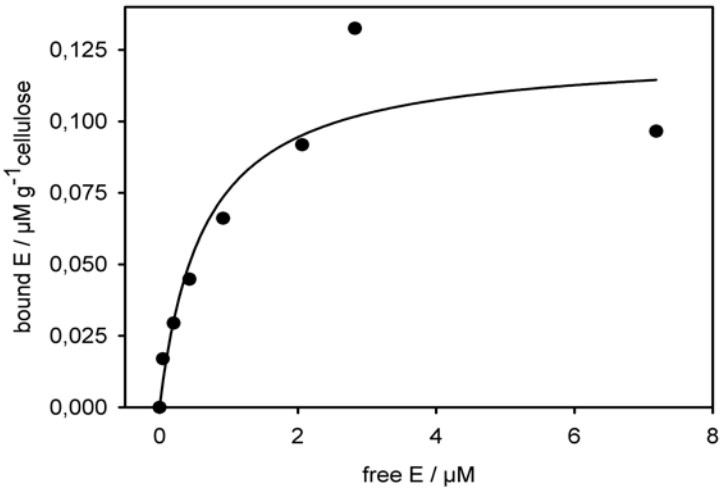

FIGURE 12: Cellulose binding isotherm of CDH.

In order to achieve saturation of cellulose, high enzyme concentrations (up to 1350 U L^{-1}) had to be used, whereas the concentration of α-cellulose was comparatively low (5 g L^{-1}). Therefore, little errors in the activity measurement result in a high deviation of the data, which explains the mismatch of data points in the saturated section of the curve.

4.7 Effect of sonication on CDH activity

Since aliquots of the culture medium had to be sonicated in order to measure the quantity of cellulose-bound CDH, the effect of sonication on CDH activity was evaluated. Purified CDH in 50 mM sodium acetate buffer (pH 4.0) was therefore treated with 5 consecutive pulses of ultrasound waves and immediately chilled on ice for 1 minute. The CDH activity of the sample was measured according to the cyt *c* assay along with a negative control (Figure 13) The power output of the sonicator was set to 80 %.

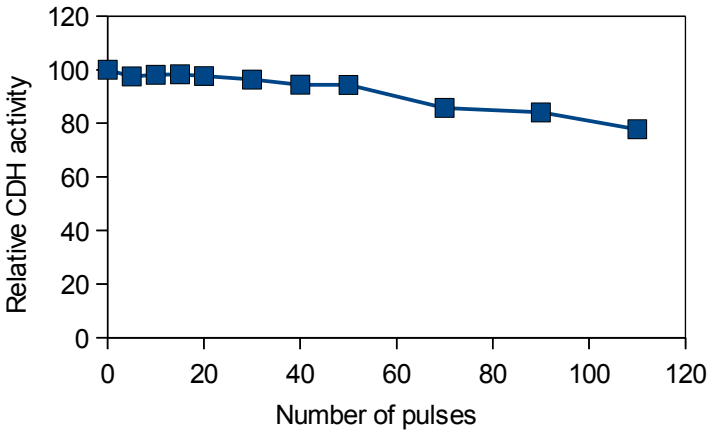

FIGURE 13: Effect of sonication on CDH activity.

CDH proved to be highly resistant to ultrasonic waves. After applying 30 pulses, the overall activity was 96 %. After 110 pulses 22.8 % of the total activity was lost.

4.8 Measurement of cellulose-bound CDH *in vivo*

The quantity of CDH adsorbed on cellulose was estimated with a new assay procedure based on the conventional cyt *c* assay. The supernatant from an aliquot of the culture medium was removed by centrifugation and the residual, insoluble compounds (biomass and cellulose) were washed, homogenized by sonication and directly tested for CDH activity in a stirred cuvette. In Figure 14 the CDH activity measured in the supernatant is compared to the quantity of cellulose bound CDH.

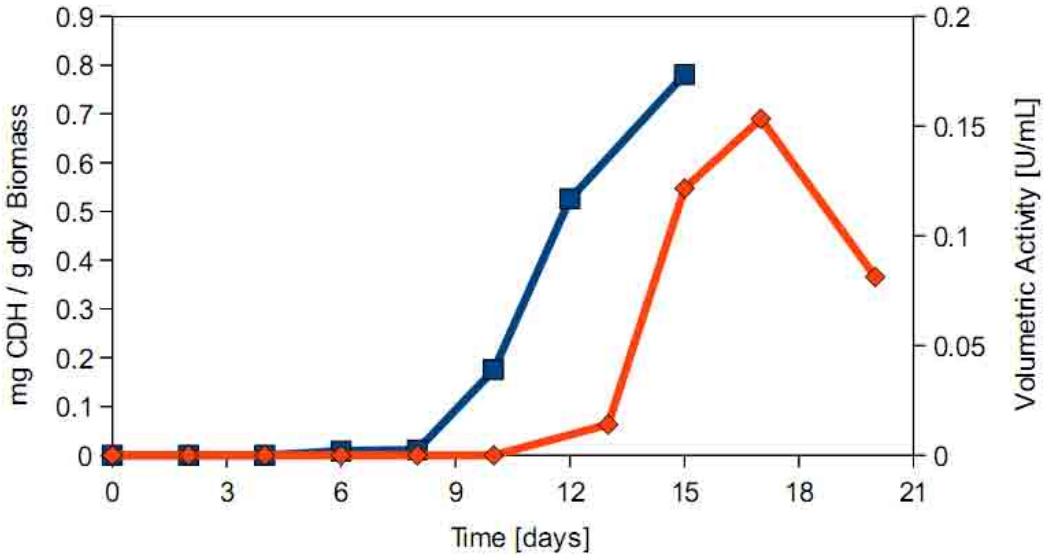

FIGURE 14: Comparison of cellulose-bound CDH (blue line) and CDH measured in the culture supernatant (red line).

Notable concentrations of cellulose-bound CDH were already found after 10 days of cultivation, whereas the appearance of CDH in the supernatant was delayed by several days. After 15 days measurement of α-cellulose-bound CDH was stopped due to the perturbing signal background, caused by the high amount of biomass. The highest amount of α-cellulose-bound CDH was calculated as 0.87 mg CDH g^{-1} dry biomass and the peak activity in the supernatant was 150 U L^{-1}.

5 DISCUSSION

5.1 Optimisation of the cultivation medium of *C. subvermispora*

In order to determine the optimal conditions for the production of CDH in the *C. subvermispora* strain FP-90031, the fungus was grown under different culture conditions. The initial pH value of the cultivation is essential for CDH production in this white-rot fungi. The highest CDH activity (219 U L^{-1}) was measured in a cultivation medium with an initial pH value of 5.5. In cultures with initial pH values of 8.5 and 4.0, the rather extreme conditions seemed to exceeded the ability of the fungus to produce CDH. Additionally the extracellular protein level was significantly lower compared to the moderate pH conditions, which indicates that the fungus could not adapt well to these conditions. Considering the pH variation in time (Figure 3), in cultures of *C. subvermispora,* best growth was found to be around pH 4.5. It was observed that within the first days of cultivation the pH value is shifted towards 6, followed by a rapid decrease. At pH values close to 6, the fungus grew in small pellets which formed around the agar plugs. Decrease in pH was accompanied by the disintegration of the pellets and the homogeneous distribution of the mycelium within the flask. These observations suggest that the fungus initially grows on the agar pieces, using residual substrates which are available without much effort and utilizes cellulose in the end. As confirmed by microscopic observation, fungal hyphae dispersed in the culture broth and enclosed the cellulose fibres when the pH was shifted to the preferred acidic milieu.
To draw a conclusion, for efficient CDH production as well as for fungal growth, it is important that the fungus itself lowers the pH to the optimal milieu. Otherwise no significant CDH production occurs.

The optimal growth temperature for *C. subvermispora* was found to be 25°C. At 30°C the growth of the fungus was slightly accelerated as indicated by the pH value and the protein concentration, but the CDH activity was generally lower. No apparent growth could be observed at 35°C, neither microscopically nor by the macroscopic appearance of the culture broth.

In general, cellulose is known to stimulate the CDH formation. The amount of α-cellulose in the medium is a second parameter which strongly influences the production of CDH in *C.*

subvermispora. The optimal concentration of α-cellulose was determined with 20 g L^{-1}.
Higher concentrations of α-cellulose significantly extended the cultivation time. Cultures grown on 30 g L^{-1} α-cellulose produced as much CDH as measured in the optimal medium, but the maximum activity was delayed 8 days. At concentrations of 40 g L^{-1} CDH activity was as low as 11 U L^{-1}, but judging from the pH value and from microscopic examination (amount of mycelium, residual α-cellulose), the culture was still viable when the experiment was terminated after 28 days.
Moreover the concentration of yeast extract and peptone from meat had no significant effect on the overall CDH production but on the cultivation time. In view of these data, the concentration of α-cellulose seems to be the most important factor for CDH production, beside the initial pH value. Upon reduction or omission of either yeast extract or peptone the fungus is still capable of producing high amounts of CDH, thus the production of the enzyme appears to be mainly dependent on the amount of the obtainable α-cellulose.

5.2 Cultivation in a stirred bioreactor and purification of CDH

A 30 L scale cultivation was performed in a stirred bioreactor. Unfortunately, an infection with rod-shaped bacteria occurred after four days of growth, which immediately alkalified the cultivation medium. As soon as the contamination was discovered, the pH was slowly lowered to 4.35 with phosphoric acid which suppressed the bacterial growth. The occurrence of the infections in bioreactors was a recurring problem in the laboratory and rod-shaped bacteria appeared in previous cultivations. The infection was tested for antibiotic resistance on agar plates and it was found that the contaminating bacteria exhibited resistances against the antibiotics ampicillin, kanamycin and chloramphenicol. Since these substances are routinely used in molecular biology, this might point to a lab-internal strain. However, there never occurred an infection with rod-shaped bacteria in experiments conducted in shaking flasks. Striking was the fact, that the infection appeared several days after the inoculation, thus it is most likely that the organism penetrated the bioreactor through the aeration system. Nevertheless, the cultivation was successful and a total activity of 3400 U was measured previous to the purification. Harreither *et al.* (2009) performed a cultivation in a 30 L scale under the same conditions and reported a volumetric activity of 170 U L^{-1} after 5 days of cultivation and a total activity of 5640 U.

During hydrophobic interaction chromatography (HIC), elution of CDH from the column failed to work and a main fraction of the enzyme was lost. Even washing steps with PEG and HQ water could not elute the presumably bound CDH. However, when washing the column with 0.5 M NaOH, a notable increase in the absorption at 420 nm (haem domain) was detected which might be the result of CDH denaturing. The remaining fraction could nonetheless be purified to apparent homogeneity.

Deglycosylation of the purified CDH worked out fine, although almost 25 % of the enzyme was lost during the process and another 20% were lost after the final polishing step with a strong anion exchange column. The purity of the obtained CDH was evaluated with a denaturing SDS gel.

5.3 Induction with manganese sulfate

A broad range of compounds and heavy metals were tested for their CDH inducing effect in *C. subvermispora*. However, only manganese sulfate supplemented cultures exhibited increased levels of CDH. The optimal Mn^{2+} concentration for CDH production was found to be 0.5 mM. It was observed that the concentration of the provided inducer is a crucial factor. In the case of indulin AT, a concentration of 200 mg L^{-1} lowered the yield of CDH to 48% compared to the blank values, whereas half the concentration of indulin AT resulted in 82% activity. This was also observed with manganese sulfate, where concentrations above 1 mM reduced the volumetric CDH activity. However, it was not possible to test a broader concentration range for each inducer in the available time, since cultivation times where rather long (up to three weeks) and due to the experimental set-up all samples were tested in doubles.

In manganese supplemented cultures, highest CDH activity was not only found to be increased by 20 %, but was also reached several days earlier. On the whole, fungal growth seemed to be accelerated as judged from the pH value and the level of extracellular proteins. This assumption was strongly supported by the fact that in the presence of manganese also the overall activity of endo- and exoglucanases was elevated. Activity of both endo- and exoglucanases was found to rise much faster in induced cultures, reaching a maximum at 12

and 13 days while cellulase concentrations in non induced cultures were still increasing when the experiment was terminated after 16 days. Striking is the fact that endoglucanases exhibited a much higher activity than exoglucanases. This might be the effect of different substrates, e.g. carboxymethyl cellulase was provided as soluble sodium salt while α-cellulose is insoluble. These experiments clearly show that due to the influence of manganese the degradation of α-cellulose is accelerated. Therefore, manganese sulfate seems to have a beneficial effect on the extracellular lignocellulolytic degradation pathway.

However, the inducing function of manganese remains to be investigated in detail. Previously, it was reported that in cultures supplied with Mn^{2+} (10 μM) the transcription level of a gene encoding laccase, an enzyme involved in lignin degradation (Manubens *et al.* 2007), was significantly increased. Furthermore, a cooperative catalytic mechanism of CDH and manganese dependent peroxidase (MnP) during wood degradation was proposed (Hilden *et al.* 2000). Along with these findings, the results presented in this thesis give further evidence for the crucial role of manganese in the ligninocellulolytic degradation system. During these experiments, manganese induced cultures were also tested for MnP activity employing two different assays, but unfortunately no activity could be measured (data not shown). However, it should be noted that MnP is believed to be involved in the degradation of lignin, and since all cultures were grown on α-cellulose as main carbon source, the fungus might not have produced any MnP. To test for cooperative correlations between manganese and CDH further investigations employing substrates like ligninocellulose or parts thereof is suggested.

5.4 Binding studies of CDH to α-cellulose

In vitro characterisations regarding the binding of purified CDH to α-cellulose revealed a high dependence of the binding capacity of CDH on the pH milieu. It was found that above pH 5 the binding of CDH is neglectable, whereas maximal binding was observed at pH 3.5. Since the binding of CDH is believed to be essential for the enzyme's *in vivo* function, it appears that CDH can fulfil its biological role only at low pH values. This agrees with the observation that growth of the fungus is best and degradation of α-cellulose is highest when the culture milieu is shifted towards an acidic milieu.

Moreover, the binding isotherm of CDH towards α-cellulose was determined. The dissociation constant (K_d) was calculated with 0.64 μM, the maximum binding capacity (K_B)

was 0.12 µM g^{-1} cellulose. These values are in good agreement with previously published data. For *Phanerochaete chrysosporium* CDH the dissociation constant for bacterial α-cellulose was 0.67 µM (Henriksson *et al.* 1997b) and 0.02 µM for Valonia cellulose (Samejima *et al.* 1997).

5.5 Measurement of cellulose-bound CDH *in vivo*

In order to assess the time-course of CDH production during a cultivation, the conventional cyt *c* assay procedure was adapted in order to measure the α-cellulose bound CDH *in vivo*. Therefore, α-cellulose along with fungal biomass was homogenised by sonication and tested for activity in a stirred cuvette. With this procedure it could be shown that significant amounts of CDH are produced before the enzyme appears in the culture supernatant. These findings contradict previous reports, which speculated that CDH is produced in the latter growth phase of the fungus (Zamocky *et al.* 2006).
But in view of the low dissociation constant of 0.64 µM, which reflects the high affinity of CDH to cellulose, the enzyme is likely to be rapidly bound to cellulose after secretion. Upon exhaustive cellulose degradation CDH might be released again from cellulose into the culture supernatant. Moreover, the pH in substrate-depleted cultures is rapidly rising to pH values of 6-7, which is probably caused by cell lysis. As aforementioned, this pH milieu might also contribute to the the release of CDH from α-cellulose.

In conclusion, these data indicate the important role of CDH in the degradation of lignocellulosic biomass. *C. subvermispora,* as CDH producer and selective delignifier could therefore provide a further basis for research and a mean to optimize the industrial application of this fungus.

6 SUMMARY

Cellobiose dehydrogenase (CDH) is an extracellular flavocytochrome that is produced by many wood degrading fungi. Despite its non-hydrolytic character CDH seems to play a highly important role in lignin degradation and is believed to aid the lignin-degrading system. To date, the biological role of CDH is not known with certainty. Recently, CDH was detected in the white rot fungus *Ceriporiopsis subvermispora*, which selectively delignifies wood, while leaving the valuable cellulose untouched. Thus *C. subvermispora* is an ideal candidate for the biological ligninolysis in the pulp and paper industry and the cellulose based bioethanol generation. The aim of this study was to investigate the unique hydrolytic system of *C. subvermispora* with respect to the role of CDH.

The presented data support the concept of a cooperative mechanism of interaction of CDH and other ligninocellulolytic enzymes like manganese peroxidase (MnP). It was shown that the addition of Mn^{2+} at a concentration of 0.5 mM significantly accelerates the growth of the fungus and elevates the level of CDH along with extracellular endo- and exoglucanases. However, the biological function of Mn^{2+} remains to be investigated in detail. Furthermore, it was shown that an acidic pH milieu (pH<5) created by the fungus is from crucial importance for α-cellulose degradation. Macroscopic and microscopic observations of the culture broth along with the determination of the pH and protein concentration showed that degradation of α-cellulose is strongly favoured under these conditions. Investigation of the binding characteristics of CDH to α-cellulose revealed a strong pH dependence. CDH can only bind to α-cellulose if the pH is decreased to values below 5, which is in good accordance with the conditions found *in vivo*. The dissociation constant (K_D) for CDH binding was found to be 0.64 µM, which reflects the high affinity of CDH to α-cellulose. This is the explanation why CDH could to date only be detected in the culture supernatant within the terminal growth phase of the fungus. Due to the strong interaction of CDH and α-cellulose the enzyme might rapidly bind to α-cellulose and be released upon exhaustive cellulose degradation. Applying a new assay procedure, it was indeed shown that CDH is produced in earlier stages of cultivation, but remains bound to α-cellulose until its release in the latter growth phase.

These data underline the importance of CDH in the degradation of wood biomass and provide a basis for further investigations. Especially the manganese-related induction might be a promising field of research in respect to industrial application.

7 ZUSAMMENFASSUNG

Cellobiose Dehydrogenase (CDH) ist ein extrazelluläres Flavocytochrom, das in vielen holzabbauenden Pilzen nachgewiesen werden konnte. Obwohl CDH selbst über keinerlei hydrolytische Eigenschaften verfügt, scheint es dennoch eine wichtige Funktion beim Abbau von Ligninbestandteilen zu erfüllen und andere ligninabbauende Enzyme, wie z.B. die Manganperoxidase (MnP) kooperativ zu unterstützen. Die biologische Funktion von CDH ist bis jetzt nicht geklärt.

Vor kurzem wurde CDH in dem Weißfäulepilz *Ceriporiopsis subvermispora* entdeckt, der über die einzigartige Eigenschaft verfügt, selektiv Lignin abzubauen, während wertvolle Zellulosebestandteile nicht zersetzt werden. Aus diesem Grund ließe sich *C. subvermispora* ideal in der Papierindustrie ("Biopulping") und für die Bioethanolproduktion aus Pflanzenbiomasse einsetzen. Das Ziel dieser Arbeit war daher die Untersuchung des hydrolytischen Systems von *C. subvermispora* im Hinblick auf die natürliche Rolle der CDH.

Die vorliegenden Daten unterstützen das Konzept eines synergetischen Wirkmechanismus von CDH mit anderen lignin- und zelluloseabbauenden Enzymen wie MnP. Es zeigte sich, dass der Zusatz von 0.5 mM Mn^{2+} in das Kulturmedium nicht nur das Wachstum des Pilzes beschleunigte, sondern auch die Aktivität von CDH und extrazellulären Endo- und Endoglucanasen erhöhte. Die Induktion durch Mn^{2+} ist bis dato allerdings unklar und bedarf weiterer Untersuchungen.

Des weiteren konnte gezeigt werden, dass ein saures pH-Milieu wichtig für den Abbau von α-Zellulose ist. Die makroskopische sowie mikroskopische Beurteilung von Schüttelkulturen sowie das fortlaufende Beobachten des pH-Wertes und der extrazellulären Proteinkonzentration verdeutlichte, dass der Zellulose-Abbau nur unter diesen Bedingungen ablaufen kann. Untersuchungen der Bindung von CDH an α-Zellulose zeigten auch hier eine starke pH-Abhängigkeit. So wurde eine nennenswerte Bindung nur bei pH-Werten unter 5 beobachtet, was den *in vivo* Bedingungen entspricht. Für die CDH-Bindung wurde eine Dissoziationskonstante von 0.64 µM bestimmt, was die starke Affinität von CDH zu α-Zellulose verdeutlicht. Dies könnte auch erklären, weshalb CDH erst in der letzten Wachstumsphase des Pilzes im Überstand nachgewiesen werden konnte. Wahrscheinlich ist die unmittelbare Bindung von CDH an α-Zellulose, sobald das Enzym sekretiert wird. Erst wenn α-Zellulose abgebaut ist oder der pH-Wert steigt, wird das Enzym freigesetzt. Durch ein neues Nachweisverfahren konnte nachgewiesen werden, dass CDH früher als bisher

angenommen produziert wird, aber an die α-Zellulose gebunden ist.

Die vorliegenden Daten verdeutlichen jedenfalls die wichtige biologische Rolle der CDH im Holzabbau und bieten eine Basis für weitere Untersuchungen. Vor allem die Mangan-assoziierte Induktion bedarf weiterer Forschungsarbeit und bietet einen vielversprechenden Ansatz für die industrielle Anwendung von *C. subvermispora*.

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