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Screening for Cellobiose Dehydrogenase in Ascomycetes

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ABSTRACT

In search of novel cellobiose dehydrogenases (CDH) 33 different ascomycetes were screened. In the screening, the more sensitive cytochrome *c* activity assay was used to determine extracellular CDH of shaking flask cultured fungi. In addition to lactose, the poor substrate glucose was tested for conversion with the novel found CDHs. Six of the 33 screened ascomycetes have been discovered as novel CDH secreting fungi. The presence of extracellular CDH in *Chaetomium atrobrunneum* and *Monilia brunnea* was confirmed. Moreover, it was possible to induce the expression of CDH in the cultures of *Neurospora crassa*, known as a *cdh* gene carrying fungus, by cultivation of this ascomycete in cellulose-containing media. In culture supernatants of *Fusarium oxysporum*, a plant pathogen, which is reported to carry a gene resembling the flavin domain of CDH, enzyme activity was only detected with cytochrome *c* but not with DCIP as electron acceptor. The detected enzyme activity in *F. oxysporum* culture supernatants is most probably not the result of a CDH. In addition to the screening in shaking flasks a new screening method was developed, by cultivation of ascomycetes on cellulose-containing agar plates. Different cultivation methods, in big Petri dishes and in bioreactors, were tested. By varying the concentration of cellulose and peptone, the effect of the carbon and the nitrogen source on CDH production was investigated for nine selected ascomycete strains. Finally, this work included the purification, determination of relevant enzyme characteristics and kinetic studies of two CDHs of selected novel detected CDH secreting fungi. The isoelectric point and the molecular mass of these two CDHs were determined. The effect of different pHs and temperatures to the enzyme activities were investigated. The substrate specificities at different pH values for four carbohydrates, cellobiose, lactose, maltose and glucose, in combination with the electron acceptors cytochrome c and DCIP were investigated by steady state kinetic measurements.

KURZFASSUNG

Auf der Suche nach neuen Cellobiose-Dehydrogenasen (CDH) wurden 33 verschiedene Pilze der Gattung Ascomycota untersucht. Im Screening für die Bestimmung von extrazellulärer CDH in Schüttelflaschen-Kulturen, wurde der sensitive Cytochrom *c* Enzymaktivitätstest angewendet. Neben Laktose wurde auch die Oxidation von Glukose, einem für CDH weniger geeignetem Substrat getestet. Von den 33 getesteten Ascomyceten konnten sechs, bislang unbekannte, CDH produzierende Stämme gefunden werden. Die CDH Produktion bei Kultivierung in cellulosehaltigem Medium konnte für *Chaetomium atrobrunneum* und *Monilia brunnea* bestätigt werden. Auch bei *Neurospora crassa*, einem Stamm der CDH kodierende Gene trägt, dessen Enzymproduktion aber noch nicht untersucht worden war, konnte die Sekretion von CDH nachgewiesen werden. *Fusarium oxysporum*, ein pflanzen-pathogener Ascomycet, welcher homologe Gene zur Flavindomäne von CDH trägt, zeigte Enzymaktivität ausschließlich mit Cytochrom *c*, nicht aber mit DCIP als Elektronenakzeptor. Da aber ausschließlich die Flavindomäne mit DCIP regeneriert werden kann, nicht jedoch mit Cytochrom *c*, handelt es sich bei dem abgesonderten Enzym von *F. oxysporum* höchstwahrscheinlich um keine CDH. Zusätzlich wurde eine weitere Screening-Methode entwickelt, wobei Ascomyceten auf cellulosehaltigen Agar-Platten kultiviert wurden. Neben Schüttelkolben wurde auch die Kultivierung in großen Petri-Schalen und in Bioreaktoren getestet. Der Effekt der Kohlenstoff- und Stickstoffmenge im Kultivierungsmedium auf die CDH Produktion wurde bei 9 Ascomyceten untersucht. Schlussendlich wurden zwei CDHs von den neu entdeckten Ascomyceten gereinigt und charakterisiert. Bestimmt wurden der isoelektrische Punkt und die molekulare Masse. Weiters wurde der Effekt unterschiedlicher pH-Werte und Temperaturen auf die Enzymaktivität untersucht, sowie die katalytischen Konstanten von vier Kohlenhydraten: Cellobiose, Laktose, Maltose und Glukose. Die Messungen wurden bei verschiedenen pH-Werten und mit zwei unterschiedlichen Redoxmediatoren, Cytochrom *c* und DCIP, durchgeführt.

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ABBREVIATIONS

CDH	cellobiose dehydrogenase
CYT_{cdh}	cytochrome domain of CDH
DCIP	2,6-dichloroindophenol
DET	direct electron transfer
DH_{cdh}	flavindehydrogenase domain of CDH
*Ds*CDH	CDH of *Dichomera saubinetii*
E	enzyme
ES	enzyme-substrate-complex
FAD	flavin adenine dinucleotide
G	Gibbs free energy
ΔG	free energy difference
*Hh*CDH	CDH of *Hypoxylon haematostroma*
HIC	hydrophobic interaction chromatography
AIEX	anion exchange chromatography
IEF	isoelectric focusing
IET	internal electron transfer
k_1 k_{-1} k_2 k_{-2}	reaction rate coefficients
k_{cat}	catalytic constant
k_{cat} K_M^{-1}	catalytic efficiency
kDa	kilo Dalton
K_I	inhibitor constant
K_M	Michaelis-Menten constant
NAD	nicotinamide adenine dinucleotide
P	product
S	substrate concentration
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
v_0	initial reaction rate
V_{max}	maximum velocity

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1 INTRODUCTION

1.1 Enzyme screening

Screening is a method to find certain features in a large number of samples. What is the reason for a classical microbial screening? Looking for enzymes with suitable properties, in respect to pH optimum, temperature stability, substrate specificity, or even to find enzymes for new reactions. A standard procedure for a classical microbial screening contains the following steps: the collection of samples from suitable habitats, or alternatively, a taxonomy-based approach of known producers for sourcing from culture collections. The next step is the cultivation and accumulation of the mixed culture sample for the isolation of positive strains to obtain pure cultures. The development of the screening assay is the most important part of the complete screening procedure. There is a simple rule for screening: "You get what you screen for!" Therefore, a specific reaction for a reliable detection of the enzymatic activity has to be found. Some common methods are the formation of halos in cultivated agar plates due to certain additives, or color reactions based on specific enzyme activity assays, evaluated with spectrophotometers. A small number of samples are usually detected in cuvettes as reaction vessels, but for large numbers a high throughput screening must be performed, for example in micro-titer plates. Once the target is found, an important next step is the optimization of culture conditions to accumulate the enzyme in larger scales, e.g. in shaking flasks, to perform enzyme purification experiments. A purified, homogenous enzyme solution is the basis for characterization and kinetic studies.

Some screening techniques replace a classical microbial screening, for example "in silico screening". This method is based on computer-assisted searching of databases for homologous DNA or RNA sequences. As a limitation of "in silico screening" has to be considered, that only known sequences can be screened.

The biggest disadvantage of a classical microbial screening is the fact that the majority of naturally occurring microorganisms, up to 99%, cannot be cultured in vitro and therefore are not accessible with conventional techniques.

An approach to avoid this problem is termed "metagenomics". This is the study of genetic material, DNA and RNA, recovered directly from environment samples without the necessity of lab-scaled cultivations under artificial and probably insufficient conditions. The entirety of the genetic information of the microbial biocoenosis in a specific habitat is referred to as metagenome. A number of genetic methods are necessary to conduct research in this field. The origin of the found genes is hardly assigned to the corresponding microorganism strain [1].

The aim of this thesis was the screening for a well-known enzyme in a barely investigated phylum of fungi. A suitable enzyme activity assay was available and most different strains of ascomycetes were present in culture collections of the institute. Therefore, a classical microbial screening was chosen to be the preferred method.

1.2 Enzymes

Enzymes are molecules that increase the rates of specific chemical reactions. This process is called catalysis. Chemical substances, which can affect the velocity of a reaction, may be composed of inorganic or organic compounds. Organic catalysts are classified as biocatalysts and the most common biocatalysts are enzymes. Enzymes are proteins, built up from amino acids connected in a linear chain and folded into a secondary and tertiary structure. As all catalysts, enzymes are not able to alter the position of a chemical equilibrium of a reaction. Chemical reactions can also occur in the same way as they would without the presence of a catalyst, until the equilibrium is achieved, but at decidedly lower rates, which would not be sufficient to create life. A chemical equilibrium is the status in which the chemical activities of the reactants and products have no net change over time, because the chemical process proceeds at the same rate as their reverse reaction [2]. During a chemical reaction, reactants change their free energies when they are transformed into products. The shift of the energy levels of reactants and products can be displayed in an energy profile diagram by plotting the reaction coordinate against the free energy, shown in figure 1.1.

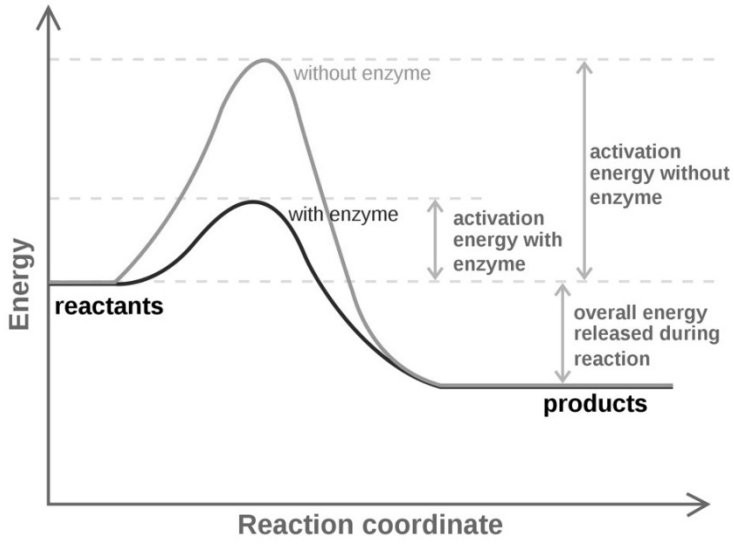

Figure 1.1 Energy profile diagram of an idealized reaction [3]

Chemical reactions are obedient to the laws of thermodynamic, which describes all different forms of energy and reveal anything about whether, under what conditions and in which extend a transformation of involved substances take place, independent of the active participation of catalysts. To understand how a chemical reaction proceeds this thermodynamic properties have to be regarded. Two thermodynamic properties are fundamentally for a successful chemical reaction. Here, the free-energy, also termed Gibbs free energy (G), is the key criterion. The difference in free-energy (ΔG) between the reactants and the products determines whether the reaction is spontaneous or disfavored. Disfavored reactions cannot proceed in a closed system under stable thermodynamic conditions. Enzymes have no influence to the extent in free energy of the reactants. An endergonic reaction in biological systems can only proceed, if it is coupled to a strongly exergonic reaction, like the coupled hydrolysis of ATP, to have a negative difference in free energy of the overall reaction. Reactions with a negative difference in free energy can proceed spontaneously. But the highest energy position represents the activation energy, the energy of the transition state, which has to be overcome and which is responsible for the velocity of the reaction. As seen in the energy profile diagram (figure 1.1), a catalyzed reaction has a lower rate-limiting free energy change to the transition state than their corresponding uncatalyzed reaction, resulting in a larger reaction rate at the same thermodynamical conditions. Hence, catalysts can enable reactions that would otherwise be

blocked or slowed by the high activation energy which act like a kinetic barrier. It is important to note that a catalyst increases the rate of a reaction, but without being consumed by it, which is also valid for enzymes. The three-dimensional structure of proteinogenic catalysts causes the enzymatic activity and different models have been developed to explain the catalytic effect of enzymes. Emil Fischer suggested already in 1894 the "Lock and Key model", shown in figure 1.2, whereas the enzyme and the substrate possesses specific complementary geometric shapes that fit exactly into one another. While this model explains enzyme specificity, it fails to explain the stabilization of the transition state that enzymes achieve.

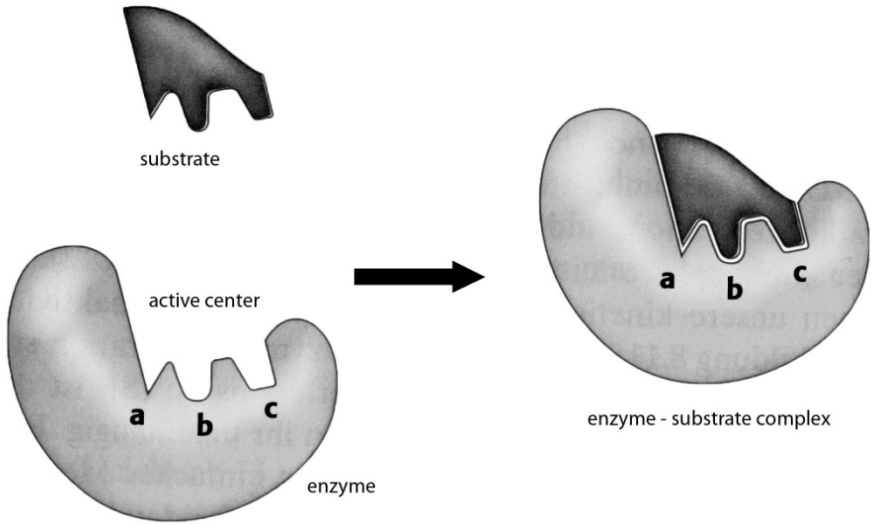

Figure 1.2 "Lock and key model" [2]

Meanwhile, it is known that substrates do not simply bind to a rigid area in the enzymes, like suggested by E. Fischer. The active site of an enzyme is reshaped by interactions with the substrate. When enzymes bind substrates in the correct orientation, the entropy of the reactants is reduced and an enzyme-substrate complex is formed. The enzyme changes its shape slightly as the substrate binds, which stabilizes the transition state. By stabilization of the transition state, the activation energy will be reduced, which increases the probability to continue the reaction in direction of enzyme-product complex, against the dissociation of the enzyme-substrate complex. This modification to the "Lock and Key" hypothesis, called "Induced-fit" model, was suggested by Daniel Koshland in 1958 and provides a better explanation of the functioning of enzymes (figure 1.3).

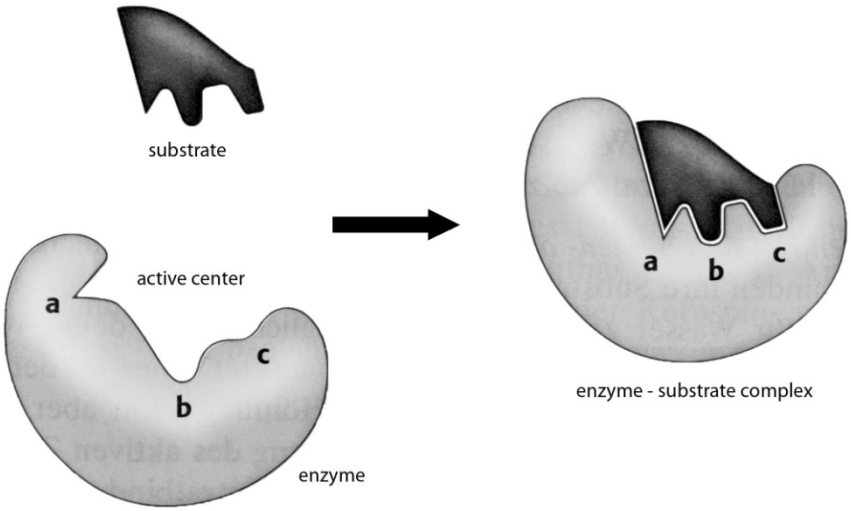

Figure 1.3 “Induced-fit model" [2]

In connection with enzymes additional components, called cofactors, which are necessary for many enzymes to develop catalytic activity, have to be mentioned. The first family of cofactors features non-protein molecules of organic origin, classified depending on the binding strength to the enzyme, into coenzymes or prosthetic groups. The loosely bound cofactors are termed coenzymes and tightly bound prosthetic groups, but there is no exact definition of these two groups, because the binding strength can alter depending on the combination of different enzymes with the cofactor. An enzyme without the corresponding cofactor is inactive and called apoenzyme, while the active enzyme with the bond cofactor is termed holoenzyme. Prosthetic groups are generally regenerated during the same reaction cycle of the enzymes catalyzed conversion and remain within the enzyme. Coenzymes are released from the enzyme's active site during the conversion and are regenerated in a subsequent reaction, regardless of the main catalysis. In this case, a cofactor acts like a substrate and can be called cosubstrate. Often vitamins can serve as coenzymes or as precursors to many organic cofactors. Some organic cofactors contain a nucleotide, like the prosthetic group flavin adenine dinucleotide (FAD) and the coenzyme Nicotinamide adenine dinucleotide (NAD), which both acts as electron carriers for many redox reactions. The probably best-known coenzyme is the nucleotide Adenosine-5'-triphosphate (ATP), which is involved in near all intracellular procedures, starting with the transport of chemical energy up to the usage as substrate in signal transduction pathways. The second big family of cofactors consists of inorganic substances and includes essential metal ions for the use in so-

called metalloenzymes, the generic term for a protein that contains a metal ion cofactor for a catalyzed reaction, like copper, zinc, iron, cobalt, to name just a few.

In order to distinguish enzymes, the enzyme commission assigns to each enzyme a systematic name that uniquely defines the catalyzed reaction. Six different enzyme classes constitute the first digit of a total four digits EC number for each individual enzyme. The six major enzyme groups, EC 1 Oxidoreductases, EC 2 Transferases, EC 3 Hydrolases, EC 4 Lyases, EC 5 Isomerases and EC 6 Ligases are classified according to the reaction type. The rest of the numbers represent a progressively finer classification of the enzyme. The second number indicates the subclass for different donor groups, the third indicates the types of acceptors and the fourth number is an arbitrary serial number.

1.2.1 Enzyme kinetics

Enzyme kinetics is the study of the enzyme-catalyzed reaction. It is useful to determine the enzymatic mechanism and to study the enzyme's activity under the influence of all factors affecting the reaction.

If the conversion of a single substrate (S) to a single product (P), catalyzed by an enzyme (E) is considered. This reaction scheme is given in (1).

(1) $$\mathrm{E} + \mathrm{S} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{ES} \xrightarrow{k_2} \mathrm{E} + \mathrm{P}$$

The amount of product is increasing with time until the chemical equilibrium is achieved. At this time, the enzyme does not stop its conversion, but rather the reaction runs in both directions equally quickly. It is hard to study enzyme conversion rates when the back reaction occurs. For this reason, Leonor Michaelis and Maud Menten introduced a simpler type of reaction scheme for one substrate to one product conversion in the steady-state (2).

(2) $$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E + P$$

The Enzyme binds the substrate by creating an ES-complex at the rate constant k_1. This created ES-complex has only two options: to dissociate to substrate and enzyme at the rate constant k_{-1}, or the substrate is converted into the product at rate constant k_2 and leaves therefore the enzyme. The enzymatic reaction is assumed to be irreversible.

Under such conditions, the Michaelis-Menten equation (3) relates the initial reaction rate (v_0) to the substrate concentration [S].

(3) $$V_0 = V_{max} \frac{[S]}{[S] + K_M}$$

To determine the initial rates at respective substrate concentrations, a series of experiments has to be carried out with constant amount of enzyme, but varied substrate concentrations, from very limiting to excess concentrations. The initial rate of the conversion process is measured. This is the reaction rate in a relatively short time period at the beginning of the conversion, whereas the substrate concentration remains approximately constant and nearly no product is formed, which could interfere and the concentration of the substrate bound enzyme complex [ES] keeps constant (steady-state conditions). Only under these conditions the Michaelis-Menten equation is valid. The reaction rate depends only on the intermediate complex concentration, which is formed under steady state conditions and is converted into product at the speed limiting rate constant k_2. The measurements can be fitted by least-square regression to the Michaelis-Menten equation, to determine V_{max} and K_M values. A plot of the Michaelis-Menten equation is shown in figure 1.4.

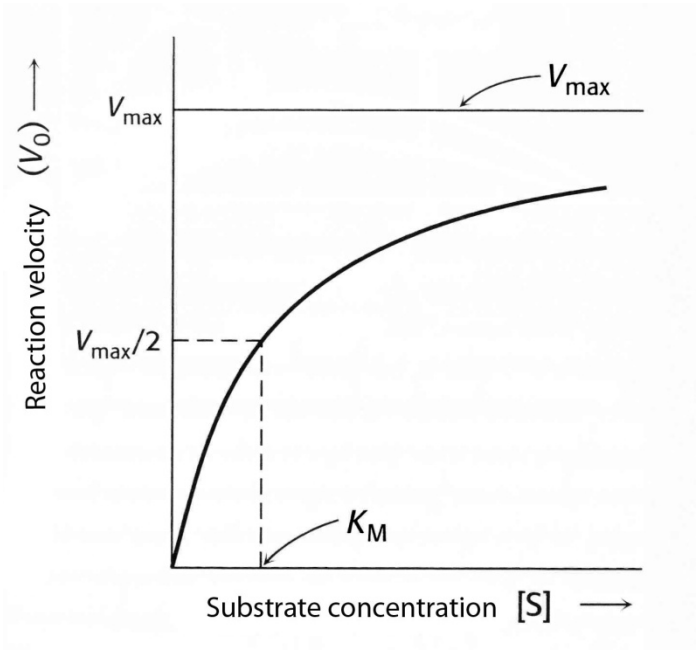

Figure 1.4 Plot of the Michaelis-Menten equation [2]

Important parameters derived from the Michaelis-Menten equation are:

1. The Michaelis-Menten constant (K_M value). The reaction rate increases with growing substrate concentration, asymptotically approaching the maximum in conversion velocity (V_{max}) as seen in figure 1.4. V_{max} can only be calculated using the Michaelis-Menten equation, but would only be reached under the infinite substrate concentration. The equation includes a further unknown parameter, the K_M value. The K_M value is derived as the ratio of complex dissociation velocities and complex building velocity. This ratio is equal to the substrate concentration at which the reaction rate reaches half of its maximum value (V_{max}^{-2}). Hence, the Michaelis-Menten constant is a unit of quantity (mol), a substrate quantity at which the half of the enzyme's active centers have formed an enzyme-substrate complex. The K_M value is a characteristic value for an enzyme to substrate combination and indicates the affinity, but is not equal to a dissociation constant.

2. The catalytic constant (k_{cat} value). The catalytic constant is the maximum number of conversions per enzyme and per second at complete enzyme saturation with the corresponding substrate. It is a useful parameter to compare maximum substrate conversion rates between different enzymes.

3. The catalytic efficiency (k_{cat} K_M^{-1}). At excessive substrate concentrations, the velocity of the enzyme's catalyzed reaction runs near V_{max}, but *in vivo* the substrate concentration is limited and found in the range of the K_M value or lower. For this reason a further significant parameter can be calculated, the catalytic efficiency. This parameter combines the maximum of possible conversion rate with the affinity to the substrate, by building the ratio of k_{cat} and K_M. The catalytic efficiency indicates the enzyme's preference to different substrates. In other words, the substrate with a higher catalytic efficiency is more suitable for the enzyme.

These three values are used to describe and compare enzymes in their interaction with substrates. It has to be considered that the Michaelis-Menten kinetic model is based on some key assumptions. The enzyme-catalyzed reaction is a one substrate to one product conversion without intermediate steps, except the enzyme-substrate complex. The first reaction is reversible and much faster than the second, which ensures a constant concentration of the enzyme-substrate complex and the conversion of the intermediate complex into product and enzyme is assumed to be irreversible.
Other parameters that have big influence to enzyme activity including Michaelis-Menten parameters are the chemical environment and thermodynamic properties that must be declared. By chemical environment mainly the pH value, the nature of the ion and the ionic strength of the assay are meant. Temperature is the most important thermodynamical factor. These factors also affect the stability and the half-life of enzymes.

1.3 Ascomycetes

Ascomycota (Ascomycetes and also known as Sac Fungi) are eukaryotic organisms and classified into the kingdom fungi and subkingdom dikarya. Dikarya includes the phyla ascomycota and basidiomycota. The defining characteristic of all ascomycota is the ascus, a sexual spore-bearing cell. In common with all fungi, ascomycetes are chemoheterotrophic organisms and need organic carbon as energy source and for growth. Ascomycetes secrete digestive enzymes to break down organic substances into smaller molecules, which can be ingested. They are adapted to many habitats and living conditions, like constantly wet conditions, a high carbon/nitrogen ratio (as present in soil), or high pH and high temperature typically occurring during composting processes. Most of these conditions are unsuitable for

basidiomycetes. Moreover, ascomycetes exhibit substantial differences in their rot decay mechanisms compared to basidiomycetes [4]. Ascomycetes, as well as basidiomycetes, the largest group of wood-decaying fungi, play an important role in degradation of wood. Both phyla feature CDH producing strains.

1.4 Cellobiose dehydrogenase

1.4.1 Classification

Cellobiose dehydrogenase belongs to the enzyme main class of oxidoreductases (EC 1), specifically to the sub-class EC 1.1. An oxidoreductase catalyzes the transfer of electrons from a molecule, acting as electron donor to another molecule, the electron acceptor. Enzymes belong to the enzyme classification EC 1.1 contains all oxidoreductases, acting on the CH-OH group of donors. This sub-class contains so-called dehydrogenases, an enzyme family into the oxidoreductases, that oxidizes a substrate by transferring on or more hydride ions (H^-) to an acceptor. Eligible donor groups for dehydrogenases are CH-OH groups, as already mentioned, like primary alcohols, secondary alcohols and hemi-acetals. The possible acceptor molecules subdivide dehydrogenases into six sub-sub-classes (EC 1.1.1 to EC 1.1.5 and EC 1.1.99). CDH reacts with others acceptors than dehydrogenases subdivided into 1 to 5, therefore it is allocated to the sub-sub-classes 99. CDH is marked with 18 at the fourth EC number, an arbitrary serial number. The complete enzyme classification number of CDH is [EC 1.1.99.18], the systematic name [cellobiose: (acceptor) 1-oxidoreductase] and an accepted common name: cellobiose dehydrogenase [5].

1.4.2 History of CDH

CDH is an extracellular flavocytochrome secreted by some white- and brown-rot, plant pathogen and saprotrophic fungi from the dicaryotic phyla basidiomycota and ascomycota under cellulolytic culture conditions [6]. The enzyme was first isolated from the fungi *Sporotrichum pulverulentum*, an imperfect form of *Phanerochaete chrysosporium* and *Trametes versicolor* [7], by Westernmark *et al.* (1974) [8, 9]. They found that the discoloration of ligno-agar plates was minimized by both cultures when the nutrient carbon source was cellulose or cellobiose. Both strains refer to the phylum Basidiomycota. The enzyme was purified, carried a flavin group and was named cellobiose quinone

oxidoreductase (CBQ) and classified as E.C. 1.1.5.1., because it was believed to have found a discrete enzyme. CBQ reduces quinones in the presence of cellobiose but not of glucose. Ayers *et al.* (1978) [10] purified a form containing a flavin and a heme domain, which was named cellobiose oxidase. These two types of flavoprotein, both of them capable of oxidizing cellobiose, were separately investigated for several years in a numerous CDH producing organisms. In 1993, it was proposed that the name cellobiose oxidase is replaced by the more appropriate term CDH, because of the catalytic preference for electron acceptors other than molecular oxygen [11]. It was also demonstrated that CBQ is a breakdown product of CDH including both domains. The formerly enzyme commission number of CBQ was finally deleted in 2002 [6].

Ascomycetes are the second important group of lignocellulose degrading fungi beside basidiomycetes. The production of CDH by ascomycetes has been less intensively investigated and the knowledge about their cellulose degrading system is limited, although CDH has already been detected in the soft rot fungus *Monilia* sp. (ascomycete) in the late 80's [12]. Up to now only some cultures within the phyla ascomycota are known for its CDH secretion (*Monilia* sp., *Sporotrichum thermophilum* [13], *Chaetomium cellulolyticum* [14], *Neurospora crassa* [15], *Humicola insulens* [16], *Chaetomium* sp. [17], *Myriococcum thermophilum* [18] and *Fusarium concolor* [19].

1.4.3 Structure and cofactors

Currently all known CDHs are monomeric proteins divided into two distinct domains with a total average weight of approximately 90 kDa, consisting of about 750 amino acids. The amino acids add 80 kDa to the atomic mass of the protein [20, 21] and the remaining 10 kDa is due to glycosylation, mostly consisting of mannose [22]. The two domains are a flavindehydrogenase domain (DH_{cdh}), which contains FAD, and a cytochrome domain (CYT_{cdh}) containing a cytochrome *b* type heme.[23] The flavin and the heme domain are connected by a protease-sensitive linker region [24, 25]. The cleavage of intact CDH leads to an inactive heme domain, but to an active FAD fragment (DH_{cdh}), which was formerly believed to be a separate enzyme. A theoretical *in silico* model of full-length *P. chrysosporium* CDH is shown in figure 1.5.

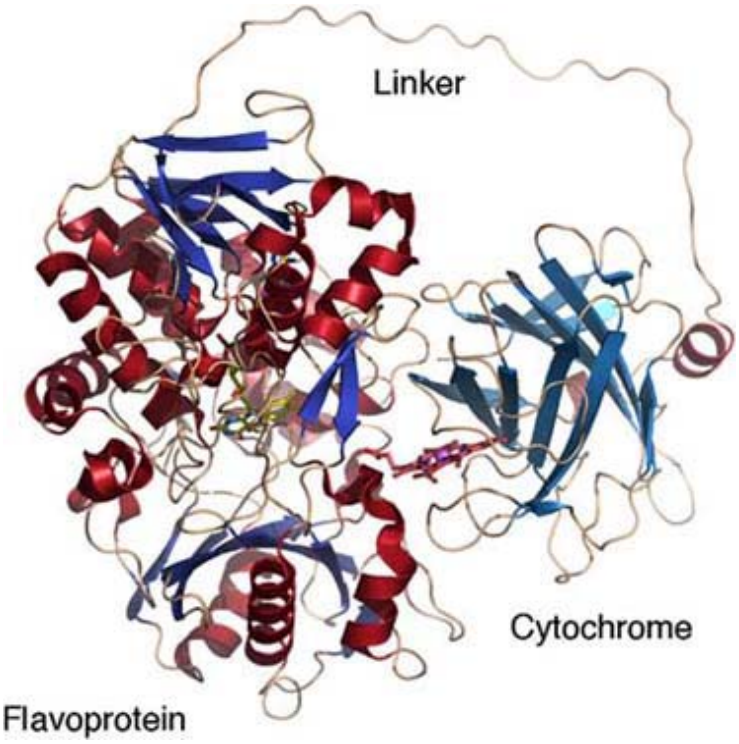

Figure 1.5 Structure of an *in silico* model of CDH [6]

1.4.3.1 Flavine adenine dinucleotide cofactor

The prosthetic group FAD (figure 1.6) is in addition to the coenzyme nicotinamide adenine dinucleotide (NAD^+/NADH) a second major electron carrier. FAD is derived from riboflavin (vitamin B_2), which is bound to the phosphate group of adenosine diphosphate (ADP).

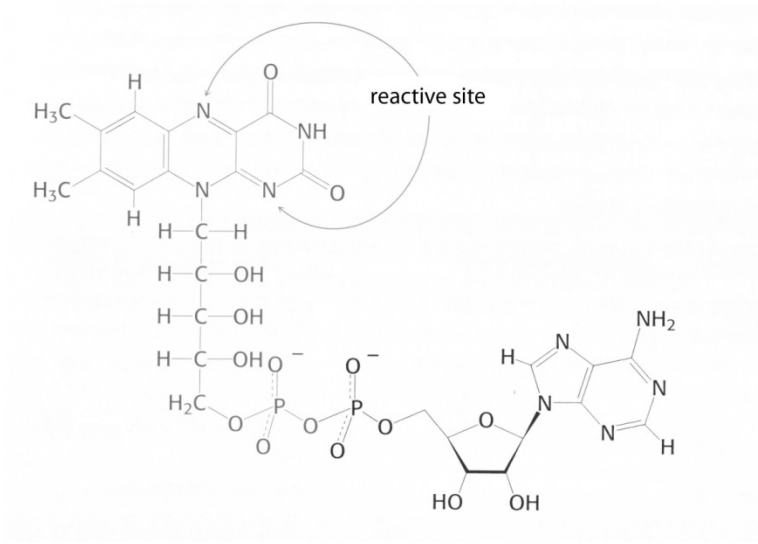

Figure 1.6 Structure of FAD [2]

The redox active center of FAD is the isoalloxazine ring. This anomeric ring system is able to accept and to donate electrons and hydrogen. Like NAD^+, FAD can accept two electrons, but unlike to NAD^+, it can carry both instead of one of the corresponding hydrogen ions in form of $FADH_2$. FAD can interact with two- and one-electron acceptors, because it features an intermediate in form of a semiquinone radical, carrying only one additive electron and one proton, compared to the oxidized form [2]. The extracellular flavocytochrome CDH carries the flavine adenine dinucleotide cofactor in the flavodehydrogenase domain (DH_{cdh}).

1.4.3.2 Heme *b* cofactor

Heme cofactors are cyclic tetrapyrrole ligands featuring the basic shape of a porphyrin and form complexes by binding of a chelat-ion as central ion. The most common heme type is heme *b*, which is also found in CDH and differs from other heme types only in the side chains of the porphyrin. The coordinated iron in the heme is capable of undergoing oxidation and reduction, usually between the oxidation states +2 (ferrous) and +3 (ferric). Hemeproteins have a lot of biological functions including carriage of gases, for example the oxygen transport by hemoglobin and myoglobin, detection of gases, up to redox reactions and electron transfers by the bound metal cation, performed for example by cytochromes [2]. CDH features this second cofactor in the *b*-type cytochrome domain (CYT_{cdh}). The structure of heme *b* is shown in figure 1.7.

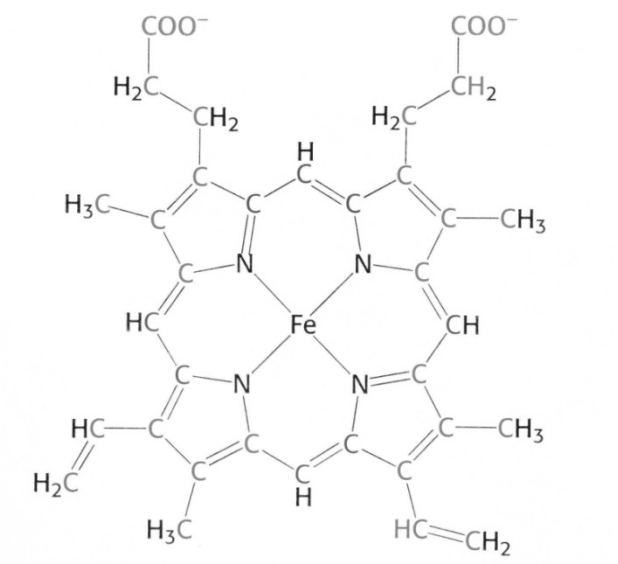

Figure 1.7 Structure of heme *b* [2]

1.4.4 Reaction mechanism

The overall reaction of CDH can be divided into a reductive half-reaction and an oxidative half-reaction. During the reductive half-reaction DH_{cdh} catalyzes a two-electron oxidation at the anomeric C1 position of the substrate (cellobiose) to yield the corresponding lactone (cellobiono-1,5-lactone), while FAD is reduced to $FADH_2$ [11]. The cellobiono-1,5-lactone is spontaneously hydrolysed to cellobionic acid in the bulk water. During the oxidative half-reaction, the cofactor of DH_{cdh}, $FADH_2$, is reoxidized to FAD by an electron acceptor, either directly, or via CYT_{cdh}. Figure 1.8 shows the reaction scheme for cellobiose oxidation with CDH.

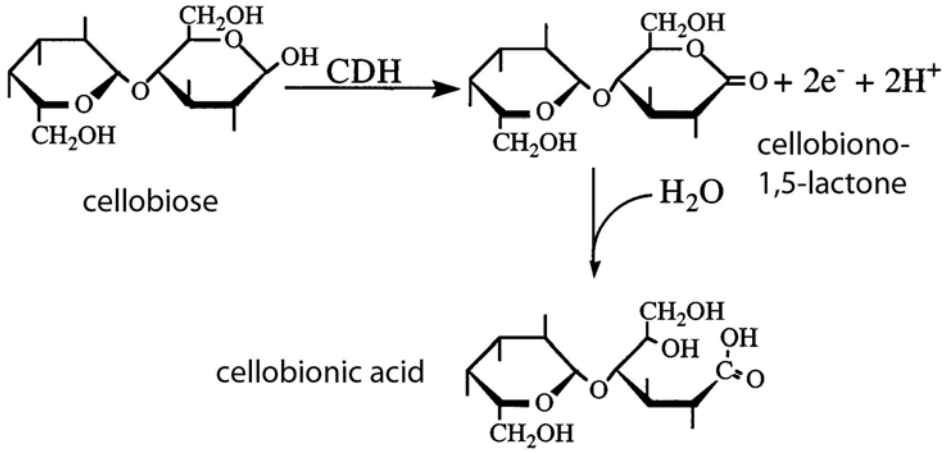

Figure 1.8 Oxidation of cellobiose by the FAD-domain

Two substrate-binding sites in the active center of CDH were suggested [26]. One that binds the reducing glucosyl moiety of cellobiose, the position where the sugar is oxidized, and another site that accounts for binding of the glucosyl unit at the non-reducing end. The crystal-structure of the basidiomycete *P. chrysosporium* flavodehydrogenase domain with a bound substrate analogue, cellobionolactam, was solved and shows the structural determinants for substrate binding in the two subsites [27]. In agreement with the structural determination of the substrate binding, kinetic data [26] showed that the β-1,4 linked disaccharides cellobiose, lactose and mannobiose are good or acceptable substrates, whereas the α-1,4 linked disaccharide maltose as well as monosaccharides are poor or no substrates.

The supposed kinetic mechanism of CDH is the Ping-Pong mechanism [28]. Two different substrates are necessary to perform the overall reaction of the enzyme. The substrate

(electron donor) binds to the enzyme, forming an intermediate, which is converted to the product. Only after the first product is released, the second substrate (electron acceptor) can bind and reacts with the modified enzyme, regenerating the unmodified active enzyme form. This means that there is only one substrate at the same time inside the enzyme's active site. A Ping-Pong mechanism can be detected by measuring the reaction velocity of a set of concentrations of the first substrate and varied concentrations of the second substrate. Results plotted in a Lineweaver-Burk diagram produce a set of parallel lines.

1.4.5 Electron transfer mechanism

As already mentioned CDH features two redox centers. DH_{cdh}, responsible for substrate oxidation, is able to interact directly with redox mediators for reoxidation. The second redox center, CYT_{cdh}, can interact with the flavodehydrogenase domain as well as with redox mediators for reoxidation, but it shows no catalytic activity in the case of cleavage into the two separate domains at the protease-sensitive linker.

For the catalytic role of the heme domain two different models were suggested [28, 29]. The "electron-chain" model states that the electrons, one by one, are shuttled from FAD to heme *b* via an internal electron transfer process (IET) before reduction of an external one-electron acceptor occurs via the electron carrying heme domain. The other model, the "electron-sink" model, suggests that the role of the heme *b* is to store electrons in order to ensure specific reaction conditions at the FAD for the reduction of electron acceptors. Both models may be valid for different electron acceptors. Unfortunately, the lack of kinetic data prevents more in-depth discussion on this subject. [6]

The oxidative half-reaction for reoxidation of the electron carrying cofactor proceeds by electron transfer to either a two-electron acceptor or two equivalents of a one-electron acceptor [30]. The natural electron acceptors for CDH are uncertain, also due to the fact of the existing second redox center in the enzyme. *In vitro* a wide range of electron acceptors are reduced by the holoenzyme or DH_{cdh}. Reduced CDH is reoxidized by electron acceptors such as 2,6-dichloroindophenol (DCIP), 1,2- or 1,4-benzoquinone and their derivatives, the ABTS cation radical, complexed metal ions such as Fe(III), Cu(II) and Mn(III), or oxygen [7] The reduction of these electron acceptors usually takes place at DH_{cdh} [31], but due to the pH dependent IET process to the heme cofactor it is obvious that one-electron acceptors can also be reduced at CYT_{cdh}. It is common belief that two-electron acceptors are reduced

directly at DH_{cdh}, but opinions differ pertaining to the reduction of one-electron acceptors. Cytochrome *c*, which can solely interact with the heme domain of CDH and thereby transfer electrons from heme *b* to heme *c*, can be used in enzyme assays to distinguish between the electron transfer via DH_{cdh}, or through the holoenzyme, obligatory involving CYT_{cdh}. Therefore, the preferred model for the electron transfer from CDH to the redox mediator cytochrome *c* is the electron-chain model.

The heme domain of CDH can also be oxidized at a polarized electrode that acts as an artificial one-electron acceptor via a reversible direct electron transfer (DET). This exceptional property opened the way for preliminary flow injection measurements for the development of biosensors based on CDH [18].

1.4.6 Phylogeny of *cdh* genes

By sequence analysis of *cdh*-encoding genes, all known CDHs were classified in two related subgroups. Class-I comprise basidiomycete CDHs that lack a characteristic cellulose-binding domain but nevertheless bind strongly to cellulose through an as yet unidentified mechanism. Whereas class-II comprise longer, more complex sequences from ascomycete fungi whose CDH carry a fungal type-1 carbohydrate-binding module (CBM1) [6]. At any given pH, the FAD of the basidiomycete *P. chrysosporium* CDH is more reductive than the heme. Thus, during the reductive half-reaction, there will be a flow of electrons from the catalytic center to the heme domain, which supports the electron-chain model. Such a flow is observed for class-I CDHs only below pH 6, but for some class-II CDHs also under neutral or slightly alkaline conditions.[16, 17, 32]. The few investigated class-II CDHs feature slightly, but nonetheless important differences in substrate specificity, e.g. the distinct discrimination against maltose and glucose of class-I CDHs cannot be assigned for ascomycete CDHs and quite high turnover rates for maltose and especially for glucose are possible. Compared to class-I CDHs the pH optima for class-II CDHs are in less acidic pH regions [6].

1.4.7 Biological function of CDH

Wood is an exceptionally difficult substrate to degrade in terms of its physical and chemical properties. Fungi are the most important and widespread group of organisms contributing massively to wood decay. Wood-decay fungi fall into three types according to their mode of attack on the woody cell walls. Brown-rot fungi are predominantly members of the Basidiomycota. The fungi that cause soft rots include Ascomycota, whereas white-rot fungi include both Basidiomycota and some Ascomycota.

The biological function of the secreted flavocytochrome CDH is not yet fully understood and many hypotheses have been published over the past years. One of the leading hypotheses of its biological role is that it assists in the degradation and modification of cellulose, hemicelluloses and lignin by generating hydroxyl radicals. This is a highly reactive chemical compound, which is able to depolymerize cellulose, hemicellulose and lignin, the major compounds of woody plants [6]. In the hydroxyl-radical hypothesis, the electron acceptor is a ferric complex, probably Fe(III)-oxalate, since oxalic acid is excreted by the fungus in large amounts during cellulose degradation. The resulting ferrous ions can then participate in a Fenton reaction in the presence of H_2O_2 forming hydroxyl-radicals [6].

1.4.8 Applications of CDH

CDH has many interesting features, which can be used in different fields. The applications can be split in two main areas: analytical- and biotechnological applications.

1.4.8.1 Analytical applications

Due to the narrow specificity in the conversion of sugars and the multitude of suitable electron acceptors, CDH can be used in several analytic assays and biosensors. By the use of CDH in analytic assays and biosensors, electron donors as well as electron acceptors can be detected. CDH can quantify lactose in milk, by the use of a colorimetric activity assay [33]. There are biosensors with CDH from *P. chrysosporium* on a rotary disc electrode for the detection of cellobiose, cellooligosaccharides, lactose and maltose [34]. A CDH biosensor in combination with other biosensors, such as glucose oxidase, or oligosaccharide dehydrogenase, can be used in flow cells or as selective dual-enzyme electrode cells for the detection of certain mono-, di- and oligosaccharides [35]. Electron acceptors, like highly

toxic *ortho*- and *para*-diphenolic compounds can be detected by CDH modified biosensors [36]. For screening of phenolic compounds in water [37] and for the detection of catecholamines [38], a CDH based biosensor was evaluated. An important research field is the development of biosensors using the DET of CDH with electrodes. The promising results from these new studies might provide new input for the application of CDH in biosensors [6].

1.4.8.2 Biotechnological applications

The use of *P. chrysosporium*, grown under CDH induced conditions, for waste removal and bioremediation is documented [CPPS]. Biodegradation of two super absorbent polymers, a cross linked insoluble polyacrylate and an insoluble polyacrylate/polyacrylamide copolymer were reported [39]. Another application in bioremediation might be to degrade hexahydro-1,3.5-trinitro-1,3,5-triazine, a military high explosive compound. The observed results in biodegradation are the result of the action of several enzymes including CDH.

Several autors suggested that CDH could be used as a part of an enzyme complex to modify or break down cellulose and lignin in the pulp and paper industry [6].

The production of lactobionic acid with CDH and a redox mediator, regenerated by laccases, was suggested [40]. The use of the same system of oxidoreductases for the production of lactose-free galacto-oligosaccharide mixtures was also proposed. The application of CDH in processes for the enzymatic conversion of carbohydrates in food industry requires the purified enzyme.

2 AIMS OF THE THESIS

The production of ascomycete class-II CDHs has been less intensively investigated than class-I basidiomycete CDHs and up to now, few ascomycetes are known for their CDH secretion. Class-II CDHs feature slightly different, but nonetheless important changes in substrate specificity

CDHs with pH optima in the neutral and alkaline range and good conversion rates from glucose were of particular interest. Furthermore, it would be important to find stable holoenzymes for the use in third generation biosensors, based on the DET between the active site of the enzyme and the electrode, without needing mediators. The use of CDH in third generation biosensors is currently an advanced field of research at the Department for Food Science and Technology.

In the event that CDH production will be observed in screened strains, optimized media should be developed to provide a good yield of the desired enzyme. Furthermore, CDH of selected positive strains should be produced in larger scales for purification.
Finally, enzyme characterisation and kinetic studies of the most promising CDHs were planned.

3 MATERIAL AND METHODS

3.1 Chemicals

All used chemicals were of analytical grade and purchased from Sigma Aldrich Chemicals (Steinheim, Germany), Fluka (Buchs, Switzerland,), VWR (Darmstadt, Germany) and Roth (Graz, Austria). Media components were obtained from Fluka and Sigma Aldrich Chemicals. For the kinetic measurements, the following electron acceptors were used: 2,6-dichlorindophenol (DCIP) and cytochrome *c* (from horse heart) from Sigma Aldrich Chemicals.

3.2 Instrumentation

Activity Measurements: Diode Array Photometer Agilent 8453, Agilent Technologies (Böblingen, Germany)
Bradford Test: Beckman Coulter DU 800 Spectrophotometer (Munich, Germany)
Kinetic Measurements: Perkin Elmer Lambda II Spectrophotometer (USA)
Chromatography: Äkta Purifier System, Amersham Biosciences (Uppsala, Sweden)
Bioreactor: Applikon Pilot-System 40 and Applikon Pilot-System 70, Applikon Biotechnology (Schiedam, Netherlands)
Centrifuges: Eppifuge 5415R, Eppendorf (Hamburg, Germany); HERAEUS Megafuge 1.0R (Hanau, Germany); SORVALL Evolution RC, SLC 6000 Rotor, SORVALL RC26Plus, SLA 3000 Rotor, Thermo Scientific (USA)
IEF: Multiphor II System, Amersham Biosciences
IEF: Phast System Separation and Controll Unit, Pharmacia (Stockholm, Sweden)
SDS-PAGE: Hoefer Mighty Small SE 250 dual minislab gel apparatus, Amersham Biosciences

3.3 Organisms

Strains were obtained from CBS (fungal biodiversity centre - Utrecht, Netherlands), from DSMZ (German Collection of Microorganisms and Cell Cultures) and the Austrian Center of Biological Resources and Applied Mycology (MA). All used fungi are listed in table 3.1.

Table 3.1 Table of organisms

Acremonium strictum	DSM 3567	*Melanocarpus albomyces*	CBS 638.94
Botrytis aclada	DSM 62081	*Monilia brunnea*	CBS 240.33
Capnodium salicinum	CBS 131.34	*Monilinia fructigena*	DSM 2678
Cenococcum geophila	DSM 4284	*Myceliophthora thermophila*	CBS 663.74
Ceramothyrium linnaeae	CBS 742.94	*Myriococcum thermophilum*	CBS 208.89
Chaetomium atrobrunneum	CBS 238.71	*Neurospora crassa*	CBS 232.56
Cheilymenia pulcherrima	CBS 607.70	*Paecilomyces variotii*	CBS 372.70
Coniosporium apollinis	CBS 352.97	*Rhizosphaera kalkhoffii*	DSM 5143
Corynascus thermophilus	CBS 174.70	*Sclerotium cepivorum*	CBS 326.66
Corynespora cassiicola	DSM 2496	*Scytalidium lignicola*	DSM 2694
Dichomera saubinetii	CBS 990.70	*Scytalidium thermophilum*	CBS 619.91
Fusarium oxysporium	MA 2516	*Stachybotrys bisbyi*	DSM 63042
Geopyxis carbonaria	CBS 655.92	*Stigmina compacta*	CBS 700.70
Glomerella cingulata	DSM 62728	*Talaromyces thermophilus*	CBS 236.58
Humicola grisea	DSM 2691	*Thermoascus aurantiacus*	CBS 396.78
Hypoxylon bipapillatum	CBS 375.86	*Thermoascus thermophilus*	CBS 624.74
Hypoxylon haematostroma	CBS 255.63	*Thermomyces lanuginosus*	CBS 288.54
Hypoxylon vogesiacum	CBS 266.63	*Thielavia terrestris*	CBS 456.75
Lamprospora wrightii	CBS 600.29	*Trametes villosa**	CBS 334.49

* Basidiomycete

3.4 Media and buffer systems

3.4.1 Preparation of media

All media components were dissolved in reverse osmosis (RO) water and autoclaved at 121°C for at least 20 minutes. After sterilisation agar containing media were cooled down to 55°C in a water bath before pouring aseptically into sterile Petri dishes in a sterile bench and stored at 4°C. In all cultivation media except PDA, 0.3 mL L^{-1} of trace element solution was applied. The trace element solution contained: 1 g L^{-1} $ZnSO_4 \cdot 7H_2O$, 0.3 g L^{-1} $MnCl_2 \cdot 4H_2O$, 3 g L^{-1} H_3BO_3, 2 g L^{-1} $CoCl_2 \cdot 6H_2O$, 0.1 g L^{-1} $CuSO_4 \cdot 5H_2O$, 0.2 g L^{-1} $NiCl_2 \cdot 6H_2O$, 4 mL L^{-1} H_2SO_4 [41].

3.4.2 List of different media

The following table contains all different cultivation media.

Table 3.2 Cultivation media

PDA-agar: small Petri dishes 39 g L^{-1} of ready-mix Potato-Glucose-Agar.
100C-5P-agar: small Petri dishes 100 g L^{-1} α-cellulose, 5 g L^{-1} peptone from meat, 0.3 mL L^{-1} trace element solution, 5 g L^{-1} agar
5 cotton wool - 5P-medium: big Petri dishes 5 g L^{-1} cotton wool, 5 g L^{-1} peptone from meat, 0.3 mL L^{-1} trace element solution
20 cotton wool - 5P-medium: big Petri dishes 20 g L^{-1} cotton wool, 5 g L^{-1} peptone from meat, 0.3 mL L^{-1} trace element solution
20 cotton wool - 20P-medium: big Petri dishes 20 g L^{-1} cotton wool, 20 g L^{-1} peptone from meat, 0.3 mL L^{-1} trace element solution
20C-5P-medium: shaking flasks and big Petri dishes 20 g L^{-1} α-cellulose, 5 g L^{-1} peptone from meat, 0.3 mL L^{-1} trace element solution
5C-5P-medium: shaking flasks and big Petri dishes 5 g L^{-1} α-cellulose, 5 g L^{-1} peptone from meat, 0.3 mL L^{-1} trace element solution
5C-20P-medium: shaking flasks 5 g L^{-1} α-cellulose, 20 g L^{-1} peptone from meat, 0.3 mL L^{-1} trace element solution
20C-20P-medium: shaking flasks 20 g L^{-1} α-cellulose, 20 g L^{-1} peptone from meat, 0.3 mL L^{-1} trace element solution
10C-10P-medium: bioreactor cultivation 10 g L^{-1} micro-crystalline cellulose, 10 g L^{-1} peptone from meat, 0.3 mL L^{-1} trace element solution
20C-5P-medium: bioreactor cultivation 20 g L^{-1} micro-crystalline cellulose, 5 g L^{-1} peptone from meat, 0.3 mL L^{-1} trace element solution

3.4.3 Buffers

The following buffers were used in the experiments: 100 mM sodium acetate (pH 4.0), adjusted with 4 M NaOH; 50 mM citric acid (from pH 2.0 to pH 6.5), adjusted with 4 M NaOH; 50 mM sodium hydrogen phosphate (from pH 5.5 to 8.0), adjusted with 4 M NaOH. The buffers were prepared using HQ water (≥ 18 MΩ cm).

3.5 Cultivation

All inoculation steps were done aseptically in a sterile bench to minimize contamination. The first step was the recultivation of stock cultures from resting state. Fresh PDA plates were inoculated aseptically with a small amount of mycelium of the stock organism, using a platinum loop. For better results with poorly growing strains, a bigger surface area between fungus and medium was achieved, by scratching the agar with the platinum loop. Cultures were incubated at their recommended cultivation temperature (25 and 37°C).

3.5.1 Subculturing

To maintain active mycelium from the stock cultures, all grown organisms were regularly subcultured every three to four weeks, depending on their rate of growth.

3.5.2 Cultivation on cellulose-agar plates

CDH production on solid medium was performed with α-cellulose containing agar plates. These α-cellulose agar plates were inoculated in the same way than PDA-agar plates, with small plugs from the mature culture grown on PDA-agar. Plates were incubated at 25°C or 37°C. Sampling was done four days after incubation.

3.5.3 Cultivation in shaking flasks

Medium containing Erlenmeyer flasks were autoclaved at 121°C for 20 minutes. After cooling to room temperature, the media were inoculated with several mycelium plugs of overgrown PDA plates. Shaking flask cultures were incubated in rotary shakers at recommended temperatures (25 or 37°C) with continuous agitation (110 rpm, eccentricity = 1.25 cm). Cultivation was performed up to 19 days.

3.5.4 Cultivation in big Petri dishes

Selected strains were cultivated in big Petri dishes (diameter = 20 cm) filled with medium at about 50% of total volume without agitation. Based on optimization tests with shaking flask medium, five different media containing α-cellulose and cotton wool as carbon source were used for cultivation. Petri dishes filled with 400 mL medium were autoclaved at 121°C for 20 minutes and cooled to room temperature. Inoculations were done by transferring of about 100 mL of 6 days old shaking flasks culture media. Petri dishes were incubated without shaking at 25°C, the preferred temperature of the selected strains.

3.5.5 Cultivation in the bioreactor

Laboratory-scale cultivations were carried out in a 42-L and 70-L stirred tank bioreactor with a working volume of about 75-80% of the total volume. Both bioreactors were equipped with instrumentation for measurement and control of agitation, temperature, pH, dissolved oxygen concentration and foam level. The culture medium except micro-crystalline cellulose, which was autoclaved separately, was sterilised *in situ* at 121°C for 30 min. The bioreactors were inoculated with one week old precultures (about 5% vol. vol.$^{-1}$) grown in shaking flasks. During cultivation, the temperature was maintained at 25°C and the pH was allowed to float freely. In the first study (*D. saubinetii and H. haematostroma*), agitation and aeration were set to 120 rpm and 20 L vol. air min^{-1} in the beginning and were increased to 150 rpm and 40 L vol. air min^{-1} at the third day. During the second bioreactor cultivation (*D. saubinetii and C. atrobrunneum*), settings were regulated constant at 90 rpm agitation and at 7.5 L vol. air min^{-1} aeration.

3.5.6 CDH production from selected ascomycetes

CDH production for enzyme purification was performed in big shaking flasks (1 L, 2 L and 3 L flasks) with a working volume of about 30% of total volume. For elevated CDH secretion, the optimized α-cellulose media were used. Cultures were incubated until high amounts of free heme-containing intact enzyme were measured. The shaking flasks were harvested immediately after reaching expected catalytic activity values, which were known from screening and media optimization tests.

3.6 Sampling

3.6.1 Submersed cultivation

Around 2 mL of the crude culture medium were pipetted aseptically into a sample tube. Insolvable components like cellulose and mycelium, which would disturb photometric measurements, were separated by centrifugation at 10.000 rpm for about 10 min. The supernatant was then analyzed to detect enzyme activity and protein amount. All pH measurements were carried out directly in crude medium, sampled in extra tubes.

3.6.2 Solid state cultivation

Small plugs from the overgrown α-cellulose agar plates were cut out, transferred into a sample tube and filled with 1 mL buffer (100 mM sodium acetate buffer, pH 4). To mesh solid medium and to dissolve CDH in the buffer the sample tubes were mixed intensely. To separate insolvable components, the mixed sample tubes were centrifuged at 10.000 rpm for about 10 min. The supernatant was then analyzed for enzyme activity and protein content.

3.7 Enzyme activity assay and protein determination

3.7.1 DCIP assay

Enzyme activity assay with DCIP was used as a standard method to determine CDH activity in crude extracts or partially purified preparations. The natural substrate cellobiose, which may exhibit substrate inhibition, was for routine measurements replaced by lactose, a much cheaper and less problematic electron donor. The reaction was detected by the concomitant reduction of the electron acceptor DCIP which decolorizes the initially red-blue assay. To eliminate laccase activity, which would cover part of the CDH activity by reoxidizing DCIP, sodium fluoride was added to the DCIP activity assay. The DCIP assay was performed by recording the time dependent reduction of DCIP at 30°C by following the decreasing absorption at 520 nm [42]. The obtained result is a negative slope which is converted into U mL^{-1} by using the enzyme factor (EF = 7.25) calculated from the absorption coefficient of DCIP (ε_{520} = 6.9 $mM^{-1}cm^{-1}$). The detection limit for the DCIP assay is about 0.03 U mL^{-1}.

3.7.2 Cytochrome *c* assay

The CDH activity assay with cytochrome *c* was used as a reference method to determine CDH solely in crude extracts or partially purified preparations. In contrast to the DCIP method, it determines only the catalytic activity of the holoenzyme CDH and not of DH_{cdh}. For standard assays lactose was used as substrate due to possible substrate inhibition by cellobiose. The reaction was detected by the concomitant reduction of the electron acceptor cytochrome *c* that changes the color from initially orange to a more reddish-pink tone. Laccase cannot interfere with this assay. The cytochrome *c* assay was performed by recording the time dependent reduction of this redox mediator at 30°C by following the increasing absorption at 550 nm. The obtained result is a negative slope which is converted into U mL^{-1} by using the enzyme factor (EF = 2.55) calculated from the absorption coefficient of cytochrome *c* (ε_{550} = 19,6 $mM^{-1}cm^{-1}$) [43]. The detection limit for the cytochrome *c* assay is 3-fold more sensitive than for the DCIP assay (0.01 U mL^{-1}).

$$EF = \frac{1000 * F}{20 * \varepsilon} \text{ [mM cm]}$$

1000	cuvette volume [µL]
F	sample dilution factor
20	sample volume [µL]
ε	[$mM^{-1}cm^{-1}$]

3.7.3 Pipetting schemes

All assay contents except the CDH sample were pipetted in a 1 mL microcuvettes and temperature was maintained to 30°C in a water bath. The cuvettes were transferred to spectrophotometer and the reaction was started when the enzyme solution was added to the assay. The enzyme solution was added by a small plunger carrying the samples with a simultaneous short intensive mixing of the assay before recording of the time dependent reduction of the electron acceptor was immediately started.

The following stock solutions were prepared for enzyme activity measurements: 300 mM D(+)-lactose monohydrate, diluted in water; 300 mM D(+)-cellobiose, diluted in water; 3 M D(+)-glucose, diluted in water; 3 M maltose, diluted in water, 3 mM 2,6-dichlorindophenol (DCIP), diluted in 10 vol.-% ethanol, 1 mM cytochrome *c*, diluted in the same buffer system

as used for following measurements; 200 mM sodium fluoride, diluted in purified water (≥ 18 MΩ cm). The following tables show the substance quantities and final concentrations in the different enzyme activity assays.

Table 3.3 Protocol of the DCIP enzyme activity assay

Substance	Stock solution	Cuvette sub-quantities	Final concentration
DCIP solution	3 mM (in 10% ethanol)	100 µL	0.3 mM
Lactose solution	300 mM	100 µL	30 mM
NaF solution	200 mM	20 µL	4 mM
Buffer	100 mM / 50 mM	760 µL	76 mM / 38 mM
Sample	clear supernatant	20 µL	
	Total volume	1000 µL	

Table 3.4 Protocol of the cytochrome *c* activity assay

Substance	Stock solution	Cuvette sub-quantities	Final concentration
Cytochrome *c* solution	1 mM	20 µL	0.02 mM
Lactose solution	300 mM	100 µL	30 mM
Buffer	100 mM / 50 mM	860 µL	86 mM / 43 mM
Sample	clear supernatant	20 µL	
	Total volume	1000 µL	

3.7.4 Protein determination

Protein was determined according to Bradford [44] The Protein Assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs. Protein concentration was measured spectrophotometrically at 595 nm at room temperature. Bovine serum albumin (BSA) was used as standard in the range of 0.1–1.0 mg mL^{-1}. To 1 mL of Bradford reagent 20 µL sample was added and incubated for 15 minutes at room temperature prior to measurement.

3.8 Purification

CDH was purified by a two-step column chromatography. After each purification step CDH activity and protein concentration were determined. All chromatographic steps were carried out at room temperature using the Äkta Explorer system.

3.8.1 Centrifugation

Immediately after harvesting, the crude medium was filtered through a strainer to separate the mycelium. The remaining insoluble components were removed by centrifugation for 30 min at 6000 rpm and 10°C. After centrifugation, the supernatant was immediately used, stored at 4°C when purification procedure started within a few days, or frozen to -80°C for later use.

3.8.2 Cross-flow filtration

The supernatant was concentrated using a 30-kDa polysulfone ultrafiltration membrane. To lower ion concentration, additionally deionized water was added to the concentrate. Cross-flow filtration was used to concentrate the supernatant and to lower the salt concentration.

3.8.3 Anion exchange chromatography

In order to purify proteins with an isoelectric point (pI) in the acidic range, a positively charged stationary phase is applied in anion exchange chromatography (AIEX). The mobile phase is chosen in a pH range higher than the protein's pI to create negatively charged protein surfaces that bind to the stationary phase. The higher the difference between pI of the protein and the pH of the used buffer, the higher is the binding strength to the gel matrix. Too big difference in pI and pH creates many other negatively charged proteins, which also bind to the matrix and the purification efficiency suffers. The used pH is a compromise between high enough binding strength (not to lose target protein) and an inefficient purification. Good results are obtained by using a pI to pH difference of at least one log value or a little bit more. In addition, ions bind to the charged matrix and abate the protein binding capacity of the column. The ion concentration can be measured as electrical conductivity in mS cm^{-1} and should be reduced for example with diafiltration, dialyses or cross-flow filtration, if necessary.

The enzyme solution was applied to a DEAE (Diethylaminoethyl) Sepharose fast flow column (XK 50, 130-mL gel volume, GE Healthcare) preequilibrated with buffer A. The used buffers are listed in table 3.5. Absorbance at 280 nm, 420 nm and 450 nm was detected on-line. Afterwards, the column was washed with buffer A until non-binding proteins were washed out completely, which is indicated by negligible protein absorbance at 280 nm in the flow through. Bound proteins eluted with a linear salt gradient from 0–1M NaCl within 5 column volumes. Fractions with significant absorption at 420 and 450 nm were collected and CDH activity and protein content was measured.

Table 3.5 Buffers for anion exchange chromatography

***Hypoxylon haematostroma* CDH purification**
Conditions 1:
Buffer A: 50 mM sodium citrate buffer, pH 6.0
Buffer B: 50 mM sodium citrate buffer, 1 M NaCl, pH 6.0
Conditions 2:
Buffer A: 25 mM Tris-acetate buffer, pH 8.0
Buffer B: 25 mM Tris-acetate buffer, 1 M NaCl, pH 8.0
***Dichomera saubinetii* CDH purification**
Buffer A: 20 mM sodium citrate buffer, pH 6.0
Buffer B: 50 mM sodium citrate buffer, 1 M NaCl, pH 6.0

3.8.4 Hydrophobic interaction chromatography

Hydrophobic interaction chromatography (HIC) exhibits the same characteristic feature than reversed-phase chromatography (RPC), based on interactions between patches on the surface of biomolecules and the hydrophobic surface of the stationary phase. However, a RPC stationary phase is usually more hydrophobic than the surface of a HIC medium. This leads to stronger interactions that, for successful elution, must be reversed by using non-polar, organic solvents. HIC media offer an alternative way of exploiting the different hydrophobic properties of biomolecules by working in a more polar and less denaturing environment.

Pooled fractions of AIEX were set to 20% ammonium sulfate concentration by adding of 20% saturated ammonium sulfate solution under constant stirring. The enriched solution was applied to the Phenyl-Source fast flow column (XK 16, 24-mL gel volume, GE Healthcare) preequilibrated with buffer A. The buffers for HIC are listed in table 3.6. Absorbance at 280 nm, 420 nm and 450 nm was detected on-line. Afterwards the column was washed with buffer A until excessive non-binding proteins were washed out completely, which is indicated by negligible protein absorbance at 280 nm in the flow through. Bound proteins eluted by decreasing conductivity using a gradient from 0–100% buffer B within 5 column volumes. Fractions with significant absorbance at 420 and 450 nm were collected and CDH activity and protein content was measured. Fractions with highest specific activities were pooled, concentrated and washed, using centrifugal filter units (Amicon Ultra Centrifugal Filter Devices) and 50 mM sodium citrate buffer, pH 6. The enzymes were removed and dissolved in a small amount of the same buffer. These enzyme concentrates were used for all further analyzes and for enzyme characterization.

Table 3.6 Buffers for hydrophobic interaction chromatography

***Hypoxylon haematostroma* CDH purification**
Conditions 1:
Buffer A: 40 mM sodium citrate buffer, containing $(NH_4)_2SO_4$ (20% saturation), pH 6.0
Buffer B: 10 mM sodium citrate buffer, pH 6.0
Conditions 2:
Buffer A: 25 mM Tris-acetate buffer, containing $(NH_4)_2SO_4$ (20% saturation), pH 8.0
Buffer B: 10 mM Tris-acetate buffer, pH 8.0
***Dichomera saubinetii* CDH purification**
Buffer A: 40 mM sodium citrate buffer, containing $(NH_4)_2SO_4$ (20% saturation), pH 6.0
Buffer B: 10 mM sodium citrate buffer, pH 6.0

3.9 Enzyme characterization

3.9.1 Isoelectric focusing

Isoelectric focusing (IEF) is a technique for separating proteins according to their net electric charge, because protein's charge changes with the pH of the surrounding solution. Proteins show very different electric charges due to the functional groups of the free amino acid residues, which can act according to their characteristic protonation constants as acids or bases. For this reason, the net charge on the molecule is affected by pH of their surrounding environment and becomes charged more positively or negatively due to the loss or gain of protons. The pH at which a particular protein carries a neutral net charge (the number of negative and positive charges are equal) is called isoelectric point. The IEF technique utilizes this characteristic. An electric current is passed through an ampholyte-saturated gel, usually a polyacrylamide gel, to separate this mixture of polyanionic and polycationic molecules according to their net charges and a continuous gradient is built. In this continuous gradient, a protein will be charged positively or negatively and migrate towards the cathode or anode because of the applied voltage, until the point where the protein reaches the pH region that corresponds to its isoelectric point. Exactly in the region of same gradient pH and protein pI, the protein movement stops because of its zero net charge and the electric field has no more influence.

Isoelectric focusing was performed two times. In first run, a gradient range of pH 2.5 to pH 10 with a Multiphor II system using precast gels (CleanGel IEF, Amersham Biosciences) was applied. Analyzed were supernatants of CDH positive ascomycete crude media, collected from the shaking flask screening and stored at -80°C. The ampholytes were separated at 300V for 30 min to obtain a focused gradient. A low pI marker protein kit (pH 2.8-6.6) and a broad pI marker protein kit (pH 3.5–9.6), shown in table 3.7, were used to determine pI values. CDH solutions with a conductivity of more than 10 mS cm^{-1} were diluted with RO-water to reduce the salt concentration. About 20 µL sample and 10 µL marker were applied to the sample application pieces. The separation procedure started by loading for 1 h at 500V and 8mA. Afterwards the sample application pieces were removed and isoelectric focusing was carried out for 3 more hours at the same voltage to a total of 120000 V min.

Table 3.7 Low and broad pI calibration standard

Low pI (pH 2.5-6.5)		Broad pI (pH 3-10)	
Protein	**pI**	**Protein**	**pI**
Pepsinogen	2.80		
Amyloglucosidase	3.50	Amyloglucosidase	3.50
Methyl red	3.75	Methyl red	3.75
Glucose oxidase	4.15		
Trypsin inhibitor	4.55	Trypsin inhibitor	4.55
β-Lactoglobulin A	5.20	β-Lactoglobulin A	5.20
Carbonic anhydrase B (bovine)	5.85	Carbonic anhydrase B (bovine)	5.85
Carbonic anhydrase B (human)	6.55	Carbonic anhydrase B (human)	6.55
		Myoglobin, acidic band	6.85
		Myoglobin, basic band	7.35
		Lentil lectin, acidic band	8.15
		Lentil lectin, middle band	8.45
		Lentil lectin, basic band	8.65
		Trypsinogen	9.30

The second IEF was performed using the Phast System from Pharmacia with precast dry gels (CleanGel IEF, Amersham Biosciences). This second analysis was provided for the purified CDH solutions of *D. saubinetii* and *H. haematostroma*. To estimate the pI values a protein kit in the range of pH 3.6 to 9.3 (IEF mix 3.6-9.3, Sigma) was used, shown in table 3.8.

Table 3.8 IEF mix pI calibration standard

IEF mix 3.6-9.3

Protein	**pI**
Amyloglucosidase from *Aspergillus niger*	3.6
Trypsin inhibitor from *Glycine max* (soybean)	4.6
β-Lactoglobulin A from bovine milk	5.1
Carbonic Anhydrase Isoenzyme II from bovine erythrocytes	5.9
C Carbonic Anhydrase Isoenzyme I from human erythrocytes	6.6
Myoglobin from equine heart	6.8, 7.2
Lectin from *Lens culinaris* (lentil)	8.2, 8.6, 8.8
Trypsinogen from bovine pancreas	9.3

3.9.2 Molecular mass determination by SDS-PAGE

SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) is an advanced variation of gelelectrophoresis. It uses also an electric field to move the molecules through the gel matrix, most commonly a cross-linked polymer like polyacrylamide gel, but a different sample preparation. The molecules will move through the matrix at different rates, determined largely by their mass when the charge-to-mass ratio of all species is uniform, toward the anode, if negatively charged, or toward the cathode, if positively charged. Proteins show varying charges, depending on pH value, but also their complex shapes influence the moving rate, why gelelectrophoresis is no adequate method for their separation based on molecular weight. By generating denatured proteins with 2-Mercaptoethanol, a reducing agent, which possesses the ability for cleaving disulfide bridges and a second detergent, SDS (sodium dodecyl sulphate), which binds relatively to the size of the protein, a determination on the basis of different molecular weights is possible. The resulting polypeptide chain has an overall negative charge with a similar charge to mass ratio.

SDS-PAGE was carried out using a Hoefer Mighty Small SE 250 dual minislab gel apparatus (Amersham Biosciences). The contents for separating and stacking gel are listed in table 3.9. All separating gel solutions were mixed and filled between the fixed two glass plates. The

filled separating gel was immediately covered with a layer of 2-butanol to smoothen the surface for an exactly horizontal dividing line between separating and stacking gel. After about 30 minutes, the gel polymerized and the 2-butanol layer was removed completely by washing with water and dried with small paper stripes. The separating gel was covered with a layer of freshly prepared stacking gel, whereon the slot-comb was placed immediately. After polymerization of the stacking gel, about 30 minutes after preparation, the slot-comb was removed and to finalize the gel, it was covered with running buffer.

The purified CDH samples were prepared for SDS-PAGE by 1:2 dilution with sample buffer containing SDS for denaturation according to Laemmli *et al.* (1970) [45] and an incubation step at 90°C for 5 min. 5 µL of the molecular weight standard mixture (BioRad Precision Plus ProteinTMStandards or Fermentas Page Ruler Prestained Protein Ladder SM0671) as well as 20 µL of each sample were applied to the gel and run at 200 V and 40-50 mA. The electrophoresis was stopped when the brompenol blue front reached the opposite side of the separating gel. Afterwards, the protein bands were visualised by coomassie blue staining. The molecular masses of the bands were determined by comparison with the standard markers.

Table 3.9 Preparation of separating and stacking gel for SDS-PAGE

Chemicals	**Separating gel**	**Stacking gel**
H_2O	4.8 mL	3.4 mL
Acrylamid 30%	3.96 mL	0.83 mL
1.5 M Tris pH 8.8	3 mL	-
1 M Tris pH 6.8	-	0.63 mL
SDS 10%	0.12 mL	50 µL
APS* 10%	0.12 mL	50 µL
TEMED**	5 µL	5 µL

* Ammonium persulfate
** N,N,N',N'-Tetramethylethylendiamine

3.9.3 Active-, silver- and Coomassie blue-staining

Active Staining

After isoelectric focusing, CDH bands were visualized by active staining using the same color reaction used in photometric activity measurements. IEF is a native electrophoresis method and separation is done in non-denaturing conditions. During the separation procedure, enzymes move to the pH point according their pI, but they are still active, for which reason catalytic activity testing is performed directly on the gel. The DCIP enzyme activity assay show more intensive color alterations during enzymatic reaction than the cytochrome *c* assay and determination of CDH bands with the naked eye is possible. To this end, the gel was covered with pure DCIP solution (3 mM DCIP in 10% ethanol), until uniformly stained. Excess DCIP solution on the gel was removed. After the addition of a few drops of 300 mM lactose solution onto the surface of the gel, active bands developed as white bands on a blue stained background.

Silver staining

Silver staining is a method to visualize proteins on electrophoresis gels. The principle of silver staining is to denaturate proteins in the gel with the fixing solution. Silver ions, added as silver nitrate, adsorbs to the proteins and are reduced to elementary silver with formaldehyde. Elementary silver is dark colored and uncovers proteins in dyed bands.

According to Ansorge *et al.* (1985) [46] the solutions for silver staining are shown in table 3.10.

Table 3.10 Solutions and incubation conditions for silver staining

Preparation		**Incubation**	
Solution	**Composition**	**Solution**	**Time**
Fixing solution	30% methanol	Fixing solution	15 min
	10% acetic acid	$CuCl_2$ solution	15 min
$CuCl_2$ solution	50% methanol	Washing solution I	15 min
	15% acetic acid	$KMnO_4$ solution	15 min
	2% copper(II)chloride	Washing solution I	15 min
Washing solution I	10% ethanol	Washing solution II	15 min
	5% acetic acid	H_2O	15 min
$KMnO_4$ solution	0.002% $KMnO_4$	Silver dyeing solution	15 min
Washing solution II	10% ethanol	H_2O	2 x 20 sec
Silver dyeing solution	0.2% $AgNO_3$	Developer	bands visible
Developer	2% K_2CO_3 + 20 µL	H_2O	15 min
	37% formaldehyde/100 mL H_2O		

Coomassie Blue staining

Coomassie dyes are used to stain proteins in gel electrophoresis gels and are responsible for coloriometric protein quantification in Bradford method.

The IEF gel was stained with coomassie brilliant blue staining solution and destained automatically by the use of the Phast System from Pharmacia, until blue bands were visible on the gel.

The SDS-PAGE gel was covered with coomassie brilliant blue staining solution and incubated for 2 hours, while softly agitated. Afterwards, the stain was decanted and the gel was rinsed with water. This was followed by a destaining step, until only blue bands were visible on the gel. Afterwards the gel was soaked in conservation solution. All solutions were prepared according to the manufacturer's manual (Pharmacia Biosciences).

3.10 Kinetic characterization

For kinetic studies, the high purity CDH concentrates, deep-frozen after the purification procedure, were defrosted. Several dilutions in 50 mM sodium citrate buffer pH 5.0 were prepared, fractioned in small tubes and again deep-frozen to -80°C. These CDH stock dilutions were used for all enzyme kinetic studies.

3.10.1 pH profiles

The pH dependency of the lactose conversion of CDH was determined with the one-electron acceptor cytochrome *c* and the two-electron acceptor DCIP. Two different buffer systems were prepared in 0.5 pH steps. The 50 mM sodium citrate buffer was used from pH 2 to pH 6.5 and a 50 mM sodium phosphate buffer in a pH range of pH 5.5 to 8.0 (pH of both buffers was adjusted with 4 M NaOH). To determine the true pH conditions for the enzyme reaction, the activity assay, except the small amounts of enzyme solution, was prepared in cuvettes and the formed pH was measured. All experiments were performed according to the standard DCIP and cytochrome *c* activity assays. Substrate was converted with two CDH dilutions, appropriate to each electron acceptors and detected in duplicate. The results of the CDH dilutions are averaged and shown in the graphs as normalized velocity versus pH. Reaction velocity at 30°C, the chosen standard temperature for catalytic activity measurements, was used as point of reference (100%).

3.10.2 Temperature profiles

Activity was measured at different temperatures using the DCIP and cytochrome *c* enzyme activity assay at pH 5.0 (50 mM sodium citrate) with lactose as substrate. During these tests, the initial substrate conversion rates at different temperatures, beginning at 20°C up to 70°C, were tested. All assay contents except the CDH sample were pipetted in 1 mL microcuvettes, which were incubated in a water bath until the desired temperature was reached. The exact assay temperatures were detected by measuring the temperature in a blank assay directly before the reaction was started in the photometer. The temperature of the assays immediately after the reaction was measured again by a resistance thermometer. The used Perkin Elmer Lambda II spectrophotometer featured additionally a device for continuous heating of the cell holder by using the water of the water bath, which is helpful

for maintaining higher temperatures of the assay during the whole reaction time. Nevertheless, at higher temperature steps, heat is lost during the recorded time of 300 seconds. For this reason, the assays were heated to a some degree higher temperature to obtain the desired reaction temperature as an average.

3.10.3 Substrate and redox mediator specificity

Reductive half-reaction

For kinetic measurements the disaccharides maltose and cellobiose (natural substrate) and the monosaccharide glucose were investigated in addition to the standard lactose as substrate for activity determinations. The conversions were performed with the one-electron acceptor cytochrome *c* and the two-electron acceptor DCIP, using 50 mM sodium citrate buffer at pH 5.0 and 50 mM sodium phosphate buffer at pH 6.5 and 8.0. All sugar stock solutions were prepared with the appropriate buffers at pH 5.0, 6.5 and 8.0 to guarantee the purposed pH in the cuvette also for catalytic activity measurements near the V_{max}, where substrates were presented in excess to the added buffer. Carbohydrate stock solutions were allowed to rest for 1 hour to allow for mutarotation.

Oxidative half-reaction

The effect of electron acceptors to steady state kinetic constants were investigated by using the two-electron acceptor DCIP and the one-electron acceptor cytochrome *c* with the electron donor lactose at three different pH conditions. The same two buffer systems, sodium citrate and sodium phosphate buffer, each 50 mM, were used to adjust the same three pH steps, pH 5.0, 6.5 and 8.0.

Reaction velocities for the calculation of the Michaelis-Menten constants (K_M), the inhibitor constants (K_I) and maximum reaction velocities (V_{max}) were determined photometrically at 30°C, in initial rate experiments using a Perkin Elmer Lambda II spectrophotometer. All kinetic parameters were calculated by non-linear least square regression, fitting the observed data to the Michaelis-Menten equation using the program SigmaPlot. By including enzyme molecular mass, obtained by SDS-PAGE, the catalytic constants (k_{cat}) and the catalytic efficiencies ($k_{cat}\ K_M^{-1}$) were calculated. The k_{cat} represents the turnover number of the respective electron acceptor, not the electron donor (substrate). The reaction stoichiometry is 1 for two-electron acceptors and 2 for one-electron acceptors.

4 RESULTS AND DISCUSSION

4.1 Screening

For the first cultivation, potato dextrose agar was chosen as a universal medium. Nevertheless, two strains, *Thermoascus aurantiacus* and *Thermoascus thermophilus*, were not able to grow under these conditions. Most other strains grew as expected on PDA-agar. *Lamprospora wrightii* was the fastest growing strain of the selected ascomycetes. On the other hand, some ascomycetes like *Rhizosphaera kalkhoffi* and *Talaromyces thermophilus* needed more than one week to indicate even growth.

4.1.1 Screening in shaking flasks

The extracellular enzyme CDH is only produced by lignocellulose-degrading fungi under cellulolytic culture conditions [6]. As basic medium for shaking flask cultivation in screening, 20 g L^{-1} α-cellulose, 5 g L^{-1} peptone from meat and 0.3 mL L^{-1} trace element solution were chosen. To handle these quantity of various strains, small Erlenmeyer flasks (300 mL) were used. Periodically sampling was performed to monitor the CDH production and the pH change in culture broth. In order not to miss a possible producing strain, the more sensitive one-electron acceptor cytochrome *c* was used to detect already quite low enzyme activity. The most efficient substrate for CDH is cellobiose, but substrate-inhibition has been reported [6]. Therefore, in enzyme activity assays the β-linked disaccharide lactose acted as electron donor, which differs from cellobiose only in the orientation of the C4 hydroxyl group on the second, non-reducing glucose moiety. Lactose is a near adequate substrate to cellobiose and no substrate inhibition has been reported so far. Additionally, glucose, a monosaccharide, was tested with the new found CDHs. The pH optima of new ascomycete CDHs were expected in the acidic to neutral range. Therefore, additional to the standardized pH of 4.0 a second activity assay at pH 6.0 were applied for catalytic activity tests.

The detection limit for CDH activity measurements by using the cytochrome *c* assay is 0.01 U mL^{-1}. All strains achieving an enzyme activity higher than 0.01 U mL^{-1} were classified as CDH producing strains. The first strain (*D. saubinetii)* crossed the detection limit after five days of fermentation and reached highest detected catalytic activity after 18 days with the

enzyme assay at pH 6. Generally, the highest activities were measured 2 to 3 weeks after inoculation. After 3 weeks of cultivation the screening was stopped, because no further increase in catalytic activity and protein content was noticed. Detectable CDH production after three weeks was not expected.

The results of the screening are shown in table 4.1. Ten of the screened ascomycetes (*A. strictum*, *C. atrobrunneum*, *D. saubinetii*, *F. oxysporum*[i], *H. haematostroma*, *M. albomyces*, *M. brunnea*, *N. crassa*, S. thermophilum and *S. bisbyi*) showed significant CDH activity under the chosen culture conditions. *Monilia* sp. [12] and *Chaetomium* sp. [17] were already known as CDH producers. *N. crassa* is known as CDH gene carrying fungus due to a sequencing project of this species [15]. Catalytic activity measurements of *H. bipapillatum*, *H. vogesiacum* and *M. thermophila* crude media indicated CDH presence but below the detection limit. Most CDHs of positive screened ascomycetes offered higher catalytic activity with the enzyme activity assay at pH 6.0 than at pH 4.0. Only CDHs from *A. strictum, F. oxysporum*[i] and *M. albomyces* showed pH optima in the more acidic range, see table 4.2.

([i]4.1.1.1 Extracellular enzyme activity of *F. oxysporum*")

Table 4.1 Screening results

Species	Strain number	Growth (PDA-agar)	Activity (U mL^{-1}) [a]		Protein (mg mL^{-1})[b]	pH	Day	Temperatur (°C)
			pH 4.0	pH 6.0				
Acremonium strictum	DSM 3567	fast	0.021	0.005	0.08	7.3	18	25
Botrytis aclada	DSM 62081	fast	n.d.	n.d.	< 0.05	5.6	12	25
Capnodium salicinum	CBS 131.34	slow	n.d.	n.d.	n.d.	7.7	-	25
Cenococcum geophilum	DSM 4284	slow	n.d.	n.d.	n.d.	7.9	-	25
Ceramothyrium linnaeae	CBS 742.94	slow	n.d.	n.d.	0.06	8.4	19	25
Chaetomium atrobrunneum	CBS 238.71	fast	0.036	0.049	0.13	7.6	15	25
Cheilymenia pulcherrima	CBS 607.70	fast	n.d.	n.d.	n.d.	8.6	-	25
Coniosporium apollinis	CBS 352.97	slow	n.d.	n.d.	< 0.05	8.7	19	25
Corynespora cassiicola	DSM 2496	slow	n.d.	n.d.	< 0.05	9.1	19	25
Dichomera saubinetii	CBS 990.70	fast	0.106	0.155	0.28	8.6	18	25
Fusarium oxysporum	MA 2516	fast	0.031	0.004	0.28	7.3	17	25
Geopyxis carbonaria	CBS 655.92	slow	n.d.	n.d.	0.04	8.7	19	25
Glomerella cingulata	DSM 62728	fast	n.d.	n.d.	< 0.05	8.4	19	25
Humicola grisea	DSM 2691	fast	n.d.	n.d.	< 0.05	8.1	19	25
Hypoxylon bipapillatum	CBS 375.86	slow	0.007	0.007	< 0.05	8.6	17	25
Hypoxylon haematostroma	CBS 255.63	fast	0.015	0.028	0.29	8.0	19	25
Hypoxylon vogesiacum	CBS 266.63	fast	0.006	0.005	0.05	8.6	19	25

Lamprospora wrightii	CBS 600.69	very fast	n.d.	n.d.	0.06	8.9	19	25
Melanocarpus albomyces	CBS 638.94	fast	0.076	0.036	0.41	8.8	15	40
Monilia brunnea	CBS 240.33	fast	0.015	0.057	0.19	7.9	12	25
Monilinia fructigena	DSM 2678	fast	n.d.	n.d.	< 0.05	8.7	15	25
Myceliophthora thermophila	CBS 663.74	fast	0.006	0.006	< 0.05	8.9	19	40
Neurospora crassa	CBS 232.56	fast	0.019	0.098	0.28	7.7	19	25
Paecilomyces variotii	CBS 372.70	slow	n.d.	n.d.	n.d.	8.7	-	25
Rhizosphaera kalkhoffii	DSM 5143	very slow	n.d.	n.d.	n.d.	8.6	-	25
Sclerotium cepivorum	CBS 326.66	fast	n.d.	n.d.	< 0.05	4.4	15	25
Scytalidium lignicola	DSM 2694	slow	n.d.	n.d.	< 0.05	8.7	15	25
Scytalidium thermophilum	CBS 619.91	slow	0.009	0.017	0.17	7.8	15	40
Stachybotrys bisbyi	DSM 63042	fast	0.014	0.026	0.16	7.3	19	25
Stigmina compacta	CBS 700.70	slow	n.d.	n.d.	0.13	8.9	19	25
Talaromyces thermophilus	CBS 236.58	very slow	n.d.	n.d.	n.d.	7.8	-	40
Thermomyces lanuginosus	CBS 288.54	fast	n.d.	n.d.	< 0.05	8.8	15	40
Thielavia terrestris	CBS 456.75	slow	n.d.	n.d.	< 0.05	8.2	15	25
Thermoascus aurantiacus	CBS 396.78	n.d.	-	-	-	-	-	40
Thermoascus thermophilus	CBS 624.74	n.d.	-	-	-	-	-	40

n.d. ... not detectable

[a] enzyme activity was measured using the cytochrom *c* assay with lactose

[b] extracellular protein was measured according to Bradford

Table 4.2. A summary of lactose and glucose conversion rates of CDH positive screened ascomycetes. By changing the substrate to glucose, all strains except *S. thermophilum* showed significant catalytic activities, approximately similar as with lactose as substrate.

Table 4.2 Positive screened ascomycetes

Species	Protein (mg mL^{-1})[a]	Activity (U mL^{-1})[b]		Activity (U mL^{-1})[c]		pH
		pH 4.0	pH 6.0	pH 4.0	pH 6.0	
A. strictum	0.08	0.021	<0.010	0.015	n.d.	7.3
C. atrobrunneum	0.13	0.036	0.049	0.010	0.028	7.6
D. saubinetii	0.23	0.146	0.155	0.026	0.084	8.6
F. oxysporum	0.25	0.031	<0.010	0.027	<0.010	7.9
H. haematostroma	0.29	0.015	0.028	<0.010	0.011	8.0
M. albomyces	0.44	0.076	0.035	0.035	0.031	8.8
M. brunnea	0.20	0.015	0.057	<0.010	0.024	7.9
*N.crassa**	0.28	0.019	0.098	*	*	7.7
S. thermophilum	0.17	<0.010	0.017	<0.010	<0.010	7.8
S. bisbyi	0.16	0.014	0.026	<0.010	0.012	7.3

* *N. crassa* CDH was not tested with glucose as substrate
[a] extracellular protein was measured according to Bradford
[b] enzyme activity was measured using the cytochrome *c* assay with lactose
[c] enzyme activity was measured using the cytochrome *c* assay with glucose

4.1.1.1 Extracellular enzyme activity of *F. oxysporum*

F. oxysporum is a plant pathogen and secrets several cell wall degrading enzymes. This fungus is reported to carry FCD1, a gene encoding a protein homologous to DH_{cdh} (CBQ). DH_{cdh} contains the flavin domain of a CDH generated by proteolysis. CDH is believed to be the necessary precursor of DH_{cdh}, but the DH_{cdh} similar gene FCD1 is encoded directly. [47] However, CDH activity in cultures of this fungus are not reported, but in cultures of *F. concolor* [19]. Therefore *F. oxysporum* was added to the list of species for screening. *F. oxysporum* culture media showed detectable activities using the cytochrome *c* enzyme activity assay. Furthermore, in media optimization tests this strain showed clearly preferences to the standard medium and achieved activities similarly to *Ds*CDH, but with much lower specific activity, because of the high protein content detected in the culture

supernatant. Remarkable was the fact, that the enzymatic activity was only detected by using the cytochrome *c* assay. DCIP was never reduced by this enzyme (no bands in IEF active staining with DCIP as electron acceptor). When *F. oxysporum* carries a sequence coding DH_{cdh}, this protein should accept DCIP for reoxidation, but not the one-electron acceptor cytochrome *c*. Despite the fact that enzyme activity tests using cytochrome *c* indicated the expression of an extracellular enzyme, which was able to convert lactose and near the detection limit also glucose, this activity could not be identified as DH_{cdh} or CDH.

4.1.2 Screening on cellulose-agar

A standard method for lab-scale extracellular enzyme production and screening is cultivation in shaking flasks. However, a screening on agar plates is faster and easier, without the necessity of shakers. This method does not allow quantitative results, but by cultivation of fungi on agar plates containing cellulose, detection of significant CDH activity should be possible. Three well-known CDH producer, *Trametes villosa, Corynascus thermophilus, and Myriocuccum thermophilum* acted as reference strains to validate this method. All plates were inoculated in the same way than PDA-agar plates. To monitor the CDH production status, sampling should be done periodically, but an aseptic sampling is hardly feasible in agar plates. Due to good growth during subculturing on PDA and also during this test on cellulose plates, it was decided to sample after four days of cultivation, regardless of the production status of the different fungi. One of the reference strains, *Trametes villosa*, showed no CDH activity, although in past studies all screened *Trametes* sp. exhibited significant CDH activity by cultivation in a cellulose-containing medium.[48] The two other reference strains, both with higher temperature optimum, showed low enzyme activity after 4 days of cultivation. According to the results with the reference strains, the detection limits were chosen very low at 0.015 U mL^{-1} using the DCIP assay and at 0.005 U mL^{-1} by the more sensitive cytochrome *c* assay. However, it must be pointed out that measured results have no quantitative evidence. According to the very low limits, 19 of the screened ascomycetes were shown to exhibit CDH activity, see table 4.3, but lower than the reference strain with highest catalytic activity at 0.115 U mL^{-1} (DCIP assay) and 0.036 U mL^{-1} (cytochrome *c* assay). Based on the very low achieved activities by cultivation on cellulose agar plates and one negative reference strain, this method seems to be improperly for correct determination of new CDH producing ascomycetes, or must be improved to meet the demands. In shaking

flask cultivations, CDH production of screened ascomycetes was detected after 5 days of inoculation at the earliest. Nevertheless, all cellulose plates were screened after four days of cultivation, because most of the plates were overgrown at the surface. Probably more time for production of CDH would have been needed and a full overgrown plate is not the only indication for the production of extracellular enzymes. Very likely, this misinterpretation caused the imprecise results of this screening.

However, it was possible to induce CDH secretion by growing on α-cellulose-agar plates. This cultivation method could be at least useful to obtain well active mycelium, adapted to cellulose as only carbon source.

Table 4.3 CDH screening on cellulose-agar

Fungi	Activity (U mL^{-1})		Results
	DCIP[A]	Cytochrome *c*[B]	
Acremonium strictum	0.020	0.004	~
Botrytis aclada	0.013	0.000	-
Capnodium salicinum	0.017	0.006	+
Cenococcum geophila	0.014	0.006	~
Ceramothyrium linnaeae	0.017	0.005	~
Chaetomium atrobrunneum	0.015	0.008	~
Cheilymenia pulcherrima	0.004	0.005	-
Coniosporium apollinis	0.012	0.001	-
Corynespora cassiicola	0.020	0.006	+
Dichomera saubinetii	0.004	0.009	~
Fusarium oxysporium	0.005	0.007	~
Geopyxis carbonaria	0.012	0.005	-
Glomerella cingulata	0.020	0.005	~
Humicola grisea	0.005	0.006	~
Hypoxylon bipapillatum	0.017	0.006	+
Hypoxylon haematostroma	0.004	0.007	~
Hypoxylon vogesiacum	0.047	0.057	+
Lamprospora wrightii	0.012	0.004	-
Melanocarpus albomyces	0.027	0.011	+

Monilia brunnea	0.000	0.001	-
Monilinia fructigena	0.012	0.006	~
Myceliophthora thermophila	0.018	0.016	+
Paecilomyces variotii	0.004	0.004	-
Phoma destructiva	0.005	0.000	-
Rhizosphaera kalkhoffii	0.002	0.002	-
Sclerotium cepivorum	0.004	0.004	-
Scytalidium lignicola	0.006	0.004	-
Scytalidium thermophilum	0.010	0.008	~
Stachybotrys bisbyi	0.002	0.006	~
Stigmina compacta	0.017	0.002	~
Talaromyces thermophilus	0.009	0.001	-
Thermomyces lanuginosus	0.014	0.004	-
Thielavia terrestris	0.014	0.006	-
*Trametes villosa**	0.000	0.005	-
*Corynascus thermophilus**	0.115	0.036	+
*Myriocuccum thermophilum**	0.025	0.013	+

A Enzyme activity was measured using the DCIP assay [DCIP (pH 5) > 0,015 U/mL^{-1} ~ (propably)]
B Enzyme activity was measured using the cytochrom *c* assay [cyt c (pH 4) > 0,005 U/mL^{-1} ~ (propably)]
Two ~ declared as + (positive)
* Reference strains

4.2 Media optimization

By modifying the proportions of carbon and nitrogen-source, the effect on CDH secretion in the screened ascomycetes were investigated. The basic medium (20 g L^{-1} α-cellulose, 5 g L^{-1} peptone from meat), used in the shaking flasks screening, was modified by decreasing both main components to a level of 5 g L^{-1}, or by increasing both components to a level of 20 g L^{-1} and at last by inverting the carbon and nitrogen proportion (5 g L^{-1} α-cellulose, 20 g L^{-1} peptone)

The cultivations were carried out similarly to cultivation in shaking flasks at 25°C or 37°C, depending on the growth temperature optimum of the strains. The media contained no buffer and the pH was allowed to float freely to study the effects of the different media on

the pH value. The cytochrome *c* assay was used for detection of catalytic activity, either at pH 4.0 or at pH 6.0, depending on the source of CDH.

Two different groups of media were preferred (see table 4.4). One group, including *A. strictum, D. saubinetii, H. haematostroma, F. oxysporium*[i] *, S. bisbyi* and *N. crassa,* show highest enzyme activity and content of extracellular CDH by growing in the basic medium (C20-P5). The other group with *S. thermophilum, C. atrobrunneum* and *M. brunnea* prefer the same proportion of carbon and nitrogen (C5-P5 and C20-P20), whereas their quantity seems to be no crucial factor for CDH expression, but for specific activity. In general, a clearly visible effect on ascomycete cultivation in media with the high nitrogen content is the stimulation of the expression of several extracellular proteins and the specific activities of CDHs suffered.

Furthermore, different carbon and nitrogen concentrations had a large influence on the pH value in the media, but no correlation between CDH production and pH value was seen. The pH in cultures grown in the basic medium (C20-P5) remained in the range between 6.8 to 7.8, increased in the low content medium up to pH 8.8 and in media with high nitrogen concentration up to pH 9.1.

([i]4.1.1.1 Extracellular enzyme activity of *F. oxysporum)*

Table 4.4 Media optimization

Species	Medium	pH Assay*	Days	Activity [U mL^{-1}]^{a}	Proteine [mg mL^{-1}]^{b}	Specific activity [U mg^{-1}]	pH
Acremonium strictum	C5-P5	4	15	0.011	0.01	1.22	8.4
	C20-P5		15	0.054	0.06	0.95	6.8
	C5-P20		15	0.021	0.02	0.91	9.0
	C20-P20		15	0.021	0.08	0.26	9.1
Dichomera saubinetii	C5-P5	6	7	0.007	0.02	0.33	8.7
	C20-P5		15	0.107	0.24	0.45	7.0
	C5-P20		17	0.005	0.13	0.04	9.1
	C20-P20		7	0.019	0.13	0.15	8.5

	C5-P5		9	0.004	n.d.	-	8.8
Hypoxylon	C20-P5		9	0.065	0.13	0.49	7.5
haematostroma	C5-P20	6	9	0.002	0.09	0.02	9.1
	C20-P20		9	0.014	0.12	0.12	9.0
	C5-P5		12	0.017	0.01	1.21	8.8
Scytalidium	C20-P5		17	0.011	0.10	0.11	7.9
thermophilum	C5-P20	6	9	0.008	0.31	0.03	9.0
	C20-P20		12	0.022	0.46	0.05	8.9
	C5-P5		8	0.083	0.08	1.00	7.8
Chaetomium	C20-P5		15	0.051	0.12	0.43	7.0
atrobrunneum	C5-P20	6	15	0.025	0.13	0.19	8.9
	C20-P20		15	0.109	0.31	0.36	8.4
	C5-P5		8	0.057	0.04	1.58	8.3
Fusarium	C20-P5		20	0.098	0.42	0.23	6.4
oxysporium	C5-P20	4	13	0.019	0.01	3.17	9.1
	C20-P20		13	0.014	n.d.	-	9.1
	C5-P5		15	0.070	0.04	2.00	8.5
Monilia	C20-P5		8	0.049	0.10	0.51	7.6
brunnea	C5-P20	6	8	0.043	0.09	0.46	8.3
	C20-P20		13	0.072	0.10	0.69	8.9
	C5-P5		13	0.018	n.d.	-	8.6
Stachybotrys	C20-P5		20	0.054	0.18	0.29	7.8
bisbyi	C5-P20	6	13	0.020	0.04	0.54	8.8
	C20-P20		13	0.015	0.02	0.79	8.7
	C5-P5		20	0.008	0.14	-	8.1
Neurospora	C20-P5		20	0.098	0.28	0.35	7.7
crassa	C5-P20	6	20	0.001	0.11	0.01	7.7
	C20-P20		20	0.014	0.84	0.02	7.6

[a] enzyme activity was measured using the cytochrom c assay
[b] extracellular protein was measured according to Bradford
* sodium acetate buffer pH 4 and sodium citrate buffer pH 6

Very interesting was the specific color development in the .cultivation broth in dependence of the peptone and cellulose concentrations, but also in connection with CDH production. The color in the optimized media differed considerably from those with less enzyme production. *N. crassa* was a good example for this color development during cultivation in different media. (Figure 4.1) The preferred medium for CDH production was C20-P5 medium.

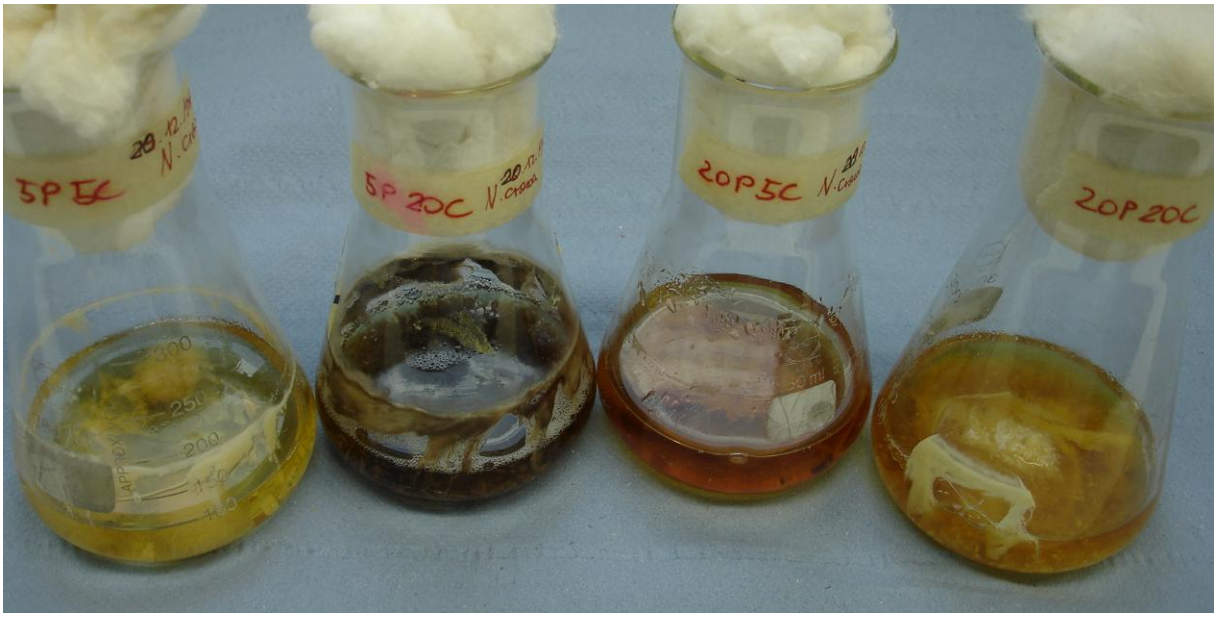

Figure 4.1 *Neurospora crassa* cultures during media optimization studies
*from left to right: *N. crassa* cultivated in C5-P5, C20-P5, C5-P20 and C20-P20 medium.

4.3 CDH production

4.3.1 Cultivation in big Petri dishes

Beside the cultivation in shaking flasks, further methods for CDH production were tested. First, cultivation of CDH producing ascomycetes in cellulose containing liquid media in big Petri dishes without agitation was evaluated. Three CDH positive strains, namely *C. atrobrunneum*, *D. saubinetii* and *M. brunnea*, were chosen for CDH production in big Petri dishes. In addition to cellulose media, cotton wool as carbon source was tested in same concentrations as cellulose. All inoculated Petri dishes were cultivated up to four weeks and sampling was done periodically, starting after 10 days of inoculation.

Cytochrome *c* at pH 6 was used as enzyme activity assay. Sampling and treatment of the crude culture medium were done in the same way than all liquid media tests.

After 10 days of cultivation, all three strains were able to produce CDH higher than the detection limit, shown in table 4.5. *C. atrobrunneum* achieved its maximum catalytic activity of 0.050 U mL^{-1} by growing in the high-level cotton wool medium after 10 days with a decreasing trend to 0.012 U mL^{-1} within 4 weeks. *M. brunnea* showed stable CDH catalytic activity during cultivation at about 0.01 U mL^{-1} in low-level cotton wool medium. However, this cultivation was not suitable for the production of CDH in *D. saubinetii*. By using the Petri dishes cultivation approach, enzymatic activity of only 3 to 5 U L^{-1} was reached instead of more than 100 U L^{-1} in shaking flasks. The pH shift in the media to the more alkaline range during cultivation were observed in the same way than in shaking flask cultivations.

All three strains were able to grown on cotton wool and showed higher activities than by the use of α-cellulose medium. Ascomycetes are aerobic organism and submerse cultivation without active aeration may limit growth, by the low oxygen solubility. Big Petri dishes feature a bigger area to volume ratio than flasks, but without agitation this system can be regarded as unsuitable for submerse cultivation of aerobic organisms. A solid-state fermentation using moist cotton wool could be a more effective procedure for cultivation of ascomycetes in big Petri dishes.

Table 4.5 Cultivation in big Petri dishes

Species	Medium	Activity [U mL^{-1}] [a]	Protein [mg mL^{-1}][b]	pH *	Days
Chaetomium atrobrunneum	C5-P5 α-cellulose	0.014	0.03	8.3	22
	C5-P5 cotton wool	0.017	0.01	8.5	22
	C20-P20 cotton wool	0.050	0.45	8.6	10
Dichomera saubinetii	C20-P5 α-cellulose	0.003	0.02	8.3	22
	C20-P5 cotton wool	0.005	0.03	8.5	10
Monilia brunnea	C5-P5 α-cellulose	0.003	0.02	8.3	10
	C5-P5 cotton wool	0.010	0.02	8.4	13
	C20-P20 cotton wool	0.007	0.05	8.5	13

[a] enzyme activity was measured using the cytochrom *c* assay at pH 6,0
[b] extracellular protein was measured according to Bradford
P...Pepton (g L^{-1})
C... celluloseulose or cotton wool (g L^{-1})
* pH measurment after 22 days of cultivation

4.3.2 Bioreactor cultivation

D. saubinetii, *H. haematostroma* and *C. atrobrunneum* were selected as candidates for bioreactor cultivations. Stirred tank reactor cultivation is so far the method of choice for CDH production in large amounts. In the first bioreactor study (*D. saubinetii and H. haematostroma*) a medium with equal level of carbon and nitrogen (C10-P10) was used. Agitation was set to 120 rpm and aeration to 20 L vol. air min^{-1}, respectively. When the oxygen saturation dropped from 96% to 15% after two days of cultivation, the aeration and agitation were adjusted to higher values (150 rpm and 40 L vol. air min^{-1}). Unfortunately, no CDH activity was present and the cultivation was stopped after one week. Maybe the high stirrer velocity and aeration bubbles caused too much shear stress to the mycelia. A second bioreactor cultivation (*D. saubinetii and C. atrobrunneum*) test was performed with lower adjustments for aeration and stirring speed (90 rpm and 7.5 L vol. air min^{-1}), using optimized composition of media. CDH was detected in the culture supernatant after 96 h of cultivation. The highest catalytic activity was measured after 6 days in *D. saubinetii* and after 10 days in *C. atrobrunneum* cultivations, but at a very low level compared to enzyme production in shaking flasks. CDH of *C. atrobrunneum* only showed a very low catalytic activity near the detection limit and highest catalytic activity of *D. saubintetti* CDH was 10-fold lower with half of the specific activity compared to shaking flask cultivation. Enzyme purification starting with such low activities would not make sense and therefore the cultivations were stopped.

4.3.3 CDH production in shaking flasks

After unsatisfactory CDH production rates in bioreactor and big Petri dishes cultivations, CDH production was done in shaking flasks. A scale-up from small 300 mL shaking flasks, used in screening and media optimization, to bigger ones (1 L) should not cause severe changes in CDH production, because of the similar conditions, contrary e.g. to bioreactor cultivations. The harvesting was performed at maximum enzymatic activity, when the sharp activity increase attenuated, preferably before the catalytic activity decrease started.

Extracellular enzyme degradation in *H. haematostroma* cultures run rapidly and the optimal time for harvesting was critical. *H. haematostroma* was cultivated in a total volume of six liter medium for nine days. CDH was harvested with 50 U L^{-1}, this is only about 75% of maximum enzyme activity, measured in media optimization tests. The CDH supernatant was

frozen at -30°C for one month, before purification started. About 15% of catalytic activity was lost by freezing.

H. haematostroma was cultivated a second time in 7.5 liter medium to repeat the purification procedure of this enzyme. Despite the earlier harvesting time after five, six and seven days of cultivation, enzyme activity was lower (40 U L^{-1}) than in first production. Most CDH activity was measured at day five with a considerable decline the days after. Purification started immediately after harvesting.

D. saubinetii was cultivated in a total volume of four liter medium. After 14 days, CDH was harvested at 0.1 U mL^{-1} enzyme activity, also lower than achieved in media optimization studies. Immediately after harvesting the purification procedure started.

Compared to small shaking flask cultivations, in big shaking flasks fewer CDH was produced, despite the same working volume of 30% of total volume. Probably the smaller surface to volume ratio in big shaking flasks was responsible for this result.

4.4 Enzyme purification

4.4.1 *Hypoxylon haematostroma* CDH

The supernatant of the first *Hh*CDH production were concentrated to 40% of total volume using cross-flow filtration. More than 15% of the total enzyme activity was lost because of this filtration step. Cross-flow filtration is only necessary at high ionic concentration in the enzyme solution, which would disturb ion exchange chromatography, or to reduce high volumes. However, not to lose valuable units in the preparation of the enzyme solution, cross-flow filtration was not used again in following enzyme purifications. In the IEF of culture supernatants, the pI of *Hh*CDH was about 4.5. The pH value of the enzyme concentrate was about pH 8, high enough to bind to the anion exchange column even at high salt concentrations. At first, a sodium citrate buffer at pH 6.0 was used as washing buffer. With a difference of more than one between the pI of the enzyme and the pH of the washing buffer, CDH should bind evenly to the matrix, but most of CDH was found in the flow trough. In the second approach, Tris-acetate buffer, pH 8.0 ensured strong adsorption of negatively charged CDH to the matrix, but protein separation quality suffered. Also for hydrophobic interaction chromatography two buffer systems were tried and better performance was obtained with the Tris-acetate buffer at pH 8.0 than with the sodium-

citrate buffer system at pH 6.0. These chromatography experiments were performed with partial volumes of the available CDH solutions. For this reason, only one result for both chromatography steps was available, shown in table 4.6. As expected, the yield was moderate at 32%. *Hh*CDH was purified 18-times and had a final specific activity of 8.3 U mg^{-1}.

Table 4.6 Purification parameters of *Hypoxylon haematostroma* CDH

Purification stage	Total activity [U]	Total protein [mg]	Specific activity [U mg^{-1}]	Purification [level]	Yield [%]
Culture supernatant	230	501	0.5	1.0	100
Defrosted solution	198	984	0.2	0.4	86
Cross-flow filtrate	165	641	0.3	0.6	72
Pool chromatography	61	7	9.4	20.4	27
Concentrate	73	9	8.3	18.0	32

The second batch of *H. haematostroma* was purified by the optimized purification protocol. AIEX was performed with the Tris-acetate buffer system at pH 8.0 for stronger binding of the CDH during the washing step. The obtained eluate showed 100% yield and a slightly higher specific activity. CDH was purified about two-folds, see table 4.7. This not really reflects the effort of a chromatography step. Caused by the bigger difference of buffer pH and enzyme's pI too many proteins, mostly also negatively charged at pH 8, bound strongly to the column too and resolution suffered, seen in a broad eluation peak. The sodium citrate buffer at pH 6 did not work very well for *H. haematostroma* CDH, but in principle, a buffer system lower than pH 8.0 should significantly improve the separation performance of the AIEX. The HIC worked very well with the Tris-acetate buffer system at pH 8.0. CDH was purified more than 20-fold from the extracellular liquid with a very high yield (table 4.7). During the concentration step with the centrifugal filter devices a mistake happened. Probably the wrong solution for washing of the concentrate was used. Most catalytic activity was lost but nearly all protein was recovered in the concentrate. However, due to the loss of most specific activity during the concentrating step, CDH of the first purification was used for further enzyme studies.

Table 4.7 Purification parameters of *Hypoxylon haematostroma* CDH 2nd

Purification stage	Total activity [U]	Total protein [mg]	Specific activity [U mg^{-1}]	Purification [level]	Yield [%]
Culture supernatant	258	899	0.3	1.0	100
DEAE Sepharose	259	502	0.5	1.8	100
Phenyl Source	244	41	6.0	20.7	95
Concentrate	64	36	1.8	6.2	25

4.4.2 *Dichomera saubinetii* CDH

The enzyme was purified using a two-step procedure based on AIEX followed by HIC. In the IEF of culture supernatants, the pI of *Ds*CDH was determined to pH 4.0, a little bit lower than *Hh*CDH. The use of a sodium citrate buffer system at pH 6.0 was decided for both chromatography steps. *Ds*CDH showed a stable adsorption to the anion exchange matrix, unlike *Hh*CDH, which was washed out under these conditions. Because of the lower set pH in AIEX more foreign proteins were eliminated during the washing procedure and a more efficient separation up to a purification level of 10-fold (table 4.8) was achieved. The following HIC purified the enzyme solution additionally 18-folds but with a loss of 50% total enzyme activity. However, the enzyme was purified more than 32-fold from the extracellular liquid, to a specific activity twice than that obtained in *Hh*CDH purification, with an acceptable yield of more than 40% of the starting total enzyme activity.

Table 4.8 Purification parameters of *Dichomera saubinetii* CDH

Purification stage	Total activity [U]	Total protein [mg]	Specific activity [U mg^{-1}]	Purification [level]	Yield [%]
Culture supernatant	380	713	0.5	1.0	100
DEAE Sepharose	361	68	5.3	10.0	95
Phenyl Source	154	10	14.9	28.0	41
Concentrate	159	9	17.5	32.8	42

4.5 Enzyme characterization

4.5.1 Isoelectric focusing

The first isoelectric focusing was performed using the Multiphor II system to analyze all CDH positive strains, collected during screening in shaking flasks. In the first place, the pIs of *D. saubinetii* and *H. haematostroma* were mainly of interest for pH adjustments in AIEX. Catalytic and specific activities of the samples were very low (table 4.1), because they were not purified and concentrated. Conductivity of most samples was about 8 mS cm^{-1} and CDH supernatants with more than 10 mS cm^{-1} conductivity were diluted with distilled water to lower the ionic concentration, which interferes in the electric field. However, a dilution of all samples was avoided, because of the very low CDH content and weak anticipated bands. The isoelectric focusing gel exhibited a horizontal uneven distribution of the marker bands, shown in figure 4.2 and figure 4.3. Also the proteins of the samples moved not uniformly, which was due to the high conductivities in the samples. An exact evaluation of the bands was difficult, because of the imprecise assignment of CDH bands to the corresponded pHs of the marker proteins. As expected some CDH bands in the active staining were very weak and hardly visible (figure 4.1). Silver staining (figure 4.2) showed many bands in the crude samples. Nevertheless, it was possible to detect differences between fungi. Most CDHs were visible as single bands but *N. crassa* and *S. thermophilum* CDHs showed double bands during active staining, which is an indication of the presence of isoenzymes (figure 4.2). In activity measurements, isoenzymes are not distinguishable from one another, because their conversion adds up to a common total catalytic activity, while their attendance remains unidentified. The pI of *C. atrobrunneum*, *D. saubinetii* and *S. bisby* CDH was estimated to about pH 4. A weak band of *H. haematostroma* CDH was found at about 4.5, as well as the band of *M. brunnea* CDH. The isoenzymes of *N. crassa* were found in the area of pH 4.5 and these of *S. thermophilum* in the more acidic range slightly lower than pH 4. Bands in *A. strictum* bands were hardly visible and a pI determination was not possible. *F. oxysporum*[i] showed no bands in active staining, even though enzyme activity with cytochrome *c* was detected in screening and media. ([i]4.1.1.1 Extracellular enzyme activity of *F. oxysporum*).

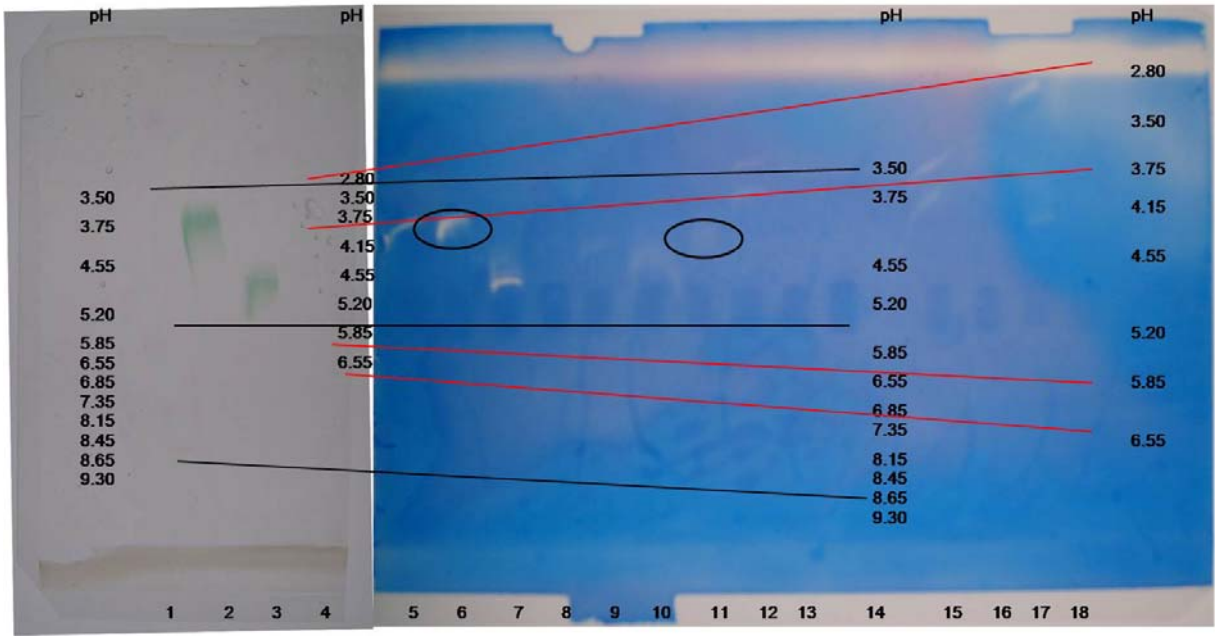

Figure 4.2 IEF of CDHs – active staining

Lane	Applied sample	Determined pI
1	standard broad pI	-
2	(*L. wrightii*)	-
3	(*B. aclada*)	-
4	standard low pI	-
5	*C. atrobrunneum*	~ 4
6	*D. saubinetii**	~ 4
7	*N. crassa*	~ 4.5
8	*F. oxysporium*	n.d.
9	*S. bisbyi*	~ 4
10	*M. brunnea*	~ 4
11	*H. haematostroma**	~ 4.5
12	*S. thermophilum*	≤ 4
13	*A. strictum* (dilluted)	n.d.
14	standard broad pI	-
15	*S. thermophilum*	≤ 4
16	*A. strictum*	n.d.
17	*S. bisbyi*	~ 4
18	standard low pI	-

n.d. … not detectable

* CDH bands of *D. saubinetii* and *H. haematostroma* are shown encircled

Figure 4.3 shows the results in silver staining to determine pH values. As expected, lots of bands were found in each sample lane, because samples were not purified.

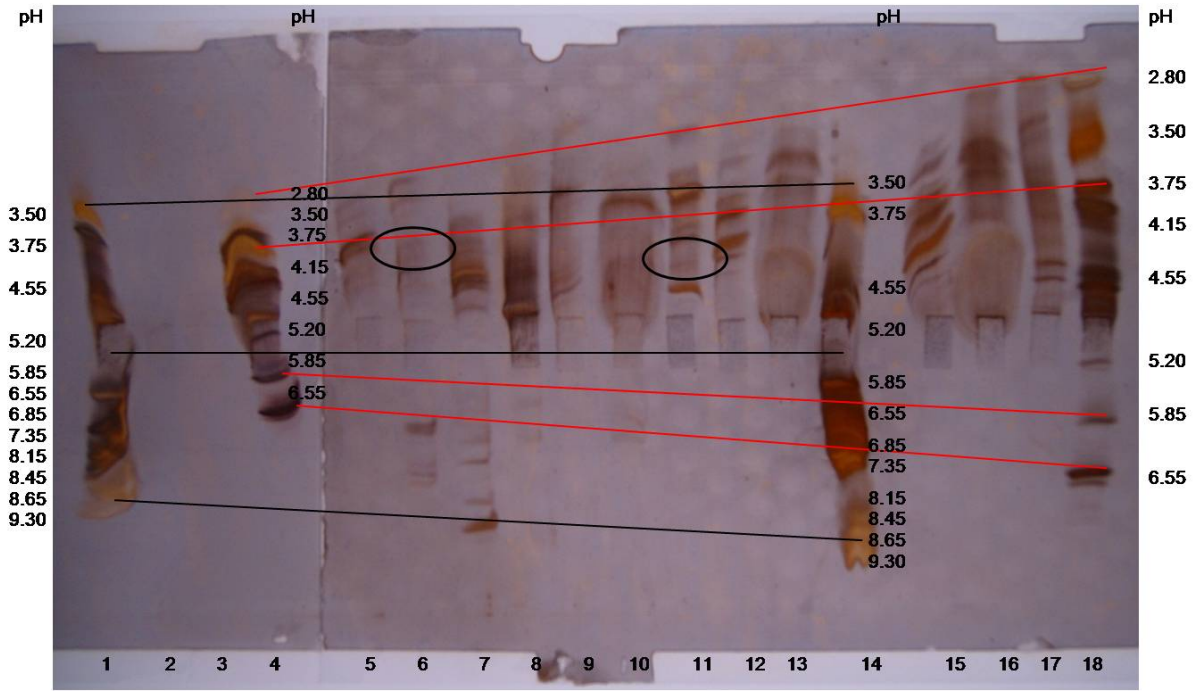

Figure 4.3 IEF of CDHs – silver staining

The second IEF was performed using the Phast System from Pharmacia with precast dry gels to determine the pI of the two purified CDH solutions of *D. saubinetii* and *H. haematostroma*. The Coomassie Blue stained gel is shown in figure 4.4. The pI of *Hh*CDH was determined at pH 4.5. The diluted CDH lane showed no band. The pI of *Ds*CDH was found in the more acidic range at pH 4.1. CDHs exhibit a pI in the acidic range [6], also these two ascomycete CDHs.

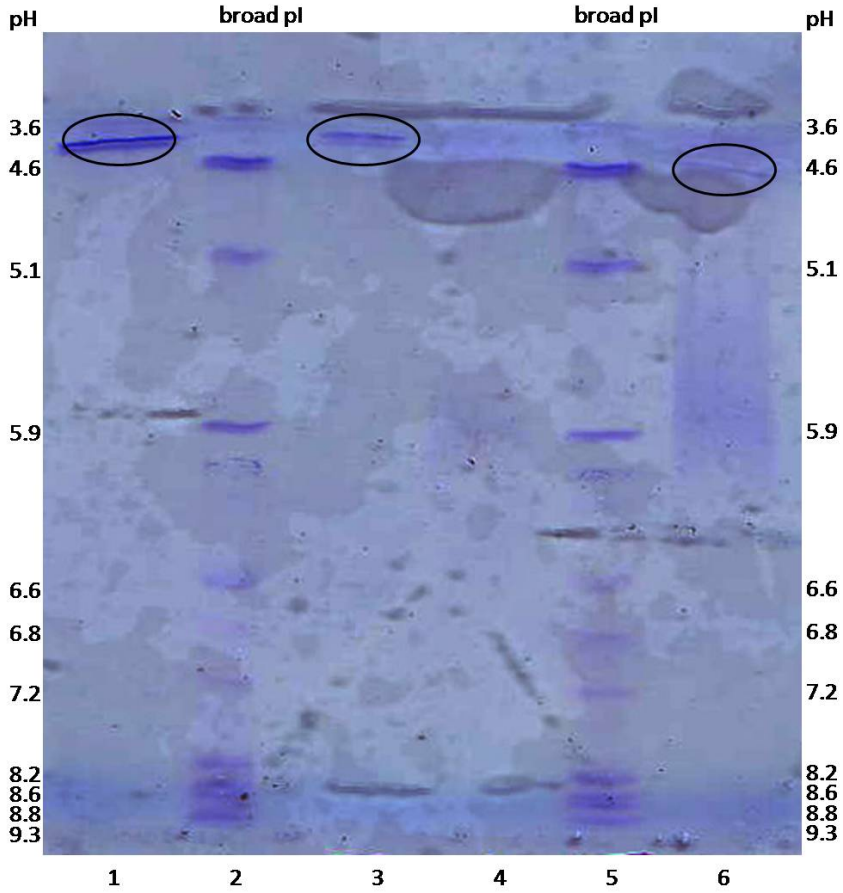

Figure 4.4 IEF of purified *Dichomera saubinetii* and *Hypoxylon haematostroma CDH*

Lane 1 *D. saubinetii* CDH
Lane 2 Standard broad pI
Lane 3 *D. saubinetii* CDH*
Lane 4 *H. haematostroma* CDH*
Lane 5 Standard broad pI
Lane 6 *H. haematostroma* CDH
* Dilution 1:2

4.5.2 Molecular mass determination by SDS-PAGE

A typical CDH is a monomeric glycoprotein with a molecular mass of approximately 80–115 kDa and an acidic pI [6]. By the molecular mass determination CDH of *C. thermophilus*, an ascomycete with a molecular mass of 80 kDa [49] was used as reference, lane 2 in figure 4.5. The molecular mass of *C. thermophilus* was determined at 80 kDa, that of *Hh*CDH was identified to be 82 kDa, similar to *C. thermophilus* CDH. In the lane of *Hh*CDH some impurities with lower masses can be seen, which corresponds with the lower specific activity in *Hh*CDH purification. *Ds*CDH showed a much higher molecular mass at 114 kDa.

Most determined CDHs are smaller than DsCDH, only *Coniophora puteana*, a basidiomycete, was found with a higher molecular weight of 115 kDa [6]. The results of SDS-PAGE are shown in figure 4.5.

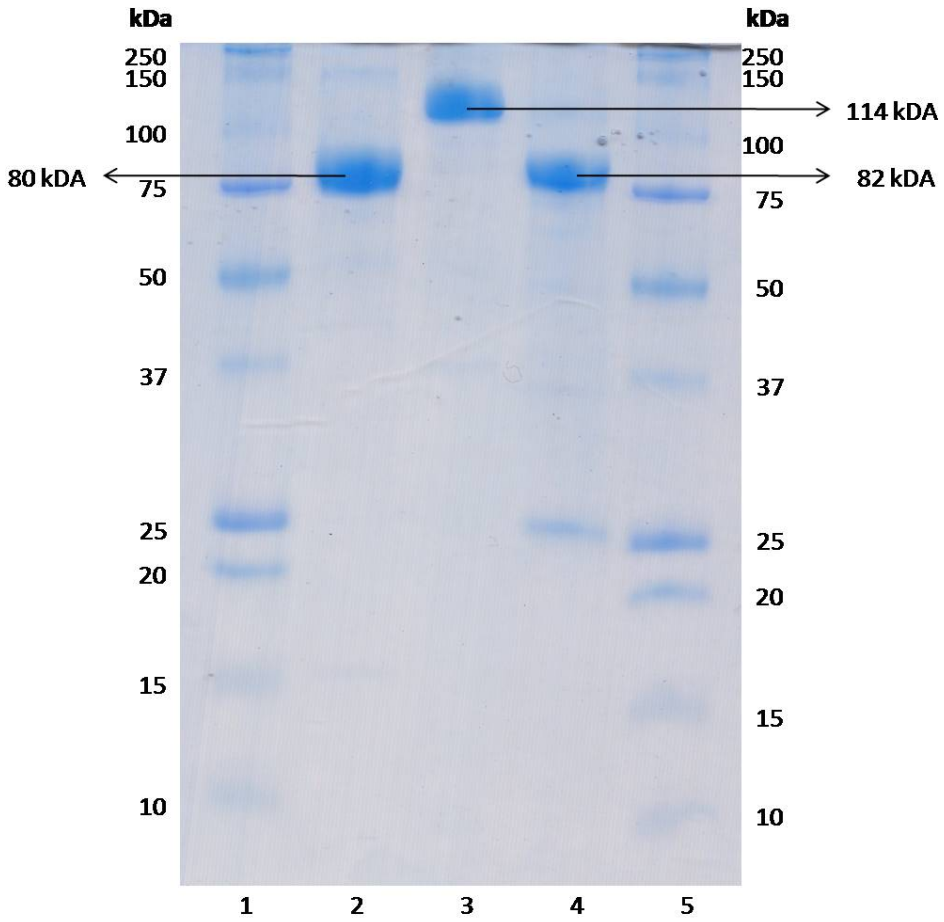

Figure 4.5 SDS-PAGE gel of *Dichomera saubinetii* and *Hypoxylon haematostroma* CDH

Lane 1 Standard
Lane 2 *C. thermophilus*
Lane 3 *D. saubinetii*
Lane 4 *H. haematostroma*
Lane 5 Standard

4.6 Kinetic studies

4.6.1 pH profiles

CDH from the ascomycetes *D. saubinetii* and *H. haematostroma* was compared in their pH characteristics with both electron acceptors and lactose as substrate. The two used buffer systems overlapped at pH 5.5, 6.0 and 6.5 to investigate the influence of different ions on the activity of CDH. The graphs show the normalized velocities versus pH conditions.

The pH optimum for *Ds*CDH using cytochrome *c* as electron acceptor was determined between pH 5.0 and 5.5 (figure 4.6). The curve is bell-shaped with near the same catalytic activity decrease by leaving the optimum towards the more acidic or more alkaline range. The different buffer systems have no influence to the velocity of the reaction.

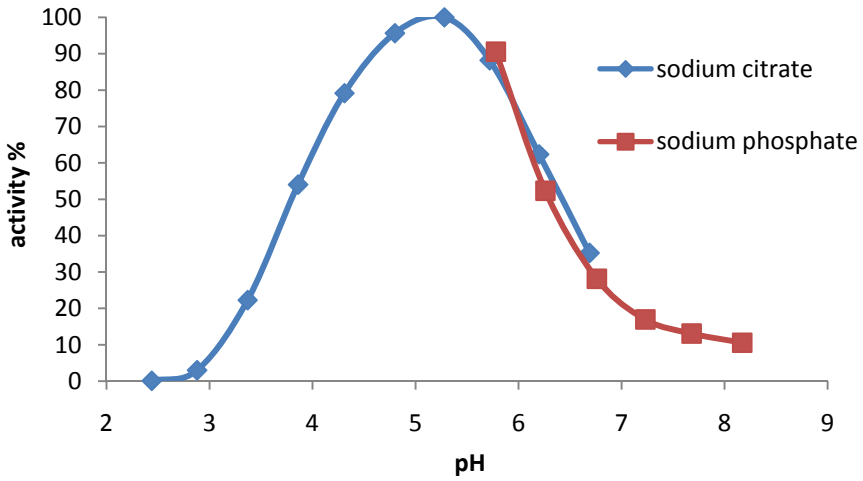

Figure 4.6 pH profile of *Dichomera saubinetii* CDH using cytochrome *c*

In figure 4.7 the pH profile for *Ds*CDH with the two-electron acceptor DCIP is shown. In contrast to the pH profile with cytochrome *c*, there is no well defined optimum, instead a plateau from pH 4 to 6 was observed. Thus, the working range with DCIP, reoxidizing DH_{cdh}, is much broader than the reoxidation via the IEF. The buffer systems showed no significant differences.

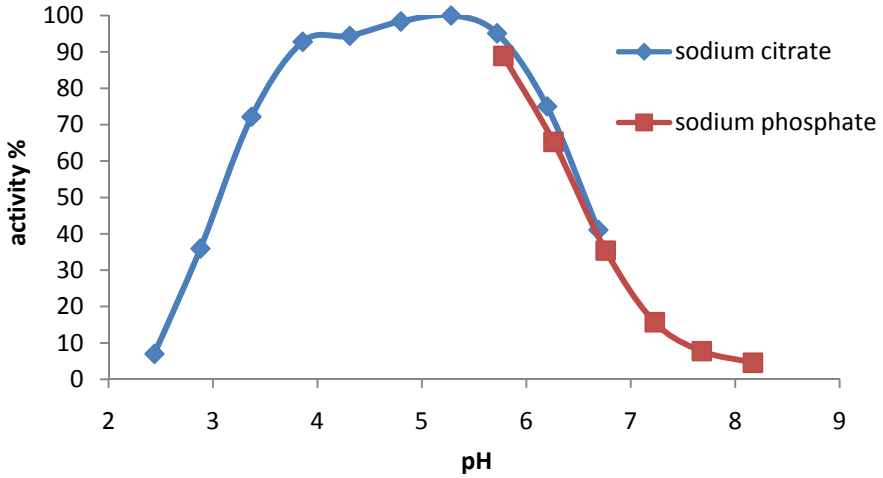

Figure 4.7 pH profile of *Dichomera saubinetii* CDH using DCIP

The pH profile of *Hh*CDH is shown in figure 4.8. *Hh*CDH exhibited a more distinct optimum with DCIP compared to *Ds*CDH. The optimum pH for the reoxidation of DH_{cdh} with DCIP was at pH 5.0. The different buffer systems have no influence to the velocity of the reaction.

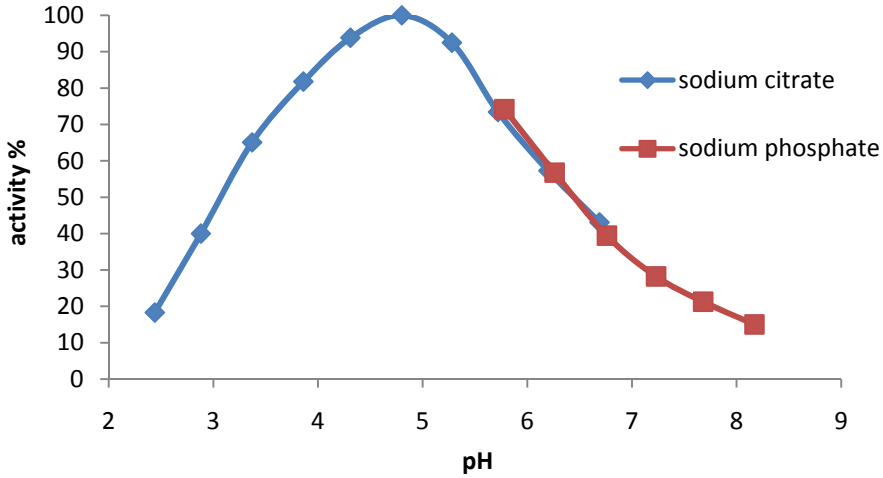

Figure 4.8 pH profile of *Hypoxylon haematostroma* CDH using DCIP

The pH optimum for *Hh*CDH using cytochrome *c* as electron acceptor was determined to be pH 5.5 by using the sodium citrate buffer, but pH 6.0 with the sodium phosphate buffer (figure 4.9). In the alkaline range, *Hh*CDH exhibits a shoulder up to pH 8 with more than 50% enzyme activity.

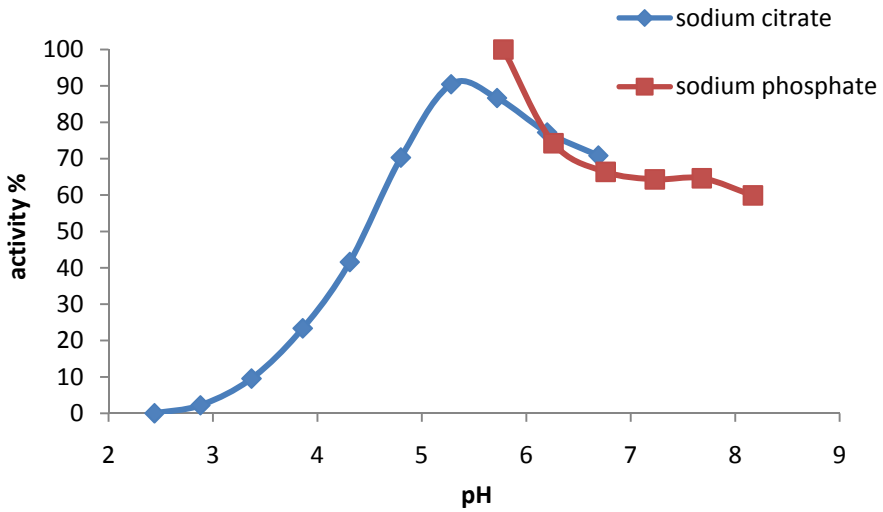

Figure 4.9 pH profile of *Hypoxylon haematostroma* CDH using cytochrome *c*

It is interesting that both CDHs were inactive at pH 2.5 when the enzyme is reoxidized at CYT_{cdh}., whereas small CDH activity was retained by using the two-electron acceptor DCIP. In general, CDH of both strains showed an expanded range of application in the more acidic range down to pH 3 of at least more than 40% of maximum catalytic activity by using the DCIP electron acceptor. In contrast, in the alkaline range higher CDH activity rates are obtained by using cytochrome *c* against DCIP as electron acceptor with an exceptionally expanded working range of *Hh*CDH in this area.

4.6.2 Temperature optimum

Lactose conversion velocities with both electron acceptors depending on the temperature were analyzed. Consequently, the enzyme activity rates (U mL^{-1}) in this test were normalized to the standard temperature of 30°C. To ensure reaction conditions at exact temperatures, a thermostated cell holder in the photometer were necessary. Temperatures of the start and endpoint of the reaction were always detected by a resistance thermometer and values in the graphs refer to the averaged temperatures.

The results of temperature dependent reaction velocities are shown in figure 4.10 for *Ds*CDH and in figure 4.11 for *Hh*CDH. Enzymes of both strains showed more than two-fold increased reaction velocities at 60°C, by using DCIP, bypassing the IET to CYT_{cdh}. At minor higher temperatures, their catalytic activity dropped down rapidly to about the same results than

obtained at standard temperature. By using the internal electron transfer to the heme domain with cytochrome *c*, the gradient of the curve is much lower, with a catalytic activity increase of not more than 50%. The lowest enzyme activity was measured at room temperature (20°C). At the highest measured temperature (67°C), about half enzyme activity was detected, compared to 30°C. It seems that the IET between DH_{cdh} and CYT_{cdh} is more sensitive to higher temperatures. The enzyme stability against heat inactivation was not tested but with the detected thermal optimum at 60°C, a slightly thermophilic nature can be expected for *Ds*CDH and *Hh*CDH.

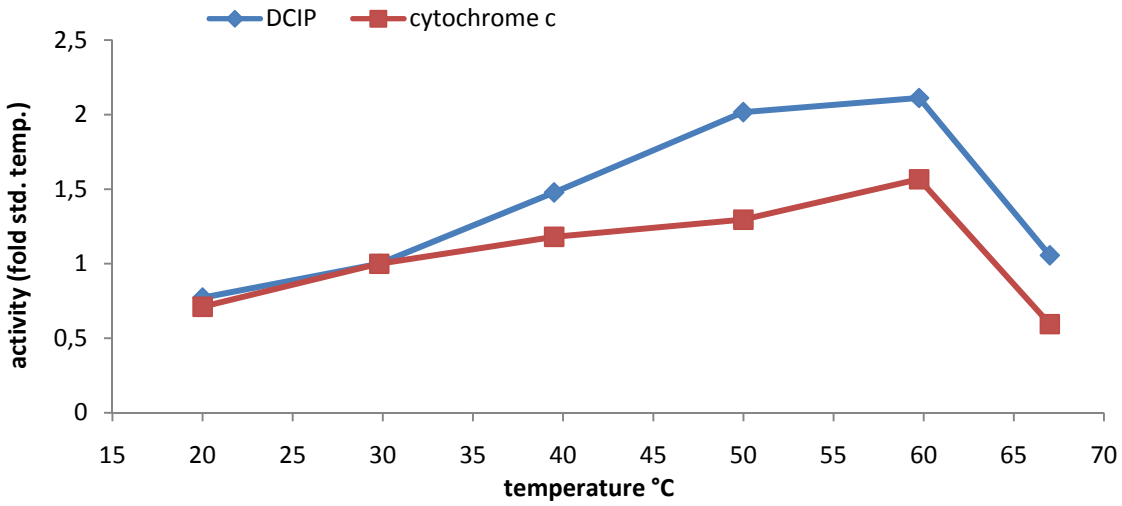

Figure 4.10 Effect of temperature on *Dichomera saubinetii* CDH activity

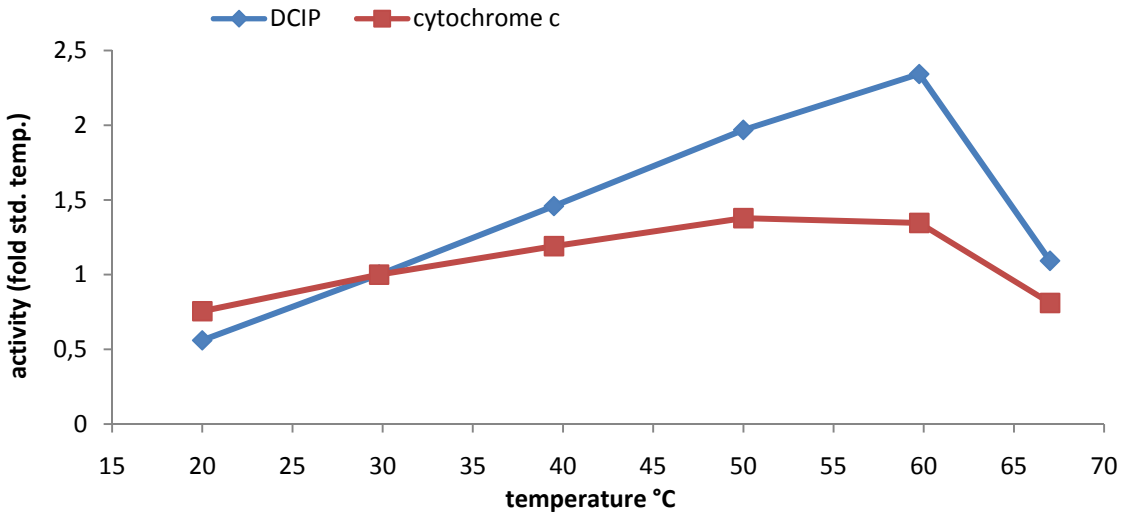

Figure 4.11 Effect of temperature on *Hypoxylon haematostroma* CDH activity

The activation energy (E_a) for lactose as substrate and the electron acceptors DCIP and cytochrome *c* were calculated from the Arrhenius plot in the linear temperature range of 20–50°C. E_a for *Ds*CDH with DCIP was 25.8 kJ mol^{-1} and with cytochrome *c* 15.6 kJ mol^{-1}. *Hh*CDH showed a higher activation energy than *Ds*CDH when DCIP was used for reoxidation of the enzyme at 32.8 kJ mol^{-1}. By using the cytochrome *c* electron acceptor the activation energy for *Hh*CDH was found at the same level than *Ds*CDH at 15.6 kJ mol^{-1}.

4.6.3 Substrate specificity

Kinetic parameters were investigated for four electron donors with two different electron acceptors. As electron donors the disaccharides cellobiose (glucose linked by a β(1→4) glycosidic bond), lactose (galactose and glucose bonded through a β(1→4) glycosidic linkage), maltose (glucose joined by α(1→4) glycosidic bond) and the monosaccharide glucose were tested at pH 5. Due to the expanded pH working range of each enzyme, the sugars lactose and glucose, the most interesting substrates in CDH applications, were also measured at pH 6.5 with both electron donors. Additionally, *Hh*CDH activity at pH 8 was tested with cytochrome *c*.

CDH features two redox centers, DH_{cdh} and CYT_{cdh} , with different functions during the reaction. The flavodehydrogenase domain is responsible for substrate oxidation. Its reduced form is able to interact directly with redox mediators for regeneration, as well as with the heme domain via an internal electron transfer. It is common belief that two-electron acceptors, like DCIP, are reduced directly at DH_{cdh}, whereas the redox mediator cytochrome *c* exclusively regenerates CYT_{cdh}. In the first case when DH_{cdh} interacts with external redox mediators, CYT_{cdh} can be seen as a storage for electrons, or maybe has no participation to the reaction, because the reaction would also occur when the holoenzyme is cleaved. In the second case, when cytochrome *c* is used as electron acceptor, the electrons of substrate oxidation are shuttled from DH_{cdh} to CYT_{cdh} via the IET and CYT_{cdh} is re-oxidized by the one-electron acceptor cytochrome *c*. In the latter case, the holoenzyme with both active domains is required to perform the reaction.

Reductive half-reaction

The reductive half-reaction of CDH affects the oxidation of the substrates. Kinetic constants for different sugars were determined. The redox mediators, necessary for the reaction cycle, were added in adequate amounts, as used in the standard assay and were not the limiting substrates. Results of *Ds*CDH reductive half-reactions are shown in table 4.11, results of *Hh*CDH in table 4.12.

The β-1,4 linked substrates cellobiose and lactose were the preferred substrate for the new ascomycetes, emphasized by the highest catalytic efficiencies. Cellobiose featured the lowest K_M values among all tested sugars and was the most efficiently converted substrate, closely followed by lactose. The redox mediator seemed to have influence on the enzymes substrate affinity. K_M values of all substrates were nearly ten-times lower when cytochrome *c* was used as electron acceptor, but the turnover numbers were only the half of DCIP mediated reactions. That means that the enzyme regeneration via CYT_{cdh}, using the internal electron transfer, was only rate limiting when substrate was converted at high velocities, but the internal electron transfer was preferred at low substrate concentrations. Nevertheless, both CDHs showed higher catalytic efficiency with all tested sugars when cytochrome *c* acted as redox mediator. *Hh*CDH had a ten-fold higher affinity to cellobiose than to lactose when cytochrome *c* was used, resulting in a much higher catalytic efficiency with the natural substrate. An exception was glucose, the substrate with worst enzyme binding properties and K_M values in the triple-digit milimolar range, but the obtained k_{cat} could keep pace with that of preferred β-1,4 linked substrates. However, glucose was the most unfavored substrate, followed by the α-1,4 linked disaccharide maltose. Slight substrate inhibition was obtained in the high milimolar range for both β-1,4 linked substrates, but only at the DCIP assay. The strong substrate inhibition by cellobiose, often reported in the past, could not be found, as the inhibition was near the molar range and applied to lactose at the same extend.

Table 4.9 Substrate specificity of *Dichomera saubinetii* CDH

Electron-donor	Assay pH	Electron-acceptor	K_M [mM]	K_I [mM]	k_{cat} [s^{-1}]	k_{cat}/K_M [$s^{-1}M^{-1}$]
Cellobiose	5.0	DCIP	0.127	473	26.54	2.1×10^5
Lactose			0.213	434	28.08	1.3×10^5
Maltose			8.0	-	1.32	160
Glucose			792	-	22.88	29
Cellobiose	5.0	Cytochrome *c*	0.012	-	10.24	8.3×10^5
Lactose			0.048	7.6×10^6	10.05	2.1×10^5
Maltose			13.2	877	1.30	98
Glucose			772	1.6×10^3	17.75	23
Lactose	6.5	DCIP	0.111	128	17.50	1.6×10^5
Glucose			750	921	22.70	30
Lactose	6.5	Cytochrome *c*	0.015	408	4.36	2.9×10^5
Glucose			90.2	-	4.20	47

At pH 6.5, the affinities to the substrates increased slightly, also substrate inhibition. In combination with marginal lower turnover numbers, the catalytic efficiency remained approximately the same. The turnover numbers of glucose were hardly affected by the higher pH.

*Hh*CDH measurements at pH 8 with lactose and glucose, using only the one-electron acceptor cytochrome *c*, provided nearly equal results than at lower pHs. Only positive changes in substrate affinity, turnover number and catalytic efficiency could be noted, except substrate inhibition, which seemed to increase with pH.

*Ds*CDH and *Hh*CDH follow the same trend than shown in pH optimum studies. *Ds*CDH had a broad working spectrum by interacting with DCIP and could maintain its turnover numbers at increased pH. *Hh*CDH, which appears to be well adapted to alkaline pH, showed no loss in turnover numbers up to pH 8, but only with the cytochrome *c* mediator, using the IET.

Table 4.10 Substrate specificity of *Hypoxylon haematostroma* CDH

Electron-donor	Assay pH	Electron-acceptor	K_M [mM]	K_I [mM]	k_{cat} [s^{-1}]	k_{cat}/K_M [$s^{-1}M^{-1}$]
Cellobiose	5	DCIP	0.162	746	15.68	9.7×10^4
Lactose			0.230	698	17.18	7.5×10^4
Maltose			18.0	-	1.10	60
Glucose			709	-	13.05	18
Cellobiose	5	Cytochrome *c*	0.005	-	4.59	1.0×10^6
Lactose			0.050	-	4.25	8.4×10^4
Maltose			7.1	877	0.57	80
Glucose			258	3.3×10^3	4.42	17
Lactose	6.5	DCIP	0.151	247	11.02	7.3×10^4
Glucose			315	-	4.82	15
Lactose	6.5	Cytochrome *c*	0.024	362	2.84	1.2×10^5
Glucose			219	-	4.05	18
Lactose	8	Cytochrome *c*	0.020	195	4.89	2.5×10^5
Glucose			229	4.7×10^3	4.53	20

4.6.4 Redox mediator specificity

Kinetic parameters were investigated at pH 5.0 and pH 6.5 for the two different electron acceptors DCIP and cytochrome *c* with lactose as substrate. Additionally, *Hh*CDH activity at pH 8 was tested with cytochrome *c* and lactose. Results of *Ds*CDH oxidative half-reactions are shown in table 4.13, results of *Hh*CDH in table 4.14.

Oxidative half-reaction

The binding abilities of *Ds*CDH and *Hh*CDH for both electron acceptors were generally high, considering the low K_M values, but highly pH dependent. The turnover of lactose and cellobiose was slightly faster than that of DCIP, indicating that near the V_{max} DH_{cdh} reoxidation with DCIP could be rate limiting in cellobiose and lactose conversions. This might apply for *Ds*CDH as well as for *Hh*CDH. A general tendency was the decrease of turnover numbers of electron acceptors with increasing pH. Even the alkaline adapted

*Hh*CDH lost half of conversion rates when the pH value was increased to 8. However, more interesting is the strong K_M shift when the pH value was altered. *Ds*CDH exhibited a narrow pH optimum near pH 5 with cytochrome *c* and a broad working area when DCIP was used, but the K_M of cytochrome *c* decreased, whereas the K_M of DCIP increased five-fold at higher pH of 6.5. The K_M values of *Hh*CDH were shifted in opposite direction, compared to *Ds*CDH, but similar to its pH profile. At pH 5, the preferred electron acceptor was DCIP, but at higher pH the affinity to cytochrome *c* was higher. This pH dependent affinity is maybe important for enzyme activities at limiting redox mediator concentrations. The catalytic efficiency is for both electron acceptors at a similar level. Note that the reaction stoichiometry is 1 for two-electron acceptors and 2 for one-electron acceptors. Each mol oxidized sugar requires the same amount of DCIP, but twice the quantity of cytochrome *c.* The k_{cat} values of cytochrome *c* were, at all measured pH steps, at least two-times higher than its electron donor counterparts and additionally showed low K_M values. This indicates that in the case of substrate oxidation and electron transfer to CYT_{cdh} its regeneration with cytochrome *c* is not rate limiting.

Table 4.11 Kinetic constants of *Dichomera saubinetii* CDH for electron acceptors

Electron-acceptor	Assay pH	Stoichio-metry[a]	K_M [µM]	K_I [mM]	k_{cat} [s^{-1}]	k_{cat}/K_M [$s^{-1}M^{-1}$]
DCIP	5.0	1	5.78	-	24.69	4.3×10^6
Cytochrome *c*		2	5.70	-	27.63	4.8×10^6
DCIP	6.5	1	32.10	-	12.98	4.0×10^5
Cytochrome *c*		2	1.70	135	8.84	5.1×10^6

[a] Mol of electron acceptor reduced per mol of lactose oxidized

Table 4.12 Kinetic constants of *Hypoxylon haematostroma* CDH for electron acceptors

Electron-acceptor	Assay pH	Stoichio-metry[a]	K_M [µM]	K_I [mM]	k_{cat} [s^{-1}]	k_{cat}/K_M [$s^{-1}M^{-1}$]
DCIP	5.0	1	5.60	-	15.99	2.8×10^6
Cytochrome *c*		2	25.90	-	21.32	8.2×10^5
DCIP	6.5	1	14.60	-	8.80	6.0×10^5
Cytochrome *c*		2	4.90	70.3	14.90	3.0×10^6
Cytochrome *c*	8.0	2	4.40	-	9.64	2.2×10^6

[a] Mol of electron acceptor reduced per mol of lactose oxidized

5 CONCLUSIONS

One third of the screened ascomycetes showed significant CDH activity under the chosen culture conditions. This reflects how wide spread and important CDH under cellulolytic culture conditions is. Moreover, it underlines again a proposed function of CDH in the cellulose degradation system of ascomycetes [6]

F. oxysporum is reported to carry FCD1, a gene encoding a protein homologous to DH_{cdh} [47]. The culture media of this fungus showed only enzyme activities using the cytochrome *c* assay, but DCIP was never reduced by the secreted enzyme. For this reason, the detected enzyme could not be identified as DH_{cdh} or CDH.

The outstanding CDH producer among the screened ascomycetes is *D. saubinetii*, which produced more than 100 U L^{-1} under appropriate culture conditions. This is much less compared to the highest reported values of some other CDH producing fungus, but a respectable result for an ascomycete.

With the exception of a few strains, all ascomycete strains raised the pH of the culture liquid during cultivation to neutral or alkaline pH milieu. Also in their natural habitats, similar pH conditions were reported. For example, the pH of soil typically ranges from 5.5 to 7.0 or during the compost process; the pH is normally raised from pH 5.0 to 7.5 [4]. While white rot fungi acidify the culture liquid during cultivation and show poor growth at pH values above pH 7.5, ascomycetes are not limited in growth or even grow better under such neutral or alkaline pH milieus.

In all selected ascomycete strains, the CDH production was increased by higher concentrations of cellulose. In previous studies, it has been shown that CDH production was enhanced in medium containing a complex nitrogen source [48, 50]. This cannot be said from the investigated ascomycetes. Higher nitrogen content stimulated the secretion of extracellular protein, but CDH secretion was not raised by nitrogen rich media.

A fast screening method for CDH producing fungi was tested. Ascomycete strains were cultivated on α-cellulose containing agar plates, similar to a solid-state fermentation, and tested for protein content and enzyme activity. It was possible to induce CDH secretion and to detect catalytic activity already after four days of cultivation. The screening after four days of cultivation did not achieved the desired results for a reliable differentiation between

CDH producer and non-producing ascomycetes. Nevertheless, this method could be improved for the detection of CDH.

In addition to shaking flask and bioreactor cultivations, CDH production in stationary vessels (big Petri dishes) was tested with an additional substrate cotton wool. It was shown that the tested ascomycetes secreted CDH when cotton wool or cellulose were added as substrates, but this method is not suitable for CDH production, mostly due to the very low oxygen saturation in liquid media without agitation.

Two bioreactor cultivation studies with different adjustments and different media for CDH production at large scale were performed with three ascomycetes. The first test with higher stirring velocity and aeration rate remained without results. At the second study, lower settings of stirrer velocity and aeration rate were chosen and the two cultivated strains produced CDH, but at very low levels compared to shaking flask cultivation. A scale-up in mycelium production of ascomycetes from shaking flask to stirred tank reactor cultivation seems to be very difficult, because of completely different systems in aeration and mixing. Not all microorganisms can be successfully cultured under these different conditions.

The two purified CDHs (*Ds*CDH and *Hh*CDH) showed a great variety in their pH profiles for DCIP and cytochrome *c* as electron acceptors. The optimal pH range for the two-electron acceptor DCIP varied between pH 4.0 and pH 6.0 and for the one-electron acceptor cytochrome *c* between pH 5.0 and 7.0. The preferred electron acceptor was strongly pH dependent and arranged differently for the two investigated CDHs. Compared to basidiomycete CDHs the pH optima for these two ascomycete CDHs are in a less acidic pH region. *Hh*CDH was able to shuttle electrons from the FAD domain to the heme domain even under alkaline pH conditions. In contrast to these ascomycetes CDHs basidiomycete CDHs show the most efficient reoxidation via the heme domain by using cytochrome *c* in more acidic pH range between pH 3.5 and 4.5 [6]. The different optimal pH conditions of at least these two investigated ascomycete CDHs reflect their probably broad distribution in nature and their adaption to different environments, whereas basidiomycete CDHs are restricted to acidic pH milieu related to wood degradation.

Class-I CDHs are known do have high substrate specificity for β-1,4 linked substrates with a glucose residue at the reducing end and a very pronounced discrimination against glucose and maltose [6]. The substrate specificities of the two new ascomycete CDHs represent a

typical substrate spectrum for a class-II CDH and show a strong affinity to cellobiose and lactose and relatively high conversion rates with glucose concentration.

It is very interesting that the redox mediators seemed to influence the substrate affinity of the enzymes. K_M values of all substrates were nearly ten-times lower when cytochrome *c* was used as electron acceptor, but the turnover numbers were only the half level of DCIP mediated reactions. It seems that the IET and regeneration of the enzyme via the electron-chain-model was preferred at lower substrate concentrations, also seen in higher catalytic efficiency of all sugars with cytochrome *c* as redox mediator, instead of DCIP.

The reported pH dependent substrate-inhibition mechanism [6], which is pronounced at higher pH values was affirmed. However, substrate inhibition was detected for both, cellobiose and lactose, but only in the high millimolar range. Kinetic constants of *Ds*CDH and *Hh*CDH followed the same trend than shown in pH optimum studies. *Ds*CDH had a broad working spectrum by interacting with DCIP and could maintain its turnover numbers at increased pH. *Hh*CDH, which appears to be well adapted to alkaline pH, showed no loss in turnover numbers up to pH 8, but only with the cytochrome *c* mediator, using the IET to CYT_{cdh} for reoxidation.

Regarding the oxidative half-reaction, the affinities to the electron acceptors were generally high, but in the same way pH dependent. The catalytic efficiency was for both electron acceptors at a similar level.

The altered substrate specificity, the pH working range in the more neutral range and the effective IET make these two investigated CDHs highly interesting for biosensors.

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